JOURNAL OF MICROBIOLOGY, EPIDEMIOLOGY AND IMMUNOBIOLOGY. 2022; 99(5)
DOI: https://doi.org/10.36233/0372-9311-296
ORIGINAL RESEARCHES

Original article
https://doi.org/10.36233/0372-9311-296

Optimized multilocus sequence analysis for laboratory identification of pathogens of ixodid tick-borne borreliosis

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Abstract

Introduction. The most common etiological agents of ixodid tick-borne borreliosis (ITBB) in Russia are *Borrelia garinii*, *B. afzelii*, *B. bavariensis.* Multilocus sequence typing and multilocus sequence analysis (MLSA) have been used in recent studies for *Borrelia* species identification. The results of using the MLSA scheme for identification of pathogens causing erythemic forms of ITBB have been presented earlier.

The **purpose** of the study was to explore the possibility of MLSA optimization for laboratory identification of ITBB pathogens. Objectives: comparative analysis of nucleotide sequences of 6 conserved genes (*rrs*, *hbb*, *fla*, *groEL*, *recA*, *ospA*) and the *rrfA-rrlB* intergenic spacer, which are recommended by the MLSA protocol; identification of the minimum set of genes, the concatenated sequences of which are essential for species identification of *Borrelia* isolates.

Materials and methods. The sequences of the above loci of 23 reference isolates collected from patients with ITBB and assigned, using MLSA, to *B. bavariensis* were compared with the sequences of similar genes of other *Borrelia* species available in international databases. The UPGMA method was used to build and analyze dendrograms based on the obtained data.

Results. The sequences of *ospA* gene loci of reference species demonstrated the greatest difference (not less than 8.5%) from the sequences of the above gene in other analyzed species of *Borrelia*; approximately similar species-related differences (not less than 6.7%) were demonstrated by the comparison of *recA* gene sequences. The sequences of the identified variants of these two genes in *B. bavariensis* differed from the sequences of the similar genes in the most closely related species — *B. garinii.* The dendrogram of the concatenated nucleotide sequences of *recA* and *ospA* genes demonstrated that it was totally consistent with the results of identification of the isolates based on the MLSA protocol.

Conclusion. The optimized approach to MLSA of the *B. burgdorferi* sensu lato group suggests that species identification should be based on the concatenated analysis of loci of only two genes (*recA* and *ospA*) out of 7 loci recommended by the MLSA protocol.

Keywords: *ixodid tick-borne borrelioses, MLSA, identification of a pathogen, laboratory diagnostics*

Ethics approval. The study was conducted with the informed consent of the patients. The research protocol was approved by the Ethics Committee of the National Research Center for Epidemiology and Microbiology named after the honorary academician N.F. Gamaleya (protocol No. 18, February 21, 2022).

Acknowledgement. The authors extend their gratitude to their Institute and laboratory associates who provided consulting, support, and assistance in their work.

Funding source. The study was supported by funds received for the Federal Task commissioned to the laboratory.

Conflict of interest. The authors declare no apparent or potential conflicts of interest related to the publication of this article.

For citation: Golidonova K.A., Korenberg E.I., Gintsburg A.L. Optimized multilocus sequence analysis for laboratory identification of pathogens of ixodid tick-borne borreliosis. *Journal of microbiology, epidemiology and immunobiology = Zhurnal mikrobiologii, èpidemiologii i immunobiologii.* 2022;99(5):514–524.
DOI: https://doi.org/10.36233/0372-9311-296

Научная статья
https://doi.org/10.36233/0372-9311-296

Оптимизация мультилокусного сиквенс-анализа для лабораторной идентификации возбудителей иксодового клещевого боррелиоза

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Аннотация

Введение. Наиболее широко распространённые этиологические агенты иксодового клещевого боррелиоза (ИКБ) в России — *Borrelia garinii*, *B. afzelii*, *B. bavariensis.* Для определения видовой принадлежности боррелий в современных исследованиях используют методы мультилокусного сиквенс-типирования и сиквенс-анализа (МЛСА). Ранее были продемонстрированы результаты применения схемы МЛСА для идентификации возбудителей эритемных форм ИКБ.

Цель работы — изучить возможность оптимизации МЛСА для практической лабораторной идентификации возбудителей ИКБ. Задачи: сравнительный анализ нуклеотидных последовательностей локусов 6 консервативных генов (*rrs*, *hbb*, *fla*, *groEL*, *recA*, *ospA*) и межгенного спейсера *rrfA-rrlB*, рекомендованных протоколом МЛСА; выявление минимальной совокупности генов, сцепленные сиквенсы которых позволяют идентифицировать видовую принадлежность изолята боррелий.

