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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Modifications in serum Amyloid A and Haptoglobin in mice following oral inoculation of graded doses of *P. multocida* type B: 2 and its lipopolysaccharide

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Abstract

This study was conducted to investigate the serum Amyloid A (SAA) and Haptoglobin (Hp) in the mice infected with *Pasteurella multocida* type B: 2 and its lipopolysacheride (LPS) inoculated through the oral route with graded doses (10^1, 10^3, 10^5, 10^7 and 10^9 cfu). Sixty healthy Balb c mice were placed in twelve plastic cages each one containing five mice. The mice were divided into three major groups (A, B and C). Group A is the control group (n = 10) and these were inoculated with 0.4 ml of PBS (pH 7.4) orally. The treatment groups (B; n = 25 and C; n = 25) were inoculated with *P. multocida* type B: 2 and its LPS respectively. The mice in group B and C were further divided into five subgroups. The subgroups were designated based on the graded doses as B10^1, B10^3, B10^5, B10^7 and B10^9 for *Pasteurella multocida* and C10^1, C10^3, C10^5, C10^7 and C10^9 for LPS. The mice were observed for 120 hours post-inoculation. The concentration of Hp was significantly higher ($P<0.05$) in the B10^9 cfu of *P. multocida* type B: 2 and LPS compared to the control group and the other treatments. In conclusion, *Pasteurella multocida* type B: 2 and its LPS are able to increase Hp in mice during acute infections.

Keywords: Lipopolysaccharides; *Pasteurella multocida* type B2; Acute phase proteins; graded doses; oral route; mice

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Introduction

Pasteurella multocida is a capsulated, Gram negative coccobacillus bacterium in the family Pasteurellacea that causes serious disease in a wide range of birds and mammals (Abdullah et al., 2013a). Hemorrhagic septicaemia (HS) is an acute septicaemia disease of cattle and water buffaloes caused by fastidious serotypes of *Pasteurella multocida* type B: 2 (Abdullah et al., 2013b). HS cause high morbidity and mortality leading to huge economic loss in the bovine industry in these parts of tropical Asia and Africa (Jesse et al., 2013a). Experimental transmission of *Pasteurella multocida* in cattle and buffalo happened successfully through intranasal and oral routes producing a syndrome with clinical signs and lesion which indicates that these may be the natural routes of infection (Bankirane and De Alwis, 2002; Shafarin et al., 2009; Ataei et al., 2009). The clinical symptom of this disease is often distinguished by rapid course, high fever, loud and stertorous breathing, profuse salivation, severe depression followed by death within 24 h of infection (Boyce et al., 2010; Abubakar and Zamri-Saad 2011; Jesse et al., 2013ab).

Pathogenesis of *Pasteurella multocida* is a complex interaction between host specific factors and specific bacterial virulence factors (Boyce and Adler 2006). There are varieties of virulence factors identified

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which include capsule, lipopolysaccharides, surface adhesions, iron regulated and iron acquisition proteins (Harper et al., 2006). Lipopolysaccharide (LPS) is an important virulence factor (Harper et al., 2011). LPS is the primary antigen for the identification of strains and is an integral component of the outer membrane essential for cell survival of Gram negative bacteria (Raetz and Whitfield, 2002; Peng et al., 2005; Moffatt et al., 2010). LPS has a dominant role during the host immune response (Horadagoda et al., 2001; Jesse et al., 2013abc; Faezet al., 2013a). In pathogens, LPS plays an important role in the disease process by interacting directly with innate host immune defence, leading to the activation of a range of host immune cells including the release of acute phase proteins (Raetz and Whitfield, 2002; Jesse et al., 2013bc).

