Research Article

Evaluation Of Protective Effects Of Graded Doses Of Lipopolysaccharide Extracted From *Pasteurella multocida* Type B:2 Against Hemorrhagic Septicemia In Mice

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Abstract | Haemorrhagic septicaemia (HS) is one of the most leading cause of death in buffalos and cattle in Malaysia. Lipopolysaccharide (LPS) is one of the major immunogen of *P. multocida* serotype B:2. This study was designed to evaluate the protective effect of LPS derived from *P. multocida* B: 2 against haemorrhagic septicaemia in mice. Twenty five mice were divided into five groups consisting of five animals in each group. The control group was inoculated orally with 0.2 mL of phosphate buffer saline (PBS) pH 6.8, whereas groups 1, 2, 3 and 4 were orally inoculated with 0.2 mL LPS extracted from 10^3, 10^5, 10^7 and 10^9 colony forming units (CFU) of *Pasteurella multocida* serotypes B: 2, respectively. The experimental animals were observed for clinical signs for seventeen days. All the groups were further challenged with 0.2 mL of 10^7 wild type *Pasteurella multocida* B: 2 17 days post LPS inoculation. The groups were observed for clinical signs for seven days post challenge and surviving mice were euthanized and their organs harvested for histopathological examination and bacterial isolation and identification. The results of clinical observation showed 60% of the mice from all group had diarrhoea, 38.5% had severe ocular discharge and all mice had laboured breathing. Mild to moderate histopathological lesions were observed in the heart, lungs, liver, spleen, kidney, small intestine, large intestine and stomach of all groups. The study showed that oral inoculation of LPS extracted from *P. multocida*, both in low and high concentrations failed to give protection against *P. multocida* infection in mice.

Keywords | Haemorrhagic septicaemia, Lipopolysaccharides, Histopathological lesions, Immunization

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INTRODUCTION

Pasteurella multocida serotypes B: 2 and E:2 is one of the major cause of an acute, fatal and septicaemic disease of cattle and buffaloes, called haemorrhagic septicaemia (HS) (Sivachandra et al., 2011). *Pasteurella multocida* Asian serotype B:2 and the African serotype E:2 (Carter and Heddleston system), which correspond to 6:B and

6:E (Namioka-carter system), are organisms mainly responsible for the disease (De Alwis, 1999; OIE, 2012). Serotype B:2 have been identified in most endemic areas of the world, whereas E:2 is only found in Africa (Mosier, 2014). The most common hosts of HS are cattle and buffalo, however, other animals such as goats, pigs, deer, sheep and camels are also susceptible to the disease (Asraf, 2014). Haemorrhagic septicaemia is one of the most widely distributed disease of cattle and buffalo, particularly in tropical countries in Africa and Asia, where it occurs as catastrophic epizootics resulting into high mortality and morbidity (Mustafa et al., 1978; Singh et al., 1996; Benkirane and De Alwis, 2012). Haemorrhagic septicaemia causes severe economic devastation in Asia, resulting not only in reduction in productivity but also loss of animal traction in harvest of agricultural produce (Benkirane and De Alwis, 2012).

Haemorrhagic septicaemia (HS) is an acute, febrile and fatal disease, causing death in susceptible animals in less than 36 to 48 hours after exposure to the organism (Jamal et al., 2013). The disease is mainly seen in cattle and water buffalo with progressive clinical manifestations typified by dullness, fever, respiratory distress with severe nasal discharge, and frothing from the mouth, recumbency and death within hours (OIE, 2012). *Pasteurella multocida* is a normal inhabitant of the nasopharynx and gastrointestinal tract of many animals. Studies have shown that up to 5% of tonsils in apparently healthy water buffalo and cattle are colonized by *P. multocida* serotype B:2 or E:2, which can be shed during periods of stress (Klein and Cunha, 1997). In Malaysia, about 3% of cattle and buffaloes sampled from HS endemic areas showed positive for *P. multocida* type B (Saharee et al., 1992).

