IMMUNOBLOT REACTIVITIES OF DIFFERENT LYME DISEASE SPECIFIC ANTIGENS TO ANTI-BORRELIA BURGDORFERI IGG ANTIBODIES

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ABSTRACT

Background

Lyme disease is a vector-borne disease with increasing incidences in last years. It is the most common tick-borne infection in Bulgaria, caused by *Borrelia burgdorferi* sensu lato (*Bb*sl) complex after a bite from *Ixodes ricinus* tick. The aim of this study is to identify the most common antigens targeted by specific IgG antibodies produced in response to*Bb*sl infection in humans.

Material and methods

Serum samples (n=220), pre-tested as positive or borderline by ELISA for specific anti-*Borrelia* IgG antibodies, were tested with a commercially available confirmatory immunoblot kit.

Results

The most common specific anti-*Borrelia* IgG antibodies reacted against: p41 (97.1%), VlsE (average of 62.1% across the three variants: VlsE Bg, VlsE Bb and VlsE Ba), p83 (32.4%), p39 (30.4%) and OspC (22.6%) among the positively interpreted immunoblot tests. The weak positive bands among the positively interpreted blot tests were: OspC (17.7%), VlsE (average of 10.5% across the three variants), p21 (9.8%), p39 (8.8%), p83 (6.9%).

Conclusions

This study analysed the most common antigens targeted by specific IgG antibodies synthesised after *Bb*sl infection in humans from all 28 provinces of Bulgaria. It was probably cross-reactive antibodies that appeared in some cases with weak positive bands. The data obtained from the confirmed cases of Lyme disease, based on the different distribution of immunoblot bands allow for a better understanding the peculiarities of this disease. The use of new specific antigens for confirmatory tests based on selected conserved fragments within *Bb*sl leads to development of more sensitive and specific serological analyses.

Keywords: Lyme disease, immunoblot, antibodies

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INTRODUCTION

Lyme disease (LD) is recognized as the most common tick-borne infection in Europe, North America and Asia with increasing geographic distribution in association with global warming (1). It is a multisystem, chronic disease transmitted by infected *Ixodes* spp. ticks, and its aetiological agents are spirochetes of the *Borrelia burgdorferi* sensu lato complex (*Bb*sl). LD incidence has been ever-increasing in the last years and this can have significant consequences for public health.

The laboratory diagnosis of the disease mainly relies on serological tests for detection of specific IgM and IgG antibodies against *Bb*sl. There is a great diversity of *Bbsl* in Europe and most of the immunogenic proteins are heterogeneous, which is why the use of whole cell lysate with only one genospecies as antigen carries the risk of obtaining a false-negative result (2, 3, 4, 5, 6). On the other hand, *Bbsl* produces many proteins homologous among microorganisms, which may lead to false-positive results. The insufficient specificity of diagnostic tests for LD required the introduction of a standard two-tiered testing in 1995, and updated later, based on: an enzyme immunoassay (EIA), or an indirect immunofluorescent antibody assay (IFA) or chemiluminiscent assay; followed

by a second-tier orthogonal EIA (modified two-tiered testing, MTTT) or immunoblot assay confirmation of positive and equivocal results from first-tier level (2, 7, 8, 9, 10, 11).

The aim of the study is to identify the most common antigens targeted by specific IgG antibodies produced in response to *Bb*sl infection in humans.

MATERIAL AND METHODS

Serum samples (n=220), pre-tested as positive or borderline with ELISA for the presence of anti-*Borrelia*-specific IgG antibodies, were collected in December 2023, from all 28 provinces of Bulgaria. Adults over 18 years of age were included. No additional patient information was available.

The samples were tested using the immunoblot method with a commercially available kit: Anti-Borrelia EUROLINE-RN-AT (IgG), DN 2131-3201 G according to the manufacturer's instructions. Immunoblot strips included the following fourteen specific antigens: VlsE Ba, VlsE Bb, VlsE Bg, p18, p19, p20, p21, p41, p58, OspC (p25), p39, p83, Lipid Bb, and Lipid Ba. After completion of the test procedure, the stained test strips were evaluated with EUROLineScan Software v. 3.4.37 (YG 0006-0101, Euroimmun). The evaluation of the bands was as follows: positive if the intensity units were ≥19; ≥ 12 but < 19 were interpreted as borderline; and negative if the intensity units were <12, according to the set software of Anti-Borrelia EUROLINE-RN-AT (IgG), Euroimmun, Germany. A positive result was indicated by one of the following: one positive VlsE band; one borderline VlsE band and one positive non-VlsE band; two or more positive non-VlsE bands.