Материалы и методы. Сиквенсы вышепредставленных локусов 23 «контрольных» изолятов, полученных от больных ИКБ и предварительно типированных методом МЛСА как *B. bavariensis*, использованы для сравнительного анализа с последовательностями аналогичных генов других видов боррелий, имеющимися в международных базах данных. На основе этого материала методом UPGMA построена и проанализирована серия дендрограмм.

Результаты. Сиквенсы локусов гена *ospA* контрольного вида показали наибольшее отличие (не менее 8,5%) от последовательностей этого гена у других сравниваемых видов боррелий; близкие показатели видовых отличий (не менее 6,7%) продемонстрировало сравнение сиквенсов гена *recA*. Сиквенсы выявленных вариантов двух этих генов у *B. bavariensis* отличались от последовательностей аналогичных генов у наиболее близкого вида — *B. garinii.* Дендрограмма сцепленных нуклеотидных последовательностей генов *recA* и *ospA* продемонстрировала её идентичность результатам идентификации изолятов по полному протоколу МЛСА.

Заключение. Предложен оптимизированный подход к МЛСА боррелий группы *B. burgdorferi* sensu lato, который сводится к выявлению их видовой принадлежности на основании специфики результата сцепленного анализа локусов только двух генов (*recA* и *ospA*) из 7 локусов, рекомендованных протоколом этого метода.

Ключевые слова: *иксодовые клещевые боррелиозы, МЛСА, идентификация возбудителя, лабораторная диагностика*

Этическое утверждение. Исследование проводилось при добровольном информированном согласии пациентов. Протокол исследования одобрен Этическим комитетом Национальный исследовательский центр эпидемиологии и микробиологии имени почётного академика Н.Ф. Гамалеи (протокол № 18 от 21.02.2022).

Благодарность. Авторы выражают признательность институту и коллегам лаборатории, оказывавшим консультирование, поддержку и помощь в работе.

Источник финансирования. Исследование финансировалось за счёт средств, полученных для выполнения Государственного задания лаборатории.

Конфликт интересов. Авторы декларируют отсутствие явных и потенциальных конфликтов интересов, связанных с публикацией настоящей статьи.

Для цитирования: Голидонова К.А., Коренберг Э.И., Гинцбург А.Л. Оптимизация мультилокусного сиквенс-анализа для лабораторной идентификации возбудителей иксодового клещевого боррелиоза. *Журнал микробиологии, эпидемиологии и иммунобиологии.* 2022;99(5):514–524.
DOI: https://doi.org/10.36233/0372-9311-296

Introduction

Ixodid tick-borne borrelioses (ITBB) is a group of etiologically independent chronic or recurrent spirochetal natural and focal transmissible infections that can affect different systems and organs. Natural foci of ITBB are commonly found in forest areas of Russia. Borrelioses rank among the natural and focal zoonotic diseases with the highest incidence rates [1, 2].

Pathogens of ITBB are spirochetes of the *Borrelia burgdorferi* sensu lato complex that includes more than 20 species [3], 8 out of which have been found in Russia [4]. The most common etiological agents

DOI: https://doi.org/10.36233/0372-9311-296

of ITBB in Russia and in most regions of Eurasia are *B. garinii*, *B. afzelii* and *B. bavariensis* [1, 5]. *B. garinii* frequently causes moderate types of infection with common symptoms, and erythema migrans accounts for nearly 60% of cases. The infection process caused by *B. afzelii* is often mild and is accompanied by the erythema migrans skin lesion that develops at the tick bite site [6, 7]. ITBB-associated erythema migrans with diverse clinical manifestations can be also caused by *B. bavariensis* [5]. Therefore, erythema migrans, which is seen by clinicians as the only pathognomonic sign of ITBB, more or less frequently can be caused by any of the most common etiological agents of the diseases of this group.

Even when the patient has erythema, the severity and size of which can depend on several reasons, including similar skin manifestations of different etiology, in most cases the clinical diagnosis of ITBB must be confirmed by laboratory tests. Addressing the problem, different serological test methods are generally used: indirect immunofluorescence assays, enzyme-linked immunosorbent assays, immunoblotting, multiplex assays (including a phosphorescence immunoassay) and others. These methods are characterized by different specificity and sensitivity levels, which tend to vary during the periods following the onset of the ITBB disease. They are usually less effective during the first weeks of the disease and are completely useless in seronegative cases that are not uncommon. The method of direct isolation of a pathogen by seeding samples onto the medium is highly time-limited; besides, the pathogen can be absent (especially during the acute stage of the infection process) in an organ, tissues and biological fluids of the patient. In addition, cultures of biomaterials cannot be used for large-scale laboratory diagnostic ITBB tests due to the length of time required for *Borrelia* growth in positive samples. However, polymerase chain reaction (PCR) can be used to amplify *Borrelia* DNA from different materials collected from patients. Its clinical sensitivity in cerebrospinal fluid tests is as high as 20%; in plasma tests it ranges from 30% to 50%; in synovial fluid tests it can be higher than 70%, and in tests of skin biopsy samples, it reaches 80% [1, 8].

