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EVALUATION OF IMMUNOBLOT RESULTS FOR DETERMINATION OF ANTIBODIES TO LYME DISEASE PATHOGENS IN CHILDREN OF TERNOPIL REGION

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Background. *Lyme disease (LD) is a multisystem disorder caused by Borrelia burgdorferi and other similar tick-borne Borrelia.*

Objective. *The aim of the research was to compare the results of the serological examination of children with different forms of Lyme disease.*

Methods. *We observed the group of children (n=178) aged 1 to 14 years who were bitten by ticks. The control group consisted of 30 healthy children. Ticks were identified using a stereomicroscopic SEO system which included a stereomicroscope, a colour digital camera and a photoadapter. B. burgdorferi sensu lato (sl) (B. burgdorferi sensu stricto, B. afzelii and B. garinii), B. miyamotoi, A. phagocytophilum DNA in blood were determined by real-time PCR. Baseline investigations related to clinical and immunological studies, including ELISA and Immunoblot, were performed.*

Results. *The survey covered 178 child parents bitten by ticks. Borrelia burgdorferi sensu lato (B. afzelii, B. burgdorferi sensu stricto and B. garinii), B. miyamotoi and A. phagocytophilum were identified. Serological results in children with different forms of Lyme disease were compared.*

Conclusions. *It is established that B. burgdorferi sensu lato; B. miyamotoi; and A. phagocytophilum are pathogens that cause erythema migrans in children. The presence of specific IgG (only positive results) to B. burgdorferi s.l. by immunoblot was confirmed in 83.8% of individuals who had positive and intermediate results in the ELISA test.*

KEYWORDS: Lyme disease; borreliosis; ELISA; immunoblot; tick bite.

Introduction

Lyme disease (LD) is a multisystem disorder caused by Borrelia burgdorferi and other similar tick-borne Borrelia.

This acute systemic disease often occurs in children and is characterized by the presence of erythema migrans (EM), and in some untreated patients of inflammatory arthritis, erythema migrans as well.

Lyme disease, caused by *Borrelia burgdorferi*, is the most common vector-borne disease. In Western Europe it is caused by *B. afzelii ma B. garinii*, [2] whereas in the United States – by *B.burgdorferi* [3]. The epidemics of Lyme disease is challenging in Poland and Ukraine. In western Ukraine, *B. burgdorferi s.l* were revealed in 14.2-17.2% of adult *Ixodes scapularis* ticks [4]. In 2020-2021, the disease incidence in Ukraine and Ternopil region was 10.62 and 20.05 per 100 thousand population, respectively (Public Health Center.org.ua).

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On the basis of MKH-10 allocate "Lyme disease" A69.2. Clinical manifestations of Lyme disease can be divided into three stages: the early localized stage characterized by erythema migrans at the site of the tick bite, multiple Erythema migrans**,** Borrelia lymphocytoma; early disseminated form with early symptoms confined to peripheral nervous system, carditis or arthritis, late disseminated form – late symptoms confined to peripheral nervous and central nervous system, manifestations of late arthritis, cardiac complications. The pathogenesis, ecology, and epidemiology of Lyme disease are well described; the developed and suggested antimicrobial treatment is very effective [2, 5].

The study aimed to assess the incidence of clinical suspicion of LD among children in Ternopil region (Western Ukraine) by serological examination of children bitten by ticks.

Methods

The children's blood tests were studied in the laboratory of the Centre for the Study of

Lyme Disease and Other Tick-Borne Infections of I. Horbachevsky Ternopil National Medical University. Ticks were identified using a stereomicroscopic SEO-IMAGLAB system.

Special defining tables were used for identification of ticks [6]. Databases regarding the incidence of LD in children of Ternopil region in 2017-2021 were used to evaluate retrospective results.

This study consists of two parts : the first part describes the data from the questionnaire and clinical examination of the patients, episodes of tick bites, and the second part is serological examination of the blood by ELISA and immunoblot.

