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MOLECULAR AND GENETIC CHARACTERIZATION OF *LEPTOSPIRA* spp. COLLECTION STRAINS FROM THE ST. PETERSBURG PASTEUR INSTITUTE BASED ON 16S rRNA GENE SEQUENCING DATA

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Abstract. Leptospirosis is a zoonotic disease found virtually worldwide. Microscopic Agglutination Test with live leptospira (MAT) is the reference method for the serological diagnosis of leptospirosis. MAT is based on assessing serum potential to agglutinate live reference serovar *Leptospira* maintained at a reference laboratory. At some laboratories having own collections of isolated and reference *Leptospira* strains applicable for serological diagnosis, those microorganisms are maintained for many years by repeated subculturing, that increases markedly a chance of strain cross-contamination. The lack of adequate quality control for reference strains may affect data of epidemiological studies. Control of *Leptospira* spp. reference strains purity and stability of their antigenic composition is very important for diagnosis of leptospirosis. The study objective was to compare the 16S rRNA gene nucleotide sequences of some *Leptospira* strains from the collection of the St. Petersburg Pasteur Institute to with relevant sequences uploaded to GenBank. In this study, 38 *Leptospira* strains were investigated. Nucleotide sequences of 36 strains were deposited in the international GenBank database, inconsistencies were revealed in two strains. The study found that the control *Leptospira* strains from the collection of the St. Petersburg Pasteur Institute had minimal dissimilarities from international control strains. The analysis of the resultant 16S rRNA sequences has shown the presence of point mutations, transitions, deletions and insertions, regardless of the strain species. The open leptospira pan-genome demonstrates high genomic variability in species due to the capability of leptospira for lateral gene transfer in order to adapt to changing environmental conditions. The massive acquisition and loss of genes give rise to an increased species diversity. The 16S rRNA gene is suitable for screening diagnostics; however, high level of the fragment similarity and close phylogenetic relationship between different species put bounds to its use in genotyping. The presence of point nucleotide mutations is most likely associated with the evolutionary mechanisms of leptospira, their ability to horizontal gene transfer and crossing-over, including ribosomal genes, but this assumption necessitates additional research. For specimen genotyping it is necessary to select alternative genes with high specificity and sufficient level of nucleotide divergence. The study shows a need for genetic analysis of collection strains in order to control the purity of cultures.

Key words: *leptospirosis, leptospira, 16S rRNA, collection, zoonotic disease, cultivating.*

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МОЛЕКУЛЯРНО-ГЕНЕТИЧЕСКАЯ ХАРАКТЕРИСТИКА КОЛЛЕКЦИОННЫХ ШТАММОВ *LEPTOSPIRA* spp. САНКТ-ПЕТЕРБУРГСКОГО ИНСТИТУТА ПАСТЕРА НА ОСНОВЕ ДАННЫХ СЕКВЕНИРОВАНИЯ ГЕНА 16S рРНК

