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Polymerase Chain Reaction Detection of *Pasteurella multocida* Type B:2 in Mice Following Oral Inoculation

[1]Faez Firdaus Jesse Abdullah, [1]Abdinasir Yusuf Osman, [1]Lawan Adamu, [1]Mohd Syamil Mohd Yusof, [1]Abdul Rahman Omar, [1]Abdul Aziz Saharee, [1]Abd Wahid Haron, [1]Rasedee Abdullah and [2]Mohd Zamri-Saad

[1]Department of Clinical Studies, [2]Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia

Corresponding Author: Faez Firdaus Jesse Abdullah, Department of Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400, Serdang, Selangor, Malaysia Tel: +60389463924

ABSTRACT

Haemorrhagic Septicaemia (HS) is an acute, fatal, septicaemic disease of cattle and water buffaloes caused by *Pasteurella multocida*, serotype B:2 in tropical countries. The limitations associated with accurate predictions of mortality, survival levels and the detection of the presence of the organism from various organs of infected animals. Hence, this study used mouse model to evaluate the pattern of mortality and bacterial recovery from organs. Twenty-four mice were randomly divided into two groups. Infected group were inoculated orally with 10^9 colony forming unit of *P. multocida* type B, the group 2 were negative controls. The mice were observed for 5 days post-inoculation. At necropsy, visceral organs of dead animals were subjected for the confirmation using Polymerase Chain Reaction (PCR). The results showed that mortality rate was significantly different ($p<0.05$) between the infected and control groups. Within infected group, highly significant difference ($p<0.05$) was observed where 12.5% of the mortality rate was recorded within 24 h and 62.5% within 48 h post-infection. The survival rate, in infected group, was found to be around 25%. In diagnosis, *P. multocida* type B was detected from all organs of animals that did not survive. In contrast, *P. multocida* type B was neither recovered nor detected from the organs of mice which survived until the end of the experimental period (120 h). The results of this study indicated that manipulation of the organism in experimental animals provided clear information of the incidence of the disease in the field.

Key words: *Pasteurella multocida* type B, oral inoculation, polymerase chain reaction, mice, organs

INTRODUCTION

Haemorrhagic Septicaemia (HS) is an acute and highly fatal septicaemic disease of ruminants in tropical regions of the world; especially in African and Asian countries (De Alwis, 1999; Kamarudin, 2005). It is caused by *P. multocida* which is the causative agent of wide range of serious infections in animals, including fowl cholera in poultry, atrophic rhinitis in swine and respiratory disease in ungulates and rabbits and Haemorrhagic Septicaemia (HS) in cattle and buffalo (Boyce *et al*., 2010). Even though the organism has a variety of serotypes, *P. multocida* serotype B:2 has been reported to be responsible of recurrent outbreaks of HS in tropical areas

(Ataei *et al*., 2009). Natural hosts that specifically prone to the disease (HS) include cattle and buffalo. However, in the context of susceptibility, buffaloes are believed to be more susceptible to the disease infection in comparison to cattle (Bain *et al*., 1982). The clinical manifestation of this disease is often characterized by rapid course, high fever, loud and stertorous breathing, profuse salivation, severe depression and followed by death usually within twenty-four hours of infection. In animals, the development of the disease has been frequently reported to occur following exposure of the susceptible hosts to stressful conditions where infections usually occur by inhalation or ingestion (Shafarin *et al*., 2009). The clinical manifestation of HS can also happen through the ingestion of contaminated foodstuff (Radostits *et al*., 2000). The source of infection has been postulated to be in relation to the carcasses which are dumped into river, tanks and channels (Joseph, 1979; De Alwis, 1999). The outbreaks usually follow the course of the river contaminated with the carcasses of infected animals. A recent study of experimental nature has confirmed the development of typical clinical changes of HS following oral route inoculation of *P. multocida* type B in buffaloes (Abubakar and Zamri-Saad, 2001). In disease diagnosis, identification of post-mortem lesions had considerably aided in HS diagnosis where lesions such as typical swelling of the neck due to severe blood-tinged oedema and other lesions in the respiratory tract have been frequently observed in many cases infected with HS. However, there is still insufficient information about the pathogenicity and the epidemiology of HS. The present study, therefore, investigates whether experimental models (mouse model) could enhance our understanding both the natural route of transmission and the characteristic of this fatal disease in these models.

