

Rational Design of Antigens to Improve the Serodiagnosis of Tick-Borne Borreliosis in Central Regions of Russia

Evgenia Baranova, Pavel Solov'ev, Evgeny Panfertsev,
Anastasia Baranova, Galina Feduykina, Liubov Kolombet,
Muhammad G. Morshed and Sergey Biketov

Abstract Tick-borne borreliosis (Lyme disease-LD) is caused by pathogenic *Borrelia* spirochetes that is transmitted through bite of *Ixodes* ticks to humans and animals. In the Russian Federation, borreliosis registered with an index of 6–7 per 100,000 people annually. In reality, LD morbidity in Russia is much higher because Russian strains develop less erythematous rashes compared to North American strains, thus missed by physicians in most of the early cases, and current serology tests have insufficient sensitivity as well. The aim of this work was to improve the sensitivity and specificity of serology tests for LD in Russia using rationale-designed *Borrelia* antigens. It was anticipated that sensitivity of LD serodiagnosis will be higher if antigen for test-systems are derived from a strain that is circulated in a geographical region of test application. A large portion of the Russian population lives in the Central region. Thus, effort has been made to create a serological test using antigens from Moscow region, Tula and Ul'janovsk areas. In this study we included wild strains (ultrasonic-treated spirochetes *B. garinii* H19, *B. afzelii* P1, *B. afzelii* P1H13, *B. burgdorferi* s.s. 39/40, *B. burgdorferi* s.s. B31), recombinant (expressed in *E.coli* DbpA, Bgp, Bbk *B. garinii*, and *B. afzelii*) antigens and some of their combinations were produced and tested against LD patients and donors serum collected in hospitals of Central regions of Russia by ELISA and Western blotting. Considering sensitivity and specificity, DbpA *B. afzelii* and DbpA *B. garinii* recombinant antigens were selected among all probed

E. Baranova (✉) · P. Solov'ev · E. Panfertsev · A. Baranova · G. Feduykina ·
L. Kolombet · S. Biketov
State Research Center for Applied Microbiology and Biotechnology, Obolensk,
Moscow region, Russia 142279
e-mail: Givanova1@yandex.ru

M. G. Morshed
British Columbia Centre for Disease Control Public Health Microbiology and Reference
Laboratory, Vancouver, BC V5Z 4R4, Canada

Department of Pathology and Laboratory Medicine, University of British Columbia,
Vancouver, BC, Canada

antigens for regional serology test. As long as DbpA *B. afzelii* and DbpA *B. garinii* antigens interacted with LD patient's serum in a complementary mode, it is possible to combine epitopes DbpA *B. afzelii* and *B. garinii* in a single antigen for improving sensitivity. We created recombinant fusion protein DbpA *B. afzelii*/B using dbpA genes from Russian isolates of *B. afzelii* and *B. garinii* in *E. coli*. Fusion DbpA A + G protein was then used for formulation of fast immuno-chromatographic serodiagnosis test (LF) in a "deep-stick" format. The trials of LF-test were conducted separately at Institute of Rheumatology Russian Academy of Medical Science (using 325 sera) and at the Borreliosis Reference Center of Ministry of Health RF (using 120 reference sera). The average sensitivity and specificity of LF-test was 80.5 and 100 %, respectively.

Keywords Lyme disease • *Borrelia garinii* • *Borrelia afzelii* • DbpA • Serology • LF-test

