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Original Article

Molecular characterization of the *lipL41* gene of *Leptospira interrogans* vaccinal serovars in Iran

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ABSTRACT

Leptospirosis caused by infection with pathogenic leptospires, which is the most prevalent zoonotic disease in the world. The outer membrane proteins (OMPs) of pathogenic leptospires such as LipL41 play a crucial role in pathogenesis of this disease. Therefore a major challenge to develop an effective vaccine against leptospirosis is application of basic research on the OMPs of leptospires to improve vaccine development. The aim of this study was cloning and analyzing of the *lipL41* gene from vaccinal serovars of leptospires in Iran, in order to identify genetic conservation of this gene. Three vaccinal serovars of *Leptospira* were used in this study. The *lipL41* gene of these serovars were amplified and cloned in the pTZ57R/T vector. The recombinant clones were confirmed by colony-PCR and sequencing. The sequenced genes were analyzed for their homology between them and other submitted sequences in Genbank database using the BLAST and MegAlign program. PCR amplification of the *lipL41* gene resulted in the 1065 bp gene product in vaccinal serovars tested. In our study, nucleotide sequencing results showed high similarity (>94%) within the leptospiral vaccinal serovars. The genetic conservation of the *lipL41*gene among different serovars of *Leptospira* indicated the capacity of utilization of this gene for development of recombinant vaccine against leptospirosis.

Keywords: Leptospirosis, *lipL41* gene, Molecular characterization, Sequencing, Vaccinal serovars of *Leptospira*

INTRODUCTION

Leptospirosis is an important re-emerging infectious disease and is considered to be the most widespread zoonotic disease in the world (Levett 2001, McBride *et al* 2005). The disease is caused by the pathogenic leptospires (Bharti *et al* 2003). The pathogenic *Leptospira* species can be classified into different serogroups and serovars that have been identified with endemicities that vary geographically (Bharti *et al* 2003, Vijayachari & Sugunan 2008). In domestic animals such as cattle, leptospirosis is responsible for serious economic losses, due to abortion, mastitis, and a decline in milk production (Quinn *et al* 2011). Infected cattle may not show any clinical signs of disease, but excrete the organisms in their urine. Therefore they play an important role in spreading the infection to other susceptible animals and to human population at risk, such as farmers and veterinarians (Levett 2001, Vijayachari & Sugunan 2008). Current vaccines against leptospirosis are mainly multivalent inactivated whole-cell, but these vaccines are not very effective

and they cannot provide cross-protection against infection with all different leptospiral serovars, and may lead to incomplete and short term immunity (McBride *et al* 2005, Meites *et al* 2004). It has been reported that these vaccines induce protective immunity against challenge with homologous but not heterologous leptospires, and their efficacy is limited when *Leptospira* of a different serovar is circulating (Sonrier *et al* 2000). Furthermore, vaccination with the whole cell leptospiral vaccine frequently results in serious adverse effects that are considered to be caused by the lipopolysaccharide fractions (Thongboonkerd 2008, Luo *et al* 2009). Despite vaccination, the disease still exists in some parts of the Iran (Khaki *et al* 2005). Therefore, construction of an efficient recombinant vaccine for leptospirosis control is very important. Demonstrating that ideally an effective vaccine should have conserved and protective antigens which consists in all pathogenic leptospires, the focus of research on protective antigens has been shifted toward the identification of conserved outer membrane proteins (OMPs) that are potentially associated with pathogenesis (Cullen *et al* 2004, Vedhagiri *et al* 2009). The OMPs play crucial roles in pathogen virulence mechanisms because these proteins are at the interface between the pathogen and the mammalian host immune responses (Haake *et al* 1993, Pinne & Haake 2013, Shang *et al* 1996). They may also be able to stimulate heterologous immunity (Gamberini *et al* 2005). *LipL41* is one of the immunogenic OMPs that is surface-exposed and is expressed during infection (Shang *et al* 1996, Senthilkumar *et al* 2007). It has been identified as a genus-specific protein antigen (Cullen *et al* 2004, Cullen *et al* 2002, Palaniappan *et al* 2007, Wang *et al* 2007). Other studies have also shown that *lipL41* is highly conserved among pathogenic species of *Leptospira* (Luo *et al* 2009, Senthilkumar *et al* 2007, Haake *et al* 2004). Therefore there is a necessity for molecular analysis of the *lipL41* gene in vaccinal serovars. The present investigation was carried out to cloning and characterization of the *lipL41* gene in order to identify the genetic conservation of this gene among the vaccinal serovars in Iran, to make a pilot step toward the utilization of this gene for development of effective recombinant vaccine against leptospirosis.

MATERIALS AND METHODS

Bacterial Serovars and Culture Conditions. Three vaccinal serovars of *Leptospira interrogans*, including serovars: Canicola (RTCC2805), Grippotyphosa (RTCC2808), Sejroe hardjo (RTCC2821) and a saprophytic serovar *L. biflexa* (RTCC2819) maintained in the *Leptospira* Reference Laboratory, Razi Vaccine and Serum Research Institute, Karaj, Iran were used. The bacteria were inoculated into the selective culture (EMJH) medium (Difco, Sparks, USA) containing 2% rabbit serum.