The disease is transmitted via direct contact with infected oral or nasal secretions from either healthy carrier animals or animals showing clinical disease and/or on fomites and by ingestion or inhalation of the organism in contaminated feed or water (Verma and Jaiswal, 1998). Prevention and control of the disease can be achieved by keeping animals in well ventilated sheds. Chemoprophylaxis can also be done, however, the results are not satisfactory due to the intrinsic resistance of *P. multocida* to most antimicrobial agents used (Mosier, 2014). Vaccination have been shown to greatly reduce the incidence of HS in endemic areas. For instance, the use of broth bacterin, oil adjuvant vaccine, double emulsion vaccine and a live vaccine have been reported to be the most significant control measure in the face of an outbreak (Verma and Jaiswal, 1998; De Alwis, 1999; Benkirane and De Alwis, 2012). In addition, oil-adjuvant vaccine (OAV), applied parenterally, has been reported as the commonly used prophylactic agent in Malaysia against HS infection (Chung et al., 2015). However, only 17% of Malaysian buffaloes were vaccinated due to difficulty of vaccine administration (Zamri-Saad, 2013). Animals need to be individually restrained to apply parenteral vaccination, making vaccine application difficult. Previous experimental infection studies by Abubakar and Zamri-Saad (2011) had shown that oral route inoculation of whole bacteria of *P. multocida* in buffaloes caused less severe clinical signs, compared to other routes, therefore, it was suggested that oral route might be a suitable route for effective vaccine administration (Chung et al., 2015).

The immunologically protective potentials of purified lipopolysaccharide (LPS) of *Pasteurella multocida* type B:2 against experimentally induced pasteurellosis in mice has previously been described (Muniandy and Mukkur, 1993; Muniandy et al., 1998). The purified LPS was found to be a good immunogen which can be used for subunit vaccine development vaccine development (Ashraf, 2014; Sarangi et al., 2014). However, there are no studies that evaluated the protective effects of *P. multocida* B: 2 lipopolysaccharide against HS. Thus, this study was designed to evaluate the protective effect of graded doses of immunogenic LPS extracted from *P. multocida* type B: 2 against HS disease. The findings of this study will provide information that can be used as preventive measures in HS disease, and the development of oral subunit vaccine against HS.

MATERIALS AND METHOD

Ethical Statement

This research was approved by the Animal Care and Use Committee of Universiti Putra Malaysia (approval number: R048/2015) as legally required in Malaysia. Animals were humanely handled and euthanized according to standard protocols stipulated in the laboratory animal care and use manual.

Laboratory Animal Selection

A total of twenty five (25) clinically healthy male mice, three to four weeks old were used in this study. Prior to experimentation, the mice were observed and acclimatised for seven days in the laboratory. The mice were placed into five plastic cages padded with dry clean wood shavings. In addition, they were provided with water and pellets *ad libitum*.

Preparation of *Pasteurella Multocida* and its LPS Inoculums

Two types of inoculums were used in this study namely; wild type P. *multocida* type B: 2 and its lipopolysaccharides. The *P. multocida* isolate used in this study was obtained from stock cultures collected from previous outbreak of HS in Kelantan, Malaysia (Ali et al., 2015). Pure cultures of *P. multocida* were prepared McFarland Nephelometer as decribed earlier (Ali et al., 2015; Ibrahim et al., 2016a,

2016b) to produce bacterial suspension with vial cell count of 10^3, 10^5, 10^7 and 10^9CFU/mL of *P. multocida*. The *P. multocida* LPS were extracted from each bacterial suspension concentrations (10^3, 10^5, 10^7 and 10^9 CFU/mL) using iNtRON LPS extraction kit (iNtRON Biotechnology, Korea) as previously described (Ali et al., 2015; Ibrahim et al., 2016b).

Study Designs

The twenty-five mice were divided into five groups consisting of five mice each. Groups 1, 2, 3 and 4 were inoculated orally with LPS extracted from different concentrations of *P. multocida* type B:2 (10^3, 10^5, 10^7 and 10^9 CFU/mL), while group 5 served as the control group and were inoculated orally with 0.2 mL of phosphate buffer solution (PBS) pH 6.8. The animals were observed for clinical signs daily for 17 days.

