1. Introduction

Office International des Epizooties (OIE) has classified haemorrhagic septicaemia (HS) in the List B category of disease due to its high mortality rate in infected cattle especially in Africa and Asia regions [1]. The Pasteurella multocida serotype B:2 is a gram negative bacteria that causes HS in bovines [2,3]. The disease resulted to low production of meat and milk from healthy bovines which will cause serious economic loss and destructive welfare problems around the world [4].

Commercial vaccines that are currently available for HS, unable to provide a long-lasting immunity and their field of efficacy is unknown [5]. Improper vaccine administration techniques, poor potent of vaccine usage and improper vaccine storage condition are the contributing factors for the failure of an effective HS vaccines in cattle [5,6]. ABA392 clone was isolated from P. multocida B:2 and it harbors the virulence factor of the bacteria which caused HS [7]. The expression and potentials of the recombinant clone ABA392 as an expressed protein vaccine candidate against HS has been described and explored in previous studies which proved to be immunogenic [8,9]. The use of ABA392 clone which harbour only the sequence that code for a virulent
factor of *P. multocida* B:2 is much safer, precise and efficient than taking the whole genome and even the whole bacteria [10]. Thus, it is expected that the expressed recombinant clone of ABA392/pET30a protein vaccine could elicit proper antibody titer production and able to protect the domestic bovines against HS attack through mucosal immunization.

HS attack is acute and therefore prevention is preferable rather than treatment of the disease. Mucosal vaccination, is the most preferable defense for shielding the host from *P. multocida* B:2 infection in bovines, since this method reduces the susceptibility of bovines to the pathogen [11]. Transmission of disease of *P. multocida* B:2 bacteria is through intranasal and oral routes of bovines [12]. Thus, intranasal administration of mucosal protein vaccine is chosen since it mimics the oronasal route of infection and able to provoke both systemic and mucosal immunity. This type of administration also prevents the induction of unfavorable immune reactions against allergens and self-antigens [13].

In this study, ABA392/pET30a protein was subjected on Sprague dawley (SD) rats to determine the efficacy of the purified recombinant ABA392/pET30a vaccine against HS disease via intranasal administration. The findings were further analysed using bioinformatics strategies via software and programs on allergenicity.

2. Material and methods

2.1. Bacterial strains

The *P. multocida* subsp. multocida serotype B:2 (PMB2) (ATCC® 43137™) was employed as a live challenged strain bacterium. Isolates of bacterium *P. multocida* serotype B:2 were reactivated in blood agar at 37 °C for 24 h from a glycerol stock which were kept at −20 °C from the Molecular Bacteriology and Toxicology Laboratory, Institute of Biological Sciences, Faculty of Science, University of Malaya.

2.2. Preparation of vaccine and challenge strains

The recombinant protein vaccines used were expressed and purified by Ref. [14]. To prepare the recombinant protein vaccines, an ABA392 clone from *P. multocida* B:2 was ligated into the expressing vector of pET30a in the Molecular Bacteriology and Toxicology Laboratory, Faculty of Science, University of Malaya and transformed into the competent cell, *Escherichia coli* BL21 (DE3) pLysS strain. After transformation, the bacteria were grown in LB media containing kanamycin and chloramphenicol at 37 °C until it reached an absorbance of 0.6 at 600 nm. Protein was expressed using 1 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) induction. The induced cells were harvested by centrifugation at 3500 × g for 1 h at 4 °C and kept at −80 °C prior for lysis. The induced pellet cell was weighed and mixed with the bacterial extraction reagent (B-PER, Thermo Scientific) at the ratio of 1:4 to get a homogenous solution. The solution was incubated for 15 min followed by centrifugation at 3500 rpm for 30 min of the lysate for the separation of the soluble protein [15]. Protein detection was identified using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Tricine-SDS. The expressed protein consisted of the 6xHis-tagged protein thus the purification of the expressed protein was conducted by Dynabeads® His-Tag Isolation and Pulldown (Novex life Technologies) technique according to the manufacturer’s protocol. Eluted His-tagged protein was further confirmed by subjected to SDS-PAGE with Tricine-SDS and western blot. Subsequently, the nucleotide sequences obtained from the sequencing result were analysed using nucleotide basic local alignment search tool (BLAST) by Ref. [14]. Bradford assay was performed to analyse the concentration of the purified recombinant expressed protein vaccine (ABA392/pET30a) and adjusted to doses 50 μg/mL and 100 μg/mL, respectively, for vaccination purpose.