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Резюме. Лептоспироз является распространенным практически по всему миру зооантропонозным заболеванием. Эталонным методом для серологической диагностики лептоспироза является реакция микроскопической агглютинации с живыми лептоспирами (РМА). Этот метод основан на оценке способности сыворотки агглютинировать живые бактерии *Leptospira* эталонных сероваров, поддерживаемых в референс-лаборатории. В лабораториях, которые имеют коллекцию собственных изолированных и референсных штаммов *Leptospira*, используемых для серологической диагностики, эти микроорганизмы сохраняются в течение многих лет путем повторного пассирования, что значительно увеличивает шансы кросс-контаминации штаммов. Отсутствие адекватного контроля качества референсных штаммов может отрицательно повлиять на эпидемиологические исследования. Контроль чистоты и постоянства антигенного состава референсных штаммов *Leptospira* spp. имеет большое значение для диагностики лептоспироза. Целью данного исследования было сравнение нуклеотидных последовательностей гена 16S, некоторых коллекционных штаммов лептоспир Санкт-Петербургского Института им. Пастера, с последовательностями, загруженными в международную базу данных. В работе были изучены 38 штаммов лептоспир. Нуклеотидные последовательности 36 штаммов были депонированы в международную базу данных GenBank, в двух штаммах были обнаружены несоответствия. В результате исследования установлено, что контрольные штаммы *Leptospira* из коллекции Санкт-Петербургского института Пастера имеют минимальные отличия от международных контрольных штаммов. Анализ полученных последовательностей региона 16S рРНК, показал наличие точечных мутаций, транзиций, делеций и инсерций, независимо от видовой принадлежности штамма. Открытый пангеном лептоспир демонстрирует высокую геномную вариабельность у видов, что обусловлено способностью лептоспир к латеральному переносу генов, с целью приспособления к изменяющимся условиям среды. Массовое приобретение и потеря генов ведут к увеличению разнообразия видов. Ген 16S рРНК подходит для скрининговой диагностики, однако высокое сходство данного фрагмента и тесное филогенетическое родство разных видов ограничивает его использование для генотипирования. Наличие точечных нуклеотидных мутаций вероятнее всего связано с эволюционными механизмами лептоспир, их способностью к горизонтальному переносу генов и кроссинговеру, в том числе и рибосомальных генов, однако это предположение обуславливает необходимость проведения дополнительных исследований. Для генотипирования образцов необходим подбор альтернативных генов, с высокой специфичностью и достаточным уровнем дивергенции нуклеотидов. Проведенное исследование показывает необходимость проведения генетического анализа коллекционных штаммов с целью контроля чистоты культур.

Ключевые слова: *лептоспироз, лептоспира, 16S рРНК, коллекции, зоонозные заболевания, культивирование.*

Introduction

Leptospirosis is a zoonotic disease occurring worldwide. The manifestations of its epidemic process are most typical for countries with humid, especially tropical and subtropical climates, as well as for the so-called developing countries, however, the disease cases occur as well in countries with temperate climates [21, 43]. Leptospirosis is endemic in tropical regions of Asia, Africa, Central and South America [45]. Numerous animal species including those synanthropic and farm livestock are considered as reservoir hosts of pathogenic *Leptospira*.

The epidemic state of leptospirosis varies significantly in European countries. In recent years, in non-endemic countries, there was an increase in imported leptospirosis due to the rise in popularity of ecotourism in regions with humid subtropical or tropical climates [1]. In Russia leptospirosis remains one of widespread zoonotic infections due to the existence of both natural and anthropurgic foci of leptospirosis in many administrative regions [2, 3, 4, 5, 6]. Some assessments come to more than 1 million severe cases of leptospirosis and about 60 000 fatal outcomes occur annually worldwide [16].

According to the serological classification, all currently recognized species of pathogenic *Leptospira* are categorized into more than 260 serovars [10]. Based on the phylogenetic classification, the genus *Leptospira* is divided into 64 species. In line with genome-wide analysis, the genus is divided into 2 clades: pathogens and saprophytes, which, in turn, are divided into 4 subclades (P1, P2, S1, S2). Subclades P1 and P2 involve 17 pathogenic and 21 intermediate species, respectively. Subclade S1 includes 22 saprophytic species, while subclade S2 includes 4 [37].

Leptospirosis in humans is mostly caused by *Leptospira interrogans*, *Leptospira borgpetersenii* or *Leptospira kirschneri* [9, 26, 35]. The pathogen can circulate in the environment for a long time thanks

to sensitive or reservoir hosts that shed bacteria in their urine, contaminating soil and water. Humans usually get infected through direct contacts of their damaged skin or mucous membranes with the urine of infected wild or domestic animals, or through indirect contacts with contaminated environment [8, 29, 36]. In humans, there is a variety of possible clinical manifestations of the disease, ranging from subclinical infection to severe progressive forms that can lead to death [19, 29, 39].

Laboratory confirmation of suspected cases is essential for the diagnosis of leptospirosis, as there is significant polymorphism of its clinical manifestations. Most cases are diagnosed through serological tests of patients' blood. Microscopic Agglutination Test with live *Leptospira* (MAT) is the reference method for the serological diagnosis of leptospirosis. MAT is based on the assessment of the serum ability to agglutinate live *Leptospira* of reference serovars maintained at a reference laboratory [22, 32, 42, 44]. At some laboratories that have their own collections of isolated and reference *Leptospira* strains applicable for serological diagnosis, those microorganisms are maintained for many years by repeated subculturing, that increases significantly the chance of strain cross-contamination [13, 14, 19, 26].

