

Characterization of recombinant protein of *Pasteurella multocida* serotype BJamal Hussaini^{1,2}, Noor Masyitah Jumahat¹¹ Faculty of Medicine, Universiti Teknologi MARA, Sungai Buloh Campus, Jalan Hospital, 47000 Sungai Buloh Selangor, Malaysia.² DDH CoRe, Universiti Teknologi MARA (UiTM), 40450 Shah Alam, Selangor Darul Ehsan, Malaysia.
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Abstract: Haemorrhagic septicaemia (HS) is an acute, fatal septicaemic bacterial disease, mainly in South and Southeast Asia, Africa and India which caused by *Pasteurella multocida*, a gram negative coccobacilli bacterium. The effectiveness and safety of available treatment and vaccines are limited due to antimicrobial resistance. The objective of this study was to characterize recombinant protein of *Pasteurella multocida* in developing vaccine against HS. **Results:** Bacterial culture strain was cultured in brain heart infusion (BHI) medium. Soluble proteins were extracted and separated electrophoretically using 12% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Immunogenic soluble proteins were detected by western blotting using anti-*Pasteurella* serum raised in rabbit against whole cell antigens of *Pasteurella multocida* and anti-rabbit antibody and identified. Recombinant protein was then expressed and transformed in *E.coli*. Expression and purification of recombinant protein was analyzed using SDS-Page. The separation of soluble proteins showed various molecular weights on the gel, ranging from 10kDa to 170kDa. According to the western blot analysis, the most intense band detected was of approximately 28kDa and identified as lipoprotein B. Immunogenicity study of soluble protein will be carried out in response to immunogenic roles against HS and potential role in vaccine development. Purified recombinant protein size was obtained approximately 39kDa after electrophoretically separated using 12% SDS-Page. **Conclusion:** According to the immunoblotting analysis, intensity band of purified recombinant protein treated with antisera from immunized rabbit detected on film was 5398.78 with p-value equal to 0.0006 (p-value < 0.05). Expression and purification of recombinant protein of *P. multocida* was characterized and indicated. Further study on the immunogenicity study of recombinant protein will be carried out in response to immunogenic roles against HS and potential role in vaccine development.

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1. Introduction

Pasteurella multocida (*P. multocida*) is a gram negative coccobacilli bacterium which is conscientious for diseases in livestock, domestic animals and poultry such as Haemorrhagic septicaemia (HS) in cattle and buffalo and fowl cholera in chicken and birds [1,2] The range of animals infected by *P. multocida* is very wide with different manifestations. The bacteria have a broad range of host but the uncharacteristic property is still not understood. *P. multocida* has an almost unlimited host range in mammals and birds [3].

P. multocida have five capsular type groups which range from type A, B, D, E and F and each types of anatomical structure type give rise different kind of illness among animals. *P. multocida* type A is known for chloral in gallinacean and pneumonia in oxen, sheep and vulgarian meanwhile structure type D produces atrophic rhinitis in pig and snuffles in leporid [4]. Capsular type F is predominantly associated with poultry disease, particularly turkeys, although it has occasionally been reported in ruminants. In cattle, capsular serotypes B and E are

allied with hemorrhagic septicaemia in Asian (serotype B) and African (serotype B) countries [5-7].

P. multocida may reside as normal flora in the upper respiratory tract, or can act as a primary or secondary pathogen depending on the taxonomic group of animal. In kine; *P. multocida* is normally set-apart from the lungs of separate affected by enzootic respiratory disorder, although isolation is generally considered to be secondary to respiratory viral linguistic process and situation agent. It was first explained by Revole'e in 1877 and further describes by Pasteur in 1880 [8].

Furthermore, treatments of infected animals with *P. multocida* are complex and unsuccessful due to increasing antibiotic resistance strains. Moreover, the efficacy and safety of available vaccines are limited [9].

In this present study, characterization of recombinant protein of *P. multocida* was carried out to indicate the potential possibilities to use recombinant protein in developing vaccine against HS.

