



Original article

Cloning and periplasmic expression of peptidoglycan-associated lipoprotein (PAL) protein of *Legionella pneumophila* in *Escherichia coli* **Abolfazl Gholipour¹, Mojtaba Moosavian¹, Hamid Galehdari², Manoochehr Makvandi¹, Hamid Rajabi Memari³, Amirhoushang Alvandi¹**

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Abstract

Introduction and objective: *Legionella pneumophila*, the etiological agent of Legionnaires' disease, is an important cause of both community-acquired and nosocomial pneumonia; therefore, rapid diagnosis and early antibiotic treatment of pneumonia are required. Urinary antigen testing to detect *Legionella* antigen has proven to be the most powerful diagnostic method. Peptidoglycan-associated lipoprotein (PAL) protein of *L. pneumophila*, as a component of *Legionella* antigens, will be detected efficiently by the PAL antigen capture assay and is considered as useful diagnostic antigen to diagnose *Legionella* infection. Because of the transfer of protein to the periplasmic region of *Escherichia coli* has numerous advantages including separation from cytoplasmic proteins and the concentration of recombinant proteins in periplasm, the aim of this study was to produce periplasmic PAL protein of *L. pneumophila* in *E. coli*.

Materials and methods: The pal gene of *L. pneumophila* serogroup 1 was amplified with specific primers, cloned and expressed under *pelB* signal sequence and T7 lac promoter in pET26b+ plasmid.

Results: The cloning was confirmed with digestion and sequencing of recombinant pET-26b-pal plasmid. The expression of r-PAL protein in cytoplasm and periplasmic space of *E. coli* was approved by SDS-PAGE and western blotting.

Conclusion: The results of this study demonstrated that the r-PAL protein successfully expressed in *E. coli*.

Keywords: *Legionella pneumophila*, Legionnaires' disease, Peptidoglycan-associated lipoprotein, Expression, *Escherichia coli*

Introduction

Legionellae are part of the microbial community of aquatic ecosystems (natural as well as manmade), which explain why legionellosis occurs worldwide [1]. Results of the studies in Iran showed that *Legionella pneumophila* isolated from different aquatic and therapeutic centers [2-4]. *L. pneumophila*, the etiological agent of Legionnaires' disease (LD), is an important cause of both community-acquired and nosocomial pneumonia infection. LD still occurs, both in sporadic and epidemic forms, sometimes involving hundreds of deaths and can cause high morbidity and mortality if treated improperly [5,6]. In addition, *Legionella pneumonia* may be severe and is potentially fatal in elderly and immunocompromised patients, and therefore, rapid diagnosis, early antibiotic treatment and primary prevention of pneumonia are required [7,8]. Over the intervening years, *Legionella* urinary antigen detection methods have proven to be the most powerful diagnostic methods [9,10].

Bacterial lipoproteins that are useful diagnostic antigens have been a focus of vaccine designs, as they are potent B-cell mitogens, immunoadjuvants and can be T-cells inducers [11-13]. The cell envelope of *L. pneumophila* has several interesting antigens, including 19-kDa peptidoglycan-associated lipoprotein (PAL) protein. The PAL protein is an immunodominant component of *Legionella* antigens and is a highly conserved antigen among *Legionella* species. This supports the idea that it could be used as a potential urinary antigen to diagnose LD and also as sub-unit vaccine against *Legionella* infections in humans. The PAL protein excretes in urine as a general component of *Legionella* antigens and could be used as an alternative diagnostic urinary antigen [14].

Escherichia coli is the best characterized and most widely used bacterial host for the production of recombinant proteins. *E. coli*

expression systems imply a rapid generation of biomass, low-cost culture conditions, and are very versatile, due to the availability of an increasingly large number of cloning vectors and host strains. However, most recombinant proteins applied in therapy and diagnosis are secreted proteins, and will form inclusion bodies when expressed in the cytoplasm of *E. coli*. Inclusion bodies are formed during cytoplasmic expression of recombinant proteins are insoluble protein deposits which demand isolation, solubilization and proper refolding [15,16]. To avoid problems associated with cytoplasmic expression; the aim of this study was periplasmic expression of r-PAL protein in *E. coli*.