Introduction

Lyme disease (Lyme borreliosis) is an emerging global public health problem. Over 10,000 cases are registered in the USA, 50,000 cases in Europe, and 8,000 cases in Russia annually. The causative agent of borreliosis is *Borrelia burgdorferi sensu lato* transmissible by *Ixodes* ticks [1–4]. The group of *B. burgdorferi sensu lato* divided further into 13 species (genotypes). This classification has been based on outer surface proteins (Osp A, Osp B, Osp C), flageller gene structures, other protein profiles, metabolic activities, and genetic diversity determined through molecular typing and/or sequencing [4]. Direct detection of Lyme *Borrelia* in infected vectors, host tissues, and clinical specimens from patients include microscope-based assays, antigen detection assays, in vitro cultivation, and molecular tests. Lyme borreliosis is caused by mostly three species: *Borrelia burgdorferi sensu stricto* (s.s.), *B. afzelii*, and *B. garinii* found in Europe and Asia [5] and North America [6, 7]. Lyme borreliosis may be caused by other species of *Borrelia burgdorferi sensu lato* as well and can be diagnosed using both clinical and laboratory information such as *B. bissettii sp. nov.*, *B. vailaisiana*, and *B. miyamotoi* may also cause infection in humans [8, 9]. *B. afzelii* and *B. garinii* are predominantly found in Central (European) regions of the Russian Federation. Antibiotics could effectively treat Lyme disease during its early stage, however, illness may persist in host for years if not treated and the person may end up with a long-term disability. Undoubtedly, early diagnostics is necessary for treatment and to prevent *Borrelia*-related complications. Diagnostic strategies may vary between early and late disease manifestations and are usually determined through clinical information and serological testing. Erythema migrans is pathognomonic and does not require any further laboratory investigations. By contrast, the diagnosis of neuroborreliosis requires the assessment of serum and cerebrospinal fluid. Lyme arthritis is diagnosed in the presence of newly recognized arthritis and high-titer serum IgG antibodies against *B. burgdorferi* [10]. It was shown in Russian hospital

laboratories that foreign ELISA tests for serodiagnosis of Lyme disease detected that 30–70 % of cases depend on the geographic location and stages of disease. It may be due to the fact that antigenic determinants of *Borrelia* could be significantly different in geographical zones and “regional” strains need to be used as antigen sources for serological testing [11]. Earlier, we showed that sensitivity of LD serodiagnosis can be increased if whole cell ultrasonic antigen for ELISA test-systems was received from strains *B. afzelii* predominantly circulating in geographical regions of test application [12]. However, such test-systems demonstrated insufficient specificity (mainly due to cross-reaction with syphilis patient’s sera) and more specific recombinant antigens were made using DNA from “regional” strains. DbpAB were selected as viable alternatives to original antigens. The aim of this study was to improve the sensitivity and specificity of serology tests for LD in Russia using rationally designed *Borrelia* antigens considering geographical region of test application. This fast, simple, relatively cheap, and field-applicable diagnosis of LD would greatly benefit monitoring LD programs. Our focus targeted to constructing LD serological tests in an immunochromatographic format which will improve laboratory testing of LD in Russia.

Materials and Methods

Human Sera

Human sera were obtained from patients with disseminated and late stages of LD (164 patients), autoimmune disease (26 patients), syphilis (42 patients), and also from healthy blood donors who live in endemic areas (105 donors) and healthy blood donors who live in non-endemic area (100 donors). The LD patients’ sera were derived from recently diagnosed and previously untreated individuals who were recruited in 2012 at the Institute of Rheumatology Russian Academy of Medical Science, Moscow. All serum specimens were obtained after blood was drawn and stored at -70°C prior to assay. Additionally, in this study the patients’ (58 patients) and donors’ (62 donors) reference sera stored at Borrelioses Reference Center of Ministry of Health RF, Obolensk, were used during trial of LF serological tests.

Strains Isolation and Typing

Borrelia Cultures were obtained from intestines of ticks *I. persulcatus* and *I. ricinus*, which have been collected in Ul’yanovsk and Tula regions. Briefly, *Borrelia* were cultured in modified BSK II medium and incubated at 33°C for 12 weeks. Spirochete grown in BSK II media were confirmed as *Borrelia* species through molecular testing (RFLP). Spirochaetal DNA was extracted from cultures during late log phase and subsequently intergenic spacer between 5S and 23S genes of ribosomal RNA

was amplified using primers RS1(5'-CTGCGATTTCGCGGGAGA-3') and RS2 (5'-TCCTAGGCATTACCATA-3'). Amplicons were treated with endonucleases MseI and DraI and digested. DNA were run through gel electrophoresis. Finally, speciation was confirmed using banding pattern.

Whole Cell Ultrasonic Antigen

Borrelia cell grown in 0.5 l BSK II media were precipitated by centrifugation (5 min at 5,000 g). Pellet was washed 3 × with PBS, suspended in 3 ml PBS, and treated with Ultrasonic Homogenizer Bandelin Sonopuls GM 3200 using horn MS72 with the following parameters: amplitude 308 μmss, frequency 20 kHz, pulsation ON cycle 5 s, OFF cycle 30 s three times. Whole cell ultrasonic antigen was stored at -70 °C prior to assay.

