Original Article

A Sensitive and Specific IgM-ELISA for the Serological Diagnosis of Human Leptospirosis Using a rLipL32/1-LipL21-OmpL1/2 Fusion Protein*

SUN AiHua[1,2], WANG Yuan[3], DU Peng[1], WU ShengLing[1], and YAN Jie[2,#]

1.Zhejiang Medical College, Hangzhou 310053, Zhejiang, China; 2.Department of Medical Microbiology and Parasitology, College of Medicine, Zhejiang University, Hangzhou 310058, Zhejiang, China; 3.The First Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou 310006, Zhejiang, China

Abstract

Objective To construct a *lipL32/1-lipL21-OmpL1/2* fusion gene and its prokaryotic expression system, and to establish an enzyme-linked immunosorbent assay (ELISA) using the rLipL32/1-LipL21-OmpL1/2 fusion antigen of *Leptospira interrogans* for sensitive and specific detection of IgM in the serum of patients with leptospirosis.

Methods *lipL32/1-lipL21-OmpL1/2* fusion genes were constructed using a primer-linking PCR. The target recombinant protein antigens, rLipL32/1, rLipL21, rOmpL1/2 and rLipL32/1-LipL21-OmpL1/2, were expressed and the purified antigens were then immobilized to the surface of microplate wells for ELISA-based detection of IgM in the sera of leptospirosis patients.

Results Of 493 acute leptospirosis patients, 95.7% and 97.8% were positive by rLipL32/1-LipL21-OmpL1/2-IgM-ELISA using different serum dilutions, which was higher than the rLipL32/1-IgM-ELISA (93.1% and 90.3%), rLipL21-IgM-ELISA (90.3% and 87.0%), and rOmpL1-IgM-ELISA (85.6% and 81.1%) (P<0.01). All IgM-ELISAs tested negative against 56 non-leptospirosis patients with typhoid fever, hemorrhagic fever or dengue fever.

Conclusion Trigeminal fusion antigen increases ELISA sensitivity and the rLipL32/1-LipL21-OmpL1/2-IgM-ELISA is a sensitive and specific serological diagnostic method for clinical leptospirosis.

Key words: Leptospira; Outer membrane protein; Fusion antigen; Recombinant expression; IgM-ELISA

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INTRODUCTION

Leptospirosis is considered to be the most widespread zoonotic disease in the world[1-4]. In recent years, this disease has become increasingly prevalent in many areas of North and South America and represents an emerging public health problem[5-6]. Leptospirosis in humans is predominantly contracted, often through skin wounds, after contact with soil or water contaminated by the urine of infected animals[7-9]. Disease severity varies from mild to rapidly fatal. Mild cases typically display common infectious symptoms such as fever, lymphadenectasis and myalgia[10-11]. However, severe forms are characterized by visible jaundice involving hepatic injury, acute renal failure, carditis and hemorrhagic syndrome, with case fatality varying from a few percent up to 25%[10-12]. Thus, laboratory examination is of considerable importance in the clinical diagnosis of leptospirosis because of the variable and often complicated manifestations observed in infected patients[12-13].

Many leptospiral species (spp), such as *Leptospira interrogans*, *Leptospira borgpetersenii*, *Leptospira noguchii*, *Leptospira weilii,* and *Leptospira*

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#Correspondence should be addressed to Prof. YAN Jie, Tel: 86-571-88208297. Fax: 86-571-88208294. E-mail: Med_bp@zju.edu.cn

Biographical note of the first author: SUN AiHua, female, born in 1968, Professor, majoring in molecular mechanism of bacterial drug resistance and laboratory diagnosis of infectious diseases.

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kirschner, can cause leptospirosis in humans[14]. Among the pathogenic *Leptospira* spp, *L. interrogans* is the most common cause of leptospirosis, which can be classified into many serogoups and serovars based on serological examination[10,14]. Although several different serological diagnostic methods have been developed to identify patients that are suffering from leptospirosis, only the microscopic agglutination test (MAT) is used as the gold standard for diagnosis of this disease in most countries worldwide[14-15]. Despite of the high specificity and sensitivity of MAT, the complex operation and low sensitivity limit its clinical application. For example, in China at least fifteen officially appointed representative strains, belonging to fifteen different serogroups of pathogenic *Leptospira* spp, must be applied to the MAT. In addition, the use of fresh and living *Leptospira* in the MAT represents a biohazard to staff operating the test[10]. Therefore, it is important to develop a rapid, and convenient serological method for leptospirosis diagnosis that is safe, while also specific and sensitive.

