

Original Research Paper

Alterations in Interleukin-1 β and Interleukin-6 in Mice Inoculated Through the Oral Routes Using Graded Doses of *P. Multocida* Type b: 2 and its Lipopolysaccharide

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Abstract: The gram-negative bacterium *Pasteurella multocida* serotype B: 2 is an important veterinary and opportunistic human pathogen that causes deplorable welfare problems and colossal economic production losses in bovine and other animal species throughout the world. *Pasteurella multocida* type B: 2 and its LPS are associated with Haemorrhagic Septicaemia (HS) in mice as animal 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 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 lipopolysaccharide respectively. The mice in group B and C were further divided into five subgroups. The subgroups were designated based on the graded doses as B10¹, B10³, B10⁵, B10⁷ and B10⁹ for *Pasteurella multocida* and C10¹, C10³, C10⁵, C10⁷ and C10⁹ for LPS respectively. The mice were observed for 120 h post-inoculation. The concentration of IL-1 β was significantly higher (p<0.001) in the B10⁷ of *P. multocida* type B: 2 and C10⁹ of LPS compared to the control group and the other treatment groups. The concentration of IL-6 was significantly higher (p<0.001) in the B10³ cfu of *P. multocida* type B: 2 and C10⁹ cfu of LPS compared to the control group and the other treatment groups. Interleukin-1 β and interleukin-6 evaluated in this study were predominantly associated with HS in varying doses of 10¹, 10³, 10⁵, 10⁷ and 10⁹ in the whole cell and its LPS respectively. Therefore, the target of this study was to investigate on pro-inflammatory response in mice infected with graded doses of *Pasteurella multocida* serotype B: 2 and its LPS via the oral route of inoculation.

Keywords: Lipopolysaccharides, *Pasteurella Multocida* Type B2, Cytokines, Oral Route, Graded Doses, Mice

Introduction

Pasteurella multocida sero type B: 2 is a Gram-negative capsulated, cocco-bacillus which causes numerous deplorable diseases in a wild, domestic animals and humans (Jesse *et al.*, 2013a). The organism is the

etiological agent of Haemorrhagic septicaemia in cattle and water buffalo and it is predominant in South and South East Asia and to most parts of tropical Africa (Abubakar and Zamri-Saad, 2011; Kharb and Charan, 2013; Khaleel *et al.*, 2013; Abdullah *et al.*, 2013a; 2013b; Jesse *et al.*, 2013a; 2013b). HS is a peracute disease and

is considered to be one of the most economically important diseases in Asia particularly in South and South East Asia leading to huge economic loss in livestock industry (Jesse *et al.*, 2013a). Cattle and buffalo are the usual host of HS (Jesse *et al.*, 2013a; 2013b). *Pasteurella multocida* type B: 2 assumed to be transmitted between the animals by aerosol infection and ingestion of contaminated river water or material with *P. multocida* type B: 2 especially during the HS outbreak (Shafarin *et al.*, 2009; Ataei *et al.*, 2009; Khaleel *et al.*, 2013). The clinical indication of this disease is often characterised by rapid course, high fever, loud and stertorous breathing, profuse salivation, severe depression and followed by death generally within a day of infection (Boyce *et al.*, 2010; Jesse *et al.*, 2013b).

Pathogenesis of *P. multocida* is a complex interaction between host specific factors and specific bacterial virulence factors; therefore, understanding the disease pathogenesis is complex and depends on the bacterial strain, the animal model and their interactions (Hawari *et al.*, 2014). There are several disease models of infection with *P. multocida* using mice and show the suitability of mice to study the pathogenesis of *P. multocida* to produce HS with same symptoms and lesion in cattle and water buffalo (Affandi *et al.*, 2012; Jamal *et al.*, 2013; Faez *et al.*, 2013). In pure chemical form endotoxin is a Lipopolysaccharide (LPS) which has widely variable composition (or chemotype) and exerts a wide range of often opposing influences on host pathogen interactions (Khaleel *et al.*, 2013; Kharb *et al.*, 2013). The key virulence factors identified in *Pasteurella multocida* type B: 2 include capsule, lipopolysaccharides, surface adhesions, iron-regulated and iron acquisition proteins (Affandi *et al.*, 2012; Kharb *et al.*, 2013). The protein toxin from *Pasteurella multocida* type B: 2 experimentally induce all of the major symptoms of a number of economically important diseases in domestic livestock (Jamal *et al.*, 2013; Ataei *et al.*, 2009; Faez *et al.*, 2013). However, *Pasteurella multocida* is nonetheless an effective immunogen and mutant derivatives which showed potential for vaccine development (Ataei *et al.*, 2009). Despite continuing investigations for several decades, the bacterial components LPS have been reported as virulence factor responsible for immuno-pathological changes (Jesse *et al.*, 2013a; 2013b; 2013c). LPS is an integral component of the outer membrane of most gram-negative bacteria which is essential for cell survival and the humoral immune response to LPS varies and is dependent upon the host animal and on variations within the LPS structure (Moffatt *et al.*, 2010).