Recombinant Antigens

DNA encoding selected *Borrelia* proteins were amplified by PCR from *B. garinii* H19 and *B. afzelii* P1 genomic DNA using Taq-polymerase (Promega, USA). Gene-specific PCR primers were designed to amplify the gene coding sequence with restriction enzyme sites at the 5' and 3' ends. The PCR products were digested by restriction enzymes and directionally cloned into expression vector pET32b (Novagen, Madison, WI) with a six-His tag at the N terminus. Each sequence-verified expression construct was transformed into strain BL21(DE3) to produce a recombinant protein. The next synthetic oligonucleotides were used for DNA cloning and sequencing were as follows: forward AFZ 5'-aacatatgtgtagttaacaggaaaagctag-3' forward GAR 5'-aaaagcttggatgtggcttaacaggagaa act-3'; reverse AFZ 5'-ggaagcttttttgatttttagttgtt tt-3'; reverse GAR 5'-aaaagctttagtagcagcagcagtggtggc-3'; T7 forward primer 5'-taatacgaactcactataggg-3'; T7 reverse primer 5'-tatgctagtattgctcagcgg-3 (Novagen). PCR reaction cycles were: 95 °C\30 s; 45 °C\30 s; 72 °C\35 s; 30 cycles. The PCR reaction comprised of 1.5 mM MgCl₂, 0.2 mM dNTP, and 10 pM of each primer, 20 ng of total DNA of *B. garinii* H19 or *B. afzelii* P1 were used as a matrix in PCR. The PCR products were analyzed by electrophoresis in 1 % agarose gel in the presence of ethidium bromide. The target DNA fragments were cloned into vector plasmid pET32b on restriction sites NdeI-HindIII. DNA sequence was determined by the CEQtm2000XL DNA Analysis System from Beckman Coulter. Hybrid proteins were found to be synthesized in *E. coli*. For the isolation of fusion protein DbpA(A + G)-his *Borrelia*, standard methods of refolding were used. Recombinant proteins were purified by metal-chelating Sepharose CL4B. Recombinant proteins were purified with Ni-nitrilotriacetic acid resin (Qiagen, Gaithersburg, MD) and quantified with the bicinchoninic acid protein assay (Pierce, Rockford, IL), and quality was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting

according Laemmly and Towbin [13, 14]. Soluble recombinant proteins were determined by anti-his antibodies (A7058, Sigma). Purified DbpA proteins were subjected to SDS-PAGE under reducing conditions and stained with Coomassie Brilliant Blue (10 % gel) and transferred onto a nitrocellulose membrane using 12 % gel. After blocking additional protein-binding sites, proteins on the membrane were probed with positive, negative sera, and sera from patients with syphilis and goat anti-human HRP conjugate as the secondary antibody (A8794, Sigma).

ELISA

Ninety-six-well ELISA plates (Corning Inc., Corning, NY) were coated with 100 μ l of *Borrelia* antigen (1–10 μ g/ml) per well in coating buffer (0.1 M carbonate buffer, pH 9.2) and incubated at 4 °C overnight. After two washes (3 min each) with 200 μ l of PBST (10 mM sodium phosphate, 150 mM NaCl, and 0.1 % Tween 20, pH 7.4) per well, 200 μ l of blocking buffer (PBST supplemented with 5 % nonfat dry milk, BioRad) was applied to each well. The plate was shaken at 150 rpm for 1 h at 37 °C. After three washes with PBST as described above, 100 μ l of human serum diluted 1:200 with blocking buffer was added to each well. The plate was incubated with shaking at 150 rpm for 1 h at 37 °C and then washed three times with PBST as before. Each well then received 100 μ l of goat anti-human IgG, IgM (0.5 μ g/ml) (Sigma, A8794) conjugated to horseradish peroxidase and dissolved in blocking buffer. Plates were incubated at 37 °C for 1 h with shaking at 150 rpm. After four washes with PBST for 3, 4, 5, and 6 min, respectively, 100 μ l of a solution composed of the chromogen 3,3',5,5'-tetramethylbenzidine at 0.2 mg/ml and 0.01 % hydrogen peroxide in the buffer supplied by the manufacturer (ChemBioTest, Russia) was added, and allowed 10 min to develop color. The enzyme reaction was stopped by addition of 50 μ l of 1 M H₂SO₄. The optical density (OD) was measured at 450 nm with an ELISA plate spectrophotometer model Pikon Uniplan (Russia). The cutoff OD value was defined as the mean OD plus 3 standard deviations (SDs) for 7 serum samples collected from patients of a hospital in Astrakhan' (where Lyme disease is not endemic). All the samples were assessed blindly in duplicate and repeated twice. Mean OD values from duplicate results were reported. OD values of individual samples never varied more than 5 % [3].