Currently, for species-level identification of *Borrelia*, researchers use methods of sequence typing (MLST) [9] and sequence analysis (MLSA) [1] based on identification of specific nucleotide sequences of conserved genes [10]. As applied to species identification of the ITBB etiology, each of these methods has its own distinctive features. Both methods are based on the analysis of nucleotide sequences of loci of 7–8 different conserved genes (including the *rrfA-rrlB* intergenic spacer), which are recommended for building final dendrograms based on concatenated data. In MLST, to build dendrograms, researchers use matrices of mismatches in the allelic profile, ignoring the number of nucleotide differences between alleles, which are assigned different numbers whether or not nucleotide sequences differ at one site or at many sites [10]. This method has been used successfully for a long time for species identification in different groups of pathogenic bacteria; for some of them, this method is seen as the "gold standard" [11–13]. In MLSA, on the contrary, to identify the phylogenetic relationships between the tested samples, researchers use concatenated sequences of housekeeping gene fragments [4, 10]. Depending on the source and DNA concentration, the MLST scheme recommends a single-round or a two-round PCR with amplification of longer fragments of conserved genes [14], while the MLSA scheme recommends a single-round PCR of shorter loci [10, 15]. Therefore, for species identification of most isolates of *Borrelia*, MLSA is undoubtedly less costly than MLST. The advantages and disadvantages of these methods were discussed in our previous publication [16].

We demonstrated the results of MLSA scheme application for identification of the etiological agent of ITBB-associated erythema migrans using the clinical material (more than 20 isolates collected from skin biopsy samples and plasma of patients). It was found that all isolates belonged to *B. bavariensis*; the variability of nucleotide sequences of loci of the analyzed conserved genes was identified [5]. The above findings defined the **aim** of our study: Exploration of the possibility to optimize MLSA for laboratory identification of ITBB pathogens.

The following objectives were set: The comparative quantitative analysis of intraspecific heterogeneity of nucleotide sequences of loci of 6 conserved chromosomal genes (*rrs, hbb, fla, groEL, recA, ospA*) and the intergenic spacer (*rrfA-rrlB*), which are recommended for MLSA; identification of the minimum set of genes, concatenated nucleotide sequences of which make it possible to identify species membership of the isolate of the *B. burgdorferi* sensu lato group.

Materials and methods

A total of 23 isolates (Fig. 1–3 — Hs), which are stored at the *Borrelia* Museum at the laboratory of infection transmitters at the Gamaleya National Research Center of Epidemiology and Microbiology, were used to study nucleotide sequences of loci of 6 genes (listed above) and the *rrfA-rrlB* spacer, which are recommended for MLSA [15]. The isolates were obtained from patients by seeding of skin biopsy samples onto the BSK medium; the isolates were collected from the peripheral area of erythemas (17 isolates) or from plasma (6 isolates) of patients of the Regional Clinical Hospital of Infectious Diseases in Perm (the east of Eastern Europe; 58°00' N; 56°15' E); the patients had a localized stage of manifest ITBB with erythema migrans. The studied isolates accounted for at least 30–40% of the average number of all patients with ITBB, who are annually admitted to this clinical hospital [6]. By culturing and

typing of isolates using the PCR-restriction fragment length polymorphism assay for the *rrfA-rrlB* spacer site, we assigned them earlier to *B. garinii NT29* [17, 18]. Based on the results of our study using the MLSA method, these isolates were identified as *B. bavariensis*. Nucleotide sequences of the primers, the PCR procedure and the subsequent sequencing of amplicons were described previously [5, 15, 19].

All the obtained nucleotide sequences were compared against each other (using the BLAST® platform) and with the nucleotide sequences of loci of similar genes in type or reference strains of other species of *B. burgdorferi* sensu lato spirochetes available in databases of GenBank INSDC and PubMLST *Borrelia spp.* The unweighted pair group method with arithmetic mean (UPGMA) was used to build a series of dendrograms using the MEGA-X v. 10.2.4 software with 1000 bootstrap repeats. Most of these dendrograms were analyzed earlier [5, 19]. In this article, we discuss only those that were required for validation of the conclusions and that had not been published previously.

Nucleotide sequences of 10 loci of the studied genes were deposited to the Genbank database (accession numbers MW981426, MZ005315-MZ005321, OM310938–OM310939) and 4 shorter sequences were deposited to the European Nucleotide Archive (accession numbers OD916881–OD916884).