The study involved 178 children aged 1 to 18 years, who visited to the centre for Study of Lyme Disease of Ternopil National Medical University after being bitten by ticks.

The control group consisted of 30 healthy boys and girls living in Ternopil and Ternopil region. They were not bitten by ticks and did not suffer from LD previously. The age and sex distribution in the control group corresponded to that in the control group.

Ticks were identified for transmissible infections. DNA of *B. burgdorferi sensu lato (sl) (B. burgdorferi sensu stricto, B. afzelii* and *B. garinii*), *B. miyamotoi, A. phagocytophilum* were determined by real-time PCR using Vector-Best production test system (Germany).

The serological examination of the children with LD was made by two-stage diagnosis procedures, primarily using ELISA and immunoblot for confirmation of the secondary results. Antibodies to antigens of the *B. burgdorferi s.l.* complex in blood serum were determined by ELISA using test systems by Euroimmun AG (Germany): class of IgM-test system Anti-Borrelia burgdorferi ELISA (IgM), IgG – Anti-Borrelia plus VLSE ELISA (IgG).

The results were evaluated quantitatively. The indicator > 22U/ml was considered positive, 16-22 U/ml – intermediate, < 16 U / ml – negative. To detect only IgM against Borrelia antigens, a specific line of the RN-AT system was used, which contained natural purified OspC Borrelia antigens of three species *(B. afzelii, B. burgdorferi ss, B.garinii*) and antigens p 39, p 41 and VIsE.

To diagnose specific IgG a line of the RN-AT system was used, which contained VLSE antigens of Borrelia of three species (*B. afzelii, B. burgdorferi s. s*, and *B. garinii)* and other specific antigens: p18, 19, p20, p21, p58, OspC (p25), p39, p83, Lipid Ba, Lipid Bb.

Statistical processing of the results was performed using the methods of parametric and nonparametric statistics by computer programs Microsoft Office Excel and STATISTICA, estimating the absolute (n) and relative amount (%) of the indicators.

The analysis of frequency was performed using Pearson's test χ^2 and two-sided Fisher's exact test, the statistical significance of which was $p<0.05$. All the studies were performed according to the Conclusion of the Commission on Bioethics of I. Horbachevsky Ternopil National Medical University, dated September 1, 2021 (Minutes No. 65).

Results

Erythema migrans was observed in 113 (63.4%), and arthritis in 15 (8.4%) individuals. Nervous system disorders were present in 30 (16.8%) children; 18 (9.5%) children had an erythema-free form of the disease; 2 children (1.1%) complained about the cardiovascular disorders.

All children bitten by ticks were divided into the following age groups (Table 1).

Table 1. The age groups of children affected by ticks

Age categories of children (years)				
Categories	1–3	4–7	8–12	13–18
Number of children	18	39	75	46

The largest age group was children of 8-12 years old (42.1% of the surveyed). Among the surveyed, there were 83 (46.6%) boys and 95 (53.4%) girls.

Only 143 (80.3%) children had a tick bite, while others did not remember the bite. The examination of patients with an erythematous form of LD took into account the presence of a tick bite in the anamnesis and the accompanying intoxication-inflammatory syndrome, the presence of lesions of various organs and systems.

In the clinical diagnosis of an erythematous form of LD the prevalent symptoms were: primary skin lesion, which was manifested by the erythema migrans, and the epidemiological history. Subsequent examination revealed the presence of various pathogens in this category of patients. In children with erythema migrans, i.e. in 24.7% of 113 people, tick-borne infection with identified pathogens was confirmed (Fig. 1), and in children with the disseminated form of the disease only the effects of the bite were observed.

