Original Article

Recombinant LipL32 Protein Developed Using a Synthetic Gene Detects Leptospira-specific Antibodies in Human Serum Samples

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Abstract

Background: **Synthetic biology is emerging as a viable alternative for the production of recombinant antigens for diagnostic applications. It offers a safe alternative for the synthesis of antigenic principles derived from organisms that pose a high biological risk.**

Methods: **Here, we describe an enzyme-linked immunosorbent assay (ELISA) using the synthetic recombinant LipL32 (rLipL32) protein expressed in *Escherichia coli* for the detection of Leptospira-specific antibodies in human serum samples. The rLipL32-based ELISA was compared with a microscopic agglutination test (MAT), which is currently used as the gold standard for the diagnosis of leptospirosis.**

Results: **Our results showed that all the MAT-positive serum samples were positive for Leptospira-specific IgG in an ELISA, while 65% (n = 13) of these samples were also positive for Leptospira-specific IgM. In the MAT-negative serum samples, 80% and 55% of the samples were detected as negative by an ELISA for Leptospira-specific IgM and IgG, respectively.**

Conclusion: **An ELISA using the synthetic rLipL32 antigen was able to distinguish Leptospira-specific IgM (sensitivity 65% and specificity 80%) and IgG (sensitivity 100% and specificity 55%) in human serum samples and has the potential to serve as a rapid diagnostic test for leptospirosis.**

Keywords: *leptospirosis, LipL32 protein, Enzyme-Linked Immunosorbent Assay, codon optimised, anti-Leptospira antibodies*

Introduction

Leptospirosis is a zoonotic disease caused by the pathogen *Leptospira interrogans* that affects both humans and animals. According to the World Health Organisation's Leptospirosis Burden Epidemiology References Group (LERG), there are an estimated 873,000 cases of human leptospirosis reported annually, with 48,000 fatalities (1). The countries with the highest reported incidence are located in the Caribbean, Latin America, Indian Subcontinent, Southeast Asia, Oceania and Eastern Europe (2, 3).

The timely diagnosis of leptospirosis remains a challenge, since the organism is not easily cultivable, and the available diagnostic tests show varying levels of performance (4). Even though the microscopic agglutination test (MAT) is considered to be the gold standard for the diagnosis of leptospirosis, this assay is not effective for the early detection of the disease. Attempts have been made to develop enzyme-linked immunosorbent assays (ELISAs) for

detecting *Leptospira*-specific antibodies using recombinant *Leptospira* outer-membrane proteins (OMPs) as antigens (5, 6, 7, 8). The LipL32 protein, which is found abundantly in pathogenic *Leptospira*, is one of the most important OMPs expressed during human infection (9, 10). The recombinant proteins produced using *Escherichia coli* serve as a biologically safe source of antigens, as live *Leptospira* are pathogenic and pose a risk of infection among laboratory personnel. Since there are variations in the codon usage among species, codon optimisation is required for increasing the translational efficiency of the target genes by modifying their codons without changing the resulting amino acid sequences. This technology has been widely used for diagnostic purposes, such as antibody detection against swine fever virus, equine infectious anemia virus, and foot and mouth disease virus (11, 12, 13, 14, 15, 16). In this study, the synthetic gene encoding the LipL32 protein was designed and expressed in *E. coli*. The expressed protein was purified and subsequently used as an antigen in an ELISA for the detection of *Leptospira*-specific antibodies in human serum samples.

Materials and Methods

Bacterial Strain and Clinical Samples

The *E. coli* BL21 (DE3) host strain was prepared from a stock maintained in-house. Human serum samples (MAT-positive and MAT-negative samples) were provided by the Kota Kinabalu Public Health Laboratory, Sabah, Malaysia. All the serum samples were stored at -20 °C until they were tested. The study was approved by the Medical Research and Ethical Committee, Ministry of Health, Malaysia, and the study number is NMRR-13-677-15713.

