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ORIGINAL ARTICLE

Pathological study of Pasteurella Multocida Recombinant Clone ABA392

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ABSTRACT

Objective: In current study Molecular analysis of the recombinant clone ABA392 was carried out

Methodology. In this study, detailed molecular analysis of the recombinant clone ABA392 were carried out by examining plasmid stability, plasmid profiling, determination of the plasmid molecular weight, and restriction endonuclease analysis.

Results: From the experiments above, the plasmid size of ABA392 was estimated as 3.5 kb, while the size of the insert gene of ABA392 was estimated as 0.8 kb (double digested with *Pst*I and *Sma*I restriction enzymes). A Pathogenicity study of recombinant clone ABA392 was carried out via a mouse virulence test and histopathological analysis, where it was found that mice, which were challenged with ABA392 and the parent strain of *Pasteurella multocida* serotype B strain PMB202, were lethal within 24 to 72 hours upon inoculation. Based on microscopic examination, the virulence effect was observed by marked haemorrhage in lung, liver, spleen, and kidney tissues.

Conclusion: This study has also proven that the plasmid of recombinant clone ABA392 was stably maintained in the *Escherichia coli* JM101 host system as reported previously. A preliminary protein analysis via SDS-PAGE was performed and a distinct protein band was most likely to be detected, which was approximately 16kD. Nonetheless, further characterization of the recombinant clone ABA392 proteins needs to be done to determine the nature of these proteins, which are involved in the virulence mechanism of hemorrhagic septicemia.

Keywords: Plasmid profiling; Pathogenicity; recombinant clone; *Pasteurella multocida*

INTRODUCTION

Pasteurella multocida (P. multocida) is a gram-negative, capsulated coccobacillus that is nonmotile and facultatively anaerobic. It's usually serotyped using LPS and capsules [1]. *Pasteurella multocida* is an important veterinary pathogen that causes a range of animal diseases and can also be a human pathogen [2]. *Pasteurella multocida* has been recognized as an important veterinary pathogen for over a century [3] with high mortality rates and significant economic loss in Asia and Africa [4]. *Pasteurella multocida* is the etiological agent that causes a variety of diseases in a wide range of hosts all over the world, resulting in significant economic losses [5]. Its importance as a human pathogen has been increasingly recognized in the last fifty years. Two types of toxins are found in *P. multocida*, which are the endotoxin and protein toxin. This endotoxin activity is due to lip polysaccharide, an important structural component of the cell outer membrane found in all strains. In addition, the production of protein toxins by these bacteria has been recognized for some time, although it is only in the last decade that some definition has been given to the nature of these products [6].

It was found that the development of hemorrhagic septicemia is very much related to the immune status of the animals in which as soon as the immune system is depressed, the disease will appear and becomes more severe. The latest occurrence of hemorrhagic septicemia [7] was reported in Kuala Terengganu and Rantau Panjang in the year 2000 and 2001 in which the disease was confirmed to be caused by *Pasteurella multocida* serotype B. In this case, the animals were successfully vaccinated by using oil vaccine and bross vaccine to control the disease from spreading [8]. A new possibility of molecular studies has been developed in conjunction with the discovery of antibiotic resistant genes located on plasmid molecules and the construction of cloning vectors and gene transfer systems particularly for interspecies studies purpose.

However, no plasmid gene has been cloned from *Pasteurella multocida* into *E. coli* or other systems [6]. Previous study, which has been done by Peterson and Foged (1989) had cloned chromosomal gene from toxigenic strain of *Pasteurella multocida* serotype D and introduced it to the E. coli system and the extracts of these clones showed cytopathic activity identical to that of extracts of toxigenic *Pasteurella multocida* [9,10,11]. In 1997, a recombinant clone derived from *Pasteurella multocida* serotype B strain 202 was constructed by Salmah (1997) while latest study, which was also done by Salmah (2000) is more on the molecular characterization basis including sequencing the gene of interest of the constructed recombinant clone ABA392 [12].

