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Original Article

Cloning and Expression of *Mannheimia haemolytica* PlpE Gene in *Escherichia coli* and its Immunogenicity Assessment

Yektaseresht *, A., Sabet Sarvestani , F., Dordani , M., Hosseini, A.

Department of Pathobiology, School of Veterinary Medicine, Shiraz University, Shiraz, Iran

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Corresponding Author: azadeh_neisi@shirazu.ac.ir

ABSTRACT

Mannheimia haemolytica is responsible for considerable economic losses to cattle, sheep, and goat industries in many parts of the world. This bacterium is one of the causative agents of shipping fever in cattle. Current vaccines against *M. haemolytica* are moderately efficacious since they do not provide complete protection against the disease. Production of an economic vaccine for protecting farm animals against *M. haemolytica* has attracted the attention of many scientists. The outer membrane proteins (OMPs) play a major role in the pathogenesis and immunogenicity of *M. haemolytica.* Research on *M. haemolytica* OMPs has shown that antibodies to a particular OMP may be important in immune protection. In the current study, the gene for *M. haemolytica* OMP PlpE was cloned into the expression vector pET26-b, and then expressed in *Escherichia coli* BL21. The expression of the protein was carried out by the induction of cultured *Escherichia coli* Bl21 cells with 1mM isopropyl-β-D-thiogalactopyranoside. The recombinant PlpE was purified using Ni-NTA agarose resin, and then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. The identity of the expressed protein was analyzed by western blotting. It was revealed that rPlpE was expressed and produced properly. To assess the immunogenicity of the recombinant protein, the purified rPlpE was used as an antigen for antibody production in goats. The observations suggested that the produced recombinant protein can be used as a antigen for developing diagnostic tests and or as a vaccine candidate.

Keywords: *Mannheimia haemolytica,* PlpE, Cloning, Expression, Immunogenicity

Clonage et Expression du Gène PlpE de *Mannheimia haemolytica* dans *Escherichia coli* et Évaluation de son Immunogénicité

Résumé: *Mannheimia haemolytica* est responsable de pertes économiques considérables dans l'industrie agroalimentaire de nombreuses régions du monde. Cette bactérie est l'un des agents responsables de la fièvre des navires chez les bovins. Les vaccins actuels contre *M. haemolytica* sont modérément efficaces car ils ne fournissent pas une protection complète contre la maladie. La production d'un vaccin économique pour la protection des animaux d'élevage contre *M. haemolytica* a donc attiré l'attention de nombreux scientifiques. Les protéines de la membrane externe (OMP) jouent un rôle majeur dans la pathogenèse et l'immunogénicité de *M. haemolytica*. Des recherches sur les OMP de *M. haemolytica* ont montré que les anticorps dirigés contre les OMP, à savoir les PlpE, peuvent jouer un rôle important dans la protection immunitaire. Dans cette étude, le gène de *M. haemolytica*, OMP PlpE, a été cloné dans le vecteur d'expression pET26-b, puis exprimé dans *Escherichia coli* BL21. L'expression de la protéine a été menée par l'induction de cellules Bl21 d'*Escherichia coli* en culture avec 1 mM d'isopropyl-β-D-thiogalactopyranoside. La PlpE recombinante a été purifiée en

utilisant une résine d'agarose Ni-NTA, puis soumise à une électrophorèse sur gel de dodécylsulfate de sodium et de polyacrylamide. L'identité de la protéine exprimée a été analysée par Western blot montrant que la protéine rPlpE était exprimée et produite correctement. Afin d'évaluer l'immunogénicité de la protéine recombinante, la rPlpE purifiée a été utilisée comme antigène pour la production d'anticorps chez les chèvres. Les résultats obtenus suggèrent que la protéine recombinante peut être utilisée comme antigène candidat pour le développement de vaccins et/ou de tests de diagnostic.