Contamination of strains in *Leptospira* reference collections may entail severe consequences. In an outbreak investigation, the pathogen serogroup is usually identified with the help of MAT. The lack of adequate quality control of reference strains may affect the results of epidemiological studies. Conventional serogroup control of reference strains is carried out using monoclonal antibodies or control sera samples [7, 28, 46].

Control of *Leptospira* spp. reference strains purity and stability of its antigenic composition is very important for the diagnosis of leptospirosis. Sequencing of 16S rRNA gene is a reliable method for molecular characterization of bacteria species, and it is applicable to *Leptospira* spp. [15, 25, 31, 38]. The method was considered to be an effective and simple tool for *Leptospira* species identification in the clinical setting. Its important advantages are fast analysis, wide availability, and relatively low cost. The study objective was to compare the 16S rRNA gene nucleotide sequences of some collection *Leptospira* strains, including reference strains, with sequences uploaded to GenBank.

Materials and methods

In this study, we investigated 38 *Leptospira* strains from the collection of the Laboratory of Zoonotic Infections at the St. Petersburg Pasteur Institute.

The cultures were characterized by serological tests using MAT [18]. The strains have undergone repeated subculturing at the laboratory for many decades. Cultivation was carried out in a liquid nutrient medium based on distilled water containing 10% phosphate buffer with the addition of 10% rabbit blood serum. The strains were cultivated at 29°C. We used 7- to 14-days grown *Leptospira* cultures, containing at least 107 microbial cells/ml. Genomic DNA was extracted using a QiaAmp DNA Mini kit (Qiagen, Germany) in compliance with the manufacturer's instruction. For PCR, two pairs of primers flanking a 1423 bp fragment were used. Primer sequence: 16S Out F 5'-AGAGTTTGATCCTGGCTCAG-3', 16S Out R 5'-GGYTACCTTGTTACGACTT-3' [28, 33]. A clinical sample was used for a positive amplification control. The presence of *Leptospira* DNA in it was confirmed by two commercial kits: AmpliSens® Leptospira-FRT (InterLabService Ltd., Russia), and BactoReal® Kit Leptospira spp. Multiplex (16S rDNA+LipL32) (Ingenetix GmbH, Austria). ClearBand Nuclease Free Ultra-Pure Water (EcoTech Biotechnology Turkey) was used for a negative control.

PCR amplification was carried out in 25 μl of the amplification mixture with the addition of primers (15 M each) 67 mM plus Tris HCl (pH 8.8), 16.6 mM ammonium sulfate, 6.7 mM $MgCl_2$; 6.7 mM EDTA; 10 mM mercaptoethanol; 170 mg BCAA; 1.0 mM each dNTP; 1 unit Taq DNA polymerase (Fermentas). Denaturation (5 min at 94°C) was followed by 40 cycles of amplification: 30 s at 94°C, 30 s at 55°C, 1 min 20 s at 72°C, final elongation 7 min at 72°C.

The PCR products were separated on 2% agarose gel stained with ethidium bromide, in comparison with the GeneRuler 1Kb molecular weight marker (Thermo Scientific, USA). Electrophoresis was conducted for 40 minutes at 120V and visualized by UV radiation.

The amplification products were purified according to the procedure as follows: the mixture (including 2 μl of 3 M sodium acetate and 2 μl of 0.125 M EDTA, and 1 μl of glycogen) was added to 20 μl of the amplification product and incubated for 15 minutes at room temperature in the presence of chilled 96% ethanol. Centrifugation was carried out for 15 minutes at 4°C, 14 000 rpm/min. Then supernatant was removed and the precipitate was washed twice with cold 70% ethanol, repeating the cold centrifugation procedure. The washed precipitate was dried in the air at room temperature. For the quality analysis, the purified precipitate was dissolved in 30 μl of TE buffer and visualized on an agarose gel. The purified fragment of sufficient concentration was used to set up a sequence of reactions with forward and reverse primers. For analysis, the reaction product was dissolved in formamide and placed in an ABI Prism 3500 genetic analyzer (Applied Biosystems, USA).