Synthesis of Codon-Optimised lipl32 Gene and Expression of LipL32 Protein

The LipL32 amino acid sequence was retrieved from the NCBI (accession number ACZ73827.1). This sequence was codon-optimised for expression in *E. coli* BL21 (DE3) using Gene Designer software (17). A single-stranded oligonucleotide was synthesised after reverse translation of the optimised codon, assembled and inserted into the open reading frame (ORF) of the pET22b plasmid expression vector using the *Bam*H1 and *Eco*RI restriction sites. The insert was verified by Sanger DNA sequencing and restriction digestion. The synthesis of this synthetic nucleotide sequence was carried out by GENEWIZ Inc. The pET22b-*lipl32* plasmid was transformed into the *E. coli* BL21 (DE3) strain. Recombinant clones were selected on Luria-Bertani (LB) agar plates containing ampicillin (100 mg/L) and subjected to direct-colony PCR to detect the recombinants harboring the *lipl32* insert. The positive clones were further screened using restriction enzyme digestion analysis. A single positive clone was cultured in 500 mL of LB broth containing ampicillin (100 mg/L) on a rotary incubator shaker set at 37 °C and 200 rpm until the optical density (OD_{600}) reached 0.6–0.8. The culture was then induced with 1.0 mM isopropyl *β*-D-1-thiogalactopyranoside (IPTG), and samples of 1 ml were drawn at hourly intervals. The culture was maintained under the stated conditions for up to 16 hours post-induction. The culture was harvested by centrifugation, and the pellet was resuspended in 5 mL/g of lysis buffer (20 mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, 1% Triton X-100; pH 7.4). The cells were sonicated on ice, and the lysate was separated from the cell debris by centrifugation. The pellet was resuspended in 5 ml/g of phosphate-buffered saline (PBS) at pH 12.0 for solubilisation and purified by affinity chromatography using a HisTrap FF crude column with an AKTA Pure Purification System (GE Healthcare, USA). After the adsorption of the recombinant LipL32 (rLipL32) protein to the column, the bound proteins were eluted with elution buffer (20 mM sodium phosphate, 500 mM NaCl and 500 mM imidazole; pH 7.4). The fractions were collected, analysed with 10% of SDS-PAGE and quantified by the Bradford method. Confirmation of the purified recombinant protein was performed using liquid chromatography-mass spectrophotometry (LC-MS; First Base Sdn Bhd, Malaysia) followed by a comparative analysis against the MASCOT database (Matrix Science).

Detection of Leptospira-Specific Antibodies by ELISA

Purified rLipL32 antigen (0.25 µg/50 µL well) was used for coating 96-well Maxisorp immunoassay plates (Nunc, Denmark) and incubated overnight at 4 °C. The plates were washed with washing buffer (PBS-0.05% Tween 20) and then blocked with blocking buffer (PBS-3% BSA) for at least 90 min. The plates were washed, and 50 µL of a serial two-fold serum

dilution, from 1:100 to 1:3200, were added. The plates were incubated for 4 hours at room temperature. Next, the plates were washed, and 1:1000 dilutions of alkaline phosphatase (ALP)-conjugated goat anti-human IgG or IgM antibodies (Southern Biotechnology, USA) were added to each well. The plates were incubated overnight at 4 °C and washed, and the bound secondary antibodies were detected by adding p-nitrophenyl phosphate (PNPP) in diethanolamine substrate buffer (DSB). The plates were incubated at room temperature for 10–20 min. The absorbance was read at 405 nm with a microplate reader (TECAN Infinite 200® PRO, Switzerland). The antibody concentrations were expressed as optical density (OD) values. Positive and negative controls were included in each assay. A serum sample was considered positive when the OD value was two-fold higher than the negative control. The sensitivity and specificity of the ELISA for the detection of *Leptospira*-specific IgM and IgG were calculated using MAT as the gold standard.

Results

Generation of Synthetic lipl32 Gene and Expression and Purification of rLipL32 Protein

The codon-optimised *lipl32* gene construct in the pET22b plasmid expression vector is shown in Figure 1. The gene contained additional His-tag sequences at the C-terminus, and the expression was regulated by the T7 promoter. The constructed recombinant plasmid (pET22b-*lipl32*) was introduced into the *E. coli* BL21 (DE3) host strain, and the transformants were selected in the presence of ampicillin and cultured for expression. In this study, a specific band of approximately 40 kDa, representing the rLipL32 protein, was obtained at the highest concentration after 3 hours following IPTG induction (Figure 2). The protein was detected primarily as inclusion bodies. The fractions collected after purification by immobilised metal affinity chromatography (IMAC) were analysed by SDS-PAGE (Figure 3). As shown in Figure 4, (Lanes E1 to E3), the purified rLipL32 protein was present in all three fractions. A de novo sequence of the recombinant protein spot by LC-MS revealed that the fragments were 100% identical to the *L. interrogans* LipL32 protein (data not shown).