Mots-clés: *Mannheimia haemolytica*, PlpE, Cloning, Expression, Immunogenicity

INTRODUCTION

Bovine respiratory disease might result from the interaction of some contributing factors, including physical stress associated with weaning, shipment, immoderate weather, and overcrowding, coupled with microbial infections (Rahn et al., 1989). In severe cases, the colonization of the lungs with pathogenic bacteria results in severe pneumonia (Purdy et al., 1997). *Mannheimia haemolytica* is the principal bacterium isolated from respiratory disease in feedlot cattle and a significant component of enzootic pneumonia in all neonatal calves (Zecchinon et al., 2005). *M. haemolytica* pneumonia is known as one of the main diseases in cattle industry (Rice et al., 2007). Antibiotics are employed in the cattle feedlot industry, both prophylactically and therapeutically. However, the efficacy of these antibiotics varies given the inconsistencies in diagnostic and therapeutic approaches and development of antibiotic resistance (Rice et al., 2007). The production of antibodies against surface antigens and secreted leukotoxin is essential for the establishment of protective immunity against *M. haemolytica* (Shewen and Wilkie, 1988). Numerous commercial vaccines for *M. haemolytica* serotype 1 (S1) are available and seem to be efficacious in roughly 50% of field studies (Perino et al., 1997). The specific surface antigens that are important in stimulating immunity have not been accurately determined yet. However, some researchers have presented data suggesting that the OMPs may be important in stimulating immunity against the surface antigens on *M. haemolytica* (Confer, 1993; Confer et al., 1995; Morton et al., 1995; Potter et al., 1999; Confer et al., 2003; Ayalew et al., 2004). Protease treatment and western blotting methods have resulted in the identification of 21 surface-exposed immunogenic OMPs in *M. haemolytica* S1 (Pandher et al., 1999). High antibody responses to the outer membranes and several specific OMPs, as measured by enzyme-linked immunosorbent assay (ELISA) and quantitative western blotting, constantly correlated with resistance to challenge with virulent *M. haemolytica* S1 PlpE. The *M. haemolytica* S1 PlpE, as a major ~ 45-kDa outer membrane lipoprotein, was one of OMPs in *M. haemolytica* to which high antibody responses correlated with resistance against experimental challenge (Mosier et al., 1989; Pandher et al., 1998). Gerlach et al. (1993) and Pandher et al. (1998) reported the cloning, sequencing, and characterization of the gene for PlpE, which is genetically alike to an immunogenic lipoprotein, namely OmlA, demonstrated in *Actinobacillus pleuropneumoniae* S1 and S5. With this background in mind, the current study was targeted toward the cloning and expression of PlpE gene fragment of *M. haemoltyica* in *E. coli* as the first step for the production of recombinant PlpE protein to be applied in diagnostic procedures and immunization trials.

MATERIAL AND METHODS

Ethical declaration. The investigation was conformed to the ethical guidelines established by the

Institutional Animal Care and Use Committee of Shiraz University, Shiraz, Iran.

Polymerase chain reaction and cloning. DNA of *M. haemolytica* isolated from the lung of a sheep around the Shiraz, Iran, was used as a template for the amplification of PlpE gene. The gene fragment was amplified using two primers, namely NPLEC26F (3' CTCTAATTA*GAATTC*CGGAGGAAGCGGTAGCG G) and NPLXH26R (3' GCCGGCC*CTCGAG*TTTTT TCTCGCTAACCATTAT), and cloned into the intermediate plasmid vector pTZ57R (Fermentas). Recombinant plasmid (pTZ57R/PlpE) was transformed into the XL1-blue *E. coli* competent cells using $CaCl_2$. The transformants were grown on a trypticase soy agar (TSA) plate with 100 µg/ml ampicillin. Some colonies were subjected to polymerase chain reaction (PCR) using NPLPE26 primers, and two gene fragment carrying colonies were chosen for plasmid isolation and sequencing. The purified intermediated recombinant plasmid pTZ57R/PlpE and also expression vector pET26-b were cut with restriction enzymes *EcoR*I and *Xho*I (Fermentas), and then electrophoresed on 1% agarose gel to confirm the correct size. The PlpE released fragment and cut pET26-b were extracted from the gel and ligated using T4 DNA ligase (Vivantis, Malaysia) at a vector:insert ratio of 1:3-5 at 16°C overnight. The ligation reaction was transformed into competent *E. coli* BL21 (DE3), and the transformed cells were selected on TSA plates containing 50 µg/ml kanamycin. The selected clones were further analyzed by PCR using specific and also universal T7 primers. Finally, the recombinant plasmid of two positive clones was sequenced using T7 promoter and terminator primers.

Expression and purification of PlpE. Five ml of TSA medium with 50 µg/ml kanamycin was inoculated with a fresh colony of recombinant clone and incubated at 37 °C with shaking. When the optical density of the culture at a wavelength of 600 nm reached 0.6, protein expression was induced with a final concentration of 1 mM isopropyl-*D*-1- thiogalactopyranoside (IPTG). Subsequently, the culture was incubated at 37 °C for an additional 16 h. The overnight culture was centrifuged at 5000 g for 10 min, and the supernatant was separated. To prepare cell lysate for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the pelleted cells of the culture (1 ml) were washed three times with cold phosphate-buffered saline (PBS), resuspended in 100 µl of cell sample buffer, and stored at -20 °C. Whole protein from pellet was extracted, and then his-tagged proteins were purified using the Qiagen Ni-NTA Spin Columns (Qiagen, Germany) as described by Eskandari et al. (2012). The expressed protein should consist of 377 amino acids, including 336 amino acids of PlpE. The profile of the purified protein was examined by SDS-PAGE using 4% stacking and 12% separating acrylamide gels in a vertical electrophoresis apparatus.