Formulation of LF-Test

To formulation of LF-test antigen was adsorbed on paper and protein G (or anti-human IGG) was conjugated with colloidal gold nanoparticles. For preservation of antibodies binding activity “fusion DbpA” was adsorbed on nitrocellulose paper in presence of protease inhibitors and drying-protectors. Developer reagents—conjugates protein G with gold nanoparticles with size 5, 20, or 40 nm (according Atomic Force Microscopy data) were preliminary tested concerning antibodies

binding activity and visualization accuracy. As results, a “deep-stick” tests composing from nitrocellulose strip containing sample pad with dried 40 nm colloidal gold-labeled protein G and test-line with dried capture antigen (“fusion DbpA”) were fabricated.

Results

Determination of Sensitivity and Specificity of Cell Antigens Based on Regional *Borrelia* Strains by ELISA

The strains from ticks collected in Central region of Russia (Moscow, Tula and Ul'janovsk areas) were used for whole cell antigen preparation. *Borrelia* whole cell ultrasonic antigens were received from *B. garinii* H19, *B. afzelii* P1, *B. afzelii* H13, *B. burgdorferi* s.s. 39/40, and *B. burgdorferi* s.s. B31 strains and tested with a patient's and donor's human sera.

It was shown that depending on the strains used, sensitivity for detecting by ELISA in disseminated and late stages of LD varied from 64 % (*B. burgdorferi* 39/40) to 88.0 % (*B. afzelii* P1). All strains demonstrated acceptable level of specificity with donors' sera and high-level cross-reaction with sera from syphilis patients (Table 1). Besides, the different immune reactivity with LD patients' pool sera in immunoblot for the foreign and Russian *Borrelia* strains was observed (Fig. 1). The more intensive immunoreactions with LD patients' pooled sera for Russian strains *B. garinii* H19 and *B. afzelii* P1 in the areas 40–70 and 34–18 kDa were detected.

This part of the study demonstrated that ELISA test systems for serodiagnosis of LD constructed using antigen sources corresponded to the geographical region of application demonstrated better sensitivity. These results indicated a possibility to increase sensitivity of LD serodiagnosis by using *Borrelia* strains circulated in Central region of Russia as an antigen source.

Determination of Sensitivity and Specificity of Recombinant Antigens Received Using DNA from “Regional” Strains

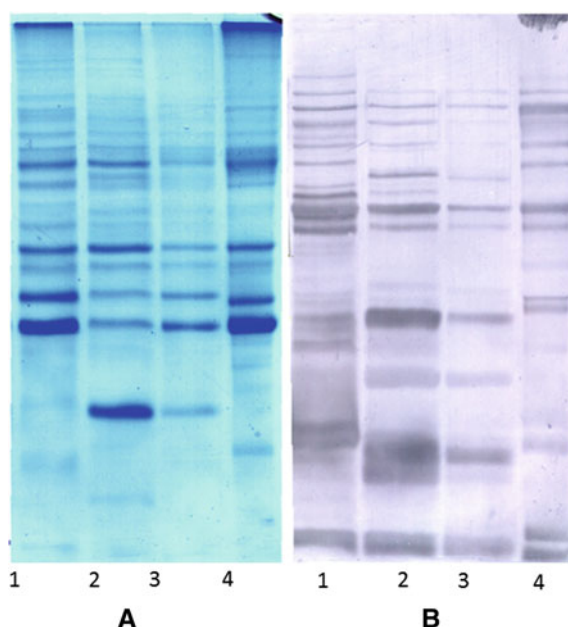
Since whole cell antigens demonstrated insufficient specificity (mainly via cross-reaction with syphilis patients sera) the more specific recombinant antigens were selected as a viable alternatives to the original whole cell antigens.

