IMMUNOGENICITY OF RECOMBINANT HS ABA392 PROTEIN VACCINE AGAINST HAEMORRHAGIC SEPTICAEMIA IN ANIMAL MODEL

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2018

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

INSTITUTE OF BIOLOGICAL SCIENCES
FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2018

UNIVERSITY OF MALAYA

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IMMUNOGENICITY OF RECOMBINANT HS ABA392 PROTEIN VACCINE AGAINST HAEMORRHAGIC SEPTICAEMIA IN ANIMAL MODEL

ABSTRACT

Haemorrhagic septicaemia (HS) outbreak has a major impact in Asian countries, where farmers encounter economic lost due to death and low milk production. HS occurred most commonly in cattle and buffaloes. The first HS outbreak reported in Malaysia was in the year 1900 which caused huge lost to the meat and dairy industry. *Pasteurella multocida* has a pathogenic potential in vertebrate animals. *P. multocida* serotype B: 2 is the etiology for HS in Asia thus contribute highest mortality rate of cattle and buffaloes. There are four types of vaccine used in all the countries namely, alum precipitated vaccine, broth bacterins, aluminum hydroxide gel vaccine and oil adjuvant vaccine (OAV). The function of DNA recombinant technology such as gene cloning and expression to produce recombinant proteins from *P. multocida*, could trigger immune response and provide protective immunity to the administered animal. The recombinant clone ABA392 which used in this study was derived from *P. multocida* serotype B strain 202 (PMB202). ABA392 gene was successfully cloned into pET-30a an expression vector and known as ABA392/pET30a. The size of expressed protein was determined as ~32 kDa and confirmed via immunoblotting. The protein was further purified using Immobilized Affinity Chromatography (IMAC) technique. The purified protein was tested on rats for the immunogenicity along with positive and negative control groups. Blood was collected weekly for 7 weeks consistently. Enzyme-Linked Immunosorbent Assay (ELISA), Total White Blood Count (TWBC), Renal Function Test (RFT) and Liver Function Test (LFT) were performed. Lung, heart, liver and kidney organs were collected and processed for histopathology analysis. No significant changes were observed in the entire organs from each group. The purified

protein from recombinant clone ABA392/pET-30a has induced high titer antibody compared to positive and negative group. Total white blood count shows leukocytosis. The RFT and LFT were in normal values in the vaccinated group. Recombinant clone ABA392/pET-30a has been expressed purified and has been tested on laboratory rat. Since the expressed protein has the capability to provoke immune response, challenge studies using *P. multocida* serotype B: 2 against this protein are required to enhance the efficient of this protein as a vaccine against HS in future.

Keywords: haemorrhagic septicaemia, *Pasteurella multocida* serotype B: 2, recombinant vaccine, recombinant clone ABA392 and protein vaccine.

KEIMUNOGENAN REKOMBINAN HS ABA392 SEBAGAI VAKSIN SUBUNIT MELAWAN HAWAR BERDARAH DALAM MODEL HAIWAN

ABSTRAK

Wabak hawar berdarah memberi kesan yang mendalam kepada negara Asia dimana di mana para penternak lembu terpaksa menghadapi kegawatan ekonomi disebabkan kematian lembu-lembu dan pengurangan dalam pengeluaran susu daripada haiwan ternakan mereka. Hawar berdarah dihidapi oleh lembu and kerbau secara umum. Wabak hawar berdarah yang pertama direkod di Malaysia pada tahun 1900 yang menyebabkan kerugian yang amat besar pada industri tenusu. *Pasteurella multocida* memberi kesan patogenik dalam haiwan vertebrata. *P. multocida* serojenis B: 2 merupakan etiologi utama yang menyebabkan hawar berdarah di Asia di samping menyumbang kadar kematian yang tinggi pada lembu dan kerbau. Terdapat empat jenis vaksin iaitu vaksin daripada bakterin sendiri, vaksin daripada alum persipitasi, vaksin gel aluminium hidroksida dan vaksin disediakan menggunakan adjuvan minyak. Fungsi teknologi rekombinan DNA seperti pengklonan gen and ekspresi protein rekombinan daripada *P. multocida* boleh mencetuskan respon imun dan memberi perlindungan imun kepada haiwan yang diberi vaksin. Rekombinan klon ABA392 yang digunakan dalam penyelidikan ini diperolehi daripada *P. multocida* serojenis B strain 202 (PMB202). Gen ABA392 telah berjaya diklonkan bersama vektor protein pET-30a dan dikenali sebagai ABA392/pET30a. Size protein yang diekspresi dapat diketahui iaitu ~32 kDa dan disahkan melalui kaedah imunoblot. Protein yang diekspresi dari rekombinan klon in telah dikaji ke atas tikus makmal untuk mengetahui tahap keimunan bersama kumpulan kawalan positif dan negatif. Darah telah diambil setiap minggu sehingga minggu ketujuh secara konsisten. ELISA ("Enzyme-Linked Immunosorbent Assay"), kiraan sel darah putih, fungsi buah pinggang dan hati turut dianalisis. Organ seperti

paru-paru, jantung, hati dan buah pinggang turut dikumpul dan diproses melalui teknik histopatologi. Tiada perubahan diperhatikan pada organ-organ yang diproses dari setiap kumpulan. Protein dari klon ABA392/pET30a dapat meningkatkan kadar antibodi titer apabila dibandingkan dengan kumpulan kawalan positif dan negatif. Kiraan sel darah putih menunjukkan leukositosis. Nilai fungsi buah pinggang dan hati berada dalam bacaan yang normal. Rekombinan klon, ABA392/pET30a telah diekspresi, disaring dan telah dikaji ke atas tikus makmal. Di mana protein dari rekombinan klon ini dapat mencetuskan tindak balas immuniti dan boleh dikaji secara mendalam sebagai vaksin. Dengan penghasilan vaksin subunit ini, dipercaya bahawa vaksin ini dapat mengurangkan kadar hawar berdarah pada haiwan-haiwan ternakan pada masa yang akan datang.

Kata kunci: hawar berdarah, *Pasteruella multocida* serojenis B: 2, vaksin rekombinan, rekombinan klon ABA392 dan vaksin subunit.

ACKNOWLEDGEMENTS

First and foremost I would like to record my gratitude to GOD Almighty for the blessing, good mentality and physical strength to face all the consequences.

I gratefully acknowledge University of Malaya for the UM by Postgraduate Research Grant (PPP)-PG236-2014B provided to perform this study.

I am heartily thankful to my supervisor, Professor Dr. Salmah Ismail whose encouragement, guidance and support from the initial to the final level enabled me to develop an understanding on my research project.

I want to acknowledgment my co-supervisor Dr Nurshamimi for her valuable guidance, advice and assistance during my thesis write up and not forgetting Dr. Hussin Alwan Rothan for his valuable advice during conducting protein studies.

I owe my deepest gratitude Molecular Bacteriology and Toxicology (MBT) members, Kelvin Swee, Tengku Iddzzan, Shamini Chelliah, Kang Tzin Lim, Kithalakshmi, Maikhudi and Dr. Arezoo for their advice, guidance and crucial contribution which made their help a backbone throughout this research were conducted.

Special thank go to Professor. Dr. Nurul Kabir for his great help to examine all my histopathology slides and not forgotten Pn. Masyitah for her guidance throughout histopathology process were carried out.

Last but not least, I would like to show my highest gratitude to my lovely parents, Mr Velappan @ Morthy and Mrs Sarasa Velappan who sailed together with me by supporting me morally and financially.

Rita Devi Velappan

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

~	:	Approximately
%	:	Percent
±	:	Plus minus
<	:	Strict inequality
>	:	Strict inequality
/	:	Sub-cloned into (e.g. ABA392/pET-30a stands for ABA392 gene clone into pET-30a protein expression vector
µg	:	Microgram
µl	:	Microliter
µm	:	Micrometer
ALP/AP	:	Alkaline phosphatase
ALT	:	Alanine aminotransferase
API	:	Analytical Profile Index
APS	:	Ammonium Persulfate
ATP	:	Adenosine Triphosphate
BCIP	:	5-Bromo-4-Chloro-3-Indoly Phosphate
BHI	:	Brain Heart Infusion
BLAST	:	Basic local alignment search tool
bp	:	Base pair
BPER	:	Bacterial protein extraction reagent
BSA	:	Bovine Serum Albumin
CBB	:	Coomassie Brilliant Blue
Da	:	Dalton
dATP	:	Deoxyadenosine Triphosphate
dCTP	:	Deoxycytidine Triphosphate

dGTP	:	Deoxyguanosine Triphosphate
dTTP	:	Deoxythymidine Triphosphate
DNA	:	Deoxyribonucleic acid
ExPASy	:	Expert Protein Analysis System
FOA	:	Food and Agriculture Organization
H&E	:	Hematoxylin & Eosin
HF	:	High Fidelity
HRP	:	Hydrogen peroxidase
HS	:	Haemorrhagic septicaemia
IACUC	:	Institutional Animal Care and Use Committee
IgG	:	Immunoglobulin G
IMAC	:	Immobilized Metal Affinity Chromatography
IPTG	:	Isopropyl-β-D-I-Thiogalactopyranoside
kb	:	Kilo base pair
KCL	:	Potassium chloride
kDa	:	Kilo Dalton
LB	:	Luria-Bertani
LPS	:	Lipopolysaccharide
LFT	:	Liver Function test
M	:	Molar
MBT	:	Molecular and Bacteriology Toxicology
MCS	:	Multiple cloning site
Mm	:	Millimolar
mABS	:	Monoclonal antibodies
ME	:	Multiple
MEV	:	Multiple Emulsion Vaccine

mg	:	Milligram
$MgCl_2$	:	Magnesium Chloride
ml	:	Milliliter
MW	:	Molecular weight
NBF	:	Neutral Buffer Formalin
NCBI	:	National Center Biotechnology
OAV	:	Oil adjuvant vaccine
OIE	:	Office International des Zooties
Omp	:	Outer membrane protein
OmpA	:	Outer membrane protein A
OmpH	:	Outer membrane protein H
PB	:	Phosphate buffer
PBS	:	Phosphate buffer saline
PCR	:	Polymerase chain reaction
PMB	:	*Pasteurella multocida* serotype B strain
PMPT	:	Passive mouse protection test
PPUM	:	Pusat Perubatan Universiti Malaya
psi	:	Per square inch
RAPD	:	Random Amplified Polymorphic DNA
RE	:	Restriction enzyme
REA	:	Restriction analysis
RFT	:	Renal Function Test
RNA	:	Ribonucleic acid
RNAP	:	Ribonucleic acid polymerase
rpm	:	Revolution per minute
SE	:	Standard error

SDS	:	Sodium Dodecyl Sulphate
PAGE	:	Polyacrylamide Gel Electrophoresis
TBE	:	Tris/Borate/EDTA
TBS	:	Tris Buffered Saline
TBST	:	Tris Buffered Saline Tween
TEMED	:	N'N'N'N-Tetramethylethylenediamine
T_m	:	Melting temperature
TMB	:	Tetramethylbenzidine
UV	:	Ultraviolet
V	:	Volt
w/v	:	Weight per volume
WBC	:	White Blood Count

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Haemorrhagic Septicaemia (HS)

Bacterial infection causes severe disease outbreak and spread rapidly among domesticated animals. Haemorrhagic septicaemia (HS) is known as a form of pasteurellosis which affect mainly water buffalo and cattle. HS outbreak has a major impact in Asian countries, where farmers encounter economic lost due to death and low milk production. HS occurred most commonly in cattle and buffaloes. HS is endemic and has a significant economic impact in Asian countries such as China, Bhutan, Indonesia, India, Myanmar, Mongolia Philippine, Malaysia and Sri Lanka since these countries own large population of cattle and buffaloes (Ghaffar *et al*., 2016). *P. multocida* causes HS in cattle and buffaloes, infect with enzootic bronchopneumonia in sheep meanwhile causes snuffles and atrophic rhinitis respectively in rabbit and swine (Harper *et al*., 2012). The first HS outbreak reported in Malaysia was in the year 1900 which caused huge lost to the meat and dairy industry (Corrongean, 1902). Over the years the outbreak of HS in the peninsular of Malaysia causing high mortality due to the lack of vaccination and inefficient veterinary services (Bisht *et al*., 2006). In a decade from 1995 to 2005 a total of 47 outbreaks of HS have been reported where 8 to 11 outbreaks were reported in Perak, Kelantan, Terengganu and Pahang, meanwhile 1 to 5 outbreaks in Penang, Kedah, Negeri Sembilan and Melaka (Annas *et al*., 2014) (Figure 1.1). HS outbreak was confirmed through post mortem among buffaloes and cattle in West Bengal (Mitra *et al*., 2013).

1.2 *P. multocida* and Current Vaccination

P. multocida has a pathogenic potential in vertebrate animals. *P. multocida* serotype B: 2 is the etiology for HS in Asia thus contribute highest mortality rate of cattle and buffaloes (Shivachandra *et al*., 2011).

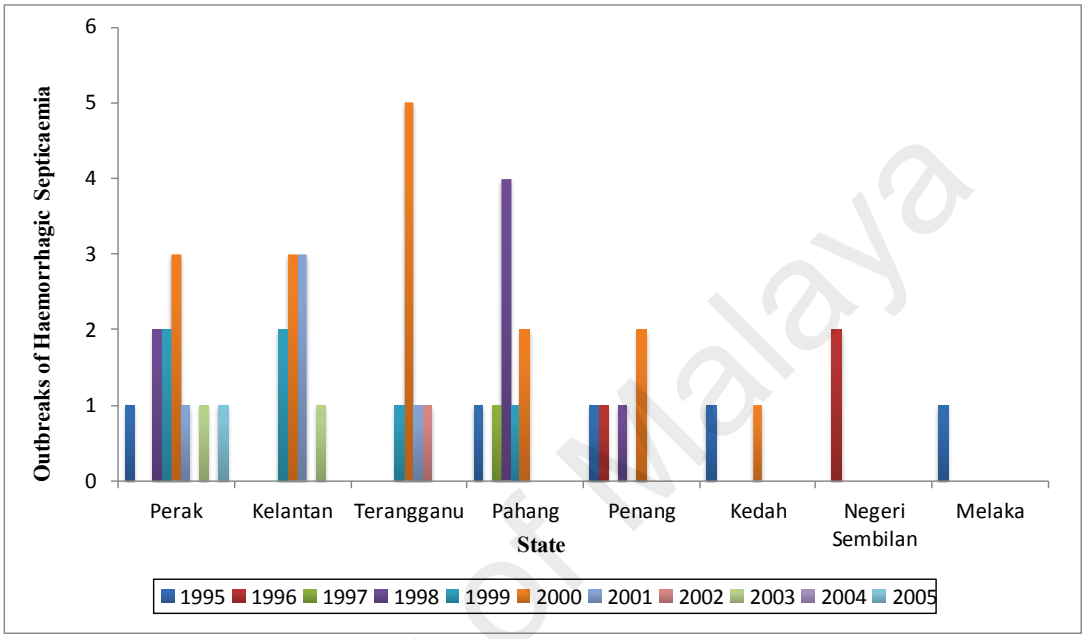


Figure 1.1: Incidence of HS outbreaks according to state and year throughout 1995 to 2005 in Peninsular Malaysia (Hussaini, 2009)

Antibiotic treatment was considered to control the infection but, the effectiveness of the antimicrobial agent was limited by the increased of antimicrobial resistance of *P. multocida* (Noor Masyitah Jumahat *et al*., 2015). Infection from *P. multocida* can cause a serious outbreak and affect domestic animals and prevention is recommended to control this disease as the mode of current treatment is complicated and ineffective. There are four types of vaccine used in all the countries namely, alum precipitated vaccine, broth bacterins, aluminum hydroxide gel vaccine and oil adjuvant vaccine (OAV) (Qureshi & Saxena, 2014). These types of vaccines consider as the first generation vaccine (Ahmad *et al*., 2014). Each of this vaccine has its advantage and limitation (Hussaini *et al*., 2012). Bacterin is the simplest form of vaccine, which consists of whole cell inactivated cultures but, the antibody response towards this

vaccine was very poor since the immunity last for less than 2 months. Alum precipitated and aluminium hydroxide gel vaccine are most extensively used but only able to provide stable immunity approximately six months. The first OAV against HS was developed in 1950s which contains water-in-oil emulsion of dense broth culture and high mineral oil. OAV gave protection up to 9 months and required annual re-vaccinations (Sotoodehnia *et al*., 2005). Thus, a long term effective vaccine is required to prevent this HS globally. Regardless of the wide availability of vaccination in the market, the quest a suitable and broadly protective with long-lasting immunity HS vaccine is still growing (Shivachandra *et al*., 2011). Recently the development of recombinant vaccine may probably reform a positive result for the next generation vaccines (Ahmad, 2014). Recombinant vaccine has proved to be safe since it conferred equitable high titer against homologous challenge and able to induce high humoral and cellular response (Ahmad *et al*., 2014). The function of DNA recombinant technology such as gene cloning and expression to produce recombinant proteins from *P. multocida*, could trigger immune response and provide protective immunity to the administered animal. Potential virulence genes in *P. multocida* can be targeted for the production of protein subunit vaccine. The current appropriation of guidelines by the Office International des Epizooties (OIE) on the detail for immunizations and analytic natural for HS ought to give the fundamental specialized stage to the generation and quality confirmation of HS antibodies (OIE, 2005). The primary trial of recombinant antibody was created against *P. multocida* that cause rhinitis in pig utilizing its own particular toxin (Bording *et al*., 1994).

1.3 The Recombinant Clone ABA392 and pET-30a Vector

The recombinant clone ABA392 which used in this study was derived from *P. multocida* serotype B strain 202 (PMB202) that was isolated by Veterinary Diagnostic Laboratory, Petaling Jaya, and Selangor in 1993 (Salmah Ismail, 1997). From the

previous results, recombinant clone ABA392 might harbor a gene that has the capability of triggering the immune system of the administrated animal and shield it from HS disease. Previously, the potential of recombinant clone ABA392 as a vaccine candidate against HS has been investigated and reported in mice model (Hussaini, 2009; Salmah Ismail *et al.,* 2010). pET-30a is a bacterial expression vector with T7 promoter, kanamycin resistant and multiple cloning sites that was establish for the cloning and expression of recombinant protein in *E. coli*. This vector was chosen for the study mainly because of its efficiency in producing high level of protein in short span of time. The produced recombinant protein will be used as one of a vaccine candidate for treating HS.

1.4 Problem Statement

HS is an acute and cause lethal in cattle and water buffaloes which cause by two specific serotype of *P. multocida* B2 and E2. HS is known as Pasteurellosis and has worldwide distribution including Malaysia and a significant loss in economically especially in dairy industry (Annas *et al*., 2014). Treatment is significant when treated once the sign of HS appeared in the early stage (Benkirane & De Alwis, 2002). There are few general antibiotic such as oxy-tetracycline, co-trimoxazole or combination of penicillin with other antibiotic like streptomycin or sulphaquinoxaline. However, *P. multocida* have developed resistant towards this antibiotic in the recent years which increase the high morbidity and mortality (Shivachandra *et al*., 2011). *P. multocida* has also gradually developed resistance to sulfadiazine on of the common antibiotic to control HS in the field (Kumar *et al*., 2009). In order to control HS, research activities begin to more concentrate on prevention trough vaccine development and vaccination in many countries including Malaysia but the outbreak of HS and death from HS is still inevitable.

Good biosecurity can be a key in controlling or preventing HS in domestic animals. A wide range of vaccine have been developed however they provide limited protection with certain disadvantage such as endotoxic shock. Thus, recombinant protein will be an effective vaccine component in treating HS since it does need adjuvant. An experimental study on animal models will be performed as to check the potential of the expressed protein from newly constructed recombinant clone ABA392/pET-30a as a protein vaccine by analyzing its immunogenicity and lethality. These studies will allow the production of protein vaccine produced to be potentially used in treating HS that is caused by *P. multocida,* not only in Malaysia but globally as well.

1.5 Objectives of the Study

It was found that the recombinant clone ABA392 has an immunogenic factor and could be used as vaccine in future (Hussaini *et al*., 2011). Since the HS determinant is yet to be reported or discovered elsewhere, it is great interest to conduct this study with the aim to evaluate the immunogenic potential and protective efficacy of recombinant protein ABA392 in rats, mice, cattle and calves. Therefore, there objection of the study are as follows:

(1) To test the DNA insert stability of recombinant clone ABA392/pET-30a

(2) To determine the lethality test of recombinant clone ABA392/pET-30a rats.

(3) To express the extracellular protein of the recombinant clone ABA392/pET-30a and test its toxicity in laboratory animals.

(4) To evaluate immunogenicity and access histopathological effects in vaccinated laboratory animals against HS

CHAPTER 2: LITERATURE REVIEW

2.1 Haemorrhagic Septicaemia (HS)

Haemorrhagic septicaemia (HS) can cause a huge loss in food industry as these ruminants are the main producer of dairy products and meats (Abdullah *et al*., 2013). HS is characterized as an acute, fatal and septicemic disease which caused by *Pasteurella multocida* serotype B: 2, a gram negative coccobacillus (Chung *et al*., 2015). HS occurred most commonly in cattle and buffaloes whereby buffaloes are more prone to this disease. Poor in management of husbandry conditions is where this disease is happened to occur (De Alwis, 1999). HS classified as a List B disease by the Office International des Epizooties (OIE) as a primary pasteurellosis with 100% mortality in infected animals in endemic areas of Africa and Asia. It arise as a major bacterial disease to economically important of livestock (Benkirane & De Alwis, 2002). It is caused by two specific serotypes of *P. multocida* designated B: 2 (Asian serotype) and E: 2 (African serotype) (Shivachandra *et al*., 2011).

2.2 Haemorrhagic Septicaemia (HS) in Worldwide Occurrence and Distribution

Haemorrhagic septicaemia (HS) can be considered as one of the important disease that affect bovines not only in South Asia but, it also occur in Middle and Eastern countries and the worst outbreak can be seen during rainy season (Khan *et al*., 2006; Mitra *et al*., 2013). There are also significant reports of HS outbreaks in several Africa regions and South America; however, there were no cases reported in Western Europe, Oceania and Canada meanwhile, in Pakistan HS is considered significantly economic loss with 34.1% of all affected animal died due to HS (FAO, 1991). The disease had been suspected in Kuwait and Qatar but the agent and serotyping has not been confirmed. However, there is record of the disease on the past in Singapore, Hong Kong

and Israel and currently these countries are free from this disease including Japan and Jordan (Benkirane & De Alwis, 2002). In Iran, this disease occurs partially in the north, north-east and south where 1,200,000 doses of vaccine are administered in cattle and buffaloes each year (Tabatabaei *et al*., 2007). There was an incident and death by HS which revealed through clinical diagnosis in some of the farm in Karachi, Pakistan (Moustafa *et al*., 2012). Mitra *et al*. (2013), has reported that there was HS outbreak at West Bengal, India on June and July 2013 where 52 of affected animals and died before treatment. *Pasteurella multocida* was isolated from nasopharyngeal and lungs of dead calves from January 2013 to March 2014 in Egypt (El-Jakee *et al*., 2015).

2.3 Haemorrhagic Septicaemia (HS) in Malaysia

HS outbreak has a major impact in Asian countries, where farmers encounter economic lost due to death and low milk production (De Alwis, 1999). Between years 1995 to 2005, a total of 47 outbreaks have been reported. This outbreak were recorded almost every year excluding the year of 2004 whereby year 1995 and 2000 show the highest outbreak occurred (Figure 2.1). In year 2000, the HS outbreaks in Terengganu and Pahang were at its peak along with Nipah virus incident. (Figure 2.1) (Kamarudin, 2005). In 2003, HS outbreak occurred in Perak due to several factors such as the immune status of herd and season changes (Moustafa *et al*., 2013). All isolates from Malaysian outbreaks were found to be *P. multocida* serotype B: 2. HS affects cattle and buffaloes where buffaloes are more affected compare to cattle as this can be seen in Figure 2.2. In February 2006 in Pasir Mas, Kelantan, HS outbreak was recorded; whereby 77 buffaloes were found dead (Lim, 2006). In May 2008, *Mingguan Malaysia* newspaper reported another HS outbreak in Rantau Panjang, Kelantan. The reason behind the outbreak was probably due to higher moisture content during the rainy season, poor living and the physical condition of the livestock. In Malaysia, an average of 360 heads of infected cattle and buffaloes are killed; approximately 1000 heads in

Philippine, Myanmar and Cambodia. Meanwhile 600 heads and 285 heads are killed in India and Sri Lanka respectively (Saad, 2013). The latest HS cases reported were occurred in Kuala Terengganu and Marang on May 2017 whereby a total of 245 including cattle and buffaloes were died of HS (Appendix A).

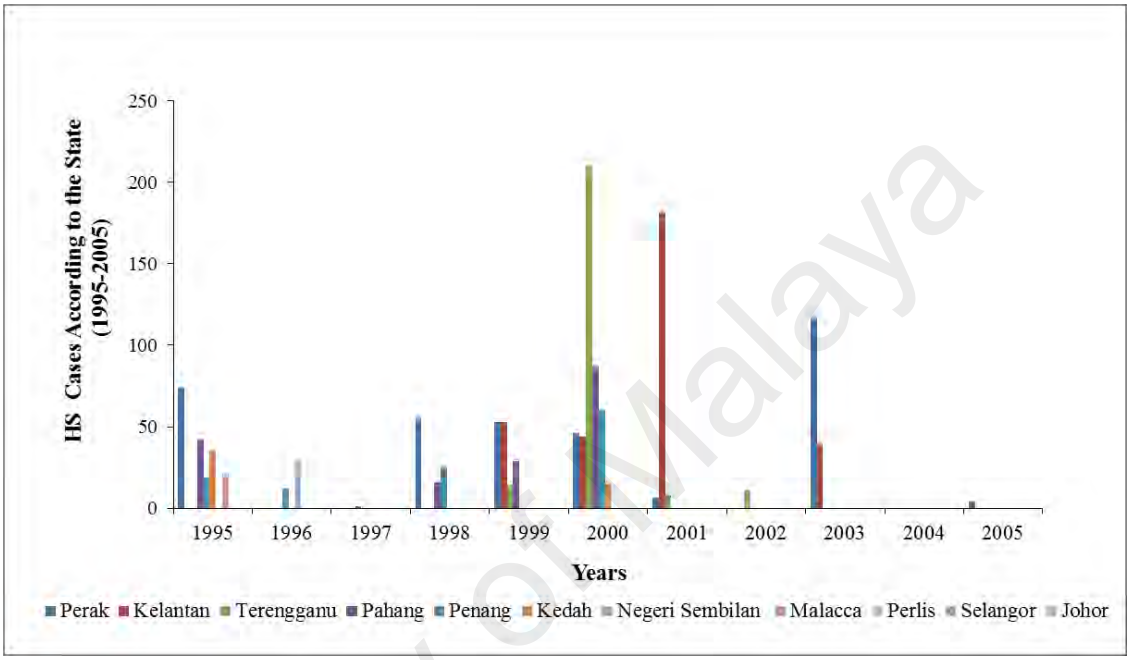


Figure 2.1: Number of HS cases according to the state throughout year 1995 to 2005 (Hussaini, 2009)

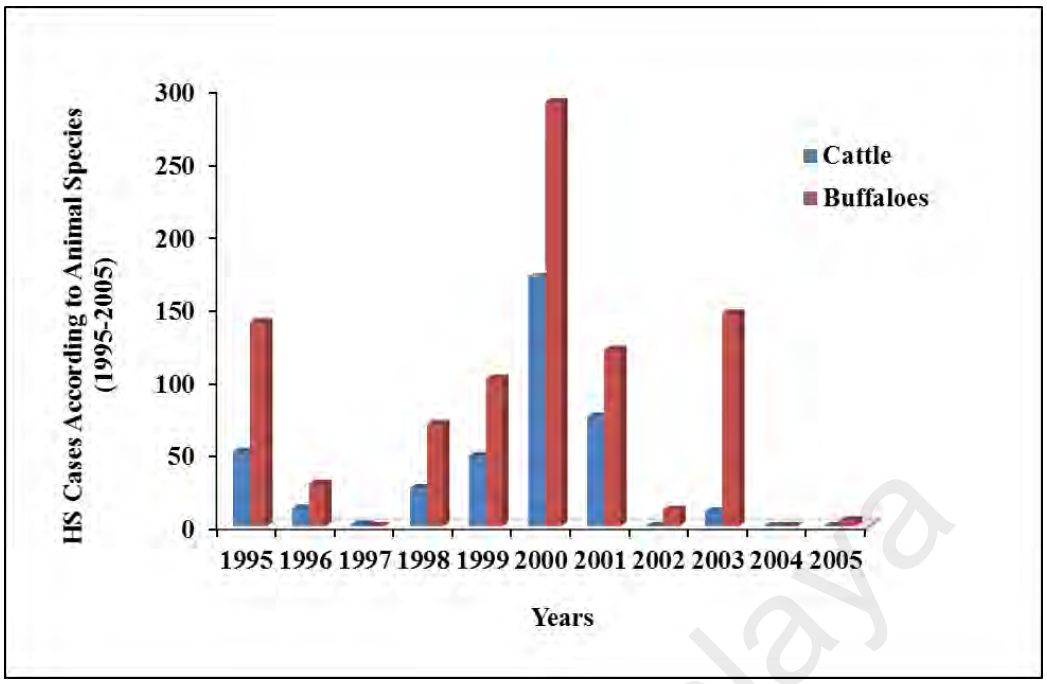


Figure 2.2: HS cases according to animal species from the year 1995 to 2005 (Hussaini, 2009)

2.4 History of Genus *Pasteurella*

P. multocida was first discovered by Rosenbusch and Merchant in 1939 and subsequently their taxonomy had been reclassified by Mutters in 1985. In 1989, Rimler and Rhoades define *P. multocida* as a pathogen in animals that colonize in birds and mammal (Mutters *et al*., 1989). *Micrococcus gallicidus* was the first scientific name given to this species which later different genera was given including "Bacterium", "Octopsis", "Coccobacillus" and "Eucystia". Trevisan has given a new generic name in 1887 as *Pasteurella* in conjunction name of Pasteur.

2.5 Colony and biochemical identification of *P. multocida*

P. multocida is a small gram negative, non-motile and non-spore forming coccobacillus showing bipolar staining. *P. multocida* is able to grow at 37°C on blood agar and produce small non-hemolytic colonies accompanied by mousy odor characteristic due to the metabolic products. The colonies appear to be whitish, opaque, circular and translucent (Haq & Abdullah, 2015). Since it is also facultative anaerobe, it is oxidase-positive, catalase-positive and ferments large number carbohydrates in

anaerobic conditions. *P. multocida* can be grown in nutrient agar but blood agar and chocolate agar is more preferable and it doesn't grow on macconkey agar, ss agar and nutrient broth which contain sodium chloride in small percentage. *P. multocida* also able to ferment mannitol, sorbitol, galactose, fructose, mannose, dextrose meanwhile, unable to ferment dulcitol, maltose and lactose. The ability to produce acid from mannitol, mannose and glucose and disability to produce gas from inositol, arabinose, dulcitol and inoline can be characterized as *P. multocida* (Haq & Abdullah, 2015). Analytical profile index (API) has been developed to identify *P. multocida*. Although this technique was rapid and easy due the misdiagnosis of strains and costly it was not use as basic test identification. (Dziva *et al*., 2008).