Fast Protein Liquid Chromatography. The fast protein liquid chromatography (FPLC) was performed using 20 mM sodium acetate buffer (pH=5.5). Two ml of bacterial suspension in this buffer was sonicated and after filtering (0.22 µm), loaded into the cation column. The column contained 5 ml sulfopropyl sepharose resin with a diameter of 34 µ (HiTrap™ SPHP, GE Health Care). Recombinant rPlpE as a negative molecule (pI=6.01, net charge at a pH of 7) should pass through the column without binding the resin. The passed solution related to the chromatogram peak was collected and concentrated using a dialyzing bag and polyethylene glycol 6000, and then subjected to SDS-PAGE and western blotting.

Western Blot Analysis. The purified rPlpE was subjected to SDS-PAGE that was performed using 12% acrylamide gel. The recombinant protein was also transferred electrophoretically onto nitrocellulose membranes using a semi-dry system with 20 V and 350 mA for 60 min. After blocking with 3% skimmed milk in tris-buffered saline (TBS) overnight and washing with TBS, the blot was incubated with 1/10000 goat horseradish peroxidase (HRP) anti-histidine Ab (Abcam, UK) for 60 min. The membrane was washed

three times with TBS and placed into substrate solution (0.5 mg/ml diaminobenzidine, 0.005% H_2O_2) to visualize the specific antigen bands.

Antigen preparation. *M. haemolytica* was used to prepare the antigen for immunological tests. Bacterial cells, grown in liquid culture, were centrifuged, and after being washing with PBS for three times, they were subjected to sonication. Cycles of 30-second sonication pulse with 20-second rest intervals were applied for cell lysis. The lysate was used in immunological tests.

Goat immunization. Two female goats were injected five times with two-week intervals. The purified rPlpE was used at a concentration of 1 mg per animal, thoroughly mixed with an equal volume of complete Freund's adjuvant (Betagen, Iran), and then injected subcutaneously (1 ml) in flanks. Two weeks later, the first booster dose was administered at the same way but with incomplete Freund's adjuvant. The blood samples were taken before each injection and also 5 days after the last injection.

Immunogenicity assessment

Enzyme-linked immunosorbent assay. A specific antibody response was determined by measuring the antibody titers via ELISA using the sera collected from the immunized goats. An indirect ELISA test was performed to detect the anti-PlpE antibodies in the sera of immunized goats. Polystyrene ELISA plates (Greiner bio-one, Austria) were coated with 15 µg/ml rPlpE (yielded from FPLC) in PBS at a pH of 7.2 overnight. After blocking with BSA, sera dilutions of 1/10 were added to the wells. The sera were examined in duplication. The HRP-conjugated anti-ruminant antibody (ID Screen® Q-fever indirect, multi-species kit, ID VET, France), and then TMB and H_2O_2 were used to detect the anti-PlpE antibodies and color forming. The plates were read at 450 nm to determine optical density on a microtiter plate reader.

Agglutination. Sixty µl of immunized goat blood serum (taken at the end of immunization period) was separately mixed with an equal volume of *M. haemolytica* Ag and also with PBS buffer and *E. coli* Ag as negative controls on a glass plate. The outcome was observed under a light for 2 min.

RESULTS

Gene fragment sequencing in intermediate and expression vectors revealed 100% identity with the sequence of PlpE of *M. haemolytica*. The sequence was submitted to the gene bank in National Center for Biotechnology Information with the accession number of KY795962.

Expression and purification of recombinant PlpE. The SDS-PAGE showed that rPlpE (with a calculated molecular weight of 41.32 KD) was expressed properly in *E. coli* after induction. Purification of protein using nickel resin yielded an about 41 KD protein; however, some unwanted protein bands presented in the final eluted solution (Figure 1).

