

EXPRESSION AND CHARACTERIZATION OF RECOMBINANT LEPTOSPIRAL OUTER MEMBRANE PROTEIN LipL32 FROM *LEPTOSPIRA INTERROGANS* SEROVAR *AUTUMNALIS*

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Abstract. *Leptospira interrogans* serovar *autumnalis*, a causative agent of leptospirosis in Thailand, was isolated from a patient for DNA extraction and amplification of LipL32 gene by polymerase chain reaction (PCR). The 782 bp PCR product was obtained, which was inserted into pAE plasmid with polyhistidine (His₆ tag) to construct pAE-LipL32. This recombinant plasmid was transfected into *E. coli* BL21 (DE3). His₆-LipL32 was purified by Ni-NTA affinity chromatography. The recombinant protein was used as antigen for testing with sera from leptospirosis and syphilis patients by dot-ELISA technique. It reacted positively with leptospirosis patient sera and negatively with syphilis and healthy sera.

INTRODUCTION

Leptospirosis is a zoonotic infectious disease caused by spirochetes belonging to the genus *Leptospira*. Pathogenic leptospires are classified as members of the species *Leptospira interrogans*, divided into numerous serogroups and more than 250 serovars. Leptospirosis in humans is transmitted by direct contact with infected animals or by indirect contact with water, moist soil or vegetation contaminated with urine from chronically infected animals such as rats, dogs and farm animals (Farr, 1995; Fain *et al*, 1999; Ribotta *et al*, 1999). Although leptospirosis has a worldwide distribution, it

is most common in tropical and rural areas (Farr, 1995; Fain *et al*, 1999). Early diagnosis of human *Leptospira* infection is very important because the symptoms may resemble those of other febrile illnesses including influenza, dengue fever, meningitis or hepatitis. Therefore, rapid and appropriate laboratory diagnostic tests are needed to aid clinical case identification for optimal treatment and patient management and to facilitate the implementation of rapid outbreak investigations. Severe leptospirosis may be associated with high mortality if not treated adequately.

The laboratory diagnosis of leptospirosis is mainly based on serological methods, since culture is both less sensitive and time consuming (Tanganakul and Kingnate, 1998; Ajay *et al*, 2003). The microscopic agglutination test (MAT), the reference method, is serogroup specific but inadequate for rapid case identification since it can only be per-

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formed in a few reference laboratories and requires analysis of paired sera to achieve sufficient accuracy.

In Thailand, leptospirosis is also one of the public health problems in the northeastern part of the country where most of the people are farmers (Tanganakul and Kingnate, 1998). A previous report showed that the major causative agent in the northeast of country identified from cultivation method is *Leptospira interrogans* serovars *autumnalis* (Dounghawee *et al*, 2005).

In a previous study, we constructed the recombinant LipL32, a major leptospiral outer membrane protein, whose expression during mammalian infection is restricted to pathogenic *Leptospira* species (Haake *et al*, 2000), from locally prevalent serovars to develop an antigen. This antigen will be a raw material for the production of a leptospirosis diagnostic test kit for the detection of the leptospira antibody in human sera.

MATERIALS AND METHODS

Bacterial strains, plasmid and sera

All *L. interrogans* and *L. biflexa* serovars *patoc* I were kindly provided by P Naigowit, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand. The pAE expression vector, a plasmid derived from pRSET (Farr, 1995), was kindly provided by Dr Albert Icksang Ko, Division of International Medicine and Infectious Disease, Weill Medical College, Cornell University, USA.

Leptospirosis patient sera that were positive for antibody to *Leptospira* by IFA were obtained from the Regional Medical Science Center, Udon Thani, located in Northeast Thailand. Syphilis patients sera that were positive for antibody to *T. pallidum* by TPHA, HIV patient sera and sera from healthy donors were obtained from the Phranangklao Hospital located in Central Thailand.

PCR amplification and cloning of LipL32 gene

A local strain of *L. interrogans* serovar *autumnalis* was used in this study. Genomic DNA of *Leptospira* was prepared by the method of Yelton and Charon (Ribotta *et al*, 1999). In brief, one ml of 10^6 - 10^8 organisms was centrifuged at 13,000g for 30 minutes at 4°C. The pellet was washed twice with phosphate-buffered saline (PBS) pH 7.2-7.4, re-suspended in 100 µl of 0.1 mM Tris-HCl pH 7.0 and boiled for 10 minutes. DNA concentration was measured spectrophotometrically (OD_{260}) and stored at -20°C.

