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Amplification and Cloning of a Gene Encoding a 41 kDa Outer Membrane Protein (LipL41) of *Leptospira interrogans* Serovar Canicola

Pejvak Khaki[1*], Soheila Moradi Bidhendi[1], Yung-Fu Chang[2], Maryam Sadat Soltani[1], Kayvan Tadaion[3]

[1]National Reference Laboratory for Leptospira, Department of Microbiology, Razi Vaccine & Serum Research Institute, Karaj, IR Iran
[2]Department of Population, Medicine and Diagnostic sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA
[3] Department of Aerobic Vaccine production, Razi Vaccine & Serum Research Institute, Karaj, IR Iran

[*]*Corresponding Author:* Pejvak Khaki, National Reference Laboratory for Leptospira, Department of Microbiology, Razi Vaccine & Serum Research Institute, Karaj, IR Iran. Tel: 09123894986; Email: p.khaki@rvsri.ac.ir

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Background: Leptospirosis has been recognized as an important reemerging infectious disease caused by pathogenic *Leptospira* spp. A major challenge of this disease is the application of a basic research to improve diagnostic method. Outer membrane proteins of *Leptospira* are potential candidates that could be useful in diagnosis. Among them the *lipL*41 is an immunogenic protein which is present only in pathogenic serovars. In order to evaluate genetic conservation of the *lipL*41 gene, we cloned and sequenced this gene from *Leptospira interrogans* serovar Canicola.
Materials and Methods: Following the DNA extraction from the serovar, the *lipL*41 gene was amplified and cloned into pTZ57R/T vector and transformed into the competent *E. coli* (Top10). Recombinant clones were confirmed by colony PCR and DNA sequencing. The related sequences were then analyzed and compared with the sequences in the Genbank database.
Results: PCR amplification of the *lipL*41 gene resulted in a 1065 bp PCR product. The PCR based on the *lipL*41 gene detected all the pathogenic reference serovars of the tested *Leptospira* spp. It was revealed that in Iran the homology of the *lipL*41 gene between vaccinal and clinical serovars of Canicola was 100%. It also showed >95.9% homology with other pathogenic serovars in Genbank database, which indicates genetic conservation of this gene.
Conclusion: Because of the conservation of *lipL*41 gene among different strains of *Leptospira* and its exclusive presence in leptospira, it was revealed that the cloned gene could be further used as a good candidate for developing diagnostic methods such as ELISA and as positive control in diagnostic PCR.

Keywords: Cloning, *Leptospira*, Outer membrane proteins, *LipL*41 gene

1. Background

Leptospirosis is a reemerging infectious disease affecting both animals and humans (1, 2). The causative agent is the spirochete which belongs to the genus *Leptospira* (3). The incidence of the disease is the most common in tropical and subtropical areas with high rainfalls (4). In tropical regions of the world, leptospirosis is a widespread public health problem because of its high mortality and morbidity rate in different countries (5).

A wide range of host species including humans and wildlife such as rodents, carnivores, and domestic animals, act as the reservoirs for *Leptospira* (6). This disease affects livestock economy due to abortion, stillbirth, infertility, decreased milk production, and death in domestic animals (7).

Leptospirosis is transmitted directly or indirectly from animals to humans. Infected animals can remain asymptomatic and continue to shed infectious organisms into their urine for the entire their lifetime then humans are infected indirectly via exposure to water or soil contaminated by the urine of the infected animals (3, 8).

Various factors including the animal activity, suitability of the environment for the survival of the organisms, behavioral and occupational habits and recreational activities of human being can be accounted as the major determinants of the incidence and the prevalence of the disease (9).

Due to the broad spectrum of signs and symptoms, an important problem that the physicians face with, is the fact that the disease is often misdiagnosed or may be mistaken with other diseases such as rickettsiosis, dengue, enteric fevers, and malaria, so the clinical symptoms are not adequate in diagnosis of this disease (10-12). Thus, the laboratory confirmation and the availability of an accurate and reliable diagnostic method are essential in the early phases of this disease (13).

Recently, in efforts to develop a diagnostic test of high sensitivity, the focus has mainly been on the outer membrane proteins (OMPs) due to their location at the interface between the pathogen and the host (14). LipL41 is one among the OMPs that was reported as immunogenic antigen that is highly conserved among pathogenic leptospira serovars but not in saprophytic serovars (11, 15). It suggests that the LipL41 antigen plays an important role in pathogenesis (14-16)

2. Objectives

In the present study, we reported the amplification and cloning of a gene encoding a 41 kDa OMP (LipL41) of *Leptospira interrogans* serovar vaccinal and clinical Canicola. We also evaluated its phylogenetic relationships with different pathogenic serovars of *Leptospira* with respect to the *lipL*41 gene.