2.6 Types of Infection of *P. multocida*

P. multocida is a pathogenic bacterium that commonly found in wide range of vertebrate animal hosts and it causes the pasteurellosis infection. *P. multocida* also infect human through the bites from pets like cat or dog (Giordano *et al*., 2015 and Akahane *et al*., 2011). The *Pasteurella* species generally colonize the upper respiratory tract and oropharynx (Krol *et al*., 2011). *P. multocida* causes hemorrhagic septicemia (HS) in both cattle and buffaloes, enzootic bronchopneumonia in cattle and sheep, atrophic rhinitis in swine and snuffles in rabbit. *P. multocida* infection in human through pets like dogs or cats can cause cellulitis and lymphangitis and may get complicated with abscess formation and septic arthritis (Ewers *et al*., 2006).

2.7 Serogroups of *P. multocida*

P. multocida consist of 5 serogroup which known as A, B, D, E and F based on the antigenicity capsule and 1-16 serotype according to the Lipopolysaccharide (LPS) antigens based on somatic typing. Thus, capsular and somatic typing is designated to identify the serotype of *P. multocida* (Johnson *et al*., 2013). Each serogroup cause difference types of infection and serogroup B is generally related to cause HS (Boyce *et al*., 2000). Besides the capsular and somatic typing, serotypes are also categorized based on the host, different infection and geographical distribution. *P. multocida* serotype A is the cause of fowl cholera and the virulence factor is the capsular material which forms from the composition of hyaluronic acid. It is heavily capsulated which can resist to phagocytosis *P. multocida* serogroup B and E can found in cattle and the major cause of HS (Nsofor *et al*., 2006). *P. multocida* serogroup D is responsible for atrophic rhinitis in pigs by expressing *P. multocida* toxin (PMT) (Harper *et al*., 2006). Serogroup F is the cause of fowl cholera which was initially isolated from turkeys in USA and birds originated from North America (Jaglic *et al*., 2005).

Table 2.1: Classification of *P. multocida* based on different serogroup and its disease (Jaglic *et al*., 2005)

Serogroup	Disease	Infected animals	Geographical distribution
A	Various infection	Domestic animals	World widely
B	HS	Cattle and wild ruminants	Tropic areas
D	Various infection	Domestic animals	World widely
E	HS	Cattle and wild ruminants	Tropic areas
F	Fowl cholera	Avian	North America

2.8 Identification *P. multocida* by Serogroup

Indirect hemagglutination test is one of the tests used to identify five distinct capsular serogroup and also serological differences. Serogroup A is responsive to the activity of hyaluronidase. Nuclear magnetic resonance research indicate that major polysaccharide component in the capsule is composition of hyaluronic acid. Meanwhile, capsular material of serogroup B and F are identified through mucopolysaccharides action or production of chondroitin sulfate and heparin. Production of chondroitin or more to chondroitin-like polysaccharides capsule in *P. multocida* serogroup F can be confirmed by carbohydrate analysis. In *P. multocida* serogroup B the monosaccharide analysis shows that the capsular polysaccharide is composed by mannose, arabinose and galactose. The chemical component in serogroup E is still remaining unknown. Multiples capsular Polymerase Chain Reaction (PCR) typing system is a useful tool in identifying capsular serotyping system in *P. multocida*. This system is rapid and highly specific in analyzing capsular serotyping from the isolates of different geographical distribution (Towsend *et al*., 2001). HS-causing serogroup B-specific PCR assays is a significance tool to identify *P. multocida* serogroup B that causing HS. Toxigenic *P. multocida* can be detected rapidly through colony lift-hybridisation assay (Hunt, 2000). Restriction (REA) analysis is also used in differentiating HS isolates meanwhile PCR fingerprinting, ribotyping and pulsed-field gel electrophoresis (PFGE) can be used in identifying serogroup of *P. multocida*. *P. multocida* serogroup can be differentiated by multi-locus sequence typing (MLST) and gene sequencing based on the specific gene loci (Benkirane & De Alwis, 2002 & Shivachandra, 2011). Access to PCR facility, Random Amplified Polymorphic DNA (RAPD) or repetitive sequence PCR is also useful in identifying of *P. multocida* serogroup. Due to the low level of systemic immunity, the immunological assay was not considered as a routine in identification of *P. multocida* serogroup (Hassan *et al*., 2016). ELISA was one of immunological assay

that have been used to identify *P. m*ultocida serogroup A, B, D, E and F (Dziva *et al*., 2008). Ranjan *et al*. (2011) stated currently real time PCR become highly sensitive and specific in detecting *P. multocida* especially field sample.

2.9 Virulence Factor and Pathogenesis HS

A virulent factor has an important role in mechanism of *P. multocida* infection. *P. multocida* strains were tested based two different strain which known as virulent and avirulent. Testing was made by administering these two different strains into the animal host (Boyce & Adler, 2000). However, it shows that virulent strain survived and multiplied on infected pharyngeal mucosa or intravascular of the exposed host (Boyce & Adler, 2000). *P. multocida* consist of 5 serogroup A, B, D, E and F and each of this serogroup was determined through the antigenicity of the capsule meanwhile serotype 1-16 was classified through lipopolysaccharide (LPS) antigen (Boyce *et al*., 2000; Towsend *et al.,* 2001; Shivachandra *et al.,* 2011). Polysaccharides are highly hydrated polyanionic and linked to phospholipid. This polysaccharide is a common substance that can be found in *P. multocida*. Polysaccharides is built by repetitive monosaccharide and attached together by glycosidic linkage. Meanwhile, this glycosidic linkages is formed by monosaccharide hydroxyl groups. Moreover, when a disease is caused by *P. multocida,* polysaccharides are more likely have an important role in the mechanism of the disease.

The capsule not only protects the bacteria from dehydrating and transports it from host to host but also strengthen the bacteria to survive in the environment. (Boyce *et al*., 2000). Studies indicate among the encapsulated and un-capsulated strains, encapsulated strains are expected to be more resistant towards phagocytosis. Besides this, both encapsulated and un-capsulated *P. multocida* B: 2 show differences in sensitivity towards phagocytosis. Antiphagocytic activity can be seen in the capsule that belongs to

pathogenic bacteria. The negative charge which released from the capsule join along with the bacteria and phagocyte thus disruption in phagocytosis activity against the bacteria occurs. In most cases, Gram negative pathogenic bacteria have increased the resistance towards bactericidal complement and it is commonly related to capsule expression. Comparison between acapsular and capsular strain, the acapsular strain of *P. multocida* B: 2 appear to be less virulent. There is two types of LPS glycoform are produce by *P. multocida*, glycoform A and B. The outer core of this glycoform is similar meanwhile the inner core different. The production of third type LPS glycoform have been observed in some *P. multocida* strain but, it was expressed in low level. The interaction between LPS and host's innate immune systems can cause endotoxic shock thus leads to mortality of the host (Raetz & Whitfield, 2002). The advantage of knowing the LPS structure can be applied during the development of rapid molecular method and vaccine development as well (Harper *et al*., 2011). *P. multocida* infection can be caused by the protease that secreted bacteria itself. This happens because the secreted protease has the capability to cleave immunoglobulin G (IgG). In 2003, Cullen *et al*. (2003) stated the outer membrane lipoprotein of *P. multocida* can be considered as one of a virulence factor and has the potential to provide protective immunity. Adhesive properties which can be found in fimbrial protein on *P. multocida* help the bacteria to colonize in the host and leads to infection (Saad, 2013).

Table 2.2: Disease and virulent factor from different serogroup of *P. multocida* (Wilkie *et al*., 2012)

Disease	Serogroup	Affected Animals	Virulent Factors
Snuffles	A, D	Pigs, Rabbit	Not Established
Enzootic Pneumonia, Shipping fever	A, D , F (rare)	Cattles, sheep, pigs	Co-infection with other bacteria or viral species important
Haemorrhagic Septicaemia	B, E	Cattle, pigs, goats and buffalo	Capsule, Fimbriae, Filamentous haemaglutinin
Avian Cholera	A, F, D (rare)	Chicken, turkey, ducks	Capsule, LPS, Iron acquisition protein, Filamentous haemaglutinin
Atrophic rhinitis	D, A (rare)	Pigs, rabbit	*P.multocida* toxin (PMT)

2.10 Etiology and Pathogenesis HS

HS is characterised by terminal septicaemia and shock in cattle and buffaloes (Ataei *et al*., 2009). *P. multocida* type B: 2 and E: 5 is the main etiology that causes HS is in Asian and African countries respectively (Ranjan *et al*., 2011). Host is infected through the inhalation and ingestion of the agent resulting in septicaemia and multiple-organ haemorrhages. Transmission *P. multocida* via gastrointestinal or urinary tracts is also another possible route, but still remain without scientific confirmation (Annas *et al*., 2014). HS affect both cattle and buffaloes whereby buffaloes encounter the highest mortality rate. The outbreak of HS happens usually when latent carriers infect the nasopharynx and thus activated the virulent bacteria. *P. multocida* cannot infect lungs but stress and other factors including climate changes or malnutrition can weaken lung's defense system. The occurrence of active and inflammatory substance may lead the death of macrophage in lungs (Khan *et al*., 2011). Poor in the management of husbandry conditions is where this disease happen to occur. The clinical symptoms are usually high temperature, loss of appetite, nasal discharged, increased salivation, labored

breathing and swelling in the submandibular region meanwhile death is usually rapid and mortality once the animal is fully infected (De Alwis, 1999). An organism that causes HS doesn't survive outside the animal body and usually it tends to spread during wet season. High buffalo population, humid conditions, free grassland system of management and paddocked together is usually associated with higher incidence of HS apart than inhalation or ingestion of *P. multocida*.

2.11 Diagnosis of HS

The diagnosis of HS may include the combination of clinical signs and gross pathological lesions and these measurements are important, since they can be used as preventive measures to control the disease from spreading. Biochemical, serological test and molecular method are the methods that have been established as diagnostic test for HS. Rapid slide agglutination, indirect hemagglutintion or non-serological test including acriflavine, flocculation or hyaluronidase may be employed to identify the serogroups. Whereas, molecular method which consist of polymerase chain reaction (PCR), ribotyping or restriction endonuclease analysis (REA) can be convincing routine to diagnosis HS since these methods can be utilized to differentiate the serotypes (Benkirane & De Alwis, 2002).

2.12 Treating HS

Antibiotic can be used as a primary treatment since bacteria is the etiology of HS but, this treatment can be applied at the early of stage of infection. In the primitive husbandry system, the early detection of this disease has always been neglected. Meanwhile, well-organized farm always be alert by checking rectal temperatures regularly (Benkirane & De Alwis, 2002). Recently, antibiotic are becoming resistance towards HS and it is becoming crucial in treating HS. In order to control the resistance strain, a new antibiotic has been introduced. However, the new antibiotic is very

restricted and costly (Xiao *et al*., 2016; Shivachandara *et al*., 2011). Vaccination is still leading method to prevent HS.

2.13 Vaccination Approach as a Method of HS Prevention

In order to prevent HS, vaccination on accessible attempt have been practiced. In most of the affected countries, the various formulation of available HS vaccine has been used in order to prevent HS. In 2004, Biswas *et al*. stated that even with the proper vaccination schedule HS still can occur. Due to this report, a proper vaccination strategy using the current vaccine needs to be remodel. The first effective vaccine was introduced in 1978 (Chandrasekaran & Yeap, 1978). In early stage, traditional vaccine has been used to exterminate the HS infection or to provide long lasting immunity (De alwis, 1999). Earlier studies in 1991 have shown that monoclonal antibodies (mAbs) help in two ways to prevent HS, by increasing the immunity against OMP and prevent the bacteria from proliferating in the lungs (Lu *et al*., 1991). Besides OMP, outer membrane protein H (OmpH), ammonium sulphate perceptible protein fraction and mixture OMP's of *P. multocida* was effective against *P. multocida* infection (Dabo *et al*., 2007; Adler *et al*., 1999; Srivastava *et al*., 1999; Marandi *et al*., 1997).

Vaccination is still being an efficient method to protect farm animals against *P. multocida*. To provoke the immune response, whole inactivated or killed bacteria are injected to the farm animals and this vaccine acted as whole cell vaccine. This type of vaccine called a first generation vaccine (Ahmad *et al*., 2014).

The preparation of killed vaccine includes inactivating the bacterins using heat or chemical like formalin. This will induce the responsible antigen in the host against the immune response (Ellis, 2001). To heighten the immune response, this killed vaccine can be prepared along with adjuvants. There are few commonly used killed vaccines such as aluminium hydroxide gel vaccines, bacterins, multiple (ME) vaccines and oil

adjuvant vaccines (OAV) against pasteurellosis. These vaccines have a variable duration to induce the hosts' immunity (Chandrasekaran *et al*., 1994b and Verma and Jaiswal, 1997). Alum precipitated vaccine has been used widely in every part of the world and this vaccine provide immunity up to 6 months against *P. multocida* (Nikhiporova, 1958). In 2001, Shah *et al*. (2001) stated that the improve version of oil-adjuvant HS from serotype B: 2.5 vaccine can provide protection towards serotype E: 2.5. OAV happens to be high in viscosity which makes it crucial in administrating to the animal and form abscess at the injection site (Verma & Jaiwal, 1998).

Multiple emulsion vaccines (MEV) were prepared by re-emulsifying OAV to an equal volume of Tween-80. This modification causes the vaccine to be administered easily and effectively protect the buffaloes against HS for up to 6 month period of time (Chandrasekaran *et al*., 1994). Newly formulated bacterin vaccine with toxoid provides high immune response in buffaloes. Longest protection up to 12 months against HS was given by OAV and double emulsion vaccine (Shah *et al*., 1997). Even though preparing bacterin vaccine is economical but this vaccine can only manage to provide immunity for short span of time which leads the occurrence of outbreaks frequently (Benkirane & De Alwis, 2002). In 2005, Sotoodehnia *et al*. (2005) stated preparation OAV with adjuvant Montanide ISA-70 may provide compromising protection in vaccinated calves.

Development live vaccine *P. multocida* serotype B and other strain have been by using several chemicals and UV radiation shows that this vaccine can protect from HS infection in different types of farm animals (Verma & Jasiwal, 1998). One of a live vaccine was developed by growing *P. multocida* under iron-restricted has offered a high protective immunity in vaccinated animals (Srivastava, 1998). In Myanmar, fallow deer strain B: 3, 4 were used as live vaccine and gave protection to the cattle against *P. multocida* serotype B for one year after vaccination. On the other hand, passive

immunization against infection from *P. multocida* serotype E: 2, F: 3, 4 and A: 3, 4 was carried out in order to provide protection by using the collected serum from vaccinated cattle (Myint *et al*., 2005). Deletion of derivative *aroA* gene in *P. multocida* serotype B: 2 will prevent the bacteria from growing in the host. The function of this gene is converting shikimic acid to chorismic acid during the synthesis of aromatic acids. This aromatic acid is accessing the bacteria to grow in the host whereby deleting this gene will help to inhibit the growth of bacteria (Tabatabaei *et al*., 2002). The same deletion of *aroA* gene in *P. multocida* p52 strain was carried out in different studies with replacement kanamycin resistance gene cassette and known as mutant P52Δaro. This mutant P52Δaro was being highly attenuated in mice and rabbit can be suitable live vaccine candidate in controlling HS in cattle and buffaloes since there were no pathological lesion were reported (Chaudhuri *et al*., 2012). The advantage of live vaccine is better in provoking cellular immunity (Haesebrouck *et al*., 2004; Hopkins *et al*., 1998).

Antigenic part from *P. multocida* such as capsule, LPS, OMP or segment of cell wall can be used to develop acellular vaccine (Shivachandra *et al*., 2011). Development of acellular vaccine using ribosome and carrier protein from polysaccharides of *P. multocida* has no promising development (Verma & Jaiwal, 1998). Although, acellular vaccine appears to be safe in animals but, it provides less effective in immune response.

Oil adjuvant and multiple emulsions have a significant importance in developing vaccine against HS among all the adjuvants. Combination of HS killed vaccine long with another pathogen has helped to reduce the vaccine doses and raise the immunity towards other infection (Verma & Jaiswal, 1998).

2.13.1 Molecular Approach Towards in HS Vaccine Prevention

Recently, synthetic peptides, purified subunits, synthetic peptides, recombinant protein and chemical conjugation of polysaccharides have been used approached for vaccine production (Shivachandra *et al*., 2011). Subunit vaccine known is as second generation vaccine. This subunit vaccine consist immunogenic component of the pathogen to provoke immunity (Ahmad *et al*., 2014). Studies were carried out using capsular, one of an immunogenic component from *P. multocida*. This vaccine when given in higher dosage it was able to protect cattle against HS for one year (Maslog, 1998). In 2008, Cho *et al*. stated combination polyvalent *pasteurella* vaccine together with adjuvant has the ability to provide immunity and create resistant against *P. multocida* infection. Development of subunit vaccine using OmpH protein, which was obtained from *P. multocida* showed a high antibody titer and able to proliferate T-cells in immunized animals (Kumar *et al*., 2011). Modification in OmpA by using heat develops cellular response against *P. multocida* (Confer & Ayalew, 2013).

Recombinant protein produced by *P. multocida* triggered strong immunity (Hatfaludi *et al*., 2010). In order to produce this third generation vaccine, identification virulence and immunity genes from *P. multocida* is one of the important aspects that have been taken into account (Ahmad *et al*., 2014). The first recombinant protein vaccine which was successfully attempted was against rhinitis in pigs which was derived from *P. multocida* toxin (PMT) (Bording *et al*., 1994). Different approach have been encounter using recombinant vaccine by expressing the fimbrial protein *P. multocida* B: 2. It was found to provide significant protection and enhanced the stimulation of local and systemic immunities in goat when the vaccination was given via intranasal against HS (Yasin *et al*., 2011). Shivachandra *et al*. (2012) stated recombinant protein which was expressed using *ptfA* gene has the potential as recombinant vaccine candidate towards HS.

DNA vaccine or known as fourth generation vaccines that was produced from *P. multocida* B: 2 antigens might provide protective immunity against HS in cattle. The mechanism of this DNA vaccine was not clearly defined. Outer membrane protein (OMP) possesses a capability as DNA vaccine against HS (Singh *et al*., 2011). DNA vaccine appears to be efficient but limited by cost and technological default (Ahmad *et al*., 2014).

Recombinant technology is reforming the shape of current vaccine development against *P. multocida* infection (Ahmad, 2014). Recombinant vaccine development can be a guidance to clarify the involvement of the associated genes in the pathogenesis of the disease (Wilkie *et al*., 2012).

2.14 ABA392 Gene

The recombinant clone ABA392 was derived from a *P. multocida* serotype B202, was constructed by shotgun cloning method (Salmah Ismail, 1997, 2004) using *E. coli* as host. Previously the sequenced recombinant plasmid (Salmah Ismail, 2000) harbours a sequence that encode for a virulence factor to *P. multocida*. Genomic DNA of isolated *P. multocida* serotype B202 was digested with restriction endonuclease *Sau*3A1 which generate restriction fragment of approximately 500 to 1000 bp. Then it was inserted at the *Bam*HI site of the pUC18 vector. From 20 chosen recombinant plasmids, only three clones, namely ABA182, ABA292 and ABA392 are were found to be toxigenic in mice. Meanwhile, only one of the retrieved clone showed mortality in mice (Hussaini *et al*., 2011). The ABA392 cloned has shown virulent characteristic when injected into mice as compared from the parental strain of PMB202. The size of recombinant plasmid clone of ABA392 was estimated at 3.2 kb and the DNA insert was determined as 921 bp (Accession No. DQ334273) using DNA sequencing analysis (Salmah Ismail, 2000, 2004). Since the recombinant clone of ABA392 was capable to protect the host from

HS, it was then sub cloned into prokaryotic expression system (pQE32/M15). The potentials of the recombinant clone ABA392 as a vaccine candidate against hemorrhagic septicemia (HS) has been described and explored in mice model (Hussaini, 2009; Salmah Ismail *et al*., 2010). They have shown a significant in which 90% of the mice vaccinated with the expressed clone (ABA392/pQE32) survived the challenge producing high antibody titer, whilst all unvaccinated mice died within 36 hours of the challenge. Meanwhile passive mouse protection test (PMPT) results showed that 83% of mice injected with hyper immune sera from vaccinated mice with ABA392/pQE32 were protected from the effects of *P. multocida* serotype B202. There were no survivors in unvaccinated group (Hussaini, 2009, 2011, 2012).

CHAPTER 3: MATERIALS AND METHODS

(A) Materials

3.1 Bacteria Strain, Recombinant Clone ABA392 and Vector

The bacteria strain that used in this study is *P. multocida*. The recombinant clone ABA392 was derived from parental strain *P. multocida* serotype B202 (PMB202). This clone was constructed by shotgun cloning method (Salmah Ismail, 1997; 2004) using *E. coli* as a host. Among the twenty recombinant plasmid previously sequenced (Salmah Ismail, 2000), ABA392 were found toxigenic to mice and thus harbors a sequence that code for a virulence factor for *P. multocida*. The expression vector that was used in this research is pET-30a with the size of 5422 bp. This expression vector was used to express the protein in *E. coli* system. Bacteria, ABA392 clone and vector were provided by Molecular Bacteriology and Toxicology Laboratory (MBT Lab) and stored in fridge -20ºC.

3.2 Chemical and Reagents

All the chemicals used were of Analar grade and highest grade available commercially. All chemical solution and molecular solution were purchased from Sigma Life, Liofilchem Diagnostic, Biorad, Thermo Scientific, Friendeman Schmidtt, Fermentas, Promega, and New England Biolabs.

Luria-Bertani (LB) broth and agar powder were obtained from Liofilchem Diagnostic, Italy. Both Brain Heart Infusion (BHI) broth and agar powder were from BD, U.S.A meanwhile Acetone, acid sulfuric, sodium chloride, sodium hydroxide, tris-EDTA were from Friendeman Schmidtt, Australia. Methanol, glycerol, acid hydrochloric, acid sulfuric, glacial acetic acid and formalin were purchased from Fisher Scientific, U.S.A. Ethanol (absolute) were purchased from RCI Labscan, Australia.

Agarose powder and IPTG were purchased from Promega, U.S.A.

Gene Ruler™ 1 kb plus DNA ladder, B-per Bacterial Protein Extraction Reagent 10X Tris-Borate-EDTA (TBE) electrophoresis buffer, Tween-20 and blood agar is from Thermo Scientific, U.S.A

DNA purification and extraction kit is purchased from Macherey-Nagel, Germany.

DNA ligation solution, Restriction Enzyme (RE), Supercoiled DNA ladder, Colorplus prestained protein ladder (#P7711S & P7712S) and 6X loading dye were from New England Biolabs (NEB), U.S.A. Gel red were from Biotium, California. Blueye prestained protein ladder from Gene Direx, Malaysia (Axon Scientific). 1kb plus DNA ladder and *Escherichia coli* BL21 (DE3) pLysS were from Invitrogen, U.S.A.

Econotaq plus Green 2X mastermix is purchased from Lucigen, U.S.A.

Anti-Mouse IgG, AP-linked antibody is purchased from Cell-Signaling, U.S.A

Ampicillin, kanamycin, chloramphenicol, glycine, sodium phosphate, sodium carbonate, sodium salicylate, glycine, accustain harris hematoxylin solution, accustain Eosin Y solution, coomassie brilliant blue (CBB-250), 2-mercapthanol, skimmed milk powder and Bovine Serum Albumin (BSA) were from Sigma-Aldrich, U.S.A. Imidazole were purchased from Calbiochem, U.S.A.

Penta Anti-HIS antibody was purchased from Qiagen, Germany.

Anti-Mouse IgG, AP-linked antibody is purchased from Cell-Signaling, U.S.A

IgG antibody (HRP) conjugate were obtained from Gene Tex, U.S.A

Tetramethylbenzidine (TMB) substrate was purchased from Nacalai Tesque, Japan.

Sodium doedecyl sulphate (SDS), 1.5M TRIS-HCL 8.8, 0.5M TRIS-HCL 6.8, Ammonium persulfate (APS), 30% Acrylamide, 2x sample buffer, and Bio-Rad DC protein assay and N'N'N'N' Tetramethylethylenediaminie (TEMED), were from Bio-Rad, USA.

Potassium chloride, sodium phosphate dibasic, sodium bicarbonate, dibasic sodium, turks solution and BCIP (5-Bromo-4-Chloro-3-indolyl phosphate) AP substrate were obtained from Merck, U.S.A meanwhile potassium dihydrogen phosphate were purchased from Riedel-De-Haen, Hannover, U.S.A. Monobasic sodium obtained from BDH, U.S.A.

Xylene were purchased from R&M chemicals, Malaysia, paraffin from PC laboratory Reagent, U.S.A and DPX is obtained from Labchem, U.S.A.

3.3 Apparatus

The main machine involved were incubator, red line by binder, Germany. Weighing balance was from A&D Company Limited, Japan. Volt power supply machine (PS-300B) from AA Hoefer, U.S.A. Super bright UV trans-illuminator was from Vilber Lourmat, U.S.A. Heating block was from Chemolab Supplies Sdn Bhd, Malaysia. Polymerase chain reaction

(PCR) veriti 96 well thermal cycler, Sorvall ST 40R Centrifuge (4ºC) and incubator shaker were obtained from Thermo Scientific, U.S.A, meanwhile gel doc (EZ) imager was from Bio-Rad, U.S.A. Mini-spin (eppendorf), Fisher Scientific, U.S.A. Thermolyne vortex maxi mix ii was from Labequip, Canada. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis SDS-Page (Mini protean tetra system) was obtained from Bio-Rad, U.S.A. Bench top professional pH meter-BP3001 was from Transinstrument, Singapore. Magnetic stirrer-Cimarec 2 was from Thermolyne, Canada. Water bath-Grant was from sub-aqua, pro instrument, UK. Orbital shaker 110V was obtained from Komabiotech, Korea. Micropipettes, microcentrifuge tube and tips were all manufactured from Eppendorf, Germany. Others apparatus included are tips, tips container, petri dish, inoculate loops, ice box, floater, microcentrifuge tube rack, falcon tube, beaker and other glassware including glass and universal bottles.

3.4 Antibiotic Solutions

Antibiotic stock solution for ampicillin (100mg/ml), kanamycin (30mg/ml) and Chloramphenicol (34mg/ml) were prepared as working concentrations and sterilized by filtration through sterile membrane filters (type PES, 0.22µm pore size) Millex-GP 33mm Syringe Driven Filter Unit. The working solution were then dispensed in 1ml aliquots into sterile 1.5ml microcentrifuge tubes and stored at -20ºC. The working solution was allowed to come to room temperature once removed from the cold and desired volume is then added to the sterile medium.

3.5 Media and Broth

The essential media and broth preparation used were Luria-Bertani (LB) and Brain Heart Infusion (BHI).

3.5.1 LB Medium

Standard LB medium powder : 21g

Distilled water : 1000ml

The standard LB medium powder was dissolved and stirred using magnetic stirrer in 1000ml distilled water. Each 10ml, 50ml or acquired of the mixture were aliquot in each universal bottle and conical flask and proceed to liquid autoclave at 121ºC for 1 to 2 hours before it is used.

3.5.2 LB Agar

Standard LB agar powder : 37.0g

Distilled water : 1000ml

3.5.3 LB Agar with Ampicillin

Standard LB agar powder : 37.0g

Ampicillin : 100µg/ml

Distilled water : 1000ml

3.5.4 LB Agar with Kanamycin

Standard LB agar powder : 37.0g

Kanamycin : 30µg/ml

Distilled water : 1000ml

3.5.5 LB Agar with Chloramphenicol

Standard LB agar powder : 37.0g

Chloramphenicol : 34µg/ml

Distilled water : 1000ml

3.5.6 LB Agar with Kanamycin/ Chloramphenicol

Standard LB agar powder : 37.0g

Kanamycin : 30µg/ml

Chloramphenicol : 34µg/ml

Distilled water : 1000ml

3.5.7 LB Agar with IPTG

Standard LB agar powder : 37.0g

IPTG : 0.1M

Distilled water : 1000ml

3.5.8 LB Agar with Kanamycin/Chloramphenicol/IPTG

Standard LB agar powder : 37.0g

Kanamycin : 30µg/ml

Chloramphenicol : 34µg/ml

IPTG : 0.1M

Distilled water : 1000ml

The standard LB agar powder was dissolved and stirred using magnetic stirrer in 1000ml distilled water. The solution was cooled down to 50ºC. Once cooled down, ampicillin (100µg/ml), kanamycin (30µg/ml), chloramphenicol (34µg/ml) and IPTG (0.1M) were then added according desired concentration then the agar solution was poured into petri dishes with ~20ml each plate and cooled down. Once the agar solidified, the LB agar plates were sealed using parafilm and stored at 4ºC.

3.5.9 Luria-Bertani (LB) Slant

The preparation of LB agar slant was similar as preparation LB agar plate but the solution was poured in universal bottles and added with the appropriate concentration of ampicillin (100µg/ml), kanamycin (30µg/ml), chloramphenicol (34µg/ml) and slanted one side. The bottles were slanted overnight in purpose for the agar to solidify in slope form.

3.5.10 Brain Heart Infusion (BHI) Medium

Standard BHI medium powder : 32.0g

Distilled water : 1000ml

The BHI medium powder was dissolved and stirred using magnetic stirrer in 1000ml distilled water. Each 10ml, 50ml or acquired of the mixture were aliquot in each universal bottle and conical flask and proceed to liquid autoclave at 121ºC for 1 to 2 hours before it is used.

3.5.11 BHI Media

Standard BHI agar powder : 52.0g

Distilled water : 1000ml

The standard BHI agar powder was dissolved respectively and stirred using magnetic stirrer in 500ml distilled water. The solution was cooled down to 50ºC. The agar

solution was poured into petri dishes with ~20ml each plate and cooled down. Once the agar solidified, the BHI agar plates were sealed using parafilm and stored at 4ºC.

3.5.12 Blood Agar

Blood agar was purchased commercially and stores at 4ºC prior to use

3.6 DNA Purification and Extraction kit

There were two types of kits have been used in this research, plasmid DNA purification (Section 3.20) and PCR clean-up Gel extraction (Section 3.24).

3.6.1 NucleoSpin® Plasmid

This kit was used to purify gene and vector of interest, ABA392 and pET-30a respectively.

Resuspension Buffer A1	: 15ml
Lysis Buffer A2	: 15ml
Neutralization Buffer A3	: 20ml
Wash Buffer AW	: 30ml
Wash Buffer A4	: 2 X 6ml
Elution Buffer AE	: 15ml
RNase A	: 6mg
NucleoSpin Plasmid Columns Tubes (white rings)	: 50pc
Collection Tubes (2ml)	: 50pc

3.6.1.1 Buffer A1

1ml of Buffer A1 was added to RNase A vial and vortex. All the resulting solution was transferred into the Buffer A1 bottle and mixed thoroughly. Buffer A1 was stored at 4ºC.

3.6.1.2 Buffer A4

24mL of 96% ethanol was added into Buffer A4 and mixed thoroughly.

