

Polymerase Chain Reaction Detection of *Pasteurella multocida* Type B: 2 in Mice Infected with Contaminated River Water

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ABSTRACT

Hemorrhagic septicemia is an acute, deadly disease of cattle and buffaloes associated with colossal economic loss in the livestock industry in the Asian regions particularly Malaysia. Therefore, this study was conducted to investigate on the Polymerase chain reaction detection of *Pasteurella multocida* type B: 2 in mice inoculated through different routes with river water contaminated with infected mice carcasses. Sixty five mice were used for the study; five mice were placed in each tank containing river water for 24, 48 and 72 h. The groups comprise of five mice each made up of the control, intraperitoneal, oral and the aerosol routes. A dose of 1 mL 10^9 CFU of *Pasteurella multocida* type B: 2 obtained from the infected river water were inoculated into each group intraperitoneally and the aerosol route while, 0.4 mL of 10^9 CFU of *Pasteurella multocida* type B: 2 was inoculated orally into the group. The control group was inoculated with 1 mL buffer saline pH 7. The PCR results in the present study revealed the presence of *P. multocida* type B: 2 from the following organs brain, kidney, heart, spleen, lung and liver in the mice inoculated through intraperitoneal, oral and aerosol route. In the river water kept for 24 h *P. multocida* type B: 2 were detected in the organs through the intraperitoneal, oral and the aerosol routes. The river water kept for 48 and 72 h were positive for the isolation of *P. multocida* inoculated via the intraperitoneal and oral route, except the aerosol route where no significant *P. multocida* was detected in the organs using PCR. In conclusion, this model could be used to enhance the understanding of the progression of the disease and control of the natural disease through the various routes of the disease transmission. This study also postulated that the outbreak of HS among buffaloes and cattle could be due to the consumption of river water contaminated with infected HS carcasses.

Keywords: Detection, PCR, *Pasteurella Multocida* type B: 2, Different Routes, Contaminated River Water, Infected Mice Carcasses

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1. INTRODUCTION

Hemorrhagic Septicemia (HS) is a lethal, deadly, septicemic disease of cattle and buffaloes caused by fastidious serotypes of *Pasteurella multocida* a Gram negative coccobacillus bacteria in the family Pasteurellaceae related with multiple acute diseases in livestock (Jesse *et al.*, 2013a), *P. multocida* is an opportunistic pathogen (Ashraf *et al.*, 2011) and serotype B: 2 is the most significant reason of the disease in Asia (Abubakar and Zamri-Saad, 2011) and in some parts of Africa (Jesse *et al.*, 2013b) with momentous high morbidity and mortality leading to huge economic loss (OIE, 2008).

Nowadays, studies were carried out to investigate on the sources of infection with *Pasteurella multocida* in river water containing carcasses (Jesse *et al.*, 2013c). Jesse *et al.* (2013c) detected *Pasteurella multocida* Type B: 2 in Mice following oral inoculation using polymerase chain reaction, *P. multocida* type B was successfully isolated from the 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. There was no proof of the presence of *P. multocida* type B in the organs isolated from the surviving mice (Jesse *et al.*, 2013c).

In animals the progression of the disease has been regularly reported to occur following exposure of the vulnerable hosts to stressful states where infections generally occur by inhalation or ingestion (Shafarin *et al.*, 2009). The clinical signs of HS can also occur through the ingestion of infected foodstuff (Shafarin *et al.*, 2009; Ataei *et al.*, 2009). The clinical symptom of this disease is often distinguished by rapid course, high fever, loud and stertorous breathing, profuse salivation, severe depression and followed by death generally within 24 h of infection (Boyce *et al.*, 2010; Jesse *et al.*, 2013). A recent study of experimental nature has substantiated the progression of typical clinical changes of HS following oral route of inoculation of *Pasteurella multocida* type B in buffaloes (Abubakar and Zamri-Saad, 2011). 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, edema and other lesions in the respiratory tract have been generally detected in many cases with HS. However, there is still inadequate information about the pathogenicity and epidemiology of HS through the intraperitoneal, oral and aerosol routes with infected river water contaminated with mice carcasses kept for 24, 48 and 72 h. Therefore, the present study aims to identify *Pasteurella multocida* type B: 2 using polymerase chain reaction after infection of mice through different routes with water contaminated with infected mice carcasses.