The recombinant clone was then tested for mice lethality, which was carried out in previous study and it was suggested that the clone might contained the insert gene that encodes for hemorrhagic septicemia disease. Similarly manifested in the original species of *Pasteurella multocida* serotype B. Salmah (2000) to further characterize the unique genomic sequence, which has been proven to cause lethality in mice, constructed a clone ABA392 [6]. The main aims of the current study were the Molecular characterization of the recombinant clone ABA392, Virulence determination of the recombinant clone ABA392 and Preliminary SDS-PAGE analysis of the recombinant clone ABA392.

MATERIAL AND METHODOLOGY

Pasteurella Multocida serotype B, recombinant clone ABA392, pUC18 and *Escherichia coli* were provided by Dr. Salmah Ismail (2000) from Department of Molecular Medicine, University of Malaya [6]. These samples were cultured in Brain Heart Infusion **(**BHI) agar and broths. Plasmid extraction was done by using kit (Promega Wizard Plus) following the manufacturer's instruction. The purified plasmid was then being analyzed by agarose gel electrophoresis and later observed under UV and the molecular weight of the recombinant plasmid was determined by using 1 kb ladder.

15 ICR mice were used for the lethality test and 5 groups were made available for the mice in which each group consists of 3 mice. To observe the lethality of the recombinant clone ABA392, all of the bacteria (*Pasteurella multocida*, *Escherichia coli* JM101 and *Escherichia coli* pUC18) were cultured in 10 ml of BHI broth, which act as controls and were incubated for 24 hours, spun down (discard supernatant) and pellets were dissolved in 5 ml of phosphate buffer saline pH 7.2 to maintain cells viability. Each group of mice was inoculated with one type of bacteria mentioned above (0.5 ml) subcutaneously and also dexamethasone (0.3 ml) was administered intramuscularly. Blood samples obtained from

the tail of the mice were collected in EDTA tube continuously for 6 days including before inoculation of bacteria and total white blood cell count was done for all samples. Lethality was observed from day 1 onwards and the results were recorded. Histology slides were made by removing out specific organs from the dissected mice such as spleen, liver, lung and heart to observe the hemorrhagic effects from the recombinant clone ABA392. The organs were fixed in 10% formalin buffer; trimmed, undergone automated tissue processing that includes dehydration, clearing, infiltration and lastly embedding with paraffin. Next, the tissues in paraffin blocks were cut into thin slices by using microtome and were transferred onto glass slides. Each of them was stained with haematoxylin and eosin, mounted and finally being observed under light microscope. Any histological changes were observed and recorded. Preliminary study of the protein was done by SDS-PAGE. Protein samples for each bacterium were obtained by using medium scale preparation. Bacteria were again cultured in BHI broth, spun down and discard supernatant and the pellets were suspended in appropriate amount of lysis buffer and were sonicated for 3 times cycle. The mixture was spun down again and the supernatants were collected and stored in a -70ºC freezer until further use. Each sample was then subjected to protein quantitation, whereby Plusone 2D Quant kit was used to determine the concentration of proteins in each sample following the manufacturer's method.

Observation of distinct band(s) of the recombinant clone ABA392 was done by comparing it with the controls through SDS-PAGE. Mini gels were prepared with 12% concentration of separating gel. Methods of preparation and running condition were according to Laemmli [13]. The gels were visualized by using Coomassie blue stain. The protein bands for all samples were analyzed and any distinct band(s) in recombinant clone ABA392 was recorded.

RESULTS

Plasmid stability of Recombinant Clone ABA392: This is the crucial step taken in analyzing the plasmid of the recombinant clone ABA392 in order to ensure the stability and to determine the size of the plasmid. Plasmid purification kit method was used and had successfully extracted the plasmid of the recombinant clone ABA392 from *Escherichia coli* JM101 host cells. The plasmid ABA392 was stably maintained in the host system of *E. coli* JM101 in which the plasmid size of the clone was constantly shown as 3.5 kb even up to 10 generations similarly reported in previous study (data not shown) when analyzed by agarose gel electrophoresis (Figure 1).