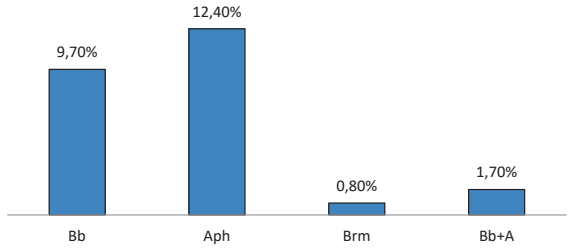

Fig. 1. *Frequency of detection of infectious agents in children with erythema migrans.*
Bbs – B. burgdorferi sensu lato; Brm – B. miyamotoi; Aph – Anaplasma phagocytophilum.

When using the western immunoblot during the first 4 weeks of the disease (an early form of LD), both immunoglobulin M (IgM) and immunoglobulin G (IgG) were determined. Since the probability of a false-positive test result for current infection is high, a positive IgM test result is not recommended when determining the active phase of the disease in people who are sick for longer than 1 month.

Verification of the presence of specific IgM antibodies was performed in the sera of 179 patients, 71 of whom had positive (56 people) or intermediate (15) results when tested by the ELISA test. (Table 2). It was found that in 56 patients with positive results, using the method of immunoblot (EUROLINE Borrelia RN-AT) also found positive results in 17 (30.4%) persons, while intermediate was not detected.

In children living in Ternopil region, a positive ELISA test was confirmed in 56 children (31.3%), an intermediate test in 15 (8.4%), and a negative test in 108 (60.3%) individuals. The results of blood screening for the presence of IgG in ELISA were positive in 28 subjects (15.6%), intermediate – in 3 (1.7%), negative – in 148 (82, 7%) (Table 2).

Subsequently, these results were confirmed by immunoblotting.

Thus, from the examined group of patients (179 people) the immunoblot confirmed the total (IgM + IgG) absolute number of positive blood results in (20 + 26) 46 children (25.7%).

To determine the etiological structure of LD, the presence of IgM antibodies to the immunogenic external surface protein OspC (a marker of the early immune response) of three species was determined: *B. garinii, B. burgdorferi, B. afzelii* separately in patients of both groups (Table 3).

Antibodies of this class to OspC *B. afzelii* were found in the sera of 11 (55%) of the 20 subjects, to OspC *B. garinii,* respectively, in 11 (55%), to OspC *B. burgdorferi s.s.* – in 6 (30%) patients.

IgM antibodies to antigens p41, p39, and VLsE were also determined in the sera of the examined patients. It was found that antibodies to antigens p41 were detected in 17 (85%) patients, to p39 antigen – in 4 (20%), respectively, to VLsE – not detected in any of the examined groups of children (Table 3).

Simultaneously, the presence of IgG antibodies (only positive results) to VLsE (recombinant highly immunogenic lipoprotein of the outer membrane, variable like sequence expressed) of Borrelia of different genes in the sera of 31 patients with ME LD was determined with a positive result in all children. To determine the etiological structure of LD, the presence of IgG antibodies to the immunogenic external

Table 2. The content of IgM and IgG to B. burgdorfery s.l. determined by different methods in children living in Ternopil region

IgM						IgG					
Elisa			EUROLINE Borrelia RN-AT			Elisa			EUROLINE Borrelia RN-AT		
Result	Total (n=179)		Result	Total (n=71)		Result	Total (n=179)		Result	Total (n=31)	
	Absolute value	%		Absolute value	%		Absolute value	%		Absolute value	%
Positive	56	31.3	Positive	17	30.4	Positive	28	15.6	Positive	25	89.3
			Intermediate	0	0				Intermediate	0	0
			Negative	39	69.6				Negative	3	10.7
Intermediate	15	8.4	Positive	3	20.0	Intermediate	3	1.7	Positive	1	33.3
			Intermediate	0	0				Intermediate	0	0
			Negative	12	80.0				Negative	2	66.7
Negative	108	60.3				Negative	148	82.7			

Table 3. Antigenic load to anti-*B. burgdorferi* IgM

Antigens	Absolute value (Ig M-20)	Relative value (%)
OspC Bg (*B. garinii)*	11	55
OspC Bb *(B. burgdorferi)*	6	30
OspC Ba (*B. afzelii)*	11	55
P39	4	20
P41	17	85
VLsE	0	0