Genomic DNA Extraction. Leptospiral genomic DNA was extracted by proteinase K treatment and Phenol-Chloroform extraction method (Sambrook J and DW, 2001). The extracted DNA was resuspended in 20µl of TE buffer (pH 8.0) and stored at -20 °C. The quantity and quality of the extracted DNA was checked by agarose gel electrophoresis and UV spectrophotometery using the Epoch system (BioTek, New York, USA).

PCR Amplification. The specific primers utilized for this study were as reported previously (Haake *et al* 1999). PCR was carried out in a 50 µl reaction mixture containing 25 µl 2× MasterMix (Ampliqon), 1 µl primer forward (10 pmol), 1 µl primer reverse (10 pmol), 3 µl DNA template (100 ng), 20 µl Nuclease free water. Amplification was performed in Thermal cycler (Eppendorf, Germany) with denaturantion at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min, and the final extension at 72 °C for 10 min. After amplification, PCR products were subjected to electrophoresis on a 1.5% agarose gel prepared and ethidium bromide staining and visualized under a UV transilluminator (National Labnet Company, USA). The gels were photographed using a gel documentation system (Bio-Rad, USA).

Gel purification and Cloning of PCR Products. The PCR products were purified using the PCR product

Purification Kit (GeneJET PCR Purification kit, Fermentas) and ligated into the pTZ57R/T vector (Fermentas, Lithuania) according to the manufacturer's instructions. Ligation was done at 4 °C for overnight. The recombinant vector was transformed into competent *E. coli* Top10 cells (Sambrook J & DW, 2001). 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 and then 1ml LB broth was added to it and incubated at 37°C for 1 h in an orbital shaker. It was centrifuged at 13000× g for 1 min and the pellet was placed on LB agar containing ampicilin (50 μg/ml) and incubated at 37°C overnight and recombinant colonies subjected to colony PCR to confirm the presence of the *lipL41* gene. Positive colonies were grown in LB agar overnight and selected transformed *E. coli* colonies were individually inoculated into LB-ampicilin (50 μg/ml) broth. The cultures were incubated in a shaking incubator at 37 °C for overnight. The bacterial cells were collected from each culture by centrifugation at 4000× g at 25 °C for 5 minutes. Plasmids were extracted from the cells by Plasmid Mini extraction kit (Roche, Germany) according to the manufacturer's instructions. The samples were analyzed by 1% agarose gel electrophoresis.

Analysis of Nucleotide Sequencing of the Gene. The extracted recombinant plasmids were sequenced by Macrogen (South Korea). The sequences were deposited in the Genbank database of NCBI with the accession numbers KJ409447, KJ409448, KJ409451. The homologies of sequences of vaccinal serovars of *Leptospira* were first evaluated with the BLAST program of NCBI. The *lipL41* gene sequences of *L. interrogans* serovar Canicola, Grippotyphosa, Sejroe Hardjo were obtained from Genbank at the National Center for Biotechnology Information (NCBI) website. The sequence alignments were performed with Clustal W. The percentage of identity and genetic divergence among our leptospiral serovars was deduced using the MegAlign Programe of DNASTAR software. On the basis of homology analysis, a phylogenetic tree (neighbor joining) was constructed using the Lasergene software by Clustal method with weighted residue table.

RESULTS

PCR Amplification and Cloning of the *lipL41* Gene. The PCR amplification of the *lipL41* gene using the specific primers resulted in the 1065 bp *lipL41* gene product in three vaccinal serovars while it was absent in saprophyte serovar *L. biflexa* (Figure 1).

The amplified gene was successfully cloned in the pTZ57R/T vector and transformed into *E. coli* TOP10 cells. The recombinants were confirmed by picking white colonies and carried out colony PCR amplification of the *lipL41* gene.

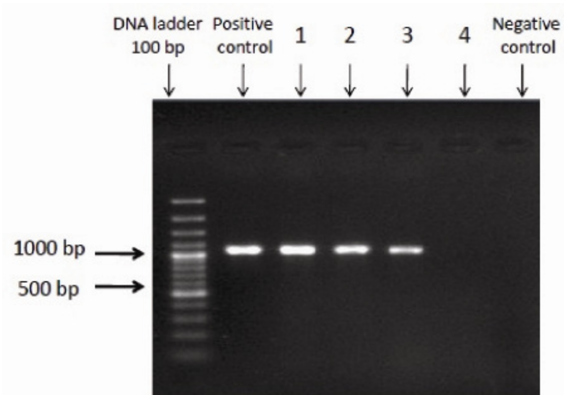

Figure1. PCR amplification of the 1065 bp *lipL41* gene of *L. interrogans* serovars.