Ethical approval for this study was obtained from the institutional review board at the National Center of Infectious and Parasitic Diseases (NCIPD) (approval number 5/17.10.2023).

RESULTS

A total of 220 samples, positive/borderline in ELISA for specific anti-*Bb*sl IgG antibodies, were tested with a confirmatory immunoblot method. 102/220 (46.4%) of the samples were positive and 118/220 (53.6%) were negative for the presence of anti-*Borrelia* IgG according to the immunoblot test results. Analysis of the reactive immunoblot bands showed that the most common anti-*Borrelia* IgG antibodies targeted p41 (flagellin) in 201/220 (91.4%), followed by VlsE Bb in 72/220 (32.7%), VlsE Ba in 66/220 (30.0%) and VlsE Bg in 58/220 (26.0%) among all tested samples (Fig. 1).

A positive p83 band was observed in 35/220 samples (15.9%). The OspC and p39 bands were each

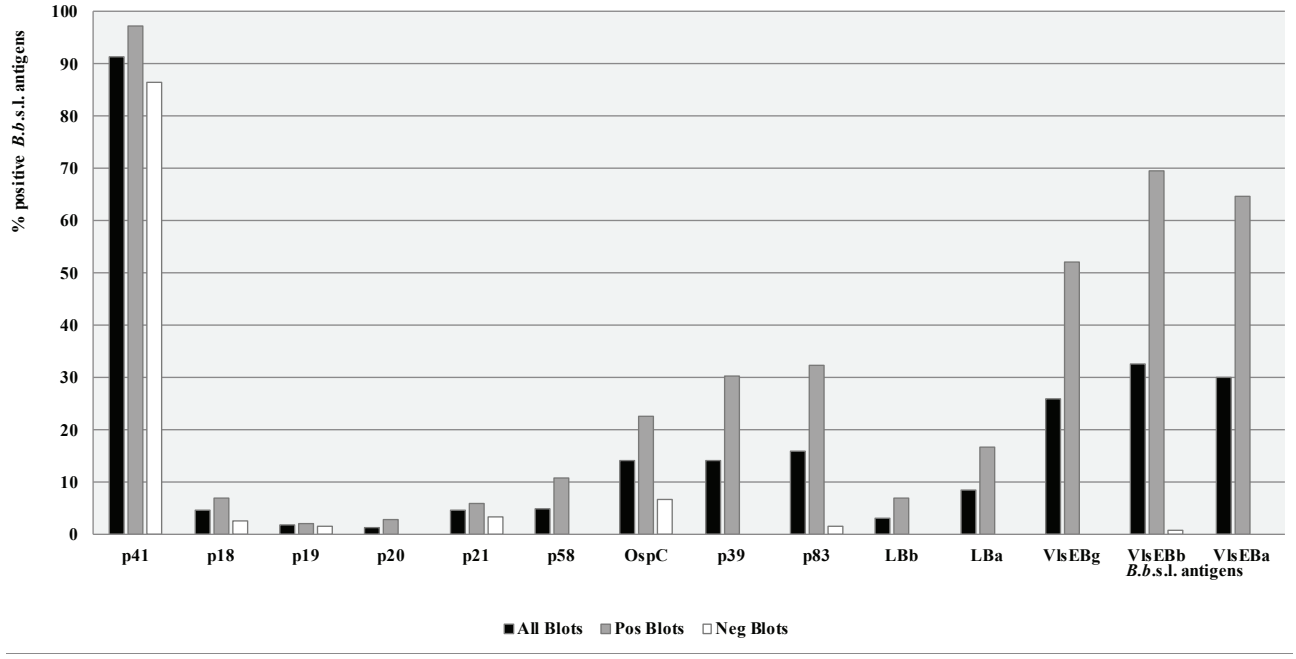

Figure 1. Antigens inducing positive IgG antibody response against of *B. burgdorferi* s.l.: frequence of detection in all blots, in blots interpreted as positive, in blots interpreted as negative

