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Clinico-pathological Features in Mice Following Oral Exposure to *Pasteurella Multocida B: 2*

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Abstract: *Haemorrhagic septicaemia (HS) is a major cause of losses to livestock production in many countries around the world. In Malaysia, more specifically, the disease yet remains a major constraint to the national industry. However, the pathogenesis of haemorrhagic septicaemia is another scenario in which the limitations still exists. Thus, the present paper provides more information on the pathogenicity and host response dynamics in a mouse model. Our study of experimental nature manipulates P. multocida serotype B:2, the bacterium responsible for the disease in Asia. In this study, sixteen mice (n=16) were divided into two groups (A & B) of 8 mice each group. Animals in group A were inoculated orally with 1.0 ml 10^9 cfu/ml of P. multocida type B while mice in group 2 were challenged orally with 1.0 ml of phosphate buffer saline (PBS). The animals were observed for clinical signs for 5 days. The mice showing severe signs and surviving mice after 5 days of post-inoculation were euthanized using cervical dislocation approach and the organs such as heart, lung, kidney, stomach, spleen, colon and small intestine were collected for microscopic examinations. The result indicated that mice inoculated with the Pasteurella multocida showed significant ($p<0.05$) severe clinical signs compared to control group.These clinical signs ranged from mild to severe in which most of individuals infected with Pasteurella multocida showed moderate to severe clinical signs of ruffled hair, laboured breathing, eye discharge and responsiveness with mean levels of 2.13±0.64, 1.88±0.99, 1.50±1.20 and 1.88±0.99 respectively in comparison to the control group. Moreover, mortality rate was recorded between 24 to 50 h post-inoculation in the group that challenged with Pasteurella multocida type B: 2. Microscopically, the extent of visceral tissue damages due to the infection was scored. The interested parameters included pulmonary oedema, presence of inflammatory cells, haemorrhage and necrosis. Of these parameters, animals in infected group showed significant ($p<0.05$) differences in all most all visceral organs. Lungs, liver and kidney were, in particular, the most predominantly affected tissues. Therefore, oral inoculation of P. multocida type B in mice showed significant clinical response and cellular changes.*

Keywords: *Pasteurella multocida type B, oral inoculation, histopathology, clinical signs, mice*

I. Introduction

Haemorrhagic septicaemia (HS) is caused by *Pasteurella multocida* in the family Pasteurellacaea (De Alwis, 1999). HS is a primary pasteurellosis in cattle and buffaloes. It occurs through the ingestion of contaminated foodstuff, infection from normal carrier animals or from clinical cases (Radostits *et al.*, 2000). Carcasses dumped into rivers, tanks and channels and carried downstream are often incriminated as a likely method of spread of the disease and it is believed that the organism can survive in animal tissues, and perhaps in decomposing carcasses, for a few days (Joseph, 1979; De Alwis, 1999). The commonly used route of inoculation for HS experiment in mice is intraperitoneal (Ramdani *et al.*, 1990). Other routes that are used, less commonly, include subcutaneous and intramuscular (OIE, 2008). Rodostits *et al.* in 2000, on the other hand, speculated that the transmission of HS can occur via ingestion of contaminated foodstuff. Studies done by Zamri and Abubakar in 2011 did answer the question that HS can be transmitted orally as inoculation using the strain of *P. multocida* type B:2 in buffaloes producing expected typical clinical changes. However, much is not yet documented where mass of knowledge about the organism is critically needed. Therefore, the disease was modelled using experimental animals to mimic the actual host in the sense of their biological relationship. Hence, we will be able to obtain more information on clinical response and pathological changes in these experimental units that associated with *Pasteurella multocida type B* infection via oral route.

II. Materials and Methods

Inoculum Preparation (*P. multocida type B*)

Stock of *P. multocida type B* was obtained from Clinical Research Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM). The organism was cultured onto 5% horse blood agar and incubated at 37°C for 18 h. Thereafter, Brain Heart Infusion broth (BHI) was seeded with single colony and incubated at 37°C for 24 h to produce the inoculums which contained 10^9 colony-forming units/mL (CFU). Mac Farland technique was used to determine the concentration of *P.multocida type B: 2*.

Animals, experimental design and inoculation

Sixteen apparently health, 2-3 weeks of old, both sexes were enrolled in this study. The experiment was carried out after 2 weeks of acclimatization period. The mice were divided into two groups (A & B). Mice of group A (treatment group; n=8) were inoculated orally with 1.0 ml of 10^9 colony forming unit (CFU) of *P. multocida type* B: 2, while group B (control group; n=8) were inoculated orally with 1.0 ml sterile phosphate buffered saline (PBS); pH 7. Within 120 h after inoculation, clinical signs and mortality rates were observed. Mice showed severe clinical signs and survived mice after 120 h post-inoculation was sacrificed via cervical dislocation approach and postmortem examination was performed. Tissue samples were obtained from heart, lung, liver, spleen, stomach small intestine and large intestine for histo-pathological purposes. 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 reference number: UPM/FPV/PS/3.2.1.551/AUP-R120.