Studies in veterinary medicine have demonstrated that the quantification of acute phase protein (APP) provides valuable clinical information in the diagnosis, prognosis and treatment monitoring of different pathologic processes (Martinez-Subiela et al., 2001; Eckersall and Bell, 2010; Tothova et al., 2013). The acute phase protein (APP) response is an innate nonspecific early response of the organism caused by various stimuli (Carroll et al., 2004; Dıaz et al., 2005; Grau-Roma et al., 2009), infection, tissue injury, neoplastic growth or immunologic disorders(Eckersall and Bell, 2010; Tothova et al., 2013; Khaleel et al., 2013). Serum Amyloid A (SAA) is a generic term for a family of apolipoproteins in mammals. The concentrations of SAA may increase during the acute-phase reaction to 1000-fold greater than those found in the non-inflammatory state within 24 hours (Khaleel et al., 2013; Jesse et al., 2013bc). The functions of SAA are thought to involve in host defence and lipid transport and metabolism (Eckersall and Bell, 2010; Tothova et al., 2013). Haptoglobin is a α2-glycoprotein. The major biologic function of Haptoglobin as a haemoglobin-binding protein conserving the iron released following red cell haemolyses and forms a stoichiometrically stable complex, to prevent haemoglobin-mediated renal parenchymal injury (kidney damage) and loss of iron following intravascular haemolysis (Lim, 2001; Cray et al., 2009). When this reaction occurs, the concentrations of Haptoglobin (Hp) and serum SAA increase.

Infection of mice with wild type *Pasteurella multocida* serotype B: 2 and its LPS in different doses in relation to the concentrations of SAA and Hp in a mice model has not been elaborated in previous studies. Therefore, the present study aims to investigate on acute phase protein responses in mice infected via oral route of inoculation with different doses (10^1, 10^3, 10^5, 10^7 and 10^9) of *Pasteurella multocida* serotype B: 2 and its LPS.

Materials and Methods

Animals

Sixty healthy Balb c mice of eight to ten weeks (both sexes) were enrolled in this study. They were obtained from the Institute of Cancer Research (ICR) and kept at the Animal Resource Centre, University Putra Malaysia. The animals were confirmed negative for *P. multocida* following culture of peripheral blood for bacterial isolations. The mice were housed in plastic cages and provided with water and pellet *ad libitum*. Five mice were kept in each plastic cage. The mice were observed for two weeks prior to the experiment to make sure that they were healthy and acclimatized.

Inoculums

Throughout the experiments, two types of inoculums were used; the whole cell and lipopoly-saccharide (LPS) extracted from *P. multocida* type B: 2.

Wild type of *P. multocida* serotype B: 2:

The wild-type *P. multocida* type B: 2 used in this study were obtained from stock culture. It was isolated from a previous 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, and Sulphur Indole Motility (SIM), Triple Sugar Iron (TSI) and citrate tests. The isolate was confirmed to be *P. multocida* 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 this experiment, LPS was extracted from different dosage of bacteria (10^1, 10^3, 10^5, 10^7 and 10^9 cfu). 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 lysed 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 1ml 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.

Experimental design in mouse model

Sixty healthy Balb c mice were placed in twelve plastic cages each one containing five mice. The mice were divided into three major groups (A, B and C). Group A was the control group (n = 10) and these were inoculated with 0.4 ml of PBS pH 7.4 orally. The treatment groups (B; n = 25 and C; n = 25) were inoculated with *P. multocida* type B: 2 and its LPS respectively. The mice in group B and C were further divided into five subgroups. The subgroups were designated based on the graded doses as $B10^1$, $B10^3$, $B10^5$, $B10^7$ and $B10^9$ for *Pasteurella multocida* and $C10^1$, $C10^3$, $C10^5$, $C10^7$ and $C10^9$ for LPS respectively. After 120 h of inoculation, clinical signs and mortality rates were observed. Mice showed severe clinical signs and survived mice after 120 h post-inoculation were sacrificed via cervical dislocation approach and post-mortem examination was performed. All procedures and experiments illustrated were undertaken under a project license approved by Animal Utilization Protocol Committee, Faculty of veterinary medicine, University Putra Malaysia, with reference number: UPM/IACUC/AUP-R017/2014

Acute Phase Proteins (APP) Analysis

The test kits were highly sensitive, two sites enzyme linked immunoassay (ELISA) obtained from Life Diagnostics Inc. (West Chester) and Tridelta Development Ltd (Ireland). The APP used in this study was the mouse serum amyloid A (SAA) and mouse Haptoglobin (Hp).