Outer membrane proteins (OMPs) are the main superficial antigens of the *Leptospira* spp[16]. Among the OMPs, lipoproteins are the most abundant proteins[17-19], of which, LipL32 and LipL21 are found in the highest concentration among the pathogenic *Leptospira* spp[20-21]. OmpL1, a transmembrane protein, is also an antigen that is only located on the surface of pathogenic leptospires[22]. In previous studies, we confirmed that all tested pathogenic *Leptospira* spp possess LipL21, LipL32, and OmpL1[23-24]. LipL21 exhibits high sequence identity between species, while LipL32 and OmpL1 have different protein types (LipL32/1 and LipL32/2, and OmpL1/1, OmpL1/2, and OmpL1/3). We also observed different levels of cross-immu noagglutination between LipLs and among OmpL1s. Thus, the three OMPs (LipL21, LipL32, and OmpL1) can be used as specific antigens for the development of novel and universal serological methods for the diagnosis of human leptospirosis.

To date, many studies have developed enzyme-linked immunosorbent assays (ELISA) using a single recombinant leptospiral OMP, such as rLipL32, rLipL41, or rOmpL1, as the antigen[25-27]. However, some reports revealed that the use of several antigens potentially increased detection sensitivity[28-29]. In this study, we aimed to construct a fusion gene containing *lipL21*, *lipL32/1*, and *ompL1/2* genes, and express the rLipL32/1-LipL21-OmpL1/2 recombinant fusion protein in *Escherichia coli* to improve the sensitivity of leptospiral OMPs-based ELISAs. Subsequently, we established an ELISA using the fusion protein as the coating-antigen (rLipL32/1-LipL21-OmpL1/2-ELISA) to detect serum antibodies in samples from 493 leptospirosis patients to explore the potential of this method to be used for routine clinical serological diagnosis.

MATERIALS AND METHODS

Leptospiral Strains and Cultures

Fifteen officially appointed representative Chinese strains, belonging to fifteen different serogroups of pathogenic *Leptospira* spp, were provided by the Chinese National Institute for Control of Pharmaceutical and Biological Products (Table 1). All leptospiral strains were grown in Ellinghausen-McCullough-Johnson-Harris liquid medium at 28 °C for 5–7 days[31- 32].

Table 1. Leptospiral Strains Used in this Study

Strains	Serovars	Serogroups	Genospecies
Lai	Lai	Icterohaemorrhagiae	*L. interrogans*
Lin	Canicola	Canicola	*L. interrogans*
65-9	Australis	Australis	*L. interrogans*
Luo	Pomona	Pomona	*L. interrogans*
P 7	Hebdomadis	Hebdomadis	*L. interrogans*
L 37	Paidjan	Bataviae	*L. interrogans*
Lin 6	Lin	Grippotyphosa	*L. interrogans*
L 183	Wolffi	Sejroe	*L. interrogans*
Lin 4	Autumnalis	Autumnalis	*L. interrogans*
Tian	Pyrogenes	Pyrogenes	*L. interrogans*
Nan 10	Mini	Mini	*L. borgpetersenii*
M 10	Javanica	Javanica	*L. borgpetersenii*
Pishu	Ballum	Ballum	*L. borgpetersenii*
55-52	Tarassovi	Tarassovi	*L. borgpetersenii*
L 105	Manhao 2	Manhao	*L. weilii*

Serum Samples

Serum samples (*n*=493) from leptospirosis patients that were clinically confirmed to be in the acute phase of infection, with fever, myalgia, headache, nausea and vomiting, jaundice and conjunctival suffusion were collected during the patients' visits to local hospitals and maintained in our laboratory. All sera were identified by MAT to verify serogroup and serovar classification (Table 2)[10]. In addition, sera from patients with other febrile illnesses (32 with typhoid fever, 18 with hemorrhagic fever and 6 with dengue fever) from Zhejiang Province were provided by the Centers for Disease Control and Prevention. Sera from 112 healthy individuals tested by routine somatoscopy were also offered by the affiliated hospitals of Zhejiang University, and used as controls. Informed written consent for sample collection was obtained from all participants, with ethical approval from the Ethics Committee of Zhejiang University.

