

Full Length Research Paper

Recombinant clone ABA392 protects laboratory animals from *PASTEURELLA MULTOCIDA* Serotype B

Jamal Hussaini¹, Nazmul M. H. M.¹, Mahmood A. Abdullah² and Salmah Ismail²

¹Faculty of Medicine, Universiti Teknologi MARA, 40450 Shah Alam, Selangor, Malaysia.

²Department of Molecular Medicine, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia.

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In this study the potential of the previously contracted recombinant clone ABA392 derived from *PASTEURELLA MULTOCIDA* serotype B to protect laboratory animal against haemorrhagic septicaemia was determined. After reconfirmation of plasmid DNA size, its stability and pathological effect of the clone, passive mice protection test and active immunization was carried out. Pooled serum sample from rats vaccinated with live and killed form of the clone was administered to mice and provided 66% protection while active immunization with the recombinant clone conferred 83% immunity to mice when challenged with lethal dose of *P. MULTOCIDA*. ELISA results were positive for presence of antibody in serum of immunized mice. Sub-cloning of the insert ABA392 into an expression vector pQE32 was carried out to express its protein. It was found that the recombinant clone ABA392 is immunogenic and could be used as vaccine in future.

Key words: Recombinant clone, immunogenicity, *Pasteurella multocida* serotype B.

INTRODUCTION

Pasteurella multocida a Gram-negative coccobacillus commonly found in respiratory tract of many domestic and wild animals. *P. multocida* is causative agent of fowl cholera, atrophic rhinitis and haemorrhagic septicaemia (HS). The organism is capable of being pathogenic to human through animal bite. Most common human *P. multocida* infections are cellulites and abscess formation, however cases leading to meningitis, pneumonia, septic arthritis and endocarditis have been reported. HS is a highly fatal disease of cattle and water buffalo. In susceptible animals, the symptoms progress rapidly from dullness and fever to death within hours and recovery is rare (De Alwis et al., 1980). HS results from infection by *P. multocida* serotypes 6B or 6E (namiokacarter classification) or serotypes 2 or E2 (carter heddleston classification). HS is an important disease in Asia, Africa, some southern European countries and the Middle East. The highest incidence is in Southeast Asia causing great economical loss to the region (Bain et al., 1982).

Pathogenicity and immunogenicity study of various components, derivatives, clones and mutants of *P. multocida* (Tabatabaei et al., 2007) has been carried out in order to find a suitable candidate for vaccine (Montserrat et al., 2004; Keith et al., 2007). In search of a promising vaccine, a recombinant vaccine would be an ideal choice of immunization. A recombinant clone ABA392 derived from HS isolate of *P. multocida* serotype B (PMB) (strain 202) constructed by Salmah (1997), carrying gene causing HS was used in this study as potential candidate for recombinant vaccine.

The aim of this study was to carry out the molecular characterization, histopathological analyses and immunological determination of the clone ABA392.

MATERIALS AND METHODS

Bacterial isolates

The bacterial strains and the recombinant clone ABA392 used in this study was constructed and provided by Dr. Salmah, Faculty of Medicine, University of Malaya, Malaysia. The clone ABA392 is derived from a *P. multocida* serotype B 202, carrying a recombinant plasmid. This clone was constructed by shotgun cloning method

*Corresponding author. E-mail: jamalh@salam.uitm.edu.my.
Tel: +603-55442886. Fax: +603-55442831.

using *E. coli* as host. Briefly, the *Pasteurella multocida* serotype B isolate, PMB202 genomic DNA was partially digested with the restriction endonuclease *Sau3A* 1, generating restriction fragments of approximately 500 to 1000 bp. were inserted at the unique *Bam*HI site in the vector plasmid pUC18 (Vieira and Messing, 1982). Of twenty selected recombinant plasmids, three of the clones obtained, ABA182, ABA282 and ABA392 were found toxigenic to mice. Only one clone, ABA392, showed mortality in mice. The recombinant plasmid previously sequenced harbours a sequence that code for a virulence factor to *P. Multocida*. The insert was found to be 921 bp. The strains were grown on brain heart infusion (BHI) blood agar incubated at 37°C overnight.