Cytokines are a diverse group of small proteins (<200 amino acids) that are secreted by a wide range of

cell types for the purpose of intercellular signalling and communication. About 30 cytokines are recognized, including the Interleukins (IL-1 β and IL-6) (Dionissopoulos *et al.*, 2012). Among the many functions of cytokines are the control of cell proliferation and differentiation and the regulation of angiogenesis and immune and inflammatory responses (Dionissopoulos *et al.*, 2012). IL-1 β is a potent pro-inflammatory cytokine produced by a variety of cell types such as white blood cells (monocytes, macrophages and neutrophils) and endothelium has been documented (Monisha and Saxena, 2012; Rania *et al.*, 2014). The principal effects of IL-1 β are: Acute-phase response, phagocyte activation, Inflammation, Haematopoiesis and fever. Under immune stimulation, IL-1 β also helps leukocytes pass through blood vessel walls to the site of infection and leads to fever by affecting areas of the brain that control body temperature (Dinarello, 2009; Monisha and Saxena, 2012; Rania *et al.*, 2014). Interleukin-6 (IL-6) is a multifunctional cytokine and produced by a variety of cell types in both tissues of immune and endocrine systems (Dionissopoulos *et al.*, 2012). It is extensively involved in promoting immune and haematopoietic bioactivities, such as interleukin gene expression, cell proliferation, B cell differentiation, T cell activation and neutrophil and macrophage differentiation (Gyenai *et al.*, 2012).

There are predominant alterations in cytokines particularly IL-1 β and IL-6 during a graded dose inoculation of mice with *P. multocida* serotype B: 2 and its LPS in the present study. To understand the pathogenesis of pasteurellosis, it is essential to investigate the profiles of host factors such as pro-inflammatory cytokines that are produced in relation to the alterations in cytokines with graded doses of *P. multocida* serotype B: 2 and its LPS. There are limited data in the literature on IL-1 β and IL-6 alterations as a result of inoculation of mice with graded doses of *P. multocida* serotype B: 2 and its LPS via the oral route. Therefore, the present study, reported the pro-inflammatory cytokine profiles of mice experimentally infected orally with varying doses of *P. multocida* serotype B: 2 and its LPS.

Materials and Methods

Animals

Sixty healthy Balb c mice of eight to ten weeks old of both sexes were enrolled 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; 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 2 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 Lipopolysaccharide (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 Hemorrhagic Septicemia (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 μ L 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 μ L of the supernatant was transferred into a new 1.5 mL centrifuge tube and 800 μ L 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 μ L 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

All mice included in the current study were approximately of the same age and strain. 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 lipopolysaccharide respectively. The mice in group B and C were further divided into five subgroups. The subgroups were designated based on the graded doses as B10¹-B10⁹ for *Pasteurella multocida* and C10¹-C10⁹ for LPS respectively. The mice were observed for 120 h post-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, Universiti Putra Malaysia, with the reference number: UPM/IACUC/AUP-R017/2014.

Analysis of Cytokines

Interleukin-1 (IL-1 β) and Interleukin-6 (IL-6) were determined from serum using high sensitivity and specificity (Cusabio®, Wuhan, China PR) mice IL-1 β and IL-6 ELISA kits in accordance to the manufacturer's specifications and protocol.

Statistical Analysis

All the data were analyzed using JMP® 9. NC: SAS Institute Inc. software Version. The data were considered significant at p<0.05.

