

Acute Phase Protein Profile and Clinico-Pathological Changes in Mice Associated with the Infection of *Pasteurella multocida* Type B and the Bacterial Lipopolysaccharide and Outer Membrane Protein Immunogens

^{1,3}Faez Firdaus Jesse Abdullah, ¹Lawan Adamu, ¹Abdinasir Yusuf Osman,

³Mohd Zamri Saad, ¹Zunita Zakaria, ^{2,4}Rasedee Abdullah and ¹Abdul Aziz Saharee

¹Department of Veterinary Clinical Studies, ²Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

³Research Centre for Ruminant Disease UPM, ⁴Institute of Bioscience, Universiti Putra Malaysia, Malaysia

Abstract: Haemorrhagic Septicaemia (HS) is a killer disease of cattle and buffalo of economic importance in Asia and Africa. There is insufficient information on the responses of Balb c mice as animal model in respect of immunogens and Acute Phase Proteins (APP) profiles. Therefore, the present study aims to evaluate the acute phase protein profiles in mice associated with the infection of *Pasteurella multocida* type B and the bacterial lipopolysaccharide and outer membrane protein immunogens. Two hundred healthy Balb/c mice of 8-10 weeks old were used in this study. They were divided into four equal groups of 50 mice each. Mice of group 1 were inoculated intra-peritoneal with 1.0 mL sterile Phosphate Buffered Saline (PBS) pH 7, group 2 were inoculated with 1.0 mL of 10⁹ colony forming unit (cfu) of *P. multocida* B: 2. Mice of groups 3 and 4 were inoculated intra-peritoneal with 1.0 mL of LPS and 1.0 mL of OMP, respectively. Acute phase proteins analysis were done using two sites Enzyme Linked Immunoassay (ELISA) highly sensitive test kits. The data was analyzed using SPSS. Haptoglobin concentration increased significantly in group 3 and 4 (p<0.05) following inoculation with immunogens compared to control group. Mice in group 3 and 4 showed significantly (p<0.000) 3 times higher concentrations of SAA and significantly (p<0.037) 1.3 times increased concentrations of SAA, respectively compared to the control group. There was no significant changes in the concentrations of fibrinogen in group 2 (p = 0.177), group 3 (p = 0.088) and group 4 (p = 0.359). C-reactive protein in groups 2 and 3 showed significantly (p<0.05) higher levels than the control group. Albumin showed significant increase (p<0.05) in group 2 compared to the control group. There were significant changes in the concentrations of acute phase proteins and clinical responses post inoculation with immunogens indicating adverse pro-inflammatory reactions in mice in the present study.

Key words: Acute phase protein, *Pasteurella multocida* type B, bacterial lipopolysaccharide, outer membrane protein immunogens, infection

INTRODUCTION

Haemorrhagic Septicaemia (HS) of cattle and buffaloes is an acute and extremely fatal septicaemic disease (Carter and de Alwis, 1989). It is caused by precisely two serotypes of *P. multocida*; serotype B: 2 in Asia (Munir *et al.*, 2007) and E: 2 in Africa (De Alwis, 1992). The pathogen consists of five capsular sero-groups specifically A, B, D, E and F with strong relationship between the capsular sero-group and disease predilection (Boyce and Adler, 2001; Chung *et al.*, 2001). At present, the Asian and African haemorrhagic septicaemia serotypes are designated as B: 2 and E: 2, respectively (De Alwis, 1990).

Most strains of *Pasteurella* form a polysaccharides capsule or envelope. It is composed of polysaccharides, Lipopolysaccharides (LPS) and a variety of proteins (Knights *et al.*, 1990). Both LPS and polysaccharides play important roles in passive haemagglutination. Prince and Smith (1996) described the three antigen complexes from bacteria namely, alpha, beta and gamma polysaccharides protein complex, a serogroup-specific polysaccharide and LPS. The surface of Gram-negative bacteria is critical for interaction of the bacterium with the host cell environment as it mediates nutrient uptake, secretion of toxins and other products as well as avoidance of the host immune system (Niemann *et al.*, 2004). In mammals including humans, activation of the innate immune system caused

by infection by Gram-negative bacteria has detrimental consequences for the function of host cells (Besnard *et al.*, 2001; Karsch *et al.*, 2002; Deb *et al.*, 2004). The immune-mediated effects of Gram-negative bacteria are caused by the Lipid A fraction of the Lipopolysaccharides (LPS) which is also known as endotoxin. This constituent of the bacterial cell wall is constantly shed into the environment of the bacteria. In mammals, induction of an immune response by administration of LPS is commonly used to mimic a bacterial infection in a host. LPS administration *in vivo* increases apoptosis of the host cells reduces the ovarian steroidogenic response to gonadotropin stimulation and impairs embryonic survival (Sancho-Tello *et al.*, 1993; Shakil *et al.*, 1994; Baines *et al.*, 1996; Besnard *et al.*, 2001; Deb *et al.*, 2004).