Table 2. Leptospirosis Serum Samples Used in this Study

Serum Samples	Classification of the Infected Leptospires		
(*n*)	Serovars	Serogroups	Genospecies
262	Lai	Icterohaemorrhagia	*L. interrogans*
53	Hebdomadis	Hebdomadis	*L. interrogans*
45	Medanesis	Sejroe	*L. interrogans*
42	Pomona	Pomona	*L. interrogans*
38	Grippotyphosa	Grippotyphosa	*L. interrogans*
23	Australis	Australis	*L. interrogans*
18	Autumnalis	Autumnalis	*L. interrogans*
12	Canicola	Canicola	*L. interrogans*

Target Fusion Gene Construction

Recombinant plasmids pUCm-T^{lipL21}, pUCm-$T^{lipL32/1}$, and pMD18-$T^{ompL1/2}$ were provided by our laboratory[23-24]. Details of all primers used for the construction of the target fusion gene *lipL32/1-lipL21-ompL1/2* are shown in Table 3. Polymerase chain reaction (PCR) reagents were obtained from TaKaRa, Dalian, China. Initially, two separate PCRs were performed to amplify the *lipL32/1* fragment using *lipL32/1* forward and *lipL32/1-lipL21* reverse primers, and the *lipL21* fragment using *lipL32/1-lipL21* forward and *lipL21* reverse primers. The *lipL32/1*and *lipL21* fragments were incubated together for 10 cycles at 94 °C for 30 s, 45 °C for 30 s and 72 °C for 90 s to form a combined *lipL32/1-lipL21* template. Subsequently, the *lipL32/1-lipL21* fragment was amplified using *lipL32/1* forward and *lipL21* reverse primers. The 1 314 bp *lipL32/1-lipL21* fragment (762 bp *lipL32/1*, 45 bp flexible peptide linker, and 507 bp *lipL21* sequences) was cloned into the pMD18-T vector to form pMD18-$T^{lipL32/1-lipL21}$ for sequencing (Invitrogen Co. Ltd, Shanghai, China).

Table 3. Primer Sequences and Product Sizes

Genes	Primer Sequences (5' to 3')
lipL32/1	F: ccg cat atg (Nde I) tgt ggt gct ttc ggt ggt ctg
	R: GCT GCC ACC GCC GCC GCT GCC ACC GCC GCC GCT GCC ACC GCC GCC ctt agt cgc gtc aga agc agc
lipL21	F: GGC GGC GGT GGC AGC GGC GGC GGT GGC AGC GGC GGC GGT GGC AGC tgt tcc agt act gac aca gga
	R: GCT GCC ACC GCC GCC GCT GCC ACC GCC GCC GCT GCC ACC GCC GCC ctg ttt gct cac ttc ctg cgc
lipL32/1-lipL21	F: ccg cat atg (Nde I) tgt ggt gct ttc ggt ggt ctg
	R: GCT GCC ACC GCC GCC GCT GCC ACC GCC GCC GCT GCC ACC GCC GCC ctg ttt gct cac ttc ctg cgc
ompL1/2	F: GGC GGC GGT GGC AGC GGC GGC GGT GGC AGC GGC GGC GGT GGC AGC aaa aca tat gca att gta gga
	R: gca ctc gag (Xho I) ttc gtg ttt ata acc gaa
lipL32/1-lipL21-ompL1/2	F: ccg cat atg (Nde I) tgt ggt gct ttc ggt ggt ctg
	R: gca ctc gag (Xho I) ttc gtg ttt ata acc gaa

Note: F: forward primers; R: reverse primers. The sequences in capital indicate the flexible peptide linker GGGGSGGGGSGGGGS.