3. Materials and Methods

Leptospira interrogans serovar vaccinal Canicola (LC_R-TCC2805), serovar clinical Canicola (LC_RTCC2824), and a saprophytic serovar *L. biflexa* were used in this study. They were obtained from the *Leptospira* Reference Laboratory, Department of Microbiology, Razi Vaccine and Serum Research Institute, Karaj, Iran.

The leptospira serovars were inoculated into the selective culture (EMJH) semisolid medium (Difco, Sparks, USA) containing 2% rabbit serum. The genomic DNA was extracted by the standard phenol-chloroform method as described by Sambrook and Russell (17). The *lipL*41gene was amplified by

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specific primers as reported previously (18). The primer sequences were: Forward 5'-TGTTACCCATGGGGAGA AAATTATCTTCTCT-3' and Reverse-5' AAAGGACTCGA GTTACTTTGCGTTGCTTTC-3'. The PCR assay and concentrations of all the reagents were optimized using 50 µl reaction mixture. It consisted of 25 µl 2X Master Mix (Ampliqon, Denmark), 10 µl (10 pmol) from each of the forward and reverse primer, and 100 ng of template DNA. The PCR was performed in Thermal cycler (Eppendorf, Germany) with the following conditions: initial denaturation at 94 ºC for 5 minutes, followed by 35 cycles of denaturation at 94 ºC for 1 minute, annealing at 57 ºC for 1minute, extension at 72 ºC for 1 minute, and a final extension at 72 ºC for 10 minutes.

The PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide. Gels were photographed using a gel documentation system (Bio-Rad, USA). The PCR products were purified using the Gene JET PCR purification kit (Fermentas, Lithuania) and ligated into the pTZ57R/T cloning vector (Fermentas, Lithuania). The ligation mixture was incubated overnight at 4 ºC followed by 10 minutes at 22 ºC and subsequently used for transformation. The recombinant plasmid transformed into *Escherichia coli* (TOP10) competent cells. It was incubated on ice for 30 min. The cells were exposed to heat shock at 42 °C for 90 s in a water bath and immediately transferred to ice for 2 min. Then 1mL LB broth was added to it and incubated at 37 °C for 1 hr in an orbital shaker. It was then centrifuged at 13000 g for 1 min.

The transformants were then plated on LB ampicillin agar and incubated at 37 ºC overnight. The confirmation of the positive recombinant clones was done by colony PCR, and related plasmids were isolated from bacterial cells using the Plasmid Mini extraction kit (Roche, Germany) as described by manufacturer. The recombinant plasmids were sequenced by Macrogen (South Korea). The sequenced gene was analyzed for its homology with the already reported sequences of other *Leptospira* serovars in Genbank database using the BLAST program. The percentage identity and divergence table and phylogenetic tree were constructed using the MegAlign program of Lasergene software DNA star (Table 1 and Fig. 2).

Table1. Sequence pair distances of the *lipl*41 gene sequences of different leptospiral serovars.

Percent Identity

Divergence	1	2	3	4	5	6	7	8	9	10		
1		100.0	99.9	100.0	99.6	96.3	99.6	99.6	99.4	99.6	1	LC_RTCC2824
2	0.0		99.9	100.0	99.6	96.3	99.6	99.6	99.4	99.6	2	LC_RTCC2805
3	0.1	0.1		99.9	99.5	96.2	99.5	99.5	99.3	99.6	3	LC(AY622675)
4	0.0	0.0	0.1		99.6	96.3	99.6	99.6	99.4	99.6	4	LC(AY642287)
5	0.4	0.4	0.5	0.4		96.3	99.8	99.3	99.6	99.3	5	LG(AY622681)
6	3.9	3.9	4.0	3.9	3.9		96.3	95.9	96.1	96.0	6	LG(JQ690557)
7	0.4	0.4	0.5	0.4	0.2	3.9		99.3	99.8	99.3	7	LH(AY642286)
8	0.3	0.3	0.4	0.3	0.7	4.2	0.7		99.1	99.6	8	LI(GQ502197)
9	0.6	0.6	0.7	0.6	0.4	4.1	0.2	0.8		99.3	9	LA(AY622678)
10	0.4	0.4	0.4	0.4	0.7	4.1	0.7	0.4	0.7		10	LP(AY776298)
	1	2	3	4	5	6	7	8	9	10		

4. Results

PCR amplification of the *lipL*41 gene resulted in the 1065 bp PCR product, while it was absent in saprophyte serovar *L. biflexa* (Figure 1). The sequences were deposited in the Genbank database under the accession numbers KJ409447, KJ398169.

