Identification of immunogenic soluble protein of Pasteurella multocida

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ABSTRACT
Background and objective: Pasteurella multocida is a gram negative bacterium, causative agent of economically significant diseases in domestic animals. Treatments of infected animals are complex and ineffectual due to increasing antibiotic resistance strains. Moreover, the efficacy and safety of available vaccines are limited. The objective of this study is to determine immunogenic soluble protein of Pasteurella multocida capable of protecting animals from infection with acute pasteurellosis.

Methods: 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.

Result: 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.

Conclusion: The detected protein band will be identified and immunogenicity study of soluble protein will be carried out in response to immunogenic roles against pasteurellosis and potential role in vaccine development.

Keywords: Pasteurella multocida; Pasteurollosis; Protein; Immunogenic protein.

1. Introduction

Pasteurella multocida (P. multocida) is a gram negative bacterium which is causative agent of Haemorrhagic septicaemia and pathogenic in domestic animals. The infection is well known over a century and termed as pasteurellosis. 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 (Dziva et al, 2008). P. multocida have five types of capsular serotypes which range from type A, B, D, E and F and each types of capsular type produce different kind of disease among animals. P. multocida type A is known for chloral in fowl and pneumonia in cattle, sheep and pig meanwhile capsular type D produces atrophic rhinitis in pig and snuffles in rabbits (Ranjan et al, 2011). 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 associated with hemorrhagic septicaemia in Asian (serotype B) and African (serotype B) countries. Under the Carter- Heddleston
naming system B:2 and E:2 are the B and E serotypes, respectively, considered to cause typical haemorrhagic septicaemia. *P. multocida* strains are generally classified using a combination of capsular and somatic typing. Serotypes are designated by the capsular type followed by the somatic type under the Carter-Heddleston naming system (McFadden et al, 2011, Hussaini et al, 2011). *P. multocida* may reside as normal flora in the upper respiratory tract, or can act as a primary or secondary pathogen depending on the species of animal. In cattle; *P. multocida* is commonly isolated from the lungs of calves affected by enzootic pneumonia, although isolation is generally considered to be secondary to respiratory viral infection and environmental stressors. It was first explained by Revoèle in 1877 and further describes by Pasteur in 1880 (Baillot et al, 2011). 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 (Hussaini et al, 2012). The aim of this study is to determine immunogenic soluble protein of *P. multocida* capable of protecting animals from infection with acute pasteurellosis.

2. Materials and method

2.1 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 Pasteurella 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.

2.2 Extraction of soluble proteins: 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.

2.3 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).
2.4 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 peroxidise 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).

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

3. Results

3.1 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.

3.2 SDS-PAGE: Soluble proteins of *P. multocida* was separated electropherically using 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) 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 (Figure 1).

Figure 1: SDS-PAGE gel of soluble proteins of *P. multocida*; Lane 1 and Lane 2: Soluble proteins of *P. multocida*; Land 3 (M): Protein ladder marker; It showed various molecular weights ranging from 10 to 170kDa
3.3 Immunoblotting: After electrophoresis, the gel with separated soluble proteins was effectively transferred onto nitrocellulose membrane using wet transfer (Figure 2). The immunoblot treated with antisera from immunized rabbit showed cross reactivity whereby there were bands appeared on the film after the detection (Figure 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) (Figure 4 and figure 5).

![Figure 2: Transferred membrane of soluble proteins of *P. multocida*](image1)

Figure 3: Immunoblot of soluble proteins of Pasteurella multocida treated with Pre-immune sera (Blot 1) and immune sera (Blot 2); most intense band of approximately 28 kDA molecular weight size was detected in Blot 2 as compared to Blot 1

![Figure 3](image2)
Figure 4: Graph Intensity of soluble proteins of *P. multocida*. Immunoblot treated with immune Sera showed significantly detected (p-value < 0.05); soluble protein size of 28 kDa was determined as most intense band.

![Graph Intensity of soluble proteins of P. multocida](image)

Figure 5: Graph Intensity of immunoblotting treated with pre-immune sera and immune-sera against 28 kDa molecular weight size soluble proteins of *P. multocida* showed significantly immunogenic (p-value < 0.05).

![Graph Intensity of immunoblotting treated with pre-immune sera and immune-sera](image)
4. Discussion

*P. multocida* serotype B was commonly associated with hemorrhagic septicaemia in Asian countries (McFadden et al., 2011). 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 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 usual host for HS, although pigs, sheep and goats are also susceptible (Ramdani et al, 1990; Hotchkiss et al, 2011; Hussaini et al, 2013). 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 (Al-Hasani et al., 2007). 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). Moreover, there were also similar study conducted by Mullika et al in year 2009, found this immunogenic protein of *P. multocida*. It was also supported by study carried out by Tabatabai and Zehr in year 2004. The first report of HS in Malaysia was recorded in the year 1900 (Correngean, 1902) which showed that outbreak of HS disease has been occurred since long time ago. Furthermore, Malaysia has a cattle and buffaloes population of about 735000 and 186000 respectively which involved in economically contribution to the country. Thus, outbreaks have had very serious economical effects with an estimated RM2.4 million loss annually (Benkirane and De Alwis, 2002). 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 (Kamarudin, 2005). All isolates from Malaysian outbreaks were found to be *P. 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 (2011) 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. (Khan et al., 2013).
Fraz Munir Khan, 2011 stated that an extensive outbreak of a HS occurred in dromedary population of Greater Cholistan from mid of November 2010 to the mid of December 2010. Although *P. multocida* is not a common respiratory tract pathogen among dromedary yet, HS is regarded as one of the five important camel diseases and serious economic losses acquired by it. HS and anthrax are the main exotic differential diagnoses in New Zealand for an acute outbreak of septicaemia in cattle resulting in high mortality. An outbreak of hemorrhagic septicaemia in a naive population results in all ages of cattle, often with 100% mortality (McFadden et al., 2011). With that, identification of immunogens of *P. multocida* is still needed as an effort towards vaccine development in protecting animals from infection with pasteurellosis.

5. Conclusion

This study was focused on immunoblotting in determining immunogenic protein of *P. multocida* serotype B to protect animals from infection with acute pasteurellosis. This strain of *P. multocida* serotype B is commonly associated with HS disease endemically occurring in South and Southeast Asia, Africa, India and other Asian countries. Pathogenesis of HS disease most likely transmits through inhalation entering respiratory tract of infected animal with several environmental factors enhancement towards it. It is merely diagnosed using PCR method and eventually 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. 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). In conclusion, immunogenic soluble protein later will be identified. Further study on the immunogenicity study of soluble protein will be carried out in response to immunogenic roles against pasteurrollosis and potential role in vaccine development.

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