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Development and application of the technique for identification of *Borrelia miyamotoi* surface antigens

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Abstract

Introduction. *Borrelia miyamotoi* is a pathogen causing erythema-free ixodid tick-borne borreliosis (ITBB), a disease widespread in Russia. The genome of *B. miyamotoi* contains genes of multiple variable major proteins (Vmps). Vmps fall into two families — Vsps and Vlps (with subfamilies δ, γ, α and β). At a particular time, a single *B. miyamotoi* expresses only one variant of *Vmp* gene.

The purpose of the work is to develop a technique for identification of the *Vmp* present at the expression site.

Materials and methods. The technique is designed in the format of a real-time multiplex PCR. It was tested by using *B. miyamotoi* DNA samples extracted from blood collected from 172 ITBB patients and 109 ticks. The samples were collected in 14 regions of Russia.

Results. The new technique made it possible to identify the expressed Vmp in 82% of the examined samples, thus having demonstrated its efficiency. Negative results were much less often observed with samples from patients than with samples from ticks. At the same time, the percentage of samples with one type of Vmp is identical for clinical samples and ticks, while the percentage of samples containing concurrently two types of Vmps is significantly higher among samples from patients with the most frequent occurrence of the Vlp-δ and Vsp combination.

Discussion. The frequent occurrence of the combination of two Vmp types in the blood samples can indicate the concurrent presence of several subpopulations of *B. miyamotoi* in ITBB patients. A new antigenic Vmp variant is synthesized after protective antibodies have been produced for the major protein of the strain transmitted by a tick. This phenomenon known as immune evasion allows the pathogen to persist within a host.

Conclusion. The developed technique of real-time multiplex PCR allows to simultaneous detect of several antigenic variants of the variable basic surface proteins of *B. miyamotoi.* The study of the antigenic spectrum of *B. miyamotoi* strains in comparison with the characteristics of conserved regions of the genome by the method of multilocus sequencing will clarify the stages of evolution and distribution of *B. miyamotoi sensu lato*.

Keywords: *ixodid tick-borne borreliosis, Borrelia miyamotoi, PCR, variable major proteins Vmps, variable large proteins Vlps, variable small proteins Vsps, immune evasion*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ethics Committees of the Central Research Institute of Epidemiology (protocol N 83, 26.06.2018) and Izhevsk State Medical Academy (protocol N 17, 24.12.2012).

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Научная статья
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Разработка и практическое применение методики для идентификации поверхностных антигенов *Borrelia miyamotoi*

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Аннотация

Введение. *Borrelia miyamotoi* — возбудитель безэритемной формы иксодового клещевого боррелиоза (ИКБ) — заболевания, широко распространённого в России. В геноме *B. miyamotoi* присутствуют гены десятков вариабельных основных поверхностных белков (Vmp). Vmp разделяют на два семейства — Vsp и Vlp (с подсемействами δ, γ, α и β). В конкретный момент времени отдельная *B. miyamotoi* экспрессирует единственный вариант гена *Vmp*.

Цель работы — создание методики для идентификации гена *Vmp*, находящегося в сайте экспрессии.

Материалы и методы. Методика реализована в формате мультиплексной ПЦР в режиме реального времени. Для её испытания были использованы образцы ДНК *B. miyamotoi,* выделенные из крови 172 больных ИКБ и 109 клещей. Образцы были собраны в 14 регионах России.

Результаты. Разработанная методика позволила идентифицировать экспрессирующийся Vmp в 82% исследованных проб, т.е. показала достаточную эффективность. Отрицательные результаты значимо реже наблюдались среди проб от больных, чем среди проб клещей. При этом доля проб с Vmp только одного типа одинакова среди клинических образцов и клещей, а доля проб, в которых детектируются одновременно два типа Vmp, значимо выше среди образцов от больных, где наиболее часто встречалась комбинация Vlp-δ и Vsp.