Sequence Analysis. Analysis of the sequences by using the NCBI database and BLAST revealed homology with vaccinal serovars of leptospires. It showed >96% identity among vaccinal serovars of *Leptospira* (Figure 2) but *lipL41* sequence had no homology with other bacterial sequences listed in NCBI database. Then these sequences were compared by multi alignment and dendrogram was constructed (Figure 3). In our study the *lipL41* gene from three vaccinal serovars was highly conserved, ranging from 96% to 99.9%. This gene also was found to be conserved with reference sequences submitted in Genbank.

Percent Identity (upper triangle) / Divergence (lower triangle)

	1	2	3	4	5	6	7	8		
1		99.9	99.5	96.2	99.5	99.7	96.2	99.4	1	LC(AY622675)
2	0.1		99.6	96.3	99.6	99.8	96.3	99.5	2	LC(AY642287)
3	0.5	0.4		96.3	99.8	99.4	96.3	99.3	3	LG(AY622681)
4	4.0	3.9	3.9		96.3	96.1	99.6	96.0	4	LG(JQ690557)
5	0.5	0.4	0.2	3.9		99.4	96.3	99.5	5	LH(AY642286)
6	0.3	0.2	0.6	4.1	0.6		96.5	99.7	6	LC_RTCC2805
7	4.0	3.9	3.9	0.4	3.9	3.7		96.4	7	LG_RTCC2808
8	0.6	0.5	0.7	4.2	0.5	0.3	3.8		8	LSH_RTC2821
	1	2	3	4	5	6	7	8		

Figure2. Sequence pair distances of *lipL41* gene sequences of different Leptospiral serovars.

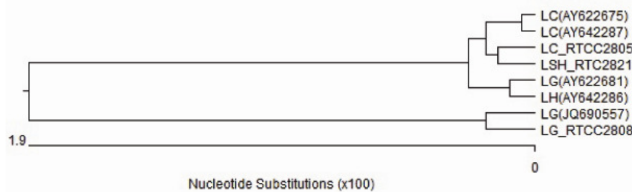

Figure3. Phylogenetic tree analysis of the *lipL41* gene.

DISCUSSION

The currently available vaccines are composed of trivalent inactivated dominant local serovars that cannot provide cross-immunity against any other serovars. Any of the *L.interrogans* serovars not included in the vaccine can still cause fulminant epidemic of leptospirosis (Luo *et al* 2009). Hence the local variability in serovars of endemic leptospiral strains complicated the development of a vaccine that could be used worldwide (Levett 2001, Meites *et al* 2004). Molecular pathogenic mechanisms of leptospirosis appear complex. Several candidate virulence factors such as lipopolysaccharide (LPS), outer membrane proteins (OMPs) and adhesion molecules have been identified that may contribute to the pathogenesis of *Leptospira* infections. Among these, the OMPs may be potential targets to induce and enhance immune responses against leptospirosis, and genetic characterization of them is an important approach in the development of subunit vaccines. According to the results of other researchers, leptospiral lipoprotein LipL41 has been evaluated as a potential vaccine candidate (Cullen *et al* 2002, Haake *et al* 1999, Felix *et al* 2009, Feng *et al* 2009). Considering that the efficient vaccine for control of leptospirosis should have immunogenic antigen that is also present in all pathogenic serovars, we investigated the presence and genetic similarity of the *lipL41* gene among vaccinal serovars of *Leptospira* in Iran which have been identified by molecular analysis. At the present study, our results indicated that the *lipL41* gene was highly conserved among our vaccinal serovars (94% > identity), in accordance with previous studies (Haake *et al* 2004, Haake *et al* 1999, Feng *et al* 2009). Haake *et al*. (2004) showed high levels of sequence conservation of the *lipL41* gene among different pathogenic leptospiral serovars and indicated that monovalent LipL41 based vaccine has the potential for being broadly protective. In a similar study, it was revealed that LipL41 could provide significant protection against homologous challenge (Haake *et al* 1999). It has been reported that the level of protection could be increased significantly by combining multiple rLipLs immunogens (Haake *et al* 1999). Another study demonstrated that the *lipL41* gene is present in pathogenic leptospires and the recombinant of this gene is a protective antigen against *Leptospira* and also resulted in better immune responses than single-component, OMPs, or single DNA or protein immunization (Feng *et al* 2009). The analysis of the *lipL41* gene sequence of our vaccinal serovars *L.* Canicola (RTCC2805), *L.* Grippotyphosa (RTCC2808), *L.* Sejroe hardjo (RTCC2821) showed the high similarity of the *lipL41* gene sequence between them and other submitted sequences in Genbank database. According to our result, the *lipL41* gene is highly conserved among vaccinal leptospiral serovars and possesses extensive sequence homology. Thus, LipL41 may contribute to the efficiency of recombinant multi-epitope vaccines. Hence these results may shed light on the use of this gene as a potential suitable platform in the development of recombinant vaccines that could generate cross protection against a wide range of *Leptospira* serovars.

Ethics

I hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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