The Outer Membrane Proteins (OMPs) of Gram-negative bacteria have a role in disease processes as they act at an interface between the host and pathogen (Lin *et al.*, 2002). The OMPs of *P. multocida* play a significant role in the pathogenesis of pasteurellosis and have been identified as potent immunogens (Ullah *et al.*, 2008). Bacterial surface proteins have been shown to be important for conferring protective immunity in a range of infection models (Brown *et al.*, 2001; Frazer *et al.*, 2006). OMPs are key mediators of bacterial interaction with the host environment (Lin *et al.*, 2002). Recently, investigations on characterized OMPs which include major proteins Omp A and Omp H and a limited number of minor OMPs showed that these Omps are virulent factors or immunogens (Boyce *et al.*, 2006).

Ullah *et al.* (2008) has determined the differences in OMP, LPS and whole cell extracts from two vaccine strains of *P. multocida*. SDS-PAGE was used to locate the major proteins in OMP, LPS and whole cell. There were attempts of using capsules, LPS and OMP components of *P. multocida* as vaccine (Confer, 1993). Administration of LPS in fish increases the phagocytic activity of leukocytes and plasma concentration of lysozyme (Salati *et al.*, 1987; Paulsen *et al.*, 2003). LPS also acts directly on host macrophages to stimulate the expression of typical pro-inflammatory cytokines such as tumor necrosis factor and interleukins (Zou *et al.*, 2002; Iliev *et al.*, 2005). Boyce and Adler (2001) stated that LPS and OMPs can be the major protective antigens for the host. Boyce and Adler (2000) had challenged mice with wild type, mutant and complemented strains of *P. multocida* B: 2 to look for crucial virulent determinant. In another study, *P. multocida* infection in mice was shown to resemble the infection of HS in cattle and buffaloes (Dawkins *et al.*, 1990). Clinical signs and gross pathology changes in the organs of mice after inoculation with *P. multocida* B: 2 were evident.

However, there is insufficient information on the response of mice in relation to immunogens, particularly OMP and LPS and also the associated changes of APP profiles and clinical responses. Therefore, the aim of this study was to comparatively observe changes in APP parameters and clinical responses in mice infected with *P. multocida* B: 2 and the immunogens.

MATERIALS AND METHODS

Mice: Two hundred healthy Balb/c mice of 8-10 weeks old were used in this study. They were obtained from the Institute of Cancer Research (ICR) and kept at the Animal Resource Centre, Universiti Putra Malaysia. The animals were confirmed negative for *P. multocida* following culture of peripheral blood for bacterial isolations, housed in plastic cages and provided with water and pellet *ad libitum*. Ten Balb/c mice were kept in each plastic cage. The mice were observed for 2 weeks prior to the experiment to make sure that they were healthy.

Inoculums: Throughout the study, three types of inoculums were used.

Wild-type *P. multocida* B: 2: The wild-type *P. multocida* B: 2 used in this study were obtained from stock culture. It was isolated from a earlier outbreak of HS in the state of Kelantan, Malaysia. Identification of *P. multocida* was made using the Gram-staining method and biochemical characterization of oxidase, urea broth, Sulphur Indole Motility (SIM), Triple Sugar Iron (TSI) and citrate tests. The isolate was confirmed to be type B: 2 by the Veterinary Research Institute (VRI) Ipoh, Perak. Pure stock culture that was stored on nutrient agar slants was sub-cultured onto 5% horse blood agar and incubated at 37°C for 18 h. A single colony of *P. multocida* was selected and grown in Brain Heart Infusion broth (BHI), incubated in shaker incubator at 37°C for 24 h before the concentration was determined by McFarland Nephelometer Barium Sulfate Standards.

The Lipopolysaccharide (LPS) *P. multocida* B: 2: The LPS Extraction kit (Intron Biotechnology) was used to prepare the inoculums of LPS. For mice experiment, LPS was extracted from 10⁹ cfu of organisms. The whole cells were centrifuged for approximately 30 sec at 13,000 rpm at room temperature. Then, the supernatant was removed before 1 mL of lysis buffer was added and vortexed vigorously to lyse the bacterial cells. This was followed by adding 200 µL of chloroform and vortexed vigorously. The mixture was incubated for 5 min at room temperature before centrifuged at 13,000 rpm for 10 min at 4°C. Then, 400 µL of the supernatant was transferred into a new

1.5 mL centrifuge tube and 800 µL of purification buffer was added. The mixture was incubated for 10 min at -20°C. This was followed by another centrifugation at 13,000 rpm for 15 min at 4°C. Lastly, the LPS pellet was washed with 1 mL of 70% ethanol and dried completely. Following that 70 µL of 10 mM Tris-HCl (pH 8.0) (Sigma®) was added into the LPS pellet and was dissolved by boiling for 1 min. The LPS extraction obtained was subjected to SDS-PAGE to confirm that no protein was present in the extracted LPS.

The Outer Membrane Proteins (OMP) of *P. multocida*

B: 2: The Qproteome™ Bacterial Protein Extraction kit was used to prepare the inoculums of OMP. For mice experiment, the OMP extracted from 10⁹ cfu of the organism. Initially, freshly harvested cell pellets were frozen using liquid nitrogen 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, the supernatant containing the soluble fraction of the bacterial outer membrane proteins was retained and subjected to SDS-PAGE to locate the range of protein bands present in the extraction.