Results

The concentration of IL-1 β was 1.45 ng mL⁻¹ and significantly higher (p<0.001) in the B10⁷ of *P. multocida* type B: 2 and 0.81 ng mL⁻¹ in C10⁹ of LPS compared to the control group and the other treatment groups. The concentration of IL-6 was 0.16 ng mL⁻¹ and significantly higher (p<0.001) in the B10³ of *P. multocida* type B: 2 and 0.14 ng mL⁻¹ in C10⁹ of LPS compared to the control group and the other treatment groups (Fig. 1-4).

Discussion

Haemorrhagic Septicaemia (HS) is ranked as the primary fatal disease in water buffaloes and is a cause

of major economic losses in cattle and water buffalo in Asian countries. The current understandings on bacterial interaction with the host suggest that *P. multocida* establishes a complex interaction in host tissues and utilizes available niches effectively to grow rapidly and cause diseases at favourable circumstances (Khaleel *et al.*, 2013). The mechanisms of acute disease pathogenesis and role of bacterial, LPS or host factors that induce pathology and the nature of the immune response to *Pasteurella multocida* are poorly understood. The present study investigated the role of the whole cell of *Pasteurella multocida* and its surface component (LPS) inoculated orally to observe the changes in pro-inflammatory cytokines (IL-1 β and IL-6) using mice as an experimental animal model *in vivo*.

The results of the present study describe infection of mice with the wild type *P. multocida* strain B: 2 and its LPS via oral route of inoculation in different doses of (10^1 , 10^3 , 10^5 , 10^7 and 10^9) cfu respectively. Jesse *et al.* (2013a; 2013b; 2013c) inoculated *Pasteurella multocida* via different routes to elicit infections in the animal models suitably. Similarly, in the present study graded doses of *Pasteurella multocida* and its LPS elicit immune responses in mice as a result of the infections. If the infection is to result in disease, the pathogen might also release toxins or toxic by-products, or interact with the host immune system (Harper *et al.*, 2011). The findings of (Harper *et al.*, 2011) is in agreement to the findings of the present study in which the inoculation of the bacterial LPS induces significant alterations in the concentrations of IL-1 β and IL-6 as a result of graded doses of (10^1 , 10^3 , 10^5 , 10^7 and 10^9) cfu respectively.

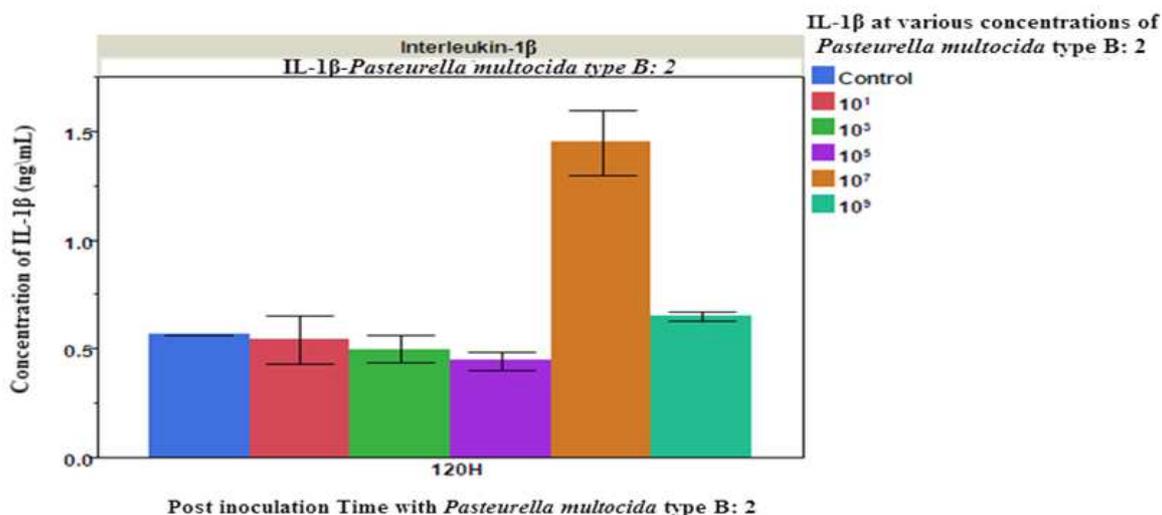


Fig. 1. Various concentrations of IL-1 β via oral inoculation with *Pasteurella multocida* type B: 2