Study results

Comparative analysis of nucleotide sequences of gene loci recommended for MLSA

Table 1 shows similarities between nucleotide sequences of loci of each gene in all the studied isolates identified as *B. bavariensis* based on the MLSA results, including the earlier published data for *rrs*, *fla*, *hbb* and *recA* genes [5, 19]. The similarity was significant (up to 99.5–100%) and did not demonstrate any difference in isolates from skin biopsy samples and in isolates from plasma. Some sequences of the *ospA* gene had minimum similarity (95.3%) with loci of other genes. However, the sequences of this gene differed by 9.5–10.9% from the sequences of the same gene *B. garinii 20047T* (the closest species by the overall genome structure [20]) and by 8.5% (more than the other genes) from the set of sequences of commonly circulating *Borrelia* of the *B. burgdorferi* sensu lato group. The similar variables for sequences of the *recA* gene were slightly lower compared to the *ospA* gene, though higher than in the other studied genes (Table 1). The differences between the nucleotide sequences of the sequenced loci of these two genes and the sequence of the similar genome site of the reference PBi strain of the European subgroup of *B. bavariensis* were significant (up to 8.1%). The above results prompted us to conduct a more detailed analysis of the intraspecific heterogeneity of nucleotide sequences of *recA* and *ospA* genes using our isolates of *B. bavariensis*, including the analysis of their differences from sequences of similar genes in other *Borrelia* species of the *B. burgdorferi* sensu lato complex.

Detailed analysis of the dendrograms of sequences of recA and ospA gene loci and comparison of the above data with the MLSA results

The sequences of *recA* genes in the analyzed isolates showed that all of them fall into a large cluster with three different allelic variants of the Eurasian genetic subgroup of *B. bavariensis*: The nucleotide sequences of most of the isolates (19 of 23) are identical with the SZ variant, 3 are identical with the Prm7504-11 variant and 1 is identical with the Hiratsuka variant (the GenBank accession numbers; **Fig. 1**). This explains a certain dissimilarity among the nucleotide sequences of loci of this gene in different isolates (Table 1). Nevertheless, all sequences of this gene are clearly different from those of *B. garinii* and reference strains of the

Table 1. Variability of the nucleotide sequences of the set of loci (according to the results of 23 studied isolates of *B. bavariensis*) of each gene

Gene (target)	Nucleotide sequence similarity between skin biopsy and plasma isolates, %	Difference from (in %)		
		B. bavariensis PBi	*B. garinii* 20047T	other *Borrelia* species *B. burgdorferi* s. l, not less than
rrs	100	0,4	0,2	0,8
hbb	98,5–100	1,2–1,5	1,2–1,5	4,3
fla	99,5–100	0,6–1,2	1,2–1,8	5,8
groEL	98,7–100	2,2–2,7	2,2–2,7	3,6
recA	97,3–100	0,7–2	2–3,4	6,7
ospA	95,3–100	4,7–8,1	9,5–10,9	8,5
Spacer *rrfA-rrlB*	98,8–100	1,7–2,3	1,7–2,3	4,8
Concatenated sequences of *recA* and *ospA* gene loci	96,1–100	3,6–5,6	6,4–7,8	8,3