Experimental design in mouse model: The two hundred mice were divided into four equal groups of 50 mice each. Mice of group 1 were inoculated intra-peritoneal with 1.0 mL sterile Phosphate Buffered Saline (PBS) pH 7, group 2 were similarly inoculated with 1.0 mL of 10⁹ colony forming unit (cfu) of *P. multocida* B: 2. Mice of groups 3 and 4 were inoculated intra-peritoneal with 1.0 mL of LPS and 1.0 mL of OMP, respectively. Clinical signs and mortality of the mice were observed for 48 h. The clinical signs that were observed are ruffled fur, movement, dullness and whether eyes are closed and the discharges from the eyes. Clinical scoring was done and the data was analyzed using SPSS. The observations were recorded using Sony Handycam 25x Optical Zoom-DCR DVD 708 (Sony Corporation) and Nikon Digital Camera 7.1 Megapixels (Coolpix) (Nikon Corporation, Japan). Blood samples were collected into plain blood tubes, EDTA-containing tubes and sodium citrate containing tubes from the moribund animals. The data was analyzed using SPSS. All procedures and experiments demonstrated were undertaken under a project license approved by animal utilization protocol committee with reference number: UPM/FPV/PS/3.2.1.551/AUP-R120.

Acute Phase Proteins (APP) analysis: The test kits were highly sensitive, two sites Enzyme Linked Immunoassay (ELISA) obtained from Life Diagnostics Inc. (West

Chester) info@lifediagnosics.com and from Tridelta Development Ltd. (Ireland) info@trideltaltd.com. The APP used in this study was mouse fibrinogen, mouse C-reactive, mouse Serum Amyloid A (SAA) and mouse haptoglobin.

Determination of mouse haptoglobin: Serum samples were used to determine the Haptoglobin (Hp) levels by ELISA technique. Briefly, the microtiter plates were coated with affinity purified anti-mouse Hp (Life Diagnostics Inc.). The mouse Hp standards were prepared as recommended by the manufacturer. Then, 100 µL of serum (1:10,000 dilutions) sample was introduced into each well and incubated for 15 min before being washed 4 times with an ELISA washer (BioRad). After that 100 µL of conjugate (1:100 dilutions) was dispersed into the wells and incubated for 15 min and washed 4 times with the ELISA washer (BioRad). The substrate (Life Diagnostics Inc.) was then introduced before incubation at 37°C. The reaction was stopped by 0.3 M sulfuric acid and the optical density values were measured at absorbance 450 nm wavelengths in an ELISA Reader (BioRad).

Determination of mouse fibrinogen: Serum samples were used to determine the Fibrinogen (Fb) levels by ELISA technique. Briefly, the microtiter plates were coated with affinity of purified anti-mouse Fb (Life Diagnostics Inc.). The mouse Fp standards were prepared as recommended by the manufacturer. Then, 100 µL of serum (1:100 dilutions) sample was introduced into each well and incubated at 37°C for 60 min before being washed 4 times with an ELISA washer (BioRad). After that 100 µL of conjugate (1:100 dilutions) was dispersed into the wells and incubated at 37°C for 30 min and washed 4 times with the ELISA washer (BioRad). The substrate (Life Diagnostics Inc.) was then introduced before incubation. The reaction was stopped by 0.3 M sulfuric acid and the optical density values were measured at absorbance 450 nm wavelengths in an ELISA Reader (BioRad).

Determination of mouse C-reactive: Serum samples were used to determine the mouse C-reactive (CRP) levels by ELISA technique. Briefly, the microtiter plates were coated with affinity purified anti-mouse CRP (Life Diagnostics Inc.). The mouse CRP standards were prepared as recommended by the manufacturer. Then, 100 µL of serum (1:20 dilutions) sample was introduced into each well and incubated at 37°C for 10 min before being washed 4 times with an ELISA washer (BioRad). After that 100 µL conjugate (1:100 dilutions) was dispersed into the wells and incubated at 37°C for 10 min and washed 4 times with the ELISA washer (BioRad). The substrate (Life Diagnostics Inc.) was then introduced before incubation.

The reaction was stopped by 0.3 M sulfuric acid and the optical density values were measured at absorbance 450 nm wavelengths in an ELISA Reader (BioRad).

Determination of mouse Serum Amyloid A (SAA): Serum samples were used to determine the mouse Serum Amyloid A (SAA) levels by ELISA technique. Briefly, the microtiter plates were coated with affinity purified anti-mouse SAA (Life Diagnostics Inc). The mouse SAA standards were prepared as recommended by the manufacturer. Then, 100 µL of serum (1:1000 dilutions) sample was introduced into each well and incubated at 37°C for 60 min before being washed 4 times with an ELISA washer (BioRad). After that 100 µL of conjugate (1:100 dilutions) was dispersed into the wells and incubated at 37°C for 30 min and washed 4 times with the ELISA washer (BioRad). The substrate (Life Diagnostics Inc.) was then introduced before incubation at 37°C. The reaction was stopped by 0.3 M sulfuric acid and the optical density values were measured at absorbance.

