GENETICS

Interspecific Polymorphism of Gene *lipL32* Restriction Profiles of Pathogenic Leptospires

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We analyzed restriction fragment length polymorphism of *lipL32* gene encoding outer membrane protein in three leprospira genomic species (*L. interrogans, L. kirschneri,* and *L. borgpetersenii*) using Bsa29 I and Bam HI restriction endonucleases. It was found that restriction profiles of the studied gene were similar at both the intraspecific and serogroup levels; variability was revealed only at the interspecific level, which can be explained by relatively low variability of *lipL32* gene.

Key Words: *Leptospira; genotyping; restriction; polymorphism*

Leptospirosis is the most prevalent zooanthroponosis in the world. This infection caused by microorganisms of *Leptospira* genus runs a severe, sometimes lethal course in humans, induces diseases in farm animals, and causes considerable economic damage [1].

Various molecular methods can be used for differentiation of pathogenic leptospira at both specific and intraspecific levels, including PCR with primers to encoding sequences flanking different fragments of minisatellite DNA (VNTR, variable number tandem repeats); this method determines belonging of the strain to a certain serogroup and species by the length of formed amplicons [7,11].

Various modifications of PCR can be used for Leptospira strains, including LSSP-PCR (low-stringency single specific primer) consisting of two stages [8] and multiplex PCR [13].

Restriction fragment length polymorphism (RFLP) analysis along with other methods can be used for Leptospira genotyping. By analogy with the test system for specific identification of *Borrelia* microorganisms based on RFLP analysis of *flaB* gene, a test-system for Leptospira typing at the specific level was created [6].

The differences in restriction profiles of rRNA genes allow differentiation of some Leptospira serovars [9]. Analysis of restriction profiles of 16S and 23S rRNA after restriction with various endonucleases revealed intraspecific variability by this sign [10]. There is also a possibility of using rRNA gene RFLP for differentiation of pathogenic and non-pathogenic Leptospira [12].

We chose *lipL32* gene encoding LipL32 lipoprotein, a component of the outer membrane of pathogenic Leptospira [4]. Since the presence of *lipL32* gene is typical of only pathogenic Leptospira, they can be differentiated from saprophyte bacteria by PCR with primers recognizing this gene sequence [2]. Due to its immunogenic properties, LipL32 protein can be considered as a prospective component of ELISA-based diagnostic systems and vaccines [5,14]. DNA containing *lipL32* gene injected to experimental animals exhibits a protective effect and can be a component of

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DNA vaccines [3]. Here we undertook a first attempt to analyze sequence of a gene unique for pathogenic Leptospira and playing an important role in vital functional of this pathogen.

The aim of this study is RFLP analysis of *lipL32* gene and evaluation of the possibility of using this gene as a marker for genotyping of pathogenic Leptospira.

MATERIALS AND METHODS

We used DNA of 46 original strains of *Leptospira interrogans* (*Leptospira interrogans* genomic species, Pomona serogroup 7 strains, Canicola 10 strains, and Icterohaemorrhagia 21 strains, *Leptospira kirschneri,* Grippotyphosa serogroup 5 strains, and *Leptospira borgpetersenii* Tarassovi serogroup 3 strains) and reference strains of the above-listed serogroups from the collection of Pasteur Institute of Epidemiology and Microbiology. All original strains were isolated from patients (45 samples from the blood and 1 sample from the urine) within the Northwestern Federal Region. The pathogen was cultured on Terskikh medium [3].

DNA was isolated by thermolytic disintegration of cells at 95°C for 20 min.

For isolation of amplicons of Leptospira DNA containing the studied gene, we used LipL32 F and LipL32 R primers, which were previously employed for sequencing of the studied site [7]: LipL32: F 5'-ATGAAAAAACTTTCGATTTTG-3', LipL32: R 5'-TTACTTAGTCGCGTCAGAAGC-3'.

A fragment of the genome containing the studied gene can be completely amplified with these primers. The length of the obtained amplicons is 819 b.p.

PCR was performed in automatic replication mode using a Tertsik thermocycler (DNA Technology) and DNA Amplification kit (Sileks) according to manufacturer's instruction. The volume of the reaction mixture was 30 μl with 4 μl DNA from each sample (~450 ng DNA). PCR protocol included preliminary heating of the reaction mixture at 94°C for 90 sec followed by 37 amplification cycles: denaturation at 94°C for 1 min; annealing 52°C for 1 min, synthesis at 72°C for 90 sec, and final elongation at 72°C for 10 min. This primer annealing temperature allowed us to perform PCR with sufficient accumulation, but without generation of non-specific products, which are formed at higher temperatures. PCR products were analyzed by electrophoresis in 1.5% agarose gel stained with ethidium bromide in a concentration of 0.5 μg/ml. The samples containing specific amplicons ~819 b.p. were selected for further analysis.

The amplified DNA was restricted with endonucleases Bsa29 I (restriction site AT^CGAT) and Bam HI (restriction site G^GATCC) manufactured by Sibenzim company using buffer G. Endonucleases were selected on the basis of analysis of *lipL32* gene sequences presented in Gene Bank. We chose restriction endonucleases for which the location of the recognition sites varied depending on systematic belonging of the organism. Since the study was aimed at choosing the marker for differentiation of pathogenic Leptospira, the use of rare cutting restriction enzymes (recognition site >4 b.p.) is advisable, because this reduces the possibility of obtaining polymorphism within one serovar. Moreover, the selected restriction endonucleases should yield well-discernible restriction profiles; in particular, the formation of small fragments should be excluded, because it hampers the application of this method for practical purposes. Endonucleases Bsa29 I and Bam HI meeting these requirements were chosen for the analysis.