JOURNAL OF MICROBIOLOGY, EPIDEMIOLOGY AND IMMUNOBIOLOGY. 2022; 99(5)
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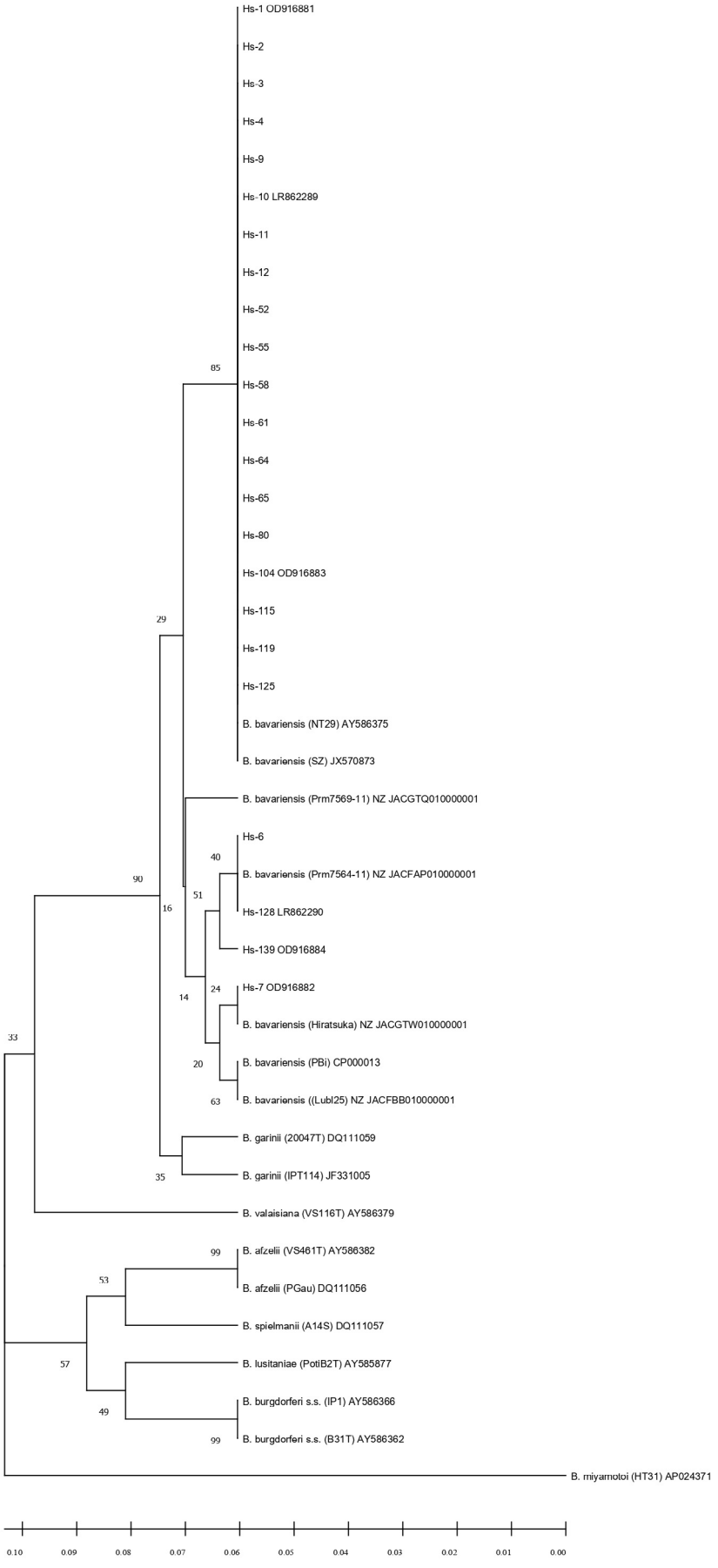

Fig. 1. Dendrogram of nucleotide sequences of the *recA* gene in the studied isolates.

Here and in Fig. 2, 3, the names of strains according to the PubMLST *Borrelia* spp. database are given in parentheses; the square brackets were used to show accession numbers assigned by Genbank or ENA. Hs — isolates from patients. The bootstrap (1000) values are shown as percentage near the respective node.

To identify the congruence between matrices of genetic distances of *recA* and *ospA* genes, both separately and against the matrix of concatenated sequences, we used the Mantel test [24] in Excel with the GenALEx add-in — $R = 0.499$ (*recA*/*ospA*).