The resulting strain sequences were compared with those available from the GenBank database.

For phylogenetic analysis, the MEGA11 software was used, the resulting sequences were aligned using the ClustalW algorithm. The sequence coverage length was 1144 bp. When constructing the tree,

the sequences of 16S region of *Borrelia* spp. from the international GenBank database, were chosen to be used for the outgroup. Based on the alignment, a tree was built using the Neighbor-joining method, bootstrap N = 1000.

Results

Nucleotide sequences of the 16S rRNA gene of 38 strains were obtained. The length of the sequences ranged from 1186 to 1423 bp. The sequences of 36 strains were deposited in the international GenBank database. For some details, such as serogroup of strain, date of isolation, origin and location see Table. The overall sequence similarity is 99%, the number of polyform variants of the gene among the obtained sequences ranges from 1 to 20 nt, which is consistent with the results of previous studies [34].

Strain sequences were identified and validated using NCBI BLAST and by alignment in MEGA 11 (Fig. 1).

The sequence of the *L. borgpetersenii* strain Perepelicin, obtained at our laboratory, forms a cluster with foreign reference strains, although it differs by one nucleotide.

Reference strains of *L. kirschneri* and *L. interrogans* species, obtained by foreign researchers, are clustered together, indicating their high similarity and inapplicability of 16S rRNA gene to typing.

The results of comparison with nucleotide sequences of the 16S rRNA gene, obtained by other researchers, were mostly concordant, although some discrepancies were observed.

Table. Leptospira strain sequences deposited in GenBank

No. of strain sequence deposited in GenBank	Serogroup	Date of isolation	Origin	Location
OL703051	*Canicola*	1985	Human	Leningrad
OL703052	*Icterohaemorrhagiae*	1999	Human	Saint Petersburg
OL703053	*Canicola*	1962	Human	Kaliningrad
OL703054	*Canicola*	1969	Human	Kaliningrad
OL703055	*Icterohaemorrhagiae*	1992	Human	Leningrad
OL703056	*Icterohaemorrhagiae*	1991	Human	Leningrad
OL703057	*Icterohaemorrhagiae*	1992	Human	Leningrad
OL703058	*Canicola*	2008	Human	Saint Petersburg
OL703059	*Icterohaemorrhagiae*	1968	Human	Leningrad
OL703060	*Canicola*	1968	Human	Kaliningrad
OL703061	*Canicola*	1985	Human	Leningrad
OL703062	*Icterohaemorrhagiae*	1990	Human	Leningrad
OL703063	*Icterohaemorrhagiae*	1990	Human	Leningrad
OL703064	*Autumnalis*	1925	Human	Japan
OL703065	*Australis*	1951	*Erinaceus europaeus*	Moscow
OL703068	*Icterohaemorrhagiae*	2018	Human	Saint Petersburg
OL703069	*Icterohaemorrhagiae*	1990	Human	Leningrad
OL825730	*Icterohaemorrhagiae*	2009	Human	Saint Petersburg
OL825731	*Icterohaemorrhagiae*	1980	*Rattus norvegicus*	Leningrad
OL825732	*Icterohaemorrhagiae*	1954	*Rattus norvegicus*	Leningrad
OL825733	*Icterohaemorrhagiae*	1961	*Rattus norvegicus*	Leningrad
OL825734	*Icterohaemorrhagiae*	1991	Human	Leningrad
OL825735	*Icterohaemorrhagiae*	1997	Human	Saint Petersburg
OL825736	*Icterohaemorrhagiae*	1990	Human	Leningrad
OL825737	*Icterohaemorrhagiae*	1991	Human	Leningrad
KR080516	*Canicola*	1931	*Canis lupus familiaris*	Holland
KY075908	*Tarassovi*	1938	Human	Moscow
KY075909	*Pyrogenes*	1923	Human	Indonesia
KY075910	*Javanica*	1938	*Rattus rattus*	Indonesia
KY075911	*Australis*	1937	Human	Australia
KY075912	*Bataviae*	1938	Human	Indonesia
KY075913	*Autumnalis*	1938	Human	Congo
KR107202	*Sejroe*	1944	*Apodemus flavicollis*	Denmark
KR091970	*Icterohaemorrhagiae*	1935	Human	Denmark
KR091971	*Pomona*	1936	Human	Australia
KR091973	*Grippotyphosa*	1929	Human	Moscow

The analysis of the resultant sequences of 16S rRNA region has shown the presence of point mutations, transitions, deletions and insertions, regardless of the strain species.