The challenged strain of *P. multocida* B:2 was revived in blood agar (BA) and grew in bulk in the Brain Heart Infusion (BHI) broth. The number of bacterium was adjusted to 10^5 CFU/mL for challenge infection purpose and 10^6 CFU/mL for vaccination as positive control.

For the positive control, 10^5 CFU/mL of the *P. multocida* B:2 were treated with 20% buffered formalin at a ratio of 1:1 for 1 h at room temperature to inactivate the living bacteria. After centrifugation, the pellet was washed thrice with Phosphate Buffered Saline (PBS) to fully eliminate formalin residues from the bacterial pellet. The pellet was then resuspended in PBS at 1:1 ratio for the initial amount. One milliliter of the inoculums was spreaded onto a BHI agar plate for cell viability confirmation. The formalin-killed bacteria were then aliquoted and kept at −20 °C prior to use.

2.3. Prediction of allergenicity of expressed protein

Allergenicity of the expressed protein was determine with allergen prediction software (http://www.imtech.res.in/raghava/algpred/submission.html) (Algpred) based on the similarity of known epitope with any region of protein [16].

2.4. Animal housing

Animal Use Protocol (AUP) was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Malaya, Faculty of Medicine (FOM) with the ethics reference number 2016-191103/IBS/R/KTL. A total number of 48 pathogen free SD rats were divided into two experiment, active immunization study and passive immunization study with 24 SD rats each respectively. All animals were housed in Individually Ventilated Cages (IVC) at the Animal Experimental Unit (AEU), Faculty of Medicine, University of Malaya.

2.5. Active immunization

A total number of 24 pathogen free SD rats were divided into 4 groups with 6 animals (n = 6) in each group. There were 2 tested groups which consisted of 2 different concentrations at 50 μg/mL and 100 μg/mL purified expressed protein vaccine, respectively; 1 negative control group with normal saline and 1 positive control group with 10^6 CFU/mL formalin-killed *P. multocida* B:2 (see Table 1).

Altogether, there were three doses of treatment administered intranasally at the volume of 50 μL each in two weeks interval based on each group respectively. The changes in behavior, movement, diet and development were observed twice daily. Serum were collected from all animals by tail venous blood sampling at the volume of 0.5 mL weekly for immunogenicity studies. On Day 42 which was two weeks after the

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<tr>
<td>Group 1 (Test Group 1)</td>
<td>50 μg/mL purified expressed protein vaccine</td>
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<tr>
<td>Group 2 (Test Group 2)</td>
<td>100 μg/mL purified expressed protein vaccine</td>
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<td>Group 3 (Negative Control)</td>
<td>Normal Saline</td>
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<td>Group 4 (Positive Control)</td>
<td>10^6 CFU/mL formalin-killed <em>P. multocida</em> B:2</td>
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<tr>
<td>Group 3 (Negative Control)</td>
<td>Serum from rats treated with normal saline</td>
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<tr>
<td>Group 4 (Positive Control)</td>
<td>Serum from rats vaccinated with 10^6 CFU/mL formalin-killed <em>P. multocida</em> B:2</td>
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last vaccination, all animals were subjected to challenge infection with the live strains of $10^5$ CFU/mL P. multocida B:2 intraperitoneally at the volume of 0.5 mL. The animals were kept for observation and euthanized by anesthetic overdose according to the animal ethics requirements on Day 56 for histopathogenicity studies.