Обсуждение. Тот факт, что в образцах крови чаще выявляется сочетание двух типов Vmp, может говорить об одновременном присутствии нескольких субпопуляций *B. miyamotoi* в организме больных ИКБ. После того как к основному поверхностному белку штамма, занесённого клещом, вырабатываются протективные антитела, происходит переключение на синтез нового антигенного варианта Vmp. Такой феномен, называемый «иммунным избеганием», позволяет патогену персистировать в организме хозяина.

Заключение. Созданная методика мультиплексной ПЦР в режиме реального времени позволяет проводить одновременную детекцию нескольких антигенных вариантов вариабельных основных поверхностных белков *B. miyamotoi. И*зучение антигенного спектра штаммов *B. miyamotoi* в сопоставлении с характеристикой консервативных участков генома методом мультилокусного секвенирования позволит прояснить этапы эволюции и распространения *B. miyamotoi sensu lato*.

Ключевые слова: *иксодовый клещевой боррелиоз, Borrelia miyamotoi, ПЦР, поверхностные основные вариабельные белки Vmp, вариабельные большие белки Vlp, вариабельные малые белки Vsp, иммунное избегание*

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Introduction

Borrelia miyamotoi is a pathogen causing erythema-free ixodid tick-borne borreliosis (ITBB). The first cultures of *B. miyamotoi* were obtained from the *Ixodes persulcatus* tick (strain HT31) and the *Apodemus argenteus* mouse (strain FR64b) in Japan in 1992 [1–3]. The detection of *B. miyamotoi* DNA in the blood of patients with erythema-free ITBB by scientists of the Central Research Institute of Epidemiology in Udmurtia in 2003 triggered studies of the biological properties of the pathogen, the specific features of the clinical course and epidemiology of ITBB caused by *B. miyamotoi* (ITBB-BM) [3–10].

The main problem in studying of the ITBB-BM pathogenesis was associated with the failing attempts to cultivate the *B. miyamotoi* obtained from a sick person, thus making it difficult to perform a whole genome analysis and full-featured genetic assessment of pathogens. Eventually, the problem was successfully solved [11–13]; by the time of the study, there were complete nucleotide sequences of chromosomes and sequences of all or some plasmids of 10 strains of *B. miyamotoi*: Strain FR64b, 3 strains CT13–2396, CA17–2241 and LB-2001 isolated from ticks in the United States [14, 15] and 6 strains Izh-4, Izh-5, Izh-14, Izh-16, Yekat-1, and Yekat-6 isolated from blood of ITBB-BM patients in Russia [11, 12, 16, 17].

B. miyamotoi express variable major proteins (Vmps), against which the affected host develops protective IgM and IgG antibodies during the infection. Based on the homology of amino acid sequences, Vmps are divided into two families: variable large proteins (Vlps) and variable small proteins (Vsps) approximately 330 and 200 amino acids in length, respectively. Vlps are further divided into 4 subfamilies or clusters: alpha (α), beta (β), gamma (γ), and delta (δ). Russian strains demonstrated several variants of expression of Vmps and their combinations [14, 17, 18]. Further on, we will refer to these 5 protein variants (Vlp-δ, Vsp, Vlp-γ, Vlp-α, and Vlp-β) as Vmp types. Note that previously, some authors referred to 4 types (or their representatives) as Vlp15/16, Vsp1, Vlp5, and Vlp18, respectively. The presence of the *Vlp*-β gene in the *B. miyamotoi* genome was first mentioned in the study [17]; we named it similarly to the homologous protein expressed by *B. hermsii* and other pathogens of tick-borne relapsing fevers. The mean difference in terms of amino acid sequences within the Vmp type is 20–40% [19], between the types — 55–80% (see Fig. 4 and Fig. 5 [17]).

Vmp genes are not located on the chromosome; they are located on plasmids, the number of which exceeds ten in the genome of the studied strains of *B. miyamotoi* [17]. It is assumed that at a particular time, a single borrelia expresses only one *Vmp* gene present at the expression site in the immediate vicinity to the bacterial promoter. However, it has a "stock" of *Vmp* genes (from 30 to 50), which, under certain conditions, can replace the former one at the expression site and, therefore, change the antigenic properties of the strain [14, 17].