2. MATERIALS AND METHODS

Sixty five healthy male mice of eight to ten weeks old were used 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 isolation, housed in plastic cages and provided with water and pellet ad libitum. Five mice were kept in each plastic cage for the control and treatment groups. The mice were observed for 2 weeks prior to the experiment to make sure that they acclimatize to the environment and were healthy.

2.1. Inoculums

Throughout the experiments, the wild-type *P. multocida* B: 2 used in this study were obtained from stock culture. It was isolated from a previous outbreak of 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, 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.

2.2. Experimental Design in Mouse Model

The river water was cultured to confirm that it was free from *P. multocida* type B: 2, the river water was obtained from Hulu Langat. Fifteen mice were initially inoculated with 1.0 mL of 10^9 colony forming unit (cfu) of *P. multocida* type B: 2 intraperitoneally. After 7-8 h of post inoculation survived mice were euthanized by cervical dislocation and the carcasses were placed in a tank containing river water. Five mice were placed in each tank for 24, 48 and 72 h and 1 mL of the pure colony of 10^9 of *P. multocida* type B: 2 were inoculated into five mice intraperitoneally and another five mice via the aerosol routes while 0.4 mL of 10^9 of *P. Multocida* type B: 2 was inoculated into mice five orally, after 48 h the mice were euthanized by cervical dislocation. The fourth group consists of the control group which had five mice and was inoculated with 1.0 mL of sterile Phosphate Buffered Saline (PBS) pH7. Thereafter, the moribund mice and surviving mice after 48 h were euthanized and Post mortem was conducted and the

following organs were sampled namely the brain, kidney, heart, spleen, lungs and liver. All the organs were cultured on the blood agar and incubated at 37°C for 24 h. PCR was performed on the chosen samples according to the organs from all the mice.

2.3. DNA Extraction

Extraction in the present study was performed using boiling method. A few colonies from the cultures were transferred into an Eppendorf tube containing 50 µL distilled water and the suspension was boiled at 100°C for 15 min. After boiling, the suspension was immediately cooled on ice for 2 min. Then, the suspension was centrifuged at 13, 000 rpm for 5 min. The upper phase was carefully transferred into another Eppendorf tube to be use as DNA template.

2.4. PCR Condition

The PCR was performed in a touchdown thermocycler in a total reaction volume 10 uL of PCR buffer, MgCl₂, 250 uM of deoxynucleotide triphosphate, 2 U of Taq DNA polymerase and 1 uM of each forward and reverse primer and 5 uL of template DNA. Amplification was performed with 30 cycles following an initial denaturing step at 94°C for 5 min. Each cycle involved denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 5 min.

2.5. Primer Design

The primer for the amplification of the *P. multocida* was referenced to (OIE, 2012). The forward primer used was KMT1SP6: 5'-GCT-GTA-AAC-GAA-CTC-GCC-AC-3' while the reverse primer used was KMT1T7: 5'-ATC-CGC-TAT-TTA-CCC-AGT-GG-3' for *P. multocida* in general. The base pair for *P. multocida* in general was 460. The forward primer used was KTSP61: 5'-ATC-CGC-TAA-CAC-ACT-CTC-3' while the reverse primer used was KTT72: 5'-AGG-CTC-GTT-TGG-ATT-ATG-AAG-3' for *P. multocida* type B: 2. The base pair for *P. multocida* in general was 620. PCR amplification with the primer pair design during the sequencing of clone 6b (KMT1SP6-KMT1T7) specifically produced a product approximately 460bp from HS- causing *P. multocida*. PCR amplification with the primer pair design during the sequencing of clone 6b (KTSP61-KTT72) specifically produced a product approximately 620bp from HS- causing *P. multocida* type B. These primers were unable to amplify DNA from

other *P. Multocida* species and other numbers of pasteurellacea family or unrelated bacteria.

2.6. Agarose Gel Preparation

About 1.5% agarose gel was prepared; 1.5 g agarose gel powder was poured into 100 mL bijour bottle, then top up with 1% TAE to 100 mL. The mixture was heated in microwave oven for about 3-5 min until all the precipitate melted, liquid form gel was then leave to cool down to 60°C. After that it was poured into suitable size cast. Wait for the gel to solidify for about 30 min. After the gel was solidified and turns to cloudy white color the gel was then ready for PCR loading and electrophoresis.