The recombinant clone ABA392 showed two plasmid DNA bands with the esti-mation sizes of 5.5 kb (open circular plasmid) and 3.5 kb (covalently closed circular plasmid) as shown in Figure 1. Meanwhile, the estimated size of the vector plasmid of E. coli pUC18 was 2.7 kb (covalently closed circular plasmid). The 4.9 kb DNA band of E. coli pUC18 observed was an open circular plasmid (Figure 1). The plasmid sizes were determined by comparing them with the supercoiled DNA ladder (Promega, U.S.A) of known molecular weights that ranges from 2 kb to 10 kb. These estimated plasmid sizes were derived by plotting a logarithm graph of the standard molecular weights versus the distance traveled (mm) by the plasmid DNA.

Restriction Endonuclease Analysis (REA): From the agarose gel result shown in Figure 2, digestion of plasmid ABA392 with EcoRI, PstI and SmaI had produced one fragment with similar size of 3.5 kb. However, the recombinant clone ABA392 plasmid that was double digested with PstI and SmaI had produced two fragments, which consist of vector pUC18 fragment and also the insert gene fragment of the clone itself. The size of the insert gene of recombinant clone ABA392 was estimated as 0.8 kb while the size of the vector pUC18 was estimated as 2.7 kb (Figure 2; Lane 4). Lane 2 and 3 were of the nonrelated fragments.

Mice virulence test: The virulence effect of *Pasteurella multocida* serotype B strain PMB202 and recombinant clone ABA392 was determined in this study. The result above demonstrated that mice challenged with *Pasteurella multocida* serotype B strain PMB202 and recombinant clone ABA392 were lethal, whereas mice challenged with *E. coli JM101, E. coli pUC18* and phosphate buffer saline pH 7.2 were remained viable (Table 1). It was found that both PMB202 and ABA392 were virulent to mice at the concentration of 10^7 colony forming units (CFU), treated with dexamethasone and had caused death within 24 to 72 hours of post infection. Dexamethasone used in this experiment was meant to suppress the immune system of the mice in order to enhance the development of bacterial infections.

Histopathological analysis of the tissues: Pathological changes were observed in the tissues obtained from dissected mice. The results demonstrated were observed under X400 magnification to produce better histological visualization of the tissues. The result of liver, spleen, kidney and lung tissues showed marked hemorrhage affected with the recombinant clone ABA392 and parental strain of *Pasteurella multocida* serotype B strain PMB202. No hemorrhage was seen in the tissues affected with *Escherichia coli* JM101 and *Escherichia coli* pUC18 and also phosphate buffer saline (PBS) pH 7.2. The histology result of the normal control tissues were of similar to the tissues affected with *Escherichia coli* JM101 and *Escherichia coli* pUC18 (data not shown). Effect of hemorrhage was absent in the heart tissues although affected with *Pasteurella multocida* serotype B strain PMB202 and recombinant clone ABA392 (Figure 3 a,b,c,d,e).

SDS-PAGE Analysis: It was most likely to be seen that a distinct band was present in Lane 4 with an approximately protein mass of 16kD. No distinct band similar to Lane 4 was detected in Lane 1, 2 and 3 (Figure 4).

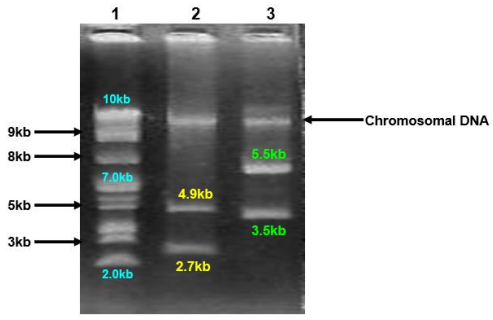

Figure 1. Agarose gel (1.0%) showing plasmid DNA extracted from the recombinant clone ABA392. *E. coli* pUC18 was used as control and supercoil DNA ladder was used as standard molecular weight marker. Lane 1: Supercoil DNA ladder, Lane 2: *E. coli* pUC18, Lane 3: Recombinant clone ABA392.