In epidemiology, the identification of a specific-class protein inherent in a particular strain is instrumental for evaluation of the intraspecific diversity of pathogens circulating at different times within different territories and in different sources (reservoirs). Information about antigenic properties of pathogens can be used in clinical practice for a prognosis of the course and outcome of the infection process as well as for development of preventive immunization products to enhance the epidemiological control of infectious diseases. In case of *B. miyamotoi* and other borrelia causing tick-borne relapsing fevers and Lyme disease, the major proteins can become the target for antigenic typing.

The direct serotyping of borrelia causing ITBB-BM offers poor information so far, due to the limited number of isolated strains of *B. miyamotoi*. Identification of antigen specific antibodies produced during the disease is theoretically possible [20, 21], though it requires creating a set of antigens, which may be not commercially available so far, as well as additional equipment when using planar protein-based immunochips; such equipment is not available in most of the diagnostic laboratories [7, 22–26]. Furthermore, in some cases, antibodies against Vmp can produce cross-reactions; for example, antibodies against Vlp-δ can partially interact with antigens of Vlp-γ and vice versa.

In evaluation of the antigenic diversity of *B. miyamotoi*, new avenues can be opened up by PCR-based molecular techniques targeted at detection or sequencing of nucleotide sequences encoding antigens. Similar approaches have been developed and are successfully used in Russian practical epidemiology for other pathogenic microorganisms [27], for example, pneumococci [28].

Considering the above, **the purpose** of this work was to create a PCR-based technique and to use it for identification of variable major proteins expressed by *B. miyamotoi* strains circulating in Russia.

Materials and methods

DNA sequences

To analyze sequences of *Vmp* gene fragments and to select oligonucleotides for a real-time PCR (rtPCR), we used nucleotide sequences of strains Izh-4, Izh-5, Izh-14, Izh-16, Yekat-1, and Yekat-6 deposited in the GenBank (accession numbers CP024390-CP024407, CP024205-CP024222, CP024371-CP024389, CP024351-CP024370, CP024333-CP024350, and CP024316-CP024332, respectively) [16, 17] along with the analysis of primary sequences of *Vmp* gene promoters [14, 18].

DNA extraction, PCR, and sequencing

The DNA was extracted from biological samples

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Table 1. Characteristics of biological samples containing *B. miyamotoi* DNA

DNA source	Territory	Years	Number of samples
Blood of patients with ixodes tick-borne borreliosis caused by *B. miyamotoi*	Sverdlovsk region	2015–2018	84
	Udmurt Republic	2016	6
	Chelyabinsk region	2019	3
	Vologda region	2017	1
	Novosibirsk region	2012, 2017, 2018	44
	Kemerovo region	2018	16
	Krasnoyarsk Territory	2017–2019	14
	Irkutsk region	2014	2
	Khabarovsk Territory	2014	2
	Total		172
Suspensions of ticks of *Ixodes* genus	Novosibirsk region	2012, 2014, 2017	58
	Tomsk region	2014	17
	Altai Republic	2016	6
	Sverdlovsk region	2013, 2014	18
	Amur region	2016	10
	Total		109

collected from ITBB-BM patients (leukocyte residue in 100 μl of the supernatant, i.e. blood plasma) by using a RIBO-prep kit in accordance with the instructions applicable to the AmpliSens® *Borrelia miyamotoi*-FL reagent kit (registration certificate RZN 2018/7316 dated 3/7/2018). The samples from ticks collected in the Novosibirsk Region and in the Far East were provided by E.I. Bondarenko. The presence of the *B. miyamotoi* DNA (the *glpQ* gene region) was confirmed with a RealBest DNA *Borrelia miyamotoi* diagnostic PCR test (Vector-Best JSC, Novosibirsk), registration certificate No. RZN 2014/1405 dated 26/5/2017 [29]. S.Yu. Kovalev provided DNA-containing *B. miyamotoi* samples from ticks collected in the Sverdlovsk and Tomsk Regions.

