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Expression and Comparative Analysis of Genes Encoding Outer Membrane Proteins LipL21, LipL32 and OmpL1 in Epidemic Leptospires

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Abstract Leptospiral outer membrane proteins (OMPs) are highly conserved in different species, and play an essential role in the development of new immunoprotection and serodiagnosis strategies. The genes encoding LipL21, LipL32 and OmpL1 were cloned from the complete genome sequence of *Leptospira interrogans* serovar lai strain Lai and expressed *in vitro*. Sequence comparison analysis revealed that the three genes were highly conserved among distinct epidemic leptospires, including three major epidemic species *Leptospira interrogans*, *Leptospira borgpetersenii* and *Leptospira weilii*, in China. Immunoblot analysis was further performed to scrutinize 15 epidemic *Leptospira* reference strains using the antisera of the recombinant OMPs. Both immunoblot assay and reverse transcription-polymerase chain reaction demonstrated that these three OMPs were conservatively expressed in pathogenic *L. interrogans* strains and other pathogenic leptospires. Additionally, the use of these recombinant OMPs as antigens in enzyme-linked immunosorbent assay (ELISA) for serodiagnosis of leptospirosis was evaluated. The recombinant LipL32 and OmpL1 proteins showed a high degree of ELISA reactivity with sera from patients infected with *L. interrogans* strain Lai and other pathogenic leptospires. These results may contribute to the identification of candidates for broad-range vaccines and immunodiagnostic antigens in further research.

Key words outer membrane protein; expression; comparative analysis; epidemic leptospire

Leptospirosis is one of the most important zoonoses with worldwide distribution. Protective immunity elicited by leptospiral lipopolysaccharide is generally serovar-specific [1]. The current available whole-cell vaccines can not provide cross-protection against infection with more than 250 different *Leptospira* serovars known to exist [2,3]. Thus characterization of leptospiral outer membrane proteins (OMPs) has emerged as an important approach.

So far three classes of leptospiral OMPs have been identified: lipoproteins, the most abundant class comprising LipL32, LipL36, LipL41, LipL48, LipL21 [3–7] and the temperature-regulated Qlp42 [8]; transmembrane protein OmpL1 [9]; and peripheral membrane proteins such as LipL45 [10]. Some spirochaetal outer membrane proteins have been characterized by a lipoprotein structure called lipobox [11,12].

The classic Triton X-114 method and the traditional approaches for isolation of the outer membrane protein from *Leptospira* species have met with some challenges [3,6,13]. On the other hand, the complete genomic DNA sequence of pathogenic *Leptospira interrogans* serovar lai strain Lai represents a new unexploited field for the design of novel vaccines and development of serodiagnosis [14], as well as for prediction of the immunoreaction of the outer membrane lipoproteins with the host environment.

Three OMP genes from the genome sequence of *L.*

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interrogans serovar lai strain Lai, encoding LipL21, LipL32 and transmembrane protein OmpL1 separately, were cloned and expressed *in vitro*. Primarily, they were identified and annotated by bioinformatics tools on primary structure, transmembrane structure, hydrophobicity, protein domain and protein family [11]. Standard nucleotide sequencing analysis of these corresponding OMP genes of different epidemic *Leptospira* strains which are of medical importance in China [15,16] were performed. To characterize and validate the potential roles of these recombinant OMPs (rOMPs) as target antigens in the host humoral immune response of leptospirosis [17], and to clarify whether these OMP genes are highly conserved in various epidemic leptospires in China, the three OMP genes were analyzed in detail by both immunoblot and reverse transcription-polymerase chain reaction (RT-PCR). The immunoreaction of purified recombinant OMPs with patients' sera of leptospirosis caused by *L. interrogans* serovar lai and other pathogenic leptospires were evaluated by enzyme-linked immunosorbent assay (ELISA).