Table 4. Antigenic load to *B. Burgdorferi* IgG

Antigens	Absolutely value (IgG-31)	Relative value (%)
OspC *B. afzelii*	0	0
VlsE *B. burgdorferi ss*	16	51,6
VlsE *B. afzelii*,	13	41,9
VlsE *B. garinii*	12	38,7
p39	0	0
P41	21	67,7
Lipid Ba	4	12,9
Lipid Bb	2	6,4
P21	4	12,9
P18	3	9,6
P58	3	9,6

surface protein VLsE (a marker of the early immune response) of three species was determined: *B. garinii, B. burgdorferi, B. afzelii* separately in the patients of both groups (Table 4).

OsC B. *garinii* antigens of IgM immunoglobulin predominated over OspC *B. afzelii*, OspC *B. burgdorteri* in Ternopil region.

Vlse *B.burgdorteri* antigens of immunoglobulin IgG prevailed over Vlse *B.afzelii*, Vlse *B. garinii* in Ternopil region.

Discussion

Lyme disease (LD) is an endemic disease in many countries. In Europe, North America, and Asia, it is the most common vector disease [8,9]. It is caused by *B. burgdorferi sensu lato* and is transmitted to humans by ticks of the Ixodes ricinus mite complex; up to 20% of them are infected with this bacterium. Only 2-4% of bites are clinically manifested that is one of the diagnostic challenges [10, 11].

In the presence of erythema migrans, there was a significant difference in the ELISA results for immunoglobulin M, in particular a significant predominance of negative IgM values. At the same time, the erythematous form of ME was characterized by positive results of IgM. In a small amount of IgM to flagellin (41 kB) and membrane protein OspC Borrelia begin to appear in the first days of the disease. Their titres increase within 4-6 weeks, and a little longer in untreated patients. During the generalization of the infectious process, IgG antibodies appear against several proteins, e.g. P39 and P58 [7]. The frequency of various pathogens that caused erythema migrans was established: the leading pathogen was *Anaplasma phagocytophilym* in (12.4%) cases, *B. burgdorferi sensu lato* in 9.7% of cases.

It is proved that if erythema migrans develops in a patient after a tick bite in an endemic area [12], treatment tactics should be suggested immediately. However, if the diagnosis of Lyme disease is uncertain, it is recommended to first determine the sensitivity of the ELISA reaction. Only ELISA-positive cases should be confirmed with a more specific immunoblot results [7].

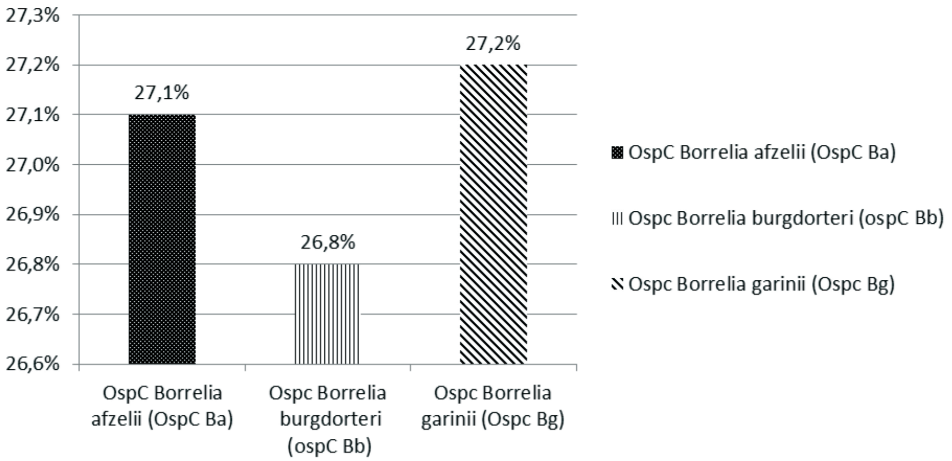

Fig. 2. *Average rate of antigenic load to anti-B. burgdorferi IgM antibodies in Ternopil region.*