MATERIALS AND METHODS

Inoculum preparation (*P. multocida* B:2): A stock culture of *P. multocida* B:2 isolated from previous outbreak of HS in the state of Kelantan, Malaysia was used to prepare the inoculums. The organism was cultured onto 5% horse blood agar and incubated at 37°C for 18 h. After the culture, Brain Heart Infusion broth (BHI) was seeded with single uniformly sized colonies and placed in shaker incubator at 37°C for 24 h to produce the inoculum which contained 10^9 colony-forming units/mL (CFU). McFarland Nephelometer Barium Sulfate Standards was used to determine the concentration.

Animals and experimental design and inoculation: Twenty four apparently healthy, 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 and B). Mice of group A (treatment group; n = 16) 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. After inoculation, the mice were observed daily for clinical signs and followed for a period over time (5 days). Surviving mice were euthanized by cervical dislocation after 120 h post-infection (p.i). Post mortem examination was performed on all dead animals. During post mortem examination, visceral organs (heart, lung, liver, spleen, stomach small intestine and large intestine) were collected for bacterial isolation. Samples for bacterial isolation were then cultured on blood agar (Oxoid, UK) and incubated at 37°C for 24 h. After incubation period, isolates were biochemically identified as *P. multocida* on the basis of criteria and biochemical tests used previously (Carter and Chengappa, 1980). All procedures and experiments illustrated were undertaken under a project license approved by Animal Utilization Protocol Committee with reference number: UPM/FPV/PS/3.2.1.551/AUP-R120.

Preparation of antigen for the PCR

DNA extraction: Boiling method was used in this study for DNA extraction. Colonies (3 to 5) of *P. multocida* on blood agar (Oxoid, UK) were transferred into a 1.5 mL Eppendorf tube containing 50 μL distilled water. After homogenizing the mixture, the suspension was kept in a boiling water bath at 100°C for 15 min. After cooling on ice for 2 min, the samples were centrifuged at 13,000 rpm for 5 min and 2 μL of the supernatant was used directly for the PCR.

PCR system and amplification conditions: The primer pairs to be used were chosen for their specificity among those reported in the literature. The composition for the sample coming from bacterial cultures was as follows: primer (reverse and forward) 10 ng μL^{-1} of each, 0.5 to 1 μL of extracted DNA as template, PCR buffer 10 μL, $MgCl_2$ 1.5 mM, *Taq* DNA polymerase 2.0 IU, dNTPs 200 μM. Thermal cycler conditions (FTS-32 thermal sequencer) were as follows: preincubation at 94°C for 5 min; 30 cycles consisting of dsDNA denaturation at 94°C for 1 min, primer annealing at 56°C for 1 min, primer extension at 72°C for 2 min; final elongation at 72°C for 5 min. PCR products were visualized following gel electrophoresis on 2% agarose gels using 1X TAE (Tris-acetate-EDTA) at 81 V for 30 min and ethidium bromide staining. DNA fragments were viewed by UV illumination and photographed.

Primer design: The primer for the amplification of the *Pasteurella multocida* was referred from Townsend *et al.* (1998). The primer sequences are as follows: forward primer (KTSP61), 5'-ACCGCACTTTAGTGTGTGTG-3' (internal sequencing primer for 6b); and the reverse primer (KTT72) 5'-TCTCTACGCCGATCTTGTAT-3' (internal sequencing primer for 6b). PCR amplification with the primer pair designed during the sequencing of clone 6b (KTSP61-KTT72) specifically produced a product approximately 590 bp from HS-causing type B isolates of *P. multocida*. These primers were unable to amplify DNA from other *P. multocida* serotypes, other *pasteurella* species and other members of the pasteurellacea family or unrelated bacteria.

Agarose gel preparation: Of 2% agarose gel with volume of 150 mL was prepared by mixing 3 g of agarose agar powder with a 150 mL 1% TAE buffer. The mixture was heated in microwave oven about 3-5 min and until the mixture became crystal clear. After cooling to approximately 60°C, the mixture was poured into suitable size gel cassette and solidified (about 15 min) for PCR loading and electrophoresis.

Electrophoresis: Agarose gel is placed carefully in a gel holder tank and submerged with 1% of TAE buffer. About 6 μL of 100 bp dyed marker (Promega®) was then used as the ladder and 5 μL of PCR product was loaded into the well carefully. The electrophoresis of 2% agarose gel is run for about 30 min at 81 V. Following this, the gel was stained with ethidium bromide (0.5 μg mL^{-1}) solution and stirred for 10-15 min. The gel was placed under UV gel imaging capturing machine and the results was recorded.

Statistical analysis: Chi-square test was used for comparison between PCR and bacteriological results. The level of significance applied to data was $p<0.05$.

