Haematological and Histopathological Vicissitudes Following Oral Inoculation of Graded Doses of *Pasteurella multocida* Type B: 2 and its Lipopolysaccharide in Mice

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Abstract

*Pasteurella multocida* type B: 2 is responsible for major animal diseases of economic importance in both developed and developing countries. Haemorrhagic Septicaemia (HS) could inflict devastating effects on blood tissues and organs in the host animal. Therefore, the current study aims to investigate the haematological and clinico-pathological responses in mice following oral inoculation of graded doses of *Pasteurella multocida* type B: 2 and its lipopolysaccharide. 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 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 hours post-inoculation. The clinical signs (Ruffled fur, Ocular discharges, Level of alertness and Laboured breathing) were significantly different (p<0.0001) in mice inoculated orally with variable doses of *Pasteurella multocida* type B: 2 and its LPS. RBC, PCV, haemoglobin concentrations, PT, APTT, Thromboocyte, WBC, Lymphocytes, monocytes, plasma proteins, band and segmented neutrophils were significantly different (p<0.0001) in mice inoculated with graded doses of *Pasteurella multocida* type B: 2 and its LPS. In conclusion, 109 cfu of *Pasteurella multocida* type B: 2 and its lipopolysaccharide have devastating effects on organs and blood tissues.

Keywords: *Pasteurella multocida* type B: 2; Lipopolysaccharides; Vital organs; Blood; Oral route; Mice

Introduction

The Gram-negative bacterium *Pasteurella multocida* is of substantial economic significance in the livestock industry around the world and it is an opportunistic human pathogen [1]. Haemorrhagic Septicaemia (HS) is an acute high mortality systemic disease of cattle and water buffaloes [2,3] leading to huge economic loss in the bovine industry particularly in South East Asia [4,5]. However, in the context of susceptibility buffaloes were found to be more susceptible to the disease in comparison to others [6-8]. The common HS serotypes which have been reported to be responsible of recurrent outbreaks in Asia are the serotypes B: 2 [1,5,9]. In Malaysia, the stressful condition developed and developing countries. Haemorrhagic Septicaemia (HS) could inflict devastating effects on organs and blood tissues. Therefore, the current study aims to investigate the haematological and clinico-pathological responses in mice following oral inoculation of graded doses of *Pasteurella multocida* type B: 2 and its lipopolysaccharide. 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 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 hours post-inoculation. The clinical signs (Ruffled fur, Ocular discharges, Level of alertness and Laboured breathing) were significantly different (p<0.0001) in mice inoculated orally with variable doses of *Pasteurella multocida* type B: 2 and its LPS. RBC, PCV, haemoglobin concentrations, PT, APTT, Thromboocyte, WBC, Lymphocytes, monocytes, plasma proteins, band and segmented neutrophils were significantly different (p<0.0001) in mice inoculated with graded doses of *Pasteurella multocida* type B: 2 and its LPS. In conclusion, 109 cfu of *Pasteurella multocida* type B: 2 and its lipopolysaccharide have devastating effects on organs and blood tissues.

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can result in immuno-histopathological changes in the vital organs and blood tissue of death hosts [22]. A recent study of experimental nature has confirmed the development of typical HS following inoculation of Pasteurella multocida type B: 2 and its LPS in mice and calves [3,5,8].

Material 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 Pasteurella 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 Pasteurella multocida serotype B: 2

The wild-type Pasteurella multocida type B: 2 used in this study were obtained from stock culture. It was isolated from a previous outbreak of Hemorrhagic septicaemia (HS) in the state of Kelantan, Malaysia. Identification of Pasteurella multocida type B: 2 were 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 Pasteurella 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 hours. A single colony of Pasteurella multocida type B: 2 was selected and sub-cultured onto 5% horse blood agar and incubated at 37°C for 18 hours. A single colony of Pasteurella multocida type B: 2 was selected for moderate (60% of abnormality), 3 for severe (more than 60% of abnormality) of clinical signs observed, 1 for mild (30% of abnormality), and 0 represent no abnormality of clinical signs observed.