On the 17thday post-inoculation with LPS, all the animals were challenged with 0.2 mL of 10^7 CFU/ml suspension of wild type *P. multocida,* orally. The animals were observed for clinical signs which include ruffled hair coat, alertness, ocular discharges, inappetance and mortality, for seven days. The observations were done hourly for the first 48 hours post challenge and twice daily afterwards. Surviving mice were euthanized by cervical dislocation and post mortem examination was conducted on the animals. Visceral organs were collected in 10% buffered formalin and processed for histopathological examination, while fresh organ samples were utilized for bacterial isolation and identification. Organ smears were used to identify bacteria using gram stain and microscopic examination.

Clinical Signs

Six parameters were assessed; food and water intake, ruffled hair coat, diarrhoea, responsiveness, ocular discharges and mortality.

Histopathological Lesions Scoring

Immediately after the mice were euthanized, tissue samples from the heart, lungs, liver, kidney, spleen, stomach, small and large intestine were collected for histopathology. Three types of lesion were examined, namely; presence of inflammatory cells, degeneration and necrosis, and haemorrhage and congestion. For each tissue sample, six regions were examined for the stated lesion. Each region was scored 0-3, with '0' as absence of lesion, '1' for mild which less than 30% of the tissue region showing lesion, '2' for moderate which 30-50% of the tissue region showing lesion, and '3' is for severe which more than 50% is showing lesion (Othman et al., 2016). The results obtained were analysed using IBM® SPSS® Statistics Version 20.

Bacterial Isolation and Identification

Organs such as heart, lungs, liver, spleen, kidney, small intestine, large intestine and stomach, were smeared properly unto blood agars media using the three-phase streaking pattern and were incubated at 37^{o}C for 24 h. Then, the presence of bacterial growth on the media was observed. Pure colonies with smooth, non-haemolytic, greyish glistening translucent colonies of approximately 1 mm in diameter on the media was assumed as the causative agent. The bacterial colonies observed were stained with gram stain and Wright's stain, to identify the organism.

RESULTS

Clinical Signs

Initial observation of clinical findings after inoculation with LPS, showed that there was no difference (P>0.05) in clinical signs between control group and inoculation groups. However, after a few hours of challenge with the whole bacteria, 60% of animals from all groups showed signs of diarrhoea. After 24 hours of inoculation, all mice with severe clinical signs showed signs of laboured breathing and ruffled fur coat. Moreover, 38.5% of the mice from all groups showed ocular discharge. Food and water intake and responsiveness were present in animals from all groups. The rate of mortality following first inoculation with LPS and challenge with *P. multocida* B:2 are shown in Table 1.

Table 1: The mortality of the mice following LPS inoculation and wild type *P. multocida* challenge

Group	No. mice that died post LPS inoculation (%)	No. of mice that died post P. multocida inoculation (%)	No. of mice that survived (%)
Control	-	2 (40)	3(60)
Group 1	1 (20)	1 (20)	3(60)
Group 2	0	4(80)	1 (20)
Group 3	0	3(60)	2(40)
Group 4	1 (20)	3(60)	2(40)

Mortality was only observed in groups 1 and 4 during initial inoculation of LPS. However, after challenge with *P. multocida*, mortality was higher in groups 2, 3 and 4.

Histopathology

In the liver, all histopathological lesions observed were comparable among all the groups after challenge with *P. multocida*. However, while the distribution of inflammatory cells and circulatory changes (haemorrhage and congestion) were mild, degeneration and necrosis is moderate to severe in this organ.

In the spleen, inflammatory cell infiltration, and degeneration and necrosis were mild to moderate in distribution in all groups and not different (P>0.05), while haemorrhage and congestion was moderate to severe. There were no differences (P>0.05) in all histopathological changes among

Table 2: Mean score of histopathological lesions observed in organs of mice inoculated with graded doses of LPS and secondary challenge with *P.multocida*