Clinical signs and Scoring

The clinical observations presented by the diseased and non-diseased groups were regularly documented from the start of the study until the end point of the experiment. Because the scoring systems require capture of signs and symptoms since the beginning of the experiment, the information collected from the two groups (A & B) was based on the individual presentation of the clinical signs. In summary, the clinical signs of the two groups (A & B) were scored in scale of 0-3 based on the presence of following parameters: ruffled hair, eye discharge, movement and responsiveness. The score 0 represented no abnormality of clinical signs observed, 1 for mild (30% abnormality), 2 for moderate (60% abnormality), 3 for severe (more than 60% abnormality).The evaluation method of the scoring system was summarized in Table I.

Table I
Clinical Observation Scoring System

Parameters	Clinical Score			
	0	1	2	3
Ruffled hair	Normal fur	Ruffled fur by 30% of the body	Ruffled fur by 60% of the body	Ruffled fur more than 60% of the body
Eye discharge	No discharge from the eyes	Eye discharge by 30%	Eye discharge by 60%	Eye discharge more than 60%
Movement	Normal movement and appetite	Reduced movement by 30%	Reduce movement by 60%	reduce movement more than 60%
Responsiveness	Normal responsiveness	Reduced responsiveness by 30%	reduce responsiveness by 60%	reduce responsiveness more than 60%

Histopathology and lesion scoring

Mice that survived after 120 hours of inoculation were euthanized by using cervical dislocation and postmortem examination was performed on them as well. Tissues were collected from the heart, lung, liver, spleen, small intestine and large intestine for histopathological evaluation. These samples were fixed with 10% formalin and processed in an automatic tissue processor. Tissues were processed into paraffin blocks and each section was routinely stained by haematoxylin and eosion (H&E). Microscopically, cellular changes were scored following observation of 5 slides per organ. Six microscopic fields for each slide were observed at 200x magnification. Lesion scoring was divided into 4 scores namely: score 0: normal, score 1: mild (less than 30% of field involved), score 2: moderate (30-60% of field involved) and score 3: severe (more than 60% of field involved).

Statistical analysis

The findings of the study were analyzed suing statistical software R version 3.0.1 for Windows. Statistical test (independent T-test) was performed and an error level of 0.05 was used (α=0.05).

III. Results

Clinical Signs

The result of the present study revealed that mice inoculated with the *Pasteurella multocida* showed significant severe clinical signs ($p<0.05$) compared to control group. Mice in *Pasteurella multocida* group showed mortality rates between 24 to 50 h post-inoculation. Of eight mice inoculated with *P. multocida*type B, none developed observable clinical symptoms within 12 h post-inoculation, when suddenly 1 (12.5%) of them died within 24 h (Table II), and 4 others (57.14%) died within 48 h (Table II) after showing observable clinical signs. The remaining 3 (37.5 %) animals survived through the experimental period and were culled after 120 h. Therefore, the lethality rate of *P. multocida*type B: 2 was 69.64% in treatment group (Table II). Mice in *Pasteurella multocida* group showed significant ($p<0.05$) moderate to severe clinical signs of ruffled hair compared to control group with mean score 2.13±0.64 (Table III). All mice in *Pasteurella multocida* group showed significant ($p<0.05$) mild to moderate laboured breathing compared to control group with mean score 1.88±0.99 (Table III). Mice in this group (*Pasteurella multocida)* also exhibited mild to moderate eye discharges with mean score 1.50±1.20 (Table III). Animals challenged with *Pasteurella multocida* presented mild to moderate for level of responsiveness in comparison to the control group with mean score of 1.88±0.99 (Table III).

Table II
Comparison of lethality rate between mice infected orally with 10^9 cfu *P. multocida* B:2 and control group

	Control Group		Treatment Group	
Time (hours)	Number of animas died	Lethality rate (%)	Number of animals died	Lethality rate (%)
12	0	0	0	0
24	0	0	1	12.5
48	0	0	4	57.14
72	0	0	0	0
96	0	0	0	0
120	0	0	0	0
Total	0	0	5	69.64

Table III
Mean score of clinical signs showed in mice after 120 hours post-inoculation with *P. Multocida* type B

Parameters	Control group	*P. multocida* type B:2
Ruffled fur	0.00±0.00	*2.13±0.64
Eye closed and discharges	0.00±0.00	*1.50±1.20
Laboured breathing	0.00±0.00	*1.88±0.99
Level of responsiveness	0.00±0.00	*1.88±0.99

*Significant value p<0.05 (Independent-samples T test). Comparison between control group and infected group.