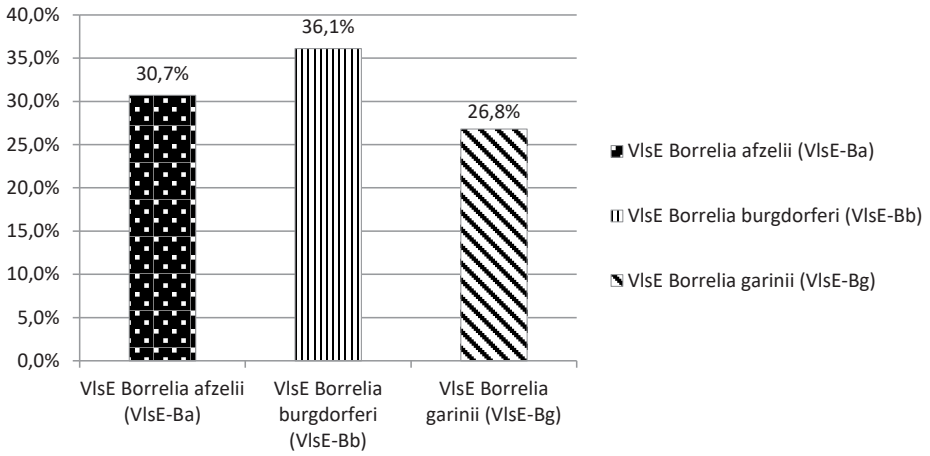

Fig. 3. *Average rate of antigenic load to anti-B. burgdorferi IgG antibodies in Ternopil.*

It is established that the differentiation between septic arthritis and Lyme arthritis in endemic areas can be a difficult task, and therefore it causes serious consequences for the treatment of the patient [14]. In the acute and late stages, Lyme disease can be difficult to distinguish from other painful processes . To establish a prediction algorithm for the differentiation of septic arthritis from Lyme disease in children with knee pain and exhaustion [13], a two-stage diagnosis is recommended. The main surface antigens of OspA, OspB, OspC proteins, which determine the difference of individual strains [14], can vary significantly; thus determining the possibility of long-term (for many years) persistence of the pathogen in the human body [14, 15].

Many antigenic determinants of the outer shell of Borrelia of different species are similar to each other and even to some bacteria of other genera, which explains the possibility of cross-immune reactions [7, 16].

Serum samples from children with disseminated or late stage LD almost always have a strong IgG response to *Borrelia burgdorferi* antigens [7, 8, 15].

In the evaluation and interpretation of serological test results, both the class of antibodies to specific *B. burgdorferi* antigenic proteins and the type of bacterial antigen, for which these antibodies are produced, are important [14]. External surface proteins (Osp) are important in the immune response to infection because they are highly immunogenic. The aantibodies to OspC are characteristic of recent infection. According to the manufacturer's recommendations, the presence of specific IgM antibodies was considered positive, intermediate, or negative, depending on the combinations of OspC antigens of three species of Borrelia (*B. afzelii, B. burgdorferi ss,* and *B. garinii*), p39, and VLsE Bb. At the same time, the presence of IgG was considered positive or negative depending on the combinations of VLsE antigens of three species of Borrelia (*B. afzelii, B. burgdorferi ss,* and *B. garinii*) and other specific antigens: p18, p19, p20, p21, p58, OspC (p25), p39, p83, Lipid Ba, Lipid Bb. In our immunological study, the genotype *B. burgdorferi sensu stricto* was detected in children with erythema migrans, arthritis, and neurolaim, which have statistically significant results (Table 4).