3.6.2 NucleoSpin® Gel and PCR Clean Up

This kit was use for DNA extraction of ABA392 gene and pET-30Aa from the agarose gel.

Binding Buffer NTI	: 2x25ml
Wash Buffer NT3 (Concentrate)	: 20ml
Elution Buffer NE	: 15ml
NucleoSpin Gel and PCR Clean-up Columns (Yellow rings)	: 50pc
Collection Tubes (2ml)	: 50pc

3.6.2.1 Buffer NT3

80ml of 96% Ethanol was added into wash buffer NT3 and mixed thoroughly.

3.7 Agarose Gel Electrophoresis

The materials that need to prepare agarose gel electrophoresis was agarose powder, Tris/Borate/EDTA (TBE) buffer and gel red for staining purpose. The amount of agarose powder depends on the percentage agarose gel that used. The percentage of gel that were used in this research is 0.7% and 1%. This agarose powder was stored at room temperature (25 ± 2ºC).

3.7.1 Agarose Gel, 0.7%

The mass of agarose powder that need to prepare is determined by 0.7% w/x of 1X TBE buffer volume. This concentration shows better separation for PCR samples and 0.7% agarose gel is usually prepared in total amount of 50ml. The standard amount used that for preparing the gel as below.

Agarose powder	: 0.35g
TBE buffer, 1X	: 50ml

To prepare 7% agarose gel, 0.35g of the agarose powder was weigh by using analytical balance and mixed into 50ml of 1X TBE buffer in a 100ml Scott bottle. This

solution is mixed well and boiled in microwave oven for 1 minute at medium temperature with loosen cap. Once the solution was dissolved, the solution will be cooled down to 50ºC and poured into gel loader. A well comb is inserted immediately after the pouring. It was important to make sure there is no bubble. The gel was cooled for 20 to 30 minutes for solidification. The well comb was removed once the gel has solidified for electrophoresis run.

3.7.2 Agarose Gel, 1%

The mass of agarose gel powder used was 1% w/v of 1X TBE. This concentration was used for plasmid samples. The amount that needs to prepare 1% agarose gel is as below:

Agarose powder : 0.5g

TBE buffer, 1X : 50ml

1% agarose gel was prepared as described above in Section 3.7.1.

3.7.3 Tris/Borate/EDTA (TBE) Buffer, 1X

TBE (10X) buffer were purchased commercially and stored at room temperature (25 ± 2ºC). 1X TBE buffer was diluted from 10X TBE which consist of 89mM Tris, 89mM Boric Acid and 2mM EDTA to required volume and stored at room temperature (25 ± 2ºC).

3.7.4 Loading Dye and DNA Marker

The loading dye that was used is gel loading dye purple (6x) and volume is about 1µl (1 volume of the dye solution 5 volumes of the DNA sample). Three types of DNA marker have been used in this research which is 1 kb Plus DNA ladder (0.1µg/µL), Gene ruler 1 kb plus DNA ladder (0.5µg/µL) and supercoiled DNA ladder (500µg/ml). The volume that was used is about 5µl. Both loading dye and DNA ladder were stored at -20ºC.

3.7.5 Gel Red™ Staining (Post Staining)

Once the gel electrophoresis is done, gel was stained in gel red staining. The Gel Red™ 10000X stock solution was diluted 3,300 fold to make a 3X staining solution in distilled water. The staining preparation as below:

Gel red : 30µl

Sterile distilled water : 100ml

30µl of gel red was mixed with 100ml sterile water in a clean container and sealed with aluminum foil since it is light sensitivity. Gel was stained for 5 to 10 minutes and bands were viewed using UV trans-illuminator. This gel red was stored at 4ºC.

3.8 DNA Cloning

3.8.1 Restriction Endonuclease (RE)

There are two types of RE were used, *Hin*dIII-HF and *Bam*HI-HF. These enzyme were used together with restriction enzyme (RE) buffer (10X) during digestion process. Below are the RE site sequence for both enzymes:

(i) *Hin*dIII-HF: AAGCTT

(ii) *Bam*HI-HF: GGATCC

Both RE and RE buffer (10X) were stored at -20º.

3.8.2 DNA Ligation Solutions

The DNA ligation solution used was T4 DNA Ligase Reaction Buffer (10X) consists of (Tris-HCl, $MgCl_2$, ATP, and DTT) and T4 DNA Ligase with 10mm ATP. All these solutions were stored at -20ºC.

3.9 Transformation

3.9.1 Types of Cells

The competent cells that were used were *Escherichia coli* (*E. coli*) and the strain was BL21 (DE3) pLysS. This strain was used during pET-30a transformation.

3.9.2 Transformation Buffer, 0.1M

The mass of calcium chloride dehydrate to prepare transformation buffer was:

Calcium chloride, $CaCl_2$ (Mw: 110.98g/mol) : 1.1g

Distilled water : 100ml

This solution was autoclaved and stored at 4ºC. This solution was used during preparation of competent cells (Section 3.26.1).

3.10 Polymerase Chain Reaction (PCR)

3.10.1 Mastermix

The total volume of PCR mix that has been used was 25µl. Econotaq Plus Green 2X mastermix was used which consists of 0.1units/µl of Econotaq DNA polymerase, reaction buffer (pH 9.0), 400µm dATP, 400µm dGTP, 400µm dCTP, 400µm dTTP, 3mm $MgCl_2$, PCR enhancer/stabilizer and blue/yellow tracking dyes. The composition of PCR mix will be described in the method Section 3.27. This mastermix was stored at -20ºC.

3.10.2 Primer

The primer was synthesized from MyTACG Bioscience Enterprise. This primer was designed based on ABA392 gene which derived from parental strain *P. multocida* 202. This primer was use during PCR amplification, colony screening and gene insert analysis. The DNA sequence below shows the designed primers:

(i) F- 5'ATGAGTCTTTTGTTCTGTCGCTTG'3

(ii) R- 5'CTAGCGGCGGTGGAAACCCGCCAT'3

3.11 Protein Expression of ABA392/pET-30a Recombinant Clone

3.11.1 Isopropyl-β-D-thiogalactopyranoside (IPTG), 0.1M

The mass of IPTG that required is as below:

IPTG, $C_9H_{18}O_5S$ (MW: 238.298g·mol^{-1}) : 0.24g

Sterile dH_20 : 10ml

The solution is sterilized by filtration through a 0.22µm membrane and stored at -20ºC.

3.11.2 Phosphate Buffer Saline (PBS), 10X

This solution was purchased commercially and stored at room temperature. 10X PBS is diluted to 1X concentration prior to use.

3.11.3 Protein Lysis Buffer

The protein lysis buffer that was used in this research is B-PER® Bacterial Protein Extraction Reagent.

3.11.4 Lysozyme and DNAase 1

This solution was purchased commercially and stored at -20ºC.

3.12 Purification of 6xHis-tagged Protein using Dynabeads

3.12.1 Dynabeads His-Tag Isolation and Pulldown

The expressed His-tag protein from recombinant clone ABA392/pET-30a was purified using Dynabeads His-Tag Isolation and Pulldown and it was bought commercially.

3.12.2 Binding/Wash Buffer (50ml), 2X

To prepare Binding/Wash buffer, 2X:

Sodium Phosphate, NaH_2PO_4 (MW: 120g/mol), 100mM, pH 8 : 0.6g

Sodium Chloride, NaCl (MW: 58.44g/mol), 600mM : 1.7532g

Tween®-20, 0.02% : 10µl

HCl/NaOH : Adjust pH

This solution was dissolved in 50ml distilled water and pH was adjusted to 8 by using NaOH and stored at 4ºC. 2X Binding/Wash buffer was diluted to 1X concentration prior to usage.

3.12.3 His Elution Buffer (50ml)

To prepare His Elution Buffer:

Imidazole, $C_3H_4N_2$ (MW: 68.077g/mol), 300mM : 1.021g

Sodium Phosphate NaH_2PO_4 (MW: 120g/mol), 50mM, pH 8 : 0.3g

Sodium Chloride, NaCl (MW: 58.44g/mol), 50mM : 0.8766g

Tween®-20, 0.01% : 5µl

HCl/NaOH : Adjust pH

This solution was dissolved in 50ml distilled water and pH was adjusted to 8 by using NaOH and stored at 4ºC.

3.13 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.13.1 Acrylamide/Bis Solution, 30%

This solution was purchased commercially and stored at room temperature. 50ml of this is aliquot in falcon tube prior to use and stored at 4ºC.

3.13.2 Resolving Gel Buffer, 1.5M Tris-HCl, pH 8

This solution was purchased commercially and stored at room temperature. 50ml of this solution was aliquot in falcon tube prior to use and stored at 4ºC.

3.13.3 Stacking Gel Buffer, 0.5M Tris-HCl, pH 6.8

This solution was purchased commercially and stored at room temperature (25 ± 2ºC). 50ml of this solution was aliquot in falcon tube prior to use and stored at 4ºC.

3.13.4 Sodium Dodecyl Sulfate (SDS), 10%

The mass of 10% (w/v) SDS that was needed as below:

SDS, $NaC_{12}H_{25}SO_4$ (MW: 288.372g/mol) : 5.0g

Distilled water : 50.0ml

The solution was mixed and filtered and stored at room temperature (25 ± 2ºC).

3.13.5 Ammonium persulphate (APS), 10%

APS was used as polymerizing agent in gel. The mass of ammonium persulphate that needed was:

Ammonium persulphate, $(NH_4)_2S_2O_8$ (MW: 228.18g/mol) : 0.1g

Distilled water : 1.0ml

This solution was prepared freshly prior to usage.

3.13.6 N'N'N'N'-Tetramethylethylenediamine (TEMED

This solution was purchased commercially and stored at 4ºC.

3.13.7 Running Buffer (25mM Tris, 192mM Glycine, 0, 1% SDS)

Tris Base, $C_4H_{11}NO_3$ (MW: 121.14g/mol) : 3.03g

Glycine, $C_2H_5NO_2$ (MW: 75.0g/mol) : 14.4g

SDS, $NaC_{12}H_{25}SO_4$ (MW: 288.372g/mol) : 1.0g

The solution was dissolved in 1000ml distilled water and stored at room temperature (25 ± 2ºC).

3.13.8 Laemli Sample buffer, 2X

This solution was purchased commercially and stored at room temperature (25 ± 2ºC). 2X Laemli sample buffer consist of 68.5mM Tris-HCl, pH 6.8, 2.1% SDS, 26.3% (w/v) glycerol and 0.01% bromophenol blue. This solution was diluted to 1:20 with 2-mercapthenol prior to usage.

3.13.9 Protein Ladder

The protein ladder was colorplus prestained (#P7711S & P7712S) and blueye prestained protein ladder.

3.13.10 Colloidal Coomassie Staining (0.08% Coomassie Brilliant Blue (CBB) G250, 1.6% Ortho-phosphoric Acid, 8% Ammonium sulfate, 20% Methanol)

Ortho-phosphoric Acid, H_3PO_4 (MW: 98.00g/mol) : 16ml

Ammonium Sulfate, $(NH_4)_2SO_4$ (MW: 132.14g/mol) : 80g

CBB 250, 5% : 16ml

Methanol, CH_4O (MW: 32.04) : 200ml

16ml Ortho-phosphoric Acid was mix into 768ml of distilled water. 80g of ammonium sulfate and 16ml of 5% CBB G250 were then added into the previous mixture. Slowly 200ml methanol was added to the solution to give final volume of 1000ml (Neuhoff, *et al*, 1985).

3.13.11 De-staining Solution

Distilled Water : 100ml

The de-staining process was done on the gel until the background of the gel transparent was appeared (Neuhoff, *et al*, 1985).

3.13.12 Quantification of His-tag Protein

The Bio-Rad DC protein assay were bought commercially and stored at room temperature (25 ± 2ºC).

3.13.12.1 Bovine Serum Albumin (BSA)

Bovine serum albumin was purchased commercially and prepared 10mg/ml as stock solution. The stock solution was stored at -20ºC.

3.14 Western Blotting

3.14.1 Blotting Buffer (Transfer Buffer), 1X pH 8.3

Tris Base, $C_4H_{11}NO_3$ (MW: 121.14g/mol) : 3.03g

Glycine, $C_2H_5NO_2$ (MW: 75.07g/mol) : 14.4g

Methanol, CH_3OH (MW 32.04g/mol) : 200ml

Naoh/HCl : Adjust pH

Distilled water : 1000ml

3.03g of tris base and 14.4g glycine were mix into 200ml methanol. Once dissolved, distilled water was top up till 1000ml and pH was adjusted to 8.3. The solution was stored at room temperature (25 ± 2ºC).

3.14.2 Blocking Buffer, 5% (w/v)

Skimmed milk powder : 0.5g

TBS, 1X : up to 10ml

0.5g of skimmed milk was mix with 1X TBS up to 10ml. This solution was freshly prepared prior to usage.

3.14.3 Tris-Borate Saline (TBS), 5X, pH 7.5

Tris Base, $C_4H_{11}NO_3$ (MW: 121.14g/mol) : 12.11g

Sodium chloride, NaCl (MW: 58.44g/mol) : 48.85g

Distilled water : 1000ml

HCL/NaOH : Adjust pH

12.11g tris base and 48.85g NaCl were mixed into 1000ml until it dissolved. pH was adjusted to 7.5. The solution was stored at room temperature (25 ± 2ºC). This solution will be diluted to 1X prior to usage.

3.14.4 TBS –Tween 20 (TBST), 0.2%

TBS, 1X (Section 3.14.1) : 1000ml

Tween-20 : 2ml

This solution was mixed and stored at room temperature (25 ± 2ºC). This solution served as washing buffer.

3.14.5 Skimmed milk, 2.5% (w/v)

Skim milk powder : 0.5g

TBS, 1X : up to 20ml

0.5g of skimmed milk was mixed with 1X TBS up to 20ml. This solution was freshly prepared prior to usage.

3.14.6 BCIP (5-Bromo-4-Chloro-3-indolyl phosphate) AP Substrate

This solution was purchased commercially and stored at 4ºC.

3.14.7 Primary and Secondary Antibody

Penta Anti-His antibody and IgG, AP-linked antibody was bought commercially and stored at 4ºC.

3.15 Histopathology Analysis

3.15.1 Mono Basic Phosphate, 0.1M

Mono Basic Phosphate NaH_2PO_4 (MW: 156g/mol) : 15.6g

Distilled Water : 1000ml

15.6g was dissolved in 1000ml distilled water to make 0.1M solution. This stock solution was stored at room temperature (25ºC ± 2ºC).

3.15.2 Dibasic Phosphate, 0.1M

Dibasic Phosphate $Na_2HPO_4.2H_2O$ (MW: 178g/mol) :17.8g

Distilled Water : 1000ml

1000ml of distilled water was filled in beaker before stirring. Small amount of dibasic phosphate was added and make sure it was dissolved completely. The remaining dibasic phosphate was added gradually until the whole amount is dissolved. This stock solution is stored in room temperature (25 ± 2ºC).

3.15.3 Phosphate Buffer, 0.1M

Mono Basic Phosphate, NaH_2PO_4, 0.1M (Section 3.15.1) : 500ml

Di Basic Phosphate, $Na_2HPO_4.2H_2O$, 0.1M (Section 3.15.2) : Adjust pH

500ml of 0.1M Mono Basic Phosphate was added into Scott bottles. pH was adjusted using 0.1M Di Basic Phosphate until pH reach 7.3. This solution was stored at room temperature (25 ± 2ºC).

3.15.4 Neutral Buffer Formalin, 10%, pH 7.3

Phosphate Buffer, 0.1M (see Section 3.15.3) : 500ml

Formalin (37%) : 100ml

Distilled Water : 400ml

All the solution is were added together to obtain 1000ml of 10% buffered formalin. This solution was stored at room temperature (25 ± 2ºC).

3.15.5 Mayer's Albumin

Fresh egg white : 50ml

Glycerol : 50ml

Sodium Salicylate, $C_7H_5NaO_3$ (MW: 160.11g/mol) : 1g

All the solution were added together and stored at 4ºC.

3.15.6 Histopathology Staining (Hematoxylin and Eosin staining (H&E))

3.15.6.1 Harris Hematoxylin

This solution was purchased commercially and stored at room temperature (25 ± 2ºC). Harris hematoxylin was filtered and 50ml of the solution was aliquot in falcon tube prior to usage.

3.15.6.2 Eosin Y

Eosin Y : 100ml

Glacial Acetic Acid, CH_3COOH (MW: 60.05g/mol) : 0.5ml

Acidified Eosin Y solution, Aqueous was prepared by slowly adding 0.5ml of glacial acetic acid per 100mL. 50ml of Eosin Y was aliquot in falcon tube and was stored at room temperature (25 ± 2ºC).

3.16 Enzyme-Linked Immunosorbent Assay (ELISA)

3.16.1 Coating Buffer, pH 9.6

Sodium carbonate, Na_2CO_3 (MW: 105.99g/mol)	: 1.06g
Sodium Bicarbonate, $NaHCO_3$ (MW: 84.00g/mol)	: 0.84g
Naoh/HCl	: Adjust pH
Distilled water	: 200ml

3.16.2 Coating Antigen

About 30 uniform single colonies of *P. multocida* B: 2 which grown on blood agar was inoculated into 200ml of sterile BHI broth and incubated at 37ºC for 16 hours. The overnight broth containing bacteria was centrifuge and the supernatant was discarded. The pellet was washed with sterile PBS and centrifuge at 4000rpm for 30minutes respectively. The pellet was resuspended with 200ml of coating buffer (see Section 3.16.1) and boiled at 97ºC for 20 minutes. The inoculum was left to cool and was allocated into 1.5ml microcentrifuge tubes. The allocated inoculum was stored at -20ºc till prior to usage.

3.16.3 Washing Buffer (PBS-Tween 20), pH 7.4

Sodium chloride, NaCL (MW: 58.44g/mol)	: 8.0g
Potassium dihydrogen phosphate, KH_2PO_4 (MW: 136.09g/mol)	: 0.2g
Sodium phosphate dibasic, $HNa_2O_4P.2H_2O$ (MW: 141.96g/mol)	: 2.9g
Potassium chloride, KCl (MW: 74.55g/mol)	: 0.2
Tween 20(added after autoclave)	: 0.5ml
Distilled Water	: 1000ml
HCl/NaOH	: adjust pH

This solution was dissolved in 1000ml distilled water and autoclaved. Tween 20 was added later and solution pH was adjusted to 7.4 and store at room temperature (25 ± 2ºC) till prior to usage.

3.16.4 Blocking Buffer (PBS-Tween 20-BSA), pH7.4

Sodium chloride, NaCL (58.44g/mol)	: 8.0g
Potassium dihydrogen phosphate, KH_2PO_4 (136.09g/mol)	: 0.2g
Sodium phosphate dibasic, $HNa_2O_4P.2H_2O$ (141.96g/mol)	: 2.9g
Potassium chloride, KCl (MW: 74.55g/mol)	: 0.2g
Tween 20 (added after autoclave)	: 0.5ml
Bovine Serum Albumin (BSA)	: 10.0g
Distilled Water	: 1000ml
HCl/NaOH	: adjust pH

This solution was dissolved in 1000ml distilled water and autoclaved. Tween 20 and BSA were added later and solution pH was adjusted to 7.4 and store at 4ºC till prior to usage.

3.16.5 Tetramethylbenzidine (TMB)

This solution was purchased commercially stored at 4ºC.

3.16.6 Stopping Buffer

Acid sulphuric, H_2SO_4 (MW: 98.00g/mol)	: 29.36ml
Distilled Water	: 200ml

This solution was dissolved in 1000ml distilled water and stored at 4ºC till prior to use.

3.16.7 Secondary Antibody

IgG antibody (HRP) conjugate was purchased commercially and stored at 4ºC.

(B) Method

3.17 Washing Procedures

All the glassware and wash ware items were soaked in teepol solution overnight and washed thoroughly under running tap water and lastly rinsed with distilled water. All washed items then were allowed to dry by heat drying at 60ºC in oven.

3.18 Sterilization

Throughout this research conducted, sterile condition was applied. All the media, reagents, chemical, distilled water and plastic disposable such as pipettes, tips, microcentrifuge tube, polymerase chain reaction (PCR) tube were sterilized in an automated autoclave 121ºC (50psi) for approximately 45 minutes. Glassware such as conical flask, beakers, measuring cylinders, bottles (universal and bijou) was sterilized in hot air oven at 180ºC for 2 hours. Antibiotic and other solution were sterilized using Millipore filter.

3.19 Storage and Maintenance of Bacterial Culture

All the overnight cultures were mixed with equal volume of 60% sterile glycerol and stored at -20ºC. Working stock culture were sub-cultured every two week and maintained on slant agar for period of 3 to 5 months.

3.20 Purification of ABA392/pUC57 using NucleoSpin® Plasmid

A single grown colony from LB agar with ampicillin 100µg (Section 3.5.3) of ABA392/pUC57 was grown in LB broth (Section 3.5.1) which contain ampicillin with final concentration of (100µg) at overnight in 37ºC shaking incubator. Before proceeding with purification step, 600µl was aliquot in different microcentrifuge tube for glycerol stock preparation as mentioned in Section 3.19. 1 to 5ml of overnight saturated LB culture was harvested in a standard microcentrifuge for 1 minute at 11,000 x g. The supernatant was discarded and liquid was removed as much as possible. To

lysis the cell, the harvested cell was suspended in 250µl buffer A1 completely by pipetting up and down until there were no clumps remained. 250µl buffer A2 was added and mixed gently by inverting the tube 6 to 8 times and incubated at room temperature to 5 min or until the lysate appears clear. After 5 minutes, 350µl buffer A3 was added and mixed thoroughly up to 6 to 8 times and centrifuge for 10 minutes at 11,000 x g. Meanwhile, a nucleospin plasmid column was placed in a collection tube (2ml) to bind the DNA. Once centrifugation was completed, the 750µl of the supernatant was pipette onto the column and centrifuge for 1 min at 11, 000 x g. The flow through was discarded and the nucleospin plasmid column was placed back into the collection tube. To wash the cells, buffer AW was preheated to 50ºC and 500µl was added into the column and centrifuge for 1 min at 11,000 x g and followed with washing step with buffer A4. 600µl of buffer A4 was added and centrifuged for 1 min. The flow through was discard and nucleospin plasmid column was placed back into the empty collection tube. To dry the silica membrane, the column was centrifuge again for 2 min at 11,000 x g and the collection tube was discarded. To elute the DNA, the nucleospin plasmid column was placed on clean 1.5ml microcentrifuge tube and 50µl buffer AE was added and allowed to incubate at room temperature for 1 minute and centrifuge for 1 min at 11,000 x g. All the purified DNA sample was stored at -20ºC to prior use.

3.21 Isolation and Purification of pET-30a

A single grown colony of pET-30a from LB agar with kanamycin 30µg (Section 3.5.4) was grown in LB broth (Section 3.5.1) which contain kanamycin with final concentration of 30µg at overnight in 37ºC shaking incubator. Since pET-30a is a low copy plasmid, 1 up to 10mL of overnight saturated LB culture was harvested. Before proceeding with purification step, 600µl is aliquot in different microcentrifuge tube for glycerol stock preparation as Section 3.19 and followed by purification step as Section 3.20. All the purified DNA was stored at -20ºC to prior use.

3.22 Agarose Gel Electrophoresis for Purify ABA392/pUC57 and pET30a

The purify ABA392/pUC57 and pET-30a were analyzed by 1% gel electrophoresis which prepared as section 3.7.2. 4µl of purified DNA sample was mixed with 1µl loading dye and pipetted into the gel wells. 5µl of supercoiled ladder was mixed up with 1µl loading dye was used as the size reference. The gel was run for at 70V for 60 to 75 minutes. The detection of the band was made by viewing the gel under UV light.

3.23 Restriction Endonuclease Digestion of ABA392 Gene and pET-30a

Restriction endonuclease digestion was carried out to obtain the insert (ABA392) and vector (pET-30a) and to re-confirm the fragment size. The insert was prepared by treating the ABA392 with restriction digestion enzyme *Hin*dIII-HF and *Bam*HI-HF. The ABA392 gene was digested using the ingredients and condition as below (Table 3.1). The same protocol was applied for pET-30a:

Table 3.1: Restriction Enzyme (RE) digestion profile of ABA392 gene and pET-30a vector

Solutions	Volume (µl)
Cut Smart® Buffer	2.5µl
*Hin*dIII-HF (Restriction enzyme)	1.0µl
*Bam*HI-HF (Restriction enzyme)	1.0µl
DNA	20.5µl
Total	25.0µl

The solution was mixed and was incubated at 37ºC for 2 hours. The digestion was confirmed by agarose gel electrophoresis to identify the size of the ABA392 gene and pET-30a vector.

3.24 Gel Extraction of ABA392 gene and pET-30a Vector using NucleoSpin® Gel and PCR Clean Up

The DNA fragment with the band size was excised from the agarose using a clean, sharp scalpel under long wave UV light for short time. Appropriate UV precautions were taken. The gel slice was weighed in a clean microcentrifuge tube. Each 100mg of agarose gel <2%, 200µl buffer NTI was added. The tube was incubated at 50ºC for 5 to 10 minutes (until the gel slice completely dissolved) and sample was vortex briefly every 2-3 minutes during the incubation to dissolve the gel. To bind the DNA, a nucleospin gel and PCR Clean-up Column was placed in collection tube (2mL) and all dissolved sample was loaded. The tube was centrifuged for 1 min at 11, 000 x g. The flow through was discarded and the column was placed back into the collection tube. To wash the silica membrane, 700µl buffer NT3 was added into the nucleospin gel and PCR Clean-up Column and centrifuge for 1 min at 11,000 x g. The flow through was discarded and the column was placed back into the collection tube. This washing step was repeated again to minimize chatropic salt carry over. The column was centrifuged for 1 min at 11, 000 x g to remove buffer NT3. To elute the DNA, the nucleospin gel and PCR Clean-up Column was placed on microcentrifuge tube. 30µl buffer NE was added and incubated for 1 min at room temperature. The column was centrifuge for 1 min at 11, 000 x g. The extracted gel was analyzed by 1% gel electrophoresis which was prepared as section 3.7.2. 4µl of purified DNA sample was mixed with 1µl loading dye and pipetted into the gel wells. 3µl of DNA ladder was mixed up with 1µl loading dye was used as the size reference. The gel was run for at 70V for 60 to 75 minutes. The detection of the band was made by viewing the gel under UV light.

3.25 Ligation by NEB T4 DNA Ligase Reaction Buffer

The ligation of digested insert (ABA392) and vector pET-30a was carried out using T4 ligase under standard condition (Sambrook *et al*., 1989). The ligation mixture ingredients and conditions were as follow (Table 3.2):

Table 3.2: Ligation Profile of ABA392 gene and pET-30a Vector

Solutions	**Volume (µl)**
Cut Smart 10x T4 DNA ligase Buffer mart® Buffer	2µl
Vector (digested pET-30a with *Hin*dIII-HF and *Bam*HI-HF)	4 µl
Insert (digested ABA392 with *Hin*dIII-HF and *Bam*HI-HF)	13µl
T4 DNA ligase	1µl
Total	20µl

The solutions were mixed and incubated 16 hours at 16ºC before being transformed into *E. coli* as described in Section 3.26.2

3.26 Transformation of Plasmid DNA into Host Cell

3.26.1 Preparation of Competent Cells

Stock culture of *E. coli* BL21 (DE3) pLysS was streaked out on Luria-Bertani (LB) agar with chloramphenicol plate (Section 3.5.5) and was incubated at 37ºC overnight. A single colony was then inoculated into 10ml LB broth with chloramphenicol added and incubated overnight. 1ml of the overnight culture was then added into 50ml prewarmed LB flask and was shaken at 220 rpm for 2 to 3 hours until the OD_{600} reach 0.5 at 600nm. The culture was then cooled on ice for 10 minutes and centrifuged at 3500 rpm at 4ºC for 1 hour. The supernatant was discarded and the pellet was resuspended in 5ml chilled 0.1M transformation buffer (Section 3.9.2) and chilled on ice for 20 minutes. The competent of the cells was found to be its maximum when kept overnight.

3.26.2 Transformation of ABA392/pET-30a into *E. coli* BL21 D3 pLysS

5µl of the ligation reaction from Section 3.25 was transformed into 100µl of *E. coli* BL21 (DE3) pLysS competent cells in the 1.5ml microcentrifuge tube. The tube with the mixture was placed on the ice for 30 minutes and followed by placing it on the heating block at 42º for 2 minutes. The tube then chilled on ice for 5 minutes. 0.9ml of LB broth was added into the mixture and mixed well. The mixture was incubates at 37ºC in a shaking incubator for 90 minutes. After the incubation, the tube was centrifuged for 1 min at 13,000 x g and the supernatant was discarded. 50µl LB broth was added and mixed well with the remaining pellet and spread on LB agar with kanamycin/chloramphenicol plate (Section 3.5.6) for screening. Only transformed cultures survive on this plate as they contain the kanamycin/chloramphenicol resistance gene.

The transformed *E. coli* BL21 (DE3) pLysS were incubated at 37ºC overnight. The transformed colonies were analyzed by using direct colony PCR as described in Section 3.27. Positive colonies (colonies carrying the vector) were grown up in kanamycin/chloramphenicol broth to amplify the construct and for glycerol storage (Section 3.1).

3.27 Colony Library and PCR Colony Screening

In this PCR colony screening, the colonies were screened based on the resistance towards kanamycin/chloramphenicol. Before proceeding with PCR colony screening, grids were drawn in a new LB agar with kanamycin/chloramphenicol plate. Each colony from the overnight transformation plate were picked using clean and sterilized toothpick and touched with the specific grids of the library plate. The plate was incubated overnight at 37º C. PCR was performed directly without DNA purification. Colony was

picked from each grid using inoculate loop from overnight plate to detect the positive colonies that carrying the insert.

Isolated colonies were picked and mixed by pipetting with the PCR solution as in the table 3.3:

Table 3.3: PCR mastermix

Solutions	25µl Reaction	Final concentration
Econotaq Plus Green 2x mastermix (Section 3.10.1)	12.5µl	1X
Forward primer (Section 3.10.2)	0.25µl	(pmol/µl) (1µm)
Reverse primer (Section 3.10.2)	0.25µl	(pmol/µl) (1µm)
DNA Template	1.0µl	
Water nuclease free	11.0µl	

All constituents of the mixture were placed in the thin wall of PCR tube and run using PCR machine. Hot-start was performed by heating the tube at 95ºC for two minutes, 30 cycles of 95ºC for 0.30 seconds, Tm (see Table 3.4) for 0.30 seconds, 72ºC for 0.30 seconds and 72ºC for 10 minutes before cooling down to 4ºC. All the positive colonies which amplified via colony PCR were analysed through 0.7% agarose gel electrophoresis (Section 3.7.1)

Table 3.4: PCR profile

PCR	Temperature
95ºC	2 minutes
95ºC	0.30 seconds
64ºC	0.30 seconds
72ºC	0.30 seconds
72ºC	10 minutes

Positive colonies (colonies carrying the vector and gene) were grown up in kanamycin broth to amplify the construct and for storage as glycerol stock for future.