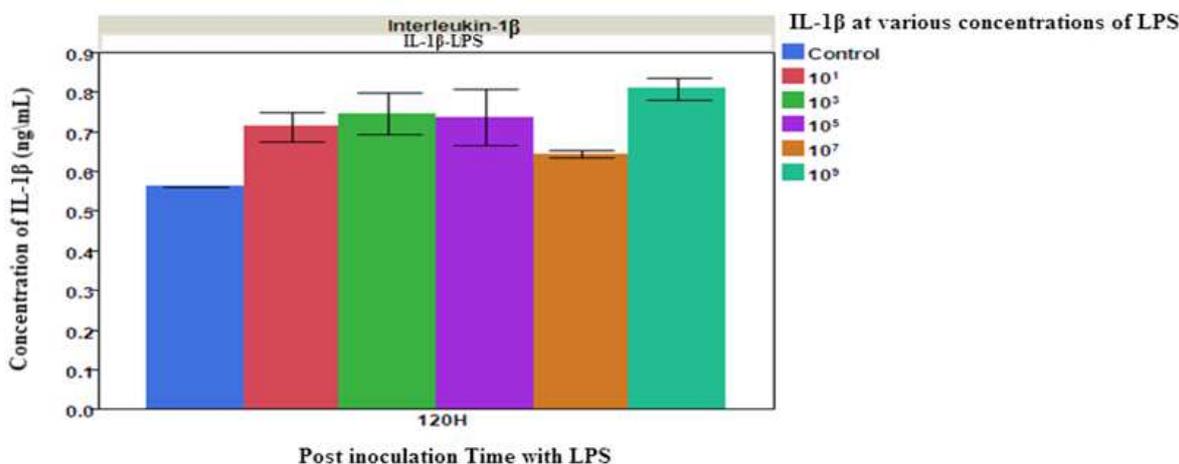


Fig. 2. Various concentrations of IL-1 β via oral inoculation of LPS

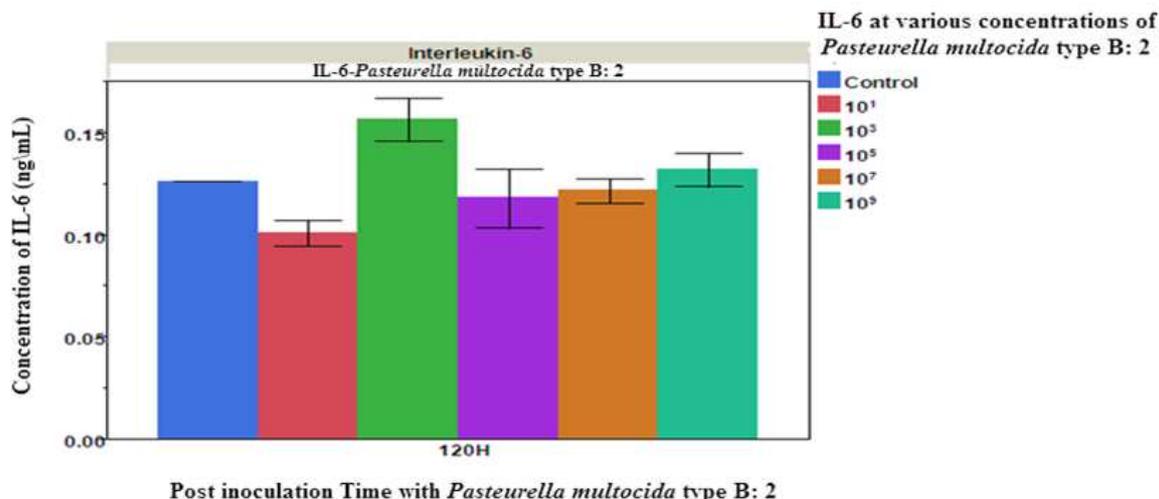


Fig. 3. Various concentrations of IL-6 via oral inoculation with *Pasteurella multocida* type B: 2