Materials and Methods

Database

The complete genomic DNA sequence of *L. interrogans* serovar lai strain Lai was obtained from GenBank at the National Centre for Biotechnology Information (NCBI) website. Homology searches with the OMP sequences of different epidemic *Leptospira* species were accomplished using the BLAST program against the GenBank/NCBI nuclear acid sequence database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=Search&DB=nucleotide).

Leptospira isolates

A panel of pathogenic *Leptospira* reference strains comprised three main epidemic species in China [15,16] including ten *L. interrogans* strains, four *Leptospira borgpetersenii* strains and one *Leptospira weilii* strain; and the nonpathogenic group composed of saprophytic *Leptospira biflexa* serovar patoc and serovar monvalerio strains (**Table 1**). All leptospira isolates were provided by the Institute for Infectious Disease Control and Prevention (Chinese Center for Disease Control and Prevention, Beijing, China). These isolates were cultivated in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium at 30 ºC [18].

Isolation of leptospiral genomic DNA

Stationary-phase culture was harvested. Samples were then extracted with an equal volume of phenol-chloroform-isoamyl alcohol. The aqueous phase was removed and extracted with chloroform-isoamyl alcohol. DNA was

Table 1 ***Leptospira*** **strains involved in this study**

Name	Strain	Genomospecies	Source	Virulence
Lep1	Serovar lai strain Lai	*L. interrogans*	CCDC	+
Lep2	Serovar canicola strain Lin	*L. interrogans*	CCDC	+
Lep3	Serovar pyrogenes strain 4	*L. interrogans*	CCDC	+
Lep4	Serovar autumnalis strain Lin 4	*L. interrogans*	CCDC	+
Lep5	Serovar australis strain 65-9	*L. interrogans*	CCDC	+
Lep6	Serovar Pomona strain Luo	*L. interrogans*	CCDC	+
Lep7	Serovar linhai strain Lin 6	*L. interrogans*	CCDC	+
Lep8	Serovar hebdomadis strain P 7	*L. interrogans*	CCDC	+
Lep9	Serovar paidjan strain L 37	*L. interrogans*	CCDC	+
Lep10	Serovar wolffi strain L 183	*L. interrogans*	CCDC	+
Lep11	Serovar javanica strain M 10	*L. borgpetersenii*	CCDC	+
Lep12	Serovar ballum strain Pishu	*L. borgpetersenii*	CCDC	+
Lep13	Serovar tarassovi strain 55-52	*L. borgpetersenii*	CCDC	+
Lep14	Serovar mini strain Nan 10	*L. borgpetersenii*	CCDC	+
Lep15	Serovar qingshui strain L 105	*L. weilii*	CCDC	+
Lep16	Serovar monvalerio	*L. biflexa*	CCDC	–
Lep17	Serovar patoc	*L. biflexa*	CCDC	–

CCDC, Chinese Center for Disease Control and Prevention.

precipitated from the aqueous phase with 2.5 volumes of 95% ethanol. DNA pellets were washed in 75% ethanol, recentrifuged, and air-dried before being resuspended in H_2O.

Isolation of total RNA

Mid-logarithmic growth phase *Leptospira* culture was harvested. Total RNA was isolated by Trizol reagent (Gibco BRL, Gaitherburg, USA). Following the chloroform-isopropyl alcohol process, the RNA pellets were washed with 1 ml of 75% ethanol and air-dried before being resuspended in RNase-free water.

Cloning and expression of OMP genes, and purification of recombinant OMPs

Selected OMP genes were amplified and expressed from pET-28b(+) (Novagen, Madison, USA) in *Escherichia coli* BL21(DE3) (Novagen). The associated gene features are listed in **Table 2**. PCR primers were designed as shown in **Table 3**. The PCR amplification reaction system was as follows: DNA was denatured at 95 °C for 10 min before 45 cycles at 94 °C for 0.5 min, 54 °C for 1 min, and 72 °C for 1 min. A final extension run for 10 min at 72 °C concluded the reaction.