3.28 Plasmid Analysis

The overnight culture was extracted as mentioned in the (Section 3.20). In order to confirm the presence of the insert of the expression clone, the plasmid DNA were subjected to restriction endonuclease digestion as described in the section (Section 3.23) using the same enzyme, *Hin*dIII-HF and *Bam*HI-HF. Both extracted plasmid and digested sample were subject to electrophoresis using 1% agarose gel (Section 3.7.2).

3.29 DNA sequencing and Basic Local Alignment Search Tool (BLAST)

The purified plasmid was sent for sequencing at MyTaccg Bioscience Enterprise as the confirmation ABA392 in pET-30a protein expression system. The sequencing service took approximately three days to obtain the chromatogram raw data with the nucleotide sequences. The obtained chromatogram raw data was analyzed through sequence scanner software known as BIOEDIT in order to retrieve the nucleotide sequence.

3.30 Blast Search

The nucleotide sequences obtained from the BIOEDIT were confirmed using BLAST search from NCBI website (www.ncbi.nih.gov).

3.31 ABA392/pET-30a DNA Stability Test

Before induction, a single positive colony of ABA392/pET-30a was cultured in 10ml broth containing kanamycin/chloramphenicol for overnight. The overnight culture was transferred to new 10ml LB broth with vigorous shaking until it reached an OD_{600} of 0.6. Serial dilution was conducted on the cell suspension from 10^1 to10^6. Cells at dilution 10^5 were plated on LB with IPTG agar (Section 3.5.7) and LB plate/ kanamycin/chloramphenicol/ IPTG agar (Section 3.5.8).

Cells at dilution 10^6 was plated LB agar plate (Section 3.5.2) and LB with kanamycin/chloramphenicol (3.5.6). Plates were than incubated overnight at 37ºC. The numbers of colonies in each plate were counted on next day.

3.32 Protein Analysis

3.32.1 Protein Induction

Colony from positive clone (ABA392/pET-30a) in LB agar with kanamycin/chloramphenicol plates (Section 3.27) was inoculated in to a 10 ml of LB broth containing kanamycin/chloramphenicol and incubated at 37ºC overnight. The 10ml overnight culture was then inoculated into 50ml pre-warmed broth with vigorous shaking until it reached an OD_{600} of 0.6 in about 60 to 120 minutes. 1ml sample was immediately taken before induction, this sample served as the non-induced control, the cells were centrifuged at 5000 rpm for 5 minutes and supernatant were removed as much as can. The cells were than resuspended with 50µl 1X PBS (Section 3.11.2) and 50ul 2x sample buffer (Section 3.13.8) and heated for 10 minutes at 95ºC on heating block. The non-induced control was frozen and stored at -20ºC until SDS-PAGE was performed. Expression was induced by adding IPTG (Section 3.11.1) to a final concentration of 0.1mM. The culture was then incubated further for an additional 4-5 hours at 37ºC. following the incubation, 1ml sample was taken, the cells were centrifuged at 5000 rpm for 5 minutes and supernatant were removed as much as can. The cells were than resuspended with 50µl PBS and 50µl 2x sample buffer and heated for 10 minutes at 95ºC on thermal block. The induced control was frozen and stored at -20ºC until SDS-PAGE was performed. The remaining induced cells were harvested by centrifugation at 3500 x g 4ºC for 60 minutes and then the cell pellets were frozen and stored overnight at -20ºC. 2µL of lysozyme (Section 3.11.4) and 2µL of DNAase 1 (Section 3.11.4) is added to 1mL of B-PER reagent (Section 3.11.3). The overnight induced frozen pellets were thawed on ice for 15 minutes. The pellets were weighed and

4ml of B-PER® reagent were added per gram of cell pellet. The suspension was pipetted up and down until it is homogenous and incubated 10-15 minutes at room temperature. The lysate was centrifuge at 3500 rpm for 30 minutes to separate soluble proteins from the insoluble proteins (Noor Masyitah Jumahat *et al*., 2015). The cell lysate supernatant was retained and the supernatant contains the soluble fraction of the recombinant protein. 5µl of the supernatant (cell lysate) is aliquot and added with 5µL 2x SDS-PAGE sample buffer and stored at -20ºC for SDS-PAGE analysis (Section 3.32.3).

3.32.2 Purification of 6xHis-tagged Proteins

350µl of Cell lysate that containing histidine-tagged protein was mixed with 350µl of 1X binding/wash buffer (Section 3.12.1) in total volume of 700µl. Dynabeads (Section 3.12.1) was thoroughly resuspended in the vial by vortex it for 30 seconds. 50µl (2mg) of Dynabeads was transferred into a 1.5ml microcentrifuge tube and placed on a magnet for 2 minutes. The supernatant was aspirated and discarded. The total 700µl of cell lysate which mixed with 1x binding/wash buffer was added to the beads and mixed well. It was then incubate on a roller for 5 minutes at room temperature. The tube was then placed on the magnet for 2 minutes and the supernatant was discarded. The beads were washed for 4 times with 300µl binding/wash buffer by placing the tube on a magnet for 2 minutes and supernatant was discarded. The beads were resuspended thoroughly between each washing step. On the washing step is done, 100µl of His-Elution buffer (Section 3.12.3) was added and incubated on a roller for 5 minutes at room temperature. The tube was applied on magnet for 2 minutes and the supernatant containing the eluted histidine-tagged protein was transferred to a clean tube. Each fraction including the elution fraction was collected in a separate 1.5ml microcentrifuge tube. 5µl of each fraction was added with 5µl 2X sample buffer and heat at 95ºC for 5 minutes before sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Section 3.32.3) was about to perform. The entire fraction was stored at -20ºC.

3.32.3 Protein Separation Technique by SDS-PAGE

Protein separation was conducted via sodium dodecyl sulfate polyacrylamide gel electrophoresis technique (SDS-PAGE) technique. SDS-PAGE separates proteins according to their respective molecular weight. The SDS-PAGE method is one dimensional gel electrophoresis. This method was based on Laemmli (1970). In this study, SDS-PAGE was performed using Mini-PROTEAN Electrophoresis units with a power supply. Gel was prepared according to below formulation (Table 3.5):

Table 3.5: SDS-PAGE gel formation

Formulation	Stacking gel (4%)	Resolving gel (12%)
Deionized H_2O	3.4ml	6.1ml
30% Acrylamide (Section 3.13.1)	4.0ml	1.3ml
1.5 Tris (pH 8.8) (Section 3.13.2)	2.5ml	-
0.5 Tris-HCl (pH 6.8) (Section 3.13.3)	-	2.5ml
10% SDS (Section 3.13.4)	0.1ml	0.1µl
Ammonium persulphate (APS) (Section 3.13.5)	50µl	50µl
TEMED (Section 3.13.6)	5µl	10µl

The protein sample was analyzed using SDS-PAGE. The apparatus for the SDS-PAGE were set by assembling one clean spacer glass plate and clean short plate. It was locked and secured by using green clamps. The prepared 12% resolving gel (Table 3.5) was poured into the glass plates using a micropipette along the edge one of the spacers to a height of 1cm below glass margin. The separating gel was covered with distilled water and the gel was left to polymerize for 30 to 60 minutes at room temperature. Once the gel polymerized, the distilled water was removed. Once all the distilled water was drained out 4% stacking gel (Table 3.5), solution were prepared and poured slowly using a micropipette along the edge of the spacer to the top of the sandwich glass plate. A 1.0mm teflon comb was inserted into layer of stacking gel solution and it was left to polymerize at room temperature for 30 to 45 minutes. On a clean flat surface, the clamping frame on the electrode assembly is opened. The electrode assembly (with the

banana plugs) is use when running only 1-2 gels. The first gel cassette (short plate facing inward) molded into the bottom of the clamping frame assembly surface. The second gel is placed on the other side of the clamping frame again by resting the gel onto the supports. The gel was pulled towards each other gently in order to make the gel rest firmly and squarely against the green gaskets in the clamping frame. The gel cassette was squeezed against the green caskets, the green arms of the clamping was slide over the gels and locked them into the place. The gel cassette was placed on the tank. The comb was removed from the stacking gel and wells were filled with running buffer (Section 3.13.7) for loading samples. The samples were mixed with sample buffer (Section 3.13.8) in ratio 1: 1. Samples mixture were incubated at 95ºC for 5 minutes and loaded into the respective wells including protein ladder (Section 3.13.9). Running buffer was poured into the gel tank and the gel tank was connected to the power supply. The electrophoresis was performed using power supply at 120 volts for 75 to 85 minutes. The power supply was turned off when the dye front reaches approximately 1 cm from the bottom of the gel. After electrophoresis, the gels were stained using colloidal coomassie staining method. The gel were placed on 100ml of colloidal coomassie solution (Section 3.13.10) in a plastic container then placed on a shaker for overnight. Once the gel stained for overnight, the de-staining process was carried out to wash out the excess dye. The coomassie blue staining was poured out and 100 ml of de-staining solution (Section 3.13.11) was added and placed on the shaker for about 30 minutes. The gel was subject to de-staining process few times until a clear background was obtained.

3.32.4 Protein Quantification

The Bio-Rad DC protein assay (Section 3.13.12) is a colorimetric assay for protein concentration following detergent solubilisation. This reaction was adapted Lowry Assay (1951) with slight modification. This assay was based on the reaction of protein

with alkaline copper tartrate solution and folin reagent. 20µl of reagent S was added to reagent A which will be needed for the run. Dilution of protein standard containing from 0.2mg/ml to 1.4mg/ml (Table 3.6) was prepared from 10mg/ml BSA solution (Section 3.13.12.1). 5µl of standards and samples were pipetted into a clean, dry microtiter plate. 25µl of reagent A was added into each well followed by 200µl of reagent B into the each well. The microtiter plate was gently agitated to mix the reagents. The absorbance was reads at 650nm 15 minutes later.

Table 3.6: Preparing of BSA Standard Curve from BSA Concentration 10mg/ml

Tube Number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Protein concentration (mg)	0	0.2	0.4	0.6	0.8	1	1.2	1.4
Volume of 10mg/ml BSA (ml)	0	0.02	0.04	0.06	0.08	0.1	0.12	0.14
Sterile Mili-Q Water (ml)	1	0.98	0.96	0.94	0.92	0.9	0.88	0.86

3.33 Western Blot Analysis

Eluted-His Tag protein and marker were run SDS- Gel as described Section 3.32.3. Four piece of filter paper and a piece of membrane (0.45µm nitrocellulose) was cut to the same size as the gel. The SDS-PAGE gel with eluted His-tag protein and protein marker was placed on blotting buffer (Section 3.14.1) for 15 minutes. The membrane was incubated in blotting buffer for 10 minutes. The filter paper and membrane was soaked in blotting buffer. 2 sheets of filter paper was placed on the fiber pad followed by the gel, membrane, 2 sheets of filter paper and finally the other fiber pad without trapping any air bubbles. Once the transfer sandwich was assembled, the blot was placed on the cathode and the gel placed on the anode. The cassette was placed on transfer tank filled with blotting buffer and ice block is placed on the tank. Blotting

transfer is done at 100V for 1 hour. The blot was soaked in 5% blocking buffer (Section 3.14.2) at 4ºC overnight. Ponceau S staining can be done in order to check the transfer quality but it is an optional step. After overnight incubation, the blot was washed with TBST (Section 3.14.4) 3 times under shaking condition for 10 minutes at room temperature each time. Primary antibody was prepared by mixing 5ml of 2.5% skimmed milk (Section 3.14.5) with 1µl of Penta Anti-His antibody (Section 3.14.7) under 1:5000 dilution. The blot was soaked in primary antibody for 1 hour at room temperature under shaking condition. Once the incubation period was over, the washing step was repeated as described previously. Secondary antibody was prepared by 5ml of 2.5% skimmed milk with 1µl anti mouse IgG, AP-linked antibody (Section 3.14.7) under 1:5000 dilution The blot was soaked in secondary antibody for 1 hour at room temperature under shaking condition. Once the incubation period was over, the washing step was repeated as described previously. Finally, the blot was soaked with 1ml of 5-Bromo-4-Chloro-3-indolyl phosphate (Section 3.14.6) under dark condition. The membrane was dried and visible band was photographed.

3.34 Animal Immunogenicity Test

3.34.1 Bacterial Suspension

The preparation of bacterial suspension for vaccine was adapted with slight modification (Hussaini *et al*., 2012). A single pure bacterium was grown overnight at 37ºC in 10 ml of BHI broth medium. The cultures were then centrifuged for 30 minutes at 3500 rpm to pellet the cells. The pelleted cells were washed three times with sterile PBS to remove all traces of the growth medium. The cells were resuspended in 5ml of PBS pH 7.2 to achieve the concentration of 10^7 colony forming units CFU. Formalin concentration 0.2% was added. The cell suspensions were left to stand for overnight and centrifuged at 3500 rpm for 30 minutes, the supernatant replaced by an equal volume of fresh 0.2% formalinized PBS. About 1ml of the inoculum was spread onto

blood agar and BHI agar as confirmation that the bacteria have been killed. The bacteria suspension was then allocated into 1.5ml microcentrifuge tube and stored at -20ºC prior to use. This formalin killed vaccine serve as positive control.

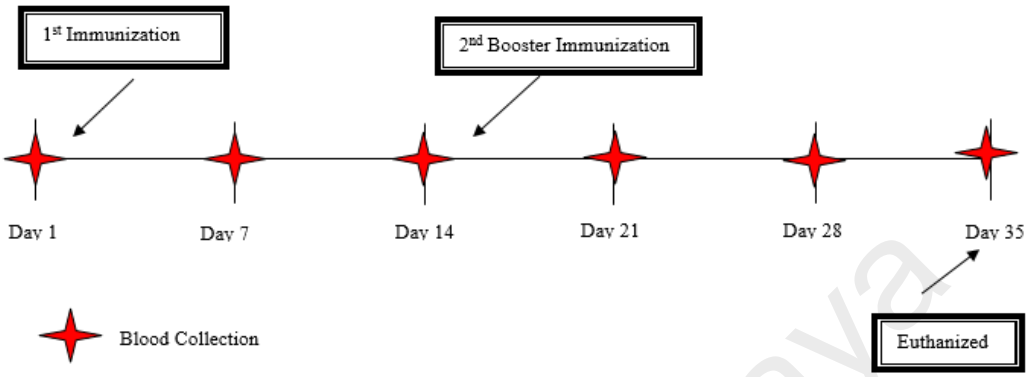
3.34.2 Experimental Animals

Twelve white rats, Sprague Dawley of female rats weighing 150g-200g were used for this part of research. Rats were purchased from Animal Experimental Unit (AEU), Faculty of Medicine, and University Malaya. The animals were housed in individual clean cage at the experimental room, Animal House, University Malaya. All the rats were feed a commercial food along with water. Rats were kept under observation for two weeks before research were conducted. This research was approved by Institutional Animal Care Use Committee (IACUC), University Malaya prior to the research under ethic number of ISB/18/11/2015/RDVM (R).

3.34.3 Immunization of the Rats

Rats were divided causally into 4 different groups where 3 rats in each group. All the rats were inoculated subcutaneously with the proper amount according to the body weight (Diehl, 2001). To determine the immunogenicity of purified protein from ABA392/pET-30a, group 1 was given purified protein of ABA392/pET-30a (50µg) and group 2 rats were treated with ABA392/pET-30a (100µg). Group 3 serve as positive control which given formalin killed vaccine meanwhile group 4 was the negative control where the rats in this group were given sterile PBS. The vaccination was carried out in two dose schedule. In this two dose schedule the rats were immunized on day zero and followed by 2nd booster were given two weeks later via subcutaneously route. Blood was collected on day 1st, 7th, 14th, 21st, 28th and 35th. At the end of the immunization, rats were sacrificed by overdose anaesthesia following the animal ethic

requirement. Serum were separated by centrifuging at 13,000 x g for 20 minutes, pooled and stored at -20ºC until it was used.



Group 1	Purify ABA392/pET-30a (50µg)
Group 2	Purify ABA392/pET-30a (100µg)
Group 3	Positive control- Formalin Killed Vaccine
Group 4	Negative control-Sterilized PBS

Figure 3.1: Timeline of animal immunization studies

3.34.4 Haematological and Biochemical Studies

For haematological studies, the blood was collected via tail and on day 35th was collected via cardiac puncture. The rats were bled on day 0, 7th, 14th, 21st, 28th and 35th (Figure 3.1). Total white blood cell (WBC) was performed as observation tools. 10µl of fresh blood was added to 190µl of Turks solution (1: 20 dilution) mixed and then loaded into a haemocytometer and the total white blood cells were counted under microscope using cell counter (Oli *et al.,* 2016). The cells were counter under microscope using differential cell counter. For biochemical analysis, blood were collected via cardiac puncture and serum separated. Serums were then send to Division of Laboratory Medicine, PPUM for liver function test (LFT) and renal function test (RFT). Results were obtained within 2 days.

3.34.5 Organ Collection

The different needed organs of the animals were collected upon dissection. Autopsy was performed on the rats and organs after the dissection. All the collected organs were placed on 10% NBF (Section 3.15.4) in a clean collection container in order to preserve for histopathological analysis.

3.34.6 Histopathological Analysis

All the organs were trimmed before they were processed. All the tissue samples were processed manually. After the overnight fixation process, the fixative solution is discarded in an appropriate waste bottle. The tissues were immersed through a series of ethanol with ascending concentration (70% >85% >95%> 100%) for 30 minutes in order to remove all the water that remained in the tissue. The tissues were immersed in 100% ethanol for 45 minutes twice. These whole processes were done in room temperature. Once the dehydration process is done, the tissues were immersed in xylene for 3 hours. Once the incubation period over, the small bottles with tissue were filled with melted from tissue embedding centre and kept at in an oven at 60ºC for 2 hours. After 2 hours, the paraffin is changed and the tissue is kept at in an oven at 60ºC for overnight. The steps involved in the processing of the tissues are shown in the Figure 3.2. Once the tissue was being processed, the tissues were embedded into paraffin blocks using tissue embedding machine (Histo Embedder Leica, Japan). The paraffin blocks were kept at room temperature for overnight to form as solid blocks. The solid blocks were trimmed before can be sectioned. The trimmed paraffin tissue blocks were section at 6µm using Leica RM2035 microtome and a sharp microtome blade for best result. The cut section of tissue were gently placed on a clean glass slide that contains a smear of Meyer's albumin (Section 3.15.5) and water in order to adhere the section tissues. It was make sure there is no air bubbles were not trapped under the paraffinized tissue section. The slides the placed on a slide warmer with 42ºC temperature for 15 to

20 minutes before placing the slides into oven for 24 to 36 hours. This will help the section adhere completely and melt the wax.

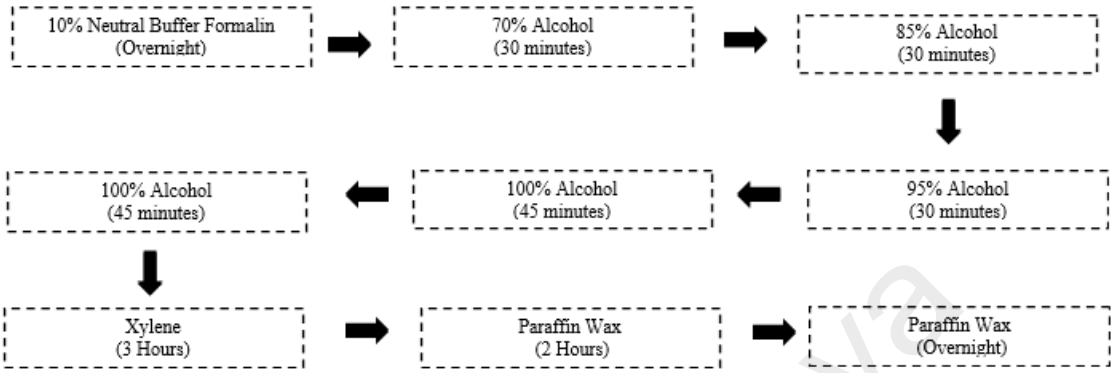


Figure 3.2: Serial steps involved during tissue processing

3.34.7 Histopathology Staining

Histopathology staining that were used in this research is Harris Haematoxylin (Section 3.15.6.1) and Eosin Y (Section 3.15.6.2) staining (H&E). The initial step of the staining is started by de-paraffin the slides followed by hydration to remove the excess of xylene. Slides were washed with distilled water before staining with haematoxylin. The washing step is repeated again before the slides were stained with Eosin. Subsequently after staining the slides, the slides were dehydrated. Finally the clearing process by passing through xylene is done followed by mounting. The H&E steps followed as Figure 3.3.

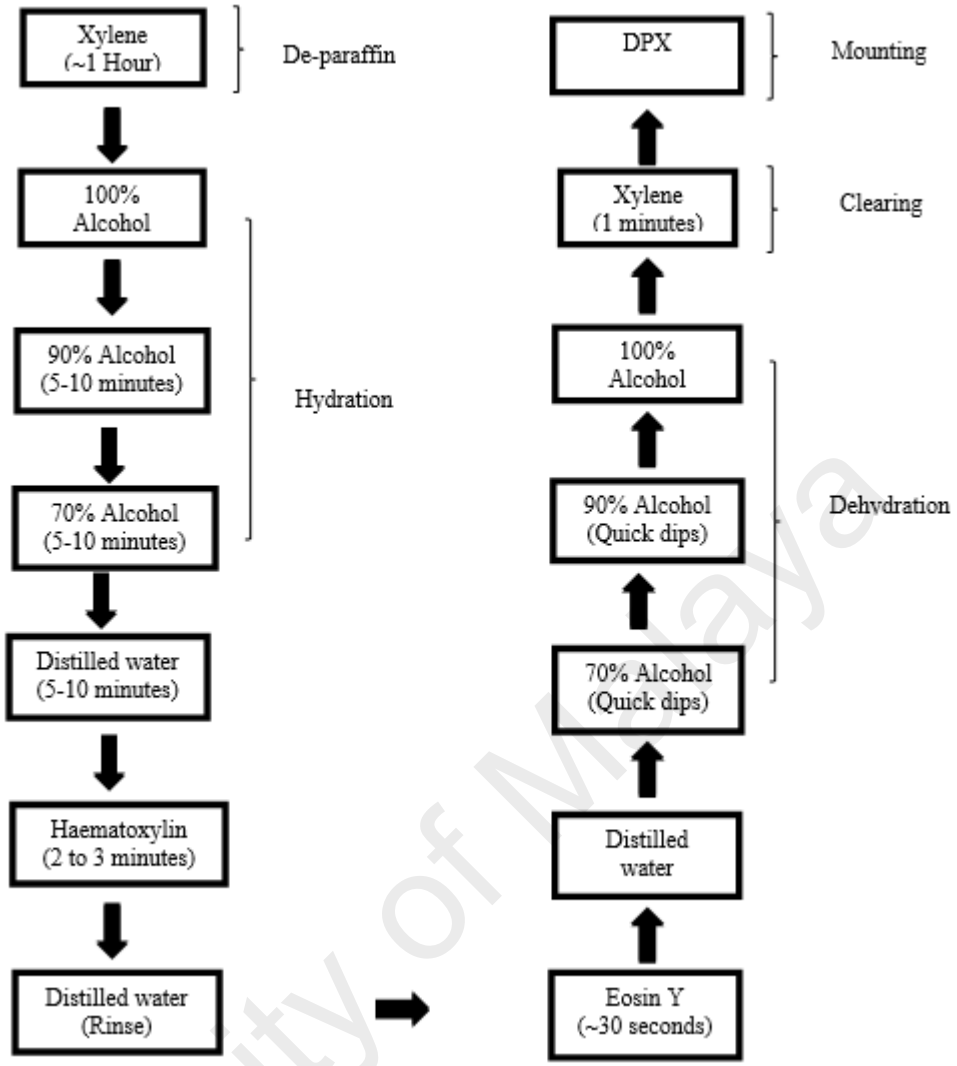


Figure 3.3: Subsequential step involved in H&E Staining

3.35 Indirect Enzyme Linked Immunosorbent Assay (ELISA)

ELISA was developed based on the procedure by Okay *et al*., (2012) with slight modification. ELISA was performed on the hyper-immune sera obtained from immunized rat for the presence of antibody. The optimization of the antibody for IgG was optimize and determined at the ratio of 1: 2,500. ELISA plate, 96-well plate with flat bottom was coated with 50µL/well of coating antigen (Section 3.16.2) and incubated at 4ºC overnight. After overnight the incubation period, the coating antigen was discarded gently and washed with 50µL/well sterile washing buffer-PBS-Tween 20, pH 7.4 (Section 3.16.3) for 2 times. Once the washing step done, 200µL/well of sterile blocking buffer-PBS-Tween 20-BSA, pH 7.4 (Section 3.16.4) was added and incubated

at 37ºC for 1 hour. Serum dilution was diluted in ratio 1:50 and was prepared during the incubation period. After the incubation time over, the blocking buffer was discarded gently and washing step was repeated as previously. About 50µL/well of serum (primary antibody) was added and the plate was incubated at 37ºC for 1 hour. After incubation period, the serum was discarded gently and washing step repeat as previously. 50µL/well of IgG antibody conjugate (Section 3.16.7) was added and incubated for 1 hour at 37ºC. Antibody conjugate was discarded gently and washing step repeated as previously. 100µL/well of tetramethylbenzidine (TMB) substrate (Section 3.16.5) was added and incubated at 37ºC for 15 minutes. As the last step and to stop the reaction, 50µL/well of stopping buffer (Section 3.16.6) was added. Finally the absorbance was read at 450nm on a microtiter plate reader. All standards, samples, positive and negative controls were used in triplicates.

3.36 Statistical Analysis

Data were analyzed by SPSS version 20 (2011) using Independent T-Test (Salmah Ismail *et al*., 2012 and Singh *et al*., 2011) and Microsoft Excel throughout this study.

CHAPTER 4: RESULT

4.1 Purification and RE Digestion of ABA392/pUC57

Figure 4.1 (a) shows purified ABA392/pUC57 where the size shows ~3.5 kb in lane 2 and 3. The appearance of nicked/relaxed circular plasmid can be seen in white box. Meanwhile figure 4.1 (b) shows the digested ABA392 from puc57 using *Hin*dIII-HF and *Bam*HI-HF restriction enzymes in lane 2 and 3. The correct digested ABA392 result is 804 bp.

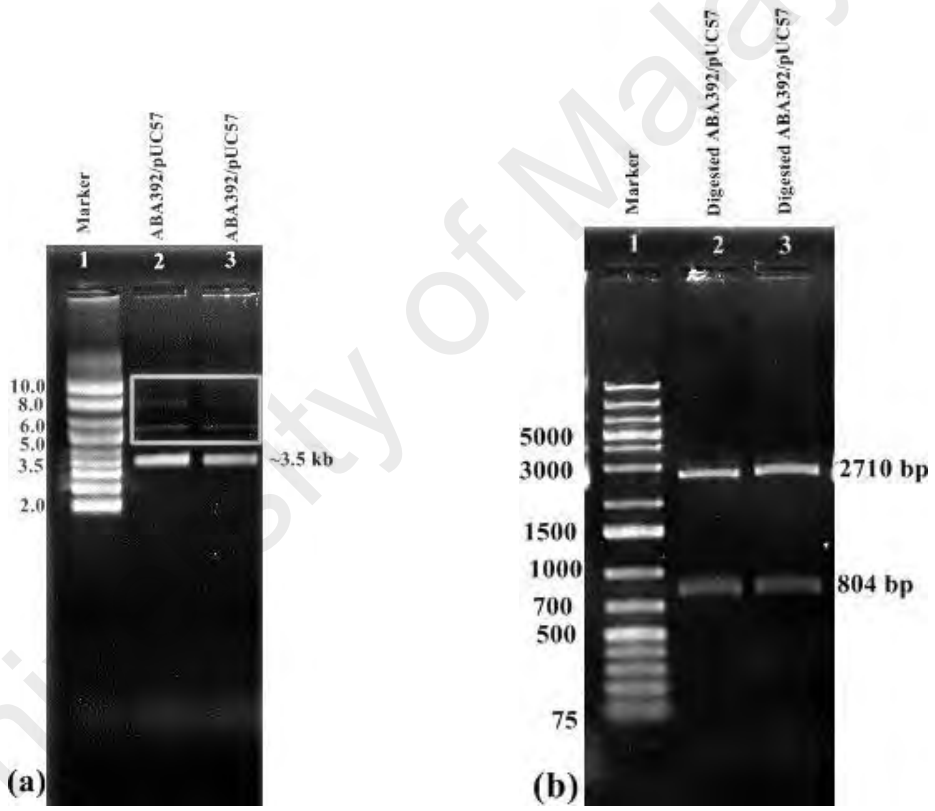


Figure 4.1: 1% Gel electrophoresis (a) shows purified ABA392 gene (b) shows digested ABA392 gene

Figure 4.1 (a) Lane 1, Invitrogen Supercoiled DNA ladder and lane 2 to 3, purified ABA392/pUC57. The purified ABA392/pUC57 size was determined approximately ~3.5 kb. Appearance of nickel/relaxed circular plasmid can been seen in white box. Figure 4.1 (b) Lane 1, GeneRuler™ 1 kb plus ladder and lane 2 to 3, digested ABA392gene with RE *Hin*dIII-HF and *Bam*HI-HF. The digested ABA392 gene size was at 804 bp.

4.2 Isolation and Digestion of Vector pET-30a

Figure 4.2 shows isolated and digested vector pET-30a. Lane 1 shows marker, lane 2 purified pET-30a vector and lane 4 and 5 show digested pET-30a vector using *Hin*dIII-HF and *Bam*HI-HF restriction enzyme at the correct size at 5422 bp. The appearance of nicked/relaxed circular plasmid can be seen in white box.

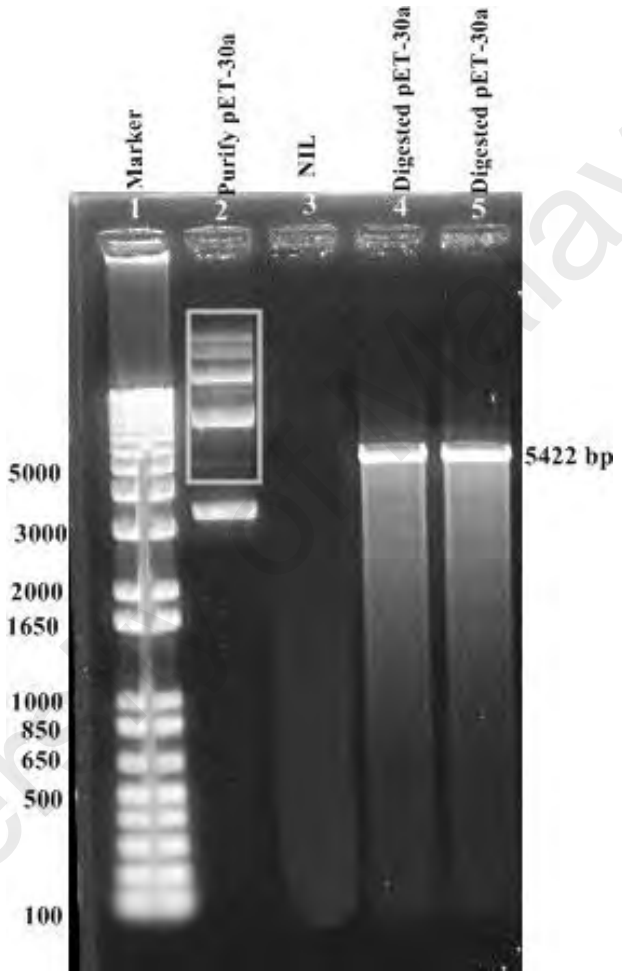


Figure 4.2: 1% Gel electrophoresis shows isolated and digested pET-30a

Lane 1, Invitrogen 1 kb plus DNA ladder; lane 2, purified pET-30a vector; lane 3, NIL; lane 4 to 5, digested pET-30a vector with RE *Hin*dIII-HF and *Bam*HI-HF. The purified and digested pET-30a vector size was determined at size of 5422 bp. There are appearance of nicked/relaxed circular plasmid that can be seen in white box.