In the PCR identifying *Vmps*, the reaction mixture in a total volume of 25 μl contained primers, probes, and dNTPs (0.44 mM) — 10 μl; Polymerase TaqF reagents — 0.5 μl and RT-PCR-mix-2 FEP/FRT – 4.5 μl; the extracted DNA — 10 μl. The multiplex rtPCR was performed by using a Rotor-Gene Q cycler (Qiagen) in accordance with the following protocol: 95ºC — 15 min (1 cycle); 95ºC — 5 sec/60ºC — 20 sec/72ºC — 10 sec (45 cycles with detection of a fluorescent signal at 60ºC). When analyzing the amplification results, the threshold line was set at 10% of the maximum value of the fluorescent signal for each of the 4 detection channels. All reagents for PCR were made at the Central Research Institute of Epidemiology.

The PCR products were sequenced by using a 3500xL genetic analyzer and the respective reagents from Applied Biosystems in accordance with the manufacturer's instructions.

Biological material (sources of B. miyamotoi DNA)

To check the efficiency of the new technique, we used biological samples collected from patients diagnosed with erythema-free ITBB (172 samples from 172 patients) [6–8, 29–31] and ixodid tick suspensions (109 samples) [32, 33]; the ticks were collected in different regions of Russia for the purpose of conducting studies focused on the epidemiology and clinical presentation of the infection caused by *B. miyamotoi* (**Table 1**). All patients signed an informed consent form in accordance with, and approval from, the institutional review boards of the Council on Bioethics of the Central Research Institute of Epidemiology, Moscow (Minutes no. 83 of 26.06.2018) and the Council on Bioethics of the Izhevsk State Medical Academy (Minutes no. 17 of 24.12.2012). Blood samples were generally collected from patients at fever peak, i.e. at the peak of spirochetemia. All samples contained *B. miyamotoi* DNA; its presence was confirmed by tests employing an AmpliSens® *Borrelia miyamotoi*-FL reagent kit.

Statistical methods

The lower and upper limits of the confidence interval for samples of a specific type were estimated by using the Wilson method [34] at 0.95 CI[1]. The IBM SPSS Statistics 19 program and Fisher's exact test were used to estimate the significance of differences in distributions of qualitative variables in the groups [35].

[1] Sergeant E.S.G. Epitools Epidemiological Calculators. Canberra: Ausvet; 2018. Available at: http://epitools.ausvet.com.au. In particular, https://epitools.ausvet.com.au/ciproportion

Table 2. Oligonucleotides for amplification of nucleotide sequences encoding expressed Vlps and Vsps

Genes	Oligonucleotide (concentration, μM)	5'–3'-nucleotide sequences
Vlp, *Vsp*	VMP-F2 (0,2)	TTATAAAgAATTTgAAAAgTAAgATTCTTgCACTAT
Vlp-δ	VLPRI-Ri (0,28)	CCCTTTCCCTAAATTAgCTATCgAAgTT
	VLPR-IZ (0,12)	FAM–CCTTTTgTggATCTTCCCCTCCTCCATTATTACATCC–BHQ1
	VLPR-IZ-L4 (0,12)**	FAM–gg+A+TC+T+TCCCC+TCC+TCC+AT–BHQ1
Vsp	I-16-2-Ri (0,28)	AATCACTgTCCCATCAgCCTTTg
	I-16-2-IIIZ (0,12)	R6G–ggTCCCCCACTTCCACAAgATATCATTAC–BHQ1
	I-16-2-IIIZ-L2 (0,12)**	R6G–CCCCCAC+TT+CC+AC+AA+gA+TA+TC–BHQ1
Vlp-γ	I-14-2-Ri (0,28)	CTgACTTTAAAAATCTACTCTgAggACTCTCT
	I-14-2-IIZ3 (0,12)**	Cy5–CCCgC+T+ACT+A+T+TACAgC+TCACg–BHQ2

Note. Note. *FAM, R6G, and Cy5 — fluorophores; BHQ1 and BHQ2 — fluorescence quenchers. **Alternative probes VLPR-IZ-L4 and I-16-2-IIIZ-L2 have improved thermodynamic parameters, as their structure, like the structure of the I-14-2-IIZ3 probe, incorporates locked nucleic acid (LNA) nucleotides preceded by the + sign. In VLPR-IZ and I-16-2-IIIZ probes, the nucleotide sequences corresponding to probes VLPR-IZ-L4 and I-16-2-IIIZ-L2 are underlined.