Statistical analysis: All the data's were analyzed using independent t-test. This was done using SPSS Software, Version 20.0. The data were considered significant at $p < 0.05$.

RESULTS

Acute phase protein: Mice of all treated groups showed significant increased in positive acute phase protein concentrations. Haptoglobin concentration increased significantly ($p < 0.05$) following inoculation with the three immunogens compared to control group 1. Mice in group 4 showed 10 times higher concentration ($p < 0.000$) of haptoglobin ($0.856 \pm 0.280 \text{ ng mL}^{-1}$) while mice in group 3 showed a significant ($p < 0.001$) 6 times increased in haptoglobin concentration ($0.505 \pm 0.262 \text{ ng mL}^{-1}$). There was no significant difference ($p = 0.158$) in haptoglobin levels in group 2 ($0.214 \pm 0.275 \text{ ng mL}^{-1}$) as compared to the control group ($0.084 \pm 0.045 \text{ ng mL}^{-1}$) (Table 1).

Similarly, mice in group 4 showed significantly ($p < 0.000$) 3 times higher concentrations of SAA ($0.662 \pm 0.205 \text{ ng mL}^{-1}$) while mice of group 3 showed a significant ($p < 0.037$) 1.3 times increased in the concentrations of SAA ($0.257 \pm 0.068 \text{ ng mL}^{-1}$) compared

to control group ($0.202 \pm 0.037 \text{ ng mL}^{-1}$). Again, there was no significant difference ($p < 0.058$) in the SAA levels of mice in group 2 ($0.258 \pm 0.081 \text{ ng mL}^{-1}$) as compared to control group (Table 1).

There was no significant change in the concentrations of fibrinogen and C-protein in mice in group 2 ($p = 0.177$), group 3 ($p = 0.088$) and group 4 ($p = 0.359$). However, C-reactive protein in groups 2 ($0.222 \pm 0.070 \text{ ng mL}^{-1}$) and 3 ($0.207 \pm 0.034 \text{ ng mL}^{-1}$) mice showed significantly ($p < 0.05$) higher levels than control group ($0.167 \pm 0.028 \text{ ng mL}^{-1}$). Group 4 ($0.196 \pm 0.391 \text{ ng mL}^{-1}$) did not show much change ($p = 0.074$) when compared with the control group (Table 1).

Clinical signs: Mice inoculated with the Outer Membrane Protein (OMP), the Lipopolysaccharide (LPS) and whole cells showed severe clinical signs. For the first 2 h post-infection (p.i.), mice in groups 2 and 4 showed severely ruffled fur at the head region and they were huddled together. Mice in group 3 showed moderate huddling. At 4 h p.i., the mice in group 2 were inactive with little respond to external stimuli. Mice in group 4 were depressed and refused to eat or drink. Group 3 mice had ruffled fur at the head region and were slightly dull while the intake of food and water was markedly reduced. All mice in group 2 died after 6 h p.i. while all mice in groups 1, 3 and 4 survived. At 18 h p.i., mice in groups 3 and 4 showed signs of inactivity and none responded to external stimuli. Fifteen mice in group 4 died at 30 h p.i. and 35 mice in this group died at 36 h p.i. Nevertheless, all 50 mice from the LPS group survived until the end of this 36 h study period (Table 2).

Mice in all treated groups showed significant changes in clinical signs compared to control group. Severe ruffled fur was observed significantly ($p < 0.05$) following inoculation with the whole cell and OMP compared to control group. Mice in group 2 showed severe ruffled fur ($p < 0.000$) with mean score of 2.84 ± 0.373 while mice in group 4 showed significant severe ruffled fur ($p < 0.000$) with mean score of 2.82 ± 0.3916 . Mice in group 3 showed significant mild ruffled fur ($p < 0.000$) with mean score of 0.67 ± 0.625 as compared to control group (Table 3). Similarly, for movement response the group 2 and 4 mice showed significant ($p < 0.05$) severe clinical

Table 1: Acute phase proteins concentration in mice after 24 h post-inoculation with immunogen

Parameters	Group 1 (Control)	Group 2 (<i>P. multocida</i>)	Group 3 (LPS)	Group 4 (OMP)
Fibrinogen (ng mL^{-1})	0.122±0.027	0.153±0.0625	0.103±0.020	0.146±0.075
C-Reactive (ng mL^{-1})	0.167±0.028	0.222±0.0700*	0.207±0.034*	0.196±0.391
SAA (ng mL^{-1})	0.202±0.037	0.258±0.0810	0.257±0.068*	0.662±0.205*
Haptoglobin (ng mL^{-1})	0.084±0.045	0.214±0.2750	0.505±0.262*	0.856±0.280*
Albumin (UL^{-1})	28.880±3.746	37.860±3.4920*	29.273±9.994	32.030±2.373

*Significant value $p < 0.05$ (Independent t-test). Comparison between immunogens groups and negative control group mice

Table 2: Comparison of lethargy and death percentage of animals in the control and treatment of groups