In this study, we found that the *lipL*41 gene of vaccinal Canicola serovar (LC-RTCC2805) and the clinical Canicola serovar (LC-RTCC2824) in Iran had 100% homology; while compared with other studies on *Leptospira* serovars, it revealed >95.9% homology (Table 1 and Figure 2).

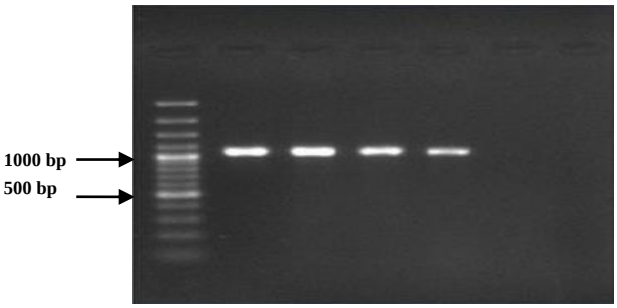

Figure 1. PCR amplification of the *lipL41* gene for detection of pathogenic serovars of leptospires.M: 100 bp DNA ladder; lane1: positive control, *L.* Sejroe hardjo; lane 2: Positive control, *L.* Pomona; lane3:*L.* Canicola (RTCC2805); lane4: *L.* Canicola (RTCC2824); lane5: *L. biflexa*; lane 6: negative control

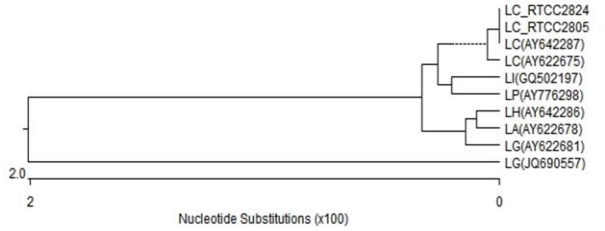

Figure 2. Phylogenetic tree of different *Leptospira* serovars constructed on the basis of *lipL*41 gene.

5. Discussion

Failure in accurate diagnosis of leptospirosis is common because of its protean manifestations and its mimicry of the clinical symptoms of many other diseases (3).

The MAT is gold to diagnose leptospirosis, but because it requires the use of living organisms as antigen, this test is risky to perform. Furthermore, the growth and maintenance of several leptospiral serovars may be difficult, expensive,.and laborious. Thus, alternative serological methods such as ELISA are assumed to be more sensitive than the conventional serological tests of leptospirosis diagnosis (19, 20). Recombinant protein based ELISA is a suitable and safe procedure for the examination of a large number of sera that need a small amount of serum, in which the diagnosis can be made in the early phases of the disease (21-23). In recent years, leptospirosis research has concerted to identify the antigens that could be effectively used in ELISA for precise diagnosis. Earlier, most of the researches on leptospiral antigens had been focused on LPS (24), identified as an immune dominant antigen; however, LPS antigens show a variety among different leptospiral serovars. In contrast to LPS, leptospiral membrane proteins are thought to be highly conserved and expressed during infection, so antigenic characterization of the OMPs is a necessary step toward the development of a diagnostic method.

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The recombinant antigen evaluated for its use in ELISA so far, was LipL41. Researches by Natarajaseenivasan and Theodoridis indicate that ELISA based on the *lipL*41 gene of pathogenic leptospira achieved better sensitivity (25, 26).

In other study, Mariya and colleagues evaluated the efficacy of a recombinant leptospiral lipoprotein LipL41 as an antigen in ELISA for the diagnosis of bovine leptospirosis. Based on their research, it was concluded that recombinant LipL41 protein could be a putative diagnostic candidate (27).

Here we analyzed the *lipL*41 gene sequences of vaccinal and clinical serovars of Canicola with other pathogenic leptospiral serovars that were previously submitted to the Genbank database. The data showed more than 95.9% similarity.

6. Conclusion

In conclusion, the nucleotide sequence analysis revealed that the amplified genes had minor nucleotide changes in comparison with the other leptospiral serovars. This finding confirms the conserved nature of the *lipL*41 gene. Therefore, the *lipL*41gene could be a proper candidate for developing serodiagnostic tests such as ELISA. However, for further studies more serovars are required.

Conflict of Interests

The authors declare they have no conflict of interests.

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Authors Contribution

All authors contributed to this study.

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