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Original Article

Etiological differences in Lyme borreliosis patients with and without localized scleroderma based on serological examination in the western Ukraine

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Abstract

Background Possible associations of localized scleroderma (LS) with Lyme borreliosis (LB) were reported in certain countries, though denied in others. Therefore, it is still under investigation, whether the association is real or fictional.

Objective The aims of the current study was to determine percentage of serologically positive patients among suspected LB patients, as well as to investigate etiological differences between confirmed LB patients with and without LS in Ternopil region.

Methods An observational study of 196 patients with complaints related to LB treated at Ternopil healthcare institutions (2017-2022) was conducted. LB serological confirmation was done using two-stage procedure (ELISA and immunoblotting). The confirmed LB patients were split into two groups, with or without localized scleroderma (LS), and a wide spectra of specific IgG were analyzed to check for significant differences between the two groups.

Results We confirmed the necessity of the two-stage procedure, as some ELISA screening results, 11.4% (95% CI 3.1−29.3) for IgM and 11.5% (95% CI 4.6−23.6) for IgG, appeared to be false positives during stage two immunoblotting tests. VlsE antigens of *B. afzelii* and p83 were significantly more often detected in the LB patients with LS compared to LB patients without LS, $p < 0.05$. VlsE antigens of *B. burgdorferi s.s.* and p39 were diagnosed more often in LB alone patients compared to LB with LS patients, $p < 0.05$.

Conclusion We can speculate, that failures to detect borreliosis-associated morphea in some countries may be related to the absence of *B. afzelii* in those areas.

Key words
Lyme borreliosis; Localized scleroderma; Borrelia afzelii.

Introduction

Infectious diseases remain an urgent problem of humanity. According to the estimates of the World Health Organization, annually more than two billion people in the world suffer from infectious and parasitic diseases, of which seventeen million die.[1] Lyme borreliosis (LB) is recognized as the most common blood infection in Europe and the USA.[2,3] The incidence of LB

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in Europe ranges from 0.001 (Italy) to 632 (Sweden, Blekinge County) per 100,000 population per year.[4] About 300,000 cases are registered annually in the USA alone.[5] Individual cases of LB were registered in all regions of Ukraine since 1994, while the official track of statistics for the incidence of this disease began in 2000.[6] In 2015, the incidence of LB in Ukraine was 7.96 per 100,000 population, and it increased to 10.62 in 2019.[7] The western part of the country, including Ternopil region, is an endemic center of LB. Moreover, Ternopil region is located in an area with fertile soils, a moderate continental climate, and forest landscapes (the total area of the forests of the region is 199.3 thousand ha with broad-leaved and mixed-broad-leaved tree species), which contributes to the preservation in nature of the main reservoir of *Borrelia* spp. - ixodes ticks.[6,8] The territories of ixodid tick-borne borreliosis were found in 57 settlements of 14 districts of the region and in the city of Ternopil itself.[9,10]

Typical skin lesions of the chronic stage of LB, also known as stage III, are chronic atrophic acrodermatitis (CAA) and possibly other scleroatrophic skin conditions, such as lichen sclerosus et atrophicus, atrophoderma Pasini-Pierini, eosinophilic fasciitis, progressive facial hemiatrophy of Parry-Romberg, as well as linear, generalized, or localized scleroderma.[11] Localized scleroderma (LS, L94.0, according to ICD-10) is a chronic disease of connective tissue with predominant damage to the skin and subordinate tissues, characterized by the appearance of foci of sclerosis against the background of inflammatory phenomena (erythema, edema) and the subsequent development of atrophy and hypo- or hyperpigmentation of the skin.[12] Trigger factors for its development can be trauma, stress, vaccination, radiation, neuroendocrine disorders, contact with chemicals, use of certain medications, insect bites, transferred viral or bacterial infections, in particular tick-borne infections caused by *Borrelia burgdorferi (s.l.).*[12-14] Contradictory findings have been reported regarding association of Lyme borreliosis with localized scleroderma, where some research groups supported such association,[15,16] while others denied.[17-19]