Organ	Histopathological lesions	Control	Group 1	Group 2	Group 3	Group 4
Liver	Inflammatory cells	0.37±0.22	0.42±0.10	0.50±0.26	0.37±0.30	0.38±0.21
	Degeneration and necrosis	2.00±1.23	2.10±0.69	1.60±0.98	2.40±0.63	1.37±0.83
	Haemorrhage and congestion	0.73±0.53	0.66±0.29	0.90±0.52	0.60±0.56	0.87±0.30
Spleen	Inflammatory cells	0.70±0.14	1.40±0.89	1.10±0.35	0.60±0.38	0.96±0.21
	Degeneration and necrosis	1.03±0.43	1.53±0.16	0.90±0.38	1.26±0.69	0.80±0.96
	Haemorrhage and congestion	2.50±0.35	2.10±0.34	2.20±0.36	2.60±0.48	2.13±0.42
Kidney	Inflammatory cells	0.20±0.22	0.03±0.08	0.05±0.04	0.07±0.09	0.17±0.14
	Degeneration and necrosis	0.13±0.18[a]	0.46±0.16[a]	0.44±0.25[a]	0.57±0.32[a,b]	1.14±0.34[b]
	Haemorrhage and congestion	0.84±0.66	0.63±0.16	0.83±0.35	0.80±0.55	0.70±0.24
Small intestine	Inflammatory cells	0.40±0.30	0.79±0.37	0.03±0.07	0.20±0.21	0.17±0.08
	Degeneration and necrosis	1.07±0.19	1.27±0.37	0.73±0.61	1.53±0.74	1.40±0.52
	Haemorrhage and congestion	0.20±0.14	0.13±0.25	0.23±0.22	0.23±0.25	0.38±0.21
Large intestine	Inflammatory cells	0.40±0.25	0.23±0.08	0.10±0.09	0.20±0.14	0.13±0.16
	Degeneration and necrosis	0.90±0.74	1.33±0.48	1.17±0.37	1.33±0.41	1.80±0.48
	Haemorrhage and congestion	0.20±0.22	0.33±0.08	0.30±0.18	0.33±0.33	0.40±0.21
Stomach	Inflammatory cells	0.37±0.27	0.50±0.08	0.10±0.15	0.23±0.28	0.13±0.17
	Degeneration and necrosis	1.00±0.72[a]	0.97±0.67[a]	1.06±0.48[a]	1.33±0.17[a]	2.25±0.29[b]
	Haemorrhage and congestion	0.33±0.35	0.33±0.24	0.40±0.25	0.57±0.30	0.33±0.28

Values are expressed as mean ± SD.[a, b] Values with different superscripts within rows are significant at P <0.05

Table 3: Mean scores of isolation and identification of *P. multocida* in organs of mice

Group	Control	Group 1	Group 2	Group 3	Group 4
Heart	0.6±0.55	0.5±0.58	0.6±0.55	0.2±0.45	0.5±0.58
Lungs	0.6±0.55	0.75±0.5	0.6±0.55	0.6±0.55	0.5±0.58
Liver	0.6±0.55	0.5±0.58	0.8±0.45	0.6±0.55	0.5±0.58
Spleen	0.4±0.55	0.00±0.00	0.6±0.55	0.6±0.55	0.25±0.5
Kidney	0.6±0.55	0.25±0.5	1.00±0.00	0.6±0.55	0.5±0.58
Small intestine	0.4±0.55	0.75±0.5	0.4±0.55	0.5±0.45	0.5±0.58
Large intestine	0.6±0.55	0.5±0.58	0.4±0.55	0.6±0.55	0.5±0.58
Stomach	0.8±0.45	0.75±0.5	0.4±0.55	0.2±0.45	0.75±0.5

Values are expressed as mean ± SD.[a, b] Values with different superscripts within rows are significant at P <0.05.

the groups.

In the kidneys, all histopathological changes observed were mild. However, degeneration and necrosis was higher (P<0.05) in group 4. In the small and large intestines, both inflammatory cell infiltration and circulatory changes (haemorrhage and congestion) were mild in distribution, while degeneration and necrosis was mild to moderate. However, there were no differences (P<0.05) in these changes among the different groups.

In the stomach, inflammatory cell infiltration and circulatory changes (haemorrhage and congestion) were both mild in distribution, while degeneration and necrosis was mild to moderate. However, degeneration and necrosis was higher (P<0.05) in group 4. There were no differenc es among the groups inflammatory cell infiltration and, haemorrhage and congestion (Table 2).