Plasmid stability testing

Plasmid stability testing was carried out as explained by Lanka and Barth with modification (Lanka and Barth, 1981). The cultures of ABA392 were grown overnight at 37°C in BHI broth. The clones were sub-cultured in BHI broth and BHI blood agar and were incubated overnight. The process of subculturing was repeated at least 10 times and after a final transfer into a fresh BHI broth and agar the clones were subjected to plasmid DNA extraction using promega wizard© miniprep plasmid DNA purification kit (USA).

Restriction endonuclease analysis (REA)

Restriction endonuclease analysis (REA) was performed as described previously by Salmah (2004). Three restriction enzymes *Eco*RI, *Pst*I and *Sma*I were used for single digestion and to release the insert gene of the recombinant clone for sizing. Double digestion of the recombinant clone plasmid DNA with restriction enzymes *Pst*I and *Sma*I was carried out. REA was done under conditions recommended by the supplier (New England Biolabs. Inc., U.S.A. and Bethesda Research Laboratories, U.S.A.). Restricted DNA (0.2 to 1.0 µg) was subjected to electrophoresis in 1.0% agarose gel.

Pathogenicity study

The cultures of ABA392 were grown overnight at 37°C in a 10 ml of BHI broth medium. The cultures were centrifuged for 5 minutes at 14000 r.p.m to pellet the cells. Cells were then resuspended in 5ml of phosphate buffered saline (PBS), pH 7.2 to achieve the concentration of 10⁷ colony forming units CFU (Yazmin, 2003). In this study, 3-4 weeks old male Sprague Dawley's (SK/WDY) rats obtained from the animal house, Faculty of Medicine, University of Malaya were used. Rats were injected intramuscularly with 0.2 ml dexamethasone (0.33 mg/kg body weight) (Gardiner et al., 1996; Salmah et al., 2003) on the first day and thereafter the rats received 0.1 ml once daily. The rats were then challenged with 0.5 ml of bacterial inoculum intraperitoneally once on the day one only. Blood was collected from rats on daily basis for haematological assays and finally the rats were dissected at the end of the experiment and their internal organs used for histopathological studies and histology slides were prepared.

Passive mice protection test (PMPT)

Pathogen free male SK/WDY rats weighing 150 to 250 g were used. Rats were divided in to different groups where each group was injected intramuscularly with 1ml of either killed or live strains of ABA392 or PMB202 prepared in Freund's complete adjuvant on 0, 2 and 4 weeks intervals. Pooled sera sample was prepared from each group of immunized rats and 0.5 ml of it was then injected into

6 to 8 weeks old ICR mice. Twenty four hours later, the mice were challenged with lethal doses of PMB and were kept under observation for 10 days (Bain et al., 1982).

Active immunization

Active immunization was carried out by immunization of 6 to 8 weeks old ICR mice as mentioned earlier. Mice were divided into different groups, where each group was injected intramuscularly with 1 ml (10⁷) of either killed or live strains of clone ABA392 or *P. multocida* serotype B202. The bacterial strains were prepared in Freund's complete adjuvant. The vaccination was carried out either with a two dose or three dose schedules. In two dose schedule the vaccines were given on day zero followed by a booster dose two weeks later. Whereas the three dose schedule, vaccination was on day 0, week 2 and week 4 intervals. Serum was collected from all the rats before vaccination and one month post vaccination. The following day the mice were injected with lethal dose of PMB (Bain et al., 1982).

Enzyme-linked immunosorbent assay (ELISA)

ELISA was developed based on the procedure described by Pati et al. (1996) with modifications. ELISA was performed on the hyper-immune sera obtained from the immunized mice to detect antibody. 100 µl of antigen was coated on the 96 well microtiter plate, sealed and kept overnight at 4°C. 100 µl of serum was then added and incubated for 1 hour at room temperature followed by 3 times wash with phosphate buffered saline with tween 20 (PBS-Tween 20) by using a multi-channel pipette. 100µl of diluted Anti-mouse IgG (H+L) Alkaline Phosphatase conjugate was then added followed by washing step as mentioned. 200 µl of the substrate was added and incubated for 30 min at room temperature and finally the reaction was stopped by adding 200 µl NaOH. The absorbance OD was read at 405 nm. All samples, including blanks, positive and negative controls were used in triplicates. The positive control used, was samples obtained from mice which had been hyper-immunized with *P. multocida* serotype B. The bacterial cells were grown in 500 ml of BHI broth at 37°C. The harvested culture were washed in PBS twice and then resuspended in fresh 0.2% formalinized PBS. This process inactivates the organism after 24 h of incubation. The density of the suspension was adjusted till a concentration of 10⁷ CFU was achieved. The negative control used was serum, collected from healthy mice which were not exposed to *P. multocida* and injected with PBS as mentioned above. Cut-off value was determined using the two-graph receiver operating characteristic (TG-ROC), a Microsoft-Excel program. The ELISA results were analyzed based on the cut-off value including sensitivity and specificity obtained from TG-ROC.