Expression of selected OMP genes was induced in mid-logarithmic growth phase *E. coli* BL21(DE3) with 1 mM isopropyl-β-*D*-thiogalactopyranoside (IPTG; Sigma, Sydney, Australia). His_6-tagged proteins were purified following the manufacturer's instructions (Novagen).

Antiserum preparation and immunization

Antisera to these OMPs were prepared as described previously [19]. New Zealand white rabbits were immunized with purified His_6-OMP fusion proteins expressed by *E. coli* BL21(DE3) transformed with the pET-28b(+) plasmid containing the OMP genes. Two hundred micrograms of purified protein was mixed with Freund's complete adjuvant and inoculated subcutaneously into one male New Zealand white rabbit. Additional immunizations with approximately 200 μg of His_6 fusion protein in Freund's incomplete adjuvant were given 4 weeks and 6 weeks after the primary immunization. The rabbits were bled 8 weeks after the primary immunization. Serum samples were collected by centrifuge. The antiserum specificity was examined using ELISA with the purified recombinant OMPs as target antigens.

Nucleotide sequencing and homological analysis

A 50 μl PCR amplification reaction system was prepared by mixing the following reagents: 5 μl 10×DNA polymerase buffer; 1 μl dNTP mixture (10 mM); 25 pmol each of upstream and downstream primers; 0.5 μl *pfu* DNA polymerase (2.5 U); 50 ng isolated genomic DNA of *Leptospira* strains evaluated as template; ion-free water to final volume. PCR primers are shown in **Table 3**.

Primer synthesis and nucleotide sequencing reaction were performed at Shanghai Shenergy Biocolor BioScience and Technology Company, Limited (http://www.biocolors.com). The sequencing procession of PCR amplified production was operated twice.

Table 2 Characterization of three outer membrane protein genes

Locus tag	Length (bp)	MW (Da) [a]	pI [a]	Pro domain [b]	Family
LA0011	561	19,661	8	–	LipL21
LA2637	819	29,613	7	PD124660	LipL32
LA3138	1014	35,393	9	PD128479	OmpL1

[a] molecular masses and isoelectric points were calculated by using the ProtParam (http://www.expasy.org/tools/protparam.html); [b] protein domains were predicted by software ProDom (http://prodes.toulouse.inra.fr/prodom/doc/blastform.html) and Pfam (http://pfam.wustl.edu/hmmsearch.shtml). bp, base pair; MW, molecular weight; pI, isoelectric point; Pro, protein.

Table 3 Primers designed for amplifying leptospiral outer membrane protein genes by polymerase chain reaction (PCR) or reverse transcription (RT)-PCR

Locus tag	Primer sequence (5′→3′)	Restriction enzyme site
LA0011	Forward: CATATGAAAGACGCAACTACTGTAG	*Nde*I
	Reverse: CTCGAGACGTTCTTCCCAGTTGT	*Xho*I
LA2637	Forward: CATATGGGTCTGCCAAGCCTAAA	*Nde*I
	Reverse: CTCGAGTTACTTAGTCGCGTCAGAA	*Xho*I
LA3138	Forward: CATATGGGATTTGGGTTACAGTTA	*Nde*I
	Reverse: CTCGAGTCTGTAGATTTGCCCACC	*Xho*I

Restriction enzyme sites were underlined.

Homological analysis was performed by BLAST against the nucleotide sequence database on the GenBank/NCBI website (http://www.ncbi.nlm.nih.gov/). Then the sequences of the genes LA0011, LA2637 and LA3138 obtained from the 15 different pathogenic leptospiral strains were submitted to GenBank/NCBI online.