Lesions scoring and statistical analysis

Histopathological changes observed (degeneration and necrosis, inflammation and congestion) were scored based on the following categorization; 0 (normal), 1: Mild (less than 1/3 of field involved), 2: Moderate (between 1/3 and 2/3 of field involved) and 3: Severe (more than 2/3 of the field involved). Six microscopic fields were examined for each lesion per slide and the mean ± standard error was calculated for each organ based on the different lesions observed.

Evaluation of clinical signs

Clinical signs such as ruffled fur, laboured breathing, alertness and ocular discharges were assessed for a period of 120 hours. In summary, the clinical signs of the twelve groups were scored on scale of 0-3 based on the presence of the following clinical signs: ruffled fur, laboured breathing, alertness and ocular discharges. The score 0 represent no abnormality of clinical signs observed, 1 for mild (30% of abnormality), 2 for moderate (60% of abnormality), 3 for severe (more than 60% of abnormality).

Statistical Analysis

All the data were analyzed using JMP® 11. NC: SAS Institute Inc. software Version. The data were considered significant at p<0.05.

Results

Inflammatory cells, congestion, degeneration and necrosis were...
significantly different (p<0.0001) in mice inoculated with graded doses of both Pasteurella multocida type B: 2 and its LPS (Figures 1 and 2). The clinical signs (Ruffled fur, Ocular discharges, Level of alertness and Laboured breathing) were significantly different (p<0.001) in mice inoculated orally with variable doses of Pasteurella multocida type B: 2 and its LPS. Red Blood Cells (RBC), Packed Cell Volume (PCV), Haemoglobin Concentrations (Hb), Prothrombin time (PT), Activated Partial Thromboplastin Time (APTT), Thrombocyte, White blood cells (WBC), Lymphocytes, monocytes, band and segmented neutrophils, plasma proteins were significantly different (p<0.0001) in

Figure 1: Histopathological changes in the Lungs (G), Intestines (I), Kidney (K), Liver (L), and Spleen (S) in mice inoculated with graded doses of Pasteurella multocida type B: 2 phosphate buffered saline (control group).

Figure 2: Histopathological changes in the Lungs (G), Intestines (I), Kidney (K), Liver (L), and Spleen (S) in mice inoculated with graded doses of LPS and phosphate buffered saline (control group).
mice inoculated with graded doses of *Pasteurella multocida* type B: 2 and its LPS (Tables 1-4).

**Discussion**

In Malaysia, the large ruminant sector is endangered by Haemorrhagic Septicaemia (HS) caused by *Pasteurella multocida* type B: 2. The protein toxin from *Pasteurella multocida* type B: 2 experimentally induces all of the major symptoms of Haemorrhagic Septicaemia (HS) in cattle and water buffaloes. The lipopolysaccharide (LPS) has a role in disease processes as they act at an interface between the host and pathogen [23]. To the researchers knowledge this is the first study to comprehensively evaluate the haematological and histopathological changes in mice following 120 hours of inoculation with graded doses of *Pasteurella multocida* type B: 2 and its LPS through the oral routes.

The commonly used route of inoculation of *Pasteurella multocida* type B: 2 and its LPS employed by most researchers in Balb c mice is the intraperitoneal route [23]. Other routes that are used, less commonly, include subcutaneous and intramuscular [4]. The routes of inoculation used in these studies differ in most cases with other research, in our research we used graded doses of *Pasteurella multocida* type B: 2 and its LPS via the oral route and observed the mice for 120 hours post inoculation. The clinical signs observed which include ruffled fur, laboured breathing, closure of the eyes and also discharge from the eye were more severe in groups inoculated with 10^9 cfu of *Pasteurella multocida* type B: 2 and it's LPS through the oral routes compared to the control and other treatment groups. These findings were similar to those reported by Jesse et al. [5] which described an experiment following intraperitoneal inoculation with the same organism. The present study showed that mice can succumb to experimental HS infection following oral inoculation of graded doses of *Pasteurella multocida* type B: 2 and its LPS, and the clinical signs observed were similar and classical to what was observed in natural HS infection in other research work [21]. All the groups of mice inoculated with *Pasteurella multocida* type B: 2 and its LPS did not die during the experiment period of 120 hours. Khamb and Charan [16] reported that regardless of the route of inoculation, mortality in mice was 80% in all the groups they studied. The absence of death in the present study could be due to the protracted time period and the route of inoculation with the graded doses of *Pasteurella multocida* type B: 2 and its LPS [24].