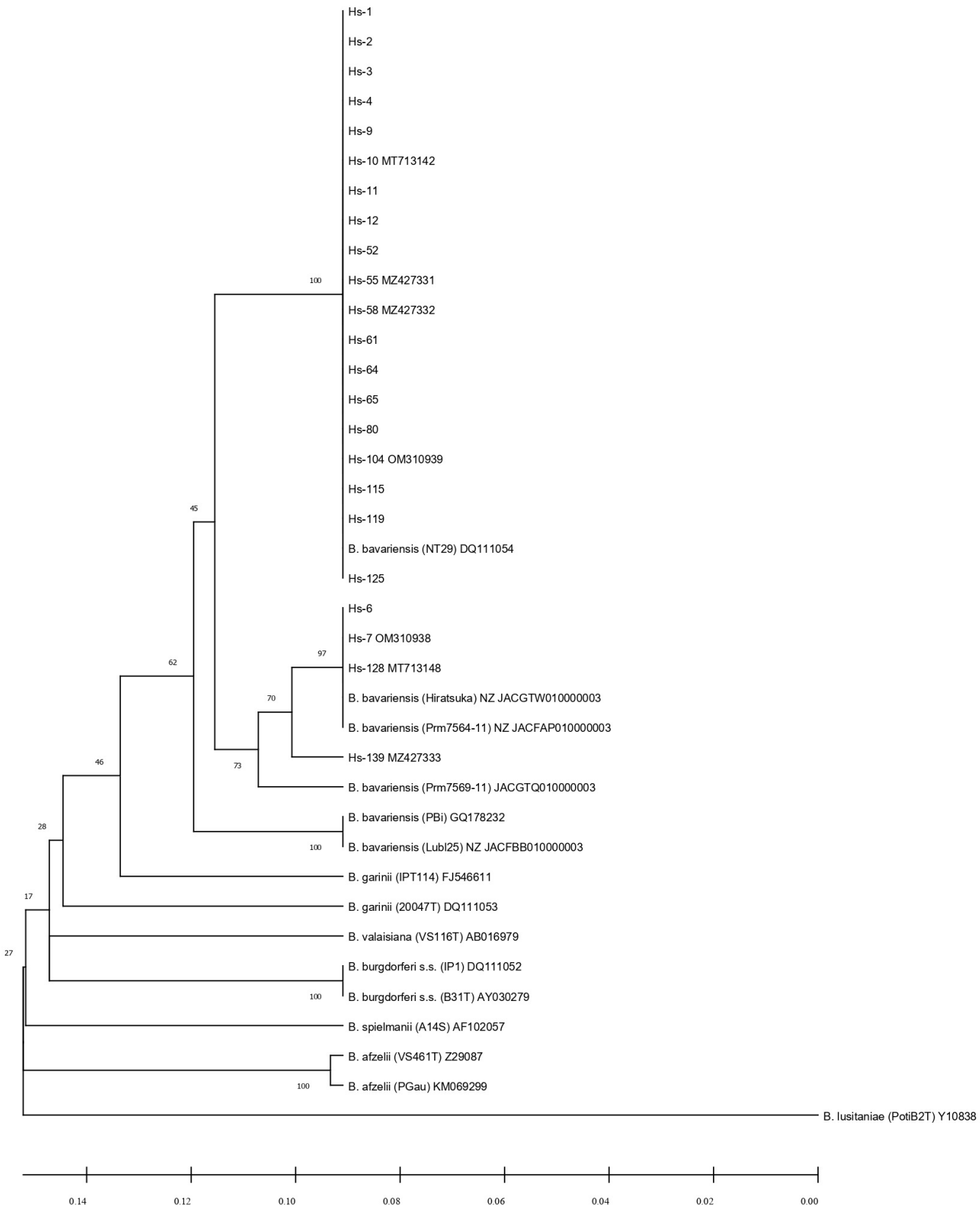

Fig. 2. Dendrogram of nucleotide sequences of the *ospA* gene.
Mantel test — $R = 0.838$ (*ospA*/*recA*).

DOI: https://doi.org/10.36233/0372-9311-296

most common species of *B. burgdorferi* sensu lato and from the sequence of this gene in the prevalent pathogenic *B. miyamotoi*, the taxonomic status of which remains debatable [1]. The similar dendrogram based on the results of sequencing of *ospA* gene loci (**Fig. 2**) does not differ significantly from the previous one.

The MLSA results for 6 selected isolates, which represent all the identified allelic variants of *recA* and *ospA* genes of *B. bavariensis* (Fig. 1, 2), showed their 98.5–100.0% identity with the sequences of different isolates from the Eurasian genetic subgroup of this *Borrelia* species (SZ, Prm7564-11, Prm7569-11, Hiratsuka); the identity was slightly lower with the European isolates, for example, with the reference PBi strain — reaching 97.8–98.4%. At the same time, all genetic variants of *B. bavariensis*, including the PBi strain, have significant differences (i.e. the similarity does not exceed 94.9%), which make it possible to differentiate them from other *Borrelia* species, using MLSA data (**Table 2**). The dendrogram of concatenated nucleotide sequences of two genes (*recA* and *ospA*) of the same isolates shows the existence of two genetic subgroups of *B. bavariensis* (Eurasian and European) and their clear differences from the respective concatenated sequences of other *Borrelia* species from the databases (**Fig. 3**). This proves that the results of identification of *Borrelia* from the *B. burgdorferi* sensu lato group using the analysis of concatenated nucleotide sequences of *recA*, *ospA* gene loci are totally identical with the MLSA results.

Discussion

As previously noted, species identification in the *B. burgdorferi* sensu lato group for *Borrelia* circulating in natural foci and (or) causing ITBB is currently performed by using molecular and biological methods. In the meantime, using one conserved gene or spacer as a target is usually not sufficient for accurate identification of the studied sample [10, 20]. The MLSA and MLST methods require sequencing of loci of several (6–8) genes; however, they are costly, time-consuming and difficult to implement [5, 10, 14–16], especially, when fast indication of the ITBB etiological agent is required at diagnostic laboratories of healthcare facilities.