Immunoblot

One nonpathogenic and 15 pathogenic leptospire whole protein preparations as samples for sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) were solubilized in final sample buffer composed of 62.5 mM Tris hydrochloride (pH 6.8), 10% glycerol, 5% 2-mercaptoethanol, and 2% SDS. Proteins were separated on a 12% gel with a discontinuous buffer system. After electrophoresis, the gel was stained with Coomassie brilliant blue or transferred to nitrocellulose (Schleicher and Schuell, Keene, USA) for immunoblotting. For antigenic detection on immunoblots, the nitrocellulose was blocked with 10% nonfat dry milk in phosphate-buffered saline (PBS; pH 7.4) containing 0.1% Tween-20 (PBS-T), incubated for 2 h with rabbit antiserum (1:5000) specific to cloned leptospiral OMPs in PBS-T, and probed with goat anti-rabbit IgG-Fc (Sigma) conjugated to alkaline phosphatase to allow colorimetric detection.

RT-PCR

The leptospiral RNA extracted was treated with DNase (Promega, Beijing, China) before reverse transcription. The RNA mixture with RNase-free DNase and reaction buffer was incubated at 37 °C for 30 min. DNase stop solution was added to terminate the reaction, then the mixture was incubated at 65 °C for 10 min. Reverse transcription was performed using the AMV reverse transcription system (Promega). The product was resuspended in 100 μl ion-free water. RT-PCR primers are shown in **Table 3**.

Recombinant OMP-based ELISA

Patients' sera were obtained from the Institute for Infectious Disease Control and Prevention. The leptospirosis group consisted of 18 patients with clinical manifestations of leptospirosis due to *L. interrogans* serovar lai, and defined by a reciprocal microscopic agglutination test [20]; there were four patients with culture-documented leptospirosis due to infection by other pathogenic *Leptospira* strains. The normal control group comprised 10 blood bank donors.

Immulon microtiter plates (Dynatech, Alexandria, USA) were coated at 37 °C overnight with purified His_6 fusion proteins, then were blocked with blocking buffer (PBS containing 0.05% Tween-20 and 1% nonfat dried milk) at 37 °C for 1 h. After washing three times with PBS-T, antiserum was added. Following incubation at 37 °C for 2 h, the mixture reacted with 1:7500 diluted goat anti-human IgG conjugated to alkaline phosphatase (Promega) at 37 °C for 1 h. Substrate solution in 20 mM carbonate buffer (pH 9.8) containing 2.5 mM *p*-nitrophenylphosphate, disodium salt and para-nitrophenyl phosphate (pNPP; Promega) was added. The reaction was terminated by 2 M H_2SO_4. The absorbance value of each well was read at 450 nm with a microplate reader (Tecan Spectra III, Sydney, Australia). Wells without a coating antigen were used as blank control. Sera of healthy individuals were analyzed as negative control. Statistical analysis was performed using GraphPad Prism 4 software.

Results

Sequencing and homological analysis of the OMP genes of different epidemic *Leptospira* strains

Three OMP genes LipL21, LipL32 and OmpL1 of the 15 pathogenic leptospires were all amplified and 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 lai strain Lai [14], *L. interrogans* serovar copenhageni strain Fiocruz L1-130 [21,22] and the corresponding sequence data of *Leptospira kirschneri* serovar grippotyphosa strain RM52 [23]. It provided significant evidence for the high conversation of the three OMP components among distinct epidemic leptospires in China. The sequence identity of the three OMP genes were found to be relatively higher in *L. interrogans* strains than in four *L. borgpetersenii* strains and one *L. weilli* strain when compared with the reported sequence data of *L. interrogans* serovar lai strain Lai [14]. Meanwhile, none of the three genes of two saprophitic *L. biflexa* strains could be amplified by PCR. Sequences of LipL32 encoding gene in three *L. borgpetersenii* strains and one *L. weilli* strain were found to be identical with reference sequences submitted in GenBank by other researchers (accession No. AY609321–AY609325, AY609327–AY609331 and AY609333; data not shown).

The sequences identified in this article were submitted to GenBank with accession No. AY688419–688431, AY634682, AY776292–776294 and AY688396–688409 (http://www.ncbi.nlm.nih.gov/entrez). Homology results with the OMP sequences from different pathogenic

Leptospira species using the special BLAST program may be searched online.

