Original Article

Molecular identification of the *ompL1* gene within *Leptospira interrogans* standard serovars

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Abstract

Introduction: Leptospirosis, caused by infection with pathogenic *Leptospira* species, is one of the most prevalent zoonotic diseases in the world. Current leptospiral vaccines are mainly multivalent dead whole-cell mixtures made of several local dominant serovars. Therefore, design and construction of an efficient recombinant vaccine for leptospirosis control is very important. OmpL1 is an immunogenic porin protein that could be of special significance in vaccination and serodiagnosis for leptospirosis.

Methodology: Three strains belonging to pathogenic *L. interrogans* were analyzed. The specific primers for proliferation of the *ompL1* gene were designed. The amplified gene was cloned. In order to investigate the *ompL1* nucleotide sequence and homological analysis of this gene, *ompL1* genes cloned from standard vaccinal *Leptospira* serovars prevalent in Iran were sequenced and cloned.

Results: PCR amplification of the *ompL1* gene using the designed primers resulted in a 963 bp *ompL1* gene product. The PCR based on the *ompL1* gene detected all pathogenic reference serovars of *Leptospira* spp. tested. Based on alignment and phylogenetic analysis, although the *ompL1* nucleotide sequence was slightly different within three vaccinal serovars (100%-85% identity), amino acid alignment of the OmpL1 proteins revealed that there would be inconsiderable difference among them.

Conclusion: The ompL1 gene of the three isolates was well conserved, differing only by a total of 6 bp and the proteins by 2 amino acids. The cloned gene could be further used for expression and recombinant OmpL1 as an efficient and conserved antigen, and may be a useful vaccine candidate against leptospirosis in our region.

Key words: leptospirosis; nucleotide sequencing; phylogenic analysis; OmpL1

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Introduction

Leptospirosis is one of the most important zoonoses with worldwide distribution. The disease occurs mostly in tropical, subtropical, temperate, and humid regions with high rainfall. Leptospirosis is characterized by hemorrhage, jaundice, myalgia, renal impairment, and aseptic meningitis [1,2]. Pulmonary diffuse hemorrhage (PDH), a serious clinical type of leptospirosis, results in death in a quarter of affected patients [3]. The genus *Leptospira* consists of a diverse group of pathogenic and saprophytic spirochetes which, based on the antigenic structure, are classified into different serovars [1]. A universal feature of pathogenic leptospires is their ability to parasitize the proximal renal tubules of a wide variety of wild and domestic animals. Infection with host-adapted leptospiral serovars can result in lifelong renal carriage and urinary shedding. In humans, exposure to infected host animals or contaminated water or soil results in this potentially lethal disease [4]. Leptospirosis eradication is difficult because there is an abundance of animal reservoirs, both wild and domestic, of *Leptospira* spp. and the long-term survival of the bacteria in the environment. Avoiding contact with animals chronically infected with *Leptospira* spp. (reservoirs) or their environments, such as soil and water contaminated with animal urine or carcasses, is the most effective means of disease prevention. However, the measure is difficult to practice, especially in the countries where agriculture is the foremost activity and environmental sanitation is compromised [5]. Protective immunity elicited by leptospiral lipopolysaccharide is generally serovar-specific [2]. The current available whole-cell vaccines cannot provide cross-protection against infection with more than 250 different *Leptospira* serovars known to

exist and may lead to incomplete, short-term immunity and serious side effects. Despite vaccination, the disease still exists in some parts of the country [6-8]. The design and construction of an efficient recombinant vaccine for leptospirosis control is very important [9,10].

Characterization of leptospiral outer membrane proteins (OMPs) has emerged as an important approach [11].

Outer membrane proteins of pathogenic bacteria species are very stable and are the foundation of communication between bacteria and the host. Therefore, many studies about the characteristics of membrane protein components have been performed.

OmpL1 is a surface genus-specific antigen of pathogenic Leptospirs that is not observed in the non-pathogenic species of *Leptospira*. This porin protein is significantly expressed during infection, demonstrating that it is an immunogenic antigen. OmpL1 can be a suitable candidate to be used in the preparation of a recombinant leptospirosis vaccine and in comprehensive serological diagnostic tests [12].