Though Lyme disease in North America is related to *Borrelia burgdorferi sensu stricto* and recently to *Borellia mayonii*, as causative agents,[20,21] European and Asian countries have Lyme disease cases associated with numerous species, namely, *Borrelia burgdorferi s.s.* itself, *Borrelia afzelii*, *Borrelia garinii*, *B. spielmanii* and four other species, generally referred to as *Borrelia burgdorferi sensu lato* complex.[22]

The purpose of the current research was to estimate the percentage of serologically positive LB patients among patients with complaints related to LB, using two-stage serological confirmation procedure, and to investigate etiological differences between serologically confirmed LB patients with and without localized scleroderma in Ternopil region, Ukraine.

Methods

A total of 196 patients aged 18 to 70 years who were receiving outpatient and inpatient treatment at the Ternopil Regional Clinical Skin and Venereological Dispensary and the Ternopil University Hospital during 2017-2022 were included in this study.

The patients had complaints likely related to LB, indicated a history of tick bites, or were in LB endemic areas. A questionnaire was conducted for these 196 patients (data not shown), who were subsequently screened for the presence of specific antibodies to LB pathogens.

Serological verification of the LB diagnosis was carried out based on the presence of antibodies to specific antigens of the *B. burgdorferi s.l.* complex. A two-stage approach was used, with initial screening studies of blood sera by the ELISA method for class M antibodies with anti-Borrelia burgdorferi ELISA (IgM) test system and for class G antibodies with anti-Borrelia plus VIsE ELISA (IgG) test system (Euroimmun AG company, Germany). According to the manufacturer's recommendations, a value of ≥22 Units/ml was considered a positive, 16 to 22 Units/ml– an intermediate, ≤16 Units/ml– a negative result. Specificity and sensitivity for Euroimmun anti-Borrelia ELISA (IgM) test kit are equal to 96.4 and 100%, respectively, while for Euroimmun anti-Borrelia plus VIsE ELISA (IgG) test kit – 90.2 and 100%, respectively.

Blood samples with intermediate and positive results from the first stage of the labwork were examined by the method of immune blotting, referred to as stage two verification. Specifically, class M antibodies were analyzed using anti-Borrelia EUROLINE Borrelia RN-AT (IgM), which contains natural purified OspC antigens of three Borrelia species (*B. afzelii*, *B. burgdorferi* s. s. and *B. garinii*), p39, VlsE and flagellar antigen (p41). The specificity of the RN-AT system line is 97 to 99% with a sensitivity of up to 88% (manufacturer's manual). As for class G antibodies, they were determined using anti-Borrelia EUROLINE RN-AT (IgG) test systems, which contain classical natural purified antigens (p83, p39), recombinant VlsE (variable like sequence expressed), natural purified Osp (outer surface proteins) C - antigens of three Borrelia species (*B. burgdorferi* s.s., *B. garinii* and *B. afzelii*), immunoreactive lipids from the Borrelia cytoplasmic membrane (Lipid Ba, Lipid Bb) and the most specific recombinant antigens p18, p19, p20, p21, p58. Compared to the traditional Western blotting system, EUROLINE Borrelia RN-AT has a much higher sensitivity (up to 89% depending on the type of antigen) and higher specificity (at least 95%).

The patients, which were tested IgG positive using the two-stage verification (ELISA and immunoblot), were also split into two groups, with or without localized scleroderma (LS) diagnosis. LS (L94.0) was established on the basis of typical clinical manifestations according to the ICD-10 classification. The obtained data by immunoblotting techniques (EUROLINE Borrelia RN-AT immunoblot systems) for a wide spectra of separate IgG (anti-VlsE *B. afzelii*, anti-VlsE *B. burgdorferi s.s.*, anti-VlsE *B. garinii* IgG, anti-LBa, anti-LBb, ant-p83, anti-p41, anti-p39, anti-OspC, anti-p58, anti-p21, anti-p20, anti-p19, anti-p18) from these patients from the two newly-defined groups were retrospectively statistically analyzed to check for significant differences.