Determination of mouse haptoglobin (Hp)

Serum samples were used to determine the Hp levels by ELISA technique. 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 of 450 nm wavelengths in an ELISA Reader (BioRad).

Determination of mouse Serum Amyloid A (SAA)

Serum samples were used to determine the mouse 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 of 450 nm wavelengths in an ELISA Reader (BioRad).

Statistical analysis

All the data were analyzed using Anova and Tukey Kramer test with JMP® 11. NC: SAS Institute Inc. software Version. The data were considered significant at P<0.05.

Results

The concentrations of Hp increased significantly in mice inoculated orally at a dose rate of 10^9 cfu *P. multocida* type B: 2 and LPS after 120 h post inoculation compared to the control (PBS), 10^1, 10^3, 10^5 and 10^7 with no effect on SAA (Table 1 &2).

Discussion

HS is an infectious disease of bovines caused by serotypes B: 2 and E: 2 of *Pasteurella multocida* in Asian and African countries respectively. The disease is

Table 1: Modifications of Hp and SAA in mice following oral inoculation of graded doses of *Pasteurella multocida*type B: 2 after 120 hours of inoculation

Parameters	Phosphate Buffered saline solution	Concentration of *Pasteurella multocida* (cfu)				
	Control	10^1	10^3	10^5	10^7	10^9
Hp (ng/ml)	$0.14^b \pm 0.05$	$0.32^{ab} \pm 0.05$	$0.16^b \pm 0.05$	$0.23^{ab} \pm 0.05$	$0.38^{ab} \pm 0.05$	$0.48^a \pm 0.05$
SAA (ng/ml)	$0.14^a \pm 0.04$	$0.16^a \pm 0.04$	$0.15^a \pm 0.04$	$0.12^a \pm 0.04$	$0.16^a \pm 0.04$	$0\ .14^a \pm 0.04$

Values with different superscript within a row are significantly different (P<0.05); Hp = Haptoglobin; SAA = Serum Amyloid A; cfu = Colony forming unit

Table 2: Modifications of Hp and SAA in mice following oral inoculation of graded doses of LPS after 120 hours of inoculation

Parameters	Phosphate Buffered saline solution	Concentration of LPS in cfu				
	Control	10^1	10^3	10^5	10^7	10^9
Hp (ng/ml)	$0.14^b \pm 0.06$	$0.49^a \pm 0.05$	$0.37^{ab} \pm 0.05$	$0.41^{ab} \pm 0.05$	$0.34^{ab} \pm 0.05$	$0.61^a \pm 0.05$
SAA (ng/ml)	$0.14^a \pm 0.05$	$0.18^a \pm 0.05$	$0.16^a \pm 0.05$	$0.15^a \pm 0.05$	$0.17^a \pm 0.05$	$0.25^a \pm 0.05$

Values with different superscript in a row are significantly different ($P<0.05$); Hp = Haptoglobin; SAA = Serum Amyloid A; LPS = Lipopolysaccharide; cfu = Colony forming unit