rLipL32 Detects Leptospira-Specific Antibodies in Human Serum Samples

The purified rLipL32 antigen was able to detect the presence of both *Leptospira*-specific IgM and IgG antibodies in the tested serum samples by an ELISA. *Leptospira*-specific IgG was detected in all 20 MAT-positive serum samples, while 65% of these samples were also positive for *Leptospira*-specific IgM. Of the 20 MAT-negative serum samples, 80% were negative for *Leptospira*-specific IgM, and only 55% were negative for *Leptospira*-specific IgG (Table 1).

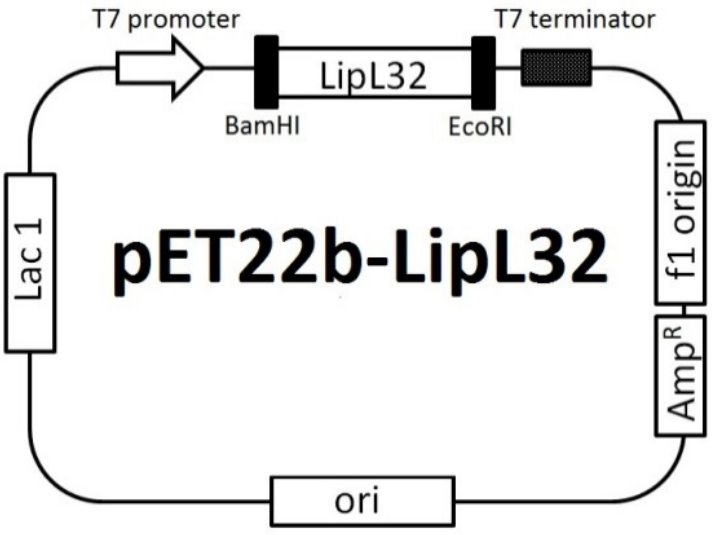

Figure 1. Schematic diagram of a codon-optimised lipl32 gene construct in a pET22b plasmid expression system

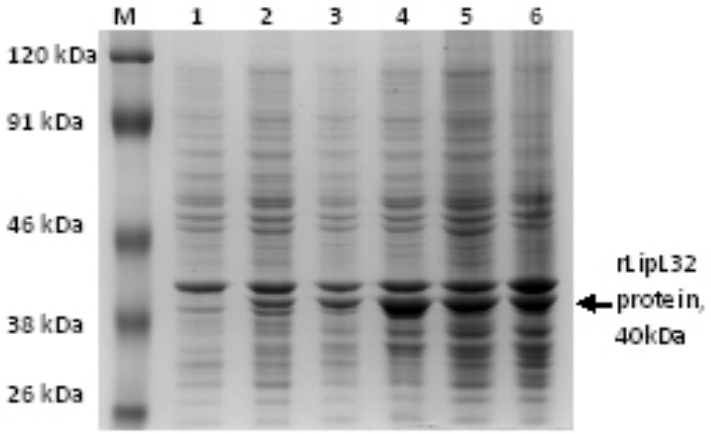

Figure 2. SDS-PAGE of total protein from *E. coli* BL21 (DE3) expressing the rLipL32 protein at different incubation times (inclusion bodies). Lane M: Low-range protein ladder (Nacalai Tesque Inc., Japan); Lanes 1–6 show the results from incubations of 0, 1, 2, 3 and 4 hours and overnight (16 hours)