Time (h)	Control group		Treatment groups						
	PBS (1)		PM (2)		LPS (3)		OMP (4)		
	DA (%)	LR (%)	DA (%)	LR (%)	DA (%)	LR (%)	DA (%)	LR (%)	
6	0	0	50	0	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0
30	0	0	0	0	0	0	15	0	0
36	0	0	0	0	0	50	35	0	0
Total	0	0	50	0	0	50	50	0	0

DA (%) = Percentage of Death Animals; LR (%) = Percentage of Lethargy Animals; PBS = Phosphate Buffered Saline; PM = *P. multocida* B: 2; LPS = Lipopolysaccharide; OMP = Outer Membrane Proteins

Table 3: Mean score of clinical signs observed in mice 48 h post-inoculation with immunogens

Parameters	Group 1 (Control)	Group 2 (<i>P. multocida</i>)	Group 3 (LPS)	Group 4 (OMP)
Ruffled fur	0.00±0.000	2.84±0.373*	0.67±0.625*	2.82±0.391*
Movement	0.00±0.000	2.84±0.330*	0.51±0.505*	2.92±0.277*
Dull and depressed	0.00±0.000	2.84±0.373*	0.31±0.466*	2.84±0.373*
Eyes closed and crust	0.00±0.000	2.86±0.354*	0.02±0.143	2.80±0.407*

*Significant value $p < 0.05$ (Independent t-test); Comparison between immunogens groups and negative control group mice

response compared to control group. Mice in group 2 showed severe immobility ($p < 0.000$) with mean score of 2.84 ± 0.330 while mice in group 4 showed significant severe immobility ($p < 0.000$) with mean score of 2.92 ± 0.277 . Mice in group 3 showed significant mild immobility ($p < 0.000$) with mean score of 0.51 ± 0.505 as compared to control group (Table 3).

For dullness response the group 2 and 4 showed significant ($p < 0.05$) severe clinical response compared to control group 1. Mice in group 2 showed severe dullness ($p < 0.000$) with mean score of 2.84 ± 0.373 while mice in group 4 showed significant severe dullness ($p < 0.000$) with mean score of 2.84 ± 0.373 . Mice in group 3 showed significant mild dullness to normal response ($p < 0.000$) with mean score of 0.31 ± 0.466 as compared to control group (Table 3).

For clinical response of closure of eyes and discharges from the eyes only the group 2 and 4 mice showed significant ($p < 0.05$) severe clinical response compared to control group. Mice in group 2 showed severe eyes closure and crust formation due to discharges from the eyes ($p < 0.000$) with mean score 2.86 ± 0.354 while mice of group 4 showed significant severe eyes closure and crust formation due to discharges from the eyes ($p < 0.000$) with mean score 2.80 ± 0.407 . Mice in group 3 showed no significant changes in the closure of the eyes and discharges ($p = 0.322$) with mean score 0.02 ± 0.143 as compared to control group (Table 3).

DISCUSSION

Mice are exquisitely susceptible to *P. multocida* (Dawkins *et al.*, 1990). Earlier, Heddleston *et al.* (1972)

also made a similar observation using *P. multocida* in Swiss Webster mice while Bain and Knox (1961) concluded that as few as two organisms could produce 100% mortality in mice. According to Dawkins *et al.* (1990), clinical disease occurred in < 6 h after infection with more than 105 bacteria. *Pasteurella multocida* was found to be pathogenic for mice and could cause death in < 24 h after infection (Hajikolaie *et al.*, 2006). Similar observation was made by Dawkins *et al.* (1990) where doses of 101 and 104 viable *P. multocida* produced fatal infections in mice and cattle in 24 h. The findings in this study for infected group 2 are in agreement with the others when mice died in < 8 h.

The symptoms of HS include high fever, shaking, depression and reluctance to move (Dawkins *et al.*, 1990). These signs and the rapid nature of the disease are similar in mice and cattle. In cattle, there is nasal discharge and excessive salivation while mice demonstrated naso-ocular involvement with a degree of crusting around the nose and eyes (Dawkins *et al.*, 1990). The crusting was found to be partly due to the excretion of *P. multocida* organisms in the murine tear glands that resulted in high bacteria counts in the peripheral blood samples during the early time points of the kinetic experiments (Dawkins *et al.*, 1990). In this study, the whole cells and OMP resulted in similar symptoms. The mice were inactive and reluctant to move while the eyes were closed due to crusting. However, the onset of clinical signs differs between whole cells and OMP groups' mice when the whole cells inoculated mice showed a more rapid onset. Severe clinical symptoms were observed at the 4th h post-inoculation with whole cells but were observed only after the 18th h post-inoculation with OMP.