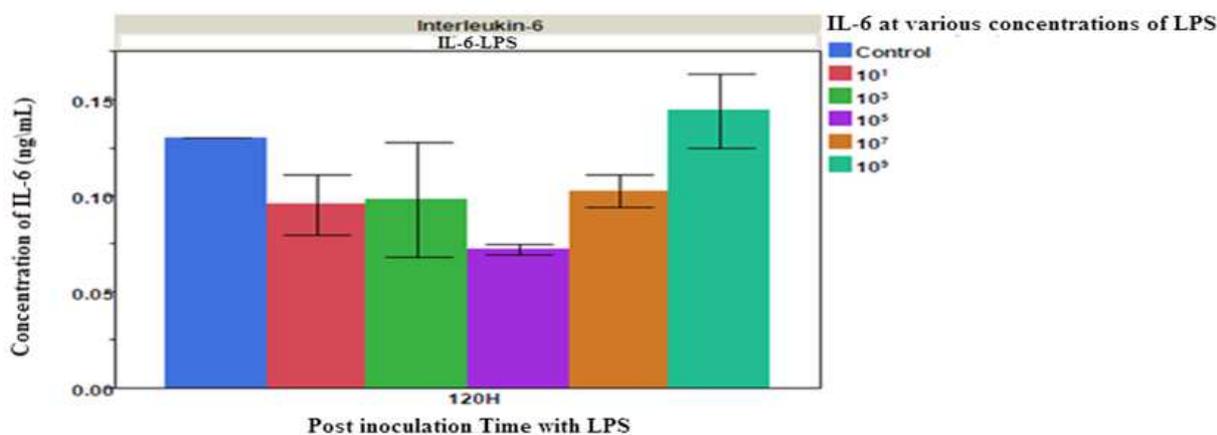


Fig. 4. Various concentrations of IL-6 via oral inoculation with LPS

In this study it seems that all the treatment groups of mice which were inoculated orally with *Pasteurella multocida* and its LPS have the same spectrum of activity in the production of acute infection of HS with significant increase in the concentration of inflammation cytokine (IL-1 β and IL-6) in the serum. This result agree with other researchers findings in the previous studies, Khaleel *et al.* (2013) showed in his study in which he challenge BALB/c mice intraperitoneally with as few as 20 cfu of *P. multocida* to produce an overwhelming septicaemia in mice within 30 h of post-inoculation. In the same manner (Jesse *et al.*, 2013b) produce clinico-pathological responses in calves inoculated with *Pasteurella multocida* and its LPS via the intramuscular and intravenous routes. Following infection, with *Pasteurella multocida* at a dose rate of 1 mL of 10⁹ cfu in a study conducted by Abdullah *et al.* (2013a) two

out 16 mice died per-acutely within 24 h while 10 died in a similar manner within 48 h. In the present study, after 120 h of post inoculation with graded doses of 0.4 mL of *Pasteurella multocida* and its LPS no death was recorded. This could be due to the protracted time period and lower dosage of 0.4 mL of 10¹, 10³, 10⁵, 10⁷ and 10⁹ cfu of *Pasteurella multocida* and its LPS respectively for the onset of infections to occur via the oral route.

Treatment groups of mice which were inoculated via oral routes with LPS showed high concentration of IL-1 β compared with groups of mice inoculated with *Pasteurella multocida* and control group at 120 h of post-inoculation. This result may be due to the route of inoculation and the potential doses used of *Pasteurella multocida* and its LPS. Furthermore, in different study by (Dionissopoulos *et al.*, 2012) found that there were increases in the concentration of IL-1 β associated with

pathogenesis of lung injury in bovine pneumonic pasteurellosis which significantly increased the levels of IL-1 β within the pneumonic lesions and in bronchoalveolar lavage cells from *P. haemolytica* infected calves and similar results were observed in the present study and those of (Praveena *et al.*, 2010) in mice infected with *Pasteurella multocida* serotype A: 1. This increase in IL-1 β in our work could be due to the effect of LPS which was inoculated orally into the mice producing the same effect of orally inoculated whole cell of *P. multocida* type B: 2 which induce HS symptoms leading to enhance immune defense to secrete high concentration of IL-1 β . Previous works widely showed that Lipopolysaccharide (LPS) and outer membrane proteins of gram negative bacteria play a dominant role during the host immune response (Harper *et al.*, 2011). The LPS of *P. multocida* has endotoxic properties, particularly in buffalo, where the injection of purified LPS from a serogroup B strain was able to mimic the clinical signs of bovine haemorrhagic septicaemia (Jesse *et al.*, 2013b; 2013c). There were increases in the concentration of IL-6 with 0.4 mL of 10³ cfu in the mice that were inoculated orally with *Pasteurella multocida* type B: 2 compared with those inoculated with LPS and the control group. Schmidt *et al.* (2014) 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 (Schmidt *et al.*, 2014) exhibiting reduced social and feeding activity, huddling behavior with piloerection and shivering, increased skin turgor and weight loss and eventually led to hypothermia before the mice became moribund. However, *Pasteurella multocida* type B: 2 exert its effect at a concentration higher with respect to LPS and the induction starts later and lasts longer. Active concentration of both *Pasteurella multocida* and its LPS can frequently be reached at infection sites either from gram-negative bacteria outer membrane blebbing (LPS) or bacterial lysis as in consequence of host defence (Harper *et al.*, 2011). LPS and *Pasteurella multocida* may initiate interaction with host cells either by adherence, either by engaging a cascade of secondary molecules that have abilities to affect host defense systems. In a study conducted by (Zhao *et al.*, 2009) in mice infected with *Pasteurella multocida* showed significantly enhanced immunity in the mice to the bacteria vaccine, which increased IL-6 in the blood of mice. This study is similar to the current study in which IL-6 was found to increase post inoculation with *Pasteurella multocida* via the oral route compared to