We cloned and expressed genes *bbk*, *bgp*, and *dbpa* from regional strains of *B. afzelii* P1 and *B. garinii* H19 in *E. coli*. Recombinant proteins were tested through ELISA using LD patient's sera (dissemination and late stages) from European region of Russia (Table 2).

Additionally the *Borrelia* recombinant antigens were tested with LD patients' sera collected in different areas of Central region—Moscow, Tula, and Ul'janovsk.

Table 1 Interaction of various *Borrelia* whole cell antigens with patients' and donors' serum collected from Central Region of Russia

Patients sera	Borrelia antigens				
	<i>B. afzelii</i> H13 (%)	<i>B. afzelii</i> P1 (%)	<i>B. garinii</i> H19 (%)	<i>B. burgdorferi</i> 39/40 (%)	<i>B. burgdorferi</i> B31 (%)
LD patients (<i>n</i> = 50)	82.0	88.0	68.0	64.0	68.0
Healthy blood donors (<i>n</i> = 100)	10.0	8.0	8.0	6.0	8.0
Autoimmune disease patients (<i>n</i> = 20)	15.0	15.0	10.0	10.0	15.0
Syphilis patients (<i>n</i> = 40)	65.0	65.0	62.5	60.5	65.0

**Fig. 1** SDS-PAGE and Western blot of whole cell ultrasonic antigens *B. garinii* H19, *B. afzelii* P1, *B. burgdorferi* 39/40, and *B. burgdorferi* B31 against pooled positive LD patient's sera. *A* SDS PAAG electrophoresis, *B* immunoblotting against pooled positive LD patient's sera. 1 *B. afzelii* P1, 2 *B. garinii* H19, 3 *B. burgdorferi* 39/40, 4 *B. burgdorferi* B31

The more frequent immunoreactions of LD patients' sera from Moscow and Tula areas for DbpA *B. afzelii* were observed (66 %). Meanwhile only for 17 % of these sera reacted with DbpA *B. garinii*. The reactivity of sera from Lyme Borreliosis patients of Ul'janovsk regions with DbpA *B. afzelii* consist of 53 % and for DbpA *B. garinii*—23 %. It is important that DbpA *B. afzelii* and DbpA *B. garinii* antigens interacted with LD patient's sera in a complementary mode that creates possibility to improve sensitivity by combining DbpA *B. afzelii* and *B. garinii* in

Table 2 The sensitivity of the ELISA serology on a base of recombinant antigens for LD patient's sera (dissemination and late stages) from European Region of Russia

Lyme disease patients' sera (n = 164)	<i>Borrelia</i> antigens				
	<i>B. afzelii</i> Strain H13 (%)	<i>B. afzelii</i> DbpA (%)	<i>B. garinii</i> DbpA (%)	<i>B. afzelii</i> Bbk (%)	<i>B. afzelii</i> Bgp (%)
Positive	71.9	73.8	24.4	11.6	15.2
Equivocal	15.2	16.4	12.8	3.0	6.0
Negative	12.9	9.8	62.8	85.4	79.8

single antigen. The analyses of dbpA *B. afzelii* and *B. garinii* sequences (Fig. 2) indicate that Russians' strains have essential variability of dbpA sequences similar or more to described European isolates.

As we can see from the alignment, there is essential interspecies/strains heterogeneity between dbpA *B. afzelii* and *B. garinii* sequences. To combine epitopes DbpA *B. afzelii* and *B. garinii* in single antigen the structural parts of genes encoding important regions of dbpA *Borrelia afzelii* and *garinii* have been obtained by PCR and fused in one ORF under control of a strong promoter. As a result, fusion protein containing structural parts of genes dbpA *B. afzelii* and *B. garinii*—DbpA(A + G) was expressed in *E. coli*. All received recombinant proteins were analyzed by immunoblotting with pooled LD patients' sera (Fig. 3).

Fusion DbpA (A + G) and other recombinant proteins were comparatively tested though ELISA using LD patient's sera (dissemination and late stages) from European Region of Russia (Tables 3 and 4).

So it was shown that in case of fusion antigen, sensitivity reaches to 84.1 % that is essentially higher than observed in case of DbpA *B. afzelii* (73.8) or DbpA *B. garinii* (24.4) individually.

ELISA test using fusion DbpA recombinant antigen had a specificity of 100 % with sera from healthy persons, sera from patients with autoimmune disease, and 96.7 % with sera from syphilis patients (Table 4).