Three points with a variable nucleotide were found in 10 *L. interrogans* sequences obtained by us. In the sequences of GenBank strains thymine is more common at position 38, however, there are some strains with cytosine. The reverse is true for the strains here studied: only 8 of 36 strains contain thymine at this point, while the rest of them contain cytosine (Fig. 2).

The sequence of the *L. kirschneri* strain is 99.0% similar to that of the *L. interrogans* species, but 99.72% sequence identity was found by NCBI BLAST analysis.

In two strains out of 38, inconsistencies were revealed. The sequences of the collection strains of the species *L. borgpetersenii* and *L. noguchii* were analyzed and proved to be identical to the strains belonging to *L. interrogans.*

Discussion

Historically, *Leptospira* strains were classified according to their virulence, all saprophytic strains were assigned to *L. biflexa*, and pathogenic strains to *L. interrogans* [41]. Further subdivision of *Leptospira*, taking into account their antigenic determinants, was based on serological techniques [28].

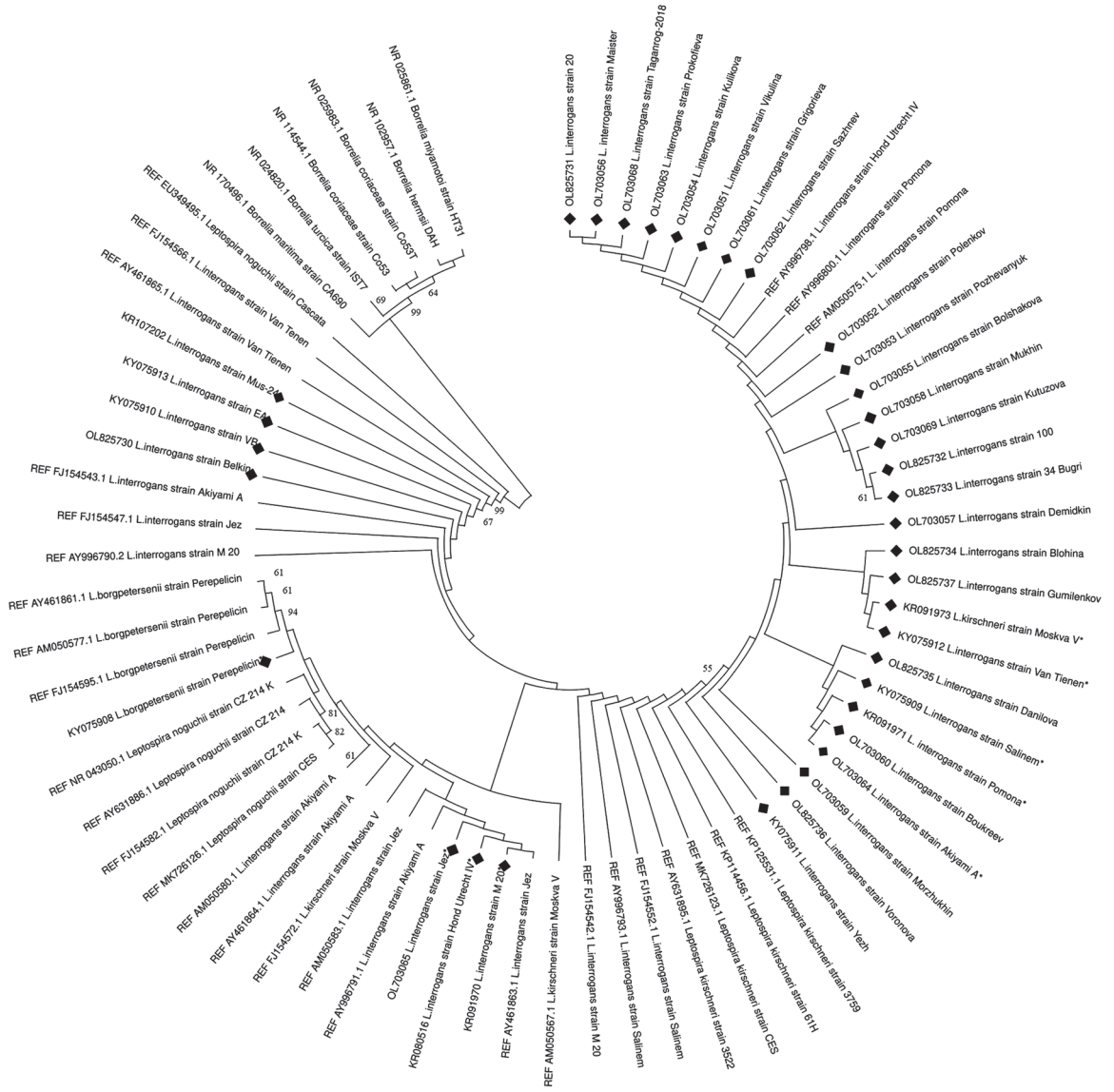

Figure 1. Phylogenetic tree built on the obtained *Leptospira* spp. 16S rRNA sequences and sequences of reference strains taken from GenBank

Note. REF — sequences of reference strains from GenBank. * — sequences of reference strains obtained at our laboratory. The studied strains are described in Table.

Leptospira classification was significantly expanded due to the introduction of such genetic techniques as DNA-DNA hybridization, pulsed-field gel electrophoresis (PFGE), and Multilocus sequence typing [11, 13, 20]. Serological characteristics do not correlate with genovariants, one species of *Leptospira* may contain several serogroups, while one serogroup may include strains of different species. The open pangenome of *Leptospira* demonstrates high genomic variability in species due to the capability of *Leptospira* for lateral gene transfer in order to adapt to changing environmental conditions. The massive acquisition and loss of genes give rise to an increase in species diversity [24, 36].