endemic in most parts of tropical Africa and Asia including Malaysia. The results of the present study describe oral route of infection of mice with the wild type *P. multocida* strain B: 2 and its LPS extract from the same bacteria in graded doses. In the present study, the concentration of Hp increased appreciably through the oral route on inoculation with both *P. multocida* and its LPS. These findings are similar to the study conducted by Murata et al. (2004) and Eckersall and Bell (2010). Several studies in mice have shown protective properties of APPs against microbial challenge. This is evident in the present study where the concentrations of Hp increased in the mice inoculated with *P. multocida* and its LPS as reported by Hochepied et al. (2000), Szalai et al. (2000), Horadagoda et al. (2001), Abubakar and Zamri-Saad (2011), Khaleel et al. (2013) and Jesse et al. (2013c) or endotoxaemia and septic shock which is also similar to the studies carried by Jesse et al. (2013abc), Khaleel et al. (2013), Abdullah et al. (2013ab) and Faez et al. (2013b). Overall, no research has been carried out on the relationship between the effect of LPS extracted of *P. multocida* via oral route of inoculation on the serum APPs, particularly Hp and SAA in mice. In our knowledge, this is the first study to evaluate the acute phase response (APR) of mice to infection with *P. multocida* and its LPS extracted from the *P. multocida* type B: 2 in varied doses via oral route of inoculation. In this study, all treated groups showed increased concentrations of APR which is consistent with results of Khaleel et al. (2013), Jesse et al. (2013b) and Jesse et al. (2013c). The increase in APP, however, varies where the inoculation with LPS led to highest increased in Hp concentration, followed by *P. multocida* whole cells. This may be due to the ability to cause different degree of severity in tissue damage and inflammation as mentioned by Horadagoda et al. (2001), Jesse et al. (2013bc), Khaleel et al. (2013), and Faez et al. (2013a).

Raised levels of Hp observed in this study is considered useful because LPS extracted from *P. multocida* produced APR in mice after infection through oral route of inoculation since their concentration is of significance in experimental animals. These results were consistent with the result which showed an increase in the concentration of Hp and SAA in mice serum after inoculation orally with river water contaminated with *P. multocida* type B:2 (Khaleel et al., 2013). It is possible that the dose of LPS inoculated was sufficient to produce its endotoxic effects to alter the APR in mice inoculated orally. One dose inoculation of LPS through oral route produces the APR in the blood that mimic a response more closely associated with clinical septicaemia in the present study. There was an evident increased concentration of Hp via oral inoculation of *P. multocida* and its LPS in the current study and was consistent with the findings of Jesse et al. (2013bc) and Khaleel et al. (2013). Al-Hasanin et al. (2007) 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 intraperitoneal injections of LPS in mice at 8 h intervals caused the mice to become ill exhibiting reduced social and feeding activity, huddling behaviour with piloerection and shivering, increased skin turgor and weight loss and eventually led to hypothermia before death. Hajikolaei et al. (2006) concluded that as few as two organisms could produce 100% mortality in <24 hours after infection in mice. Following infection, with *Pasteurella multocida* at a dose rate of 1ml of 10^9 cfu in a study conducted by Abdullah et al. (2013b) two out 16 mice died per-acutely within 24 hours while 10 died in a similar manner within 48 hours. In the present study, after 120 hours of post inoculation with graded doses of 0.4ml of *Pasteurella multocida* and its LPS, no death was recorded. This could be due to the protracted time period and lower dosage of 0.4ml of 10^1, 10^3, 10^5, 10^7 and 10^9 cfu of *Pasteurella multocida* and its LPS respectively for the onset of infections to occur via the oral route. The evidence of APPs production in the LPS group indicates a new contribution to the available database. The Hp level increased significantly in mice infected with *P. multocida* type B: 2 and also in the LPS immunogen groups. Increased concentration of serum Hp was detected in these mice which suggest an acute disease such as HS (Cray et al., 2009; Khaleel et al., 2013). Therefore, the Hp is more sensitive APP for LPS and *P. multocida* type B: 2 infections in response to the bacterial immunogens via oral route of inoculation.

Conclusion

In conclusion, this result showed the potential effect of the graded doses of LPS and *Pasteurella*

multocida which enhanced the production of different types of haptoglobin in mice. Further, haptoglobin can be used as potential biomarkers for detection of HS in susceptible animals.

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