### 2.6. Passive immunization

A total number of 24 pathogen free SD rats were divided into 4 groups with 6 animals (n = 6) in each group (see Table 1).

At Day 0, all animals were administered intranasally with the serum obtained from each group of the active immunization study at a volume of 50 μL to each group respectively. After 24 h, at Day 1, all animal was subjected to challenge infection with a live strain of $10^5$ CFU/mL P. multocida B:2 intraperitoneally at the volume of 0.5 mL. The animals were kept for observation and euthanized by anesthetic overdose according to the animal ethics requirements on Day 21 for histopathogenicity studies.

### 2.7. Immunogenicity determination

Blood was collected at 0.5 mL from the venous of the tail from the experimental animals every week after anesthetic procedure for active immunization study and 0.2 mL for passive immunization study. Serum was separated from the coagulated blood by centrifugation and stored at $-20^\circ$C prior for usage. Immunogenicity study was conducted by carrying out total white blood cell count (TWBC), differential white blood cell count and Indirect Enzyme Linked Immuno-Sorbent Assay (ELISA).

For TWBC, the collected blood was mixed immediately with the Turk's solution at a ratio of 1:20 and left for up to an hour prior to leukocytes counting using a neubaur hemacytometer. Collected blood was immediately smeared on a glass slide and stained with a Giemsa stain for differential white blood cell count.

Whereas for indirect ELISA study, immunoglobulin levels of IgA and IgG were analysed from the serum obtained for each group of the experiments at the dilution of 1:50 ratio. All samples including blanks and standards, were triplicates. Secondary antibody employed in this study was IgA and IgG anti-rat horseradish peroxidase (HRP) conjugated which were optimized at the dilution of 1:2500 and 1:1000, respectively. Tetramethylbenzidine (TMB) was used as an indicator substrate which were optimized at the dilution of 1:2500 and 1:1000, respectively. The optical density (OD) was read at an absorbance of 450 nm using an ELISA microplate reader.

### 2.8. Histopathological analysis of lung tissues

The internal organs such as lungs, heart, liver and kidneys were collected from the euthanized rats, trimmed and fixed in 10% formalin. The tissues were then processed for thin paraffin sections, Hematoxylin/Eosin stained and observed by a digital light microscope for histopathological analysis. The study of Bronchus Associated Lymphoid Tissues (BALT) formation in the lung of SD rats was carried out throughout the histopathological analysis.

### 2.9. Statistical analysis

Statistical analysis was carried out by using a software program Statistical Package for Social Sciences (SPSS) Version 20. The significant differences and comparison of the level of TBWC and specific antibody IgA and IgG formation between each group of SD rats against P. multocida B:2 were carried out using the method of independent sample t-test. Significance differences were considered when the p-value is $< 0.05$.

## 3. Results

### 3.1. Protein allergenicity

The expression of His-tag ABA392/pET30a protein showed a molecular weight of 32 kDa which is similar to the one compute using Expasy MW bioinformatics tools using sequence obtained from sequencing report. Allergen prediction result from AlgPred tool predicted that the expressed purified protein ABA392/pET30a was non-allergen, with the score value of $\sim 1.816611$ [Threshold = $-0.4$]. No experimentally proven IgE epitope was found in the protein sequence. In addition, the positive predictive value of the protein was 15.19% (values $> 35$ indicates allergenicity).

### 3.2. Active immunization

The survival rate of both test groups, G1 and G2 as well as the positive control G4 recorded as 100% (n = 6/6) even after challenge with the live $10^5$ CFU/mL P. multocida B:2 bacteria. In contrast, 3 out of 6 rats from the negative control G3 died within 48 h. All infected animals showed similar symptoms as shown previously [17,18], such as ocular and nasal discharge, lesion around eyes and nose, weak and sluggishness, hair loss and ruffled hair coat. No anaphylactic response or site inflammation was observed.