In contrast, LPS did not produce severe clinical signs and all mice survived the inoculation. When LPS is known as a potent endotoxin of *P. multocida*, inoculation of LPS is expected to produce severe signs as observed by Horadagoda *et al.* (2002). Nevertheless, failure of LPS in this study to produce the disease might be due to the fact that only one dose of LPS was inoculated and the dose was probably too low to produce the disease. Studies have indicated that endotoxin or LPS is needed to be infused continuously since endotoxin is rapidly cleared

by the mononuclear phagocytic system (Rokke *et al.*, 1988; Warner *et al.*, 1988). Waring *et al.* (1995) observed that single intravenous injection of LPS was tolerated easily by mice without any observable pathology or evidence of ill health. They also stated that repeated intra-peritoneal injections of LPS in mice at 8 h intervals caused the mice to become ill (Warning *et al.*, 1995) exhibiting reduced social and feeding activity, huddling behavior with pilo-erection and shivering, increased skin turgor and weight loss and eventually led to hypothermia before the mice became moribund.

An Acute Phase Protein (APP) is a protein which concentration either increased or decreased by >25% in cases of infection and inflammation (Kushner, 1982). The Acute Phase Response (APR) is a series of physiologic reactions initiated early in the inflammatory process (Baumann and Gauldie, 1994). It includes the release of inflammatory mediators such as cytokines, interleukin-1 and interleukin-6 which stimulate hepatocytes to produce Acute Phase Proteins (APP). Hepatic production of APPs is stimulated by pro-inflammatory cytokines (predominantly interleukin 1, interleukin 6 and tumor necrosis factor alpha) released into the circulation during acute phase response. Other factors such as growth factors, gluco-corticosteroids and anti-inflammatory cytokines have important modulating effects on APP production (Richards *et al.*, 1991; Baumann and Gauldie, 1994; Gabay and Kushner, 1999). The APPs are involved in many events during inflammation such as tissue repair, binding of bacterial components and activation of complement (Gruys *et al.*, 1994). The circulating concentrations of the APP are related to the severity of the disorder and the extent of tissue damage in the affected animals (Gruys *et al.*, 1994).

APPs are thought to participate in innate defense mechanisms and in controlling inflammatory response to infection by for examples, binding to foreign substances, having opsonizing activities and modulating phagocytic cell functions (Orro *et al.*, 2004). Several studies in mice have shown protective properties of APPs against microbial challenge (Briles *et al.*, 1989; Vogels *et al.*, 1993; Hochepped *et al.*, 2000; Szalai *et al.*, 2000) or endotoxaemia and septic shock (Lamping *et al.*, 1998; Alcorn *et al.*, 1992; Xia and Samols, 1997). Concentrations and kinetics of systemic APPs during inflammatory response appear to be related to the severity of tissue damage and time course of the inflammation process (Kent, 1992).

In this study, all treated groups showed increased in concentrations of acute phase protein, consistent with results of Kushner (1982) and Gruys *et al.* (1994). The increased in APP, however, varies where inoculation with

OMP led to highest increased in APP concentration, followed by LPS and lastly the whole cells. This may due to the ability to cause different degree of severity in tissue damage and inflammation as mentioned by Kent (1992). The haptoglobin and Serum Amyloid A (SAA) concentrations increased significantly following inoculation with LPS and OMP. The whole cells did not show significant increase due to the short time of survival of mice (8 h) that did not provide enough time for inflammation to take place. The whole cells, however, led to significant increase in the level of albumin protein, probably due to dehydration following decreased in water and feed intake.

CONCLUSION

It can be concluded that the positive control group and OMP group in mice, exhibited host cell response. The severity of host cell response differs between each group. From this study, the positive control group mice manifested severe clinical response followed by the OMP group and finally the LPS group. All the immunogen group mice showed changes in complete blood count and biochemistry profiles with some differences between the groups. All the three immunogen group mice showed changes in acute phase protein concentrations and also showed gross and cellular changes where the LPS and OMP group mice showed gross and microscopic lesions in the lung, heart and liver which resembles the positive control mice with slight differences in each group.

REFERENCES

- Alcorn, J.M., J. Fierer and M. Chojkier, 1992. The acute-phase response protects mice from D-galactosamine sensitization to endotoxin and tumor necrosis factor-alpha. *Hepatology*, 15: 122-129.
- Bain, R.V. and K.W. Knox, 1961. The antigens of *Pasteurella multocida* type I. II. Lipopolysaccharides. *Immunology*, 4: 122-129.
- Baines, M.G., A.J. Duclos, A.R. Fougérolles and R.L. Gendron, 1996. Immunological prevention of spontaneous early embryo resorption is mediated by non-specific immunosimulation. *Am. J. Reprod. Immunol.*, 35: 34-42.
- Baumann, H. and J. Gauldie, 1994. The acute phase response. *Immunol. Today*, 15: 74-80.
- Besnard, N., E.A.L. Horne and S.A. Whitehead, 2001. Prolactin and lipopolysaccharide treatment increased apoptosis and atresia in rat ovarian follicles. *Acta Physiol. Scand.*, 172: 17-25.