Characterization of purified recombinant OMPs

The outer membrane protein components expressed by OMP genes LA0011, LA2637 and LA3138 were described as cpLipL21, cpLipL32 and cpOmpL1 respectively in this article. Lanes of purified proteins were shown in PAGE gel as in **Fig. 1**, molecular weights of His_6-tagged proteins are 17.2 kDa, 29.5 kDa and 31.9 kDa respectively.

Distribution of the corresponding OMP antigens among different *Leptospira* strains by immunoblot assay

Immunoblot results showed that the antisera to the three OMPs cpLipL21, cpLipL32 and cpOmpL1 had recognized the corresponding antigens in various epidemic leptospiral strains (**Fig. 2**).

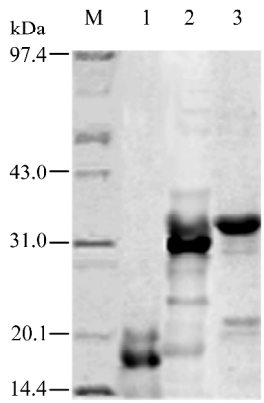

Fig. 1 Purification of recombinant *Leptospira interrogans* serovar lai strain Lai outer membrane proteins

M, marker; 1, LA0011; 2, LA2637; 3, LA3138. Molecular weights of purified products are 17.2 kDa, 29.5 kDa and 31.9 kDa respectively.

mRNA expression of OMP genes in different *Leptospira* strains

RT-PCR analysis was performed on a panel of leptospires comprising six pathogenic strains including *L. interrogans* serovar lai and two nonpathogenic *L. biflexa* strains (**Table 4** and **Fig. 3**). The expression of the three OMP genes encoding LipL21, LipL32 and OmpL1 in these leptospires differed slightly. These three genes were expressed in tested leptospiral pathogens, but not in the nonpathogenic strains *L. biflexa* serovar monvalerio or *L. biflexa* serovar patoc. This is consistent with the immunoblot results in **Fig. 2**.

Recombinant OMP-based ELISA

ELISA results showed that two recombinant proteins, cpLipL32 and cpOmpL1, reacted significantly with the sera of the 18 patients infected by *L. interrogans* serovar lai ($P<0.01$ and $P<0.05$ respectively), as well as with the sera of four patients infected by the other pathogenic leptospires (both $P<0.01$), compared with the negative control sera. No significant difference was observed between the reaction of cpLipL21 with patients' sera and with the control individuals' sera ($P>0.05$) (**Fig. 4**).

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

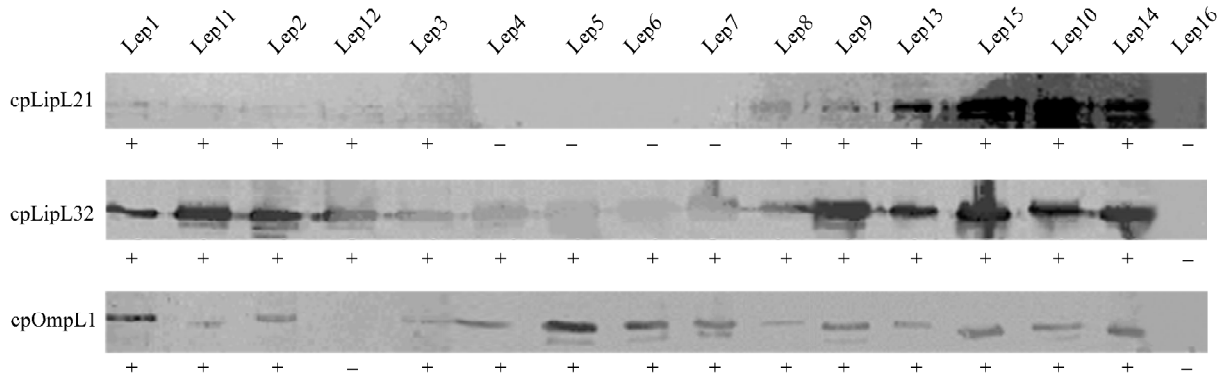

Fig. 2 Immunoblot results of different pathogenic leptospire protein antigens recognized by antisera to three recombinant outer membrane proteins, cpLipL21, cpLipL32 and cpOmpL1

Each antiserum sample reacted with the protein extract of 15 pathogenic leptospires. Details are given in **Table 1**.