RESULTS

Molecular analyses

The recombinant clone ABA392 was found to maintain its plasmid profile after it was subjected to plasmid DNA stability testing up to 10 generations. The clone carries 2 plasmid DNA, open circular plasmid (oc plasmid) with and approximate size of 5.6 kb and covalently closed plasmid (ccc plasmid) of 3.5 kb. RE analysis of the clone was carried out to determine the size of the insert. After single digestion of plasmid with *Eco*RI, *Pst*I and *Sma*I, 1

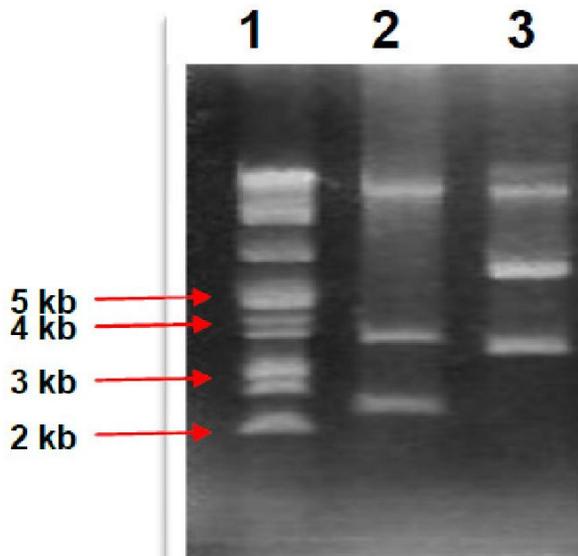


Figure 1. Agarose gel showing the insert size (ABA392). Lane 1: Supercoil DNA Ladder; Lane 2: pUC18; Lane 3: ABA392.

fragment of 3.5 kb in size was observed (Figure 1). Two linearized fragments of 2.7 and 0.9 kb in size, corresponding to pUC18 and the inserted gene respectively was produced when the double digestion with *Pst*I and *Sma*I was carried out (Figure 2).

Pathogenicity study

From histopathological examination it was observed that, when 0.5 ml of 10^7 CFU bacterial suspensions of the clone and PMB injected into immunosuppressed SK/WDY rats with dexamethasone (0.33 mg/kg body weight), typical HS symptoms are produced. Gross pathology showed severe multiple abscesses in parenchyma of lung. Upon examination of the histological slides, severe congestion of parenchyma of the lungs and infiltration of inflammatory cells were seen. Microscopic examination of histo-slides revealed severe haemorrhagic effect on spleen, lungs and liver (Figure 3).

Immunogenicity determination

On average, the hyper-immune sera obtained from vaccination of both killed and live form of the clone ABA392 (2 and 3 dose vaccination) provided 66% protection to mice challenged with *P. multocida* serotype B;2. On other hand the hyper-immune sera obtained from the parental strain PMB202 with similar vaccination, provided 100% protection. Mice that were actively vaccinated with ABA392 vaccine, when challenged with lethal dose of PMB showed 83% protection. The control

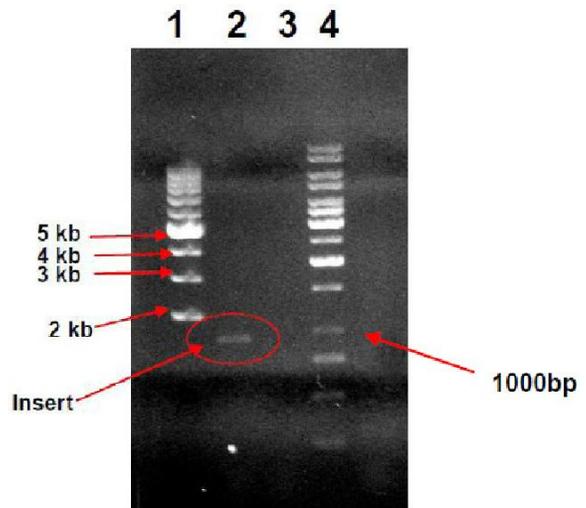


Figure 2. Plasmid Restriction Endonuclease Analysis was carried out, using the enzymes *Pst*I and *Sma*I. From the RE analysis the insert was recovered and found to be of the expected 0.9kb in size. Lane 1: Supercoil DNA ladder; Lane 2: *Pst*I/*Sma*I; Lane 3: NA; Lane 4: 1 kb DNA ladder.