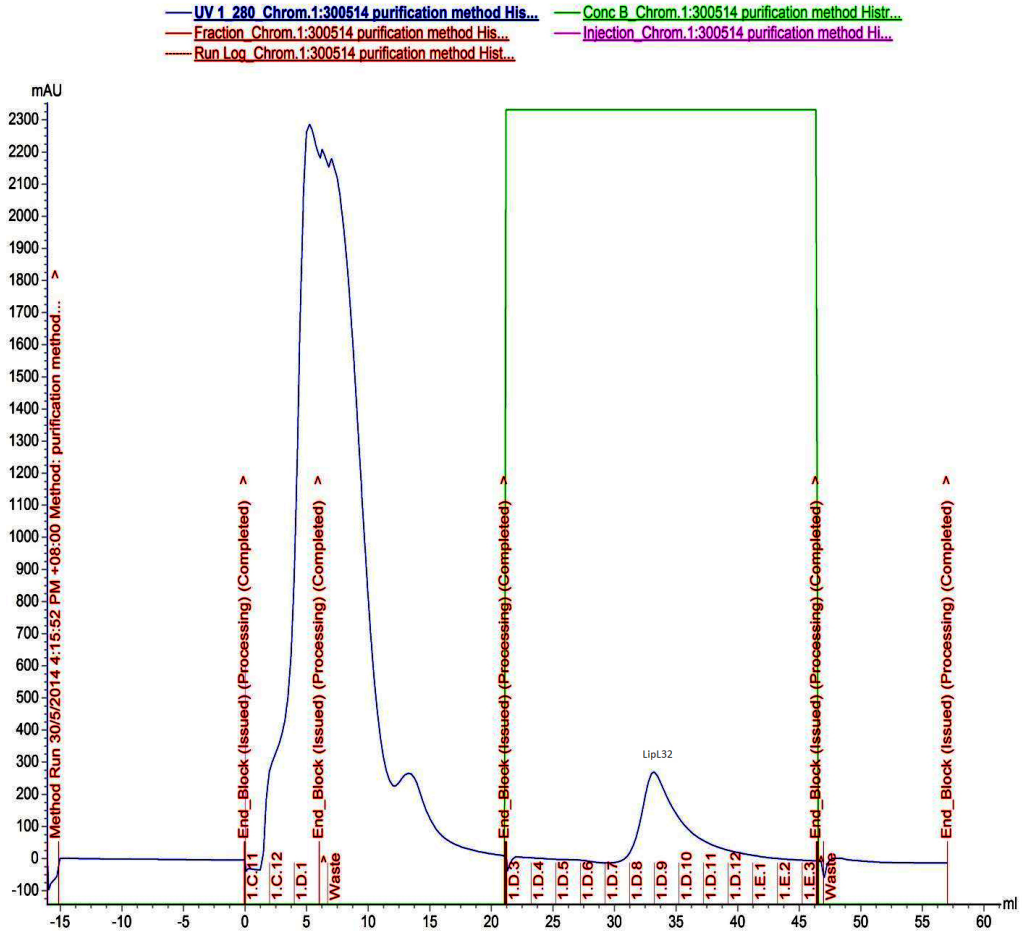

Figure 3. Chromatogram profile of the rLipL32 protein at UV 280 nm

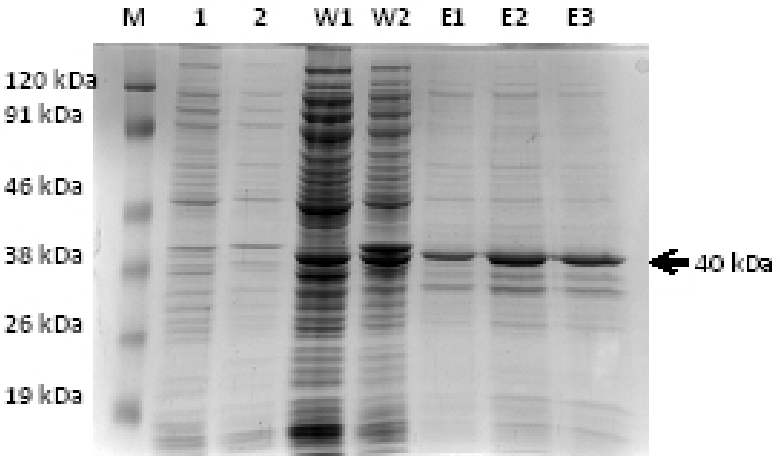

Figure 4. SDS-PAGE analysis of purified rLipL32 protein. Lane M: Low-range protein ladder; Lanes 1–2: Uninduced culture of insoluble and soluble fractions; Lane W1: Insoluble fraction washed with 1% Triton-X 100; Lane W2: Insoluble fraction washed with deionised water; Lanes E1–E3: Elution fractions of purified rLipL32 protein

