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Recombinant Multiepitope Protein for Diagnosis of Leptospirosis▽

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Leptospirosis is an emerging infectious disease and is considered to be the most widespread zoonotic disease in the world. It can be misdiagnosed because manifestations of this febrile disease vary from mild flu-like symptoms to severe illness involving vital organs such as the liver and lungs. Therefore, accurate diagnosis for differentiation of leptospirosis from other pyrogenic infections prevailing in the same locality is imperative for proper treatment. Here, we report a customized recombinant leptospirosis multiepitope protein (r-LMP) that can specifically detect the immunoglobulin class of anti-leptospirosis antibodies in patient sera. Immunodominant epitopes from leptospire outer membrane proteins OmpL1, LipL21, and LipL32 were predicted and confirmed using phage display and immunity reaction. On the basis of the sequences of the identified epitopes, five major immunodominant epitopes were selected to construct a synthetic gene, recombinant *lmp*. The recombinant *lmp* gene was doubled and expressed in *Escherichia coli*. The recombinant protein was purified and used as an antigen to develop an enzyme-linked immunosorbent assay for detection of special immunoglobulin M (IgM) or IgG in sera from patients with leptospirosis or other febrile illnesses and healthy subjects. The results showed that the r-LMP protein recognized IgG and IgM in all the sera that were microscope agglutination test positive, and there were no cross-reactions with other patient sera. This approach of creating customized antigens coupled to overexpression and simple purification offers a promising alternative option for leptospirosis diagnosis, with the potential to circumvent the drawbacks of whole-leptospirosis-antigen-based assays.

Leptospirosis is an important infectious disease; the mortality rate in the severe form can be as high as 15% (9). Leptospirosis exhibits a broad spectrum of clinical manifestations, ranging in severity from acute to chronic (with multiorgan syndromes) and fatal (13). Although leptospirosis can be treated with antibiotics, its broad clinical presentation and similarities with other febrile illnesses complicate the diagnosis (1, 8). Misdiagnosis has become a significant problem, as diseases with similar early symptoms occur (4, 10). Obviously, improving the index of clinical suspicion and developing a rapid and specific test are essential for the identification of leptospirosis.

The standard method for the diagnosis of leptospirosis, the microscopic agglutination test (MAT), is not only technically complex but also time-consuming (6). The sensitivities of other rapid and less complicated antibody-based alternatives, such as conventional enzyme-linked immunosorbent assays (ELISAs) and immunofluorescence assays, are very low during the early phase of the infection (3, 13). In recent years, several attempts have been made to overcome these diagnostic obstacles, including the development of an antigen-based test (12, 15) and molecular methods, such as PCR and real-time PCR (16). Although their rapidity and diagnostic efficacy at the acute phase of the illness may be appreciable, their use is restricted in developing countries due to the equipment cost (5). It is necessary to develop a cost-effective, safe, and efficacious diagnostic test that combines sensitivity, specificity, and laboratory as well as field applicability.

Previously, we examined the potential B-cell epitope-containing peptides of *Leptospira interrogans* OmpL1, LipL21, and LipL32 (11, 18). In the present work, we designed a recombinant leptospirosis multiepitope gene, recombinant *lmp*, on the basis of the selected epitope sequences. The expressed protein was evaluated for possible diagnostic utility in immunoblotting and ELISA by using leptospirosis specimens from early and convalescent phases of the illness.

MATERIALS AND METHODS

Materials. *Escherichia coli* host strains DH10B and BL21(DE3) plysS and plasmids pBacPAK8 and pET-28a(+) were maintained in the laboratory. The secondary antibody-enzyme conjugates (goat anti-human immunoglobulin M [IgM]- and IgG-horseradish peroxidase [HRP]) were from Jackson ImmunoResearch, and the goat anti-rabbit IgG-HRP conjugate was from Santa Cruz. Sera from patients with fever, myalgia, headache, vomiting, jaundice, conjunctival suffusion, and abdominal symptoms were collected during the patients' visits to hospitals in the Guangdong, Sichuan, and Zhejiang provinces and maintained in our laboratory. The acute and convalescent phases were defined as previously reported (7). Briefly, serum samples collected at a median of 7 days (range, 2 to 23 days) after the reported onset of the symptoms were defined as acute phase, and serum samples collected at a median of 29.5 days (range, 17 to 113 days) were defined as convalescent phase. The case definition for MAT confirmation was a fourfold rise in MAT titer between paired sera or a MAT titer of >1:80 for a single serum sample (17). Sera from patients with other febrile illnesses (18 with hemorrhagic fever and 6 with dengue) and 10 healthy counterparts were used as patient and normal controls, respectively. This study was approved by the Institutional Review Board at our institution, and informed consent was obtained from each participant.