2. Material and Methods

Bacterial culture condition

P. multocida was purchased from Institute for Medical Research (IMR, Kuala Lumpur, Malaysia). The bacterium was obtained in lyophilized form. This bacterium was identified and confirmed as *P. multocida* serotype B. Lyophilized bacteria was reconstituted with 1 to 2ml of sterile brain heart infusion broth (*Laboratories Conda S.A*) and streaked onto blood agar. The bacteria were grown at 37°C overnight. After incubation, a loopful of bacterial colonies was inoculated into brain heart infusion broth. The culture was incubated at 37°C with shaker incubator (*Vision Scientific Co Ltd., Korea*) overnight. The bacteria were harvested by centrifugation. The pellet was store at -20°C or used directly for soluble protein extraction.

Extraction of soluble protein

Soluble proteins of *P. multocida* were extracted using B-Per Bacterial Protein Extraction Reagent with Enzymes (Thermo Fisher Scientific Inc, USA). Bacteria pellet was resuspended with B-Per reagent. Lysozyme and DNase I were then added into it. The suspension (lysate) was incubated for 15 minutes at room temperature and centrifuged for 5 minutes to obtain soluble protein. Soluble protein concentration was determined using Bradford assay.

Antisera from immunized rabbit

Production of polyclonal antibody in rabbit was done using standard protocol (Florida State University Polyclonal Antibody Production Protocol - Rabbits, 2007). Soluble protein of *P. multocida* (antigen) was prepared and sterilized using 0.22 micron filter. Three months old female New Zealand white rabbit (2.5kg) was used in this study. 1ml blood was collected from the rabbit before the injection as control and then the rabbit was immunized subcutaneously with 2mL of antigen. Booster immunizations of the same dosage and route were administered every 2 weeks for 1 month. After the last immunization, blood was collected from the rabbit. Antisera were collected by centrifugation and stored at -80°C until used (Eppendorf Centrifuge, Germany).

SDS-PAGE and immunoblotting

Soluble proteins of *P. multocida* were separated electrophoretically using 12% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This method was adopted from (Lameli, 1970). The apparent molecular weight of the soluble proteins was determined using appropriate molecular weight markers (Thermo Fisher Scientific Inc, USA). After the electrophoresis, separated proteins were either stained with 0.25% Coomassie blue R-250 (Bio-Rad), 25% methanol and 10% (v/v)

acetic acid or transferred to reinforced nitrocellulose membranes (0.45 µm pore size, Whatman, Germany) in a wet transfer (Bio-Rad, USA) as described by the manufacturer. The transferred membranes were blocked with 5% skim milk (Bio-Rad) in phosphate buffered saline containing 0.05% Tween-20 (PBST). After 3 washers with PBST, the membranes were then incubated overnight at 4°C with pre-immune sera and immune sera as primary antibodies (1:500 dilutions) in PBST, respectively. After three washes, the membranes were incubated with horseradish peroxidase conjugated goat anti-rabbit IgG (1:5,000; Thermo Fisher Scientific Inc, USA) at 4°C for 1–2 h. Finally, the membranes were washed three times with PBST and detection were done using Supersignal west pico chemiluminescent substrate (Thermo Fisher Scientific Inc, USA) on CL-exposure film (KODAK, USA). The films were scanned with GS-800 calibrated densitometer (Bio-Rad, USA) and analyzed using myImage analysis (Pierce biotechnology Inc, USA).

Protein identification

Most intense immunogenic soluble protein of *P. multocida* from immunoblotting analysis were identified using electrospray mass spectrometry (LC/MS/MS)

Expression of recombinant protein

Identified immunogenic soluble protein was expressed using a LICator Ligation Scientific Cloning and Expression System (*Thermo Fisher Scientific Inc, USA*) according to the manufacturer's instructions. Primers were designed based on its LIC vector (pLATE52). The primers for gene amplification were synthesized (*Sigma Aldrich Pte Ltd, Singapore*), which has added pLATE52 vector specific sequences (*italic and underlined*) at 5' end of forward and reverse primer, respectively as follows:

Forward:

5' GGTTGGGAATTGCAACCGATCGTGGGCAATACCTTT-3';

Reverse:

5' GGAGATGGGAAGTCATTAACCGCAACAGTTGTCCCAG-3'

The PCR mixture consisting of 100ng of DNA template, 10µM of each of the forward and reverse primers, 10µl 2X Phusion Flash PCR Master Mix (*Thermo Fisher Scientific Inc, USA*) was made into 20µl with nuclease free water. PCR program was performed with the initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 45 sec, extension at 72°C for 2 min and a final extension at 72°C for 10 min. The amplified PCR product was purified and ligated to pLATE52-LIC ready vector. The

recombinant plasmid was initially transformed into *Escherichia coli* (*E. coli*) BL21 (DE3) cells [20,1].

Analysis of recombinant protein

Recombinant protein was analyzed by colony PCR screening. LIC forward and reverse sequencing primers were used which provided by the manufacturer in aLICator Ligation Scientific Cloning and Expression System (*Thermo Fisher Scientific Inc, USA*). Colony PCR screening was done according to the manufacturer. The PCR product was then run on an agarose gel for the and size of the PCR product.

Recombinant protein purification and expression analysis

Recombinant protein was purified using HisPur™ Ni-NTA Spin Columns (*Thermo Fisher Scientific Inc, USA*). Purified recombinant protein was analysed by SDS-Page which was separated electrophoretically using 12% gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The purified recombinant protein was determined using appropriate molecular weight markers (*Thermo Fisher Scientific Inc, USA*).

Statistical Analysis

SPSS version-20 and Microsoft Excel were used as statistical tool in processing and analyzing all data in this study.

3. Results

Extraction of soluble protein:

Soluble protein of *P. multocida* was successfully extracted using B-Per Bacterial Protein Extraction Reagent with Enzymes (*Thermo Fisher Scientific Inc, USA*). The concentration of soluble protein was 5.734 mg/ml.

SDS-Page and immunoblotting analysis:

Soluble proteins of *P. multocida* showed various molecular weights ranging from 10 kDa to 170 kDa which was compared with protein ladder from *Thermo Fisher Scientific Inc, USA* as a marker on SDS-Page (**Fig. 1**). After electrophoresis, the gel with separated soluble proteins was effectively transferred onto nitrocellulose membrane using wet transfer (**Fig. 2**). The immunoblot treated with antisera from immunized rabbit showed cross reactivity whereby there were bands appeared on the film after the detection (**Fig. 3**). According to the immunoblotting analysis, the most intense band of approximately 28 kDa molecular weight sizes was determined with p-value equal to 0.0017 (p-value < 0.05) (**Fig. 4 and fig. 5**).

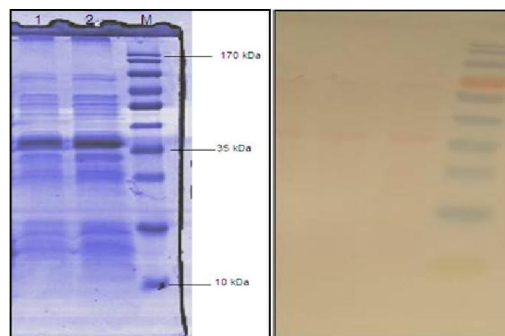


Figure 1. SDS-PAGE gel of soluble proteins of *Pasteurella multocida*; Lane 1 and Lane 2: Soluble proteins of *Pasteurella multocida*; Land 3 (M): Protein ladder marker.

Figure 2. Transferred membrane of soluble proteins of *Pasteurella multocida*.

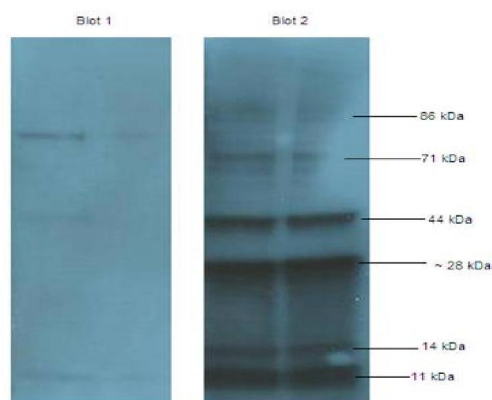


Figure 3. Immunoblot of soluble proteins of *Pasteurella multocida* treated with Pre-immune sera (Blot 1) and immune sera (Blot 2).