Like the LPS of other Gram-negative pathogens, *Pasteurella multocida* LPS 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 [25]. Oral inoculation of *Pasteurella multocida* type B: 2 and its LPS into Balb c mice in the present study was able to produce histopathological changes

<table>
<thead>
<tr>
<th>PM Parameters</th>
<th>RBC (10^12/L)</th>
<th>Hemoglobin (g/L)</th>
<th>PCV (L/L)</th>
<th>WBC (×10^9/L)</th>
<th>Neutrophils (×10^9/L)</th>
<th>Monocytes (×10^9/L)</th>
<th>Eosinophils (×10^9/L)</th>
<th>Plasma Protein (g/L)</th>
<th>PT (seconds)</th>
<th>APPT (seconds)</th>
<th>Thrombo (×10^9/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9.26 ± 0.047</td>
<td>8.89 ± 0.047</td>
<td>0.46 ± 0.005</td>
<td>7.72 ± 0.042</td>
<td>0.15 ± 0.007</td>
<td>0.52 ± 0.0067</td>
<td>0.320 ± 0.0061</td>
<td>62.66 ± 0.475</td>
<td>26.46 ± 0.361</td>
<td>53.30 ± 0.516</td>
<td>407.54 ± 1.12</td>
</tr>
<tr>
<td>10^1 (cfu)</td>
<td>8.89 ± 0.047</td>
<td>8.77 ± 0.047</td>
<td>0.44 ± 0.005</td>
<td>5.22 ± 0.042</td>
<td>0.19 ± 0.007</td>
<td>0.53 ± 0.0069</td>
<td>0.362 ± 0.0061</td>
<td>68.90 ± 0.475</td>
<td>37.72 ± 0.361</td>
<td>63.12 ± 0.516</td>
<td>549.10 ± 1.12</td>
</tr>
<tr>
<td>10^3 (cfu)</td>
<td>8.67 ± 0.047</td>
<td>8.43 ± 0.043</td>
<td>0.392 ± 0.005</td>
<td>4.92 ± 0.042</td>
<td>0.22 ± 0.007</td>
<td>0.53 ± 0.0069</td>
<td>0.408 ± 0.0061</td>
<td>70.20 ± 0.475</td>
<td>43.50 ± 0.361</td>
<td>69.30 ± 0.516</td>
<td>634.72 ± 1.12</td>
</tr>
<tr>
<td>10^5 (cfu)</td>
<td>8.47 ± 0.047</td>
<td>139.84 ± 0.243</td>
<td>0.368 ± 0.005</td>
<td>4.2 ± 0.042</td>
<td>0.22 ± 0.007</td>
<td>0.53 ± 0.0069</td>
<td>0.409 ± 0.0061</td>
<td>74.70 ± 0.475</td>
<td>49.66 ± 0.361</td>
<td>77.96 ± 0.516</td>
<td>705.76 ± 1.12</td>
</tr>
<tr>
<td>10^7 (cfu)</td>
<td>8.25 ± 0.047</td>
<td>137.02 ± 0.243</td>
<td>0.318 ± 0.005</td>
<td>3.92 ± 0.042</td>
<td>0.22 ± 0.007</td>
<td>0.53 ± 0.0069</td>
<td>0.409 ± 0.0061</td>
<td>79.64 ± 0.475</td>
<td>56.56 ± 0.361</td>
<td>84.12 ± 0.516</td>
<td>779.34 ± 1.12</td>
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<tr>
<td>10^9 (cfu)</td>
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<td>134.82 ± 0.243</td>
<td>0.306 ± 0.005</td>
<td>3.1 ± 0.042</td>
<td>0.22 ± 0.007</td>
<td>0.53 ± 0.0069</td>
<td>0.409 ± 0.0061</td>
<td>87.20 ± 0.475</td>
<td>63.34 ± 0.361</td>
<td>97.34 ± 0.516</td>
<td>837.80 ± 1.12</td>
</tr>
</tbody>
</table>