However, the diversity of *ompL1* gene sequences from different pathogenic *Leptospira* spp. and the distribution of the *ompL1* gene in vaccinal and clinical isolates have not been characterized.

In this study, for the first time, we sequenced and analyzed *ompL1* genes cloned from standard pathogenic strains of leptospires prevalent in Iran.

Methodology

Database

The *ompL1* sequence of pathogenic *Leptospira* serovars were obtained from GenBank at the National Centre for Biotechnology Information (NCBI) website. Homology searches with the *ompL1* sequences of different pathogenic *Leptospira* species were accomplished using the BLAST program against the GeneBank/NCBI nuclear acid sequence database.

Bacterial strains and media

The Microbiology Department of Razi Vaccine and Serum Research Institute in Karaj, Iran provided three standard strains belonging to three serogroups of pathogenic *L. interrogans* and one saprophytic strain of *L. biflexa* (Table 1).

L. Sejroe hardjo, *L*. Canicola, *L*. Grippotyphosa (belonging to pathogenic *L. interrogans*) and *L. biflexa* were grown in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco, Detroit, USA) containing 8% rabbit serum at 28°C under aerobic conditions [12-14].

Isolation of leptospiral genomic DNA

The culture was centrifuged at 17,000 x *g* at 4°C for 20 minutes. Genomic DNA was extracted from whole cells of samples grown to log phase growth using the phenol-chloroform extraction method [15]. The preparation was added with a lysis buffer containing 10% SDS, EDTA (50mM), NaCl (100mM), Tris (100mM), 3 µL of 20 µg/mL proteinase-K, 5 µL of 20 mg/ml RNase A and incubated at 37°C for one hour. An equal volume of phenol-chloroform-isoamyl was added to the mixture. The preparation was centrifuged at 12,000 x *g* at 25°C for 5 minutes. The top phase was transferred to a new tube and isopropanol was added to precipitate the DNA. The DNA pellet was collected after centrifugation, washed with 70% ethanol, and air-dried. The dried DNA was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). The density and purity of the extracted DNAs were detected by UV spectrophotometery and analyzed by 1% agarose gel electrophoresis, ethidium bromide staining.

Primer design and ompL1 amplification by polymerase chain reaction

The oligonucleotide primers for amplification of the *ompL1* gene by polymerase chain reaction (PCR) were designed from the DNA sequences encoding OmpL1 of *L. interrogans* strains of the GenBank database. Primer syntheses were performed at (SIGMA ALDRICH, Taufkirchen, Munich, Germany).

A gradient PCR was performed for determining the optimum primer annealing temperature. The temperature gradients were 47-58°C.

In order to provide appropriate amplicons for the TAclone method, a 50 µL PCR amplification reaction

Table 1. *Leptospira* strains included in this study

Name	Accession No.	Genomospecies	Serovar	Strain	Source	Virulence
LC	JX532093	*Leptospira interrogans*	Canicola	2805	RTCC	+
LG	JX532094	*Leptospira interrogans*	Grippothyphosa	2808	RTCC	+
LSH	JX532095	*Leptospira interrogans*	Hardjo	2821	RTCC	+
L. biflexa		*Leptospira biflexa*	Patoc	2819	RTCC	-
RTCC :		Razi Type Culture Collection				

system was prepared by mixing *Pfu-Taq* DNA polymerase to amplify the target gene. The PCR mixture consisted of 5 µL of *Pfu* buffer with MgSO4 (10x), 1 µL of dNTPs mix (10 mM), 1 µL each of upstream and downstream primers (10 pmol), 0.5 µL of *Pfu* DNA polymerase (5 U), 0.2 µL of *Taq* polymerase (5 U/µL), 1 µL of DNA template (100 ng), and DNAse-free water to final volume. The reaction mixture was initiated by incubation at 94°C for 5 minutes, followed by 35 cycles of amplification at 94°C for 1 minute, 52°C (determined by gradient PCR) for 1 minute, and 72°C for 90 seconds. A final extension run for 10 minutes at 72°C concluded the reaction.

An aliquot of each DNA amplicon was subjected to 1% agarose gel electrophoresis and ethidium bromide staining.