Results

The rtPCR technique for antigenic evaluation of B. miyamotoi

The genome of *B. miyamotoi* contains a considerable number of sequences homologous to *Vlp* and *Vsp* genes; the sequences are located on the expression plasmid or on storage plasmids. For the purpose of detection of the sequences, which presumably have corresponding protein products, the annealing site for a forward primer was located in the promoter region of the lpB plasmid specific in sequence [14]. The annealing sites for reverse primers and probes were located on the sequences corresponding to *Vlp* and *Vsp* genes. The selected oligonucleotides are shown in **Table 2**.

The annealing site for the common VMP-F2 primer is located in the promoter region. The annealing sites for other primers and probes detected in green, yellow, and red channels are located in the regions corresponding to Vlp-δ, Vsp, and Vlp-γ proteins. The reaction mixture contained an internal positive control for detection of the *B. miyamotoi* DNA via the orange channel — diagnostic primers and a probe used in the AmpliSens® *Borrelia miyamotoi*-FL reagent kit.

To validate the technique, we used DNA samples of Russian *B. miyamotoi* isolates. In all cases, the identification revealed only those types of expressed Vmps, which were inherent in these strains according to the data of whole-genome sequencing [16, 17], i.e. Vlp-δ in strains Izh-4 and Izh-5, Vlp-γ in Izh-14, Vsps in Yekat-1 and Yekat-6 as well as both Vlp-δ and Vsp in Izh-16.

Identification of expressed Vmp B. miyamotoi in biological samples

The results of identification of *B. miyamotoi* Vmp types in the biological samples collected from ITBB-BM patients and ticks are shown in **Table 3**. Out of 281 tested samples, 51 (18%) failed to demonstrate a potentially expressed *Vmp* gene: the signal was detected only in the orange channel. In other 230 samples, the positive signal was detected; the median values of the threshold cycles and their dispersion were as follows: for the green channel — 31 and 19-42, for the yellow channel — 30 and 21-42, for the red channel — 30 and 21-41. These values correlated with the amplification parameters of the internal positive control – the diagnostic target in the *B. miyamotoi* genome, which was detectable in the orange channel: the median value of the threshold cycles — 28; the dispersion — 18-37.

Negative results (Table 3, line 7) are much less often observed among samples from patients than among samples from ticks ($p = 0.0002$; two-sided Fisher's exact test). At the same time, the proportion of samples where *Vmps* of only one type are detected (line 10) is almost identical among the clinical samples and ticks ($p = 0.7$), while the proportion of the samples where two types of *Vmps* (line 11) are concurrently detected is significantly higher among the clinical samples ($p = 0.0003$), which explains the decrease in the number of negative samples. The more thorough examination of the types of the *Vmp* gene and their combinations detected at the expression site (lines 1–6) spotlights the *Vlp*-δ and *Vsp* combination (line 4) frequently detected in the clinical samples ($p = 0.00003$). Any other differences in the occurrence of *Vmp* genotypes in the samples from ITBB-BM patients and ticks are not significant.

Lines 1–6 in Table 3 clearly show that the occurrence of *Vmp* types ranges from higher to lower rates: *Vlp*-δ → *Vsp* → *Vlp*-γ. However, the occurrence assessment based on positive signals in each of the detection channels (lines 12–15) will be more visible and reliable; the signal typical of the *Vmp* type is recorded any time, no matter whether it is detected as a single signal or together with the signal from another *Vmp* type. Such analysis confirms the *Vlp*-δ → *Vsp* → *Vlp*-γ ranking

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and highlights the fact that the occurrence of the most widely spread *Vmp* types is identical among patients with acute ITBB-BM infection and in tick vectors (the probability that the null hypothesis implying there are no differences is true is 0.94).