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In silico identification and characterization of epitopes. The B-cell epitopes from *L. interrogans* outer membrane proteins OmpL1, LipL21, and LipL32 were predicted by the ANTIGENIC program in EMBOSS (http://bioinfo.hku.hk/EMBOSS/). The predicted epitopes were cloned in phage vector M13KE for phage surface display. Epitopes with strong immunogenicity were selected based on the results of Western blot analysis (11, 18) and used for the synthesis of the recombinant *lmp* gene.

Construction of the recombinant *lmp* gene expression vector. A synthetic gene, recombinant *lmp*, codon optimized for *E. coli* expression, was first generated by ligation oligonucleotides encoding five linear immunodominant epitopes of leptospire OmpL1, LipL21, and LipL32 proteins. These epitopes contained 11 to 19 amino acid residues, and adjacent epitopes were joined together by tetraglycyl linkers. In recombinant *lmp*, the BamHI site was designed at the 5′ end, and EcoRI and BglII were at the 3′ end and separated by the terminal codon TAA. The vector pBacPAK8 was digested with BglII and EcoRI, and BamHI- and EcoRI-digested recombinant *lmp* was inserted into the linearized pBacPAK8 vector to construct p8-lmp and sequenced. Then, recombinant *lmp* was inserted into BglII- and EcoRI-digested p8-lmp to construct p8-2lmp. After that, recombinant *lmp* was doubled and 10 epitopes were combined together. The 2lmp fragments were inserted into the BamHI and EcoRI sites of bacterial expression vector pET-28a(+) to generate the plasmid pET28a-2lmp. Recombinant clones were selected on kanamycin plates and subjected to direct colony PCR screening to identify recombinants harboring the constructed multiepitope gene.

Expression screening. The pET28a-2lmp plasmid was transformed into the BL21(DE3) plysS strain. Several positive clones, chosen based on restriction analysis, were inoculated into 3-ml LB cultures and allowed to grow at 37°C in a shaker at 200 rpm. When the cultures had grown for about 3 h, they were induced with 1.0 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for about 4 h at 37°C. After induction, on the basis of the optical density at 600 nm (OD_{600}), equivalent numbers of cells from the different cultures were lysed by sonication and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Noninduced and induced pET-28a(+) controls were analyzed in parallel. The clone that expressed maximal levels of the target protein was selected for further analysis.

Purification of target protein. The selected clone was inoculated into 20 ml LB medium containing 100 μg/ml kanamycin. The culture was grown overnight at 37°C, reinoculated into 1 liter of fresh medium at a dilution of 1:100, and cultured in the shaker at 37°C for 3 h before induction for 4 h with 1.0 mM IPTG. Aliquots of the induced and noninduced cell cultures were analyzed by SDS-PAGE. The induced culture was centrifuged at 3,000 × *g* for 10 min at 4°C. Cell pellets from 1 liter of *E. coli* culture were resuspended in 20 ml NTA-0 buffer, containing 20 mM Tris-HCl (pH 7.9), 500 mM NaCl, and 10% glycerol, and homogenized by sonication on ice. The lysate was clarified by centrifugation at 12,000 × *g* for 30 min at 4°C. The supernatant was gently shaken with 5 ml Ni-nitrilotriacetic acid (NTA) Superflow resin (Biocolors, Shanghai, China) that had been preequilibrated with the NTA-0 buffer and then packed into a column. After the flowthrough was collected, the column was washed extensively with the NTA-40, -100, and -1000 buffers, containing 40 mM, 100 mM, and 1,000 mM imidazole, respectively, in sequence. Fractions of 5 ml were collected and analyzed by SDS-PAGE. Peak fractions were pooled together and stored at −80°C until use.