- Boyce, J.D. and B. Adler, 2000. The capsule is a virulence determinant in the pathogenesis of *Pasteurella multocida* M1404 (B: 2). *Infect. Immun.*, 68: 3463-3468.
- Boyce, J.D. and B. Adler, 2001. Acapsular *Pasteurella multocida* B: 2 Can stimulate protective immunity against pasteurellosis. *Infect. Immun.*, 69: 1943-1946.
- Boyce, J.D., P.A. Cullen, V. Nguyen, I. Wilkie and B. Adler, 2006. Analysis of the *Pasteurella multocida* outer membrane sub-proteome and its response to the *in vivo* environment of the natural host. *Proteomics*, 6: 870-880.
- Briles, D.E., C. Forman, J.C. Horowitz, J.E. Volanakis and W.H. Benjamin *et al.*, 1989. Antipneumococcal effects of C-reactive protein and monoclonal antibodies to pneumococcal cell wall and capsular antigens. *Infect. Immun.*, 57: 1457-1464.
- Brown, J.S., A.D. Ogunniyi, M.C. Woodrow, D.W. Holden and J.C. Paton, 2001. Immunization with components of two iron uptake ABC transporters protects mice against systemic *Streptococcus pneumoniae* infection. *Infect. Immun.*, 69: 6702-6706.
- Carter, G.R. and M.C.L. de Alwis, 1989. Haemorrhagic Septicaemia. In: *Pasteurella and Pasteurellosis*, Adlam, C. and J.M. Rutter (Eds.). Academic Press, New York, USA., ISBN: 978-0120442744, pp: 37-73.
- Chung, J.Y., I. Wilkie, J.D. Boyce, K.M. Townsend, A.J. Frost, M. Ghoddusi and B. Adler, 2001. Role of capsule in the pathogenesis of fowl cholera caused by *Pasteurella multocida* serogroup A. *Infect. Immun.*, 69: 2487-2492.
- Confer, A.W., 1993. Immunogens of *Pasteurella*. *Vet. Microbiol.*, 37: 353-368.
- Dawkins, H.J.S., R.B. Johnson, T.L. Spencer and B. Adler, 1990. *Pasteurella multocida* infections in mice with reference to haemorrhagic septicaemia in cattle and buffalo. *Immunol. Cell Biol.*, 68: 57-61.
- De Alwis, M.C.L., 1990. Haemorrhagic septicaemia. ACIAR Monograph No. 57, pp: 36.
- De Alwis, M.C.L., 1992. Haemorrhagic septicaemia: A general review. *Br. Vet. J.*, 148: 99-111.
- Deb, K., M.M. Chaturvedi and Y.K. Jaiswal, 2004. A minimum dose of lipopolysaccharide required for implantation failure: Assessment of its effect on the maternal reproductive organs and interleukin-1alpha expression in the mouse. *Reproduction*, 128: 87-97.
- Frazer, L.T., N.M. O'Brien-Simpson, N. Slakeski, K.A. Walsh and P.D. Veith *et al.*, 2006. Vaccination with recombinant adhesins from the RgpA-Kgp proteinase-adhesin complex protects against *Porphyromonas gingivalis* infection. *Vaccine*, 24: 6542-6554.
- Gabay, C. and I. Kushner, 1999. Acute-phase proteins and other systemic responses to inflammation. *N. Engl. J. Med.*, 340: 448-454.
- Gruys, E., M. Obwolo and M.J. M. Toussaint, 1994. Diagnostic significance of the major acute phase proteins in veterinary clinical chemistry. *Vet. Bull.*, 64: 1009-1018.
- Hajikolaie, M.R.H., M. Ghorbanpour, M.R. Sayfi-Abadshapouri, A. Rasooli and H. Jahferian, 2006. Occurrence of *Pasteurella multocida* in the nasopharynx of healthy buffaloes and their immunity status. *Bull. Vet. Inst. Pulawy*, 50: 435-438.
- Heddleston, K.L., J.E. Gallagher and P.A. Rebers, 1972. Fowl cholera: Gel diffusion precipitin test for serotyping *Pasteurella multocida* from avian species. *Avian Dis.*, 16: 925-936.
- Hocephied, T., W. van Molle, F.G. Berger, H. Baumann and C. Libert, 2000. Involvement of the acute phase protein α 1-acid glycoprotein in nonspecific resistance to a lethal gram-negative infection. *J. Biol. Chem.*, 275: 14903-14909.
- Horadagoda, N.U., J.C. Hodgson, G.M. Moon, T.G. Wijewardana and P.D. Eckersall, 2002. Development of a clinical syndrome resembling haemorrhagic septicaemia in the buffalo following intravenous inoculation of *Pasteurella multocida* serotype B:2 endotoxin and the role of tumor necrosis factor-alpha. *Res. Vet. Sci.*, 72: 194-200.
- Iliev, D.B., C.Q. Liarte, S. MacKenzie and F.W. Goetz, 2005. Activation of rainbow trout (*Oncorhynchus mykiss*) mononuclear phagocytes by different pathogen associated molecular pattern (PAMP) bearing agents. *Mol. Immunol.*, 42: 1215-1223.
- Karsch, F.J., D.F. Battaglia, K.M. Breen, N. Debus and T.G. Harris, 2002. Mechanisms for ovarian cycle disruption by immune/inflammatory stress. *Stress*, 5: 101-112.
- Kent, J., 1992. Acute phase proteins: Their use in veterinary diagnosis (guest editorial). *Br. Vet. J.*, 148: 279-282.
- Knights, J.M., C. Adlam and P. Owen, 1990. Characterization of envelope proteins from *Pasteurella haemolytica* and *Pasteurella multocida*. *J. Gen. Microbiol.*, 136: 495-505.
- Kushner, I., 1982. The phenomenon of the acute phase response. *Ann. N. Y. Acad. Sci.*, 389: 39-48.
- Lamping, N., R. Dettmer, N.W. Schroder, D. Pfeil, W. Hallatschek, R. Burger and R.R. Schumann, 1998. LPS-binding protein protects mice from septic shock caused by LPS or gram-negative bacteria. *J. Clin. Invest.*, 101: 2065-2071.