Two additional PCRs were performed to amplify the *lipL32/1-lipL21* fragment using *lipL32/1* forward and *lipL32/1-lipL21-ompL1/2* reverse primers, and the *ompL1* fragment using *lipL32/1-lipL21-ompL1/2* forward and *ompL1/2* reverse primers. Both fragments were incubated as described above and then linked by PCR using *lipL32/1* forward and *ompL1/2* reverse primers to form a 2 247 bp *lipL32/1-lipL21-ompL1/2* fusion gene (1 314 bp *lipL32/1-lipL21*, 45 bp flexible peptide linker, 888 bp *ompL1/2* sequences). These fusion gene fragments were further cloned into the pMD18-T vector to form pMD18-T $^{lipL32/1\text{-}lipL21\text{-}ompL1/2}$ for sequencing.

Construction of a Prokaryotic Expression System for the Fusion Gene

pMD18-T $^{lipL32/1\text{-}lipL21\text{-}ompL1/2}$ constructs that gave the expected sequencing results, and the prokaryotic expression vector pET42a (Novagen, USA), were digested with Nde I and Xho I (TaKaRa), respectively. The recovered *lipL32/1-lipL21-ompL1/2* fragments were linked with linearized pET42a using T4 DNA ligase (TaKaRa) and then transformed into *E. coli* BL21DE3 (Novagen) to form the target prokaryotic expression system, *E. coli* BL21DE3$^{pET42a\text{-}lipL32/1\text{-}lipL21\text{-}ompL1/2}$. *E. coli* BL21DE3$^{pET42a\text{-}lipL32/1}$, *E. coli* BL21DE3$^{pET42a\text{-}lipL21}$, and *E. coli* BL21DE3$^{pET42a\text{-}ompL1/2}$ were provided by our laboratory[23-24].

Expression and Purification of Recombinant Protein Antigens

All four engineered bacteria described above were induced by 0.5 mmol/L isopropyl-β-D-thio-galactoside (IPTG) in LB medium in a shaker at 250 rpm (30°C). The expressed products were checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then quantified using a Gel Image Analyzer (Bio-Rad Laboratories, Milan, Italy). Bacterial cells were harvested by centrifugation at 10 000 rpm for 10 min and lysed by ultrasonication at 300 V power with 5 s bursts for 30 breaks. Each lysate was loaded into a Ni-NTA column (BioColor Inc., Shanghai, China) to extract the target recombinant proteins rLipL32/1, rLipL21, rOmpL1/2, and rLipL32/1-LipL21-OmpL1/2. Recombinant protein extract purity was determined using SDS-PAGE.

Preparation and Identification of Rabbit Antisera

New Zealand white rabbits (*n*=2; 3.0-3.5 kg) were provided by the Medical Laboratory Animal Center of Zhejiang University [Certificate No. SCXK (zhe) 2007-0030] Each rabbit was immunized intradermally four times at an interval of once a week with 2 mg of purified rLipL32/1-LipL21-OmpL1/2 pre-mixed with Freund's adjuvant. Fifteen days after the last immunization, rabbit sera were collected to determine the titers against rLipL32/1-LipL21-OmpL1/2 using an immunodiffusion test. Specificity against *Staphylococcus aureus* ATCC25923, *E. coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 was also tested using slide agglutination. Rabbit anti-rLipL32/1, anti-rLipL21, and anti-rOmpL1/2 sera were provided by our laboratory, and the specificity and immunoreactivity of each had previously been reported[23-24].

Preparation and Identification of Leptospiral OMPs

Fifteen freshly cultured standard strains of pathogenic *Leptospira* spp were precipitated by centrifugation at 12 000 rpm for 15 min. OMPs of each strain were extracted and protein concentrations were quantitated as described by Barbosa et al.[33]. OMP extracts were further examined with Coomassie Blue R-250 staining after SDS-PAGE.

Western Blot Assay

rLipL32/1, rLipL21, rOmpL1/2, rLipL32/1-Lip L21-OmpL1/2, and each OMP extract were transferred from the SDS-PAGE separation gel to a polyvinylidene difluoride membrane (Milipore, USA). By using 1:500 diluted rLipL32/1, rLipL21, rOmpL1/2, or rLipL32/1-LipL21-OmpL1/2 antiserum as the primary antibody and l:3 000 diluted goat anti-rabbit horseradish peroxidase (HRP)-1abeled IgG (Jackson ImmunoResearch, USA) as the secondary antibody, a western blot assay was performed to determine the immunoreactivity among the protein antigens and antisera. In this assay, the same dilutions of twelve serum samples from typhoid fever, hemorrhagic fever, dengue fever patients and healthy individuals were used as the negative controls.