Western blot analysis. For the detection of recombinant protein by antibodies present in sera of rabbits immunized with *L. interrogans*, the purified target protein was run on a 12% SDS-PAGE gel, along with appropriate controls and prestained protein markers, and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 6% newborn bovine serum in 1× PBST (phosphate-buffered saline [PBS], 0.1% Tween 20, pH 7.2) for 1 h at 37°C. It was then washed three times with 1× PBST and incubated with rabbit anti-leptospirosis serum (1:1,000 dilution) for 1 h at 37°C. The membrane was washed as described above and incubated with goat anti-rabbit IgG-HRP conjugate secondary antibody (1:5,000 dilution) for a further 1 h at 37°C. After the wash, the protein bands were revealed with enhanced chemiluminescence reagents.

Detection of IgM and IgG antibodies to r-LMP. ELISAs were used to detect specific binding of the recombinant protein to IgM and IgG antibodies from serum samples. A panel of 156 human serum samples from patients with suspected leptospire infections was included in this study. The ELISA was initially standardized, and a concentration of 1.0 μg of purified recombinant LMP (r-LMP) protein gave the best reading. Purified target protein, which was diluted to 10 μg/ml in 0.1 M carbonate buffer (pH 9.6), was used to coat 96-well microtiter plates (100 μl/well) at 4°C overnight. The coated wells were washed three times with 1× PBST and blocked with 5% bovine serum albumin in 1× PBS for 1 h at 37°C. The wells were washed three times with 1× PBST, and 100 μl of diluted serum from each leptospirosis patient (1:50 dilution) was added as appropriate.

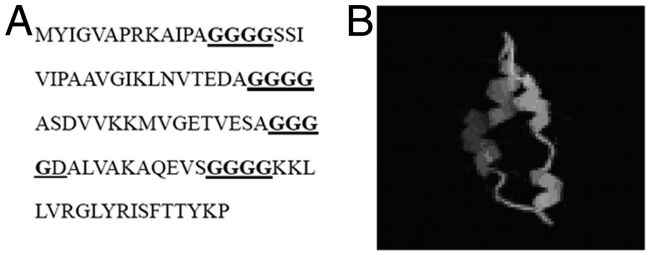

FIG. 1. Design of recombinant multiepitope protein. (A) Complete amino acid sequence of one copy of the recombinant multiepitope protein. The epitope amino acids are shown in normal font, and the tetraglyclyl linkers are in bold font and underlined. (B) Computer-generated representation of the codon protein unit. The protein amino acid sequence was imported into the three-dimensional position-specific scoring matrix Web server (3D-PSSM) and visualized using ViewerLite software.

Blank wells containing PBS and control wells containing healthy human sera or other patient sera were included in the ELISA plate. The antigen-antibody reaction was allowed to take place at 37°C for 1 h. Wells were washed using 1× PBST and incubated with goat anti-human IgM- or IgG-HRP conjugate (1:5,000 dilution). The wells were washed once again as described above and incubated with 100 μl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate for 15 min at 37°C in the dark. The color development reaction was terminated by addition of 100 μl 2 M H_2SO_4, and the absorbance was measured at 450 nm. If the OD value was more than double the negative control value, then the sample was defined as leptospirosis positive.

RESULTS

Design of the leptospirosis multiepitope protein (r-LMP). Outer membrane proteins were the focus of the current research because they are potential targets in bacterium-host interactions. We selected several immunodominant epitopes from the outer membrane proteins OmpL1, LipL21, and LipL32. Those epitopes were shown to display not only IgM but also IgG specificity and were reactive toward sera from patients infected with various leptospire serotypes. On the basis of the five selected epitopes, we synthesized the recombinant *lmp* gene and constructed the *E. coli* expression system. The amino acid sequence of one copy of the r-LMP protein is shown in Fig. 1A, and computer modeling analysis of the structure is depicted in Fig. 1B. This suggested that the proposed design of the multiepitope protein would be consistent with a structure that permits easy accessibility of all the constituent epitopes.

Expression of the r-LMP protein. Recombinant plasmid was introduced into *E. coli* host BL21(DE3) plysS; then, the transformants were selected in the presence of kanamycin and analyzed by expression screening. In this experiment, IPTG-induced cells were sonicated and analyzed by SDS-PAGE, and a specific band representing the recombinant product was obtained (Fig. 2). The protein was found to be present mainly in the soluble fraction. Induction of the fused multiepitope gene resulted in the appearance of a new band, about 23 kDa, consistent with the predicted size.