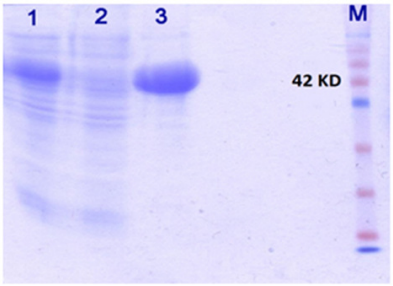

Figure 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified rPlpE using nickel resin, 1) cell lysate of isopropyl-D-1- thiogalactopyranoside-induced bacteria, 2) liquid passed through the nickel resin column, 3) eluted solution containing rPlpE and some unwanted proteins, M) Chromatein prestained protein ladder (Vivantis, PR0602).

The FPLC using 20 mM sodium acetate buffer (pH=5.5) resulted in the separation of a fraction in the non-salt phase. After the collection of liquid (associated with the chromatogram peak), it was concentrated, and then subjected to electrophoresis, and the results showed a nearly pure protein band with a size of nearly 41 KD (figures 2a, 2b). In western blotting, anti-histidine antibody detected the 41-KD protein band and also the control histidine-possessed recombinant protein (Figure 3).

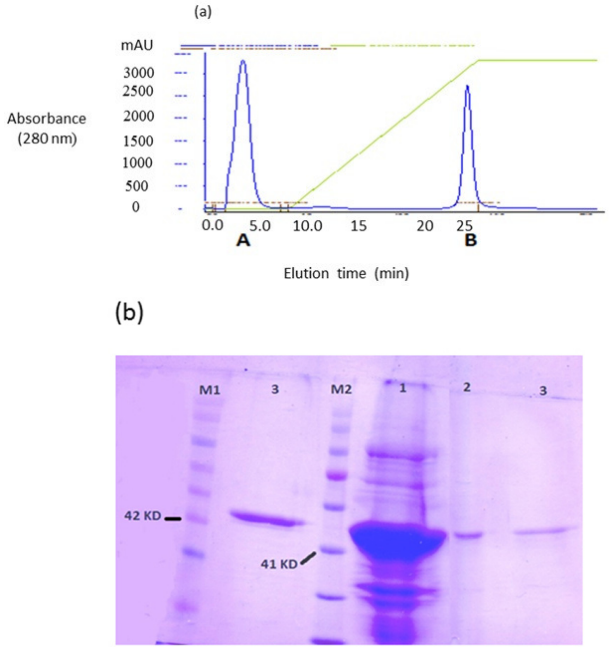

Figure 2. Purification of the rPlpE protein, (a) graph of fast protein liquid chromatography (FPLC) with sulfopropyl sepharose resin; A) molecules with negative charge passed through the column without binding to resin, B) molecules with positive charge eluted by continuous increasing the salt concentration, b) sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified rPlpE yielded from FPLC, 1) cell lysate of isopropyl-D-1- thiogalactopyranoside-induced bacteria, 2) nickel resin eluted solution containing rPlpE, 3) purified rPlpE yielded from FPLC, M1) chromatin prestained protein ladder (Vivantis, PR0602), and M2) prestained protein ladder (Cinnagen PR911654 [SL7012]).

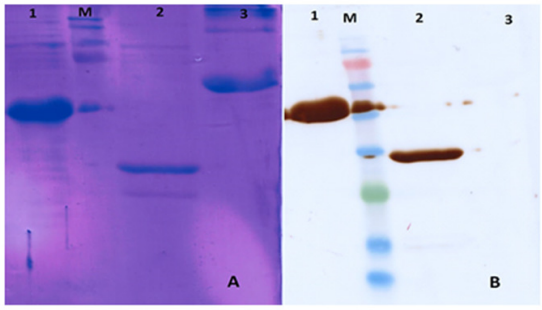

Figure 3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of rPlpE (A) and western blotting with anti-histidine antibody (B); 1) rPlpE. 2: control histidine-possessed recombinant protein, 3) Bovine serum albumin, M) prestained protein ladder (Cinnagen PR911654 [SL7012]).

Immunogenicity assessment. As shown in Figure 4, serum antibody levels in goats immunized with rPlpE increased after five injections. Indirect ELISA test showed the increasing development of anti-rPlpE antibody during the immunization period in goats. The elevation of antibody in goat 2 was irregular may be due to pregnancy (Figure 4). Precipitated particles were formed after mixing immunized goat blood serum with *M. haemolytica* antigen; however, no agglutination occurred during the reaction of serum with *E. coli* antigen or PBS buffer (Figure 5).