DNA of *L. interrogans* pathogenic strains and *L. biflexa* serovar *patoc* I, a non-pathogenic strain used as a negative control, were amplified for LipL32 gene encoding the mature protein, beginning with the first residue after the N-terminal cysteine as previously reported (Fain *et al*, 1999). The forward primer containing the nucleotide sequence coding for the six amino acids following the N-terminal cysteine of mature LipL32 and including a *Xho* I restriction endonuclease site (underlined) is 5'-TTA CCG CTC GAG GTG CTT TCG GTG GTC TGC-3'. The reverse primer containing the nucleotide sequence coding for five amino acids and LipL32 stop codon and including a *EcoR* I restriction endonuclease site (underlined) is 5'-TGT TAA GAA TTC TTA CTT AGT CGC GTC AGA -3'. PCR was carried out in a volume of 50 µl containing 200 ng of DNA, 25 nmol of each primer, 200 µM dNTPs, 2 mM MgCl₂, 1xPCR buffer and 0.25 U *Taq* DNA polymerase (Qiagen). PCR was performed using heat denaturation at 94°C for 3 minutes, followed by 35 cycles of 94°C for 1 minute, 59°C for 1 minute and 72°C for 1 minute. After the last cycle, the sample was heated at 72°C for 10 minutes. The 782-bp amplicon of *L. autumnalis* LipL32 gene was digested with *Xho* I and *EcoR* I and ligated with pAE plasmid with the same restriction

enzymes. The resulting construct, pAE-LipL32, was transfected into *E. coli* BL21 (DE3) competent cells, which were grown on LB plates containing 100 µg/ml of ampicillin, 50 µg/ml of 5-bromo-4-chloro 3-indolyl β-D-galactopyranoside (X-gal) and 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). White colonies were analyzed for the presence of pAE-LipL32 using PCR technique with specific primers for LipL32 followed by restriction endonuclease analysis.

Recombinant protein production and purification

Transformants that contains LipL32 gene were cultured and harvested for 3 hours after induction with 1mM IPTG. Bacterial cells were sonicated on ice three times (5 seconds pulses and 1 minute pause) at high intensity in lysis buffer containing 6 M guanidine HCl, 20 mM sodium phosphate pH 7.8 and 500 mM NaCl. After sedimentation, the supernatant was loaded onto affinity Ni-NTA agarose column (Qiagen), which was washed with 8 M urea, 20 mM sodium phosphate pH 7.8 and 500 mM NaCl. Protein was eluted with 8 M urea, 20 mM sodium phosphate pH 4.0 and 500 mM NaCl and then extensively dialyzed against 10 mM Tris pH 8.0 and 0.1% Triton X-100. The amount of purified recombinant protein was determined by Bradford technique (Transuphasiri *et al*, 2005).

SDS-PAGE and Western blot analysis of recombinant protein

Samples were solubilized in sample buffer consisting of 50 mM Tris-HCl pH 6.8, 100 mM dithiothreitol, 2% sodium dodecyl sulfate (SDS) and 0.1% bromophenol blue in 20% glycerol. Proteins were separated on 2.5% stacking and 12 to 15% linear gradient polyacrylamide gel using the discontinuous buffer system of Laemmli (1970) and stained with Silver stain (Bio-Rad). For immunoblotting protein bands were transferred to

nitrocellulose membrane, which was incubated overnight at 4°C with 1% bovine serum albumin in TTBS (100 mM Tris-HCl pH 7.5, 0.9% NaCl, 0.1% Tween 20). Recombinant proteins were identified incubating with rabbit anti-LipL32 antibody (kindly provided by Professor Ming Jeng Pan, Graduate Institute of Veterinary Medicine, National Taiwan University, Republic of China) followed by alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Dako) or leptospirosis patient sera followed by phosphatase-conjugated rabbit anti-human immunoglobulin G.

Characterization of expression protein by dot-ELISA

Purified protein from bacterial cell lysate was characterized for LipL32 by dot-ELISA (Tansuphasiri *et al*, 2005). Cell lysate and negative control (non-induced lysate) were dotted on nitrocellulose membrane and incubated with a solution of 1% BSA and 3% skim milk in PBS containing 0.01% Tween 20 (PBS-T) for 30 minutes with shaking at room temperature. The membrane was washed three times (5 minutes each) with PBS-T. Sera of leptospirosis patients positive by IFA test, of syphilis patients positive for *T. pallidum* antibody by TPHA, and sera of HIV patients and healthy donors were diluted 1:500 in PBS-T and incubated with membrane at room temperature for 40 minutes with shaking. The membrane was washed with PBS-T for two times. Rabbit anti-human immunoglobulin M peroxidase conjugate (Dako) diluted 1:500 in dilution buffer (1% BSA and 3% skim milk in PBS containing 0.01% Tween 20 (PBS-T) was added. The membrane was then incubated for 40 minutes at room temperature with shaking, washed 2 times with PBS-T and once more with PBS. Substrate solution of 2, 6-dichlorophenol indophenol was added and a pink color was allowed to develop with shaking for 1 minute. The reaction was stopped by adding 5% acetic acid.