Purification of the r-LMP protein. As r-LMP protein was mostly in the soluble fraction, we proceeded to purify it under native conditions to obtain maximal yields. Fractions collected during different steps of the purification were analyzed by SDS-PAGE (Fig. 3A). Extensive washing in the presence of 40

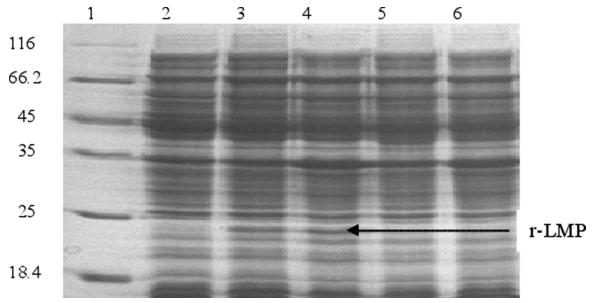

FIG. 2. Expression of recombinant protein in *E. coli* BL21(DE3) plysS. Results are shown for localization of r-LMP protein in induced cell lysates. An aliquot of the induced cell lysate prepared by sonication in 1× PBS buffer was separated into supernatant and pellet fractions and analyzed by SDS-PAGE. Lane 1, protein ladder; lane 2, pET28a; lanes 3 and 4, supernatant fractions; lanes 5 and 6, pellet fractions. The position of the r-LMP fusion protein is indicated by arrows.

mM imidazole progressively removed most of the nonspecific contaminants. This can be seen from a comparison of lanes 5 (NTA-40 buffer) and 6 (NTA-100 buffer). Elution of the bound proteins by use of 100 mM imidazole resulted in the emergence of highly purified recombinant protein from the column (lane 5). Starting from about 1 liter of induced cells, we obtained about 10.2 mg of purified recombinant protein. To characterize the bioactivity of r-LMP protein, we raised rabbit antibodies against leptospires and employed the serum in a Western blot assay. The immune rabbit serum bound well to the recombinant protein (Fig. 3B).

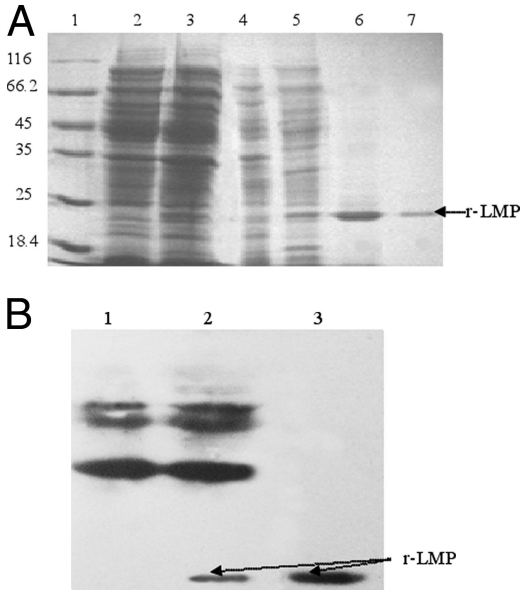

FIG. 3. Purification and characterization of r-LMP protein. (A) SDS-PAGE analysis of purified r-LMP protein. Lane 1, protein ladder; lane 2, pET28a; lane 3, pET28a-2LMP induced by 1.0 mM IPTG; lanes 4 to 7, pET28a-2LMP induced by 1.0 mM IPTG, eluted with the NTA-0, NTA-40, NTA-100, and NTA-1000 buffers, respectively. (B) Western blot analysis of r-LMP protein by use of a *Leptospira interrogans* monoclonal antibody. Lanes 1 to 3, hybridized strip of pET28a, pET28a-2lmp, and purified multiepitope protein with an anti-leptospire antibody. The positions of the hybridized strip of r-LMP fusion protein and the antibody are indicated by arrows.

TABLE 1. Detection of anti-recombinant multiepitope protein IgG and IgM antibodies in sera from leptospirosis patients by ELISA[a]

L. interrogans serogroup	No. of cases	No. positive/no. negative for:	
		IgM	IgG
Icterohaemorrhagiae	54	54/0	54/0
Autumnalis	8	8/0	8/0
Grippotyphosa	22	22/0	22/0
Australis	18	18/0	18/0
Hebdomadis	15	15/0	15/0
Pomona	28	28/0	28/0
Canicola	11	11/0	11/0

[a] Samples were designated positive when the OD_{450} values were more than double the control values.

