Original Research Paper

Volume - 7 | Issue - 8 | August - 2017 | ISSN - 2249-555X | IF : 4.894 | IC Value : 79.96

Veterinary Medicine

EXPERIMENTAL INFECTION OF POECILIA RETICULATE WITH *PASTEURELLA MULTOCIDA* TYPE B2

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ABSTRACT Haemorrhagic septicaemia (HS) is a highly fatal disease in cattle and buffaloes caused by specific serotypes of Pasteurella multocida and have emerged as a disease of considerable economic importance particularly in Malaysia. Experimental study was designed and conducted to look on the possibility of *P. multocida* B^2 to establish clinical infection in Poecillia reticulate which may become an important source of infection in HS outbreak. Twenty P. reticulate (common guppy) was divided into four equal groups namely A, B, C, D, consisting of five fish each. Thirty ml of 10^8 colony forming unit (cfu) of *P. multocida* B^2 was added in the aquarium of fish in group B. Dead mouse previously infected with 1ml 108 cfu *P. multocida* was placed in fish in group C, while fish in groups A and D act as control respectively. Bacteriology from water samples, gills and peritoneum and PCR from fish organ samples were negative for P. multocida. Therefore, an attempt of experimental infection of *P. multocida* in *P. reticulate* did not produce clinical infection. This study postulates that P. reticulate is not a susceptible host for the bacteria.

KEYWORDS : Experimental infection, Haemorrhagic septicaemia, *Pasteurella multocida, Poecilia reticulate,* Pathology.

Introduction

Pasteurella multocida is known to be associated with the upper respiratory tract as natural microflora and in the lymphatic system, where in cases of immunocompromised animals, the bacteria will proliferate and cause infection (Cynthia, 2010). However, the fact that dead cattle and buffaloes are usually found near the river, stream and natural water sources (Saharee et al., 1991; Wirdatul, 2002; Mokhtar & Yeo, 1991) suggests the possibilities of infection which can occur when the animal takes up the bacteria from the contaminated natural water sources. This is supported by the fact that the bacteria are able to survive in water. How it survive and what makes the bacterium survive are poorly understood but there is evidence regarding the ability of the virulent type of *P. multocida* to invade, replicate and escape from free-living amoeba (Matthew and Carmel, 2005) which suggest the possibilities of other aquatic life form such as fish, susceptible to the bacteria or as a carrier host that shed the pathogen and contaminating the aquatic environment. Previous experimental studies of inoculation of *P. multocida* had been conducted on many species of animal but never on fish. Thus, this study was designed to observe the possible effect and clinical changes in fish following administration of P. multocida type B^2 in water and to observe the survivability of the bacteria in the water.

Materials and Methods

Bacteria inocula: Throughout the experiments, the wild-type P. multocida B2 used in this study were obtained from a stock culture. The bacterium was isolated from a previous outbreak of HS in the state of Kelantan. The isolate was confirmed to be *P. multocida* type B2 by the Department of Veterinary Services (DVS) Kubang Kerian, Kelantan. A standard method of inocula preparation was followed to prepare a concentration of 10^8 colony forming units (CFU) and was determined by using McFarland Nephelometer Barium Sulfate Standards.

Inoculation of *P. multocida* in mice: Four Swiss Albino mice of four to six weeks old used in this study were obtained from Animal Research and Service Centre (ARASC), University Sains Malaysia (USM) Kubang Kerian, Kelantan. Each mouse was housed in different plastic cages and provided with water and pellet ad libitum for one week to make sure the mice acclimatize to the environment and were healthy. After a week, two mice were inoculated with 1.0 mL of 10^8 cfu of *P. multocida* B2 inocula intraperitoneally while the other two control mice were inoculated with 1.0mL of sterile phosphate buffered saline (PBS) pH7. After 5 hours of inoculation, blood samples were taken from the ocular vein for bacteria culture and the smears were stained with Giemsa stain. Both mice that were inoculated with *P. multocida* B 2 died eight hours post-infection.

Experimental Design of Poecillia reticulate infection: Twenty healthy male *Poecillia reticulate* fish brought from a fish shop at Padang Tembak, Kota baharu, Kelantan were used in this experiment. The fish were placed in plastic aquariums containing de-chlorinated (anti-chlorine Ki-Ki™, one ml to five litters of water) tap water and fed with fish pellet *ad libitum*. Water samples were taken every day for bacteria culture and a few extra fish was selected for post-mortem and samples of peritoneal swab and gills were taken for bacteria culture to confirm negative for *P. multocida* B 2. The fish were observed for one week prior to the experiment to make sure that they acclimatize to the environment and healthy. The fish was divided into four groups consisting of five fish each namely group A, B, C, D. All tank contained five liters of de-chlorinated tap water. 30 mL of 10^8 cfu units of *P. multocida* was added to tank B, euthanized dead mouse carcass that was not infected with *P. multocida* was placed in tank C and carcass of mouse infected with the bacteria was placed in tank D. Water samples were taken every 24 hours for routine bacteria culture. Clinical sign and motility were observed everyday for five days. Post-mortem was conducted on dead fish during the experimental period. Fish that survived was euthanized using overdose anaesthetic agent ENO™, GlaxoSmithKline (sodium bicarbonate, citric acid and anhydrous sodium carbonate) approximately 200g per five liters of water and post-mortem conducted. Peritoneal swabs and gills were sampled for bacteriology and the pooled of fish organs includes liver, kidney, bladder, gills, spleen, stomach, pyloric caeca and muscle were sampled for polymerase chain reaction (PCR).