The regional differences in terms of expression of different *Vmp* types normally do not demonstrate statistical significance, as only 10–20 samples from multiple regions have been studied so far (Table 1). Nevertheless, it should be noted that the samples from ITBB-BM patients from the Sverdlovsk Region were negative less frequently (2.4%) than the samples from the Novosibirsk Region (15.9%), $p = 0.008$. At the same time, among the positive samples from ITBB-BM patients from the Sverdlovsk and Novosibirsk Regions, the relative proportions of *Vlp*-δ, *Vsp,* and *Vlp*-γ do not differ ($p = 0.71$) and are close to the values shown in lines 12–14 in Table 3. On the other hand, the proportion of negative samples from ticks from the Sverdlovsk Region (39%) and from the Novosibirsk Region (29%) and relative proportions of *Vlp*-δ, *Vsp* and *Vlp*-γ in ticks do not differ significantly ($p > 0.45$).

Confirmation of the reliable identification of expressed B. miyamotoi Vmps

Among some samples, nucleotide sequences of *Vmp* gene fragments located at the expression site were also identified by sequencing of the respective genomic fragments. Based on the rtPCR data, the *Vsp* gene was expressed in 19 out of the sequenced samples, while the *Vlp*-δ – in 23, and the *Vlp*-γ — in 11 samples.

In all cases, the sequencing confirmed the reliability and accuracy of the rtPCR results in DNA samples from ticks and patients' blood as well as the match between the primer and probe biding sites in the target nucleotide sequences and the sequences of primers and probes, which were initially selected with reference to the data of whole-genome sequencing of 6 Russian isolates.

Discussion

The new technique demonstrated sufficient efficiency in the analysis of *B. miyamotoi* DNA-containing biological samples; it can be extensively used as it is; it also has good prospects for improvement and

Table 3. Distribution of *Vmp* genotypes expressed by *B. miyamotoi Vmp* and detected in the biological samples

No	The type of *Vmp* gene at the expression site	Number of samples from patients	Samples of a specific type, %	The lower and upper limits of the confidence interval*	Number of samples from ticks	Samples of a specific type, %	The lower and upper limits of the confidence interval*
1	*Vlp*-δ only	63	36,6		38	34,9	
2	*Vsp* only	42	24,4		28	25,7	
3	*Vlp*-γ only	12	7,0		5	4,6	
4	*Vlp*-δ + *Vsp*	30	17,4		2	1,8	
5	*Vlp*-δ + *Vlp*-γ	1	0,6		1	0,9	
6	*Vsp* + *Vlp*-γ	5	2,9		3	2,8	
7	Not identified	19	11,1	7,2–16,6	32	29,4	21,6–38,5
8	Total number of samples	172	100		109	100	
9	Total number of positive samples (from 172 or 109 samples)	153	89,0	83,4–92,8	77	70,6	61,5–78,4
10	Among them — only one *Vmp* type is identified	117	68,0	60,7–74,5	71	65,1	55,8–73,4
11	Among them — two *Vmp* types are identified##	36	20,9	15,5–27,6	6	5,5	2,6–11,5
12	*Vlp*-δ, total&	94	49,7	42,7–56,8	41	49,4	38,9–59,9
13	*Vsp*, total&	77	40,7	34,0–47,9	33	39,8	29,9–50,5
14	*Vlp*-γ, total&	18	9,5	6,1–14,6	9	10,8	5,8–19,3
15	Positive signals received, total&	189	100		83	100	

Note. *At confidence probability (the confidence level) of 0.95; **the positive signal only in the orange detection channel: the detection of *B. miyamotoi* DNA in the sample; #the positive signal in one of the green, yellow or red channel, and in the orange detection channel: the detection of the DNA encoding Vmps in the sample; ##the positive signal in 2 channels out of 3 (green, yellow, or red) and in the orange detection channel; &singly or together with another type of *Vmp*.

upgrading. There are two main paths of development: To achieve the similar efficiency in analysis of other widely spread *B. miyamotoi* genotypes – American and European — and to explore the possibility to identify and assess the expression of less common *Vmp* types — *Vlp*-α and *Vlp*-β. The work in this field has started.