Sequencing of the 16S rRNA region is currently the most accessible method for genotyping. This gene provides distinction between pathogenic, intermediate, and saprophytic clades, but its discriminatory ability is too low to distinguish between *Leptospira* genotypes. For example, it is impossible to differentiate *L. interrogans*, *L. kirschnerii*, and *L. noguchii*, since they differ by one or two bases only and belong to the same operational taxonomic unit (mOTU) that is shown by the phylogenetic tree (Fig. 1) [23]. The sequence of the *L. kirschneri* strain obtained in our study also has a minimal difference only from the type strains of *L. interrogans.*

A large number of 16S rRNA sequences uploaded to GenBank are not full-length (< 1400 bp), therefore it is difficult to identify strains correctly. Long-term subculturing of *Leptospira* in collections does not exclude erroneous identification of sequences uploaded to public databases, it also can give rise to single mutations [12].

When sequencing this gene, it was found that the control strains of *Leptospira* from the collection of the St. Petersburg Pasteur Institute have minimal dissimilarities from international control strains and are applicable in serological diagnosis of leptospirosis.

In this study, two out of 38 strains did not match the sequences of similar strains uploaded to GenBank, which may be due to its contamination or mislabeling. Long-term storage of *Leptospira* by freezing is not available at all practical laboratories owing to its high price, therefore collection strains of *Leptospira* for many years undergo repeated subculturing, possibly resulting in strain mutations and increasing the risk of cross-contamination or erroneous labeling of samples. This justifies the need for genetic analysis of collections in order to circumvent the possible risks associated with under- or overdiagnosis.

The 16S rRNA gene is suitable for screening diagnostics; however, high level of the fragment similarity and close phylogenetic relationship between different species put bounds to its use in genotyping.

The presence of point nucleotide mutations is most likely associated with the evolutionary mechanisms of *Leptospira*, their ability to horizontal gene transfer and crossingover, including ribosomal genes, but this assumption necessitates additional research [34].

For genotyping of samples, it is necessary to select alternative genes with high specificity and a sufficient level of nucleotide divergence.