Materials and methods

Bacterial strains, plasmid, media, and culture conditions

Legionella pneumophila serogroup 1 (ATCC33152); obtained from the American Type Culture Collection (ATCC), Manassas, VA was cultured on nonselective buffered charcoal-yeast extract medium supplemented with 0.1% α -ketoglutarate (BCYE α medium) and selective modified Wadowsky-Yee medium (MWY) (Oxoid) [17] at 37°C and 5%CO₂. *E. coli* strains were cultured on Luria-Bertani (LB) medium (Himedia), supplemented with kanamycin (50mg/L), if necessary. *E. coli* strain DH5 α and *E. coli* strain BL21 (DE3) (Novagen Inc., Madison, Wis.) were used for cloning and expression, respectively; and the expression vector pET26b+ (Novagen Inc.) was used for cloning the PCR products and also as the T7-based expression vector. All chemicals used in this study were from Merck (Germany) and all of the enzymes from Roche (Germany), Fermentas (Lithuania) or Cinagen (Iran) Companies.

General DNA procedures

The genomic DNA of *L. pneumophila* and the pET26b+ plasmid were extracted using Genomic DNA Purification and Gene JET Plasmid Mini prep Kits, respectively, according to the manufacturer's instructions (Fermentas, Lithuania). PCR primers were designed based on the full protein-coding region of the *L. pneumophila* pal gene (Accession No. X60543) of the 19-kDa lipoprotein [18] by the DNASIS v2.6 Primer Designer Software.

The forward primer, pal-F, (5'-CATGCCATGGCAAAAAGCCGGATCGT TTTATAAA-3') contained *NcoI* recognition site and reverse primer, pal-R, (5'-CCCAAGCTTGGGTCTTGTTCCTCAT AAATAAAC-3') contained recognition site for *HindIII*. PCR was performed on a Thermocycler TC-512 (Techne) using Expand High Fidelity PCR System (Roche) and the PCR fragment was analyzed on a 1% agarose gel. For purification purposes, DNA was extracted from the PCR reaction mixture by DNA Extraction Kit (Fermentas, Lithuania).

Construction of pET26b-pal expression vector

To form the over-expression pET26b-pal plasmid, the PCR product and the pET26b+ plasmid were digested by *NcoI* and *HindIII* restriction enzymes (Fermentas). After *NcoI* and *HindIII* thermal inactivation, the linearized plasmid and the digested PCR product were used for ligation by T4 DNA ligase (Fermentas). After ligation, *E. coli* DH5 α were transformed with the recombinant pET26b-pal plasmid and selected on LB agar plates containing kanamycin (50mg/L). A number of colonies were assayed by colony PCR. After selecting the recombinant clones, the plasmid DNA was extracted from the overnight culture and confirmed by restriction digestion with *KspAI* restriction enzyme and sequence analysis (TAG Copenhagen, Denmark). In order to raise

protein expression, the recombinant pET26b-pal plasmid was transformed into competent *E. coli* BL21 (DE3) cells [19].

Expression of recombinant PAL protein

A single colony of *E. coli* BL21 (DE3) carrying the pET26b-pal was used to inoculate culture of LB broth supplemented with kanamycin (50mg/L) (LBB-K). The culture was incubated overnight at 37°C with orbital shaking at 250rpm. The overnight culture was inoculated to fresh LBB-K in a 1:100 volumetric ratio and was placed at 37°C with orbital shaking at 250 rpm. The r-PAL protein expression was induced by adding various concentrations (0.1-1 mM) of Isopropyl- β -D-thiogalactopyranoside (IPTG) at an OD₆₀₀ of 0.4-1. To determine the time course of the r-PAL protein expression, aliquots (1ml) of the different cultures were removed just before IPTG induction and at different times after induction (1, 2, 4, 6, 8, 10, 12, and 15 hrs) and subjected for protein analysis.

Preparation of the periplasmic extract

All of the following steps were performed at 4°C. The bacterial cells were harvested by centrifugation at 5000g and the pellet was re-suspended in 100ml TES buffer (0.02M Tris-Cl, 0.5M EDTA, and 20% sucrose, pH=8.0) per liter of culture. Lysozyme (final concentration 1mg/ml, Boehringer Germany) and one tablet of complete protease inhibitor cocktail (Roche) were added to the cell suspension, followed by adding 200ml of ice cold deionized water. The suspension was agitated at 60rpm on ice for 1h and the cells were centrifuged at 16000g for 20 minutes. Both the pellet as the cytoplasmic and membrane fractions and the supernatant, which constituted the periplasmic fraction, saved for further protein analysis.

SDS-PAGE and Western blotting

SDS-PAGE was carried out according to the method of Laemmli [20]. The prepared protein samples were subjected to electrophoresis on 18% polyacrylamide gel and stained by Coomassie Blue. To perform western blot analysis, proteins were transferred from SDS-PAGE gel to a 0.45µm pore nitrocellulose membrane (Amersham Pharmacia Biotech, UK) at 300 mA for 1.5hrs in Tris-glycine buffer (0.025 M Tris, 0.2M glycine (pH=8.3) with 20% methanol) [21].