The research of the fundamentally new aspects of genetics and epidemiology of *B. miyamotoi* raised a number of new questions; however, at this stage, any possible answers can be only hypothetical. Firstly, why do some samples of *B. miyamotoi* DNA from patients' blood (11%) remain negative? The preliminary results show that when additional *Vlp*-α and *Vlp*-β genes are included in the identification system, the percentage of negative results decreases slightly. Therefore, they can be explained by the limited sensitivity of rtPCR or by a low bacterial load in blood samples or by the presence of unknown mutations at the binding sites for primers and probes. The first explanation looks more plausible, as the thorough prospective study of ITBB-BM patients in Ekaterinburg [7, 11] demonstrated that the timely selection, storage, and transportation of blood and/or DNA samples following the cold chain requirements can lead to a 3% decrease in the proportion of negative samples. The second question: Why is the proportion of negative *B. miyamotoi* DNA samples from ticks (29%) is significantly higher? It can be assumed that *B. miyamotoi* present in ticks do not need Vmp expression, at least, the expression of the most widespread Vmp types: Vlp-δ, Vsp, or Vlp-γ. Such phenomenon is typical of *B. burgdorferi sensu lato*, which start expressing OspC (outer surface protein C) only in human blood where borrelia OspC performs protective functions or, more specifically, inhibits the activation of the complement cascade on their surface [36]. Recently, it has been found that *B. miyamotoi* Vlp-δ and Vlp-α also inhibit the bypass activation of the complement cascade [37].

Finally, the most intriguing questing is why in the blood samples from ITBB-BM patients (lines 11 and 4 in Table 3) our rtPCR technique quite often identifies the expression of two *Vmp* types (21%), most frequently, *Vlp*-δ and *Vsp* concurrently (17%). If we assume that "a single borrelia expresses only one *Vmp* gene present at the expression site", we should also assume that concurrently there are several *B. miyamotoi* subpopulations coexisting in the patient's body. It is not a double infection (mixed infection) with two different borrelia strains; it is the result of quasispecies emerging from the same "ancestor" present in the infecting tick. Quasispecies can result from phase variations, i.e. protein synthesis switch, in part of the population of the pathogen colonizing the host. Phase variations are usually of adaptive significance. In case of surface proteins having antigenic properties, specifically Vmps, the alteration of the antigenic pattern may make borrelia less sensitive to bactericidal antibodies developed against the initial variants of antigens. Such phenomenon known as immune evasion helps the pathogen extend its existence in the host, at least, until antibodies against new variants of antigens are developed. Genetic mechanisms of phase variations are extensively explored for the VlsE protein of *B. burgdorferi sensu lato* [38]. The experiments on laboratory animals and/or borrelia culture showed the actual possibility of Vmp synthesis switch for *B. hermsii* [39, 40] and *B. miyamotoi* [18]. With acute ITBB-BM disease, more than half of the patients produce antibodies against several Vmp types [7, 24], thus implying the existence of immune evasion *in vivo*, similarly to the presence of several *Vmp* genotypes expressed in the same clinical sample, as it was found in this study. During the laboratory infection of small rodents with Russian strains of *B. miyamotoi*, we documented the manifestations of immune evasion by using direct genetic and serological methods [41]. Presumably, it is immune evasion that is responsible for fever relapses in ITBB-BM patients having no adequate antibiotic therapy [42]. The relapsing course is typical of tick-borne relapsing fevers and other recurrent fevers, for example, for such life-threatening disease as louse-borne relapsing fever or *Borrelia* recurrentis infection [2].

With the increasing number of clinical and biological field samples containing pathogenic DNA, the new technique can be used in further epidemiological studies in Russia and worldwide to produce a clearer picture of the antigenic spectrum of *B. miyamotoi* strains circulating in different regions and to compare it with the profile of more conserved regions of their genome, for example, by using the multilocus sequence typing method [27]. It will help understand the stages of evolution and spread of *B. miyamotoi sensu lato* – pathogens causing ixodid tick-borne borreliosis, which is very similar to the well-known Lyme disease in its epidemiological significance.

The second strand of research addresses the analysis of the relationship between the complex of clinical manifestations and complications of ITBB-BM and the Vmp type expressed at the onset of the disease, including the possibility of expression switch (immune evasion) during the infection process.

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