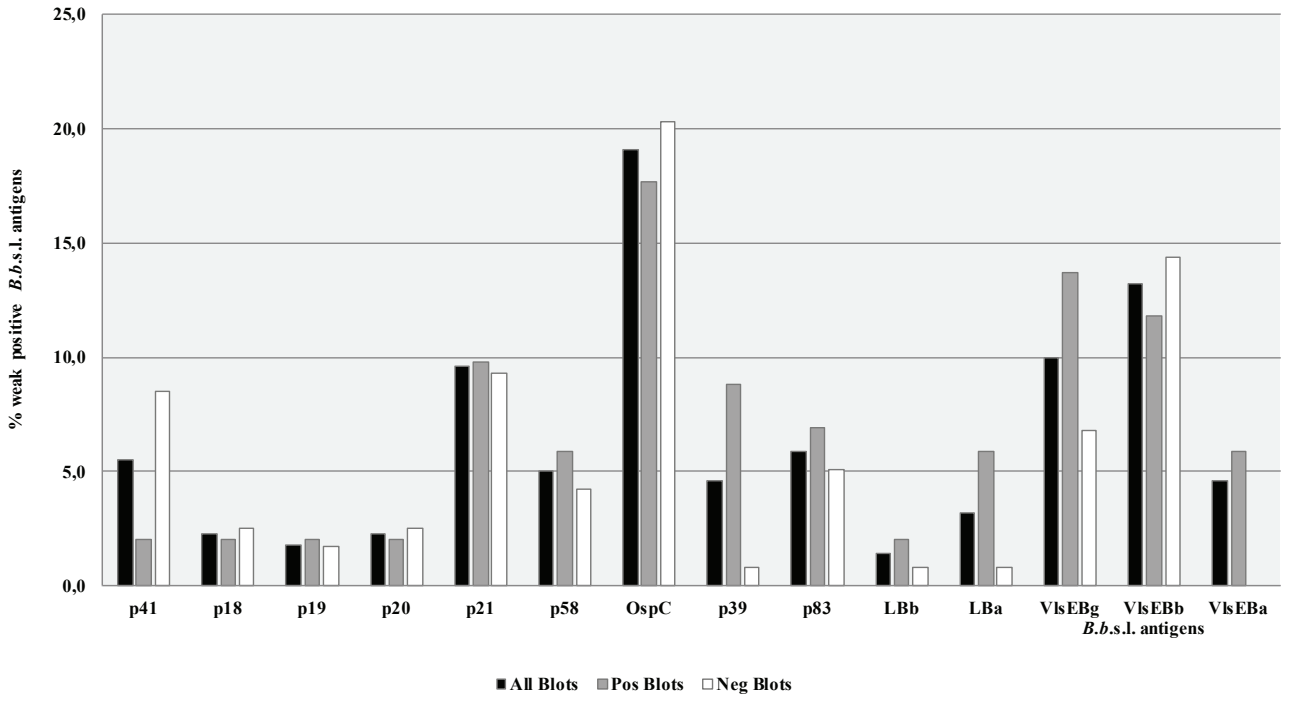

Figure 2. Antigens inducing a weak positive IgG antibody response against of *B. burgdorferi* s.l.: frequence of detection in all blots, in blots interpreted as positive, in blots interpreted as negative

detected in 31/220 samples (14.1%). A positive LBa band was identified in 19/220 samples (8.6%), while 11/220 (5.0%) had a positive p58 band. The p18 and p21 bands were each found in 10/220 samples (4.6%), and the LBb band was observed in 7/220 samples (3.2%). The least frequent were the p19 band in 4/220 samples (1.8%) and the p20 band in 3/220 samples (1.4%).

Among the positively interpreted blot tests, there were no differences with the combination of the reactive bands, compared to the set of reactive bands from all examined blot tests: 99/102 (97.1%) had a positive p41 band, followed by VlsE Bb - 71/102 (69.6%), VlsE Ba - 66/102 (64.7%), VlsE Bg - 53/102 (52.0%), p83 - 33/102 (32.4%), p39 - 31/102 (30.4%) (Fig. 1). OspC was positive in 23/102 (22.6%), LBa - in 17/102 (16.7%), and p58 - in 11/102 (10.8%). LBb and p18 were both detected in 7/102 (6.9%) and the least frequent were: p21 - in 6/102 (5.9%), p20 - in 3/102 (2.9%) and p19 - in 2/102 (2.0%).

Only seven positive bands were observed among the negatively interpreted blot tests: p41 - 102/118 (86.4%), OspC - 8/118 (6.8%), p21 - 4/118 (3.4%), p18 - 3/118 (2.5%), p19 and p83 - 2/118 (1.7%) (Fig. 1).