Nowadays numerous genetic targets have been proposed for *Leptospira* diagnosis and typing. The gene encoding the outer membrane lipoprotein of patho-

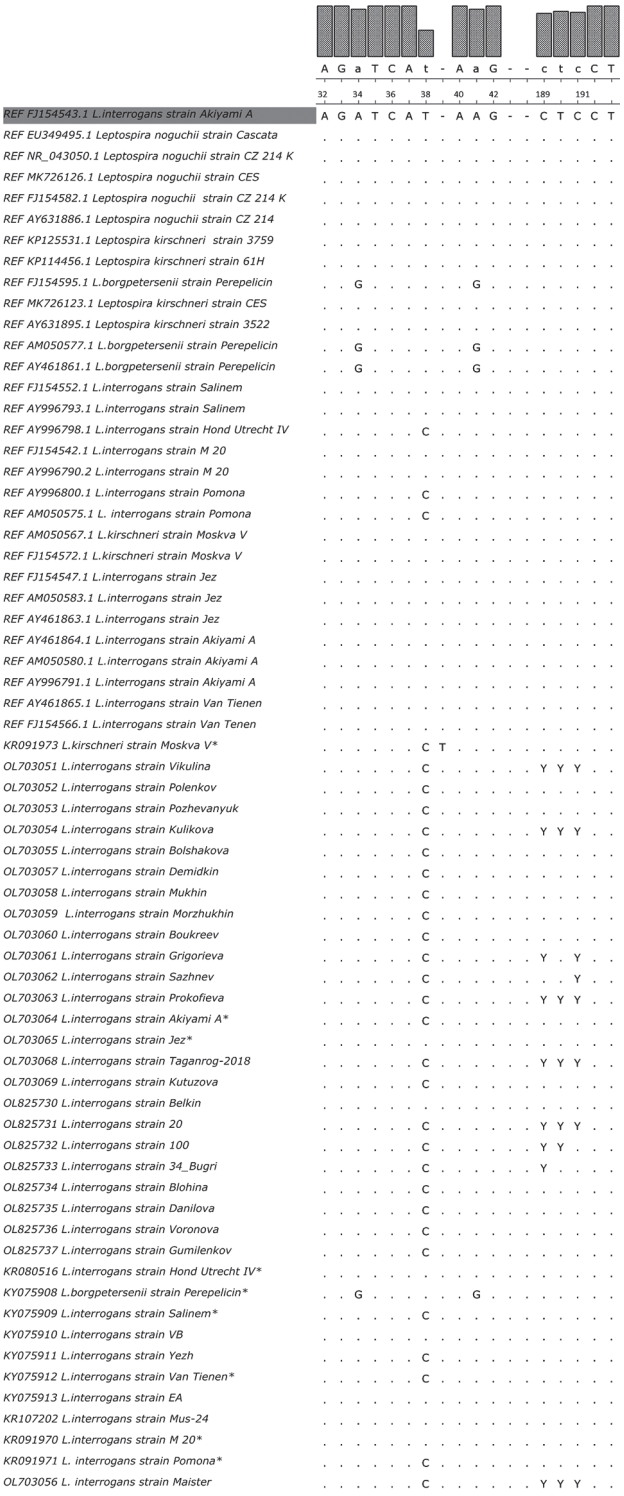

Figure 2. Multiple alignment of the obtained sequences with reference strains obtained from the international GenBank database

Note. The main differences are shown.

genic *Leptospira lipL32* is one of the most common alternatives to 16S rRNA, however, its use is also limited by its discriminating abilities, and it is mostly applied in screening diagnostics.

The housekeeping gene encoding the *secY* preprotein translocase is next in frequency of use; the use of this gene for *Leptospira* genotyping seems to be the most promising at the moment. Other proposed targets are: the gene encoding flagellin, the class B polypeptide subunit of periplasmic flagella *flaB*, the gene for the β-subunit of RNA polymerase *rpoB*, and the genes *gyrB*, *Adk*, *glmU*, *LigB*, etc. [17, 27, 30, 40].

As a variety of targets is used by many researchers, the comparison of their results is hardly possible. Therefore, the lack of standardization is currently the main restriction on the molecular diagnosis of leptospirosis.

This study shows the need for genetic analysis of collection strains in order to control the purity of cultures.

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