those mice infected with LPS and PBS orally. However, IL-6 level was found to be higher in the treatment group of mice which were inoculated with *Pasteurella multocida* with 0.4 mL of 10³ cfu compare with treatment group of mice inoculated with LPS with 0.4 mL of 10⁹ cfu which increased after 120 h post-inoculation. It has been reported that IL-6 regulates the transition from neutrophil to monocyte recruitment during inflammatory process (Praveena *et al.*, 2010). This could be due to the increased concentration of *P. multocida* which was inoculated orally. Pro-inflammatory mediators and especially (IL-1 β and IL-6) were reported to enhance leukocyte transmigration into the site of infection by favouring expression of cell adhesion molecules and production of eicosanoids and prostaglandins that perpetuate the inflammatory cascade in septicemia (Praveena *et al.*, 2010). The higher levels of (IL-1 β and IL-6) cytokines in the mice serum in this research work could also be attributed to fever, anorexia and behavioural changes observed in the infected animals (Dionissopoulos *et al.*, 2012; Gyenai *et al.*, 2012; Schmidt *et al.*, 2014). The increased in serum cytokine levels of pro-inflammatory cytokines (IL-1 β and IL-6) at 120 h post-inoculation could perchance be due to the damage of the tissue cells and infiltration of inflammatory cells into the sites of infections. The increase in the secretion of IL-1 β and IL-6 was detected in the serum of the mice in the treatment groups which were inoculated orally with different doses of LPS. It is possible that the dose of LPS inoculated was sufficient to produce its endotoxic effects to alter the pro-inflammatory cytokines (IL-1 β and IL-6) in mice inoculated orally. A one dose inoculation of LPS through oral route produces the pro-inflammatory cytokines (IL-1 β and IL-6) in the blood that mimic a response more closely associated with clinical septicaemia. The previous studies were similar to the current study where there was an evident increase in the concentration of pro-inflammatory cytokines (IL-1 β and IL-6) via oral inoculation of *P. multocida* and its lipopolysaccharide using graded doses.

Conclusion

In the current study, serum concentrations of pro-inflammatory cytokines (IL-1 β and IL-6) were affected by the graded doses of *P. multocida* and its polysaccharide and caused HS in the treatment groups of mice. The severity of host cell response differs between each group. The immunogen group of mice showed changes in pro-inflammatory cytokines (IL-1 β and IL-6) concentrations. The present study showed significant differences in the levels of the pro-inflammatory cytokines (IL-1 β and IL-6) in mice experimentally

infected with *Pasteurella multocida* and its lipopolysaccharide. It is assumed that these differences in serum concentrations of pro-inflammatory cytokines (IL-1 β and IL-6) in the present study were mainly due to the graded doses of 10¹, 10³, 10⁵, 10⁷ and 10⁹ cfu of *Pasteurella multocida* and its LPS respectively. In conclusion, this result showed the potential effect of the graded doses of LPS and *Pasteurella multocida* which enhanced the immune responses in the animal models thereby increasing the production of different types of pro-inflammatory cytokines particularly IL-1 β and IL-6 in the serum of the mice. Therefore, it is concluded that these quantitative indicators (IL-1 β and IL-6) can be used as a potential biomarkers for detection of HS in susceptible animals.

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Author's Contributions

All authors equally contributed in this work.

Ethics

This article is original and contains unpublished materials. The corresponding author confirms that all of the other authors have read and approved the manuscript and no ethical issues involved.

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