The most common weak positive bands among all tested samples were: OspC - 42/220 (19.1%), followed by: VlsE Bb - 29/220 (13.2%), VlsE Bg - 22/220 (10.0%), p21 - 21/220 (9.6%), p83 - 13/220 (5.9%), p41 - 12/220 (5.5%), p58 - 11/220 (5.0%), p39 and VlsE Ba - 10/220 (4.6%), LBa - 7/220 (3.2%), p18 and p20 - 5/220 (2.3%), p19 - 4/220 (1.8%) and LBb - 3/220 (1.4%) (Fig. 2).

The weak positive bands among the positively interpreted blot tests were as follows: OspC - 18/102 (17.7%), VlsE Bg - 14/102 (13.7%), VlsE Bb - 12/102 (11.8%), p21 - 10/102 (9.8%), p39 - 9/102 (8.8%), p83 - 7/102 (6.9%), p58, LBa and VlsE Ba - 6/102 (5.9%), p41, p18, p19, p20 and LBb - 2/102 (2.0%) (Fig. 2).

Finally, the weak positive bands among the negatively interpreted blot tests were: OspC - 24/118 (20.3%), VlsE Bb - 17/118 (14.4%), p21 - 11/118 (9.3%), p41 - 10/118 (8.5%), VlsE Bg - 8/118 (6.8%), p83 - 6/118 (5.1%), p58 - 5/118 (4.2%), p18 and p20 - 3/118 (2.5%), p19 - 2/118 (1.7%), p39, LBb and LBa - 1/118 (0.8%) (Fig. 2).

DISCUSSION

There is not yet much data related to the most common reactive immunoblot bands corresponding to antigens targeted by *Bb*sl - specific IgG in infected humans in Bulgaria. Immunoblots are not routine diagnostic tests as they require specialized laboratory

equipment. Immunoblot tests are currently available in two formats: western blot and line blot. Western blot involves electrophoretically separated proteins of *Bb*sl (typically whole-cell lysate). When whole cell lysate is used, similarly sized proteins move in parallel, which can reduce specificity. Therefore, purified recombinant proteins are currently used for the second tier of LD assays (9, 10). By utilising purified recombinant antigens, line blots reduce the potential for cross-reactions due to co-migrating proteins.

Despite the efforts in this direction, the serodiagnosis of LD is still challenging. Some of the antigens currently used for diagnosis of this disease are involved in cross-reactions. In general, this is due to the complex antigenic structure of the *Bb*sl. There is a significant heterogeneity among the *Bb*sl species prevalent in Europe (*B. afzelii*, *B. garinii*, and *B. burgdorferi*), particularly regarding the most relevant for serodiagnosis antigens (3, 11). Cross-reactivity can occur with a single antigen like flagellin (12) or with entire pathogens, such as *T. pallidum* (13, 14), *Yersinia* spp. (15, 16, 17), cytomegalovirus (CMV), Epstein-Barr virus (EBV) (2). Knowledge of these antigens - their potential for cross-reactions and the use of purified recombinant or chimeric proteins, is crucial for the correct diagnosis and treatment of LD.

*Bb*sl-specific IgM can be detected two to four weeks after the appearance of erythema migrans - a pathognomonic symptom observed during the early stage of disease, or a longer period after the tick bite in cases without erythema, while IgG rise four to six weeks after the onset of infection (18). In many cases, IgG levels peak in six months and may remain elevated for months to years. Eg, 62% of patients with lyme arthritis during the first diagnosic testing showed a presence of specific IgG antibodies even 10 years later (19).

The sensitivity of serological tests increases during the later stages of the disease, because the immune response matures with the continuation of the infection (3, 20). An extensive IgG response against multiple antigens is observed, with reactivity against DbpA (p18), OspC (p21 - p25), OspD (p28), OspA (p30), VlsE, BmpA (p39), FlaB (p41), p45, p58, p66 and p93 (3). IgG antibodies directed against specific OspC, VlsE and p41 antigens are commonly detected early in the course of infection (3). In the second stage, IgG antibodies are directed against highly specific p39 and p58 membrane antigens. Other antigens are added in the late stages of the disease: late-phase marker p83 (major extracellular protein) (21) and immunogenic lipids. The 39 kDa and 41 kDa proteins were found to be some of the most significant markers for LD (21). Some manufacturers offer kits containing engineered immunoreactive antigens designed to increase the test specificity. Such antigens (p18, p19, p20, p21 and p58), are included in the Euroimmun strips used in our study.