Cloning of ompL1 *amplicon into vectors and selection of* E. coli *transformants*

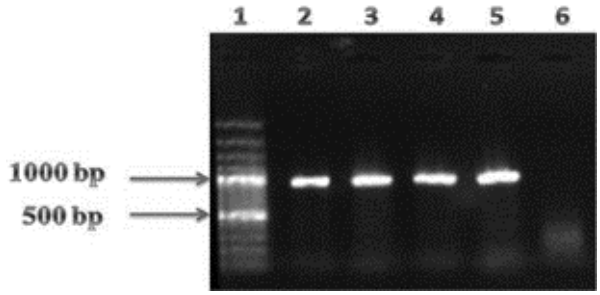
To obtain more accurate sequence data, the *ompL1* amplicons were purified using the GeneJET PCR Purification Kit (Thermo Scientific-Fermentas, Waltham, USA). The purified DNA was ligated into pTZ57R/T using the InsTAclone PCR Cloning Kit (Thermo Scientific-Fermentas, Waltham, USA) via the overhang T of the plasmid and A of the DNA. The recombinant vector was cloned into Top10 *E. coli* competent cells using heat shock protocol [15,16].

Selected transformed *E. coli* colonies (examined by Colony PCR using *ompL1* specific primers) were individually inoculated into LB-ampicillin (50 µg/mL) broth. The cultures were incubated in a shaking incubator at 37°C for 16 hours. Bacterial cells were collected from each culture by centrifugation at 5,000 x *g* at 25°C for 5 minutes. Plasmids were extracted from the cell preparations using a Plasmid Isolation Kit (CinnaGen, Tehran, Iran). The purified plasmid preparation was analyzed by 1% agarose gel electrophoresis, ethidium bromide staining, and visualization under a UV transilluminator. Furthermore, PCR using *ompL1*-specific primers was performed to confirm the accuracy of recombinant purified plasmids.

Nucleotide sequencing and homological analysis

The *ompL1* genes from all three pathogenic standard serovars were sequenced by SeqLab Co. (Germany). Homological analysis was performed by BLAST against the nucleotide sequence database on the GenBank/NCBI website (http://www.ncbi.nlm.nih.gov/). Sequence alignment was performed with the Clustal W method [17] using MEGA5 software [18].

Figure 1. PCR amplification of *Leptospira* species based on ompL1 gene



(1) 100 bp DNA Ladder; (2) positive control; (3) *L.* Canicola; (4) *L.* Grippothyphosa; (5) *L.* Sejroe hardjo; (6) saprophytic *L. biflexa*; 963 bp amplicon can be seen in all pathogenic serovars tested (lanes 3-5) but cannot be observed in saprophytic *L. biflexa* (lane 6)

The amino acid sequences of proteins were deduced from the nucleotide sequences using the EditSeq program of DNAStar software.

Amino acid sequence alignments were carried out using the Clustal W method of MegAlign program of DNAStar software package (DNASTAR, Madison, USA).

Phylogenetic analysis was done with neighbor-joining (NJ) optimality criteria of MEGA5 software and MegAlign program.

Results

Isolation of leptospiral genomic DNA and ompL1 *amplification by PCR*

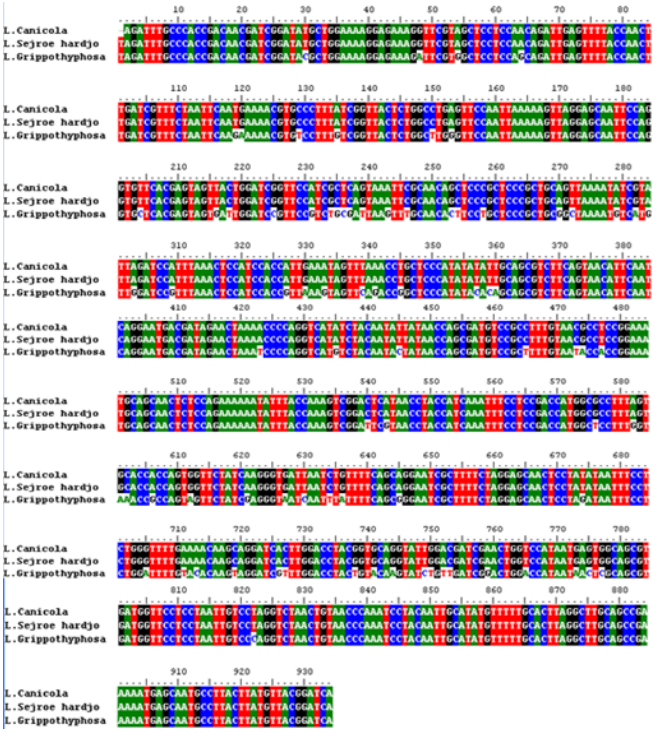
Genomic DNA encoding OmpL1 was successfully extracted from pathogenic and saprophitic serovars, as the *ompL1* could be amplified from the genomic DNA preparation. The DNA was used as a template for amplification of *ompL1* by gradient PCR and the annealing temperature at 52°C was chosen. The *ompL1* amplicon at 963 bp is shown in Figure 1.