Post-mortem Technique and Sample Collection: Before post-mortem was conducted, the carcass were observed for any lesion or abnormality and recorded. Sterile forceps was used to lift up the operculum and sterile scissors were used to cut part of the operculum to expose the gills. Abdominal exposure was made at the ventral midline starting from between the pelvic fin and extends dorsally to the anus. Sterile swabs were used to take the peritoneal samples. The fish were then cut into two halves and sampled for PCR. All samples for bacteriology were placed in BHI broth and incubated for four to six hours at 37.0^0C before bacterial isolation and identification.

Supernatant sample preparation for PCR: The pooled sample of individual fish consists of heart, liver; kidney, bladder, gills, spleen,

stomach, pyloric caeca and muscle were grinded in sterile mortars and pestles with the aid of sterile sand and one to two ml of phosphate buffered saline (PBS) pH 7, until the sample become well grinded. An additional one to two ml PBS was gradually added until a 10% suspension was obtained. The suspension was centrifuged at 4000 rpm for 15 minutes. The supernatant was collected into sterile bottles and stored at -20^{o}C untill used.

DNA Extraction: To obtain purified DNA that was covered and stored at -20 to -70 ^{o}C until used. PCR was performed in a thermocycler. 1% of agarose gel was prepared for use in electrophoresis. The PCR was operated in 1% agarose gel for 45 min at 80 V. then, the gel was placed under UV gel imaging capturing machine and the results were recorded.

Results

Two mice that were inoculated with 1ml 10^{8} cfu *P. multocida* B2 died eight hours post inoculation. Reisolations of inoculated P. multocida B2 were confirmed from liver samples. Infection in Poecilia reticulate with *P. multocida demonstrated* that all fish in tank A and B survived until day 7 of observation while all fish in tank C and D died at the end of day 5. Post-mortem revealed fish which survived until day 7 post-infection showed no gross lesion in all organs. Peritoneal swabs and gills were taken for bacterial culture and the pooled sample of fish organs were used for PCR. Several bacteria were isolated from the peritoneal swabs, gills and water samples that were taken daily but were negative for *P. multocida*. Only fish in tank B and C were taken for PCR and was negative for *P. multocida* B2 (Fig. 1).

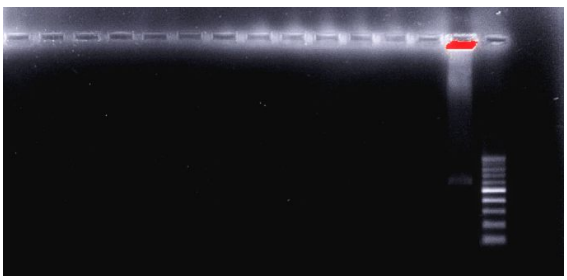

Fig.1: PCR identification of *P. multocida* B2 on selected fish sample. A = DNA ladder, B = positive control from stock culture of PMB2, 1 – 5 = fish sample from tank B, 6 – 10 fish sample from tank C.

Discussion

In this experiment, two groups of fish that were inoculated with pure culture of *P. multocida* B2 inocula did not show any clinical sign during the observation period. This result shows that the fish in tank C did not die due to *P. multocida* infection, rather it is believed to die due to the decomposing effect of the natural microflora of the fish that changed the water quality. *P. multocida* was not isolated from water samples taken daily until day 7. These findings however, contrary with other documented observation that the bacteria are able to survive in river and natural pond water. This might be due to tap water where even if it is pre-treated with anti-chlorine still contain unfavourable microenvironment compare to the river and natural pond water that do not support the growth of *P. multocida*. Both natural river water and tap water share a few mineral content in common which is calcium, magnesium, sodium, potassium where the metal concentration in river water is lower than tap water (Azrina et al., 2011). The effects of these micromineral content and metal bioavailbility on how it affects the survivability of haemorrhagic septicaemia derived-*P. multocida* in water is poorly understood. However, increasing the water soluble protein by 175 micrograms/ml and NaCL by 0.5%, greatly enhance survival of *P. multocida* (Backstrand and Botzler,1986). The other explanation of why *P. multocida* was not isolated from the water might be related to reduction of viable bacteria significantly due to overgrowth of other bacteria and contaminants from the fish that suppressed the growth of P. multocida. The result may alter if the enrichment medium (BHI broth) containing selected antimicrobial is used to eliminate other contaminants thus enhancing the growth of *P. multocida* or when the bacterium is inoculated into mice, the mice will die within 24 hours (De Alwis, 1999). In conclusion, experimental infection of *P. multocida* B2 via infected mouse carcass and bacterium inocula do not induce clinical infection in P. reticulate nor does the fish potential to become natural reservoir or carrier for the bacteria. There were also no *P. multocida* isolated from the water which means that the bacteria may not able to survive in de-chlorinated tap water. These findings rejected the hypothesis of the possibility of fish to take up the bacteria naturally, where the bacteria survive and proliferate in the fish and eventually shed through their faeces. However, it does not change the fact that the bacteria is able to survive in river, pond water or other natural fresh water.

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