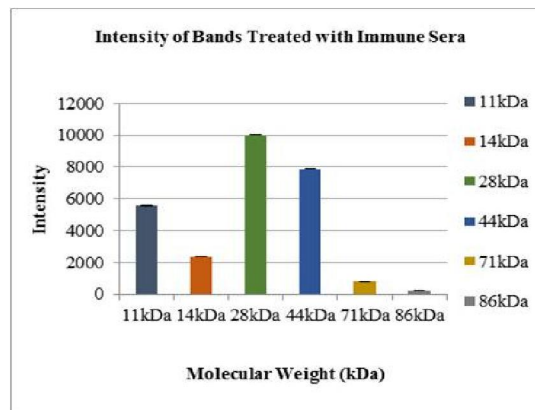


Figure 4. Graph Intensity of Bands Treated with Immune Sera.

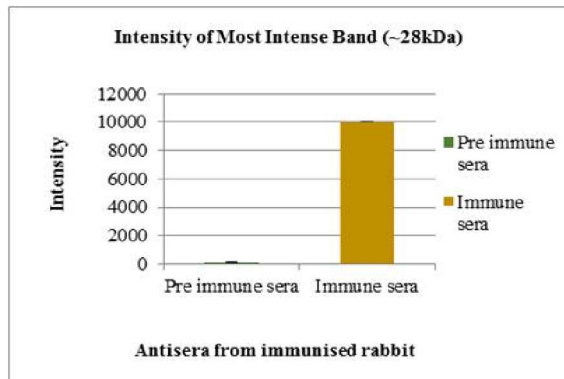


Figure 5. Graph Intensity of Most Intense Bands (~28kDa); p-value = 0.0017 (p-value < 0.05).

Protein identification and recombinant protein analysis

Immunogenic soluble protein was identified as lipoprotein B (plpB) and successfully expressed and transformed in *E.coli*. The PCR product of recombinant protein was run and analyzed on an agarose gel for the presence and size of the PCR product. PCR sequencing primers was 624bp (including insert size for Plate52 vector. Insert size and vector size were 350bp and 264 respectively (Fig. 6).

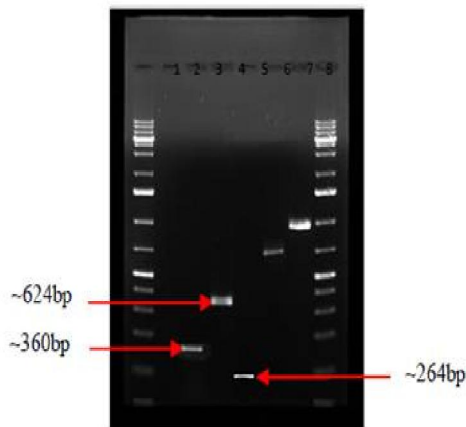


Figure 6. Gel electrophoresis of analysis recombinant protein; Lane 1 and 8: 1kb DNA Ladder (Thermo Scientific); Lane 2: PCR product negative control (Free Nucleus Water, ddH₂O); Lane 3: PCR product of *Pasteurella multocida* strain P52 serotype B: 2 lipoprotein B (plpB); Lane 4: PCR product of recombinant protein of *Pasteurella multocida* Lane 5: PCR product of vector recombinant protein (pLATE52); Lane 6: PCR product of plasmid control; Lane 7: PCR product of vector control.

Expression and purification of recombinant protein

The concentration of recombinant protein was 0.50 mg/ml. Purified recombinant protein size was approximately 39kDa on SDS-Page (Fig. 7). The control Plate21-Cm plasmid expression protein control size was run on SDS-Page and separated electropherically with approximately 30kDa size (Fig. 8).