For immunogenicity studies, total white blood cells were monitored every week starting from Day 0 until euthanization which was at Day 56. Leucocytosis was observed for both of the test groups, G1 and G2 as well as the positive control G4 after each dosage of vaccination was administered which returned to the initial levels after the bacterial challenge. Negative control G3 treated with normal saline did not show any significant differences ($p > 0.05$) in the white blood cell count. An acute increase of neutrophils or neutrophilia were observed in both test group G1 and G2 as well as positive group G4 after each of the vaccination. Increase of lymphocytes was observed slightly slower than the increment of neutrophils. No significant differences ($p > 0.05$) on the development of neutrophils and lymphocytes were observed in both test group G1 and G2 with the positive control G4. There were no significant differences ($p > 0.05$) between test groups G1 or G2 and the positive control G4.

Indirect ELISA were carried out to determine the IgA and IgG level at 450 nm. The level of IgA increased after each dose of vaccination for both test groups G1 and G2 and the positive control G4, however negative control G3 showed no IgA increase until the challenged day (Fig. 1). No significance difference ($p > 0.05$) was observed between both test groups G1 and G2 with the positive control G4 but there were significance differences ($p < 0.05$) with the negative control G3. Interestingly, it was observed that the higher concentration of purified expressed protein vaccine at 100μg/mL (G2) produced slightly higher IgA level as compared to the lower concentration at 50μg/mL (G1). Besides, it was observed that IgG development started only after the second vaccination at Day 21 which is slower and also lower in amount than the development of IgA. There was a significance difference ($p < 0.05$) in the IgG development between both test groups G1 and G2 with the negative control G3 but not with the positive control G4.

For histopathological studies, the formation of bronchus associated lymphoid tissues (BALT) was observed in both test groups G1 and G2 and the positive control G4 group as demonstrated in Fig. 2. No formation of BALT was observed in the negative control G3. With reference to Fig. 3, no severe inflammation was observed in liver, kidney or heart of any of the animals. This was supported by liver and renal function tests. For liver function tests, the aspartate aminotransferase (AST), alkaline phosphate (ALP) and Alanine Aminotransferase (ALT) are within the normal range as recorded by Johnson-Delaney in 1996 [19]. For renal functions test, the creatinine level for all animals vaccinated with protein vaccine was 29.75 μmol/L on the average which is within the normal range of between 17.68 and 70.72 μmol/L as
Passive immunization study indicated that, the survival rate of both test groups G1 and G2 as well as the positive control G4 was 100% (n = 6/6) after challenge with the live $10^9$ CFU/mL $P.\ multocida$ B:2 bacteria. However, the negative control group G3 showed 33.33% (n = 2/6) survival rate; as expected, 4 rats from the negative control group G3 died within 48 h.

For immunogenicity studies, total white blood cells were monitored every week from Day 0 until euthanization which was on Day 21. Leucocytosis was observed for both test groups G1 and G2 as well as the positive control group G4 after the immunization with serum but returned to initial level right after the bacterial challenge. Therefore, the response was equal for both of the test groups G1 and G2 and the positive control group G4. Negative control group G3 did not show any significant increase ($p > 0.05$) in the white blood cell count at any point.

Indirect ELISA was carried out to investigate the level of immunoglobulin IgA and IgG at 450 nm. The development of IgA increased for each of the test groups G1 and G2 and the positive control group G4 after immunization with serum. However negative control G3, did not indicate any IgA development until the challenged day (Fig. 1). No significance difference ($p > 0.05$) was observed between both the test groups G1 and G2 and the negative control group G3. No anaphylactic response or site inflammation were observed in any of the rats receiving the intranasal vaccination.

In this study, all infected animals showed symptoms similar to what has been recorded [17,18], such as ocular and nasal discharges, recorded [19].