Several diseases can be misdiagnosed as LD due to cross-reactivity, and these are most often syphilis, relapsing fever, EBV and CMV infections (2). Patients with elevated rheumatoid factor are also often misdiagnosed with LD (22 - 26). Studies showed that rheumatoid factor is reactive with many *Bb*sl antigens: BmpA, VlsE, OspC, OppA2, LA7 (p22), BBA64 (p35), p37 (25 - 28).

The flagellar protein FlaB (p41, 41 kDa), localized on Bbsl chromosome, is one of the first proteins targeted by antibodies after infection with *Bb*sl (29). It builds the flagellum core and is highly conserved among a large number of microorganisms (2). P41 was the most common positive reactive band against *Borrelia*-specific IgG among all blot tests in our study: 97.1% of the positively interpreted, and 86.4% of the negatively interpreted tests. P41 was very rare among the weak positive reactive bands both in the positively and negatively interpreted blot tests. Bands with intensity under 19 units are not considered signifcant in the IgG immunoblot (29). According to some studies, p41 has about 40% sequence identity with the *Treponema* spp. flagellin, the cross-reactive epitopes being located between positions 131 - 234 (2). Low levels of IgG against flagellin are present in sera from persons with infections other than LD: syphilis, human granulocytic anaplasmosis (HGA), yersiniosis, EBV and CMV. This suggests that in some cases in our study the appearance of p41 band could be due to cross-reacting antibodies (2, 29).

Outer surface-exposed protein C (OspC, 21 - 25 kDa) is an immunodominant lipoprotein antigen for *Bbsl* (30). It is necessary for *Bbsl* transmission to mammals as it binds plasminogen, as well as tick saliva protein Salp 15 (2). However, antibodies cross-reacting with OspC can be observed in some viral infections. The

prevalences of EBV and CMV among the adult human population are very high - about 90% and 83%, respectively (22, 23, 31). Studies from Poland have established that infection with these two viruses generated antibodies that cross-reacted with p41 and OspC in immunoblots (22, 32). Generally, it was observed that serum samples from patients with EBV infection cross-reacted mainly with the OspC protein of *Bbs*l, while anti-CMV IgG cross-reacted with a wider variety of *Bbs*l antigens, such as OspC, FlaB, BmpA and VlsE (2). Antigenic similarity was found between the OspC and FlaB antigens of *Bbs*l and YopD of *Yersinia* spp. and two-way cross-reactivity was presented (15, 16). It was also shown that IgG from patients with HGA cross-reacted with *Bbs*l proteins, such as OspC, p37, FlaB, OspA and OspF (25). Cross-reactions with *Bbs*l antigens, like OspC, p37, BmpA and OspF were also observed in patients with oral infections (2). The locus of OspC is probably the most genetically diverse locus in all *Bb* genospecies (33). In our study, OspC was positive in 22.6% of the positively interpreted blot tests and in 6.8% of the negatively interpreted blot tests. It was the most common weak reactive band among both positive and negative blot tests, 17.7% and 20.3% respectively. It is considered as significant for the IgG response when the intensity units are greater than 19 (29). It is possible that in our study, the observed weak positive OspC bands could, in some cases, be due to cross-reactive antibodies.

Another immunodominant antigen for *Bbsl* is the variable major protein-like sequence expressed (VlsE, 34 - 35 kDa), which is a surface-exposed outer membrane lipoprotein encoded by a gene located on the linear plasmid lp28-1 (30). VlsE helps the spirochete evade the host immune response by undergoing antigenic variations. The expressed region of the VlsE gene contains six variable regions that recombine with multiple unexpressed cassette sequences located on the same linear plasmid. This results in a high degree of antigenic variation among *Bbs*l clones infecting a host (30). VlsE contains a highly conserved IR6 region that is used in lyme immunoblots as a target antigen (2). Strong immunoblot reactivity to a recombinant VlsE protein was obtained with LD patients' sera, but not with sera from patients with syphilis and autoimmune conditions (34). Another study provided strong evidence for the key role of the vls locus for *Bbs*l persistence in the human host despite antibiotic treatment (35). Other studies have shown the importance of VlsE antigenic variation for persistent infection of natural reservoir hosts (36) and it was observed that the VlsE recombination system operates only in mammal models of infection, but not in culture or in the tick vector (33). Currently, multiple purified recombinant proteins are used for European LD immunoblot tests, as in Europe LD can be caused by different *Bbsl* species - *Borrelia afzelii, Borrelia garinii,* and *Borrelia burgdorferi* sensu stricto (9, 37). Recombinant VlsE proteins were also used in our study - VlsE Ba (*Borrelia afzelii)*, VlsE Bb (*Borrelia burgdorferi* sensu stricto) and VlsE Bg (*Borrelia garinii*). Reactive VlsE Bb bands were present in 69.6% of positive immunoblot tests, VlsE Ba - in 64.7% and VlsE Bg - in 52.0%. This shows their importance as highly specific diagnostic antigens.