Values with different superscript in a row are significantly different (P<0.05); PM: *Pasteurella multocida* type B: 2; cfu: Colony Forming Unit.

**Table 1:** Modifications in Hematological parameters in mice following oral inoculation of graded doses of *Pasteurella multocida* type B: 2 after 120 hours of inoculation.

**Table 2:** Modifications in clinical signs in mice following oral inoculation of graded doses of LPS after 120 hours of inoculation.

Values with different superscript in a row are significantly different (P<0.05); PM: *Pasteurella multocida* type B: 2; LPS: *Pasteurella multocida* type B: 2; cfu: Colony Forming Unit.

**Table 3:** Modifications in hematological parameters in mice following oral inoculation of graded doses of *Pasteurella multocida* type B: 2 after 120 hours of inoculation.
in the vital organs. These findings were similar to those reported in the experimental infection of mice and buffalo calves via oral exposure [15,26-29]. However, the use of graded doses of Pasteurella multocida type B: 2 and its LPS as used in this study was not previously reported in mice following oral inoculation. HS is a deadly infection and the extent of lesion manifestation depends on the duration of the disease and the dose of bacterial inoculums [16]. In the study, mice inoculated with 10⁹ cfu of P. multocida and its LPS showed extensive histopathological changes in most organs and the most common lesions observed were inflammation, necrosis, degeneration and congestion. This showed that Pasteurella multocida type B:2 and its LPS were more severe in the 10⁹ cfu, perhaps due to the effective doses of the bacteria and its immunogen. Nevertheless, the strong effect of LPS in this study produces an extensive histopathological changes in the vital organs in the treatment groups of mice which were inoculated with LPS extracted from 10⁹ cfu of Pasteurella multocida type B: 2. This could be due to the sufficient dose of 10⁹ cfu of LPS that was inoculated via oral route to produce the HS histopathological lesions. It is also possible that the LPS dose of 10⁹ cfu which was inoculated via the oral route was able to produce its endotoxic effects to alter the histopathological response in the Balb c mice. A dose of 10⁹ cfu of LPS inoculation through oral route produces histopathological changes in the vital organs that mimic a response more closely associated with clinical sepsis in the present study. Some studies denoted that endotoxin or LPS is needed to be infused continuously since endotoxin is rapidly cleared by the mononuclear phagocytic system [3,5,8,15,28]. Abdullah et al. [29] 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 hours intervals caused the mice to become ill [30]. For infection to occur, the bacterial pathogen must have the capability to penetrate the mucus membrane of the buccal cavity and the epithelial layers of the digestive system and multiply whilst evading the host immune system. If the infection is to result in disease and cause cellular changes, the pathogen might also interact with the host in a way which might result in disturbance of homeostasis [16]. In the present study, the infection of Balb c mice orally with graded doses of Pasteurella multocida type B: 2 and its LPS was able to mimic the natural scenario and it was observed that the bacteria and its LPS were successfully recovered and they were able to produce haematological and histopathological changes in the mice through oral routes of inoculation. The haematological and histopathological changes observed were also similar with the mice and calves inoculated with this bacteria and its LPS [15,24]. Besides that, the histopathological lesions were also similar to those of the cattle infected naturally and experimentally with Pasteurella multocida type B: 2 [12,31,32]. In a related study, Jesse et al. [5,8] observed that calves and mice inoculated intramuscularly and intravenously developed classical HS with the presence of degeneration, necrosis, hemorrhage and inflammatory cells. It was observed that the severity of histopathological lesions in the present study was moderate to severe following oral routes of inoculation of 10⁹ cfu of the bacteria. Similar observations were made by Abdullah et al. [29], Rhoades et al. [33] and Horadagoda et al. [34] following oral inoculation of Pasteurella multocida type B: 2 in mice. The least commonly observed lesion at histopathology was congestion. These lesions were mild following inoculation with 10⁴ and 10⁵ cfu and mild to moderate on inoculation with 10⁶ and 10⁷ cfu and severe with dose of 10⁹ cfu in all the organs. Jesse et al. [5,8] reported a similar finding in calves and mice inoculated with Pasteurella multocida type B: 2 and its LPS in which thrombosis was mild in all the groups.