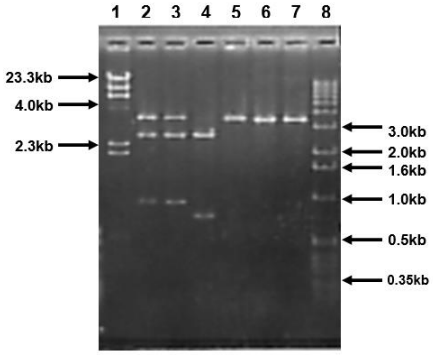

Figure 2. Agarose gel (1.0%) showing digested plasmid of recombinant clone ABA392 restricted with (*Eco*RI, *Pst*I, *Sma*I) restriction enzymes.Lane 1: *Hind*III marker, Lane 2: Non related, Lane 3: Non related, Lane 4: *Pst*I/*Sma*I-double digested plasmid ABA392, Lane 5: *Pst*I-digested plasmid ABA392, Lane 6: *Sma*I-digested plasmid ABA392, Lane 7: *Eco*RI-digested plasmid ABA392, Lane 8: 1 kb DNA ladder.

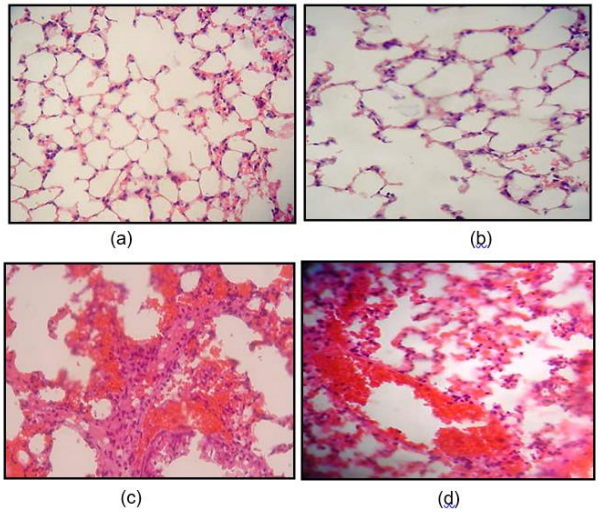

Figure 3. a. Histological section of lung tissues treated with dexamethasone and affected with (a) *E. coli* JM101, (b) *E. coli* pUC18, (c) ABA392 and (d) *Pasteurella multocida* serotype B strain PMB202, respectively.

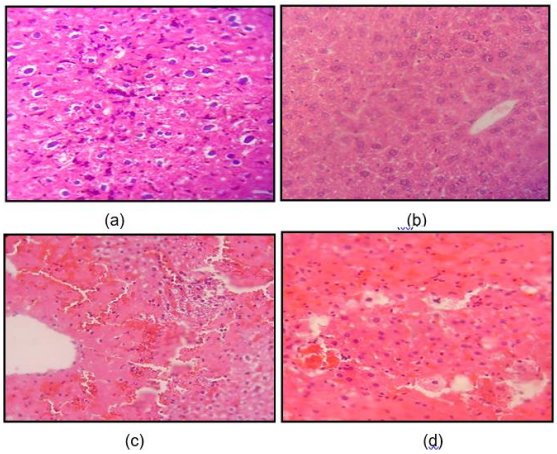

Figure 3. b. Histological section of liver tissues treated with dexamethasone and affected with (a) *E. coli* JM101, (b) *E. coli* pUC18, (c) ABA392 and (d) *Pasteurella multocida* serotype B strain PMB202, respectively.

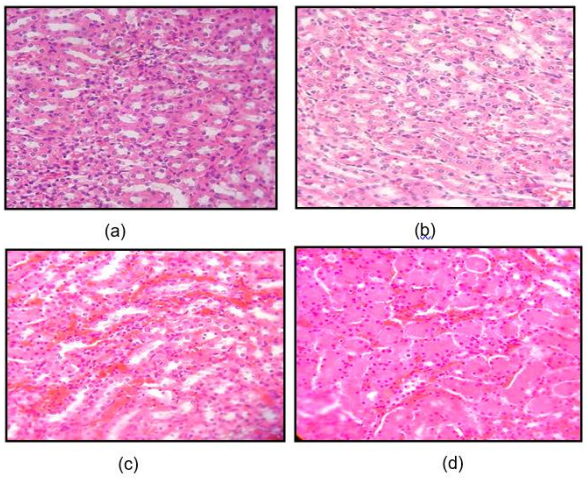

Figure 3. c. Histological sections of kidney tissues treated with dexamethasone and affected with (a) *E. coli* JM101, (b) *E. coli* pUC18, (c) ABA392 and (d) *Pasteurella multocida* serotype B strain PMB202, respectively.