Statistical analysis

The results were expressed in percentages and 95% confidence intervals (95% CI). Fisher exact two-tailed test was used to analyze contingency tables. The level of statistical significance was assumed at the level $p<0.05$. The statistical analysis was performed using commercially available software Statistica 10.0 (StatSoft, Tulsa, Okla., USA).

Results and Discussion

Currently, LB is becoming a significant problem in Ukraine not only among professional workers, such as foresters and hunters,[10,23] but also among children and adolescents, which contract the disease through recreational visits to the woody areas where borreliosis is endemic.[24] Moreover, awareness about Lyme disease among youngsters in Western Ukraine remains fairly low.[25]

The problem itself lies within observations that not all patients being serologically positive for LB develop clinical manifestations, but clinical manifestations must be confirmed by serological tests in order to avoid unnecessary treatments with antibiotics. Therefore, the target populations of our serological studies were patients with characteristic signs and symptoms of Lyme disease.

Analysis of the stage one serological examination data for the presence of specific antibodies to the *B. burgdorferi s.l.* complex by the ELISA method revealed positive results in 82 (41.8% [95% CI 33.3−51.9]) out of 196 examined patients. Specifically, 34 patients (17.4% [95% CI 12.0−24.2]) had positive IgM results, 1 (0.5% [95% CI 0.1−2.8]) had intermediate results, and 161 (82.1% [95% CI 69.9−95.9]) had negative results. As for the IgG data, there were 50 patients (25.5% [95% CI 18.9−33.6]) with positive results, 11 (5.6% [95% CI 2.8−10.0]) – with intermediate results, and 135 (68.9% [95% CI 57.8−81.5]) patients had negative results (**Table 1**).

By the method of immune blotting (stage two), positive results of the IgM were detected in 30 (88.2% [95% CI 59.5−100.0]) of 34 patients who had positive results according to the stage one, and 1 (100%) who had intermediate result according to the ELISA method.

Therefore, positive results for the presence of specific antibodies of the IgM class to *B. burgdorferi s.l.* complex was confirmed by the immunoblot method in 31 (30+1) patients, which was 88.6% (95% CI 60.2−100.0) of the 35 patients who had positive and intermediate results in the ELISA test and 15.8% (95% CI 10.8−22.5) of the total 196 examined (**Table 1**).

Concurrently, the presence of specific IgG class antibodies in the blood sera of 61 patients with

Table 1 Presence of IgM and IgG antibodies to *B. burgdorferi s. l.* in blood serum of patients (ELISA and EUROLINE Borrelia RN-AT test), n=196, %

IgM						*IgG*					
Stage 1, ELISA			*Stage 2, EUROLINE Borrelia RN-AT*			*Stage 1, ELISA*			*Stage 2, EUROLINE Borrelia RN-AT*		
Result	Total (n=196)		Result	Total (n=35)		Result	Total (n=196)		Result	Total (n=61)	
	n	%(95% CI)		n	%(95% CI)		n	%(95% CI)		n	%(95% CI)
Positive	34	17.4(12.0−24.2)	Positive	30	88.2(59.5−100.0)	Positive	50	25.5(18.9−33.6)	Positive	45	90.0(65.7−100.0)
			Intermediate	-	-				Intermediate	-	-
			Negative	4	11.8(3.2−30.1)				Negative	5	10.0(3.2−23.3)
Intermediate	1	0.5(0.01−2.8)	Positive	1	100.0	Intermediate	11	5.6(2.8−110.0)	Positive	9	81.8(37.4−100.0)
			Intermediate	-	-				Intermediate	-	-
			Negative	-	-				Negative	2	18.2(2.2−65.7)
Negative	16	82.1(69.9−95.9)	*			Negative	135	68.9(57.8−81.5)	*		
1			*						*		

Note. * – test was not performed (ELISA result was negative).