Table 1. Detection of Leptospira-specific IgM and IgG in human serum samples

		MAT Positive (*n* = 20)	Sensitivity	MAT Negative (*n* = 20)	Specificity
IgM ELISA	Positive	13 (65%)	65%	4 (20%)	80%
	Negative	7 (35%)		16(80%)	
IgG ELISA	Positive	20 (100%)	100%	9(45%)	55%
	Negative	0 (0%)		11(55%)	

n = number of samples

Discussion

We have evaluated the diagnostic potential of an rLipL32-based ELISA for its ability to detect *Leptospira*-specific antibodies in human serum samples. Our data suggest that the codon-optimised LipL32 antigen has the potential to be used in the development of immunoassays for the serodiagnosis of leptospirosis. The rLipL32 ELISA is able to detect both *Leptospira*-specific IgM and IgG antibodies and can thus be used for detecting both recent and past *Leptospira* infections in human serum samples.

The lipoprotein LipL32 is the most-studied OMP relative to the other major classes of OMPs in *Leptospira*. A study conducted by Haake et al. (9) revealed a high degree of nucleotide sequence conservation in *lipl32* genes in five out of seven *Leptospira* strains, with an average of 96.4% similarity among them. Consequently, the interest in this protein is due its presence in pathogenic strains of *Leptospira*. The development of an ELISA based on a recombinant *Leptospira* antigen is advantageous, as it bypasses the need for culturing hazardous *Leptospira* in the laboratory and produces results that can be easily interpreted.

The efficiency of the expression of the rLipL2 protein in *E. coli* was enhanced by codon optimisation. There are only a few reports relating to the use of synthetic genes encoding the LipL32 protein for the diagnosis of human leptospirosis (18). The rLipL32 was expressed with a His-Tag at the C-terminus in *E. coli* under the control of an IPTG-induced T7 promoter. SDS-PAGE analysis showed that the rLipL32 expressed was approximately 40 kDa in size, which differs from what has been reported in the literature (9, 19, 20, 21, 22, 23). The synthetic lipl32 gene in this study was designed without its own start codon (ATG), and the expression

of rLipL32 was initiated by the start codon from the pET22b expression vector, thus causing an increase in the molecular weight of the rLipL32 protein.

The sensitivity of the rLipL32 IgG ELISA was found to be 100% when compared to the MAT assay, suggesting its potential use in assessing the seroprevalence of leptospirosis in the community (24, 25). Meanwhile, the sensitivity and specificity of the rLipL32 IgM ELISA were lower than in earlier reports (8, 26, 27). One third of the MAT-positive and IgG-positive serum samples were IgM negative when tested in an ELISA, and this may have been due to past infections. The MAT assay does not distinguish between IgM and IgG, and it may not be the ideal method for comparison; thus, the lower performance calculated in this study may not be accurate, as it was compared against the MAT assay. The MAT-negative samples that showed reactivity in the ELISA could have resulted from the higher sensitivity of the ELISA, since the OD values were read with a spectrophotometer; or, they could have been due to cross-reactivity and nonspecificity. This issue needs further confirmation by PCR or testing of serum samples of healthy controls from non-endemic regions. The interpretation of the MAT assay is complicated due to subjectivity and the high degree of cross-reactions occurring among different serogroups, especially in acute-phase samples (28).

This study is being continued with a larger number of serum samples to accurately determine the sensitivity and specificity of the ELISA. Studies on the determination of the immunodominant epitopes of LipL32 is underway to reduce any cross-reactions that may occur during the detection of *Leptospira*-specific antibodies in serum samples. Further work on the validation of the epitopes will allow a wider application of this recombinant antigen for the detection of *Leptospira*-specific antibodies.

Conclusion

A synthetic rLipL32 antigen-based ELISA for the detection of *Leptospira*-specific antibodies developed in this study was able to detect the presence of *Leptospira*-specific IgM and IgG in human serum samples. The findings presented here demonstrate the capability of a synthetic rLipL32 ELISA for the early diagnosis of leptospirosis as well as its potential use in assessing the seroprevalence of leptospirosis in the community.

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