All the three standard strains of pathogenic *L. interrogans* carried the *ompL1* gene, since amplicons with the expected size were produced by PCR from these strains. However, saprophitic *L. biflexa* strains could not be amplified by PCR [11] (Figure 1).

Cloning, sequencing and homological analysis of the ompL1 *gene*

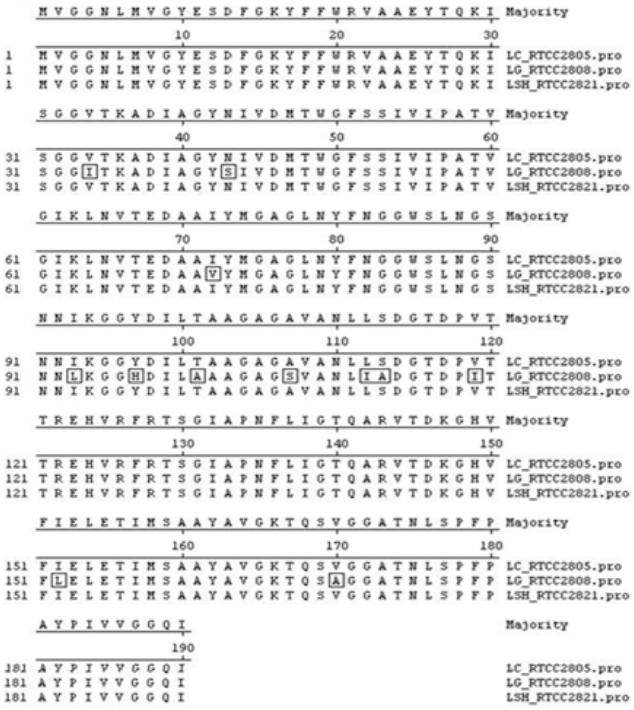
The *ompL1* gene was successfully subcloned into pTZ57R/T vector and the recombinant vector, *ompL1*-pTZ57R/T, was introduced into competent Top10 *E. coli*.

Figure 2. Nucleotide alignment of three pathogenic serovars performed with Clustal W method using MEGA5 software.



There are slightly more contraversies between *L.* Grippothyphosa and other two serovars which are entirely identical in terms of *ompL1* nucleotide sequence.

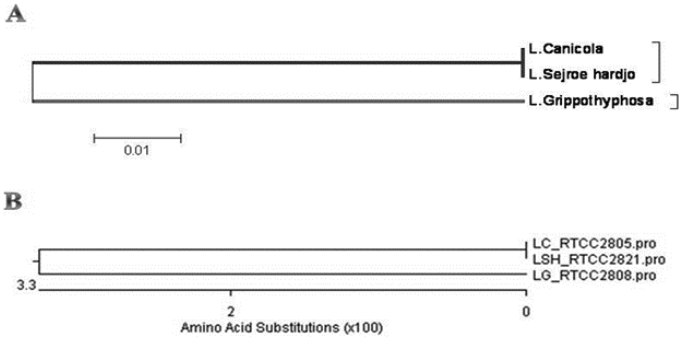
Figure 3. Amino acid alignment of three pathogenic serovars performed with Clustal W method using MegAlign program of DNAStar software package



L. Grippothyphosa has insignificant differences compared to others in its amino acid sequence, specifically in 90th-120th amino acid.