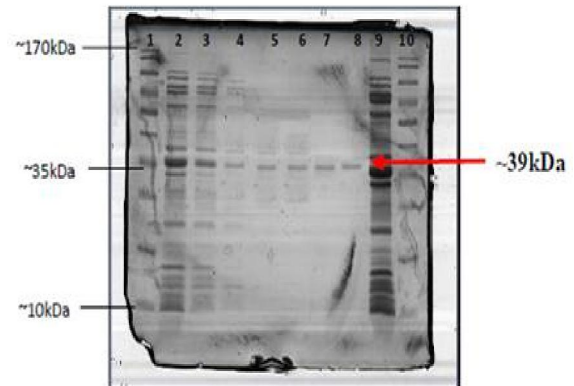


Figure 7. SDS-PAGE gel of recombinant protein of *P. multocida*; Lane 1 and 10: Protein Ladder (Thermo Scientific); Lane 2: Flow-through fraction; Lane 3: Wash fraction 1; Lane 4: Wash fraction 2; Lane 5: Wash fraction 3; Lane 6: Elution fraction 1; Lane 7: Elution fraction 2; Lane 8: Elution fraction 3; Lane 9: Cell lysate.

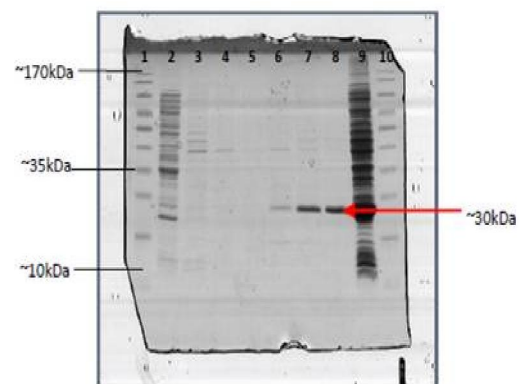


Figure 8. SDS-PAGE gel of plasmid expression protein control; Lane 1 and 10: Protein Ladder (Thermo Scientific); Lane 2: Flow-through fraction; Lane 3: Wash fraction 1; Lane 4: Wash fraction 2; Lane 5: Wash fraction 3; Lane 6: Elution fraction 1; Lane 7: Elution fraction 2; Lane 8: Elution fraction 3; Lane 9: Cell lysate.

4. Discussions

Haemorrhagic septicaemia (HS) is an acute, fatal septicaemic bacterial disease, mainly in South and Southeast Asia, Africa and India. Besides that, the route of entry is not very well defined, causing the sudden death of the animal within 24 hours and has a very high mortality rate and is considered to be one of the most economically important diseases of livestock in South-East Asia. Cattle and buffalo are the typical host for HS, although pigs, sheep and goats are also prone to it [10-13].

In addition, the first report of HS in Malaysia was recorded in the year 1900 [14] which showed that outbreak of HS disease has been occurred since long time ago. Furthermore, Malaysia has a cattle and buffalo population of about 735000 and 186000 respectively which involved in economically contribution to the country. Thus, outbreaks have had very serious economic effects with an estimated RM2.4 million loss annually [15]. A total of 48 outbreaks have been reported between the years 1994 and 2005. The outbreaks were reported almost every year excluding the year 2004. The highest numbers of outbreaks were seen between 1995 and 2000. During Nipah virus outbreak in the year 2000, the HS outbreaks were at its highest [16]. All isolates from Malaysian outbreaks were found to be *Pasteurella multocida* serotype B: 2, whilst in the last 12 years states of Perlis, Selangor and Johor were free of any HS outbreaks; about 8 to 11 outbreaks were reported in the states of Pahang, Terengganu, Kelantan and Perak. In February 2006 in Pasir Mas, Kelantan HS disease was reported where 77 buffaloes were found dead. Moreover, throughout the past four decades, HS is documented to be responsible for 45-55% of all bovine deaths in India. It is also reported about five million animals die annually in India from HS disease because the current vaccines have limitations in offering long term protection. While in Pakistan, likewise, 34.4% of all deaths in prone stock and 31.48% mortality have been reported in buffalo calves. Moreover, study done by Farooq et al [17] reported that the overall morbidity, mortality and case fatality rates were 17.39, 14.66 and 84.30%, respectively from 10 infected/outbreak villages in Pakistan with the total population of 4248 animals. It was obviously pointed out that HS is a vital hurdle in the economic uplift of the livestock sector with high incidence rates and alarming morbidity, mortality and case fatality rates [18]. HS was merely treated with wide range of antibiotics. However, prolonged usage of antibiotics has caused emergence of multi-drug resistance Strains. Therefore, attempts in vaccine development for HS disease are carried out.