LB, who had positive (50) or intermediate (11) results in the ELISA test, was confirmed by the immunoblot method (stage two). Positive results were obtained in 45 (90.0% [95% CI 65.7−100.0]), while negative - in 5 (10.0% [95% CI 9.6−36.8]) patients with positive ELISA test. In the study of the blood sera of 11 people who, according to the stage one ELISA test, had intermediate results regarding the presence of IgG class antibodies to *B. burgdorferi s.l.*, only 9 (81.8% [95% CI 37.4−100.0]) patients had positive results, 2 (18.2% [95% CI 2.2−65.7]) patients had negative results. None of the patients had an intermediate result of the presence of specific IgG according to the second stage (immunoblot) of the examination (**Table 1**).

Therefore, the presence of specific IgG class antibodies (only positive) to *B. burgdorferi s.l.* was confirmed by the immunoblot method in 54 patients (45+9), which was 88.5% (95% CI 66.5−100.0) of 61 patients who had positive and intermediate results in the stage one ELISA test and 27.6% (95% CI 20.7−35.9) of 196 all examined patients (**Table 1**).

The "golden" standard of LB serological confirmation includes two-stage verification, by both initial screening with ELISA with the next confirmation by more sensitive immunoblotting protocols.[9] Hereby, we supported this statement, as some screening results, 11.4% (95% CI 3.1−29.3) for IgM and 11.5% (95% CI 4.6−23.6) for IgG, appeared false positives after stage two immunoblotting confirmation tests.

In the follow-up experiments, the specific etiological structure of LB was studied only in those 54 patients, who were confirmed by specific *B. burgdorferi s.l.* IgG antibodies presence, using two diagnostic methods, ELISA and immunoblot. IgM positive patients were excluded from the follow-up experiments, as LS happens during chronic last stage of LB, where patient's sera no longer contain anti-borrelia IgM, but anti-borrelia IgG only.

It was established that 29 patients out of 54 examined, who were diagnosed with specific IgG antibodies to *B. burgdorferi s.l.* based on ELISA (stage one) and immunoblot (stage two), were also diagnosed with localized scleroderma. They were assigned to group 1 - patients with Lyme borreliosis (LB) and localized scleroderma (LS). Group 2 comprised the rest of the patients, specifically 25 patients with confirmed LB, but without LS manifestation. Females predominated in group 1 (79.3% [95% CI 50.3−100.0], $p<0.05$), while there were approximately equal numbers of females and males, 44% (95% CI 21.9−78.7) and 56% (95% CI 30.6−93.9), respectively, in group 2.

The evidence linking *Borrelia sp*. infection with localized scleroderma includes presence of immunoglobulins against borrelia in some patients with LS, identification of borrelial organisms in skin biopsies and histological sections in morphea, clinical and histologic similarities between LS and CAA as well their coexistence in one patient, and response to antibiotics in many cases of LS.[11] To another extreme, Meis *et al*. stated that patients with reported Borrelia-associated LS actually had atypical form of CAA misdiagnosed as LS.[18] However, a general belief now is that at least some cases of localized scleroderma are associated with LB.[26]

Initially, specific serum IgG to the VlsE antigen of different Borrelia species, namely *B. afzelii*, *B. burgdorferi s.s.*, and *B. garinii*, was analyzed in patients of both groups (**Table 2**).

It was established that in the blood serum of the group 1 patients specific IgG to VlsE *B. afzelii* significantly prevailed compared to IgG against

Table 2 Detection frequency of specific IgG (positive results) to VlsE *B. afzelii*, *B. burgdorferi s. s.* and *B. garinii* in blood sera of patients from group 1 (LB +LS) and from group 2 (LB alone), EUROLINE Borrelia RN-AT (n = 54), % (95% CI)