Figure 4. Phylogenetic trees of three standard strains using the NJ method

(A) based on ompL1 nucleotide sequences; (B) based on amino acid sequences of OmpL1 protein



These colonies were then separately grown in LB-ampicillin broth and the plasmids were extracted and purified. The purified plasmid preparation was analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. Furthermore, PCR using *ompL1*-specific primers was performed to confirm the accuracy of recombinant purified plasmids (data not shown).

The *ompL1* genes of the three pathogenic *Leptospira* serovars were all sequenced by a standard sequencing process. A BLAST search of the GenBank database revealed high nucleotide sequence identity when compared with the available complete genome sequence database of *L. interrogans* serovar strain Lai [19], *L. interrogans* serovar Copenhageni strain Fiocruz L1-130 [20,5], the corresponding sequence data of *Leptospira kirschneri* serovar Grippotyphosa strain RM52 [21] and *ompL1* sequences belonging to other pathogenic *Leptospira* strains. Alignment of the *ompL1* gene sequence of three standard serovars was also performed. The results showed that sequences of the *ompL1* gene of *L.* Canicola (RTCC2805) and *L.* Sejroe hardjo (RTCC2821) (Table 1) were exactly the same (100% identity), while *L.* Grippotyphosa (RTCC2808) was less similar (88.5%) to the other two serovars (Figures 2 and 4A).

Discussion

Highly conserved OMPs are of special significance in serodiagnosis and vaccine development for leptospirosis. The leptospiral OMPs expressed during mammalian infection may have potential immunoprotective capabilities [25,26].

Surface exposure is a key characteristic for an effective antigen. OmpL1 is an immunogenic transmembrane porin protein extensively expressed in pathogenic leptospires [27,22].

According to earlier reports, a strong interaction between the recombinant OmpL1 protein and leptospirosis patients' sera was observed by enzyme-linked immunosorbent assay. It has also been demonstrated that OmpL1 and LipL41 together could provide significant protection against homologous challenge in the hamster model of leptospirosis [26].

Moreover, a previously designed OmpL1 DNA vaccine was found to be well tolerated by the immunized animals and conferred immunity that protected some immunized hamsters against the heterologous lethal challenge. The vaccine was shown to confer the delay in the death time and reduced morbidity in the vaccinated animals [5].

OmpL1 should therefore be viewed as a potential candidate of genus-specific antigen for the development of new universal vaccines and serodiagnostic methods for leptospirosis in Iran.

We evaluated probable differences of the *ompL1* gene and its protein product between various local pathogenic serovars. We sequenced and analyzed *ompL1* genes cloned from standard pathogenic strains of leptospires prevalent in Iran and then compared the OmpL1 amino acid sequences.

In our survey, nucleotide sequencing results demonstrated that the gene encoding OmpL1 was found well conserved in all tested pathogenic *Leptospira* strains including *L*. Canicola (RTCC2805), *L*. Sejroe hardjo (RTCC2821), and *L*. Grippotyphosa (RTCC2808).

According to our alignment and phylogenetic analysis from the three standard strains of pathogenic *L. interrogans*, although the *ompL1* gene nucleotide sequence was slightly different within some of the strains, amino acid alignment (Figures 3 and 4B) of the OmpL1 proteins revealed that there was little difference among them.

There is some evidence in previous studies that the OmpL1 protein has five main putative surface-membrane regions (70–80, 115–120, 145–150, 235–245, 305–310 amino acid residuals) [22,23] and seven transmembrane regions (10–40, 120–130, 160–190, 215–225, 240–250, 270–280, and 295–305 amino acid residues) [24]. The only salient different was the presence of less alpha-helix in the N-terminal and beta-sheet in the C-terminal region of the OmpL1/3 sequence, compared to the sequences of OmpL1/1 and OmpL1/2.

Conclusion

The differences in nucleotide sequences in the *ompL1* gene types may not affect the immunogenicity of OmpL1 proteins. The cloned gene, therefore, could be further used for expression; recombinant OmpL1 as an efficient and conserved antigen may be a useful vaccine candidate against leptospirosis in Iran.

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