Table 4 mRNA expression of outer membrane protein (OMP) genes in some pathogenic and nonpathogenic leptospires

Locus tag	*Leptospira* spp.							
	Lep1	Lep3	Lep4	Lep7	Lep8	Lep12	Lep16*	Lep17*
LA0011	+	+	+	+	+	+	–	–
LA2637	+	+	+	+	+	+	–	–
LA3138	+	+	+	+	+	+	–	–

* nonpathogenic leptospires (details are given in **Table 1**).

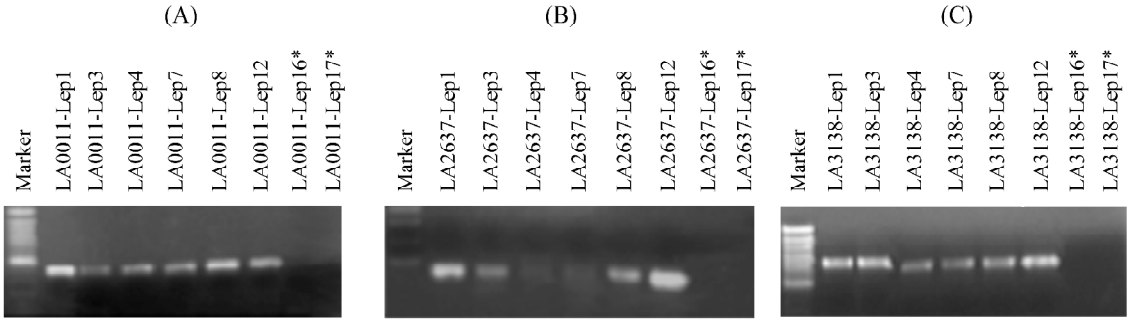

Fig. 3 mRNA expression of outer membrane protein genes in some pathogenic *Leptospira interrogans* strains and nonpathogenic leptospires by reverse transcription-polymerase chain reaction (RT-PCR)

(A) LA0011. (B) LA2637. (C) LA3138. Marker: 1800 bp, 1400 bp, 1000 bp, 800 bp, 600 bp, 400 bp, 300 bp, 200 bp and 100 bp (from top to down). * nonpathogenic leptospires (details are given in **Table 1**).

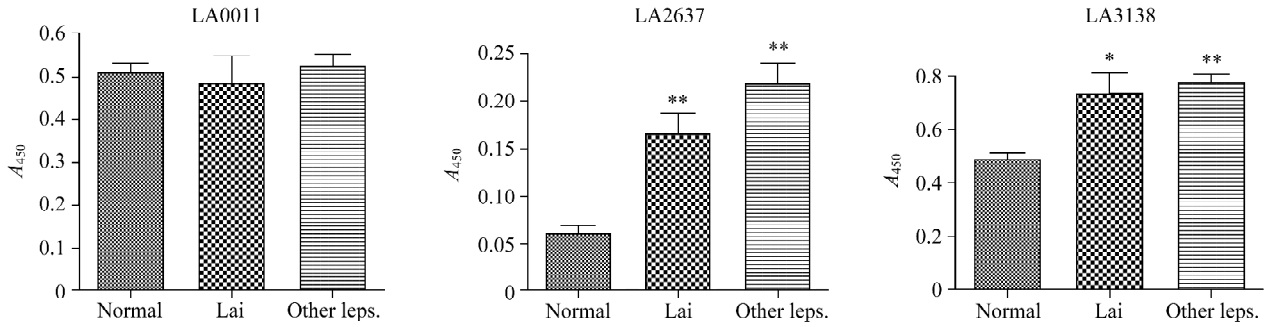

Fig. 4 Enzyme-linked immunosorbent assay (ELISA) of three recombinant outer membrane proteins with sera of leptospirosis patients