Bacterial Isolation

The result of the isolation and identification of *P. multocida* from organs of each mice showed no significant difference (P>0.05) in recovery of the whole bacterial organism between groups. This showed that bacteria were isolated from all organs in control and in the inoculation groups. Thus, indicating that mortality observed in all the mice was due to *P. multocida* (Table 3).

DISCUSSION

Pasteurellosis is a major and significantly important respiratory diseases affecting economically valuable farmed

animals such as cattle, goats, pigs, rabbits and poultry. The disease causes a severe economic devastations due to the symptoms of local to generalised fatal septicaemia. The chemotherapeutic treatment of this disease is expensive, prolonged and ineffective due to frequent resistance development and toxicity in humans. Thus, there is the need for the development of a viable potent vaccine to diminish the spread of this organism (Ahmed et al., 2014).

In Asia, haemorrhagic septicaemia is ranked as the most fatal disease and a major cause of economic losses in cattle and buffaloes; however, the nature of its immune response to *P. multocida* is poorly understood. In addition, current vaccines which are not sufficiently efficacious are administered parenterally and require repeated administration to achieve the requisite immune response to wade off the infection. In this study, oral inoculation of animals with LPS showed no significant difference (P>0.05) in clinical signs between the control and treatment groups. This finding was consistent with the findings of Abubakar and Zamri-Saad (2011), where less clinical signs of HS were produced following oral administration of *P. multocida* as compared to other routes. Hence, indicating that oral administration is the best route for administration of LPS (Khaleel et al., 2014). Furthermore, this finding is also consistent with the report of Jacobsen et al. (2004) where all the animals used in the study developed clinical signs of haemorrhagic septicaemia following inoculation of the LPS of *P. multocida*.

The clinical manifestation of the disease seen in this study when the animals were challenged with the LPS of *P. multocida* B: 2 was similar to the report of Horadagoda et al. (2002), where the author experimentally mimicked the onset of haemorrhagic septicaemia in buffaloes after inoculating the animals intravenously with the endotoxin of *P. multocida* B: 2, the most distinctive clinical features observed include anorexia, pyrexia, tachycardia, tachypnea, depression, low ruminal motility and diarrhoea (Jesse et al., 2013). In this study, clinical manifestation of the disease was observed between one to two hours after the animals were challenged with the whole bacteria and about 60% of the mice showed signs of diarrhoea. Interestingly, 38.5% of all the mice from the control and treated groups had severe ocular discharge which completely closed the animals' eyes. These findings were similar to the report of Jesse et al. (2013), where similar signs of laboured breathing, ruffled fur and severe ocular discharged where observed in experimental mice inoculated with *P. multocida* B: 2. Based on the mortality pattern observed, mean mortality within the first 24h after inoculation of the whole organism showed no significant difference between treatment and control groups. This shows that the oral inoculation of LPS and the whole organism didn't confer immunity to the animals.

The histopathological findings were generally similar between the organs and among the groups; however, in a few organs (stomach and kidney) it was more severe in group 4. These findings are consistent with the report of Ali et al. (2015), where less severe histopathological changes were observed in mice inoculated with LPS of *P. multocida*, while moderate to severe histopathological changes were observed following inoculation of *P. multocida*.

In this study, the result of the isolation and identification of *P. multocida* from organs of each animal showed that there is no significant difference (P>0.05) in recovery of the whole bacterial organism between groups. This showed that bacteria were isolated from all organs in control and in the treatment groups. However, the finding of this study differed from the report of Priadi and Natalia (2000), where the authors reported difficulty in the recovery of whole bacteria from the organs of animals after inoculation.

CONCLUSIONS

The findings of this study evaluated the importance of oral inoculation of *P. multocida* B: 2 LPS as an important immunogen against HS. However, it was observed that oral inoculation of the LPS of *P. multocida* B: 2 failed to confer immunity against HS in mice.

CONFLICT OF INTEREST

No conflict of interest to declare.

AUTHOR CONTRIBUTIONS

All authors contributed equally to this work.

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DATA AVAILABILITY

All data are presented within the manuscript.

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