group which had not received any hyper-immune sera died within 36 h. ELISA showed positive result for presence of antibody from hyper-immune sera of the rats immunized with the clone ABA392.

DISCUSSION

Haemorrhagic septicaemia is a disease which is acute, febrile and lethal. The condition sets in rapidly and is capable of killing susceptible animals in less than 36 to 48 h (Dawkins et al., 1990). Livestock, especially cattle and buffaloes are of significant economical importance to many countries in the world. In several Asian and African countries, significant foreign exchange and earning is from live stock (Kock et al., 2002). This study was based on the previously created clone ABA392 from *P. multocida* serotype B; 2, 202 (Salmah, 2000). This clone was shown to produce HS like signs and symptoms when injected into mice intra-peritoneally. Mice died within 36 h of injection. In order to further understand and study the immunogenic properties of this clone, this experiment was conducted. The clone ABA392 has shown promising results. In phase one of this study it was important to reconfirm the clone's stability although the clone has been proven to be stable up to 20 generations. The clone was found to be stable and the expected 3.5 kb plasmid was recovered. RE analysis showed the insert of 0.9 kb when subjected to electrophoresis. An effective treatment for HS is difficult, not only due to sudden onset of symptoms and death of animals but also due to the rising number of antibiotic resistance strains (Bindu et al., 2008;

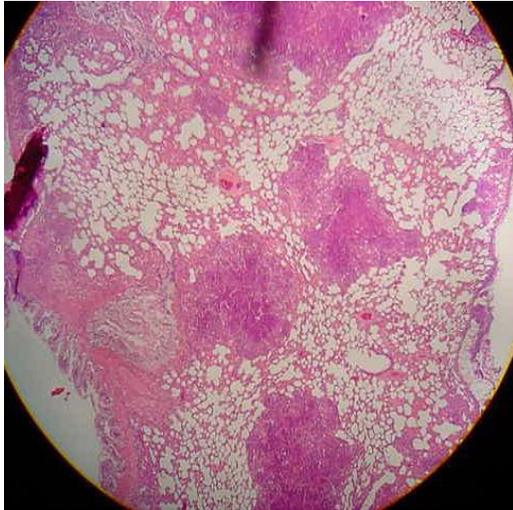


Figure 3. Histological section of Lung in animal treated with Dexamethasone and inoculated with ABA392 clone, showing severe congestion of parenchyma of the lungs and infiltration of inflammatory cells. H&E stain (40X).

Hussam et al., 2003). Hence vaccination would be a better and cheaper option in HS control. ABA392 when used as killed or live attenuated vaccine in 2 doses 2 weeks apart provided no protection to the vaccinated mice and serum of the mice showed a negative result based on the cut-off value obtained by TG-ROC analysis. The 3-dose vaccination provided 83% protection and ELISA results showed a high titer of antibody production. The serum from these mice used in PMPT study provided 66% protection as well. At the same time the similar vaccination regime was carried out with *P. multocida* serotype B:2, the parental strain. The two-dose vaccination was shown to be ineffective and the ELISA revealed a low titre of antibody while the 3-dose vaccination had provided almost a 100% protection to mice with a very high antibody titre. This study as well as previous studies (Salmah, 1997; Yazmin, 2003) has shown that the clone ABA392 is virulent. The histo-slides (Figure 3) results revealed the closeness of the lesions caused by the clone to that of the ones caused by parental strain PMB which causes HS, and the immunization tests shown that this clone not only can rise antibody when introduced as vaccine but also can provides protection when challenged with lethal doses of PMB in mice. These results provide a strong ground for creating a new, specific and a safe recombinant vaccine against HS caused by PMB. It is well known that a recombinant vaccine is the preferred choice of vaccination and this recombinant clone ABA392 seems to be a potential candidate.

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