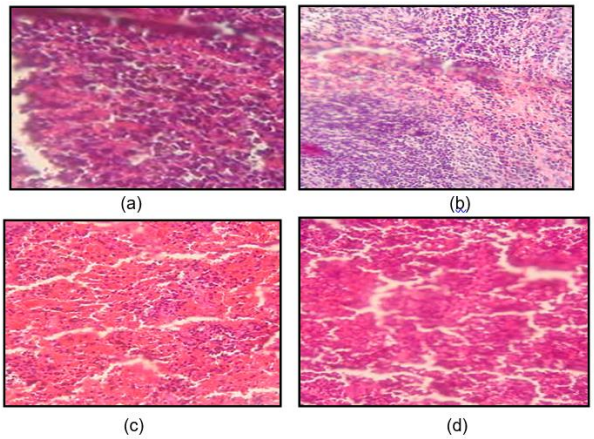

Figure 3. d. Histological sections of spleen tissues treated with dexamethasone and affected with (a) *E. coli* JM101, (b) *E. coli* pUC18, (c) ABA392 and (d) *Pasteurella multocida* serotype B strain PMB202, respectively.

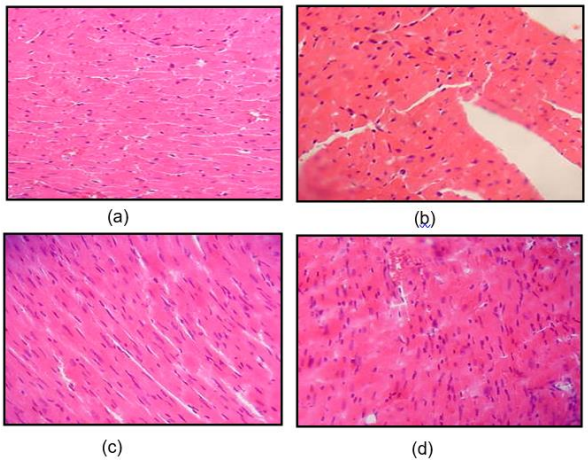

Figure 3. e. Histological sections of heart tissues treated with dexamethasone and affected with (a) *E. coli* JM101, (b) *E. coli* pUC18, (c) ABA392 and (d) *Pasteurella multocida* serotype B strain PMB202, respectively.

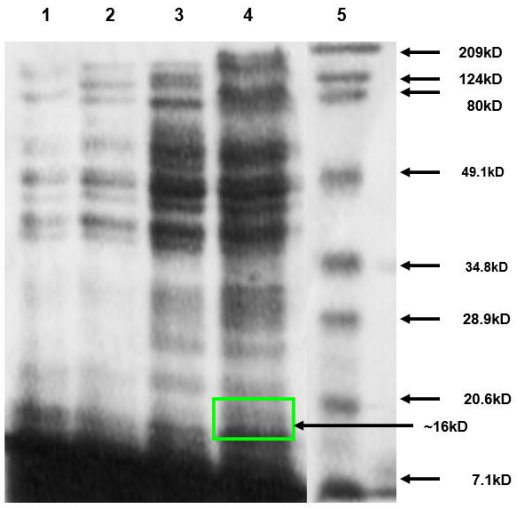

Figure 4. SDS-PAGE (12%) of medium scale bacterial cultures demonstrated by protein bands stained with Coomassie blue stain. Lane 1: *E.coli* pUC18, Lane 2: *E.coli* JM101, Lane 3: PMB202, Lane 4: Broad range protein marker.