The diagnosis of Lyme disease should be established by a laboratory (serological tests (ELISA and Western blot) investigations, indicating the presence of specific anti-*B. Burgdorferi* IgM / IgG antibodies), which confirms clinical manifestations of the disease. This is very important because physicians often seek serological evidence of *B. burgdorfery* infection in patients with undefined diffuse complaints [15,18].

According to the list of symptoms compatible with Lyme disease, the most common symptoms in children of Ternopil region were erythema migrans (84.9%). This corresponds to the results of other studies [19, 20].

Conclusions

It was estanlished that *B. burgdorfer*i sensu lato; *B. miyamotoi*; and *A. Phagocytophilum* are pathogens that cause erythema migrans in children of Ternopil region. The presence of specific IgG (only positive results) to *B. Burgdorferi* s.l. was confirmed by immunoblotting in 83.8% of individuals who had positive and intermediate results in the ELISA test.

Conflict of Interests
Authors declare no conflict of interests.
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Author's Contributions
Svitlana Oleksiivna Nykytyuk – formal analysis, writing – original draft, writing – reviewing and editing; *Sergiy Ivanovych Klymnyuk* – conceptualization, writing – original draft, writing – reviewing and editing; *Ivan Mykolayovych Klishch* – methodology, writing – reviewing and editing; *Sofia Sergiivna Levenets* – investigation, formal analysis.

ОЦІНКА РЕЗУЛЬТАТІВ ІМУНОБЛОТУ ДЛЯ ВИЗНАЧЕННЯ АНТИТІЛ ДО ПАТОГЕНІВ ХВОРОБИ ЛАЙМА У ДІТЕЙ ТЕРНОПІЛЬСЬКОЇ ОБЛАСТІ

***С.О. Никитюк, С.І. Климнюк, І.М. Кліщ, С.С. Левенець**
ТЕРНОПІЛЬСЬКИЙ НАЦІОНАЛЬНИЙ МЕДИЧНИЙ УНІВЕРСИТЕТ ІМЕНІ І. Я. ГОРБАЧЕВСЬКОГО МОЗ УКРАЇНИ, ТЕРНОПІЛЬ, УКРАЇНА

Вступ. *Вступ. Лайм бореліоз (LD) є мультисистемним захворюванням, спричиненим Borrelia burgdorferi та іншими подібними кліщовими Borrelia.*

Мета. *Визначити і порівняти серологічних результатів крові при різних формах хвороби Лайма у дітей.*

Методи. *Під нашим спостереженням знаходилась група дітей (n=178) у віці від 1 до 14 років, укушених кліщами. Контрольна група становила 30 здорових дітей. Кліщів ідентифікували за допомогою стереомікроскопічної системи SEO та визначника. Фрагменти ДНК B. burgdorferi sensu lato (sl) (B. burgdorferi sensu stricto, B. afzelii та B. garinii), B. miyamotoi, A. phagocytophilum визначали у крові методом ПЛР в реальному часі. Кліщів ідентифікували за допомогою стереомікроскопічної системи SEO. Були проведені базові дослідження, пов'язані з клінічними та імунологічними дослідженнями, включаючи дані Elisa та Immunoblot.*

Результати. *Опитування охопило 178 батьків дітей, на яких напали кліщі. Виявлено Borrelia burgdorferi sensu lato (B. afzelii, B.burgdorferi sensu stricto та B. garinii), B. miyamotoi та A. phagocytophilum. Проведено порівняння серологічних результатів крові при різних формах хвороби Лайма у дітей.*

Висновки. *Встановлено, що B. burgdorferi sensu lato; B. miyamotoi; та A. Phagocytophilum є збудниками, які викликають у дітей мігруючу еритему.*

Наявність специфічних антитіл IgG (тільки позитивні результати) до B. Burgdorferi s.l. імуноблотинг був підтверджений у 83,8% осіб, які мали позитивні та проміжні результати в тесті ІФА.

КЛЮЧОВІ СЛОВА: хвороба Лайма; бореліоз ; ІФА; імуноблот; укус кліща.

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