4.3 Gel Extraction of Digested ABA392 Gene and pET-30a

Figure 4.3 shows gel extraction of digested ABA392gene and pET-30a vector. Lane 1 was loaded with marker, lane 2 and 3 show gel extraction of digested ABA392 gene at size of 804 bp meanwhile lane 4 and 5 show gel extraction of digested pET-30a vector at size of 5422 bp. This extracted gene and vector was ligated and transformed into *E. coli* BL21 (D3) pLysS.

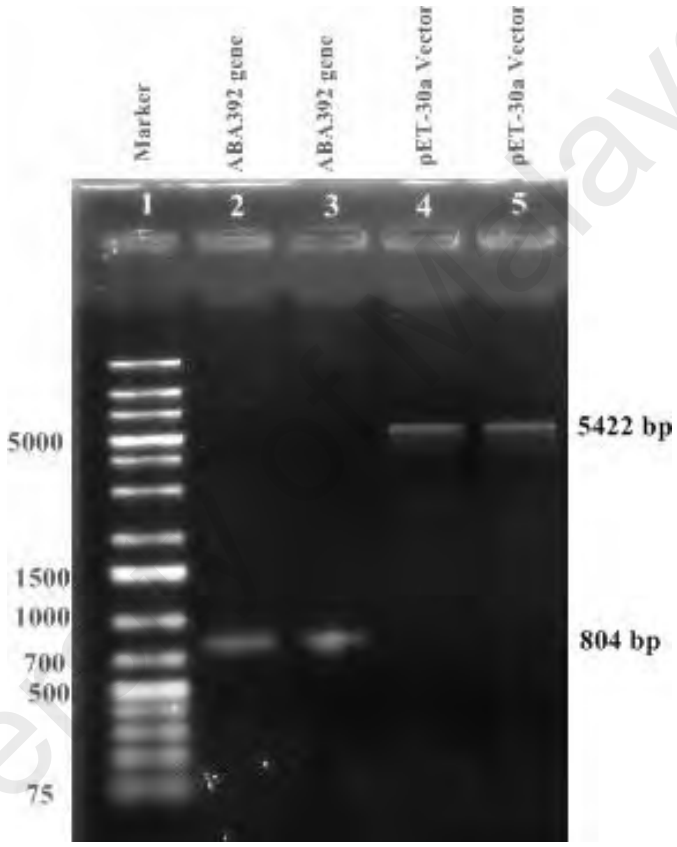


Figure 4.3: 1% Gel electrophoresis shows gel extraction of ABA392 gene and pET-30a vector

Lane 1, GeneRuler™ 1 kb plus ladder; lane 2 to 3, gel extraction of digested ABA392 gene and lane 4 to 5, gel extraction of digested pET-30a vector. The extracted of digested ABA392 gene and pET-30a vector shows size of 804 bp and 5422 bp respectively.

4.4 Cloning ABA392 Gene into pET-30a Expression Vector

The ABA392 gene and vector was double digested with the restriction enzyme *Hin*dIII-HF and *Bam*HI-HF. Figure 4.3 shows extracted digested ABA392 and pET-30 vector was ligated between *Hin*dIII-HF and *Bam*HI-HF restriction sites. The ligated vector was then transformed into *E. coli* BL21 (DE3) pLysS host to be expressed. Figure 4.4 (a) shows positive transformants of the colony had been grown in normal and concentrated LB agar with kanamycin 30µg and 34µg chloramphenicol and Figure 4.4 (b) shows negative control was done by just replacing the ligated product with a sterile distilled. Figure 4.4 (c) shows potential transformants were subsequently patch plated on fresh LB kanamycin/chloramphenicol and the positive clones were further screened using colony PCR and confirmed the presence of the insert.

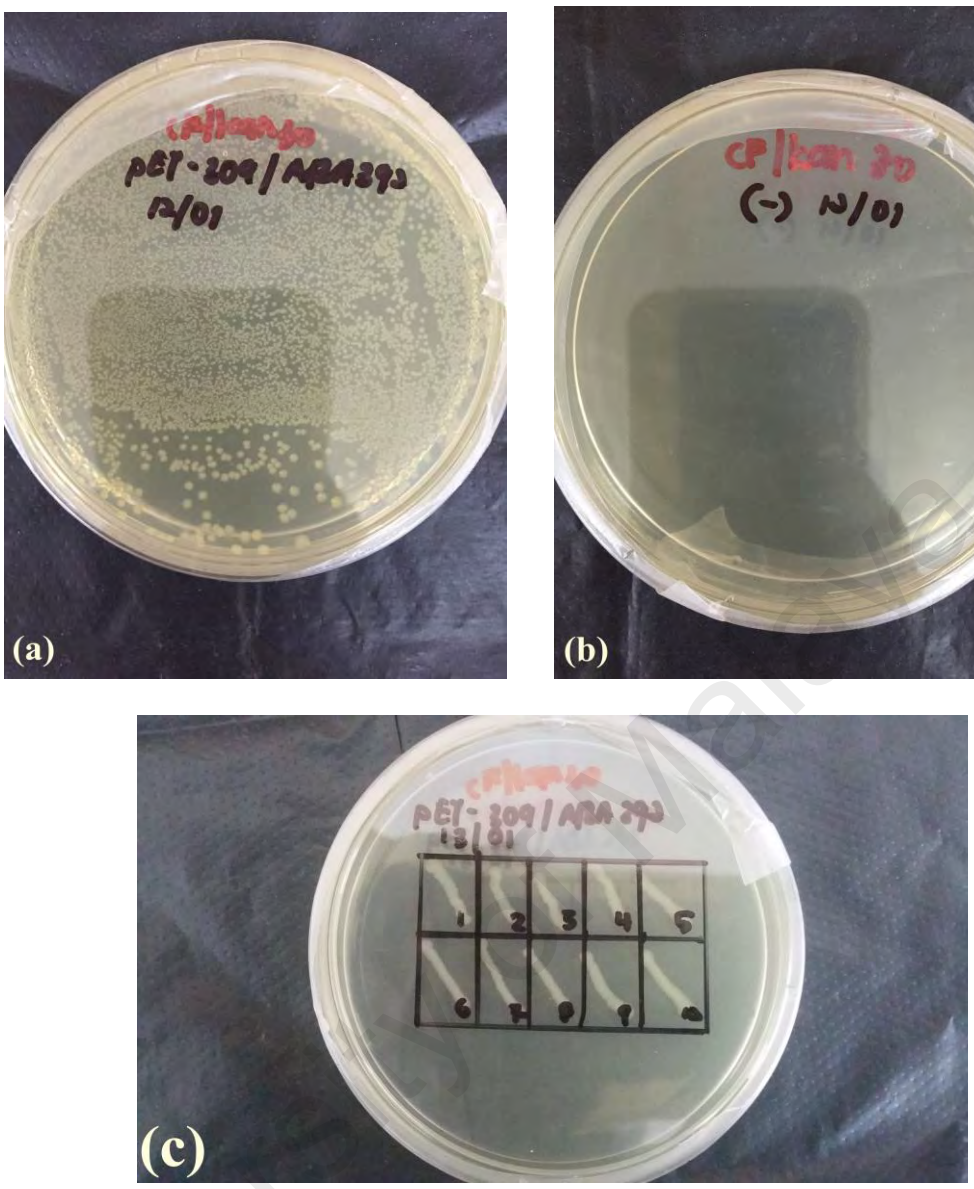


Figure 4.4: LB agar with kanamycin (30 µg) and chloramphenicol (34 µg) plate (a) shows positive tranformants of ABA392/pET-30a in *E. coli* BL21 pLysS (b) shows negative control (c) shows ABA392/pET30a colony library plate

4.5 Colony PCR Screening

The positive clones were further screened using colony PCR. The size of insert was determined through gel electrophoresis. Figure 4.5, lane 1 show marker, lane 3 to 10 shows the band with the size of around 804 bp from positive colony screening and no visible band in control lane 11

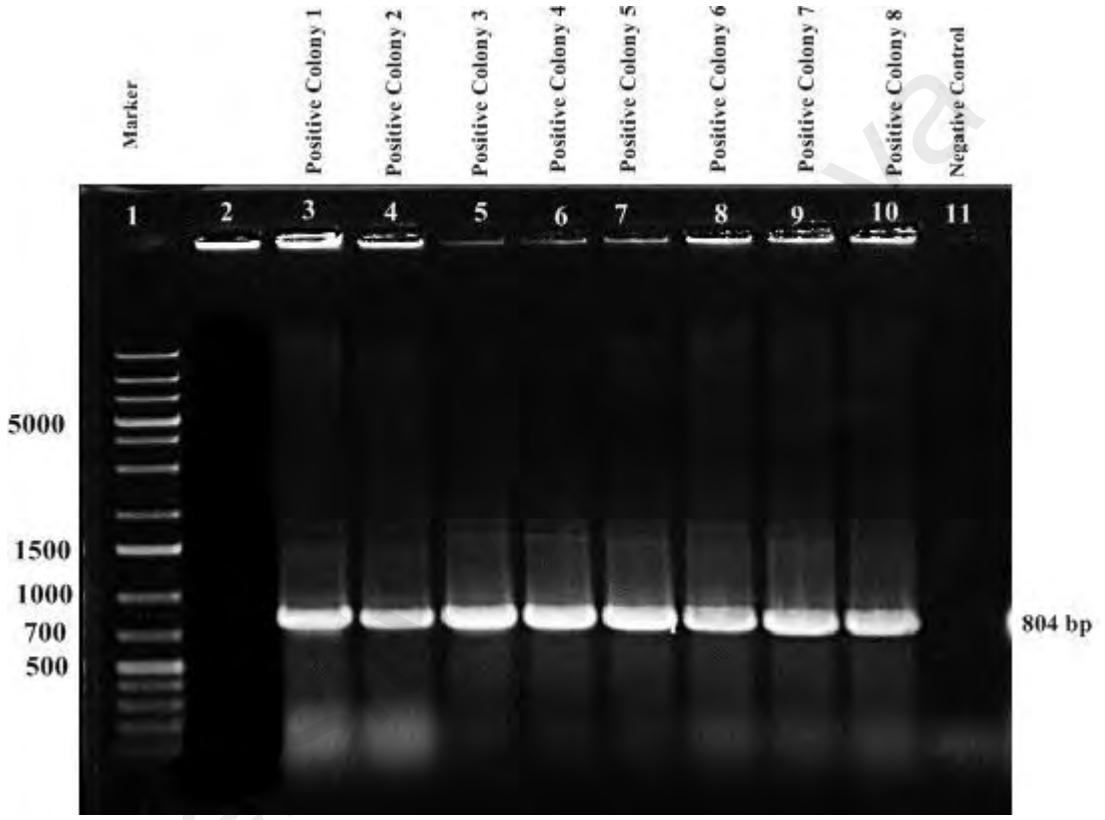


Figure 4.5: 0.7% Gel electrophoresis shows colony screening of ABA392/pET-30a through colony PCR

Lane 1, GeneRuler™ 1 kb plus ladder; lane 2, Nil; lane 3 to 10, PCR product positive colony which contain ABA392 gene and lane 11, negative control. PCR product show positive colony that contain ABA392 gene at size 804 bp..

4.6 Recombinant Plasmid Isolation

The plasmid was than extracted from the positive transformants. RE digestion was carried out as reconfirming the presence of the insert and sent for sequencing. Double digestion of the clone with the two RE, *Hin*dIII-HF and *Bam*HI-HF using cut smart buffer. This RE digestion generated the clone into two fragments, an insert and the vector. Figure 4.6 (a) shows ABA392/pET30a recombinant plasmid isolation, lane 1 is marker and lane 2 to 3 purified ABA392/pET-30a size of 6.2 kb. Figure 4.6 (b) shows the ABA392 gene (insert) was digested from pET-30a vector, lane 1 is marker and lane 2 to 3, digested ABA392/pET-30a. The correct digested ABA392 gene show the size of 804 bp meanwhile pET-30a vector size was 5422 bp. The appearance of nicked/relaxed circular plasmid is circled in white meanwhile circular/single stranded plasmid is circled in yellow box.

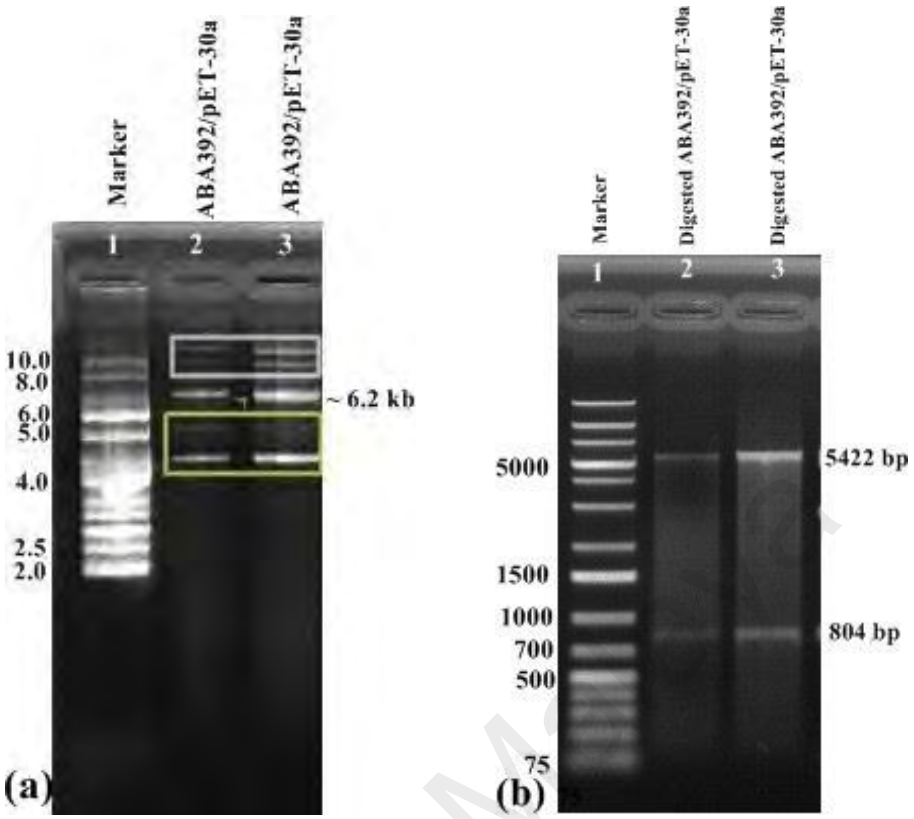
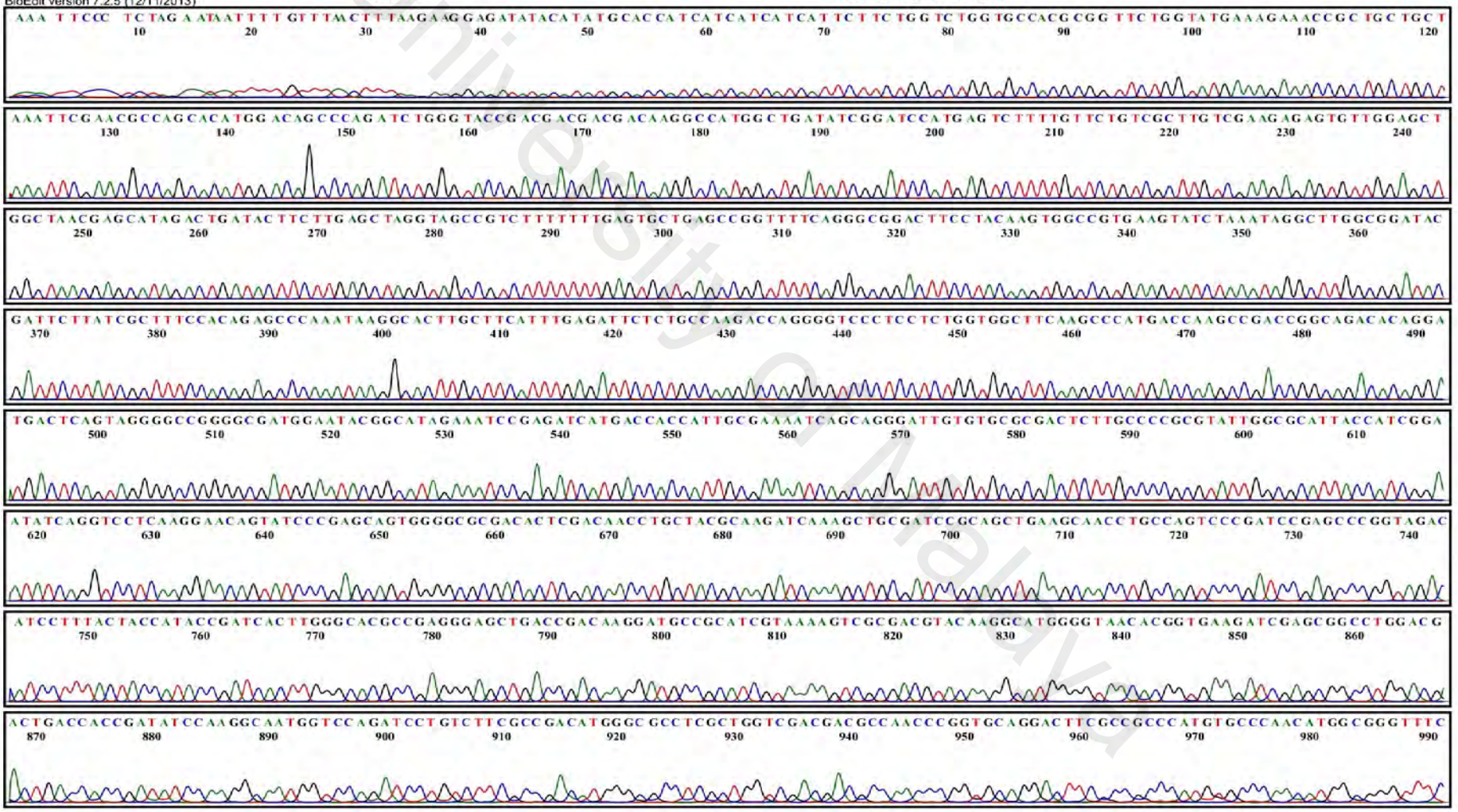


Figure 4.6: 1% Gel electrophoresis (a) shows isolation of recombinant plasmid of ABA392/pET-30a (b) shows digested of ABA392/pET392 through restriction enzyme analysis

Figure 4.6 (a) Lane 1, Invitrogen Supercoiled DNA ladder and lane 2 to 3, purified ABA392/pET-30a. The purified ABA392/pET-30a size shows approximately ~ 6.2 kb. There are appearance of nicked/relaxed circular plasmid that can be seen (white box) and circular/single stranded plasmid that can be seen in (yellow box). Figure 4.6 (b) Lane 1, Gene Ruler 1 kb plus ladder and lane 2 to 3, digested ABA392/pET-30a. The digested ABA392 shows size at 804 bp and pET-30a vector show size at 5422 bp.

4.7 DNA Sequencing of ABA392/pET-30a

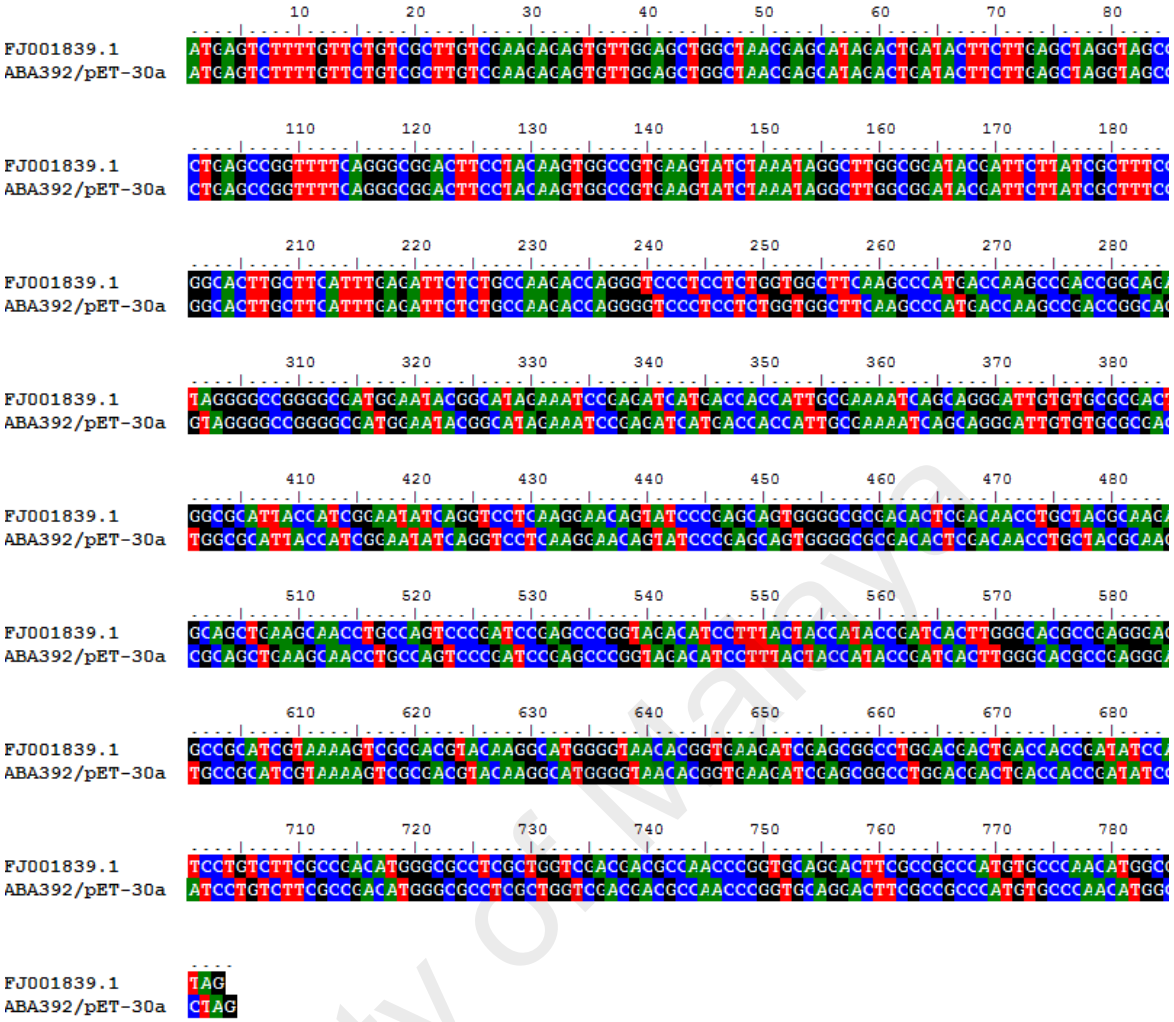
As to reconfirm the presence of the insert ABA392 gene, the isolated was send for sequencing. The sequencing was than analysed using BIOEDIT software and obtained sequence was compared using the available database. BLAST (Basic Local Alignment Search Tool) was one of the tool used to compare between pairs of obtained sequences and search for region of local similarity. BLAST was accessed from National Centre for Biotechnology Information (NCBI) similarity with the native ABA392 recombinant clone. Figure 4.7 (a) show chromatogram raw data from the sequencing result. Figure 4.7 (b) show the sequence found from the chromatogram raw data. The sequence highlighted in bold and underlined is the alignment of nucleotide of recombinant plasmid ABA392/pET-30a which has the same identical sequence PMB202 through BLAST application at NCBI (Accession No. DQ334273). Figure 4.7 (c) and (d) shows the query sequence that had 100% similarity with the reference sequence FJ001839.1 in BLAST.



(a)

GAG ATG GCA **TAT GAG TCT TTT GTT CTG TCG CTT GTC GAA GAG AGT GTT GAG CTG GCT AAC GAG CAT AGA CTG ATA CTT CTT GAG CTA GGT AGC CGT CTT TTT TTG AGT GCT GAG CCG GTT TTC AGG GCG GAC TTC CTA CAA GTG GCC GAG AAG TAT CTA AAT AGG CTT GGC TGA TAC GAT TCT TAT CGC TTT CCA CAG AGC CCA AAT AAG GCA CTT GCT TCA TTT GAG ATT CTC TGC CAA GAC TAG GGC TCC CTC CTC TGA TCG CTT CAA GCC CAT GAC CAA GCC GAC CGG CAG ACA CAG GAT GAC TCA GTA GGG GCC GGG GCG ATG GAA TAC GGC ATA GAA ATC GAG ATC ATG ACC GCC ATT GCG AAA ATC AGC AGG GAT TGT GTG CGC GAC TCT TGC CCC GCG TAT TGG CGC ATT ACC ATC GGA ATA TCA GGT CCT CAA GGA ACA GTA TCC CGA GCA GTA GGG CGC GAC ACT CGA CAA CCT GCT ACG CAA GAT AGC AGC TGC GAT CCG CAG CTG AAG CAA CCT GCC AGT CCC GAT AAG AGC CCG GTA GAC ATC CTT TAC CAT ACC GAT CAC TTG GGC ACG CCG AGG GAG CTG ACC GAC AAG GAT GCC GCA GTC TAA GTC GCG ACG TAC AAG GCA TGG GGT AAC ACG GTG AAG ATC GAG CGG CCT GGA CGA CTG ACC ACC GAT ATC CAA GGC AAT GGT CCA GAT CCT GTC TTC GCC GAC ATG GGC GCC TCG CTG GTC GAC GAC GCC AAC CCG GTG CAG GAC TTC GCC GCC CAT GTG CCC AAC ATG GCG GGT TTC CAC CGC CGC TAG** ATG GCC AGC AAC CGC GCC TGC CCT TCT GGG TGC AGA TGG TGC GCG AGA TGG ACG TGG CCA TGA CGT CCC CAG CAC GGG CCG GCC CTT CGT CGG GTG GCG AGA TGA TC

(b)



(c)

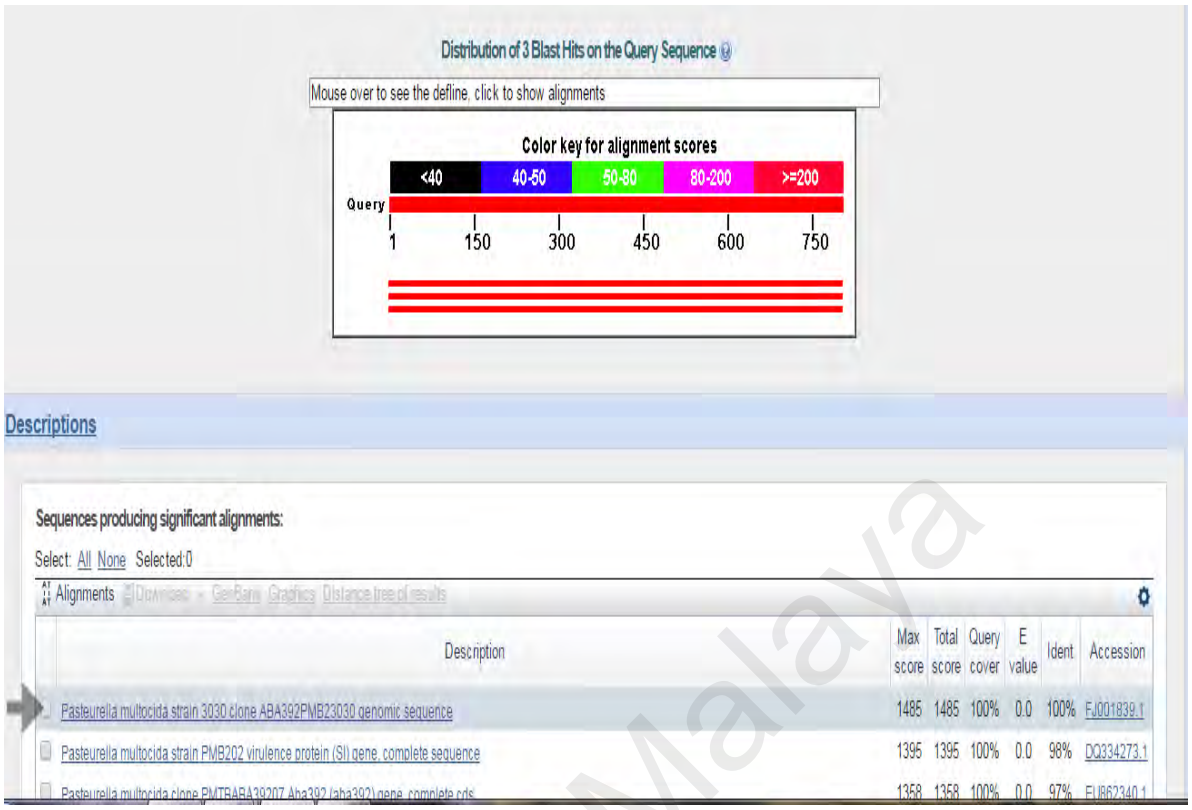


Figure 4.7: Sequencing Analysis of Recombinant Clone ABA392/pET-30a (a) shows chromatogram raw data from the sequencing results (b) shows the sequence found from the chromatogram raw data (c) and (d) shows the query sequence that had 100% similarity with the reference sequence FJ001839.1 in BLAST

4.8 Recombinant Plasmid ABA392/pET-30a DNA Stability Test

Before induction, fractions of cells that carry the target plasmid were determined. Plating of recombinant plasmid ABA392/pET-30a was done in four different plates with two replicates for each plate. Figure 4.8 shows cells grown on four different plates. Plate 1 shows cells were grown on LB agar plate, plate 2 show cells were grown on LB agar with kanamycin/chloramphenicol, plate 3 shows cells were grown on LB agar with IPTG and plate 4 shows cells were grown on LB agar with kanamycin/chloramphenicol/IPTG. Table 4.1 shows the number of cells that were grown in each plate.