Criterion	*Group 1 patients, LB+LS (n=29)*			*Group 2 patients, LB alone (n=25)*			*Fisher exact p, two-tailed*
	n	*%*	*95% CI*	*n*	*%*	*95% CI*	
VlsE *B. afzelii*	23	79.3#	50.3−100.0	8	32.0	13.8−63.1	<0.001*
VlsE *B. burgdorferi s. s.*	9	31.0	14.2−58.9	19	76.0#	45.8−100.0	0.003*
VlsE *B. garinii*	11	37.9	18.9−67.9	7	28.0	11.3−57.7	0.565

Note 1.* – the difference is significant regarding VlsE of a single *Borrelia* sp. between patients from group 1 and group 2, $p < 0.05$.
Note 2. # – the difference is significant regarding VlsE of a different *Borrelia* spp. between patients of the same group, $p < 0.05$.

VlsE *B. burgdorferi s.s.* and *B. garinii*: 23 (79.3 % [95% CI 50.3−100.0]) versus 9 (31.0 % [95% CI 14.2−58.9]) and 11 (37.9 % 18.9−67.9), respectively, $p < 0.05$. Whereas, IgG to VlsE *B. burgdorferi s.s.* predominated in group 2 patients, compared with antibodies of this class to VlsE of *B. afzelii* and *B. garinii*: 19 (76.0% [95% CI 45.8−100.0]) versus 8 (32.0% [95% CI 13.8−63.1]) and 7 (28.0% [95% CI 11.3−57.7]), respectively, $p < 0.05$ (**Table 2**).

The analysis of the content of IgG to VlsE (*B. burgdorferi s.s.*, *B. garinii* and *B. afzelii*) between patients of the two groups revealed that in the blood serum of group 1 patients compared to group 2 patients, IgG to VlsE of *B. afzelii* were detected significantly more often: 23 (79.3% [95% CI 50.3−100.00]) versus 8 (32.0% [95% CI 13.8−63.1]), $p < 0.05$. Whereas in the examined group 2 patients, comparing to group 1 patients, IgG to VlsE *B. burgdorferi s.s.* occurred significantly more often, 19 patients (76.0% [95% CI 45.8−100.0]) versus 9 patients (31.0% [95% CI 14.2−58.9]), respectively, $p < 0.05$. However, serum immunoglobulins of class G to VlsE *B. garinii* were detected equally often in patients of both groups (**Table 2**).

Therefore, borreliosis related to *B. afzelii* was more frequently present in the patients with LB accompanied by LS, while *B. burgdorferi s.s.* was more likely to be present in the patients with LB without LS, $p < 0.05$.

Aberer and Wutte noted a strong relationship between specific geographic regions with Lyme disease-associated localized scleroderma cases and with the predominant genotype of *Borrelia afzelii*.[26] For example, such countries of middle Europe as Austria, Italy, Poland, Slovakia, Hungary, Switzerland, as well as Puerto-Rico and Turkey, reported *B. burgdorgeri s.l.*–associated scleroatrophic changes, while Netherlands, Denmark, England, Finland, France, Spain and the USA - did not.[27] It was also hypothesized that such geographical distribution of subset of Borrelia-associated LS is caused by a special subspecies of *B. burgdorferi* that is present in Asia and certain European countries, but does not occur in the USA.[28] Interestingly, Aberer and Wutte further proposed, that scleroatrophic skin lesions may be caused by LB in specific countries where *B. afzelii* is endemic (2016), which is also supported by our findings.

Similarly, a group of researchers showed that among thirty-two patients with localized scleroderma, 18.8% of them were tested positive for either IgG or IgM against *Borrelia* spp., which was significantly higher then in general population of western Ukraine.[29] Our results showed higher values because we studied patients with accompanying LB signs and

Table 3 Detection frequency of specific IgG to various antigens in the blood sera of group 1 and group 2 patients, EUROLINE Borrelia RN-AT (n = 54), % (95% CI)