MAT

Different dilutions of rLipL32/1-LipL21-OmpL1/2 antiserum were mixed with the equivalent amount of each of the fifteen freshly cultured leptospiral standard strains and incubated at 37 °C for 1 h. Agglutination was examined under a dark field microscope (400×) and the MAT titer was determined using the reciprocal of the highest dilution of serum in which 50% of the leptospires were agglutinated[10,14]. Several serum samples from typhoid fever, hemorrhagic fever, dengue fever

patients and healthy individuals were used as controls for the MAT.

IgM-ELISAs

Protein samples (rLipL32/1, rLipL21, rOmpL1/2, or rLipL32/1-LipL21-OmpL1/2) were diluted to 50 μg/mL using 0.01 mol/L carbonate sodium buffer (pH9.6) before 100 μL was loaded into each well of a 96-well polystyrene microplate and incubated at 4 °C overnight. The next day, the microplate was washed three times with 0.05% Tween 20/0.01 mol/L phosphate buffered saline (PBS) (pH7.4), and then blocked using 10% bovine serum albumin (BSA) (Sigma, USA) solution. A 1:50 or 1:100 dilution of serum samples were used as the primary antibody and 1:3 000 diluted goat anti-human HRP-labeling IgM (Jackson ImmunoResearch) as the second antibody to detect infection within the 493 sera from leptospirosis patients using the rLipL32/1-IgM-ELISA, rLipL21-IgM-ELISA, rOmpL1/ 2-IgM-ELISA, and rLipL32/1-LipL21-Om pL1/2-IgM -ELISA. For all ELISAs, sera from 32 typhoid fever, 18 hemorrhagic fever and 6 dengue fever patients were diluted as described above and used as controls. Patient's serum was regarded as positive if its OD_{490} value was ⩾ to the mean plus three standard deviations (SD) of the control samples[34].

Data Analysis

ELISA data were statistically analyzed using the Chi-square test. Statistical analysis was performed using SPSS1.0 statistical software and statistical significance was defined as $P<0.05$.

RESULTS

PCR and Sequencing Results

The *lipL32/1-lipL21-ompL1/2* fusion gene fragment was successfully obtained by overlap extension PCR. Sequencing data confirmed that *lipL32/1-lipL21-ompL1/2* fusion gene sequence was identical to that of the three separate genes (GI: 7330696, 30171159, and 348937) (data not shown).

Expression and Purity of Target Recombinant Pprotein Antigens

Under IPTG induction, *E. coli* BL21DE3$^{pET42a\text{-}lipL32/1}$, *E. coli* BL21DE3$^{pET42a\text{-}lipL21}$, *E. coli* BL21DE3$^{pET42a\text{-}ompL1/2}$, and *E. coli* BL21DE3$^{pET42a\text{-}lipL32/1\text{-}lipL21\text{-}ompL1/2}$ expressed rLipL32/1, rLipL21, rOmpL1/2, and rLipL32/1-LipL 21-OmpL1/2, respectively. Each of the purified target recombinant proteins showed a single protein band in the gel after SDS-PAGE, indicating that the purified proteins were qualified as appropriate antigens for subsequent ELISAs.

Immunoreactivity and Specificity of rLipL32/1-LipL21-OmpL1/2 and its Antiserum

The rLipL32/1-LipL21-OmpL1/2 antiserum bound to rLipL32/1, rLipL21, rOmpL1/2, and rLipL32/1-LipL21-OmpL1/2, while the anti-rLipL32/1, anti-rLipL21, and anti-rOmpL1/2 serum also bound to rLipL32/1-LipL21-OmpL1/2 (Figure 1). Although the OMP extracts of fifteen standard strains of pathogenic *Leptospira* produced multiple protein bands with different sizes after SDS-PAGE, rLipL32/1-LipL21-OmpL1/2 antiserum could still specifically recognize and combine with the target protein antigens (LipL32, LipL21, and OmpL1) (Figure 2). However, the nine serum samples from typhoid fever, hemorrhagic fever or dengue fever patients, and the three serum specimens from healthy individuals gave negative results against rLipL32/1-LipL21-OmpL1/2 in the western blot assay. Additionally, the rLipL32/1-LipL21-OmpL1/2 antiser um could not agglutinate *S. aureus* ATCC25923, *E. coli* ATCC25922 or *P. aeruginosa* ATCC27853.