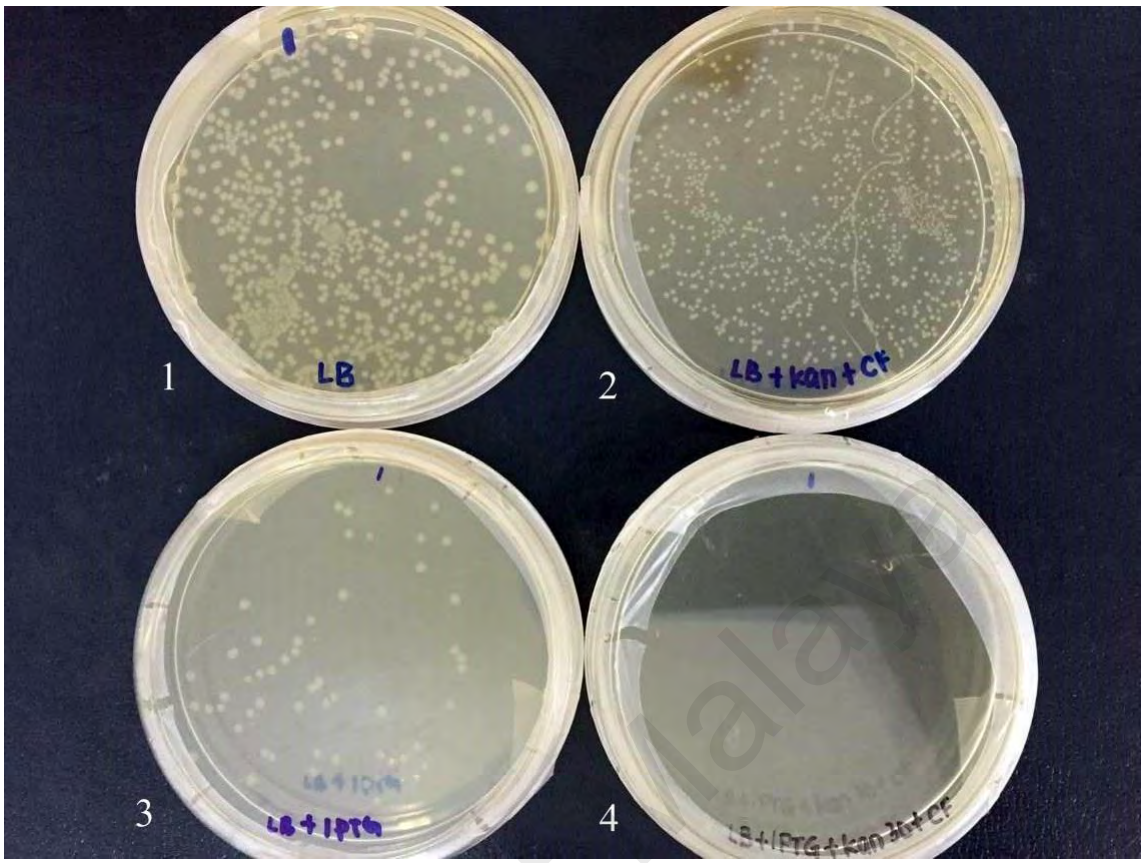


Figure 4.8: Shows ABA392/pET-30a DNA stability testing

Recombinant clone ABA392/pET-30a grown in four different plates: plate 1, LB agar plate; plate 2, LB agar with kanamycin/chloramphenicol; plate 3, LB agar with IPTG and plate 4, LB agar with kanamycin/chloramphenicol/IPTG

Table 4.1: Shows number of cells in each different four plates of ABA392/pET-30a DNA stability testing with 2 replicates for each plates

	Number of Cells		
Types of Plates	**Plate 1**	**Replicate Plate 1**	**Replicate Plate 2**
LB agar plate	423	410	420
LB agar with kanamycin/chloramphenicol	401	375	370
LB agar with IPTG	58	58	65
LB agar with kanamycin/chloramphenicol/IPTG	0	0	0

4.9 Separation of purify His-Tag Protein of Recombinant Plasmid ABA392/pET-30a by SDS-PAGE

Recombinant plasmid ABA392/pET-30a was induced and purified using His-Tag isolation and pulldown. Figure 4.9 shows purified his-tag protein of recombinant plasmid ABA392/pET-30a, lane 1 is marker, lane 2 is cell lysate of recombinant plasmid ABA392/pET-30a, lane 3 is flow through fraction of recombinant plasmid ABA392/pET-30a, lane 4 to 7 is wash fraction (1-4) of recombinant plasmid ABA392/pET-30a and lane 9 to 10 is eluted His-tag protein of recombinant plasmid ABA392/pET-30a shows the approximate size of the recombinant protein determined to be ~32 kDa

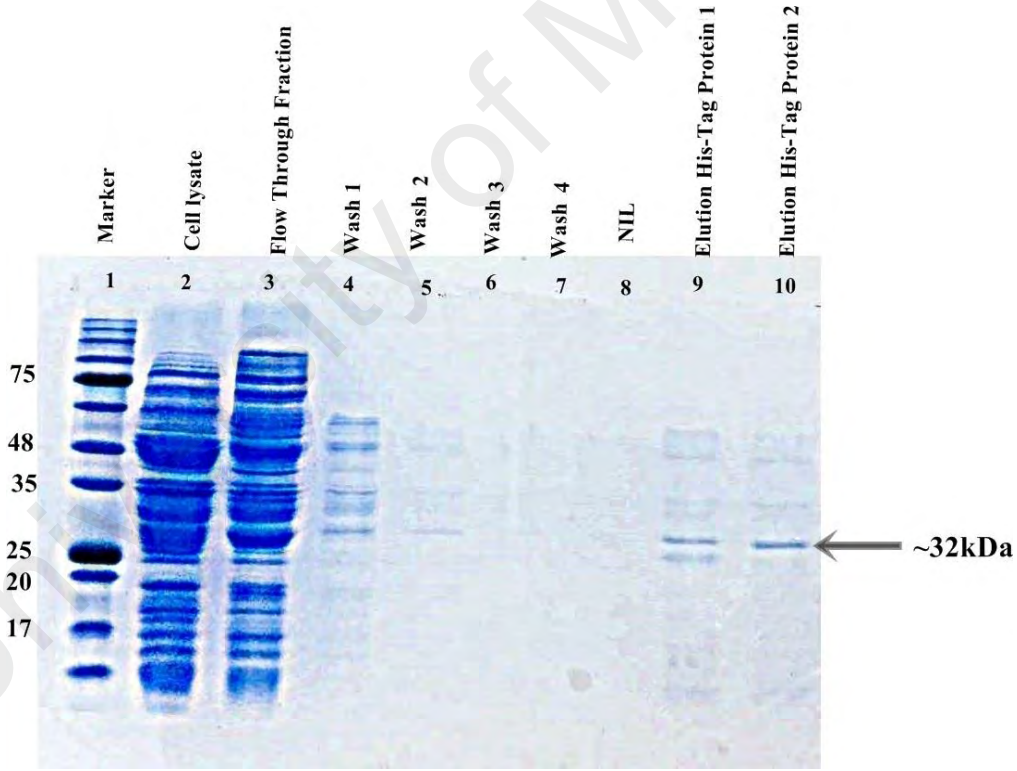


Figure 4.9: Analysis of ABA392/Pet-30a 6xHis-tagged protein expression in *E. coli* BL21 (DE3) pLysS using Dynabeads His-Tag Isolation and Pulldown

Lane 1, Protein prestained ladder; lane 2, cell lysate; lane 3, binding protein, lane 4 to 5, wash fraction 1-4 and lane 9-10, extracted His-tag recombinant protein approximately ~32 kDa (arrow). The transformed ABA392/pET-30a plasmid was induce with IPTG thus protein were expressed. The expressed was purified through affinity chromatography by using Dynabeads His-Tag Isolation and Pulldown and size were determined from SDS-PAGE, approximately ~32 kDa.

4.10 Protein Quantification of Eluted Protein of Recombinant Plasmid ABA392/pET-30a

Eluted His-tag protein from recombinant plasmid ABA392/pET-30a was quantified using DC protein assay. Lowry assay was adapted to quantify his-tag protein from recombinant plasmid ABA392/pET-30a and the concentration of 0.5mg/ml was determined.

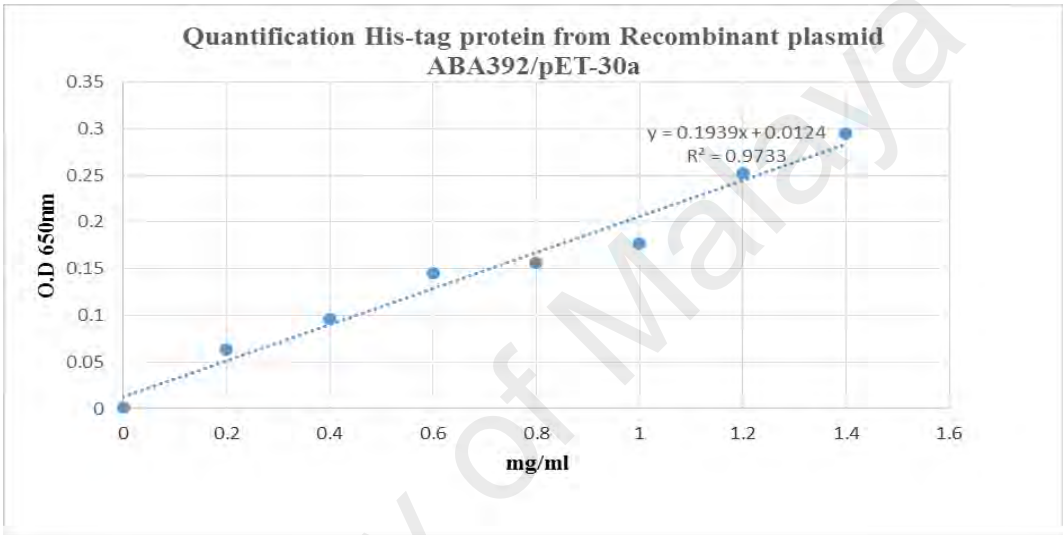


Figure 4.10: Graph showing protein standard curve constructed by DC protein assay using BSA as a standard

Table 4.2: Absorbance reading for the protein standard and protein sample

Protein Concentration (mg/ml)	Absorbance (650nm)
0	0.001
0.2	0.063
0.4	0.096
0.6	0.145
0.8	0.156
1	0.177
1.2	0.252
1.4	0.295
His-tag protein of ABA392/pET-30a	0.095

4.11 Immunoblotting of the Expressed Protein

The expressed his-tag recombinant protein was subjected to immunogenic detection by using immunoblotting method. Figure 4.11 shows expressed His-tag protein on nitrocellulose membrane, Lane 1 is marker and lane 2 shows expressed His-tag recombinant protein of ABA392/pET-30a at size of ~32 kDa.

Figure 4.11: Western blot analysis with Penta Anti-His Antibody

Lane 1, Protein prestained ladder and lane 2, expressed His-tag recombinant protein. The expressed His-tag protein on nitrocellulose membrane shows approximately ~32 kDa.

4.12 Animal Immunogenicity Test

4.12.1 Gross Pathological Analysis

Upon dissection, gross pathological analysis was done for each organ, lung, heart, kidney and liver collected from each group.

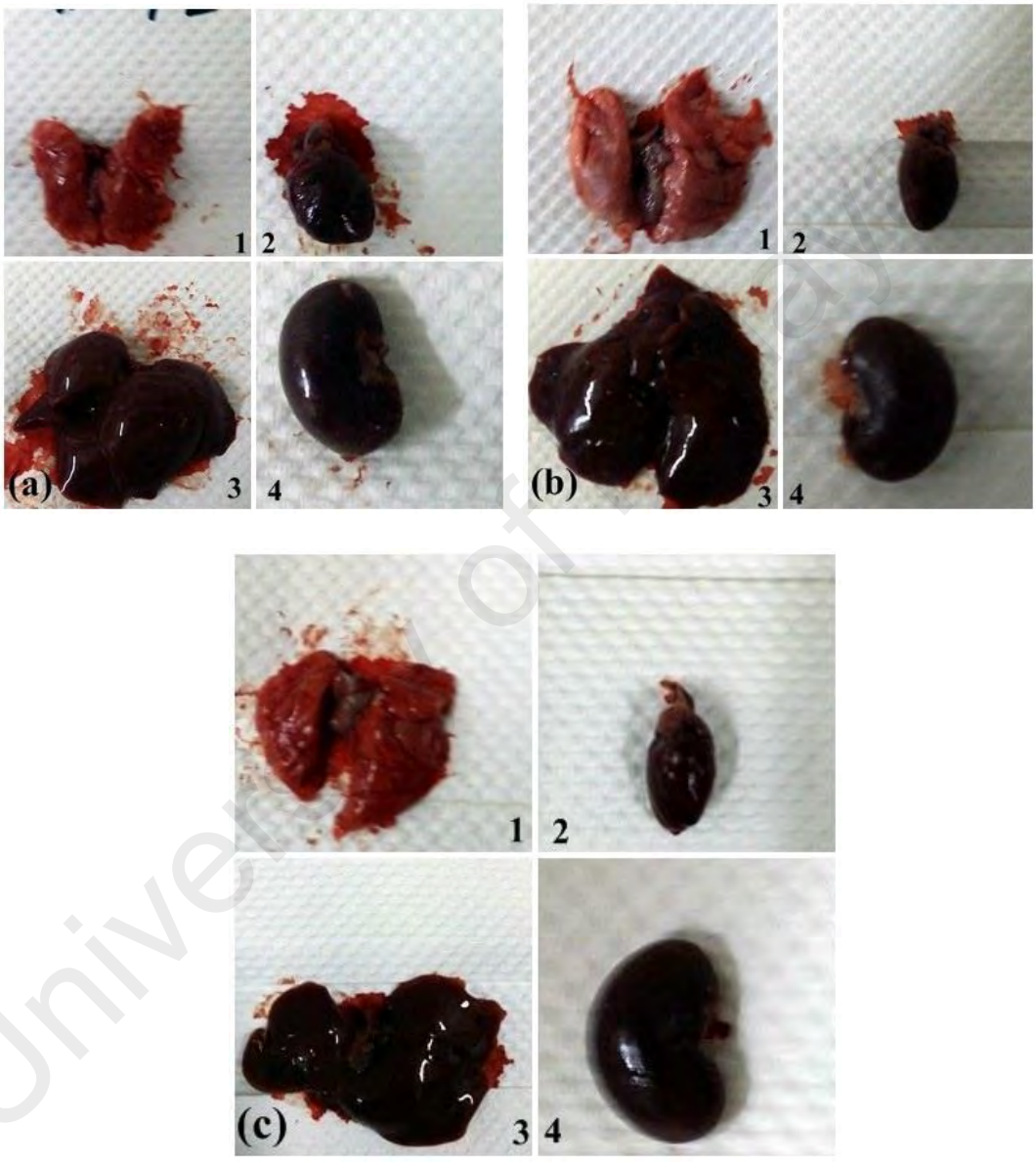


Figure 4.12: Gross pathological analysis for each organ, lung, heart, kidney and liver from (a) shows each organ from rats vaccinated with ABA392/pET-30a (50µg), (b) shows each organ from rats vaccinated with ABA392/pET-30a (100µg) and (c) shows each organ from rats vaccinated with PBS-negative control

There were no significant changes were observed in all organs from each group.

4.12.2 Histopathology Analysis

Histological slides were prepared from all the organs collected from group 1, group 2 and group 4. Each slide was stained with hematoxylin and eosin (H&E). Sign of haemorrhage, inflammation, congestion or was observed in all the tissues. Histopathological analysis of the collected organs is summarized in Table 4.3.

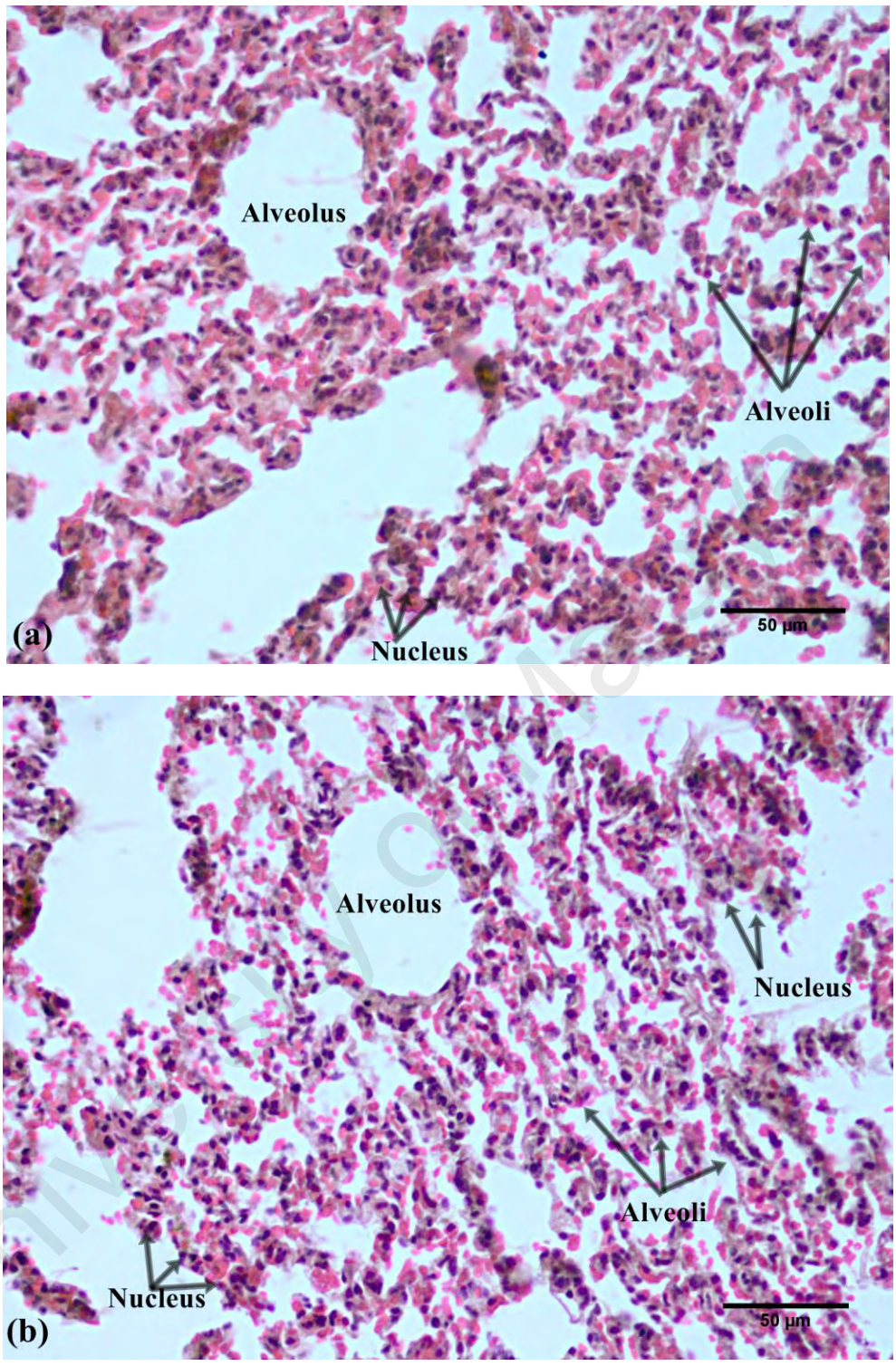


Figure 4.13: Histopathological section of the lungs from (a) rats vaccinated with PBS (negative control) and (b) rats vaccinated with purify protein from ABA392/pET-30a (50µg) with 20x magnifications, 50µm

Nucleus appears to be normal and slight thickening wall of alveoli with red blood cell (RBC) presents. No inflammation cells or haemorrhagic were observed.

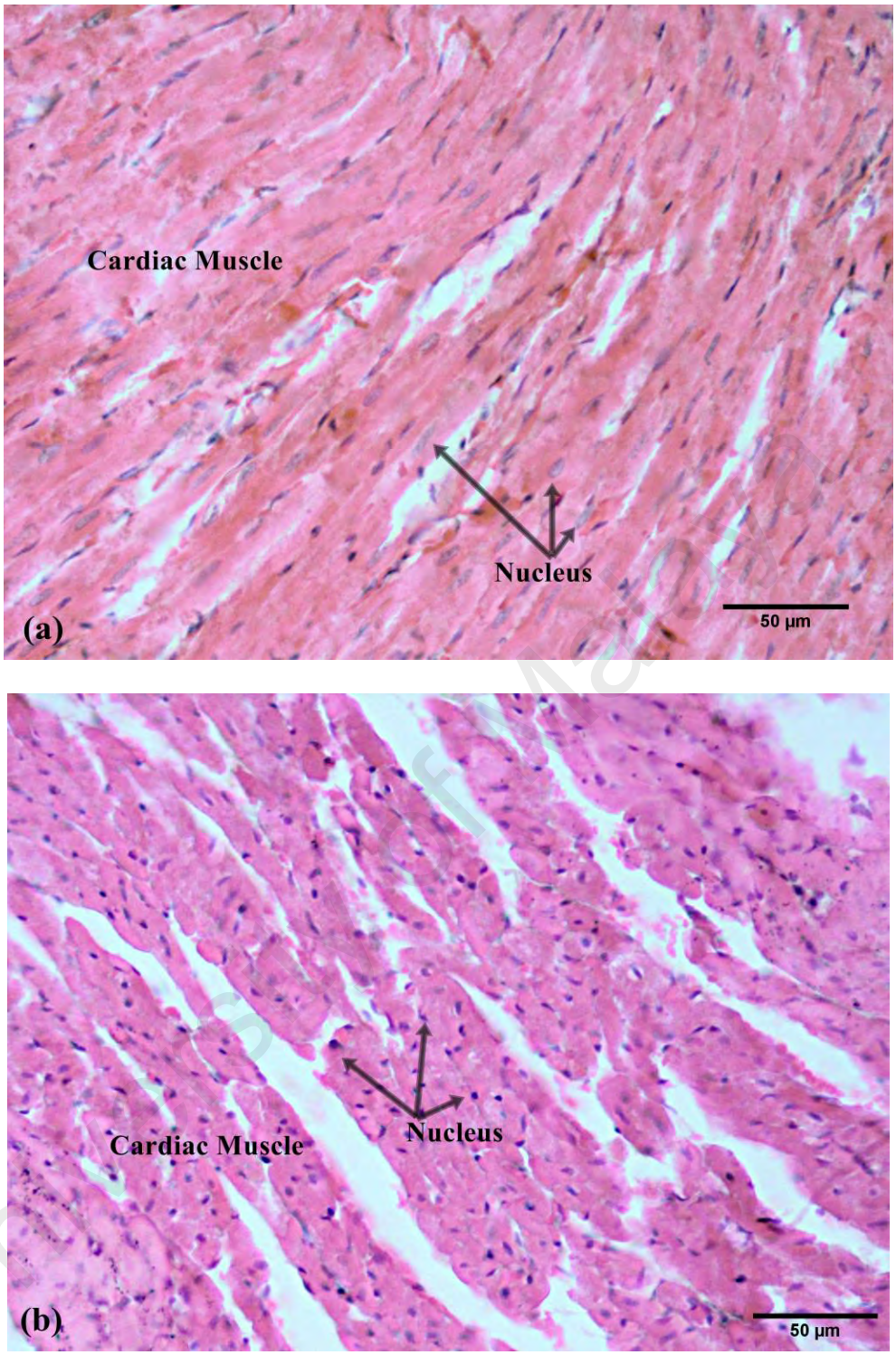


Figure 4.14: Histopathological section of the heart from (a) rats vaccinated with PBS (negative control) and (b) rats vaccinated with purify protein from ABA392/pET-30a (50µg) with 20x magnifications, 50µm

Nucleus appears to be normal. Cardiac muscle shows branches and cardiac muscle cells with normal looking nucleus. No inflammation cells or haemorrhagic were observed.

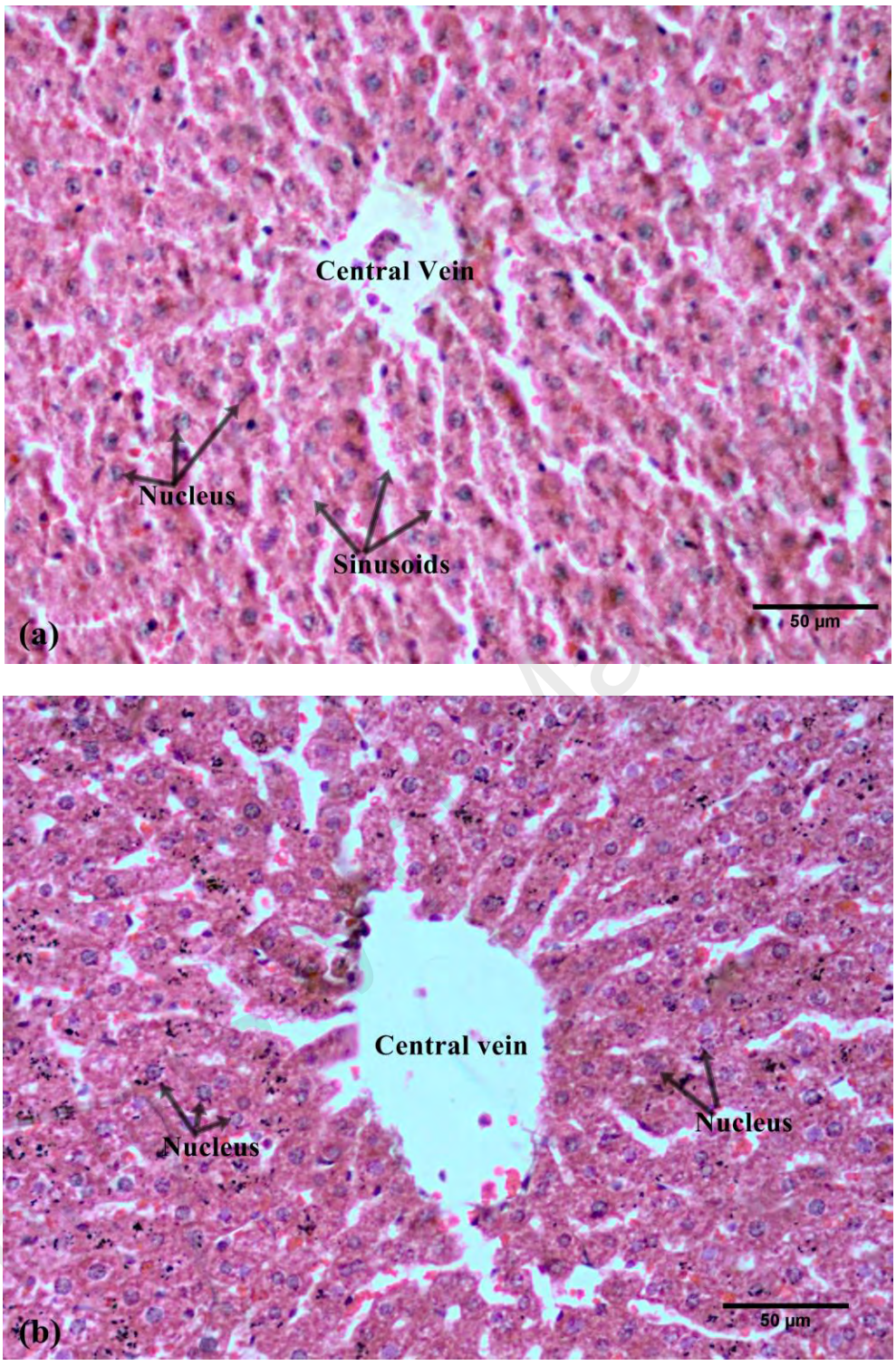


Figure 4.15: Histopathological section of the liver from (a) rats vaccinated with PBS (negative control) and (b) rats vaccinated with purify protein from ABA392/pET-30a (50µg) with 20x magnifications, 50µm

Nucleus and central vein appears to be normal. No inflammation cells or haemorrhagic were observed.

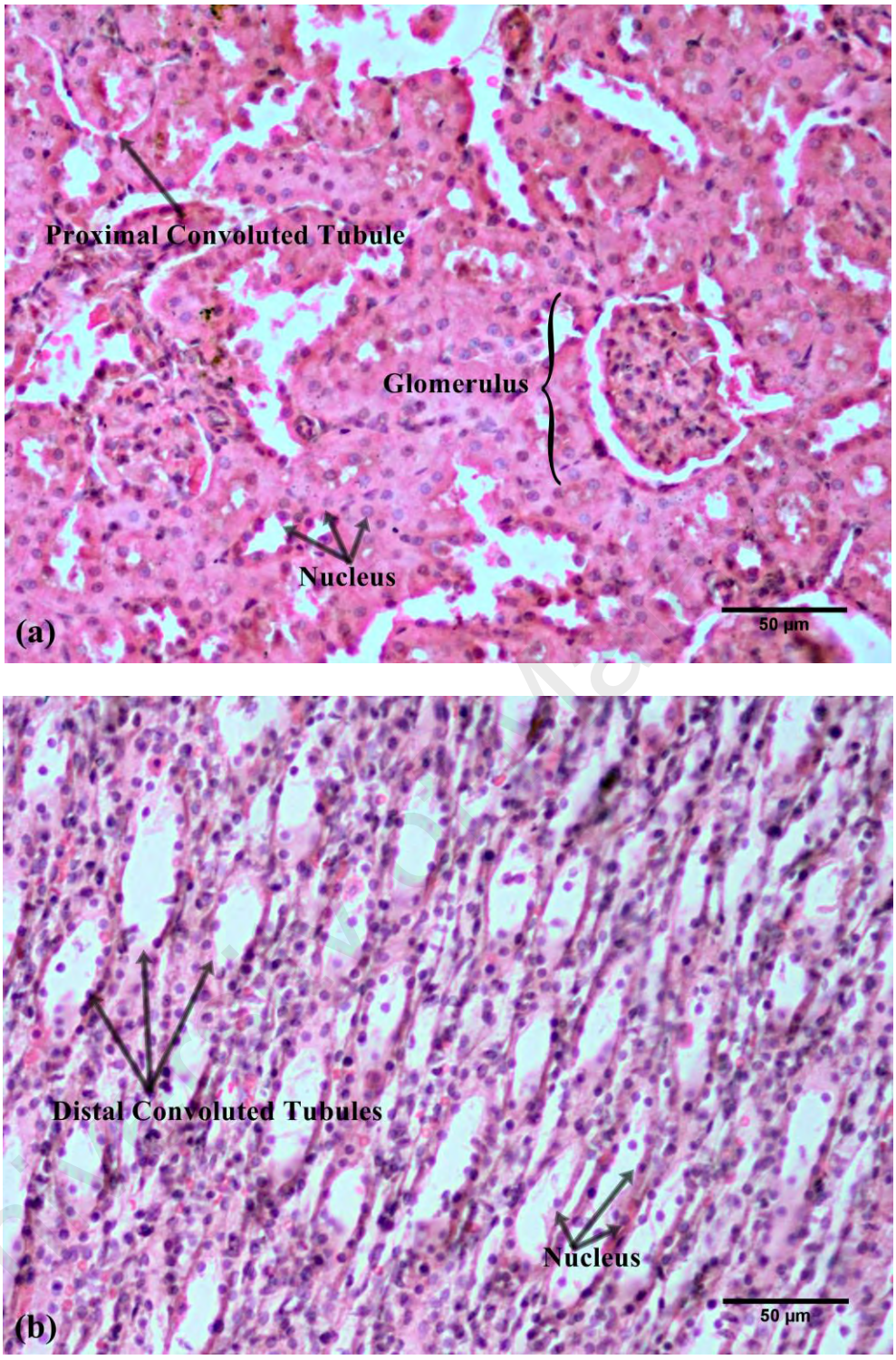


Figure 4.16: Histopathological section of the kidney from (a) rats vaccinated with PBS (negative control) and (b) rats vaccinated with purify protein from ABA392/pET-30a (50µg) with 20x magnifications, 50µm

Nucleus, proximal convoluted tubule (4. 16(a)) and distal convoluted tubule (4.16(b)) appears to be normal. No inflammation cells or haemorrhagic were observed.