Antigens IgG	*Group 1 patients, LB+LS (n=29)*			*Group 2 patients, LB alone (n=25)*			*Fisher exact p, two-tailed*
	Positive cases	*%*	*95% CI*	*Positive cases*	*%*	*95% CI*	
LBa	3	10.3	2.1−30.2	3	12.0	2.5−35.1	1.000
LBb	3	10.3	2.1−30.2	1	4.0	0.1−22.3	0.615
p83	16	55.2	31.5−89.6	6	24.0	8.8−52.2	0.028*
p41	29	100.0	−	22	88.0	55.2−100.0	0.093
p39	10	34.5	16.5−63.4	18	72.0	42.7−100.0	0.007*
OspC	7	24.1	9.7−49.7	13	52.0	27.8−88.9	0.049*
p58	8	27.6	11.9−54.4	5	20.0	6.5−46.7	0.544
p21	9	31.0	14.2−58.9	9	36.0	16.5−68.3	0.776
p20	-	-	-	-	-	-	
p19	4	13.8	3.8−35.3	3	12.0	2.5−35.1	1.000
p18	5	17.2	5.6−40.2	3	12.0	2.5−35.1	0.711

Note. * – the difference is significant regarding specific IgG to different Borrelial antigens between patients from group 1 and group 2, p <0.05.

symptoms, while Zinchuk *et al*. – all available patients with LS irrespective of other complaints, including LB-related (2016).

Also, the presence of specific anti-IgG to other borrelial antigens of the B. *burgdorferi s.l.* complex was analyzed in group 1 and group 2 patients (**Table 3**).

Interestingly, it was also found that specific IgG antibodies to flagellar antigen p41 were found in the blood serum of only 88.0% of patients with LB without LS (group 2), while 100.0% of examined patients from group 1 had it.

IgG class antibodies to the p83 antigen, which is a marker of the late immune response, typical for neuroborreliosis,[30] were also determined in the blood sera of the examined patients from both groups. It was established that antibodies to this antigen were detected more often in the group 1 patients compared to group 2 patients, 16 patients (55.2%) versus 6 patients (24.0%), respectively, p <0.05 (**Table 2**).

In addition, it was found that specific anti-p39 IgG, which are considered possible markers of Lyme arthritis,[30] were significantly more often detected in the group 2 patients than group 1 patients - 18 (72.0% [95% CI 42.7−100.0]) against 10 (34.5% [95% CI 16.5−63.4]), respectively, p <0.05 (**Table 3**).

Regarding anti-p58, anti-p21, anti-p19 and anti-p18 IgG, they were found in the blood serum of patients of both groups with the same frequency, p>0.05. It is worth noting that anti-p20 IgG was not found in the blood serum of any patient from both examined groups.

Conclusion

1. Specific anti-IgG to *B. burgdorferi s.l.* complex, using a two-stage scheme, was diagnosed in 54 (27.6% [95% CI 20.7−35.6]) patients out of 196 examined with clinical manifestations of tick-borne infections, while anti-IgM - in 31 (15.8% [95% CI 10.8−22.5]) examined patients, respectively.
2. For the first time in Ukraine, the etiological structure of Lyme borreliosis was studied based on specific IgG to antigens of the *B. burgdorferi s.l.* complex in the blood serum of patients with Lyme borreliosis accompanied by localized scleroderma.

3. It was established that IgG to VlsE antigens of *B. afzelii* and p83 (a marker of the late immune response, typical for neuroborreliosis) were significantly more often detected in the blood serum of Lyme borreliosis patients with localized scleroderma compared to Lyme borreliosis patients without localized scleroderma manifestation, $p < 0.05$.
4. In patients with Lyme borreliosis without localized scleroderma, serum IgG to VlsE antigens of *B. burgdorferi s.s.* and to p39 (Lyme arthritis marker) were diagnosed more often compared to patients with Lyme borreliosis accompanied by localized scleroderma, $p < 0.05$.
5. To summarize, Lyme borreliosis with localized scleroderma patients were more often diagnosed with *B. afzelii*, while in the patients with Lyme borreliosis without localized scleroderma - *B. burgdorferi s.s.* was the dominating causative agent, $p < 0.05$.

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