The detailed analysis of the earlier obtained data characterizing the genetic structure of the pool of *B. bavariensis* isolates [5, 19] led to the conclusion that the nucleotide sequences of *recA* and *ospA* gene loci

Table 2. Similarity (in %) of some "control" isolates with *Borrelia* of various species, the nucleotide sequences of which are available in the GenBank and PubMLST databases

		Hs-7	Hs-10	Hs-55	Hs-104	Hs-128	Hs-139	*B. garinii* 20047T	*B. bavariensis* PBi	*B. bavariensis* NT 29	*B. bavariensis* Prm7564-11	*B. bavariensis* Hiratsuka	*B. spielmanii* A14S	*B. burgdorferi* s. s. B31T	*B. lusitaniae* PotiB2T	*B. valaisiana* VS116T	*B. afzelii* VS461T
Hs-7		100															
Hs-10		98,9	100														
Hs-55		98,9	100	100													
Hs-104		98,9	100	100	100												
Hs-128		100	98,9	98,8	98,9	100											
Hs-139		99,6	98,9	98,8	98,9	99,7	100										
B. garinii 20047T		97,8	97,6	97,5	97,6	97,7	97,3	100									
B. bavariensis	PBi	98,2	98,4	98,3	98,4	98,1	97,8	98,1	100								
	NT29	98,8	100	99,9	100	98,8	98,8	97,6	98,4	100							
	Prm7564-11	100	98,9	98,8	98,9	100	99,7	97,7	98,1	98,8	100						
	Hiratsuka	100	98,9	98,9	98,9	100	99,6	97,8	98,2	98,8	100	100					
B. spielmanii A14S		94,5	94,5	94,5	94,5	94,4	94,4	94,3	94,0	94,5	94,4	94,5	100				
B. burgdorferi s. s. B31T		93,6	93,5	93,4	93,5	93,5	93,5	93,3	93,2	93,5	93,5	93,6	93,6	100			
B. lusitaniae PotiB2T		93,8	93,9	93,9	93,9	93,7	93,9	93,6	93,5	93,9	93,7	93,8	94	93,3	100		
B. valaisiana VS116T		94,5	94,9	94,9	94,9	94,5	94,4	94,2	94,4	94,6	94,5	94,5	94,3	93,8	94,3	100	
B. afzelii VS461T		93,9	93,9	93,8	93,9	93,9	94,0	94,2	94,0	93,9	93,9	93,9	94,5	94,1	93,1	94,2	100

