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Clinico-pathology and hemato-biochemistry responses in buffaloes infected with *Pasteurella multocida* type B:2 immunogen outer membrane protein

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ABSTRACT

The aim of this study was to investigate the clinico-pathology and haemato-biochemistry alterations in buffaloes inoculated with *Pasteurella multocida* type B:2 immunogen outer membrane protein via subcutaneous and oral routes. Nine buffalo heifers were divided equally into 3 treatment groups. Group 1 was inoculated orally with 10 mL of phosphate buffer saline (PBS); Group 2 and 3 were inoculated with 10 mL of outer membrane protein broth subcutaneously and orally respectively. Group 2 buffaloes showed typical haemorrhagic septicaemia clinical signs and were only able to survive for 72 h of the experiment. However, Group 3 buffaloes were able to survive throughout the stipulated time of 21 days of experiment. There were significant differences ($p < 0.05$) in the rectal temperature between the experimental and control group. In the hematology and biochemistry findings, there were significant differences ($p < 0.05$) in packed cell volume, mean corpuscular volume, mean corpuscular haemoglobin concentration, leukocytes, band neutrophils, segmented neutrophils, lymphocytes, eosinophils, basophils, gamma glutamyl transferase, total protein, and globulin between Group 2 and control group. In contrast, Group 3 and control group revealed significant differences ($p < 0.05$) in erythrocytes, haemoglobin, mean corpuscular haemoglobin concentration, segmented neutrophils, lymphocytes, monocytes, eosinophils, basophils, thrombocytes, gamma glutamyl transferase, total protein, globulin, and albumin:globulin ratio. In Group 2 buffaloes, there were gross lesions observed in the lung, trachea, heart, liver, spleen, kidney and submandibulae lymph nodes. In contrast, lesions were only observed in the lung, and liver of Group 3 buffaloes. There were significant differences ($p < 0.05$) in hemorrhage and congestion; necrosis and degeneration; and inflammatory cells infiltration between experimental groups and control group. However, there were no significant differences ($p > 0.05$) in edema between groups except for the lung. This study was a proof that oral route infection of *Pasteurella multocida* type B:2 immunogen outer membrane protein can be used to stimulate host cell.

1. Introduction

The cell envelope of a gram-negative bacteria consist of an inner cytoplasmic membrane, a thin peptidoglycan cell wall, and lipopolysaccharide containing outer membrane protein that surround the peptidoglycan layer which are considered the few virulence factors of *Pasteurella multocida* type B:2 [1]. The outer membrane is the region of gram-negative bacteria that separates between the host and the environment where 44% of the membrane is composed of the outer membrane protein [2]. Outer membrane

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protein protects the gram-negative organism against harsh unfavourable environments besides playing significant pathogenic role in protein translocation and signal transduction. Protein H or OmpH is the major outer membrane protein that surround and envelope *Pasteurella multocida* [3]. These proteins may act as virulence determinants such as the ability to acquire iron, thus mediating adherence to epithelial cells while invading the host and thwarting the immune system [1].

Experimental inoculation of outer membrane protein in mice and cattle via intraperitoneum and intramuscular routes correspondingly was found to cause clinico-pathological, hematology and biochemistry changes in both the animal models [4,5]. Mice inoculated with outer membrane proteins were having severe clinical responses, significant alteration in hemato-biochemistry results and severe pathological changes [4]. In contrary, the changes in clinical responses, hemato-biochemistry and pathological changes were milder in the cattle model after inoculation with outer membrane protein [5]. Outer membrane protein extracts were found to provide strong immune response against *Pasteurella multocida* infection and at the same time has antigenic property [6]. Nevertheless, experimental inoculations of outer membrane protein via different route of infections in buffaloes have never been done before.

The virulent factors of *Pasteurella multocida* vary in their ability to act as antigen based on their molecule size [7]. Outer membrane proteins were more effective antigens in stimulating host cell responses due to their large molecule size compared to lipopolysaccharide [7]. Thus, outer membrane protein had been recognised as immune-dominant antigens and was thought to be responsible for cross protective immunity and its role in generating bactericidal antibodies since lipopolysaccharide alone can only induce partial protection [8]. Animals immunized with an oil-adjuvant vaccine prepared from outer membrane protein were able to demonstrate high antibody titres [9]. Outer membrane protein with a molecular mass approximately 30 kDA is the major outer membrane protein in inducing immunity to haemorrhagic septicaemia [10]. Therefore, outer membrane protein could be used as new vaccine candidate for haemorrhagic septicaemia but there is lack of knowledge on the host cells response such as the clinical signs, hematology and biochemistry alterations, gross and histopathological changes in the buffaloes.