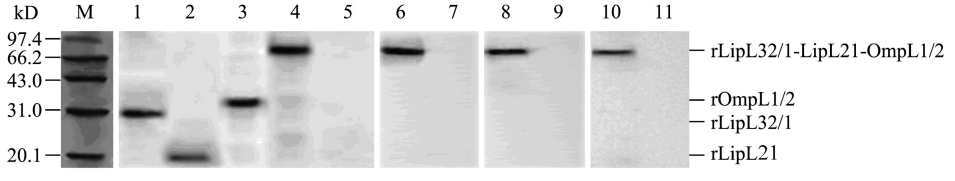

Figure 1. Immunoreactivity of rLipL32/1-LipL21-OmpL1/2 and its antiserum using western blot assay. M: protein maker (BioColor); 1 to 4: western hybridization bands of rLipL32/1-LipL21-OmpL1/2 antiserum with rLipL32/1, rLipL21, rOmpL1/2, and rLipL32/1-LipL21-OmpL1/2 proteins, respectively; 6, 8, and 10: western hybridization bands of rLipL32/1-LipL21-OmpL1/2 with rLipL32/1, rLipL21, or rOmpL1/2 antiserum, respectively; 5, 7, 9, and 11: protein-free blank controls.

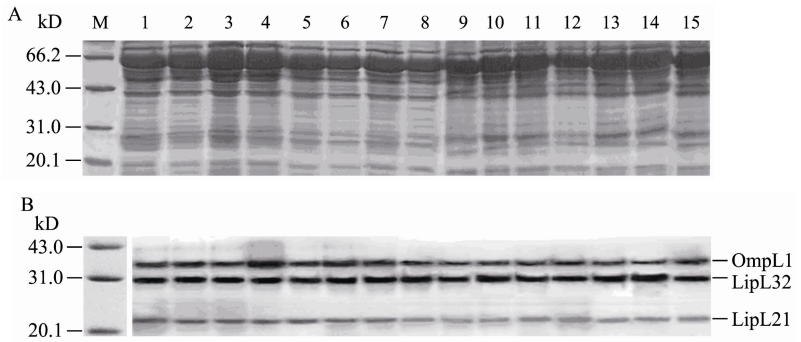

Figure 2. Western blot results using rLipL32/1-LipL21-OmpL1/2 antiserum with leptospiral OMP target protein antigens. M: protein maker (BioColor); A1 to A15: SDS-PAGE map of the OMP extracts from fifteen standard leptospiral strains Lai, Lin, 65-9, Luo, P7, L37, Lin6, L183, Lin4, Tian, Nan10, M10, Pishu, 55-52, and L105, respectively; B1 to B15: western hybridization bands of rLipL32/1-LipL21 -OmpL1/2 antiserum with LipL21s (about 21 kDa), LipL32s (about 32 kDa), and OmpL1s (about 35 kDa) in the OMP extracts, respectively.

MAT Titers of rLipL32/1-LipL21-OmpL1/2 Antiserum

The rLipL32/1-LipL21-OmpL1/2 antiserum effectively agglutinated all fifteen standard leptospiral strains with 1:100 to 1:400 titers (Table 4), while all control sera gave negative agglutination results.