RESULTS

In general, the results obtained from this study indicated significant differences ($p<0.05$) in the context of exhibition of clinical signs (data not shown), incidence of mortality rate and detection of the organism from infected animals. All these responses were significantly ($p<0.05$) associated with both time and individual variability.

Clinical observation: Of sixteen mice inoculated with *P. multocida* type B, none developed observable clinical symptoms within 12 h post inoculation (p.i.), when suddenly 2 (12.5%) of them died within 24 h (Table 1) and 10 others (62.5%) died within 48 h (Table 1) after showing observable clinical signs (data excluded). The remaining 4 (25%) animals survived through the experimental period and were culled after 120 h. Therefore, *P. multocida* type B:2 lethality rate was 75% in treatment group (Table 1). In contrast, animals served as control group neither died nor developed clinical signs (Table 1, Fig. 1).

PCR results: Following PCR assay, *P. multocida* type B was successfully isolated from heart, lung, liver, spleen, stomach, small intestine and large intestine of the mice from the treatment group which died during the 5 days of the experimental period (Fig. 2-4). There was no evidence of the

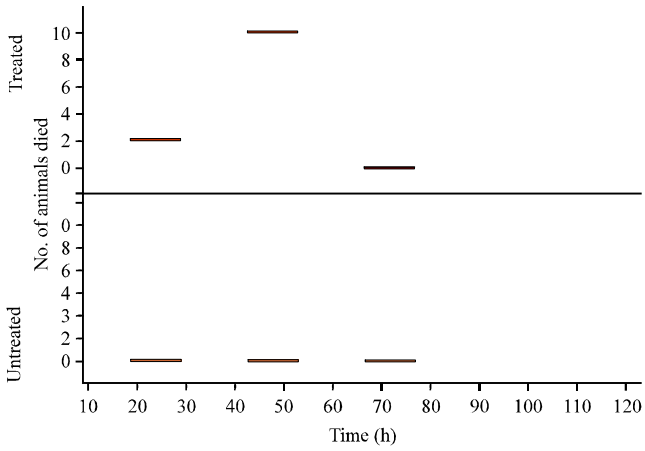

Fig. 1: Comparison of mortality rate between mice infected orally with *P. multocida* type B:2 and those served as a control group

Table 1: Comparison of lethality rate between mice infected orally with 10^9 CFU *P. Multocida* type B:2 and control group

	Control group		Treatment group	
Time (h)	No. of animals died	Lethality rate (%)	No. of animals died	Lethality rate (%)
12	0	0	0	0
24	0	0	2	12.5
48	0	0	10	62.5
72	0	0	0	0
96	0	0	0	0
120	0	0	0	0
Total	0	0	12	75

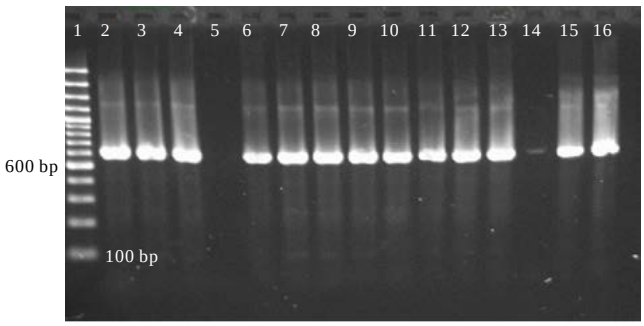

Fig. 2: PCR examination of tissue sample of mice of group 1 (*P. multocida type B*), killed within 24 h post inoculation. Bands at ca 600 bp indicate *Pasteurella multocida* B:2, 1: Ladder, 2: Positive control, 3-4: Heart, 5-6: Lung, 7-8: Liver, 9-10: Spleen, 11-12: Stomach, 13-14: Small intestine and 15-16: Large intestine

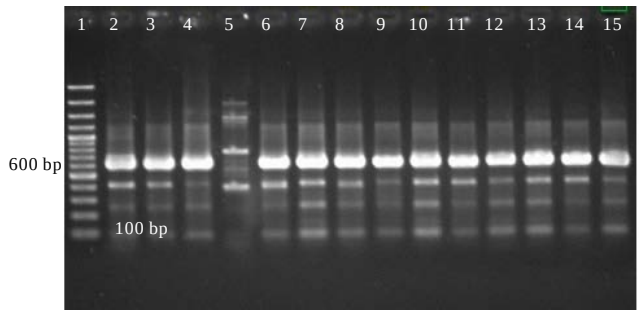

Fig. 3: PCR examination of tissue sample of mice of group 1 (*P. multocida* type B), killed within 75 h post inoculation. Bands at ca 600 bp indicate *Pasteurella multocida* B:2, 1: Ladder, 2: Positive control, 3: Heart, 4: Lung, 5: Liver, 6: Spleen, 7: Stomach, 8: Small intestine, 9: Heart, 10: Lung, 11: Liver, 12: Spleen, 13: Stomach, 14: Small intestine and 15: Large intestine