Development of a Immunochromatographic Test for Serodiagnosis of LD Based on “Fusion DBPA”

Fusion DBPAG protein was used for formulation of fast immunochromatographic serodiagnosis test in “deep-stick” format. On a base fusion antigen DbpAG experimental test was developed (Fig. 4).

The trials of deep stick were conducted with LD patient's sera from various disease stages. Deep stick was interacted with reference sera (dissemination and late stages) with sensitivity in a range 80.5 %. The test had a specificity of 100 % (no cross-reactivity was found with sera from healthy persons and syphilis patients). During clinic trials the possibility of novel recombinant protein to identifying specific anti-*Borrelia* IgG was demonstrated.

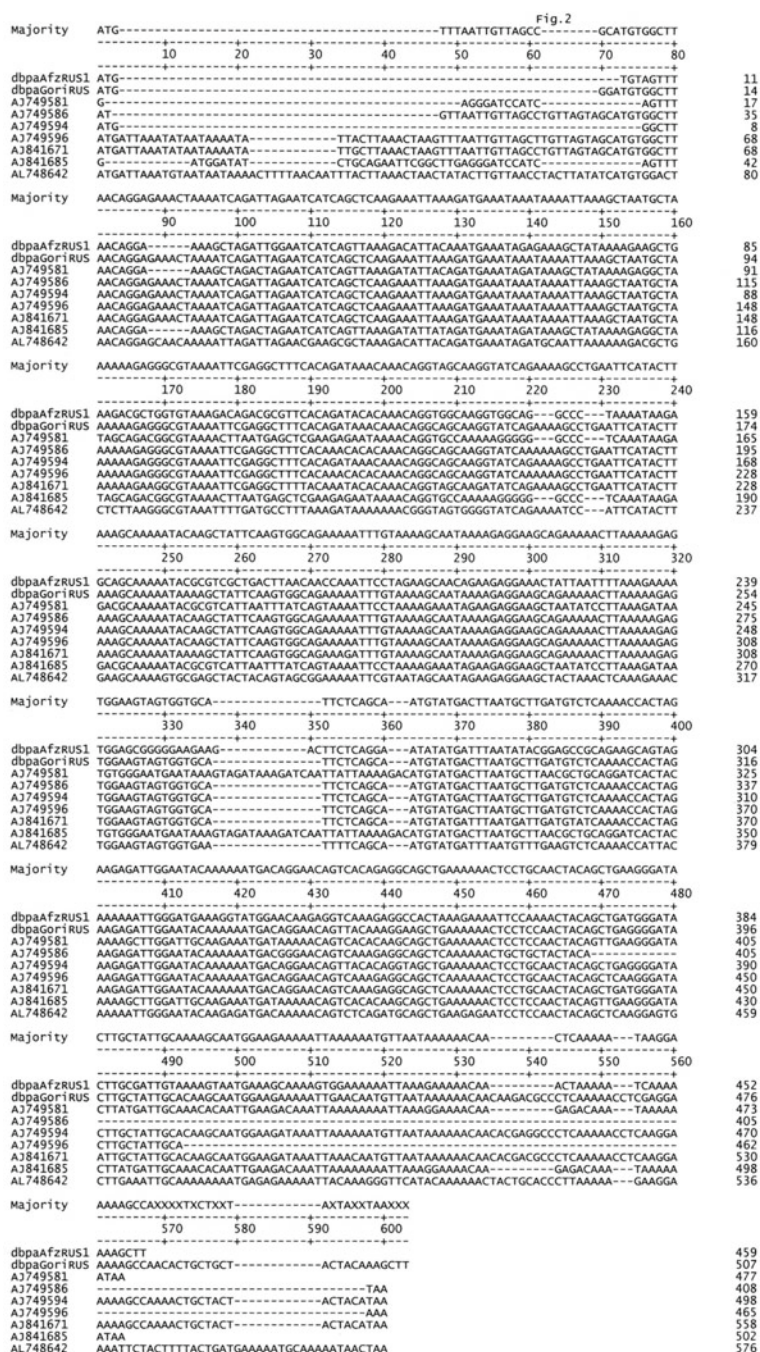


Fig. 2 Alignment of dbpa nucleotide sequences from *B. afzelii* and *B. garinii* strains isolated in different European geographic regions, including Russia