2. Material and methods

2.1. Animal selection

Nine eight-month-old, buffalo heifers weighing 100 kg were used in this study. Nasal swabs were collected from all buffaloes to ensure that the animals were free from *Pasteurella multocida* prior to the start of the experiment. The research was approved by the Animal Care and Use Committee of Universiti Putra Malaysia (approval number: R056/2014).

2.2. Inoculums preparation of outer membrane protein

Qprotome™ Bacterial Protein Extraction kits were used to prepare the inoculums of outer membrane protein from 10^{12} cfu of *Pasteurella multocida* type B:2. Firstly, freshly harvested cell pellets were frozen at −80 °C for 24 h prior to the extraction. The cell pellets were then thawed for 15 min on ice and were re-suspended in 10 mL of native lysis buffer. Then the cells were incubated on ice for 30 min followed by centrifugation at 14,000 rpm for 30 min at 4 °C. Lastly, supernatant containing the soluble fraction of the bacterial outer membrane were retained and subjected to SDS-PAGE to identify the range of protein bands.

2.3. Experimental design

All the nine buffalo heifers were divided equally into 3 treatment groups. Group 1 buffaloes were inoculated orally with 10 mL of phosphate buffer saline. Group 2 and 3 buffaloes were inoculated with 10 mL of outer membrane protein broth extracts subcutaneously and orally respectively. All buffaloes were monitored for clinical responses throughout the 21 days of the experiment. The clinical signs monitored daily were the rectal temperature, heart rate, respiratory rate, mucous membrane, rumen motility, salivation, nasal discharges, edema swelling, movement and dullness. Blood samples were collected via jugular venipuncture every 24 h for complete blood count and biochemistry analyses. Surviving buffaloes after 21 days were euthanized by severing the neck region which is a form of humane killing for post mortem examinations. Immune organs, gastro-intestinal tract organs and vital organs samples were collected for the evaluation of cellular changes.

2.4. Histopathology analysis and lesion scoring

The cellular changes observed were hemorrhage and congestion; necrosis and degeneration; inflammatory cells infiltration; and edema. These cellular changes were then scored according to establish scoring method [11]. The scores include score 0: normal (normal tissue); score 1: mild (less than 25% tissue affected); score 2: moderate (less than 50% tissue affected); and score 3: severe (more than 50% tissue affected).

2.5. Statistical analysis

JMP® Version 11. NC: SAS Institute Inc. software was used to analyze all the data collected. ANOVA with control, Dunnett's test were used to compare means between treatment groups. The data were considered significant at $p < 0.05$.

3. Results

3.1. Clinical response

Buffaloes in the control group showed normal clinical findings throughout the 21 days of the experiment. The rectal temperature, heart rate, respiratory rate, mucous membrane, and rumen motility were within the normal range. There were no salivation, nasal discharges, edema, and dullness observed. In contrast, buffaloes from Group 2 inoculated with outer membrane protein subcutaneously showed typical haemorrhagic septicaemia clinical signs and were only able to survive for 72 h of the experiment. Group 2 rectal temperatures were high throughout the experimental period where the temperatures were above 39 °C (Fig. 1). There were significant differences ($p < 0.05$) in the rectal temperature between the subcutaneous group compared to the control group. At 1 h post infection, all buffaloes started to have serous nasal discharges that persist throughout the experimental period. This was then followed by submandibular, prescapular and brisket edema after 24 h inoculation. At 48 h post infection, all buffaloes started showing sign of dullness and inappetance. All buffaloes from Group 2 were then euthanized at 72 h post infection following the Animal Welfare Guidelines where the animals were in recumbency and were having respiratory distress with labored breathing. Similar to the control group, Group 3 buffaloes inoculated orally showed no significant findings where all parameters measured were within the normal range. All buffaloes were able to survive throughout the stipulated time of 21 days. There were no significant differences ($p > 0.05$) in the rectal temperature between the oral group and the control group (Fig. 2).