### 4. Discussion

Haemorrhagic septicaemia is highly fatal disease in bovines that is caused by $P.\ multocida$ B:2. Although, several vaccines have been developed, it has been observed at field level that the cases reported for haemorrhagic septicaemia is still ongoing among cattle and buffalos [20,21]. Recently 133 clinically diagnosed HS affected farms were happened in Karnataka State, India which caused huge loss to the livestock farm community estimated mortality loss ($\$415$ per animal) during the HS outbreak [22]. Besides, a re-emergence of acute haemorrhagic septicaemia was reported with unaccountably losses in Hungary in the year 2016 [23].

Purified expressed protein vaccine from recombinant ABA392/pET30a yielded a band at 32 kDa which conformed to the molecular weight computed by EXPASY MW bioinformatics tool. The protein was found to be a non-allergen. Based on the report that a protein will be considered as an allergen once it shows > 35% identical with a familiar allergen over a window of 80 amino acids or the presence of six contiguous amino acids in a known allergen [24]. From the prediction, we proceeded to the animal studies and inoculated the purified expressed protein vaccine via intranasal route. We observed that no uncomforted occur among all the SD rats. No anaphylactic response or site inflammation were observed in any of the rats receiving the intranasal vaccination.

In this study, all infected animals showed symptoms similar to what has been recorded [17,18], such as ocular and nasal discharges,
conjunctivitis with lesions around eyes and nose, weakness and sluggishness, hair loss and ruffled hair coat after the challenge with bacterial infection. But, the conditions for the SD rats in negative group 3 worsen and then succumbed to death according to the humane endpoint of the ethic references. The death animals in negative group 3 showed severe ocular discharge, labored breathing as recorded in previous studies [25,26]. The survival rate for both the test groups with the recombinant protein vaccine and the positive control group showed 100% which showed a very promising protection. This proved that the intranasal recombinant protein vaccine allows self-vaccination contingency to happenings within the rats in contact with the mucosal vaccinated hosts and showed similar stimulation creating protection of the in-contact animals against the infection challenge and increased the vaccination coverage [27,28]. In a nutshell, intranasal inoculation of the protein vaccine would not provoke any inflammation onsite or anaphylactic responses as was mentioned to occur in previous studies [20,29,30]. On the other hand, the survival rate for SD rats in negative group 3 is quite high which is not as expected even after the lived *P. multocida* B:2 bacterial challenge. This is due to the less susceptible of rats towards HS disease as compared to mice or cattle, in addition, one of the factor contribute to HS disease infection spreading is under stress condition [31]. Therefore, the usage of dexamethasone could be used in order to suppress the immune system of SD rats before the bacterial challenge to make the SD rats more susceptible towards HS disease [17].

From the aspect of immunogenicity level, both the test groups G1 (50μg/mL protein vaccine) and G2 (100μg/mL protein vaccine) showed the same efficacy as the positive control group G4 (formalin-killed 10^6 CFU/mL *P. multocida* B:2). The protein vaccine is capable enough to play a role as an immunogen as it is able to elicit a high titre production of antibodies against HS. This is also proven by the increment of neutrophils or neutrophilia, following each of the vaccination as neutrophils are the vital first line defense against introduce antigen [32]. In addition, as noticed, the development of immunoglobulin IgA was much higher compared to IgG, this proved that the protein vaccine had induced mucosal immunity in the rats through the intranasal inoculation. Development of IgA is much faster than IgG when encountering an antigen and it is an important local immunity as it prevents the attachment of bacteria or microbes to the mucous membranes [20]. IgA is responsible for the protection against respiratory infectious diseases by providing the cross-neutralizing activity of the mucosal secretion [33]. Large amount of IgA is secreted across the mucosal lining. There has been an increase in the recognition and interest of the effects of residential microbiota in respiratory homeostasis and disease processes [34]. Previous studies have shown that intranasal vaccination is safer, provides longer lasting immunity and causes no site inflammation or endotoxic shock [35,36]. Furthermore, the serum antibodies IgA and IgG has been shown to play an important role in the protection of the rat from bacterial *P. multocida* B:2 infection challenge. This is due to the serum antibodies detectable in passive immunization. The serum antibodies IgA and IgG remain high and constant in vaccinated rat two weeks after the bacterial infection challenge.