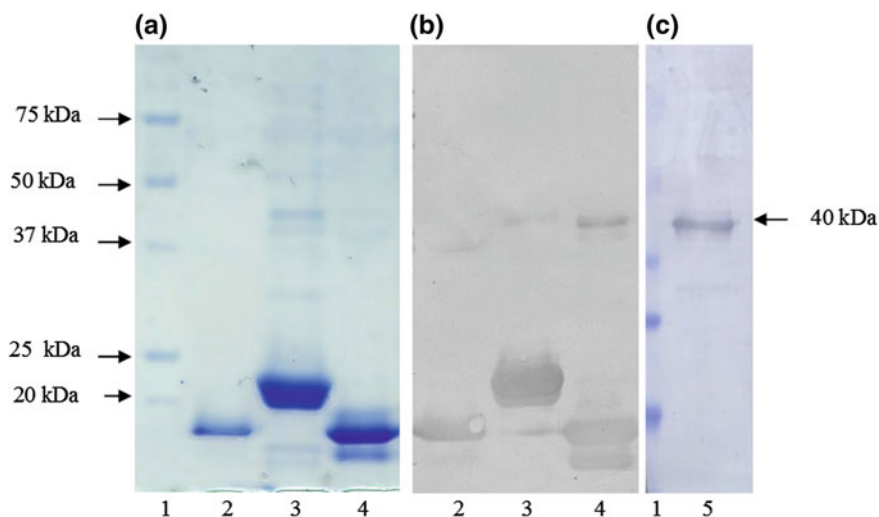


Fig. 3 SDS-PAGE and Western blot analysis of recombinant DbpA. **a** SDS PAGE electrophoresis: 1- MW markers; 2 – DbpA *B. afzelii* P1; 3 – DbpA *B. garinii* H19; 4 – DbpA *B. burgdorferi* B31 **b** and **c** - immunoblotting with pooled positive LD patient's sera: 2 – DbpA *B. afzelii*; 3 – DbpA *B. garinii*; 4 – DbpA *B. burgdorferi* B31; 5 - DbpA (A+G)

Table 3 The sensitivity of the ELISA serology on a base of natural and recombinant antigens with LD patients' sera (dissemination and late stages) from European Region of Russia

Lyme disease patients' sera (<i>n</i> = 164)	<i>B. afzelii</i> Strain H13 (%)	<i>B. afzelii</i> DbpA (%)	<i>B. garinii</i> DbpA (%)	<i>B. afzelii/B. garinii</i> fusion DbpA (%)
Positive	71.9	73.8	24.4	84.1
Equivocal	15.2	13.4	12.8	12.2
Negative	12.9	12.8	62.8	3.7

Table 4 Specificity of the ELISA on a base of natural and recombinant antigens

Patients' sera	<i>B. afzelii</i> H13 (%)	<i>B. afzelii</i> DbpA (%)	<i>B. garinii</i> DbpA (%)	<i>B. afzelii/B. garinii</i> fusion DbpA (%)
Healthy blood donors endemic areas (<i>n</i> = 105)	96.2	100.0	100.0	100.0
Healthy blood donors non-endemic areas (<i>n</i> = 100)	99.0	100.0	100.0	100.0
Autoimmune disease patients (<i>n</i> = 26)	88.5	100.0	100.0	100.0
Syphilis patients (<i>n</i> = 42)	38.1	95.2	97.6	96.7