The restriction mixture included: 10 μl PCR product, 8 μl sterile water, 2 μl respective buffer, and 0.1 μl endonuclease (Bsa29 I and Bam HI in concentrations of 40 000 and 20 000 U/μl, respectively). Restriction was performed in the following regimen: incubation at 37°C for 90 min, 20-min pause at 65°C, and 10 min at -20°C. The results were evaluated by electrophoresis in 2% agarose gel. The length of DNA fragments were evaluated visually by comparing with Gene Ruler 100 bp DNA Ladder molecular weight marker (Fermentas). For improving the reliability of the results, three repetitive observations were performed.

RESULTS

Restriction of amplicons containing *lipL32* gene of Leptospira belonging to *Leptospira borgpetersenii* genomic species (Tarassovi serogroup) by Bam HI endonuclease yields three fragments (~420, 250, and 150 b.p., Fig. 1), analogous amplicons of *Leptospira kirschneri* (Grippotyphosa serogroup) forms two fragment (420 and 400 b.p.). All restriction profiles of *Leptospira interrogans* genomic species (Pomona, Canicola, Icterohaemorrhagia serogroups) irrespective of the serogroup included two fragments with lengths of 420 and 400 b.p., the same was true for analogous amplicons obtained during amplification of *lipL32* gene of *Leptospira kirschneri*. These fragments were not separated by electrophoresis in agarose gel and form a common band corresponding to 400 b.p. molecular weight marker (Fig. 1).

Bsa29 I restriction of amplicons carrying *lipL32* gene of *Leptospira borgpetersenii* (Tarassovi serogroup) and analogous amplicons of *Leptospira kirschneri* genomic species (Grippotyphosa serogroup) yields two fragments with lengths of 600 and 220 b.p. (Fig. 2). Amplicons carrying *lipL32* gene of Pomona, Canicola, and Icterohaemorrhagia serogroups of *L.*

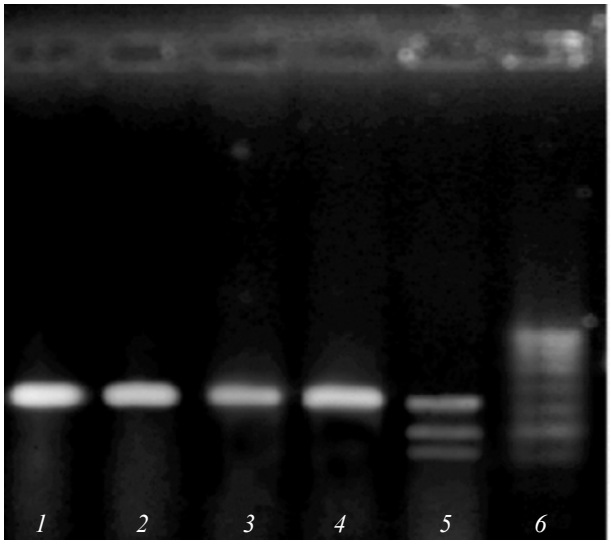

Fig. 1. Bam H I restriction profiles of *lipL32* gene from different Leptospira species. *1*) *L. interrogans*, Icterohaemorrhagia serogroup; *2*) *L. interrogans*, Canicola serogroup; *3*) *L. interrogans*, Pomona serogroup; *4*) *L. kirschneri*, Grippotyphosa serogroup; *5*) *L. borgpetersenii*, Tarassovi serogroup; *6*) molecular weight marker 10 b.p.

interrogans genomic species were not cleaved during restriction (Fig. 2).

Thus, restriction analysis of *lipL32* gene in Leptospira belonging to different species and serogroups within the studied genomic species and serogroups with Bsa29 I and Bam HI endonucleases revealed similar restriction profiles, which suggests high stability of this gene sequence. The possibility of detecting polymorphism of restriction profiles of this gene enables creation of a system for typing of pathogenic Leptospira in culture on the basis of restriction of *lipL32* gene with these endonucleases. Since the chosen endonucleases are active under similar conditions, two restriction reactions can be simultaneously carried out in the same tube.

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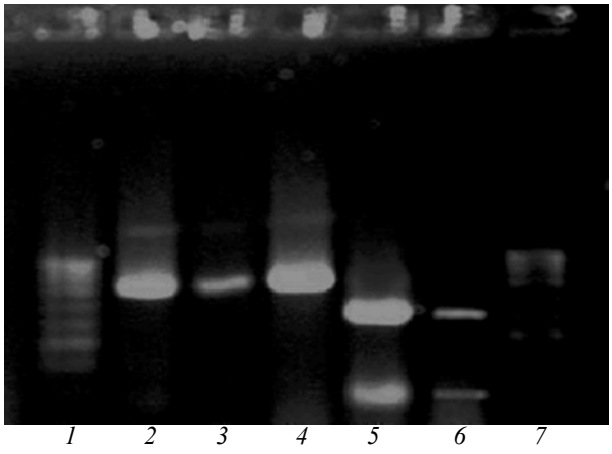

Fig. 2. Bsa29 I restriction profiles of *lipL32* gene from different ***Leptospira species***. *1)* and *7*) molecular weight marker 10 b.p.; *2*) *L. interrogans*, Icterohaemorrhagia serogroup; *3*) *L. interrogans*, Canicola serogroup; *4*) *L. interrogans*, Pomona serogroup; *5*) *L. kirschneri*, Grippotyphosa serogroup; *6*) *L. borgpetersenii*, Tarassovi serogroup.

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