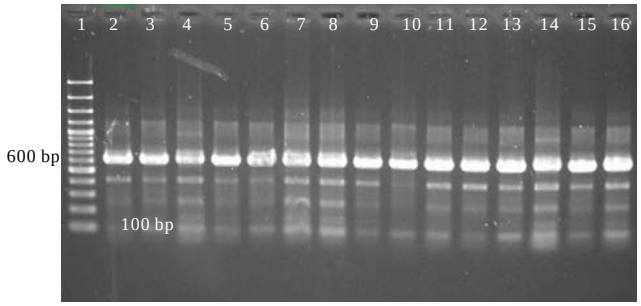

Fig. 4: PCR examination of tissue sample of mice of group 1 (*P. multocida* type B), killed within 75 h post inoculation. Bands at ca 600 bp indicate *Pasteurella multocida* B:2, 1: Ladder, 2: Positive control, 3: Heart, 4: Lung, 5: Liver, 6: Spleen, 7: Stomach, 8: Small intestine, 9: Large intestine, 10: Heart, 11; Lung, 12: Liver, 13: Spleen, 14: Stomach, 15: Small intestine and 16: Large intestine

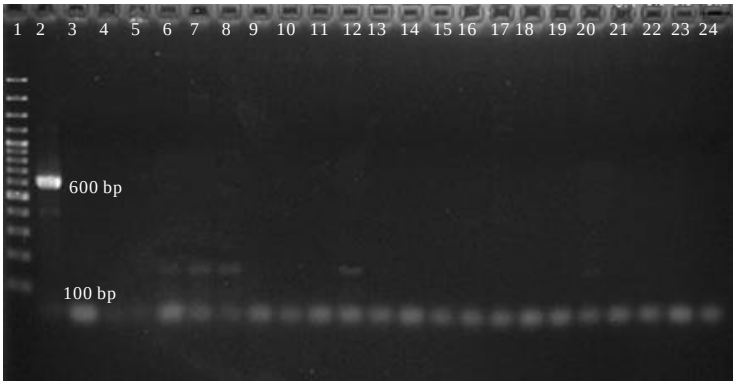

Fig. 5: PCR examination of tissue sample of survived mice of group 1 (4-8) and control group(18-24), killed after 75 h post inoculation. Bands at ca 600 bp indicate *Pasteurella multocida* B:2, 1: ladder, 2: Positive control, 3: Negative control, 4: Heart, 5: Lung, 6: Liver; 7: Spleen, 8: Stomach, 9: Small intestine, 10: Large intestine, 11: Heart, 12: lung, 13: liver, 14: Spleen, 15: Stomach, 16: Small intestine, 17: Large intestine, 18: Heart, 19: Lung, 20: Liver, 21: Spleen, 22: Stomach, 23: Small intestine and 24: Large intestine

Table 2: Detection of *P. multocida* type B from organs of mice

Animal group	No. of animals	Days p.i. (h)	Organs	Status
Treatment	2	24	Heart, Lung, Liver, Spleen, Stomach, Small intestine, Large intestine	Detected
Treatment	10	48	Heart, Lung, Liver, Spleen, Stomach, Small intestine, Large intestine.	Detected
Treatment	4	72-120	Heart, Lung, Liver, Spleen, Stomach, Small intestine, Large intestine.	Not detected
Control	8	12-120	Heart, Lung, Spleen, liver, Stomach, Small intestine, Large intestine.	N/A*

*N/A: Not applicable, p.i.: Post inoculation

Table 3: Detailed information about the figures (2-4) showing PCR detection of *P. multocida* type B from visceral organs of mice

	Lines							
No	1	2	3	4	5	6	7	8
Fig. 2	Ladder	Positive control	Heart†	Heart†	Lung†	Lung†	Liver†	Liver†
Fig. 3	Ladder	Positive control	Heart†	Lung†	Liver*	Spleen†	Stomach†	Small intestine†
Fig. 4	Ladder	Positive control	Heart†	Lung†	Liver†	Spleen†	Stomach†	Small intestine†