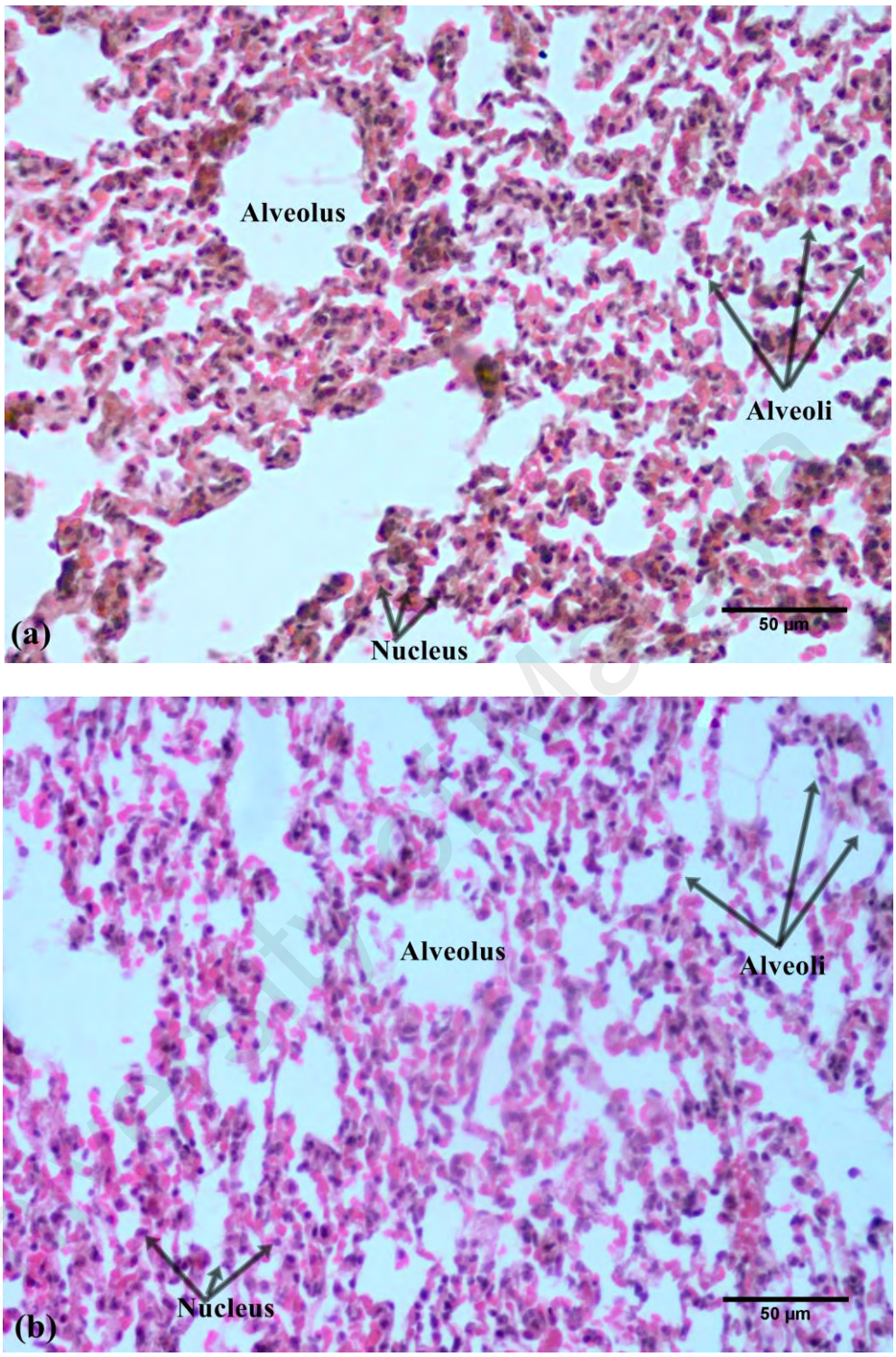


Figure 4.17: Histopathological section of the lungs from (a) rats vaccinated with PBS (negative control) and (b) rats vaccinated with purify protein from ABA392/pET-30a (100µg) with 20x magnifications, 50µm

Nucleus appears to be normal and slight thickening wall of alveoli with red blood cell (RBC) presents. No inflammation cells or haemorrhagic were observed.

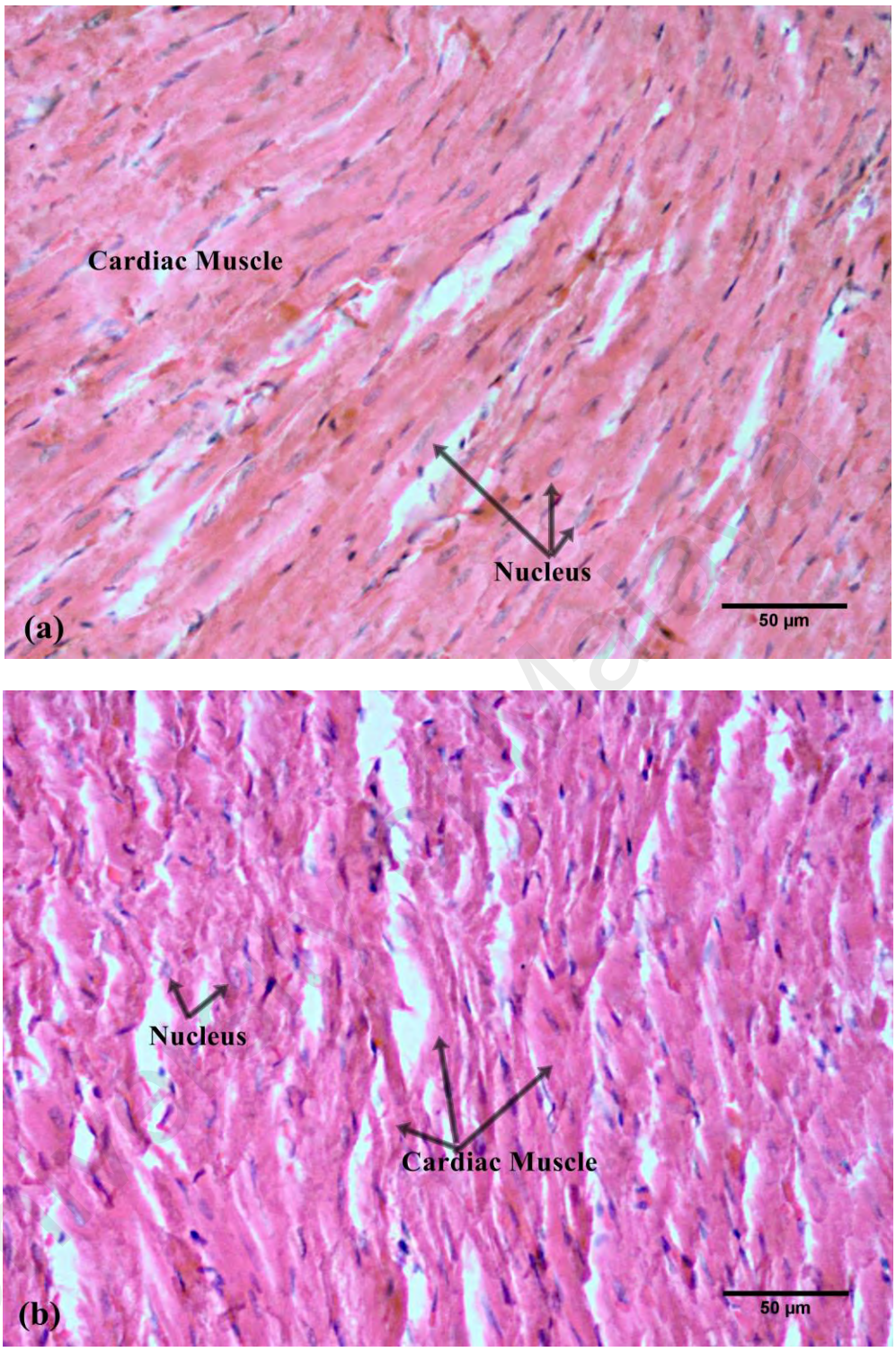


Figure 4.18: Histopathological section of the heart from (a) rats vaccinated with PBS (negative control) and (b) rats vaccinated with purify protein from ABA392/pET-30a (100µg) with 20x magnifications, 50µm

Nucleus appears to be normal. Cardiac muscle shows branches and cardiac muscle cells with normal looking nucleus. No inflammation cells or haemorrhagic were observed.

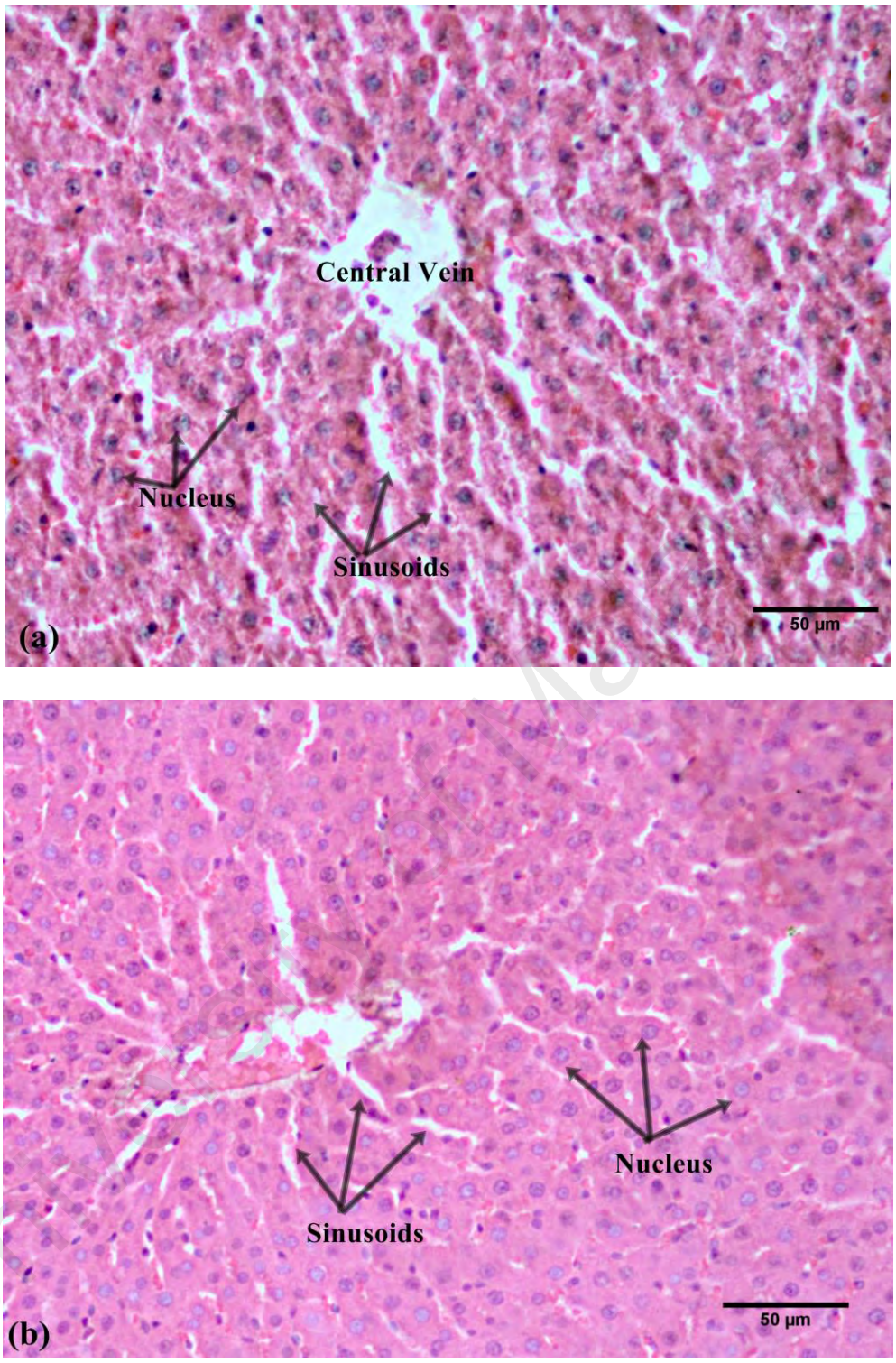


Figure 4.19: Histopathological section of the liver from (a) rats vaccinated with PBS (negative control) and (b) rats vaccinated with purify protein from ABA392/pET-30a (100µg) with 20x magnifications, 50µm

Nucleus and central vein appears to be normal. No inflammation cells or haemorrhagic were observed.

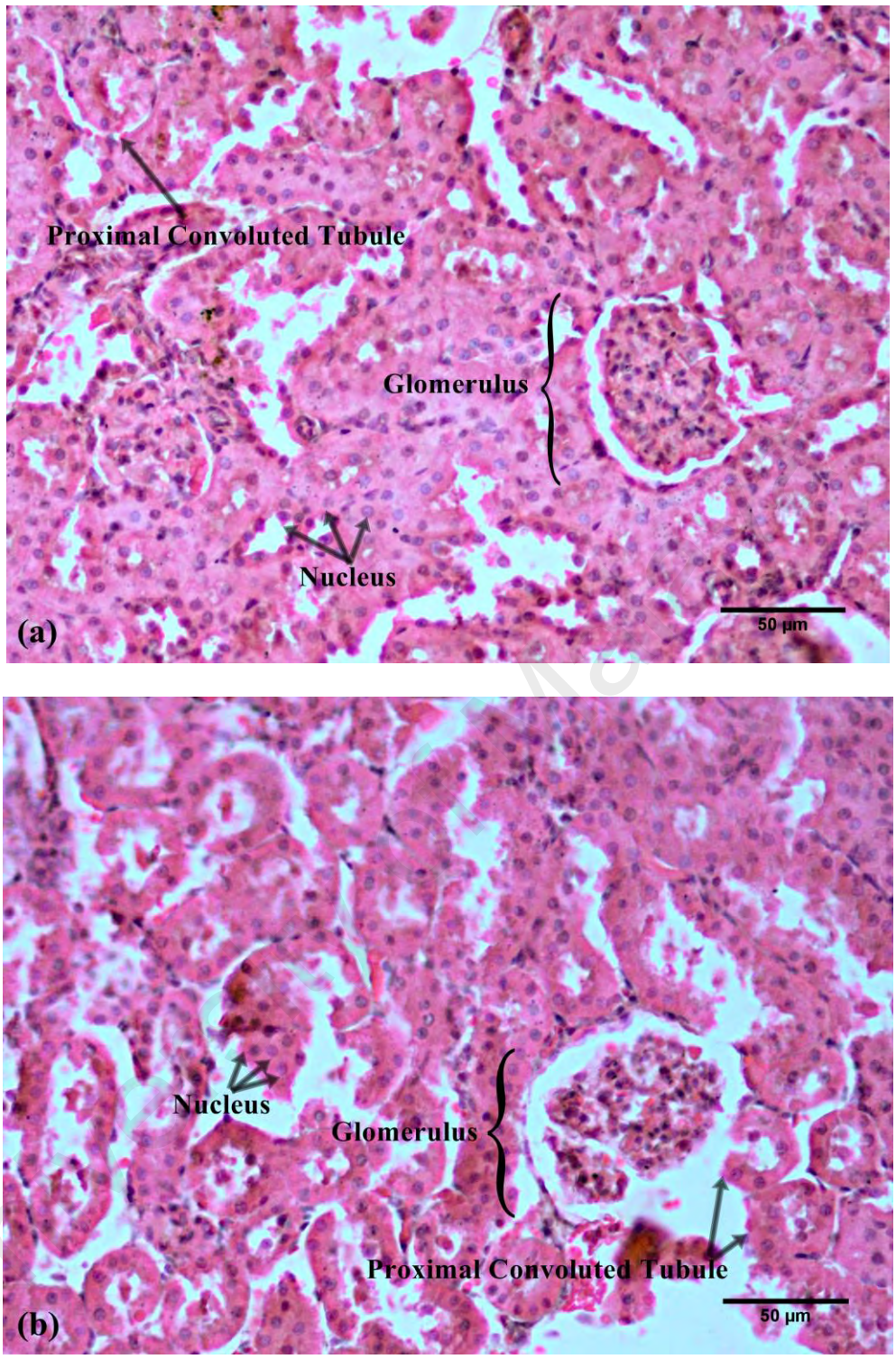


Figure 4.20: Histopathological section of the kidney from (a) rats vaccinated with PBS (negative control) and (b) rats vaccinated with purify protein from ABA392/pET-30a (100µg) with 20x magnifications, 50µm

Nucleus, glomerulus and proximal convoluted tubules appear to be normal. No inflammation cells or haemorrhagic were observed.

Table 4.3: Summary of histopathological analysis of the collected organs from rats vaccinated with ABA392/pET-30a (50µg & 100µg) and PBS (negative control)

	Animal Group of the Histopathology Analysis		
Organ	**ABA392/pET-30a (50µg)**	**Negative Control-PBS**	**ABA392/pET-30a (100µg)**
Lung	**Nucleus** -appear to be normal **Alveoli** -slight thickening with the present of Red Blood Cell (RBC) **Inflammation** -No inflammation cells were identified **Haemorrhagic** -None	**Nucleus** -appear to be normal **Alveoli** -slight thickening with the present of Red Blood Cell (RBC) **Inflammation** -No inflammation cells were identified **Haemorrhagic** -None	**Nucleus** -appear to be normal **Alveoli** -slight thickening with the present of Red Blood Cell (RBC) **Inflammation** -No inflammation cells were identified **Haemorrhagic** -None
Heart	**Nucleus** -appear to be normal **Cardiac muscle** -shows branches with normal nucleus cells **Inflammation** -No inflammation cells were identified **Haemorrhagic** -None	**Nucleus** -appear to be normal **Cardiac muscle** -shows branches with normal nucleus cells **Inflammation** -No inflammation cells were identified **Haemorrhagic** -None	**Nucleus** -appear to be normal **Cardiac muscle** -shows branches with normal nucleus cells **Inflammation** -No inflammation cells were identified **Haemorrhagic** -None
Liver	**Nucleus** -appear to be normal **Central vein** -normal **Inflammation** -No inflammation cells were identified **Haemorrhagic** -None	**Nucleus** -appear to be normal **Central vein** -normal **Inflammation** -No inflammation cells were identified **Haemorrhagic** -None	**Nucleus** -appear to be normal **Central vein** -normal **Inflammation** -No inflammation cells were identified **Haemorrhagic** -None
Kidney	**Nucleus** -appear to be normal **Distal convoluted tubules appears** -normal **Inflammation** -No inflammation cells were identified **Haemorrhagic** -None	**Nucleus** -appear to be normal **Glomerulus and proximal convoluted tubules** -normal **Inflammation** -No inflammation cells were identified **Haemorrhagic** -None	**Nucleus** -appear to be normal **Glomerulus and proximal convoluted tubules** -normal **Inflammation** -No inflammation cells were identified **Haemorrhagic** -None

4.12.3 Total White Blood Count (WBC)

Table 4.4: Total WBC count and significance of values among rats vaccinated with ABA392/pET-30a (50µg), ABA392/pET-30a (100µg), killed bacterin (positive control) and PBS (negative control)

Animal Group	No. of animal	Type of Vaccination	T. White Blood Count (Means ±SE) Significance
Group 1	3	ABA392/pET-30a (50µg)	9107(2007)[a]
Group 2	3	ABA392/pET-30a (100µg)	10250(1908)[a]
Group 3	3	Positive control-Killed bacterin	7876(1914)[a]
Group 4	3	Negative control-PBS	3760(268)[b]

All values are expressed as mean ± Standard Error (SE). n=3, mean with different super scripts are significantly differences $p<0.05$.

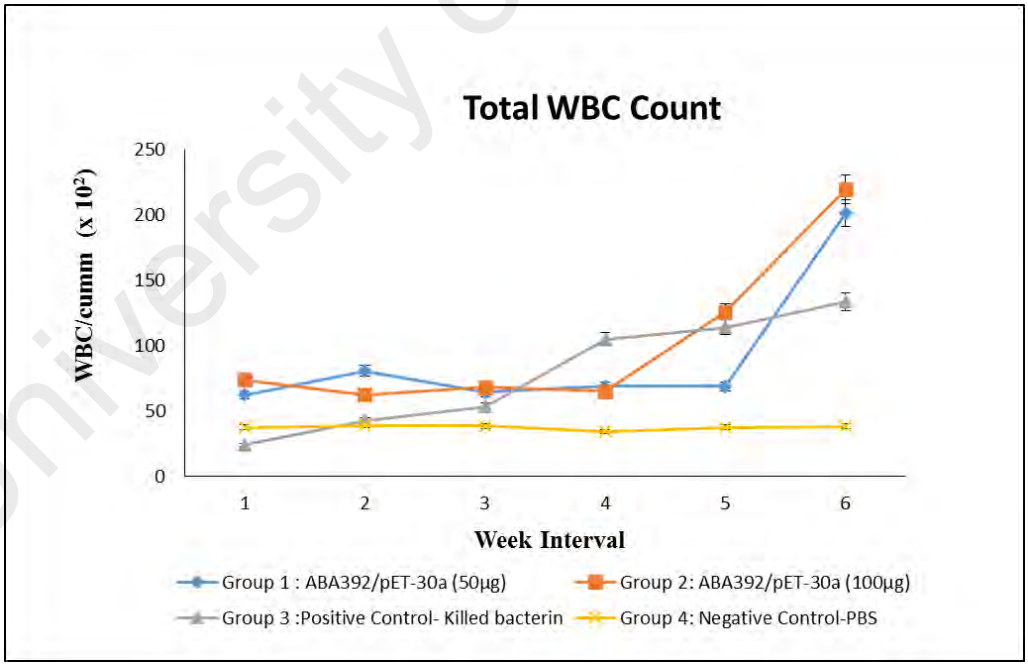


Figure 4.21: Graph shows the mean WBC count of different groups and their treatment for past 6 interval week

Comparison on total WBC from rat vaccinated with purified protein from ABA392/pET-30a (50µg and 100µg) compared with rat vaccinated with normal saline and killed bacterin *P. multocida* serotype B. Each points shows means of total WBC count of three pooled serum sample from three individual rats (n=3) and bar represents SE value ($p<0.05$).

Total WBC was performed on the blood collected from Sprague Dawley rats of the entire groups for each interval week. Figure 4.21 shows, group 1 (ABA392/pET-30a, 50μg), group 2 (ABA392/pET-30a, 100μg), group 3 (killed bacterin, positive control) and group 4 (PBS, negative control). From the graph, it shows that group 1, 2 and 3 had slight constant line increase of total white blood count compare to group 4. All the collected data was analysed using independent t-test using SPSS. Result are expressed as mean ± standard error (SE), n=3 in each group. The first immunization was given in the first week, group 1 and group 3 shows to have increased in the total white blood count, but there were no significant differences were observed till the second booster immunization was given. After the second booster immunization was given at week 3, the graph started to show leukocytosis from week 4 to 6. There were statistically significance differences ($p<0.05$) between week 5 and 6. The rats in negative control which was injected sterile PBS did not show any significance increase in leucocyte count. Table 4.4 shows, as compared between treatments groups with positive control there were no significance differences were observed meanwhile there were significantly different in comparison between treatment groups and negative control.

4.12.4 Liver Function Test (LFT)

Table 4.5: Serum level of liver function test (LFT) among rats vaccinated with ABA392/pET-30a (50µg), ABA392/pET-30a (100µg), killed bacterin (positive control) and PBS (negative control)

		Parameter				
Animal Group/ No. of animal	**Type of Vaccination**	**Albumin g/L**	**Alkaline Phosphatase (ALP) Umol/L**	**Alanine Aminotransferase (ALT) U/L**	**Total Bilirubin U/L**	**G-Glutamyl Transferase (GGT) U/L**
Group 1/ (3)	ABA392/pET-30a (50µg)	40.00±0.577[a]	203.33±19.751[a]	40.67±4.055 [a]	2.00±0.00	6.00±0.00
Group 2/ (3)	ABA392/pET-30a (100µg)	38.33±0.667[a]	179.67±.9.684[a]	36.33±3.667 [a]	2.00±0.00	6.00±0.00
Group 3/ (3)	Positive control-Killed bacterin	40.67±1.202[a]	159.00±3.055 [a]	40.67±2.963 [a]	2.00±0.00	6.00±0.00
Group 4/ (3)	Negative control-PBS	41.33±0.882[a]	158.00±24.021[a]	52.67±18.809 [b]	2.00±0.00	6.00±0.00

Results are presented as mean ± SE. $p<0.05$.
SE value for both group total bilirubin and G-Glutamyl Transferase is 0.

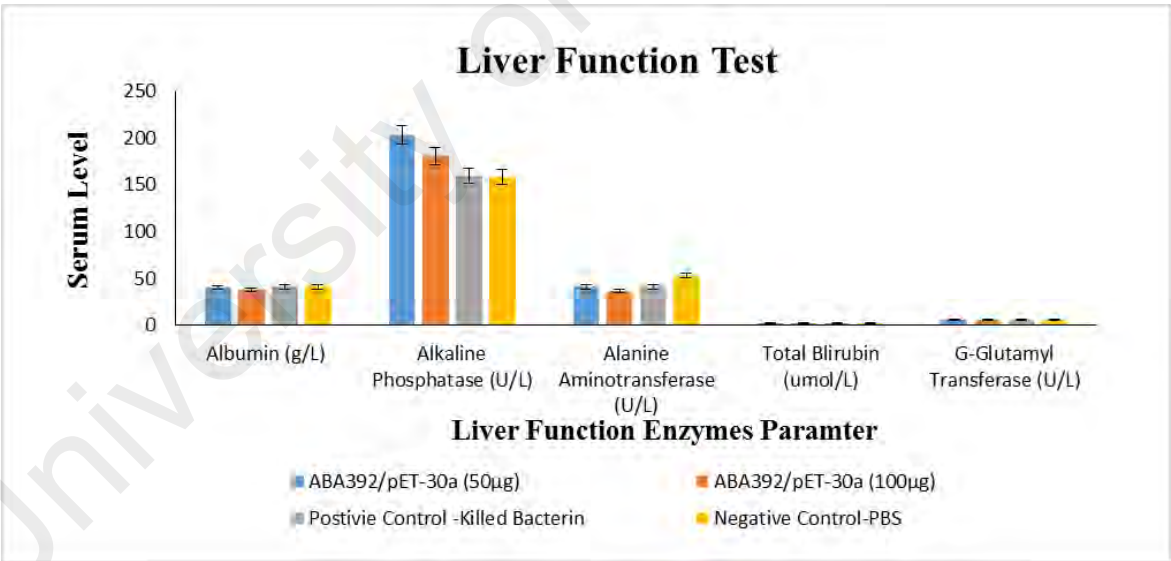


Figure 4.22: Graph shows the mean of liver function test (LFT) of different group

The liver function test parameters that were involved are albumin, Alkaline Phosphatase (ALP), Alanine Aminotransferase (ALT), total bilirubin and G-Glutamyl Transferase (GGT). Comparison on serum activities of LFT parameters from rat vaccinated with purified protein from ABA392/pET-30a (50µg and 100µg) compared with rat vaccinated with normal saline and killed bacterin *P. multocida* serotype B. Each points shows mean of serum activities of three pooled serum sample from three individual rats (n=3) and bar represents SE value ($p<0.05$).

Liver function test (LFT) was performed on the blood collected from Sprague Dawley rats of each group on day 35. Figure 4.22 shows, group 1 (ABA392/pET-30a, 50µg), group 2 (ABA392/pET-30a, 100µg), group 3 (killed bacterin, positive control) and group 4 (PBS), negative control). All the collected data was analysed using independent t-test using SPSS. Result are expressed as mean ± SE, n=3 in each group. The serum level of activities of albumin, Alkaline Phosphatase (ALP), Alanine Aminotransferase (ALT), total bilirubin and G-Glutamyl Transferase (GGT) in experimental animal is shown on Table 4.5. Serum activities of albumin, ALP, total bilirubin and GGT in all the treatment group and control groups were in normal value. There was no statistically significant differences were observed in serum activities of albumin and ALP between treatment groups and negative control groups ($p>0.05$). There were statistically significant differences were observed in serum activities of ALT between treatment groups and negative control groups ($p<0.05$). Serum activities of ALT in treatment group were in normal value meanwhile slightly higher was observed in negative control.

4.12.5 Renal Function Test (RFT)

Table 4.6: Serum level of renal function test (RFT) among rats vaccinated with ABA392/pET-30a (50µg), ABA392/pET-30a (100µg), killed bacterin (positive control) and PBS (negative control)

		Parameter				
Animal Group/ No. of animal	**Type of Vaccination**	**Sodium mmol/L**	**Potassium mmol/L**	**Chloride mmol/L**	**Urea mmol/L**	**Creatinine umol/L**
Group 1/ (3)	ABA392/pET-30a (50µg)	141.33±2.906[a]	6.133±0.5457[a]	100.00±2.082[a]	6.200±0.4163[a]	30.67±2.186[a]
Group 2/ (3)	ABA392/pET-30a (100µg)	139.67±1.453[a]	6.000±0.4163[a]	99.00±1.528[a]	6.400±0.5568[a]	28.00±1.000[a]
Group 3/ (3)	Positive control-Killed bacterin	137.67 ±3.333[a]	5.233±0.2906 [a]	97.00±2.517 [a]	6.833±0.2404[a]	27.33±0.667[a]
Group 4/ (3)	Negative control-PBS	140.67±1.764[a]	5.333±0.3180 [a]	98.33 ±2.404 [a]	7.467±0.3667[a]	32.00±1.528[a]

Results are presented as mean ± SE. p>0.05.