Histopathological findings

The number of organs examined in each group and corresponding parameters are summarized in Table IV. Mice in the treatment group showed a significant ($p<0.05$) mild to moderate pulmonary oedema compared to the control group with the mean score of 1.13±0.95.The treatment group also showed a significant ($p<0.05$) severe presence of inflammatory cells in the lung with the mean score of 2.5±0.59. There were also significant ($p<0.05$) moderate degeneration and necrosis observed in the lungs with the mean score of 2.03±0.83 followed by moderate haemorrhage with the mean score of 2.21±0.78. The treatment group also presented mild to moderate presence of inflammatory cells in the liver with the mean score of 1.75±0.90. There were also mild to moderate degeneration and necrosis observed in the liver with the mean of 1.79±0.66. There were significant ($p<0.05$) moderate to severe presence of Kupffer cells in the liver with the mean of 2.38±0.65 compared to control group. In the infected group, there were mild to moderate infiltration by the mononuclear cells in the heart with the mean score of 1.58±1.06 whereas haemorrhage were mild to moderate with the mean score of 1.96±0.95. There were also moderate degeneration and necrosis with the mean score of 2.04±1.00 in the heart compared to PBS group. For kidney, the *Pasteurella multocida* group showed significant ($p<0.05$) mild to moderate infiltration of the inflammatory cells with the mean score of 1.79±0.88. There were also moderate haemorrhage and moderate to severe degeneration and necrosis with the mean score of 2.00±0.98 and 2.25±0.79 respectively in the kidney compared to PBS group. There were significant ($p<0.05$) moderate to severe infiltration by the inflammatory cells in the small intestines and colon with the mean of 2.75±0.44 and the

haemorrhage as well as degeneration and necrosis occurred at mild to moderate with the mean score of 1.58±0.78 and 1.83±0.70 respectively compared to control group.

Table IV
Scoring system for the lesions of visceral organs of mice after 120 h post-inoculation with P.*multocida B: 2*

Organs	Groups	Parameters					
		Oedema	Inflammatory cells	Degeneration/ Necrosis	Hemorrhage	Congestion	Kupffer Cell
Lung	Control	0.11±0.32	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	n/a
	Treated	*1.13±0.95	*2.50±0.59	*2.08±0.82	*2.20±0.78	*2.08±1.02	n/a
Liver	Control	n/a	0.00±0.00	0.00±0.00	0.00±0.00	n/a	0.00±0.00
	Treated	n/a	*1.75±0.90	*1.79±0.66	*1.95±0.86	n/a	*2.38±0.65
Heart	Control	n/a	0.00±0.00	0.00±0.00	0.00±0.00	n/a	n/a
	Treated	n/a	*1.58±1.06	*2.04±1.00	*1.95±0.95	n/a	n/a
Kidney	Control	n/a	0.00±0.00	0.00±0.00	0.00±0.00	n/a	n/a
	Treated	n/a	*1.79±0.88	*2.25±0.79	*2.00±0.98	n/a	n/a
Intestine	Control	n/a	0.00±0.00	0.00±0.00	0.00±0.00	n/a	n/a
	Treated	n/a	*2.75±0.44	*1.83±0.70	*1.58±0.78	n/a	n/a

*Significant value $p<0.05$, n/a =not applicable

IV. Discussion

Oral inoculation of mice with *P. multocida* type B resulted in development of expected clinical signs as seen in the intraperitoneal inoculation with the same organism as described in an experiment done by Ramdani *et al.*, 1990 and Jesse, 2011. The signs that had been observed in the infected group included ruffled fur, laboured breathing, and closure of the eyes together with the eye discharge and less responsiveness. Five out of eight mice died in this experiment with the time of death ranged between twenty-three hours and forty-five min to fifty h post-inoculation. The histopathological changes observed were also similar with the mice inoculated intraperitoneally with this bacteria (Jesse, 2011). Besides that, the histopathological lesions were also similar to those of cattle infected naturally and experimentally with *P. multocida* type B (Rhoades *et al.*, 1967, Horadagoda *et al.*, 1991, Khin *et al.*, 2010). Nevertheless, histopathological lesions shown in the small intestine is a new contribution as none of the literature ever described the involvement of the small intestine and the changes in terms of histopathlogical lesions. It could be speculated that the bacteria have the affinity towards the enterocytes where it is triggering the infiltration of the inflammatory cells to the small intestine before the endotoxin being released and causing the septicaemic phase. However, further study are required to be done to fully understand the exact pathogenesis of oral inoculation of *P. multocida* type B:2.

V. Conclusion

This study revealed that oral exposure of live *P. multocida type B: 2* resulted relativily establishment of HS infection in a mouse model. The oral inoculation of *P.multocida type B* in mouse model was able to produce the typical clinical signs and significant histopathological changes that were characteristic of HS.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect the research, authorship, and/or publication of this article.

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