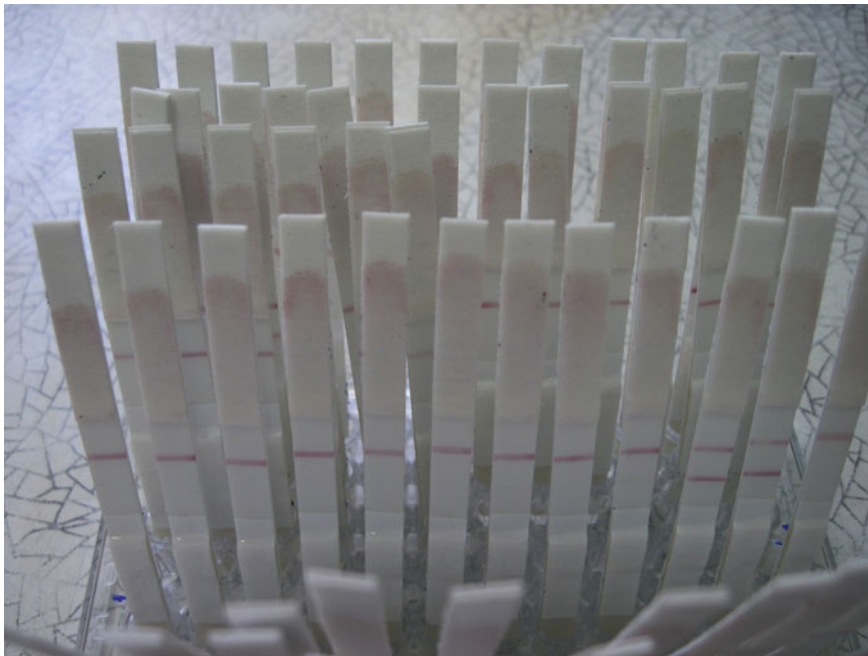


Fig. 4 Deep stick for detection of anti-Borrelia antibodies

Conclusion

We aimed to develop a serological test using local strains from the Central region of Moscow since a large portion of the Russian population lives in this area. We isolated a number of strains from ticks collected from Moscow and Ul'janovsk area and from the skin biopsy of patients with clinical diagnosis of Lyme diseases. The strains from ticks (*B. afzelii* P1, *B. afzelii* Y6 M, *B. afzelii* H13, *B. garinii* H19) and from patients (*B. garinii* Siu, *B. garinii* Kol) were isolated using standard methods and confirmed by molecular methods such as RFLP and used as antigen sources. We searched antigenic spectrums of selected isolates. All diagnostically significant bands with molecular weights 93, 80, 66, 45, 41, 39, 37, 34, 31, 28, 25, 21 kDa are well represented in our antigen preparation of those isolates. Ultrasonic cell lysates of these strains were used as antigenic basis in experimental immunochemical test-systems produced "in house." However, false-positive result levels that were relatively high was observed. Therefore, genes *dbpA*, *bgp*, and *bbk* were cloned and appropriate recombinant proteins were expressed using templates DNA from isolates *B. garinii* and *B. afzelii*.

As results, recombinant antigens expressed in *E. coli*—*DbpA*, *Bgp*, *Bbk* *B. garinii* and *B. afzelii*, and some of their combination were produced and tested by ELISA using LD patient's and donor's serum collected in hospitals of Central regions of Russia. Taking into consideration that *DbpA* *B. afzelii* and *DbpA*

B. garinii recombinant antigens demonstrate higher specificity compared to ultrasonic cell lysates, they were targetted for the specific population (regional). Decorin-binding *Borrelia* protein DbpA represents a lipoprotein, which participates in spirochete adhesion. High titer of antibodies to DbpA among infected people makes this protein suitable for serological diagnosis of Lyme disease [15, 16]. The genes encoding DbpA synthesis from *Borrelia* species are structurally highly heterogeneous. Analysis of dbpA sequences from European isolates *B. burgdorferi* s.s., *B. afzelii*, *B. garinii*, and human pathogenic genospecies A14S revealed five distinct DbpA groups. Group I comprises *B. burgdorferi* s.s. and group II *B. afzelii*, *B. garinii* is divided into groups III and IV, whereas A14S strains form group V [17]. We also found that Russian isolates demonstrates essential variability of dbpA sequences. Hence, it is important that DNA encoding selected *Borrelia* proteins should be amplified by PCR from genomic DNA of *B. afzelii* and *B. garinii* strains circulated in geographical regions of test application. As long as DbpA *B. afzelii* and DbpA *B. garinii* antigens interacted with LD patient's sera in a complementary mode, it is possible to combine epitopes DbpA *B. afzelii* and *B. garinii* in single antigen for improving the sensitivity and DbpA *B. afzelii*/*B. garinii* (DbpA A + G) recombinant fusion protein were created [18]. Fusion DBPA G protein was used for formulation of serodiagnosis test in "deep-stick" format (rapid point of care test). The trials of test were conducted separately in the Borreliosis Reference Center of Ministry of Health RF and Institute of Rheumatology Russian Academy of Medical Science. The average sensitivity and specificity of test consisted 80.5 and 100 % appropriately. This test was found to be useful as a screen test on LD and could be accurately performed and interpreted by minimally trained healthcare workers within 20 min.

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