	Lines							
No.	9	10	11	12	13	14	15	16
Fig. 2	Spleen†	Spleen†	Stomach†	Stomach†	Small intestine†	Small intestine†	Large intestine†	Large intestine†
Fig. 3	Heart†	Lung†	Liver†	Spleen†	Stomach†	Small intestine†	Large intestine†	N/A
Fig. 4	Large intestine†	Heart†	Lung†	Liver†	Spleen†	Stomach†	Small intestine†	Large intestine

†: Positive samples, *Negative samples

presence of *P. multocida* type B on the organs isolated from the surviving mice (Table 2, Fig. 5). The detailed information and interpretation of the PCR results were summarized in Table 3 and 4.

Table 4: Detailed information about PCR detection of *P. multocida* type B from visceral organs of mice (Fig. 5)

	Lines											
Figure 5	1	2	3	4	5	6	7	8	9	10	11	12
	Ladder	Positive control	Negative control	Heart*	Lung*	Liver*	Spleen*	Stomach*	Small	Large intestine*	Heart* intestine*	Lung*
	Lines											
	13	14	15	16	17	18	19	20	21	22	23	24
	Liver*	Spleen*	Stomach*	Small intestine*	Large intestine*	Heart*	Lung*	Liver*	Spleen*	Stomach*	Small intestine*	Large intestine*

*Negative samples

DISCUSSION

Following oral inoculation of *P. multocida* type B:2, two types of infections were recorded; per acute and acute infections. Similar observations have been reported in cattle, buffaloes and goats following experimental infection with *P. multocida* B:2 (Zamri-Saad and Shafarin, 2007; Horadagoda *et al.*, 2002). In reference to the findings of Zamri-Saad and Saharee (1990), they stated that the effectiveness of certain route of infection can be determined by observing the number of animals which died or were killed per-acutely following experimental infection with *P. multocida* B:2. In their study, 60% of the goats were killed per-acutely following subcutaneous route of infection. The subcutaneous route of infection was considered the best method for experimental recreation of the disease in goats. In the present study, only 2 out of 16 mice (12.5%) from the treatment group which were inoculated orally with 10^9 CFU *Pasteurella multocida* died per-acutely within 24 h, while 10 out of 16 (62.5%) mice died within 48 h post-inoculation with the bacteria. This shows that, oral route takes longer time for death to ensue and 4 out of 16 mice (25%) survived and were killed after 120 h post-inoculation.

Based on the PCR results obtained, the oral infections of mice by *Pasteurella multocida* was able to produce infections in every organ isolated from the dead mice of the treatment group and the organs that were positive included heart, lung, liver, spleen, stomach, small intestine and large intestine. The presence of *Pasteurella multocida* in the lung, liver and spleen using PCR detection were similar with the findings of Zamri-Saad and Shafarin (2007) where they also successfully isolated and detected *P. multocida* type B (by using PCR) from these organs and the mode of inoculation were subcutaneous and intra-tracheal routes in the goats. The detection of *P. multocida* from the heart, on the other hand, was similar to the study conducted by Ashraf *et al.* (2011), Khin *et al.* (2010), Zamri-Saad and Shafarin (2007) and Shafarin *et al.* (2009), where they successfully isolated and detected *P. multocida* using PCR and the mode of inoculation were intranasal, intratracheal, subcutaneous and intraperitoneal route of infection. The detection of *P. multocida* in small intestine by using PCR was similar to the findings of Khin *et al.* (2010). In that study, the *P. multocida* was challenged intra-tracheal in 8 months old calves. However, there were no documented findings of *P. multocida* type B isolated and detected from large intestine, whereas in the present study, the presence of *P. multocida* was detected using PCR in all the large intestine samples from the mice which died within 120 h period.

Therefore, in the present study *P. multocida* was isolated and detected from large intestine of the mice using PCR following oral inoculation with the pathogen.

CONCLUSION AND FUTURE PERSPECTIVES

From the result of the present study, all the mice inoculated orally with *P. multocida* type: B revealed successful demonstration of infection with the bacteria. Therefore, by using mice as a model for the study of HS will be more cost effective than the actual hosts which are cattle and buffaloes. Detection of the pathogenic organism using PCR can be more effective for the diagnosis of the disease based on the specificity and sensitivity. Further studies are required to fully understand the pathogenicity of *P. multocida* type B:2 through oral route of inoculation. The use of multiplex PCR is recommended in detection of *P. multocida* type B compared to singleplex PCR as used in this study, since it is far more sensitive and specific in providing better PCR result.

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