The blotted membrane was blocked in fresh TBS blocking buffer (50mM Tris, 150mM NaCl, pH=7.5) containing 1% bovine serum albumin (BSA) at room temperature for 1h. Expression of the r-PAL protein was monitored by immunodetection with mouse anti-His₆-peroxidase (Roche), using the manufacturer's protocol; the membrane probed with anti-His₆-peroxidase (1:10000 dilution) in TBS blocking buffer containing 1% BSA at room temperature for 1.5hrs. Following three times washing with TBST (TBS with 0.1% Tween 20), for five minutes; to visualize the r-PAL protein band, the membrane was incubated with the precipitating substrate solution (BM Blue POD substrate, precipitating) (Roche) for five mins.

Results

The *pal* gene of *L. pneumophila* was amplified using specific primers and showed a band about 0.55 kb on agarose gel (Fig. 1). In order to periplasmic expression of the r-PAL protein, the full protein-coding region of the *pal* gene was inserted between the *Nco*I and *Hind*III restriction sites of the pET26b+ plasmid. This resulted in a recombinant plasmid pET26b-pal that contained the desired *pal* gene sequence. Following the transformation and plating of

the bacterial cells on LB agar containing kanamycin (50mg/L), the transformed colonies and the extracted recombinant pET26b-pal plasmids were PCR positive against the *pal* gene specific primers. The presence of the *pal* gene in the pET26b-pal plasmid was confirmed by digestion with *Ksp*AI restriction enzyme. The pET26b-pal plasmid has two *Ksp*AI restriction sites (RS) in its sequences; the first in the pET26b+ plasmid and the second in the *pal* gene sequence. Therefore, digestion of the pET26b-pal plasmid using *Ksp*AI restriction enzyme produced two expected fragments, a 4188 bp and a 1616 bp fragment. Digestion results showed the *pal* gene had been inserted in the pET26b+ plasmid (Fig. 2). Sequencing analysis of the constructed plasmid also confirmed that there were no amplification errors in sequence of the cloned *pal* gene.

In order to examine the expression of the r-PAL protein, a single colony of *E. coli* BL21 (DE3) carrying the pET26b-pal plasmid was cultured and induced with IPTG for various post induction durations and its cytoplasmic as well as periplasmic proteins were subjected to protein analysis. SDS-PAGE analysis showed a protein band about 21 kDa; however no band detected in non-induced culture. This band observed in both cytoplasmic and periplasmic fraction of extracted total soluble protein (Fig. 3). Western blotting analysis was carried out for the cytoplasmic and periplasmic fractions and the presence of the r-PAL protein in *E. coli* BL21 (DE3) was detected (Fig. 4). The results obtained from optimization conditions showed the highest expression of the r-PAL protein was induced by the addition of 1mM IPTG induction for 15hrs.

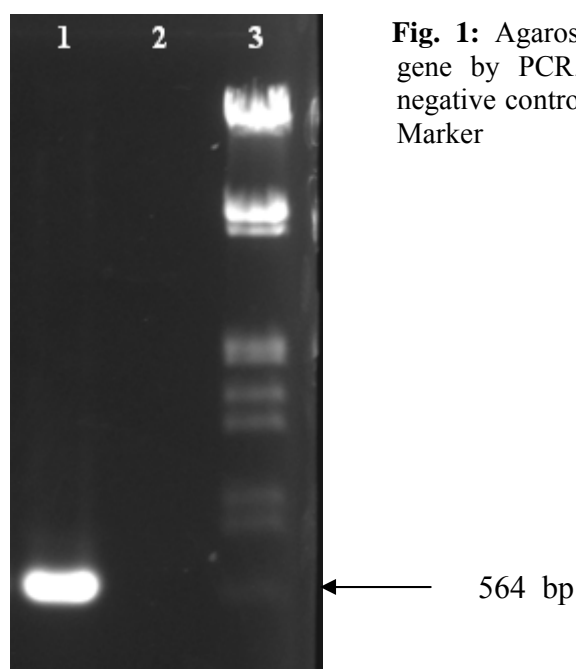


Fig. 1: Agarose gel electrophoresis of the amplified pal gene by PCR. Lane 1, 0.55kb PCR product; Lane 2, negative control and Lane 3, Lambda DNA/EcoRI+HindIII Marker