2.7. Electrophoresis

Agarose gel was placed into the gel holder tank and submerged with 1% TAE buffer and placing the holding wells near the negative terminal to allow the band to run towards the positive terminal. Make sure the positive and negative terminals are placed properly. One hundred bp marker (Promega) was used. Five uL of PCR product was loaded into the well carefully without breaking it with the pipette. One uL of loading dye was mixed with 2 uL ladders by using pipette and loaded into the first well. The PCR were run in 1.5% agarose gel for 40 min at 90 V. Than the gel was stained with ethidium bromide 0.5 ug mL⁻¹ solutions and stirred for 20 min, then the gel was dip into distilled water. Lastly, the gel was placed under UV gel imaging capturing machine and the results was recorded. Thereafter, the moribund mice and surviving mice after 48 h were euthanized and Post mortem was conducted and the following organs were sampled namely the brain, kidney, heart, spleen, lungs and liver. All the organs were cultured on the blood agar and incubated at 37°C for 24 h. PCR was performed on the chosen samples according to the organs from all the mice.

3. RESULTS

The PCR results in the present study revealed the isolation of *P. multocida* from the following organs brain, kidney, heart, spleen, lung and liver in the mice inoculated through intraperitoneally, orally and aerosol route. In the river water kept for 24 h there were positive identification of *P. multocida* inoculated through the intraperitoneal, orally and the aerosol routes (**Table 1 and Fig. 1**). The river water kept for 48 and 72 h were positive for the isolation of *P. multocida* inoculated via the intraperitoneal and oral route, except the aerosol route where no *P. multocida* was identified in the organs using PCR (**Table 2 and Fig. 2**).

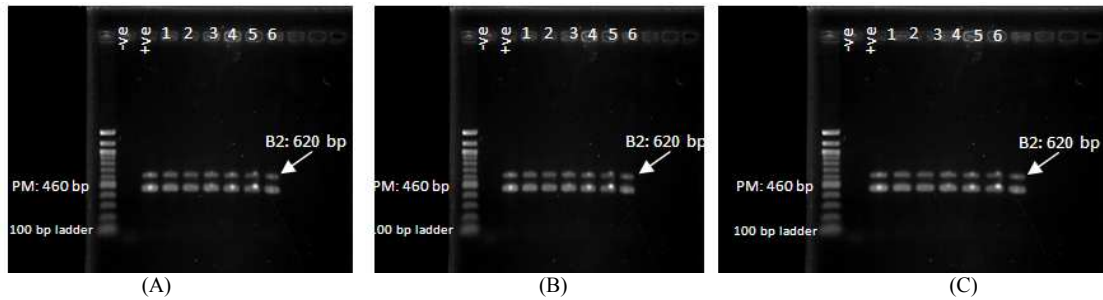


Fig. 1. PCR identification of *P. multocida* type B: 2 in organs inoculated through different routes with river water contaminated with infected mice carcasses kept for 24 h. A = Intraperitoneal route; B = Oral route; C = Aerosol route. 1 = Brain; 2 = Kidney; 3 = Heart; 4 = Spleen; 5 = Lung; 6 = Liver

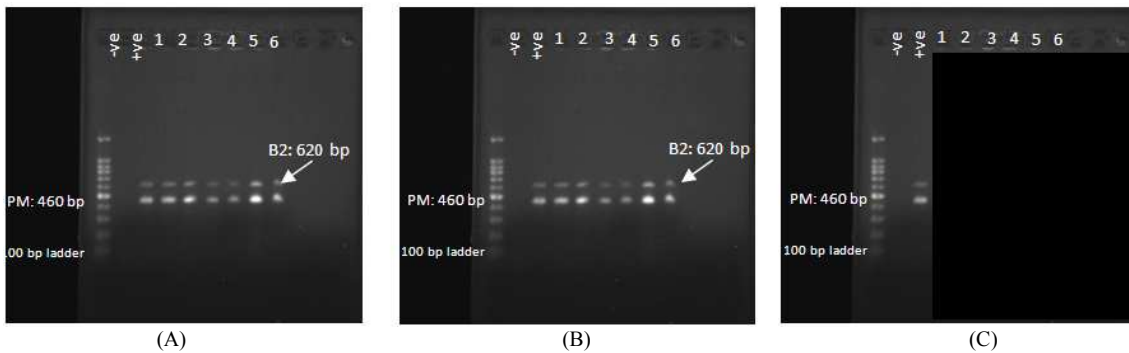


Fig. 2. PCR identification of *P. multocida* type B: 2 in organs inoculated through different routes with river water contaminated with infected mice carcasses kept for 48 h. A = Intraperitoneal route; B = Oral route; C = Aerosol route (negative); 1 = Brain; 2 = Kidney; 3 = Heart; 4 = Spleen; 5 = Lung; 6 = Liver