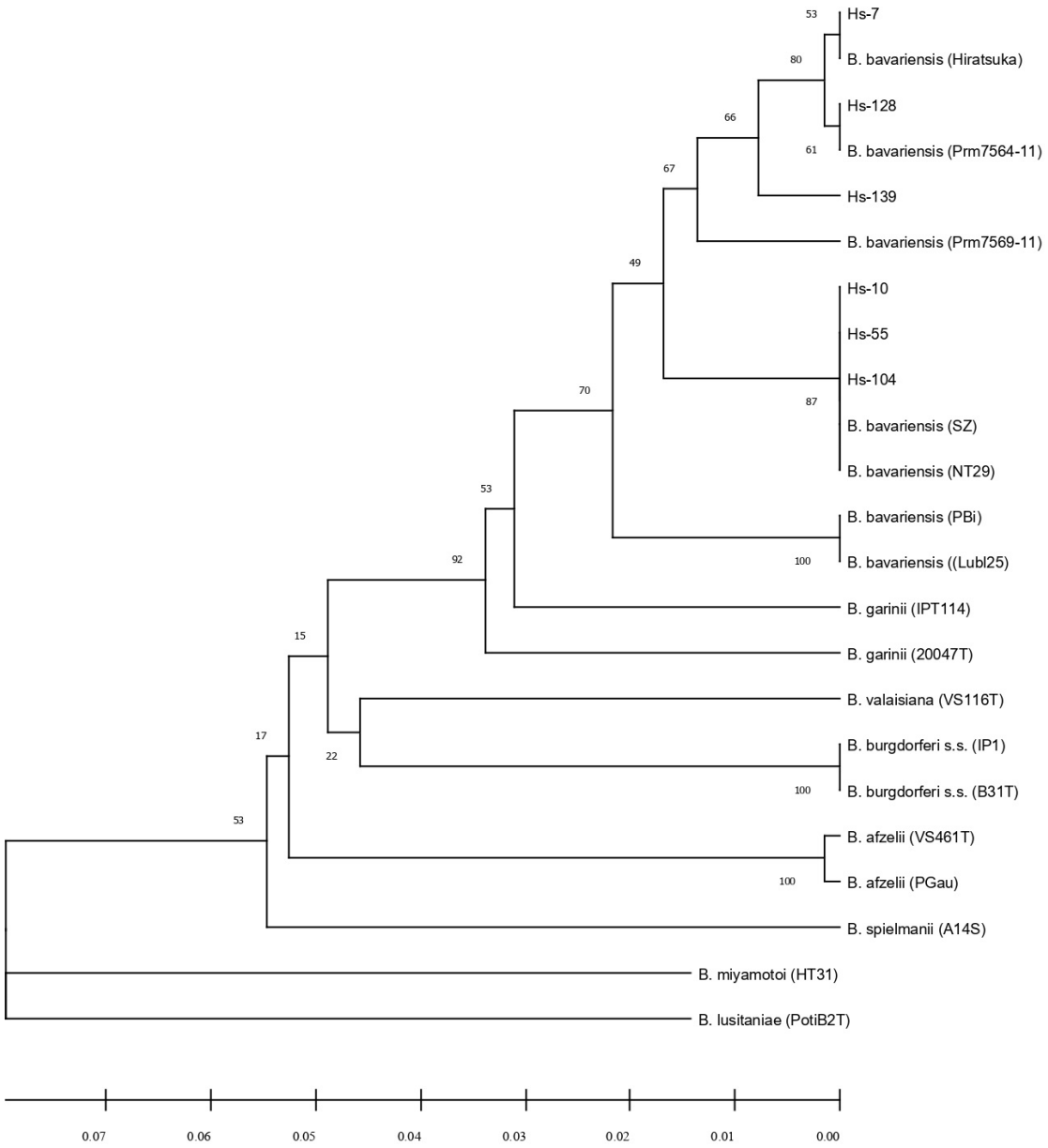

Fig. 3. Dendrogram of concatenated nucleotide sequences of *recA* and *ospA* genes.
Mantel test — R = 0.860 (concatenated/*recA*), R = 0.965 (concatenated/*ospA*).

differ significantly from the sequences of other genes recommended by the MLSA protocol for identification of prevalent species of the *B. burgdorferi* sensu lato group. The earlier attempts were based on the diversity of the structure of each of the *recA* and *ospA* genes [21, 22]. The comparative analysis of our data (Fig. 1 and 2) shows that they demonstrate very similar, but not identical results regarding the genotypes of some isolates. However, the dendrogram of concatenated nucleotide sequences of *recA* and *ospA* gene loci (Fig. 3) demonstrates the species-level and genetic variant-level profiles of the isolates, which are totally consistent with the previously obtained results regarding their typing in accordance with the MLSA protocol.

To perform the "validation" study addressing the objectives set in the article, we used a representative pool of *B. bavariensis* isolates, species and heterogeneity of which had already been studied and identified using this method [5]. The concatenated nucleotide sequences of loci of *recA* and *ospA* genes, which belong to reference strains of the most common species of the *B. burgdorferi* sensu lato group (Fig. 3), have clear differences. Therefore, the species identification of the studied sample can be performed by measuring the maximum similarity between concatenated sequences of these two genes and the concatenated sequences of specific *Borrelia* species of this group, including *B. bavariensis*, or by building a dendrogram. To opti-

mize the laboratory testing process, its template matching the one shown in Fig. 3 can be stored as a file (for example, in the MEGA or any other program). The comparison of the number of genes recommended by the MLSA and MLST protocols against their number, which, based on our data, is required and is sufficient for sequencing and identification of the known species of the ITBB pathogen, shows that the labor costs and financial expenses can be reduced approximately 3–4 times.

Further studies are needed to explore the possibility of differentiation between *Borrelia* from the *B. burgdorferi* sensu lato group and *B. miyamotoi* based on their nucleotide sequences of the *recA* gene, significant differences between which are demonstrated by our data (Fig. 1). This can be of high significance for improvement of the gene diagnostics regarding the ITBB etiology, especially, knowing that the above gene was used together with two other genes for the laboratory test to confirm the disease caused by *B. miyamotoi* [23].

Conclusion

Based on the above studies, we are offering an optimized approach to MLSA of *Borrelia* from the *B. burgdorferi* sensu lato group. It suggests their species identification based on the results of the concatenated analysis of loci only of 2 genes (*recA* and *ospA*) out of 6 and the *rrfA-rrlB* spacer, which are recommended by the protocol of this method. This approach will help decrease costs significantly and will reduce the turnaround time for laboratory tests of the studied samples.

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DOI: https://doi.org/10.36233/0372-9311-296

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Author contribution. All authors made a substantial contribution to the conception of the work, acquisition, analysis, interpretation of data for the work, drafting and revising the work, final approval of the version to be published.

The article was submitted 21.06.2022;
accepted for publication 13.09.2022;
published 30.10.2022

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Участие авторов. Все авторы внесли существенный вклад в проведение поисково-аналитической работы и подготовку статьи, прочли и одобрили финальную версию до публикации.

Статья поступила в редакцию 21.06.2022;
принята к публикации 13.09.2022;
опубликована 30.10.2022