Table 1. Virulence of *Pasteurella multocida* serotype B strain PMB202 and recombinant clone ABA392, as determined via subcutaneous challenge and treated with dexamethasone intramuscularly of female ICR mice.

Challenge strain + Dexamethasone	Colony Forming Unit (CFU) per mice	No. of survivors/ No. of Challenged
Escherichia coli JM101	10^7	3/3
Escherichia coli pUC18	10^7	3/3
PMB202	10^7	0/3
Recombinant clone ABA392	10^7	0/3
Normal saline	None	3/3

DISCUSSION

The results obtained for the plasmid sizes were constant even up to 10 generations when analyzed using agarose gel electrophoresis and indicated that the plasmid was stably maintained in the host system of *Escherichia coli* JM101. The results were also showed that the recombinant clone ABA392 possessed two plasmid DNA bands which designated it may have two different plasmids in the form of covalently closed circular (CCC) and open circular (OC) or only one plasmid in different configurations due to the nicking of the plasmid during plasmid extraction process. The presence of chromosomal DNA, which has been observed, has become the main problem during plasmid extraction process. Its presence is due to vigorous shaking of bacterial cells upon addition of the Cell Lysis Solution and its presence was due to the composition of the plasmid DNA that comprises of more than chromosomal DNA rather than the plasmid DNA itself. As a result, the chances of releasing chromosomal DNA during plasmid extraction are very high. Therefore, in order to eliminate such problem, samples must be treated gently during lysis step.

As the plasmid size of recombinant clone ABA392 has been determined, further characterization was conducted via restriction endonuclease analysis (REA) in order to confirm the insert DNA size of the clone. Restriction enzymes, which have been used in this study, were directed to the multiple cloning site of pUC 18 vector plasmid containing the insert gene of clone ABA392. This multiple cloning site were meant for various restriction enzymes including *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I and *Sma*I [14]. From the previous study, which has been conducted using restriction endonuclease analysis, no fragment was produced upon cleavage with *Bam*HI enzyme, which indicated the cleavage site for *Bam*HI has been degenerated [12].

According to the previous study, marked leucocytosis, lymphopenia and monocytopenia were observed upon injection of dexamethasone. The occurrence of leucocytosis was due to increment in circulating numbers of neutrophil, usually termed as neutrophilia. Lymphopenia and monocytopenia were probably due to severe decrease in the number of pre-cursor lymphocytes and monocyte cells in bone marrow by dexamethasone treatment that eventually leads to a decreased production of lymphocytes and monocytes [15].

However, the total white blood cell count was not performed in this study due to the early occurrence of lethality in mice although blood samples were taken for this purpose. Therefore, comparison cannot be established between the negative controls, normal control and the clone ABA392 along with the parental strain of PMB202 treated with dexamethasone. Thus, results were not available. The limitation in this part of my study was insufficient numbers of mice to perform *in vivo* study for further virulence confirmation of recombinant clone ABA392 and to overcome this limitation factor, repetition of *in vivo* study of recombinant clone ABA392 using large numbers of mice need to be done to justify the results obtained in the current study.

Confirmation study of virulence in the recombinant clone ABA392 has been done via histopathological analysis of the lung, spleen, liver, kidney and heart tissues. All tissues infected with *Pasteurella multocida* serotype B strain PMB202 and recombinant clone ABA392 were present with severe hemorrhagic effect except in the heart tissue. The effect was demonstrated as destruction of the cells alignment and presence of red blood cells in between the tissue cells (sinusoid regions) especially seen in liver tissue (Figure 3B), which occurred mostly at the centrilobular region of the hepatic cells. However, the nucleus of the cells was still intact.

Hemorrhage generally indicates extravasation of blood due to the rupture of blood vessels [16].With this evidence, it could be suggested that the recombinant clone ABA392 might carry a virulence gene, which produce proteins that are toxic to the tissues and at the same time involved in the pathogenesis of hemorrhage.