Haematological changes caused by bacterial infections are first detected during routine blood count. However, an animal’s defensive mechanisms can react quite differently to different bacteria; therefore, there is no singular pattern in complete blood count that indicates a bacterial infection. Nevertheless, there are few abnormalities that are suggestive of bacterial infection such as neutrophilia with a left shift being the hallmark of acute inflammation [35,36]. Results from the present study revealed significant reduction in the red blood cell counts in all treated groups. This is consistent with Walton et al. [37] whose findings concluded that inflammation is able to reduce red blood cell count. As part of the bacteriostatic mechanisms, inflammatory mediators such as tumor necrosis factor up-regulate ferritin and transfer macrophage receptors and thus, promote iron storage in the mononuclear phagocytic system. The shifting of iron to storage plus the use of iron by bacteria makes iron less available as erythroid precursors thus, leading to anaemia [37].

Neutrophilic leukocytes are critical component of the host system, forming the first line of cellular defense against invading organisms. Neutrophils normally are released from bone marrow as mature cells, which after a brief period in circulation transmigrate through the vascular endothelium into tissues. Their primary function is ingestion
and killing of bacteria [35,36]. To perform its functions, neutrophils employ mediators that promote inflammation and eliminate invading microorganisms. Mounting evidence indicates that neutrophils are not only end-stage effectors of the inflammatory response, but also modulate the immune response [37]. Increased peripheral blood neutrophil counts or neutrophilia reflects physiological, pathological or xenobiotic induced states [15,24]. Total blood and circulating neutrophil pools are increased in the present study in the treatment groups inoculated with graded doses of Pasteurella multocida type B: 2 and its LPS. Neutrophilia with a left shift is the classic response to inflammation, accompanied by lymphopenia, reflecting endogenous steroids release. The decrease in monocytosis in the present study may reflect a response to endogenous mediators of inflammation [15,24,35,36]. In the present study, inoculation of whole cells and its LPS resulted in significant increase in neutrophil counts, which is consistent with observations by other workers [35-37]. The increased neutrophils count in the present study denotes HS [15,24]. Prothrombin Time (PT) and Activated Partial Thromboplastin Time (APTT) are coagulation profiles. The protracted time period or the increase in PT and APTT in the current study was per chance associated to the degree of infections produced by the graded doses of Pasteurella multocida and its LPS via the oral routes. This findings agrees with a study conducted on dengue fever which also produced a protracted PT and APTT [38,39]. The increase in eosinophils and Plasma proteins in the present study could be related with the infections induced by inoculation of graded doses of Pasteurella multocida and its LPS. This is similar to the study conducted by Faez et al. [15] and Lin et al. [24] on mice and calves infected with 10^9 cfu of Pasteurella multocida and its LPS via the intraperitoneal routes. Furthermore, the increase in eosinophils in the present study could perchance be associated with the congestion observed in the treatment groups. Jesse et al. [5,8] reported a similar finding in calves and mice inoculated with Pasteurella multocida type B: 2 and its LPS in which thrombosis was mild in all the groups [40].

Conclusion

In conclusion, this result showed the possible effect of 10^9 cfu of Pasteurella multocida and its LPS in causing devastating effects on vital organs and blood tissues of susceptible animals compared to the treatment groups.

Acknowledgement

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