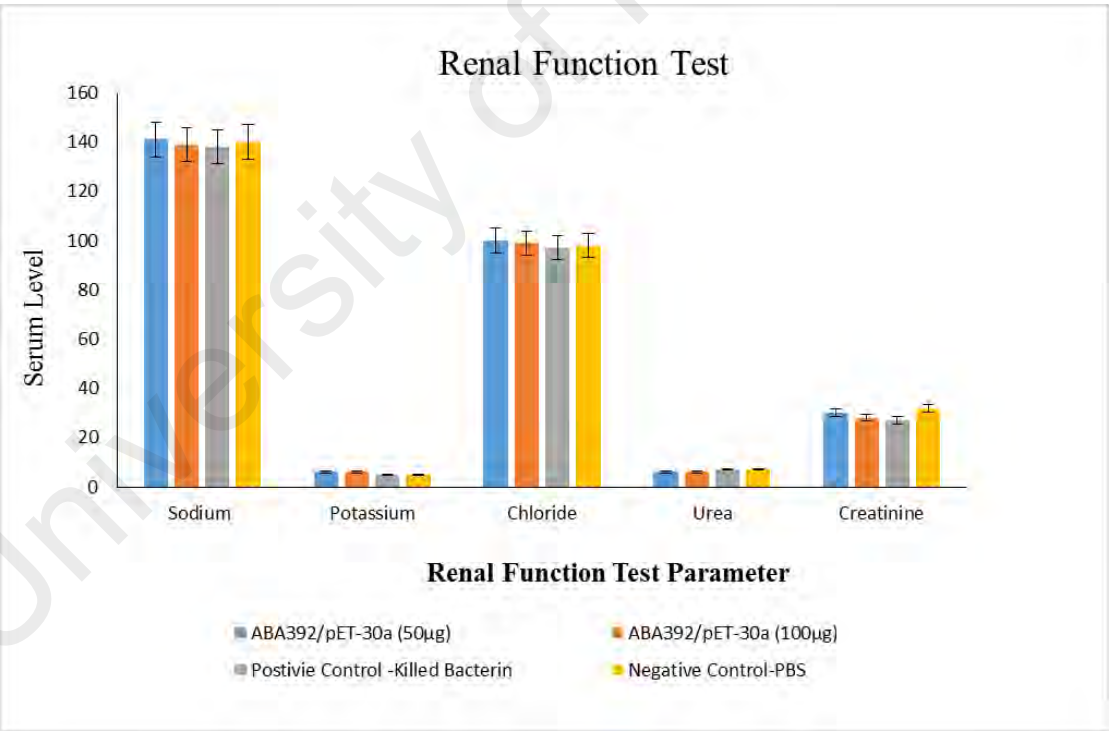


Figure 4.23: Graph shows the mean of renal function test (RFT) of different group

The renal function test (RFT) parameters that were involved are sodium, potassium, chloride, urea and creatinine. Comparison on serum activities of RFT parameters from rat vaccinated with purified protein from ABA392/pET-30a (50µg and 100µg) compared with rat vaccinated with normal saline and killed bacterin *P. multocida* serotype B Each points shows mean of serum activities of three pooled serum sample from three individual rats (n=3) and bar represents SE value (p>0.05).

Renal function test (RFT) was performed on the blood collected from Sprague Dawley rats of each group on day 35. Figure 4.23 shows, group 1 (ABA392/pET-30a, 50µg), group 2 (ABA392/pET-30a, 100µg), group 3 (killed bacterin, positive control) and group 4 (PBS, negative control). All the collected data was analysed using independent t-test using SPSS. Result are expressed as mean ± SE, n=3 in each group. The serum level of activities of sodium, potassium, chloride, urea and creatinine in experimental animal is shown on Table 4.6. Serum activities of sodium, potassium, chloride, urea and creatinine in all the treatment group and control groups were in normal value. There were no statistically significant differences ($p>0.05$) were observed in serum activities of sodium, potassium, chloride, urea and creatinine among all the groups.

4.12.6 ELISA

Table 4.7: ELISA OD reading using 450nm performed on serum from rat vaccinated ABA392/pET-30a (50µg), ABA392/pET-30a (100µg), killed bacterin (positive control) and compared with rat vaccinated PBS (negative control)

Animal Group	No. of animal	Type of Vaccination	ELISA OD (means ±S.E.M) Signifcance
Group 1	3	ABA392/pET-30a (50µg)	0.099(0.015)[a]
Group 2	3	ABA392/pET-30a (100µg)	0.081(0.007)[a]
Group 3	3	Positive control- Killed bacterin	0.071(0.012)[a]
Group 4	3	Negative control- PBS	0.045(0.009)[b]

All values are expressed as mean ± SE. Mean with different super scripts are significantly differences $p<0.05$.

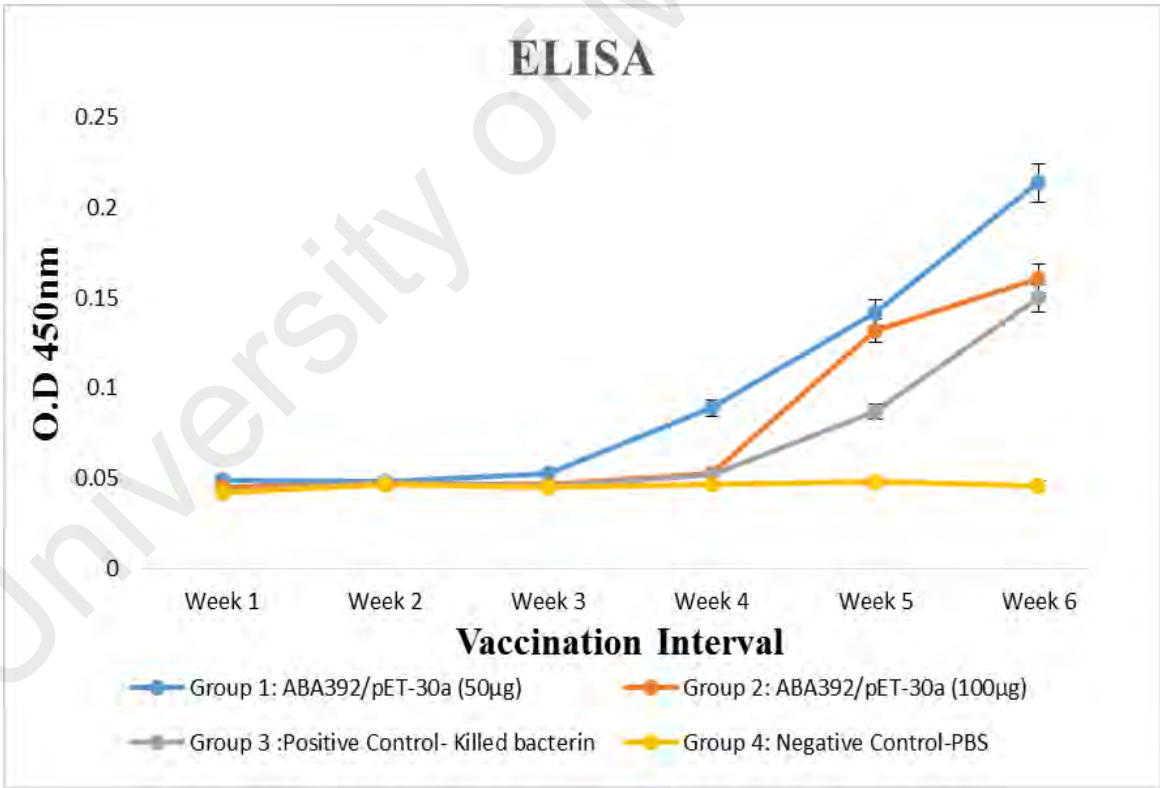


Figure 4.24: ELISA O.D Analysis Using 450nm

Comparison on serum IgG from rat vaccinated with purified protein from ABA392/pET-30a (50µg and 100µg) compared with rat vaccinated with normal saline and killed bacterin *P. multocida* serotype B. Each points shows mean of serum activities of three pooled serum from three individual rats (n=3) and bar represents SE value ($p<0.05$).

The development of the immunoglobulin (IgG) antibody response in Sprague Dawley rats was analysed using ELISA. From Figure 4.24, graph shows group 1 which was treated ABA392/pET-30a (50µg) had slightly higher development IgG compare to overall other groups. Group 3 which were treated with killed bacterin thus serve as positive control has also develops IgG from week 3 towards to week 6. Among all the groups, group 1 shows constant of development of antibody compare to other remaining groups. Once the first immunization was given on week 1st no development was observed but when the 2nd booster was given on week 3, the chart shows increase in the IgG level in all groups expect for the negative control group. All the collected data was analysed using independent t-test using SPSS. Result are expressed as mean ± SE, n=3 in each group. There were no significance differences after the 1st immunization was given. When the second booster was given on 3rd week the level IgG antibody were increase and statistically significant differences were observed from week 3 to 6 ($p<0.05$). Table 4.7 shows comparison between treatments groups (ABA392/pET-30a (50µg and 100µg) with positive control (killed bacterin). There is no significance differences were observed between the two groups. Meanwhile, there were significantly difference in comparison between treatment group (ABA392/pET-30a (50µg and 100µg)) and negative control (PBS), 0.099, 0.081 and 0.045 respectively. As compared, positive and negative treatments show significant differences in the level of IgG which can be due to non-specific immunity induced in the white rats. There were no doubts in the ELISA results obtained and rats treated with two doses of vaccine showed to be effective in developing antibody.

CHAPTER 5: DISCUSSION

5.1 Cloning ABA392 into Protein Expression Vector

ABA392 gene was successfully cloned into pET-30a, an expression vector. In order to complete this transformation process, purified ABA392 gene (Figure 4.1 (a)) and pET-30a (Figure 4.2) vector was digested using restriction enzyme (RE). The RE composition consists of two identical polypeptide subunits joined together to form a homodimer. This homodimer is known as short symmetric DNA sequences of 4 to 8 bp whereby 6 bp is ideal for cloning. The function of this enzyme is to recognize specific, short and nucleotide sequence on a double-stranded DNA molecule which is known as restriction site and cleaved it accordingly (Mullis, 1990). The RE digested ABA392 gene in Figure 4.1 (b) and pET-30a vector (Figure 4.2) were ligated using DNA ligase. DNA ligase together with ATP which function as cofactor forms a phosphodiester bond between ABA392 gene and pET-30a vector was ligated and this process known as ligation (Allison, 2007). This ligation mixture was introduced into bacterial cells (*E. coli* BL21 (DE3) pLysS) and the process known as transformation. The *E. coli* BL21 (DE3) pLysS was incubated in concentrated calcium salt solution in order to make the membrane leaky. This solution was mixed with the ligation mixture and was allowed into the bacterial cell. Successfully transformed bacteria carried both recombinant and non-recombinant plasmid DNA. The tranformants were plated on LB agar with kanamycin/chloramphenicol and those bacteria that have recombinant DNA plasmid only will grow on the plate (Figure 4.4 (a)). The selection of transformant selection was based on the growth of the colonies on plate that containing both kanamycin/chloramphenicol) (Sambrook *et al*., 1989). For the colony library) the colony PCR was conducted from patch plate (Figure 4.4 (c)) to detect the presence of ABA392 gene inserts in pET-30a vector using the synthesized primer where the

ABA392 gene were at 804 bp (Figure 4.5). Two positive colonies were extracted and show the expected size the clone carrying the insert. The clone size were determined to be 6.2 kb (Figure 4.6 (a)). RE digestion was carried out on extracted clone using *Hin*dIII-HF and *Bam*HI-HF where the insert (ABA392gene) at 804 bp and vector (pET-30a) at 5422 bp were observed (Figure 4.6 (b)). To reconfirm the presence of the insert ABA392 gene, the extracted clone was send for sequencing. The result from DNA sequencing confirmed the presence of ABA392 gene in the recombinant and raw data was used for homology and nucleotide sequence analysis by identifying the similar based through BLAST application at NCBI (Figure 4.7 (a), (b) and (c) (d).

5.2 Plasmid Stabilty testing of ABA392/pET-30a

Before induction, the culture was determined for the fragment of cells that still carry the targeted plasmid. This process started with plating the cells on four different plates and done in triplicate for each plate (Figure 4.8). Cells grown on LB agar were viable cells. Cells that grown on LB agar with kanamycin/chloramphenicol are the cells that still carry the plasmid meanwhile, cells that grown on LB agar with IPTG shows the cells that have lost the plasmids on mutants. This lost leads to the disability of expressing the targeted gene. Finally cells that grown on LB agar with kanamycin/chloramphenicol/IPTG shows only mutant that contain the plasmid which lost the ability to express the target gene. The existence of IPTG causes all the energy is being used for the production of recombinant protein instead of maintaining the cell that has the plasmid for protein production.

5.3 Protein Induction and Purification

The recombinant plasmid ABA392/pET-30a was transformed into an expression host *E. coli* Bl21 (DE3) pLysS bacterial strain which specially design for expression of genes that controlled by T7 promoter. This pET-30a expression was mainly chosen due to T7

promoter, kanamycin resistant and multiple cloning sites. pET-30a vector will be more suitable for the cloning and expression of recombinant protein in *E. coli* purpose. Since *E. coli* is a nontoxic host and this being an important reason for many recombinant protein being expressed hence it is being used as generally in research industry as an expression host (Farhangnia, 2014). The present of T7 promoter in pET-30a is very important and compromising during targeted recombinant protein production. When the interested gene is cloned behind T7 promoter and it is recognized by T7RNA polymerase (T7 RNAP) which can be found in bacterial genome. Protein from this pET-30a expression system can be expressed by inducing using IPTG (Rosano & Ceccarelli, 2014). IPTG addition will induce and prompt protein expression. T7 RNA polymerase production after the induction will particular transcribe the coding sequence of the protein of interest which is inserted into the expression vector which restraint by T7 promoter (Briand *et al*., 2016). Sequences that encoding six histidine presence at 5' of the multiple cloning sites (MCS) and this is the most significant reason on choosing this pET expression vector (Fang, 2006).

E. coli BL21 (DE3) pLysS has lysogenic cycle and λ-DE3 which encode T7RNA polymerase and control by lacUV5 promoter. The compatible plasmid pLysS carries the gene encoding T7 lysozyme which reduces the background expression level of the targeted gene. This is controlled by T7 promoter and expression level is achieved without any interference during IPTG induction (Rosano & Ceccarelli, 2014).

After induction, the expressed protein from ABA392/pET-30a was purified using cobalt-based Immobilized Metal Affinity Chromatography (IMAC). The principle of this purification is based on magnetic separation where consist a simple step. The magnetic carrier which can be consist of an immobilized affinity, magnetic biopolymer particles, hydrophobic ligand or ion exchange groups has an affinity towards to the

isolated substances. The magnetic carrier will be mixed with a sample. This sample can be from crude protein as well. During the incubation duration, the target substances which have affinity tag towards the magnet carrier will bind to each another. This magnetic complex can be eluted using magnet separator after a few washing steps. This magnetic separation technique is very simple conduction steps involved and due to the magnetic propertied it easily removed from the samples. This process is very gentle to obtain the targeted proteins and effective for large scale purification (Safarik & Safarikova, 2004). IMAC can purify highly expressed protein and can provide purified tagged protein in single purification step. Using IMAC the polyhistidine-tagged protein can be also purified under mild condition without altering the biological activity. Purified protein from IMAC can be also further purified using other chromatographic method in future (Bornhorst & Falke, 2000).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is an important method to separate protein. Protein is separated based on their movement capability which depends on the length of polypeptide chain or molecular weight when place on electrical current. SDS function as removing secondary and tertiary protein structure in order to maintain the protein as polypeptide chain (Roy & Kumar, 2014). Laemmli system is the most extensively SDS-PAGE gel system that has been used to separate the protein. It uses tris-glycine gels which composed of stacking gel and resolving gel. Both have two different principle in separating the protein where stacking gel component will aid focusing the protein into sharp bands during the initial phase of the electrophorectic run meanwhile resolving gel with the different acrylamide percentage will separate the protein bands on their the mass weight (Laemmli, 1970). To find the molecular weight of the expressed clone, ABA392/pET-30a recombinant plasmid was subjected to SDS-PAGE. Before performing induction, colony from positive clone (ABA392/pET-30a) was culture on a LB kanamycin/chloramphenicol

plates (Section 3.27) was inoculated in to a 10ml of LB broth containing kanamycin/chloramphenicol and incubated at 37ºC overnight. The 10ml overnight culture was then inoculated into 50ml pre-warmed broth with vigorous shaking until it reached an OD_{600} of 0.6 in about 60 to 120 minutes. 1 ml sample was immediately taken before induction, this sample served as the non-induced control, the cells were centrifuged at 5000 rpm for 5 minutes and supernatant were removed as much as can. The cells were then re-suspended with 50µl 1X PBS (Section 3.11.2) and 50ul 2x sample buffer (Section 3.13.7) and heat for 10 minutes at 95ºC on heating block. The non-induced control was frozen and stored at -20ºC until SDS-PAGE was performed. Expression was induced by adding IPTG (Section 3.11.1) to a final concentration of 0.1mM. The culture was then incubated further for an additional 4-5 hours at 37ºC. following the incubation, 1ml sample was taken, the cells were centrifuged at 5000 rpm for 5 minutes and supernatant were removed as much as can. The cells were then re-suspend with 50µl 1X PBS and 50µl 2x sample buffer and heat for 10 minutes at 95ºC on thermal block. The induced control were frozen and stored at -20ºC until SDS-PAGE was performed. The remaining induced cells were harvested by centrifugation at 3500 x g 4ºC for 60 minutes and then the cell pellets were frozen and stored overnight at-20ºC. The cells were lysis using B-PER reagent (Section 3.32.1). The cell lysate was then purified using Dynabeads His-Tag Isolation and Pulldown) which based on cobalt-based Immobilized Metal Affinity Chromatography (IMAC) (Section 3.32.2). 6x His-tag has been used extensively to purify recombinant protein due to applied a low metabolic burden on expression host and provide flexible condition for IMAC (Dan *et al*., 2009). Cobalt-based Immobilized Metal Affinity Chromatography (IMAC) has the ability to display less non-specific protein binding compare to nickel and due to this it has increased the purity of eluted protein (Bornhorst & Falke, 2000). The entire collected fraction was subjected to SDS-PAGE. The gel was the stained with colloidal coomassie

solution (Section 3.13.10) in a plastic container for overnight followed by destaining (Section 3.13.11). The gel was wash 2 to 3 times until a clear background was obtained. Following the SDS-PAGE analysis, size of the expressed protein from recombinant clone ABA392/pET-30a was determined to be at ~32 kDa (Figure 4.9). The nucleotide sequence (Appendix F) of ABA392/pET-30a was then translated into six reading frame of amino acid using the translation programme. This translation programme was provided by Expert Protein Analysis System (ExPASy) (Abdul Latif *et al*., 2017). Among the protein blasted result from all the frame, amino acid (267aa) from 3'5' frame 1 (Appendix G) showed 93% similarity (252/272 aligned) with ABA392 clone (*P. multocida*) (Appendix H). Translation of six histidine residue (HHHHHH) was founded along with the inserted protein. The molecular weight of the expressed recombinant protein from ABA392/pET-30a was analysed using the ExPASy pI/Mw Tool (Appendix I). It was comparable to the purified expressed recombinant protein from ABA392/pET-30a with a molecular weight approximately around 32 kDa. Protein which has more than 100 amino acids has the potential to be a functional protein (Salmah Ismail *et al*., 2012). During the protein purification, some of the non-specific protein which was not able to bind to the resin will be discharged through the flow-through solution along with the washing buffer. The existence of several protein bands at the lane of eluted His-tag protein (lane 9 & 10) in Figure 4.9 might be due to some of the contaminant proteins which were eluted together with the protein of interest. As the protein was expressed in low intensity, some background protein contamination may be observed (Abdul Latif *et al*., 2017). This may occur when the purification of low level His-Tag recombinant protein in *E. coli* system using IMAC which frequently result in co-eluted with some contaminant protein from the host cells (Robichon *et al*., 2011). The low intensity of the expressed protein with background contaminant can be referred to the instability of targeted protein which has the potential to be degraded or weak

bound to the resin. In this similar case, the recombinant might be lost easily due to the gap between chromatography resins with irreversible adsorption of non-specific region of the targeted protein. In conjunction with this, the expressed His-Tag protein might be decrease in the final elution step (Abdul Latif *et al*., 2017). Meanwhile, the non-tagged protein which from the host cell has higher chance to bind together with the resins and eluted together with background contamination in case there is a failure in targeted recombinant protein binding to the resins (Chirstensen *et al*., 2007). To overcome this problem, mass spectrometry can be conducted along to identify the targeted expressed His-tag protein as well.

5.4 Protein Quantification

To quantify the purified protein, Bio-Rad DC protein assay was used. This assay was adapted from Lowry assay with slight modification. In Lowry assay, the reaction between protein and copper occur in an alkaline medium and followed by reduction of the folin reagent by the copper-treated protein. Colour was developed due to the amino acids tryptophan and tyrosine and reduction in amount of cystine, cysteine and histidine (Lowry *et al*., 1951). The concentration of purify protein was 0.5mg/ml were obtained (Figure 4.10).

5.5 Immunoblotting of Expressed His-tag Protein of ABA392/pET-30a

Immunoblotting was carried out in order to confirm the expression of 6xHis-tagged of ABA392/pET-30a. Western blot is the most common immunoblotting technique to separate and identify proteins. First the protein was separated by molecular weight using SDS-PAGE followed by transferring the membrane which has a band for each protein. The membrane then was incubated with specific antibodies to the targeted protein. The bound antibody to the targeted protein only will be left and the unbound antibody after the washing step and these bound antibodies can be exposed in the film (Mahmood & Yang, 2012). The western blot procedure can recognize the recombinant plasmids that

express a 6xHis-tagged protein since there is short his-tag peptide sequence encoded by pET-30a protein expression vector. The eluted purify his-tag protein was run SDS-PAGE gel. After transferring the eluted his-tag protein of ABA392/pET-30a to nitrocellulose membrane, his-tagged protein had reacted positively with Penta Anti-His antibody and anti-mouse IgG, AP-linked antibody. A band was visible at ~32 kDa size on the film (Figure 4.11). Thus, His-tag protein is successfully expressed and it was ready to for further analysis.

5.6 Histopathology Analysis

Histopathological studies were performed on both groups of ABA392/pET-30a and negative control groups. Upon gross pathological examination, there were no significant changes were noticed on the site of injection of ABA392/pET-30a (50µg or 100µg) vaccinated. There were no allergy reaction or lesion arise which suggest a good tissue uptake characteristics of the protein vaccine (Figure 4.12 (a)-(b)). There was no sign of HS like haemorrhage, inflammation cells, oedema or hyperaemia were found in the lung, heart, liver or kidney (Figure 4.13 (a) - 4.20 (b)) (Chung *et al*., 2015). The histopathological analysis was summarized in Table 4.3. This finding signifies that ABA392/pET-30a protein vaccine does not cause any visible changes to the host. Invisible of any histopathological changes that observed in ABA392/pET-30a vaccinated group when compared with control group, it can be suggested a safety use of the expressed protein as a vaccine since it has not shown any toxicity effect on the tissue especially on the site of administration. Previously, Hussaini *et al*. (2011) stated that, when ABA392 clone was injected to rats, HS symptoms were observed. Gross pathology analysis demonstrated severe multiple abscess were observed in parenchyma. Meanwhile, histopathological analysis showed severe parenchyma congestion of the lungs along with the presence of inflammatory cells and haemorrhagic were observed in few vital organs like spleen, lungs and liver.

5.7 Haematology Analysis and Biochemical Analysis

The haematological analysis shows increased in WBC count from week 1 towards week 6. Total WBC shows leukocytosis (Figure 4.21). There is a significant different were observed between treatment group when compare to control group ($p<0.05$). There are two types of immunity, innate and adaptive or both. Innate immunity is known as body's first line protection which is rapid, non-specific, and presents during exposure to foreign texture. Meanwhile adaptive immunity, when there is successive exposure to foreign texture there will be increased defensive response of the immune system (Vogel, 2010). The biochemical analysis in this study is being effective parameters to reveal an impairment functional capacity of organs.

The liver function test (LFT) in this studies was conducted to see the impacts of the vaccinated rats with ABA392/pET-30a (50µg and 100µg) on the liver function enzyme parameter such as albumin, Alkaline Phosphatase (ALP), Alanine Aminotransferase (ALT), total bilirubin and G-Glutamyl Transferase (GGT) in order to assess the hepatotoxic potential of this recombinant vaccine (Figure 4.22). The obtain result of ALP, ALT, total bilirubin, and GGT of treated with recombinant protein vaccine ABA392/pET-30a (50µg and 100µg) groups were in normal value. Meanwhile, histopathology examined of liver tissue also show no degenerative changes in both treatment groups (Figure 4.15 (b) and 4.19 (b)). Hepatic injury is often correlated with alteration in the serum and liver of some enzyme particularly ALT, AST and ALP (Whitby, 1984). Renal function tests (RFT) are mandatory to examine the presence or absence lesion in the kidney. RFT in this study was conducted to see the impacts of the vaccinated rats with ABA392/pET-30a (50µg and 100µg) on the renal function test parameters of sodium, potassium, chloride, urea and creatinine (Figure 4.23). Mild hyperkalaemia were observed in both treatment groups meanwhile hypernatremia was not observed. Sodium, chloride, urea and creatinine of treated with recombinant protein

vaccine ABA392/pET-30a (50µg and 100µg) group were in normal value. Histopathology examined of kidney tissue also show no degenerative changes in both treatment groups (Figure 4.16 (b) and 4.20 (b)).

5.8 Immunogenicity Study of Expressed Protein from ABA392/pET-30a

The ABA392 gene was successfully clone into pET-30a protein expression vector, the protein was induced and his-tag protein was purified. The expressed his-tag protein were reconfirmed via immunoblotting analysis by using Penta Anti-His antibody and anti-mouse IgG, AP-linked antibody. Immunogenicity of expressed protein of ABA392/pET-30a was distinguished. Rat was chosen due to its larger size. Besides that, the availability to perform surgical procedures and the involvement of proportional size of important substructures in the organ and affected level (Lannaccone & Jacob, 2009). Moreover, rats offer the capacity to collect large amount of blood (up to1.5ml) at one time. This amount of blood allows multiple assays at one time (Lee & Goosens, 2015). According to Fish (2008), female are affected more compare to male in disease that involved with immune response and display more strong cell-mediated and humoral response to antigenic challenge. Besides that, in order to correct the exclusion of female rats in research and to reduce the bias of using male rats is one of the reasons of using female rats in this research (Beery & Zucker, 2011). 150g-200g female Sprague Dawley were used and immunized with 2 dose where first immunization were given on 1st day followed by 2nd booster on day 14th with two different 50µg and 100µg protein/rat/subcutaneous injection. Rats were observed daily and blood was collected every week for total white blood count (WBC). The vaccinated rat with expressed protein from ABA392/pET-30a (50µg and 100µg) shows significantly induced higher antibody titers compared to PBS (negative control) and formalin killed bacterin (positive control) (Figure 4.24). All the collected data were analysis using independent t-test. Further, it was observed from primary vaccination with ABA392/pET-30a, a

significant increase can be seen only after week 3 when second booster was given. It shows that IgG response is more rapid and significant by 14 day after the second booster is given on week 3 (Hodgson *et al*., 2005). The increased of antibody can only be seen after the second booster were given which begin after week 3 and started to increase for the next two weeks. Even though administration of recombinant protein vaccine has the capability to prompt immune response against pathogen after being expressed by plasmid or harmless bacteria but it shows weak or low immune response. Thus, it sometimes requires the usage of an adjuvant in order to provoke protective and last long term immunity. The main problem encountered during development of new adjuvant is not only involving to understand their molecule complexity but also the mechanism of the immune response activation (Nascimento & Leite, 2012). The same observation was made by Yasin *et al*. (2011), when using the expressed recombinant protein vaccine from fimbrial protein of *P. multocida* B: 2 whereby, the antibody level gradually rise and increased significantly for the next 2 weeks which can be seen at week 3 and 4 after the booster dose was given. This might be due the primary vaccination was unable to boost the memory cells to produce a significant level of secondary antibody thus a booster dose was required (Yasin *et al*., 2011). Another possibility, a booster is required when the vaccine might be degraded rapidly by the host defense mechanism without giving the memory cells to copy the vaccine material (Isaguliants *et al*., 2004). Further, significant difference was observed among sera from rat vaccinated ABA392/pET-30a compare to both positive and negative group ($p<0.05$) which able to provoke humoral immune response against recombinant plasmid (Singh *et al*., 2011; Salmah Ismail *et al*., 2012). Sera from formalin killed vaccine have been observed and show the significance difference when compared to the negative control. This is due to the formalin killed vaccine have the same homologus potency as inactivated vaccine. The immunity may have obtained half from host specific and

bacterium itself which cause ineffective and short immunity thus became the disadvantage of this killed vaccine. Recombinant vaccine which known as third generation vaccines and has specific aspect in producing vaccine against *P. multocida*. Identified virulence and immunity gene from *P. multocida* has a promising in the recombinant protein vaccine production (Ahmad *et al*., 2014). The first recombinant vaccine was developed in 1994 using a non-toxic recombinant derivative of the *P. multocida* toxin (rPMT) has induced response against rhinitis is pig (Bording *et al*., 1994). Okay *et al*. (2012), has stated recombinant created using 3 different fusion of gene, P1pE or OmpH and lipoprotein E (P1pE) genes have shown high competency and can be used as vaccine for cattle against *P. multocida* infection. A recombinant protein vaccine using a gene that encoding VacJ outer membrane lipoprotein of *P. multocida* B: 2 strain p52 was developed. This vaccine has provoked humoral response thus can be a potential vaccine candidate against pasteurellosis in livestock (Shivachandra *et al*., 2014)

5.9 Limitation of Study

In this study, the protective properties of this vaccine candidate were not being able to carry out to the vaccinated animals in order compare with other currently available vaccine. This is due to lack of supportive facilities to carry out the challenge tests as *P. multocida* serotype B: 2 is classified under Class 3 biohazard organism and must be treated with very much care to avoid unnecessary infection to the involved personnel.

CHAPTER 6: CONCLUSION

6.1 Conclusion

This study was carried out to express protein from ABA392/pET-30a and focused on the immune response of the expressed protein for vaccine production against HS. In order to expressed the protein and discover the capability of the gene, ABA392 gene was cloned to expression system. ABA392/pET-30a recombinant plasmid was successfully constructed by ligating ABA392 gene into pET-30a expression system and transformed into *E. coli* BL21 (DE3) pLysS bacterial strain. The 6x His-tagged recombinant protein from ABA392/pET-30a was successful expressed due to tagging ABA392 gene in N/C-Terminal of pET-30a vector. The expressed protein was then purified using cobalt-based Immobilized Metal Affinity Chromatography (IMAC) system and was confirmed by immunoblotting method. The expressed and purified protein size was determined as ~32 kDa from the SDS-PAGE. Immunogenicity of the ABA392/pET-30a recombinant plasmid was determined as discussed in Chapter 5. The purified protein from ABA392/pET-30a is being immunogenic and has capability to produce high titer antibody. Simultaneous immunization, no shock or other inflammation were observed in vaccinated animals. At the site of injection, there is no inflammation or any other changes were visible. This discovery indicates that ABA392/pET-30a as vaccine does not cause any apparent changes to the host. This characterization shows ABA392/pET-30a have a potential to be a vaccine candidate in future.

In this study protective properties of the vaccine candidate in the vaccinated animal which involving challenging with *P. multocida* serotype B: 2 was not able to carry out. This is due *P. multocida* serotype B: 2 is classified under Class 2 biohazard and there is lack of supportive facility to carry out the protective efficacy part and in

order to avoid unnecessary infection to the personnel involved and other laboratory experimental animals.

Since the expressed protein has the capability to provoke immune response, challenge studies using *P. multocida* serotype B: 2 against this protein are required to enhance the efficient of this protein as a vaccine against HS in future. Thus, the protein could be used in development of ELISA kit for identification of *P. multocida* serotype B: 2 during sudden outbreak as diagnostic tool.

To discover the full effectiveness of the clone, the assurance and efficacy of the protein in cattle or buffalo will be targeted as future study. Certain characteristics of the vaccine need to be looked into as a measurement the efficient and safety of the vaccine such as the route of immunogenicity of the vaccine under local condition, route of administration, standard of vaccine delivery method and appropriate dosage according body weight. Besides that, studies must be conducted on immune response from new vaccine and existing HS vaccine to compare the effectiveness.

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