Table 1. Status of organs kept for 24 h in river water infected with *P. multocida*

Organs	Status	
	Positive	Negative
Brain	IP,OR,AE	NA
Kidney	IP,OR,AE	NA
Heart	IP,OR,AE	NA
Spleen	IP,OR,AE	NA
Lung	IP,OR,AE	NA
Liver	IP,OR,AE	NA

IP = Intraperitoneally; OR = Orally; AE = Aerosol; NA = Not Applicable

Table 2. Status of organs kept for 48 h in river water infected with *P. multocida*

Organs	Status	
	Positive	Negative
Brain	IP,OR	AE
Kidney	IP,OR	AE
Heart	IP,OR	AE
Spleen	IP,OR	AE
Lung	IP,OR	AE
Liver	IP,OR	AE

IP = Intraperitoneally; OR = Orally; AE = Aerosol

4. DISCUSSION

Following intraperitoneal, oral and aerosol routes of *P. multocida* type B: 2 inoculations with infected river water kept for 24 h established hemorrhagic septicemia in the mouse model in the current study. Similar observations have been reported in cattle, buffaloes and goats following experimental infection with *P. multocida* B: 2 (Jesse *et al.*, 2013c; Zamri-Saad and Shafarin, 2007), they suggested that the efficacy of certain route of infection can be established by observing the number of animals that died per-acutely following experimental infection with *P. multocida* type B: 2. In the study they conducted, 60% of the goats died per-acutely following experimental subcutaneous route of infection. The subcutaneous route of infection was regarded as the best technique for experimental induction of the disease in goats. In the present study 5 mice each were inoculation with the infected river water kept for 24, 48 and 72 h orally, intraperitoneally and by the aerosol route. Subsequently, the doses of 0.4 mL of 10^9 cfu inoculated orally and 1 mL of 10^9 cfu inoculated intraperitoneally and by the aerosol routes were able to produce the disease in the

mouse model which lead to the per-acute death of the animals within 24, 48 and 72 h of post inoculation. The animals in the intraperitoneal group died faster compared to oral and the aerosol groups.

The results obtained from the current study is similar to the study carried out by Jesse *et al.* (2013c) where they detected *P. multocida* Type B: 2 in mice following oral inoculation using polymerase chain reaction, *P. multocida* type B was successfully isolated from the 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. However, there was no proof of the presence of *P. multocida* type B in the organs isolated from the surviving mice in a study conducted by Jesse *et al.* (2013c).

According to the PCR result derived from the intraperitoneal, oral and the aerosol routes within 24, 48 and 72 h of infection with infected river water with *P. multocida* type B: 2 were able to induced infections in the following organs namely brain, kidney, heart, spleen, lung and liver with the exception of the aerosol routes at 48 and 72 h where no infections were detected using PCR in all the organs. The existence of *P. multocida* type B: 2 in the organs using PCR detection technique were similar with the findings of Zamri-Saad and Shafarin (2007), through the subcutaneous and the intra-tracheal routes in goats. The isolation of *P. multocida* type B: 2 from the heart in the current study in the river water kept for 24 h was similar to the study carried out by Ashraf *et al.* (2011); Khin *et al.* (2010); Zamri-Saad and Shafarin (2007) and Shafarin *et al.* (2009) via intra-nasal, intra-tracheal, subcutaneous and the intraperitoneal routes of infections.

5. CONCLUSION

In conclusion, identification and isolation of *P. multocida* type B: 2 from infected river water kept for 24 h using PCR revealed positive results in organs of the mice inoculated intraperitoneally, orally and by the aerosol routes. Furthermore, no *P. multocida* type B: 2 were detected in the organs of the mice inoculated by the aerosol route with the river water kept for 48 and 72 h using PCR. Therefore, the intraperitoneal route is the most effective route of HS induction and it could also portray an effective and efficient route for effective and heightened immunomodulatory establishment. Additionally, this model could be used to enhance the understanding of the progression of the disease and control of the natural disease through the various routes of the disease transmission. This study also postulated that the outbreak of HS among buffaloes and cattle could be due to the consumption of river water contaminated with infected HS carcasses.

6. ACKNOWLEDGEMENT

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