From the aspect of histopathological analysis, lung was the most severely affected organs against the haemorrhagic septicaemia disease,
as this disease affects the respiratory route of natural host during the disease outbreaks [37]. Haemorrhages and congestion, thickening of interalveolar septa and inflammatory cells infiltration were observed in the negative control group G3 which is consistent in previous studies [32,37]. Formation of the bronchus associated lymphoid tissues (BALT) was observed in both the test groups G1 and G2 as well as the positive control group G4 proved that the recombinant protein vaccine could serve as an immunogen. The development of BALT served as an effector priming site of mucosal immune response to help the rats fight against the bacteria *P. multocida* B:2 challenge [38]. BALT is most consistently distributed at sites of bronchial tree bifurcation and usually found between a bronchus and an artery [38]. The development of the BALT observed in this study showed a consonance results as previous studies whereas following antigenic stimulation through the protein vaccine could results in the significantly increase of BALT formation [39,40]. No severe inflammation or lesions were observed in heart, kidney and liver indicating that this recombinant protein vaccine has no toxicity towards the host's organs.

Comparisons between active and passive immunizations showed that the development of TWBC, IgA and IgG were slightly lower and short lasting immunity in the passive immunization as compared to the active immunization. Passive immunization showed the same efficacy as the active immunization, however, the immunity was unable to last long enough until the next infection attack. Therefore, prevention is better than cure in this case of HS infection which recorded high mortality and morbidity rate once infected [41]. Therefore, the development of proper antibody level and BALT prior to the infection is crucial.

The use of recombinant protein vaccines prevents the risk of co-purification of unwanted contaminants and eliminate the chance of the toxoids reversing into their toxicogenic constitutes [42]. Recombinant clone ABA392 consists of the virulent factor of *P. multocida* B:2 and was proved to be immunogenic in this study as well as other previous studies [8]. Three doses regime or homologous prime boost were given in this study to boost the immune response for recombinant protein and improve the immunogenicity and lasting period. Intranasal vaccination approach was chosen as the preferred administration route due to its ability to provoke protective immunity at the mucosal surface in the respiratory tract to deter the entry of pathogens and restrain their spreading thus preventing the disease. Previous studies have shown that mucosal immunization via intranasal route produced better results than the parental route; for example, the vaccination with the outer membrane proteins (OMP) of *P. multocida* B:2 [29], live *P. multocida* B:2 [35,36], live gdhA derivative *P. multocida* B:2 [27] and the attenuated aroA derivatives of *P. multocida* B:2 [43]. Protein vaccine was proved to give more effective antigens in inducing the host cell responses than lipopolysaccharide based vaccine due to its larger molecular size and high antigenic property [37]. Intranasal administration has proven to induced a good and effective mucosal immune response against bacterium due to the larger surface area of the mucosa that can be easily
access directly by the vaccine [44].

5. Conclusion

In conclusion, this study showed that recombinant protein vaccine ABA392/pET30a could be a promising candidate in the prevention of haemorrhagic septicaemia disease. It is also able to act as an immunogen to provoke the mucosal immunity against the *P. multocida* B:2 antigen by developing the high immunoglobulins IgA and IgG level and inducing the formation of bronchus associated lymphoid tissues with no toxicity to the rats.

Conflicts of interest

The authors have no conflict of interests. All authors attest that they meet the ICMJE criteria for authorship.

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KTL performed the research and wrote the manuscript. SI designed and interpreted the data and revised the manuscript. JM analysed and revised the research study and revised the manuscript. NK analysed and interpreted the data and revised the manuscript. JM analysed and revised the manuscript. NK analysed and in

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