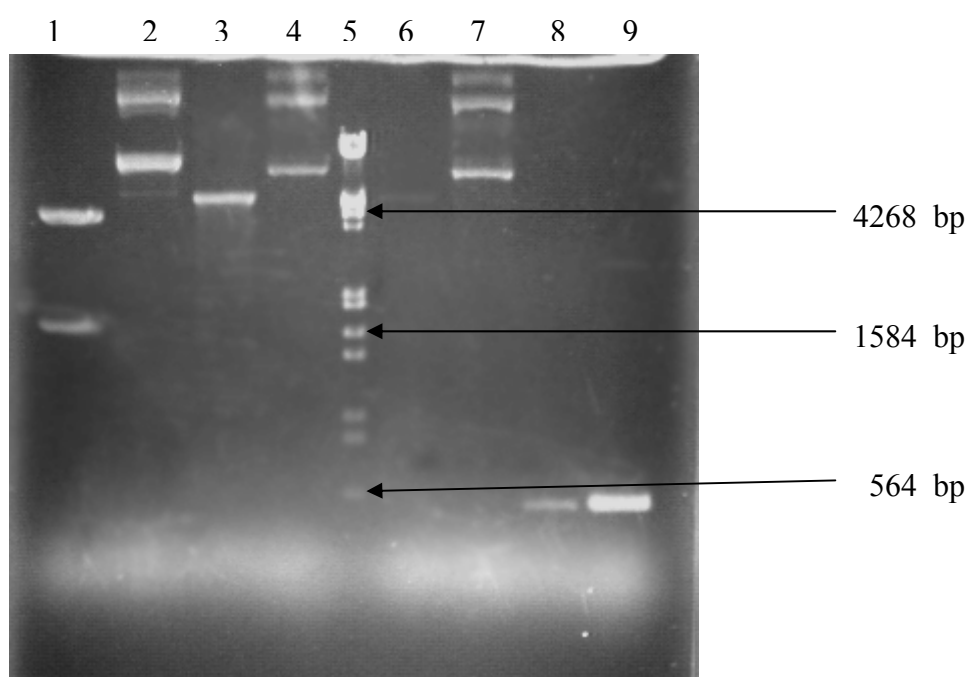


Fig. 2: Restriction enzyme analyses of pET26b-pal plasmid and pET26b+ plasmid without insert by *KspAI* restriction enzyme and comparison of digested and purified patterns of pET26b+ plasmid and the pal gene PCR product with undigested patterns. Lane 1, the pET26b-pal plasmid digested by *KspAI* (4188bp and 1616bp fragments); Lane 2, undigested pET26b-pal plasmid; Lane 3, the pET26b+ plasmid digested by *KspAI*; Lane 4, undigested pET26b+ plasmid; Lane 5 Lambda DNA/EcoRI+HindIII Marker; lane 6, digested and purified pET26b+ plasmid (by *NcoI* and *HindIII*); Lane 7, undigested pET26b+ plasmid; Lane 8, digested and purified PCR product (by *NcoI* and *HindIII*); Lane 9, 0.55kb PCR product

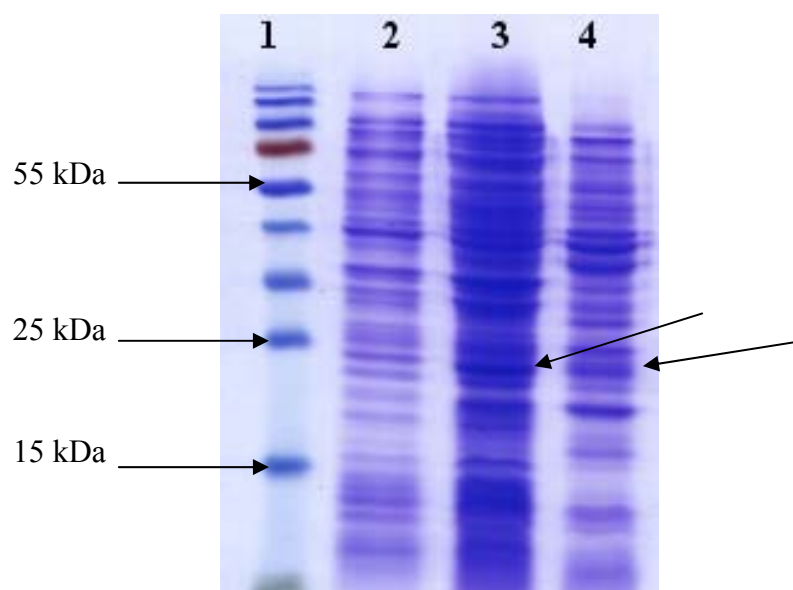


Fig. 3: The protein pattern of recombinant bacteria carrying the pET26b-pal plasmid demonstrated by SDS-PAGE. IPTG-induced r-PAL protein is marked with arrows. Lane 1, PageRuler™ Prestained Protein Ladder; Lane, 2 the cytoplasmic proteins pattern before induction; Lane 3, the cytoplasmic proteins pattern 15 hrs after induction and Lane 4, the periplasmic proteins pattern