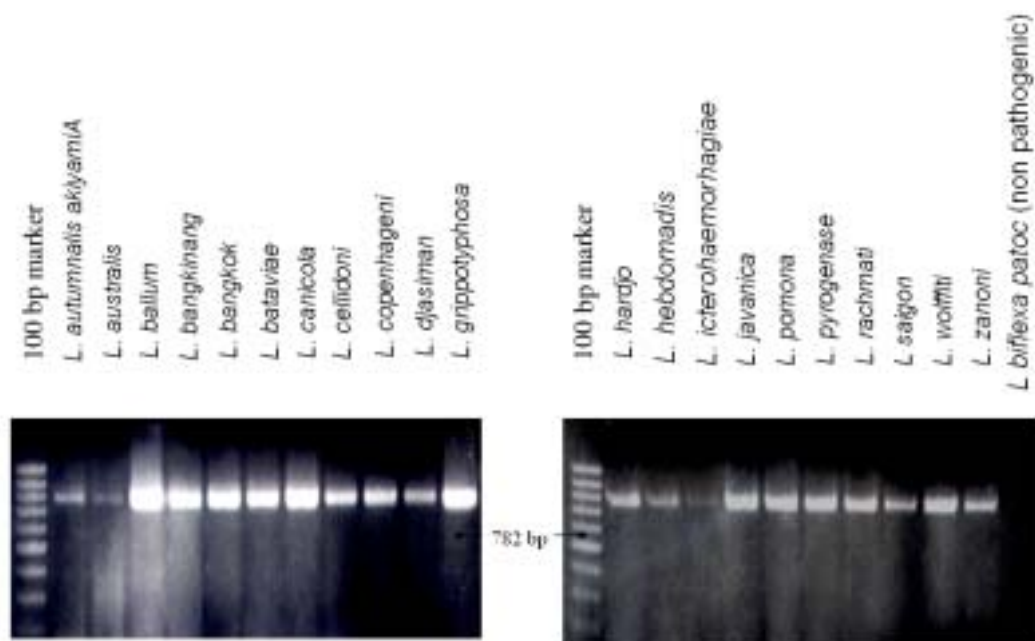


Fig 1–LipL32 gene from 21 pathogenic *Leptospira* strains and *L. biflexa* serovar *patoc* I, a non-pathogenic strain amplified by PCR; 782 bp is present only in pathogenic *Leptospira* strains.

RESULTS

LipL32 gene from 21 pathogenic *Leptospira* strains and *L. biflexa* serovar *patoc* I, a non-pathogenic strain, was amplified by PCR. Fig 1 shows that only pathogenic strains have 782 bp amplicon of LipL32 outer membrane protein. *Leptospira interrogans* serovar *autumnalis* isolated from a Thai patient was used to clone LipL32 gene. This gene was inserted into pAE plasmid vector and transfected into *E. coli* BL21 (DE3), an expression host. A transformed clone that contained the recombinant plasmid was selected and was used for protein expression (Figs 2 and 3).

The optimal condition for protein expression was determined by inducing with 1 mM IPTG at different temperatures: 37°C, 30°C and 20°C. The optimal temperature for LipL32 protein expression was 20°C. The incubation times tested for protein expression were 0 hour, 1 hour, 2 hours, 3 hours,

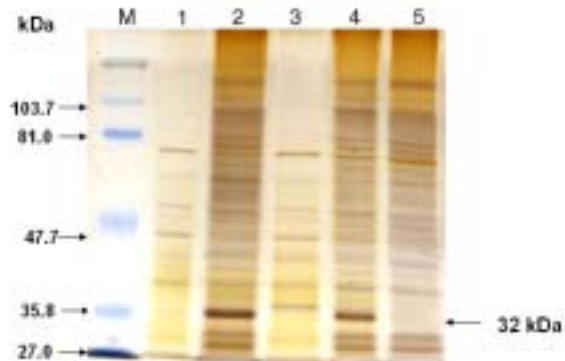


Fig 2–SDS-PAGE of cell lysates from *E. coli* BL21 (DE3) expressing LipL32 gene. SDS-PAGE was performed under reducing condition and protein bands were stained with silver. Clones no. 6 and no. 12 gave protein of molecular mass 32 kDa after induction with 1 mM IPTG overnight (lanes 2 and 4). Lanes 1 and 3 show no expression of LipL32 protein under non-induction condition. Lane 5 shows expression from cells transfected with pAE vector. Lane M contains molecular weight markers.



Fig 3–Western blot analysis of expressed proteins reacting with polyclonal antibody to leptospira. LipL32 protein was expressed in cells induction with 1mM IPTG (lanes 2 and 4). Lanes 1 and 3 show no expression of LipL32 protein under non-induction condition. Lane 5 shows no protein from cells transfected with pAE vector. Lane M contains molecular weight markers.