Detection Results of IgM-ELISAs

ELISA cut-off values (mean at OD490 + 3SD), from the sera of 112 healthy individuals at 1:50 and 1:100 dilutions, were determined as 0.34 and 0.27 for rLipL32/1, 0.36 and 0.26 for rLipL21, 0.33 and 0.24 for rOmpL1/2, and 0.36 and 0.27 for rLipL32/1-LipL21-OmpL1/2, respectively. Positive infection rates for the 493 serum samples from leptopsirosis patients using the rLipL32/1-IgM-ELISA, rLipL21-IgM-ELISA, rOmpL1-IgM-ELISA, and rLipL32/1-LipL21-OmpL1/2-IgM-ELISA were as follows: 93.1% (459/493), 90.3% (445/493), 85.6% (422/493), and 97.8% (482/493) for 1:50 serum dilution, respectively, and 90.3% (445/493), 87.0% (429/493), 81.1% (400/493), and 95.7% (472/493) for 1:100 serum dilution, respectively (Table 5). The rLipL32/1-LipL21-OmpL1/2-IgM-ELISA, using either 1:50 or 1:100 serum dilutions, showed significantly higher positive rates than the other three IgM-ELISAs (P<0.01). Although the positive rates of all the IgM-ELISAs

Table 4. MAT Titers of rLipL32/1-LipL21-OmpL1/2 Antiserum

Standard Strains	Serovars	Serogroups	Genospecies	MAT Titers
Lai	Lai	Icterohaemorrhagiae	*L. interrogans*	1:400
Lin	Canicola	Canicola	*L. interrogans*	1:400
65-9	Australis	Australis	*L. interrogans*	1:400
Luo	Pomona	Pomona	*L. interrogans*	1:400
P 7	Hebdomadis	Hebdomadis	*L. interrogans*	1:400
L 37	Paidjan	Bataviae	*L. interrogans*	1:400
Lin 6	Lin	Grippotyphosa	*L. interrogans*	1:100
L 183	Wolffi	Sejroe	*L. interrogans*	1:200
Lin 4	Autumnalis	Autumnalis	*L. interrogans*	1:200
Tian	Pyrogenes	Pyrogenes	*L. interrogans*	1:100
Nan 10	Mini	Mini	*L. borgpetersenii*	1:200
M 10	Javanica	Javanica	*L. borgpetersenii*	1:100
Pishu	Ballum	Ballum	*L. borgpetersenii*	1:100
55-52	Tarassovi	Tarassovi	*L. borgpetersenii*	1:100
L 105	Manhao 2	Manhao	*L. weilii*	1:100

Table 5. IgM-ELISAs Detection Results for Leptospirosis Patient Sera

Serum Samples	Cases (*n*)	Dilution (1: x)	IgM Positive Cases (*n*)			
			rLipL32/1	rLipL21	rOmpL1/2	rLipL32/1-LipL21-OmpL1/2
Leptospirosis patients:						
Lai	262	50	244	237	225	256
		100	239	232	218	253
Hebdomadis	53	50	50	49	45	52
		100	49	47	43	50
Medanesis	45	50	42	41	36	44
		100	40	39	34	42
Pomona	42	50	39	37	39	41
		100	37	35	36	40
Grippotyphosa	38	50	35	34	31	37
		100	33	32	28	36
Australis	23	50	21	21	21	23
		100	20	19	19	22
Autumnalis	18	50	17	16	15	17
		100	17	15	13	17
Canicola	12	50	11	10	10	12
		100	10	10	9	12
Total	493	50	459^{bc}	44^{bc}	422^{c}	482^{ac}
		100	445^{b}	429^{b}	400^{c}	472^{a}
Non-leptospirosis patients	56	50	0	0	0	0
		100	0	0	0	0

Note: a: $P<0.01$ vas the rLipL32/1, rLipL21, and rOmpL1/2; b: $P<0.01$ vas rOmpL1/2; c: $P>0.05$ between the 1:50 and 1:100 dilutions; d: $P<0.05$ between the 1:50 and 1:100 dilutions.

using 1:50 serum dilution were slightly higher than those using 1:100 serum dilution, no significant differences were observed between the two serum dilutions ($P>0.05$). Each of the IgM-ELISAs did not produce positive results against the sera from the 56 non-leptospirosis patients with typhoid fever, hemorrhagic fever or dengue fever.

DISCUSSION

A diverse range of predominant leptospiral serovars is known to exist in different geographical areas[4-8,10-14]. In China, *L. interrogans* serogroup Icterohaemorrhagia serovar Lai is the most common pathogen causing leptospirosis in humans[23-35]. Our study reports that approximately 53% (262/493) of the Chinese leptospirosis patients were infected with *L. interrogans* serovar Lai based on the MAT, and that the remaining 47% (231/493) of patients were infected with *L. interrogans* serovars Hebdomadis, Medanesis, Pomona, Grippotyphosa, Australis, Autumnalis and Canicola (Table 2). Thus, combined with previous epidemiological investigation data[23-24], these eight serovars represent the predominant pathogenic leptospires in China.