Another immunogenic antigen which may induce antibody response is p18 (Dbp A/B) surface protein. P18 is an adhesin encoded by a gene located on the linear plasmid lp54. It binds to decorin and is involved in the spread of *Bbsl* in mammals (2). P18 and VlsE are the most widely used proteins in commercial diagnostic tests, mainly for detection of IgG antibodies (2). Because of the high variability of DbpA antigens within the *Bbsl*, for diagnostic purposes they are added in different variants from different genospecies. In our study, the p18 band was positive in 4.6% of all tested samples, in 6.9% of all positive samples and in 2.5% of all negative samples.

The Basic membrane protein A (BmpA, p39) is one of the most commonly applied specific markers for LD, both on IgM and IgG immunoblot strips (29). Its gene is localised on the borrelial chromosome, it binds to mammalian lamin and is highly conserved. BmpA plays a role in the development of lyme arthritis (2). Studies have shown that BmpA cross-reacts with antibodies against syphilis, relapsing fever, CMV and parvovirus B19, and is recognized by rheumatoid factor (2). In our study, the p39 band was positive in 14.1% of all tested samples and in 30.4% of all positive samples. None of the negative samples had a reactive p39 band.

In a study on the seroprevalence of LD in Eastern Slovakia in 2018, the most commonly found antibodies were against VlsE (80.2%), p41 (66.7%), p18

(56.3%), p58 (31.3%), and p39 (30.2%) (38). According to our study, the most common specific anti-*Borrelia* IgG antibodies were established against p41 (97.1%), VlsE (average of 62.1%), p83 (32.4%), p39 (30.4%) and OspC (22.6%). In comparison, p58 and p18 were reactive in 10.8% and 6.9% of positive blots, respectively. Wide variations in different combinations of reactive proteins were observed, which could be due to multiple factors specific to host, pathogen and environment.

CONCLUSIONS

This study analysed the most common antigens targeted by specific IgG antibodies synthesized as a result of *Bb*sl infection in humans from all 28 provinces of Bulgaria. The immunoblot can provide more detailed information on antibody response patterns to different antigens of *Bb*sl and adds to the true picture of the disease in patients with Lyme borreliosis. The immunoblot might also be used to identify the immunodominant antigen, part of it and to apply chimeric molecules for the development of more sensitive and specific serological analyses. The design of new specific antigens from selected conserved fragments within *Bb*sl eliminates the problem of cross-reactivity with commonly found antigens of other pathogens. It was shown that the precise selection of protein fragments from VlsE and FlaB increased the specificity without reducing the sensitivity of the test (39, 40). The assessment of the manifested symptoms of LD and the detection of highly specific antibodies, based on purified recombinant or chimeric proteins, without cross-reactive epitopes is very important for the correct diagnosis and treatment of this disease. The study of data related to the incidence and seroprevalence of LD, together with the peculiarities of the immune response allow a better understanding of the pathogenesis of this disease and the development of key strategies for protecting human health.

ACKNOWLEDGEMENTS

This study was conducted with the support of:

PRIDST 2021-2027, BG16RFPR002-1.014 “Sustainable Development of Centers of Excellence and Centers of Competence, including specific infrastructures or their consortia from the National Roadmap for Research Infrastructure (NRRI)“ funded by Programme “Research, Innovation and Digitalisation for Smart Transformation“ and

KP-06-H73/4 from 05.12.2023 “Investigations on genetic characteristics of the etiological agents of Lyme borreliosis, biomarkers, humoral and cell-mediated immunity in patients with Lyme borreliosis”.

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