Hence, study on identification of immunogens in *P. multocida* has been carried widely since it was

required for more effective vaccines to control diseases caused by *P. multocida*. As a pace towards developing vaccines, a genomics based approach was applied for the identification of novel immunogens [19].

Besides that, expression and purification of recombinant plasmid or clone of *P. multocida* has been carried widely since it was required for more effective vaccines to control diseases caused by *P. multocida* [13, 19]. Sing et al carried out study of gene expression from *P. multocida* and revealed that DNA vaccine presents a promising approach for the prevention of HS in year 2011. Moreover, Hussaini et al., 2011 found out that recombinant clone of ABA392 was immunogenic and could be used as vaccine against HS.

Therefore, based on the immunoblotting analysis, it was found and identified that there were positive cross reactivity between soluble proteins of *P. multocida* with antisera from immunised rabbits. There were few bands ranged from 10 to 170 kDa appeared on the film after detection. It was then further analysed using myImage analysis (Pierce biotechnology Inc, USA) and found that the most intense band molecular weight size of approximately 28kDa among all positive intense bands. It was statistically significant with p-value < 0.05 (p-value = 0.0017).

The immunogenic soluble protein was identified as lipoprotein B (plpB) and was expressed using aLICator Ligation Scientific Cloning and Expression System in *E.coli* as host cell. The PCR product of recombinant plpB protein was then run and analyzed on an agarose gel for the presence and size of the PCR product. It was obtained with LIC forward and reverse sequencing primers where its size was 624bp (including insert size for Plate52 vector according to the recombinant cells analysis. While control cloning reaction was tested using the appropriate known control PCR fragment size (720bp) ligated to pLATE52-LIC ready vector as a vector control, where its size was 984bp (including known PCR fragment insert size). Both plasmid and control cloning reaction (vector control) were provided by the manufacturer in the aLICator Ligation Scientific Cloning and Expression System (*Thermo Fisher Scientific Inc, USA*). Besides that similar study on expression of recombinant 39kDa plpB protein was done by Chomnawang et al, 2009 [20] whereas in this present study 28kDa plpB protein was expressed and purified.

Furthermore, expressed purified recombinant protein size was obtained approximately 39kDa whilst plasmid control size was approximately 30kDa after electrophoretically separated using 12% SDS-Page. Thus, it was found that recombinant protein

was expressed with the presence of band on the SDS-Page.

This study was focused on identification of immunogenic soluble protein and characterization of recombinant protein of *P. multocida* to the potential possibilities to use the recombinant protein in developing vaccine against HS.

In this study cross reactivity between the soluble proteins of *P. multocida* serotype B with antisera from immunised rabbit was detected. It was found that the most intense band to be of approximately 28kDa among all positive intense bands molecular size ranged from 10 to 170 kDa. It was statistically significant with p-value < 0.05 (p-value = 0.0017). Immunogenic soluble protein later was identified as lipoprotein B (plpB) and successfully expressed in *E.coli*.

In conclusion, expression and purification of recombinant protein was characterized and indicated. Further study on the immunogenicity study of recombinant protein will be carried out in response to immunogenic roles against HS and potential role in vaccine development.

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The procedures involving animals and their care conformed to the international guideline, Principles of Laboratory Animals Care. I declared that the ethic for laboratory animals was proven by the ethical committee members of Faculty of Medicine reference number ACUC-12/11.

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