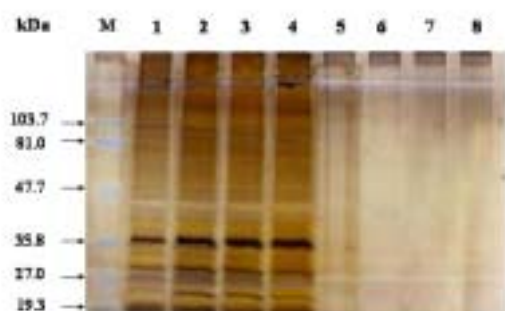


Fig 4–SDS-PAGE of cell lysates from *E. coli* BL21 (DE3) expressing LipL32 gene under various incubation times. Lanes 1-5 show results from 0, 1, 2, 3 hours and overnight incubation. Lanes 6-8 show results from expression of pAE vector at 0, 3 hours and overnight. Lane M contains molecular weight markers.

and overnight. The 3-hour incubation gave the highest protein expression (Fig 4).

The expressed intracellular protein was purified by Ni-NTA affinity chromatography obtaining a yield of 0.518 mg/ml. It was then used in dot-ELISA. Recombinant LipL32

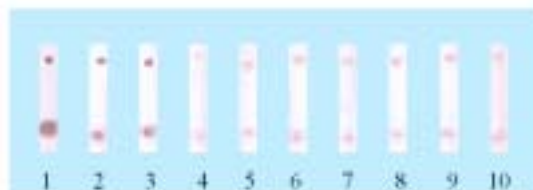


Fig 5–Characterization of purified LipL 32 protein by dot-ELISA. Purified LipL32 were dotted on nitrocellulose membrane to act as antigen to react with sera of polyclonal antibody to leptospirosis (1), leptospirosis patients sera (2 and 3), leptospirosis negative by IFA patients sera (4 and 5), healthy sera (6 and 7), syphilis patients sera (8 and 9), and HIV pateint serum (10).

protein and negative control (lysate from control cell) were dotted onto nitrocellulose membrane to act as antigen to react with sera of leptospirosis, syphilis, HIV patients and healthy donors. Only sera from leptospirosis patients reacted positively with recombinant LipL32 protein as shown in Fig 5.

DISCUSSION

Leptospirosis is considered as a re-emerging infectious disease, not only for the increase in its incidence during the past recent years but also for the increased severity of the illness (Sundharagiati *et al*, 1966; Sitprijia *et al*, 1985; Heisey *et al*, 1988; Silva *et al*, 1995; Phraisuwan *et al*, 2002). False positive reactions with other diseases such as syphilis, dengue and hepatitis obtained using traditional methods have also been reported (Turner, 1967; Sagdeeva, 1970; Sitprijia *et al*, 1985; Gussenhoven *et al*, 1997; Levett, 1999; Sanders *et al*, 1999; Smits *et al*, 1999,2000; Yang *et al*, 2002; Bajani *et al*, 2003).

Because of the requirement of a more specific test for laboratory diagnosis, purified antigens have been considered as a key factor to solve this problem. An ideal antigen should be a principal target of the host

immune response, expressed only in pathogenic *Leptospira* spp and is conserved among more than 200 serovars associated with human diseases in different geographic regions and epidemiological situations. An ideal test would need to discriminate between leptospirosis and other diseases that cause acute febrile illness and have overlapping clinical presentations. Because the burden of leptospirosis is greatest in developing countries, there is a need to develop a test which can be produced at low cost and is easily standardized for use in field settings. Recombinant protein-based serologic tests can achieve high sensitivity and specificity because of the high concentration of immunoreactive antigens used in the assays. Recombinant-antigen-based assays can also be produced at low cost, an important consideration for implementation in developing countries (Fain *et al*, 1999).

A 32 kDa protein was identified to be an immunodominant antigen with the best serodiagnostic utility (Haake *et al*, 2000). This antigen was identified as LipL32, a major leptospiral outer membrane protein whose expression is restricted to pathogenic leptospira species. It is the most prominent protein in SDS-PAGE protein profile (Haake *et al*, 2000) and is also the most frequently recognized antigen in immunoblots with patient sera.

In this study, we found that the protein expression of each cell batch was not precise and unpredictable. So, extensive quality control measures are necessary to monitor batch-to-batch variability in antigen composition inherent in growing cultures (Fain *et al*, 1999). The amount of purified protein obtained in the study (0.518 mg/ml) may be too low for further use as an antigen for the leptospirosis antibody diagnostic kit. However, a previous study indicated that this recombinant protein may not be able to be produced in large amounts because of its toxicity to *E. coli* (Shang *et al*, 1995).

In summary, recombinant LipL32 protein was produced and used to test sera from patients with leptospirosis. However, testing with a larger group of sera samples should be done for the determination of specificity and sensitivity before further use in a leptospirosis diagnostic kit.

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