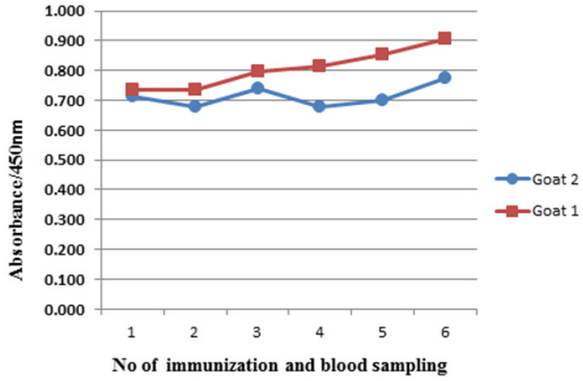

Figure 4. Serum antibody titers in the goat immunized with rPlpE, measured by enzyme-linked immunosorbent assay coating the plates with rPlpE in sera dilutions of 1/10 after five injections (1 mg/animal) with two-week intervals (Blood samples were taken before each injection and also 5 days after the last injection.).

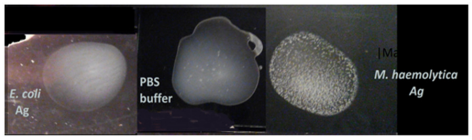

Figure 5. Slide agglutination test (Well-formed agglutination was seen in the combination of serum and Mannheimia haemolytica antigen.).

DISCUSSION

In recent years, tremendous changes have occurred in the genetic manipulation and production of recombinant proteins to build a new generation of vaccines and diagnostic techniques. Genetic recombination technology has been developed rapidly in the world. Despite the limitations of bacterial expression systems, including the inability to perform post-translational modifications, these systems are used frequently due to their rapid growth rate, capacity for

continuous fermentation, and relatively low cost (Belshe et al., 1988; Haag and Ostermeier, 2009). Since 1986, pET expression vectors, as a powerful and highly selective system, have been widely used for the quick production of a large number of proteins (Studier and Moffatt, 1986). This system is under the control of strong bacteriophage T7 transcription. In the present study, the target sequence was cloned into the pET26-b vector. The pET-26b carries an N-terminal *pelB* signal sequence for potential periplasmic localization, plus optional C-terminal histidine-Tag sequence. Furthermore, *E. coli* BL21 (DE3) strain, issued for the transformation step of the recombinant vector in this study, contains T7 polymerase upon IPTG induction (Studier, 2005; Haag and Ostermeier, 2009). In this study, the PlpE gene fragment of *M. haemolytica* was cloned into *E. coli*, and rPlpE was produced properly. In our previous experiences with pET-26b and also in the preliminary trials of this project, we could not achieve an expressed protein as an exported molecule in media. The recombinant protein was achieved from the bacterial cell pellet. Numerous attempts have been made previously to develop efficacious vaccines against *M. haemolytica,* including live *M. haemolytica*, killed *M. haemolytica* cells, components or fractions of *M. haemolytica* cells, and commercial vaccines (Confer et al., 1985; Purdy et al., 1986; Confer et al., 1987; Shewen and Wilkie, 1988; Conlon et al., 1991; Confer et al., 1995; Srinand et al., 1996a; Srinand et al., 1996b; Aubry et al., 2001; Rajeev et al., 2001). Vaccination with recombinant chimeric proteins made of the specific immunogenic regions of the leukotoxin and PlpE proteins can induce a functional immune response against *M. haemolytica* (Ayalew et al., 2008). Hodgson et al. indicated a relationship between the incidence and severity of bovine pneumonic mannheimiosis and the lipopolysaccharide (LPS) chemotype, suggesting a principal role for the LPS chemotype in determining the host species susceptibility to pulmonary infection (Hodgson et al., 2003). Significant resistant intensifications were observed in the cattle vaccinated with OMP-enriched cellular fraction from *M. haemolytica* S1 in the absence of antibodies to leukotoxin (Morton et al., 1995). In addition, Confer et al. demonstrated that the cattle vaccinated with a commercial *M. haemolytica* vaccine containing recombinant *M. haemolytica* S1 PlpE had significantly greater resistance against experimental challenges with the homologous serotype than those vaccinated with the commercial vaccine alone (Confer et al., 2003). It can be concluded that the recombinant protein rPlpE was able to stimulate the immune system of the goat to produce antibody; nonetheless, the titer of antibodies in ELISA was lower than expected. According to our previous experiences, the injection of antigen with 7 to 10-day intervals resulted in appropriate antibody formation. In this experience, 14-day intervals were applied between the injections, and this may be the reason why the results were lower than our expectation. The number of antibodies was enough to agglutinate bacterial particles. Therefore, it could be concluded that the produced anti-rPlpE was a suitable tool for the diagnosis of *M. haemolytica.* However, more field experiments are required to understand the protective efficacy of produced rPlpE.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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