In kidney tissues (Figure 3c), the nucleus of the tubules was distorted and in addition, the lumen of the tubules was absent due to the destruction of inner membrane of the tubules. This effect was seen in the kidney tissues affected with the parental strain of *Pasteurella multocida* serotype B strain PMB202. However, the outer membrane was still remained. Hemorrhage was also seen with the presence of red blood cells spreading within the sinusoid regions of the tissues.

Severity of hemorrhage and tissue necrosis seen in liver, lung, spleen, and kidney tissues infected with recombinant clone ABA392 and the parental strain of *Pasteurella multocida* serotype B strain PMB202 was induced by treatment of dexamethasone. This is due to inhibition of the proliferation of peripheral lymphocytes by down-regulating IL-2 receptor mediated signal [17] and in the absence of leucocytes as inflammatory cells, the clearing of the invading bacteria in the affected tissues has failed and therefore, this might induced the severity of infection.

No pathological changes was observed in the tissues infected with *E. coli* JM101, *E. coli* pUC18 and PBS pH7.2 with the treatment of dexamethasone, indicated that they were not contributed in the virulence mechanism of hemorrhage.

Based on the SDS-PAGE experiment that has been performed for the unknown proteins of recombinant clone ABA392, a 16kD protein band was most likely to be detected from the gel stained with Coomassie Blue compared to the protein's bands observed in the parental strain PMB202 together with the negative controls, which are the *E. coli JM101* and *E. coli pUC18*. However, further characterization via purification is needed to identify the nature of this protein derived from the capsular antigen, which was suspected to be involved in the mechanism of hemorrhagic septicemia.

It was to be suggested that the distinct protein band detected in the recombinant clone ABA392 might be present in the parental strain protein sample. However, the condition did not apply due to several factors, which are not fully understood. Therefore, analysis of the recombinant clone ABA392 novel protein(s) should be further characterized via optimization study.

The analysis protein has been done via SDS-PAGE, further characterization of the proteins from recombinant clone ABA392 need to be done to get better and promising results. This can be achieved by using 2 dimensional gel electrophoresis that produces better resolution and separation demonstrated via protein spots according to their isoelectrofocusing used in the first dimension while separation according to their respective masses by SDS-PAGE in second dimension.

As the novel protein spots has been recognized, excision of the protein spots will be carried out to further proceed to MALDI which specifically determines the exact molecular mass of the novel proteins which has been excised from the gel[18].

The recombinant proteins will be subjected to *in vivo* study in mice for further confirmation of the virulence of these proteins, which are suspected to have similar effect in the *in vivo* study using bacterial cells. Apart from the confirmation of virulence, the *in vivo* study will also be able to contribute in the production of antibody against the proteins, which act as an antigen. The antibody produced is very important in developing suitable vaccines to overcome the great economical loss of domestic animals particularly the cattle and buffaloes due to hemorrhagic septicemia disease.

In future, it is hoped to produce an improved vaccine against haemorrhagic septicaemia. These efforts have been met with

varying degrees of success, but none has yet resulted in a practical vaccine.

CONCLUSIONS

In conclusion, the recombinant clone ABA392 was determined to be stably maintained based on the presence of plasmid via agarose gel electrophoresis and confirmation has been made by restriction endonuclease analysis in which no deletion or mutation has occurred within the 0.8 kb insert gene. Upon the pathological analysis, the recombinant clone was virulent based on the lethality in mice at the concentration of 10^7 CFU within 24 to 72 hours. In addition, the clone ABA392 was confirmed to carry a virulent gene due to the severe hemorrhagic effect in lung, liver and spleen tissues with the occurrence of necrosis in some part of the tissues affected. Whereas mild hemorrhagic was observed in the kidney tissue, no effect was observed in the heart tissue. A preliminary SDS-PAGE analysis was conducted to determine distinct protein band(s) that was suspected to be virulence-associated with the production of the recombinant clone ABA392 insert gene that might cause hemorrhagic septicemia disease. The protein band(s) was most likely to be seen with an estimated molecular mass of 16kD. However, further studies have to be done in order to confirm this finding.

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Conflicts of Interest: The authors declare no conflict of interest.

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