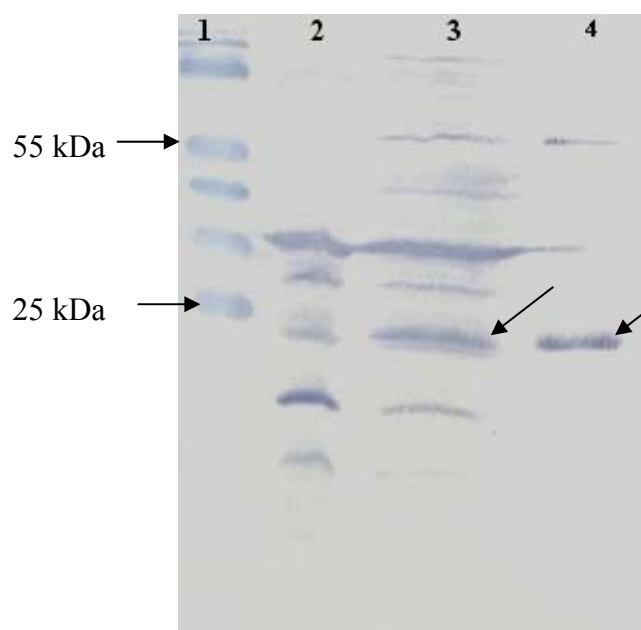


Fig. 4: The protein pattern of recombinant bacteria carrying the pET26b-pal plasmid demonstrated by western blotting analysis. Lane 1, PageRuler™ Prestained Protein Ladder; Lane 2, the cytoplasmic proteins pattern before induction; Lane 3, the cytoplasmic proteins pattern 15 hrs after induction and Lane 4, the periplasmic proteins pattern

Discussion

Legionnaires' disease is a common form of severe pneumonia, but these infections are infrequently diagnosed [1]. However, urine antigen testing has revolutionized the laboratory diagnosis of LD, making it the most common laboratory test ordered for diagnosis of this disease [6]. Several research groups have studied the characterization of different antigens of *L. pneumophila* in bacterial cells [18,22-25].

Here we reported the expression of pal gene that is the most prominent surface antigen of *L. pneumophila* [18], which can be used subsequently in diagnostic and vaccine studies. In this study, we used *E. coli* system for expression of the *L. pneumophila* r-PAL protein because of having many advantages like the short generation time, the ease of handling, the high capacity to accumulate foreign proteins, etc. Meanwhile, some disadvantages like the lack of posttranslational modification has limited using this expression system. To overcome this problem, we expressed the r-PAL protein of *L. pneumophila* in periplasmic space of *E. coli*.

The periplasmic expression causes some posttranslational modification events which can not be done in cytoplasmic expression [26,27]. In addition, secretion of recombinant proteins to the periplasm of *E. coli* has several advantages over intracellular production among which are separation from cytoplasmic proteins, enhanced biological activity, enhanced product solubility and the ease of protein purification [28,29]. There is one report on the cytoplasmic expression of the *L. pneumophila* pal gene [24]. However, there is no report on the periplasmic expression of this protein.

The pET26b+ plasmid was used in this study for overexpression of the r-PAL protein. The pET26b+ plasmid carried an N-terminal *pelB* signal sequence for

periplasmic expression and a C-terminal His-tag sequence for detection of r-PAL protein in binding assays. The His-tag generally has no significant effect on the structure of the native protein and facilitates selective binding of the expressed protein to a nickel affinity column [30]. The pET expression system is the most powerful system and because of this, it is chosen for the cloning and expression of recombinant proteins in *E. coli*. The target pal gene cloned and driven under the transcriptional/translational control of strong bacteriophage T7 signals; expression was induced by providing a source of T7 RNA polymerase in the *E. coli* BL21 host cell with an advantage that when fully induced, almost all of the cell's resources are converted to target gene expression and the desired product can comprise main part of the total cell protein within 15 hrs after induction with 1mM IPTG.

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