- Lin, J., S. Huang and Q. Zhang, 2002. Outer membrane proteins: Key players for bacterial adaptation in host niches. *Microb. Infect.*, 4: 325-331.
- Munir, R., D. Shahwar, U. Farooq, I. Nawaz, I. Shahzad and A. Khanum, 2007. Outer membrane protein profiling of *Pasteurella multocida*. *Pak. Vet. J.*, 27: 1-4.
- Niemann, H.H., W.D. Schubert and D.W. Heinz, 2004. Adhesins and invasins of pathogenic bacteria: A structural view. *Microb. Infect.*, 6: 101-112.
- Orro, T., S. Sankari, T. Pudas, A. Oksanen and T. Soveri, 2004. Acute phase response in reindeer after challenge with *Escherichia coli* endotoxin. *Comp. Immunol. Microbiol. Infect. Dis.*, 27: 413-422.
- Paulsen, S.M., H. Lunde, R.E. Engstad and B. Robertsen, 2003. *In vivo* effects of β -glucan and LPS on regulation of lysozyme activity and mRNA expression in Atlantic salmon (*Salmo salar* L.). *Fish Shellfish Immunol.*, 14: 39-54.
- Prince, G.H. and J.E. Smith, 1996. Ultracentrifugation as a means for the separation and identification of LPS. *ACS Symp. Ser.*, 419: 238-249.
- Richards, C., J. Gauldie and H. Baumann, 1991. Cytokine control of acute phase protein expression. *Eur. Cytok. Network*, 2: 89-98.
- Rokke, O., A. Revhaug, B. Osterud and K.E. Giercksky, 1988. Increased plasma levels of endotoxin and corresponding changes in circulatory performance in a porcine sepsis model: The effect of antibiotic administration. *Prog. Clin. Biol. Res.*, 272: 247-262.
- Salati, F., Y. Ikeda and R. Kusuda, 1987. Effect of *Edwardsiella tarda* lipopolysaccharide immunization on phagocytosis in the eel [*Anguilla japonica*]. *Bull. Jpn. Soc. Sci. Fish.*, 53: 201-204.
- Sancho-Tello, M., J.S. Tash, K.F. Roby and P.F. Terranova, 1993. Effects of lipopolysaccharide on ovarian function in the pregnant mare serum gonadotropin-treated immature rat. *Endocr. J.*, 1: 503-512.
- Shakil, T., A. Snell and S.A. Whitehead, 1994. Effects of lipopolysaccharide and cyclosporin on the endocrine control of ovarian function. *J. Reprod. Fertil.*, 100: 57-64.
- Szalai, A.J., J.L. VanCott, J.R. McGhee, J.E. Volanakis and W.H. Jr. Benjamin, 2000. Human C-reactive protein is protective against fatal *Salmonella enterica* serovar typhimurium infection in transgenic mice. *Infect. Immun.*, 68: 5652-5656.
- Ullah, M.I., M. Abubakr, R. Durani, R. Anjim, N. Ayub and Q. Ali, 2008. Differentiation of closely related vaccinal strains of *Pasteurella multocida* using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). *Pak. Vet. J.*, 28: 175-180.
- Vogels, M.T., L. Cantoni, M. Carelli, M. Sironi, P. Ghezzi and J.W. Van Der Meer, 1993. Role of acute-phase proteins in interleukin-1-induced nonspecific resistance to bacterial infections in mice. *Antimicrob. Agents Chemother.*, 37: 2527-2533.
- Waring, P.M., L.J. Waring, T. Billington and D. Metcalf, 1995. Leukemia inhibitory factor protects against experimental lethal *Escherichia coli* septic shock in mice. *Proc. Natl. Acad. Sci.*, 92: 1337-1341.
- Warner, A.E., M.M. DeCamp Jr., R.M. Molina and J.D. Brain, 1988. Pulmonary removal of circulating endotoxin results in acute lung injury in sheep. *Lab. Invest.*, 59: 219-230.
- Xia, D. and D. Samols, 1997. Transgenic mice expressing rabbit C-reactive protein are resistant to endotoxemia. *Proc. Natl. Acad. Sci.*, 94: 2575-2580.
- Zou, J., T. Wang, I. Hirono, T. Aoki and H. Inagawa *et al.*, 2002. Differential expression of two tumor necrosis factor genes in rainbow trout, *Oncorhynchus mykiss*. *Dev. Comp. Immunol.*, 26: 161-172.