Leptospirosis patient sera recognize r-LMP protein. For development of an ELISA using the purified r-LMP protein, a panel (n = 156) of suspected leptospirosis patient sera, which had been tested for the presence of leptospire infection by using the MAT, was used in the experiment.

To evaluate the purified r-LMP protein as a diagnostic reagent in detecting anti-leptospirosis IgM or IgG antibodies by using these well-characterized sera, an in-house ELISA protocol was developed. In this assay, r-LMP protein was used to capture the IgM or IgG class of anti-leptospirosis antibodies from patient sera, and goat anti-human IgM- or IgG-HRP conjugate was employed as a second antibody to react with anti-leptospirosis globins. On the basis of the data obtained, r-LMP recognized all of the 156 samples (Table 1). There was no cross-reaction with the control sera. The overall comparative analysis of our data with the MAT results suggests that there is excellent agreement between the r-LMP-based ELISA and the MAT.

DISCUSSION

Leptospirosis diagnosis based on antibody identification methods, such as MAT, has emerged as the most practical approach, but this test normally fails to detect the disease in the early acute phase (2). In recent years, new techniques such as PCR and real-time PCR were developed for diagnosis of leptospirosis, but these methods are expensive or prone to false-positive results. Recombinant-protein-based diagnosis offers certain advantages, such as high sensitivity and specificity, because higher antigen concentrations can be used, and nonspecific moieties are present as in the whole-cell preparations (14). Nevertheless, according to the results of conventional serological methods, one antigen is unable to detect the antiserum belonging to different serovars. Thus, there is an urgent need for a diagnostic test that not only incorporates high degrees of sensitivity and specificity for leptospirosis diagnosis but also covers the different serovars. The use of a synthesized recombinant antigen that includes the major epitopes of leptospire outer membrane proteins and can be easily overexpressed in *E. coli* may effectively address this aim. Therefore, the major objective of this study was to design an IgM- and IgG-specific recombinant multiepitope protein and evaluate its

potential application in the detection of anti-leptospire antibodies.

For this purpose, we first characterized the epitopes from sequences of the outer membrane proteins OmpL1, LipL21, and LipL32, using a phage display system. Then, we created the synthetic recombinant *lmp* gene, encoding five major screened epitopes, in which adjacent epitopes were joined using flexible tetraglycyl linkers. Three-dimensional position-specific scoring matrix analyses showed that all the epitopes were freely accessible, which suggested that each of them would be able to collectively contribute to the antibody specificity of the molecule. In order to amplify the antigenic signal, two copies of the recombinant *lmp* gene (r-2lmp) were cloned and the corresponding protein product was expressed in *E. coli*. Analysis of the Ni-NTA-purified r-LMP protein by SDS-PAGE showed that a high degree of purity had been achieved. Our data showed that 10.2 mg of r-LMP protein could be obtained from 1 liter of cultured cells.

Since the major objective of this study was to investigate the potential application of r-LMP protein in the detection of leptospire infection, we tested the ability of the r-LMP protein to detect anti-leptospire antibodies by Western blot analysis. When leptospire-infected rabbit sera were used as the primary antibody, the results showed that r-LMP reacted with antibodies present in sera. When r-LMP protein was used as the capture antigen and leptospirosis patient serum as the test sample, captured anti-leptospire IgM or IgG antibodies were detected using goat anti-human IgM- or IgG-HRP conjugate and the color development of TMB substrate. This analysis showed that r-LMP protein detected IgM and IgG antibodies in all 156 specimens, which had been classified as *L. interrogans* serovars Icterohaemorrhagiae, Hebdomadis, Pomona, Grippotyphosa, Australis, and Autumnalis in our previous work by using the MAT method and an MAT titer of >80 (17). That is to say, when the r-LMP protein was used as an antigen, the ELISA method detected most of the serogroups, which overcame the restriction of the single-serogroup specificity of single leptospire protein antigens. The diagnostic method was sensitive for the recombinant protein, which recognized both IgM and IgG in the serum samples. Our results are consistent with a previous report indicating that, in *Leptospira*, the early immune response appears to encompass both IgM and IgG antibodies (7). We consider that the recombinant multiepitope protein can be used for diagnosis of leptospirosis in the acute and convalescent phases. In conclusion, the recombinant multiepitope peptide that we generated is a useful diagnostic antigen. Particularly, r-LMP-based serology has a high potential for early diagnosis of leptospirosis at a time when culture and MAT results are not yet available.

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