Three recombinant outer membrane proteins, cpLipL21, cpLipL32 and OmpL1, encoded by genes LA0011, LA2637 and LA3138, respectively, acted as target antigens in ELISA. Sera of patients with convalescent-phase leptospirosis were tested. Absorbance was recorded at wavelength of 450 nm. Data were represented as mean±SD. **P*<0.05 vs. normal group; ** *P*<0.01 vs. normal group. Lai, sera of 18 patients infected by *Leptospira interrogans* serovar lai strain Lai; other leps., four patients infected by other pathogenic leptospires; normal, sera of individuals in the normal control group.

[24,25]. However, the lack of an effective, widely available laboratory tool remains a major problem [26] and standard serologic tests for case confirmation need to be optimized [27]. Based on bioinformatics and genomic tools, an important approach named "reverse vaccinology" has emerged [28,29]. It has played an essential role in delivering an effective and universal vaccine in the case of serogroup B *Neisseria meningitidis* [30,31].

The whole genome sequence of two pathogenic leptospires makes it possible that a considerable set of OMPs may be determined through experiments. In our study, nucleotide sequencing results validated that three OMP genes encoding LipL21, LipL32 and OmpL1 were highly conserved among various pathogenic *Leptospira*

strains, which have been identified by epidemic and molecular analysis [16,17], including three pathogenic species *L. interrogans*, *L. borgpetersenii* and *L. weilii*. Both immunoblot assay and RT-PCR results suggest that these recombinant OMPs may play a potential role in developing immunodiagnosis and recombinant vaccine candidates.

The sequencing results revealed that the LipL32 coding gene was highly conserved among all the pathogenic leptospires including three epidemic species. It was demonstrated by immunoblot assay that this gene was expressed conservatively in most cultured epidemic leptospires. Furthermore, mRNA expression of the conserved LipL32 gene was detected in six virulent *Leptospira* strains tested including five *L. interrogans* strains and one *L. borgpetersenii* strain. The results indicated that recombinant LipL32 may act as an optimal antigen molecular candidate in the serodiagnosis of leptospirosis as described [26].

LipL21 gene was found well conserved among pathogenic leptospires including three epidemic species in China, while not found in saprophytic *L. biflexa* serovar patoc, which is consistent with an earlier report [7]. On the other hand, no statistical significance was detected between the ELISA reaction of the recombinant protein with the sera of leptospirosis patients, and individuals in the control group. As no related reports are available, it remains to be testified whether the recombinant LipL21 protein has potential use in immunodiagnosis.

The transmembrane lipoprotein, OmpL1 [9,32], was reported recently to have sequences with mosaic compositions consistent with horizontal transfer of DNA between related bacterial species [22]. In this study, though less conservative than the other two OMP genes in the epidemic leptospires tested, the gene encoding OmpL1 was found well conserved in all tested pathogenic *Leptospira* strains including *L. interrogans* strains, *L. borgpetersenii* and *L. weilii*. A strong interaction between the recombinant OmpL1 protein and leptospirosis patients' sera was observed by ELISA. According to the earlier report, OmpL1 and LipL41 together could provide significant protection against homologous challenge in the hamster model of leptospirosis [25]. This reminds us that the combination of recombinant OmpL1 and LipL41 products may be applied to future immunoprotective research and serodiagnosis strategies.

Our study aimed to primarily select and evaluate the leptospiral recombinant OMPs as target antigens by the approach of reverse vaccinology. Obviously more precise and comprehensive explorations need to be done in this field, for example, the monoclonal antibodies should be more optimal as detective probes in the immunoblot assay, and whether the recombinant OMPs could produce effective protection in animal challenges still needs to be confirmed. It is firmly believed that the recombinant leptospiral proteins as antigen molecular candidates should be further examined in the succeeding research.

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