The protein antigens LipL32, LipL21, and OmpL1 are generally found in all pathogenic *Leptospira* spp[20-22]. Among these, LipL21 had been identified as an OMP with high intra-strain sequence conservation between different countries, including China[21,23]. Although LipL32 can be divided into two different protein types (LipL32/1 and LipL32/2), all fifteen Chinese officially appointed representative strains of pathogenic *Leptospira* spp contain the LipL32/1 encoding gene[23]. OmpL1 protein (OmpL1/1, OmpL1/2, and OmpL1/3) has a higher mutation rate than LipL32 in Chinese leptospiral strains[24]. However, among the leptospires prevalent in China, only *L. interrogans* serovars Grippotyphosa, Medanesis and Autumnal express the OmpL1/1 protein, with the other five prevalent serovars, such as Lai, Hebdomadis, Pomona, Australis and Canicola, expressing the OmpL1/2 protein[24]. Furthermore, the cross immunoreactions among different OmpL1 types have also been determined[24]. Therefore, in this study we chose *lipl32/1* and *ompL1/2* genotypes to construct the fusion gene *lipL32/1-lipL21-ompL1/2* to obtain a trigeminal protein antigen.

To maintain the immunoreactivity of the LipL31/1, LipL21, and OmpL1/2 protein antigens in a fusion protein molecule, we utilized flexible peptide sequences to link these three genes. Western blot analysis in our study confirmed that the fusion protein rLipL32/1-LipL21-OmpL1/2 could be recognized by rLipL32/1, rLipL21, and rOmpL1/2

antisera, and that the rLipL32/1-LipL21-OmpL1/2 antiserum also could bind to the three separate protein antigens. In addition, rLipL32/1-LipL21-OmpL1/2 antiserum could agglutinate with all fifteen Chinese officially appointed representative strains of pathogenic *Leptospira* spp. These results indicate that the LipL32/1, LipL21, and OmpL1/2 peptide segments in the fusion protein rLipL32/1-LipL21-OmpL1/2 can each maintain immunoreactivity independently. Sera from non-leptospirosis patients and healthy individuals did not recognize rLipL32/1-LipL21-OmpL1/2 and rLipL32/1-LipL21-OmpL1/2 antiserum did not agglutinate *S. aureus*, *E. coli* and *P. aeruginosa*. Thus, this fusion protein antigen and its antiserum exhibit high specificity.

MAT is generally considered as the gold standard for leptospirosis diagnosis, yet this test is ineffective during early stages of infection because it targets IgG[14-15]. To overcome this limitation, several IgM-ELISAs based on single leptospiral OMP antigens have been developed[25-27]. However, the relatively lower sensitivity of these IgM-ELISAs has restricted their clinical application[37-38]. We therefore used rLipL32/1-LipL21-OmpL1/2, a fusion protein containing three different leptospiral OMPs, as the coated antigen to establish an ELISA (rLipL32/1-LipL21-OmpL1/2-IgM-ELISA) for improved detection of specific IgM in sera from patients with acute leptospirosis. Our results demonstrated that the rLipL32/1-LipL21-OmpL1/2-IgM-ELISA was more sensitive at detecting specific IgM against leptospirosis in 493 serum samples (95.7% and 97.8% for 1:50 and 1:100 serum dilutions) than any of the other IgM-ELISAs (81.1% to 93.1% for both the serum dilutions) using single rLipL32/1, rLipL21, or rOmpL1 antigens (P<0.01). This indicated that the use of the trigeminal protein antigen, rLipL32/1-LipL21-OmpL1/2, could efficiently increase the detection sensitivity. Furthermore, since the positive rates in each of the IgM-ELISAs using a 1:50 serum dilution did not show significant differences compared to those using the 1:100 serum dilution (P>0.05), the higher serum dilution (1:100) is more suitable for detection to limit background cross-reaction.

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