# IMMUNOGENICITY OF RECOMBINANT HS ABA392 DNA 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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Matric No: SGR 120026

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

## ABSTRACT

*Pasteurella multocida* is the main cause of haemorrhagic septicaemia (HS) outbreak in livestock, such as cattle and buffaloes. This disease is caused by *P. multocida* serotype B (PMB) (strain 202), a gram-negative bacterial pathogen associated with a variety of disease in domestic animals. The acute form of HS is manifested by sudden onset and death within 24 hours. In Malaysia, HS outbreak has caused an average of 360 heads of cattle and buffaloes killed in a year. Conventional vaccines such as alum-precipitated or oil-adjuvant broth bacterins were injected subcutaneously to provide protection against HS. However, the immunity developed is only short-term and needed to be administered frequently. In previous study, a short gene fragment from *P. multocida* serotype B:2 were obtained via shotgun cloning technique and later were cloned into bacterial expression system. pQE32-ABA392 was found to possess immunogenic activity towards HS when tested in vivo in rat model. In this study, the targeted gene fragment of ABA392 was sub-cloned into a DNA expression vector pVAX1 and named as pVAX1-ABA392. The new recombinant vaccine was stable and expressed via intramuscular. Serum sample collected from a group of vaccinated rats for ELISA test shows, that the antibody in immunized rats was presence in high titer and can be tested as a vaccine candidate with challenge in further studies. Total white blood count shows leukocytosis. The RFT and LFT were in normal values in the vaccinated group. This successful recombinant vaccine is immunogenic and potentially could be used as vaccine in future against HS.

Keywords: Haemorrhagic septiceamia; *Pasteurella multocida*; DNA vector pVAX1; recombinant vaccine.

***IMUNOGENIK REKOMBINAN HS ABA392 DNA VAKSIN TERHADAP PENYAKIT SEPTISEMIA BERDARAH DALAM HAIWAN***

## ABSTRAK

*Pasteurella multocida* adalah punca utama penyakit septisemia berdarah (HS) dalam ternakan seperti lembu dan kerbau. Penyakit ini adalah disebabkan oleh *P. multocida* serotip B (PMB) (strain 202), patogen bakteria Gram-negatif yang dikaitkan dengan pelbagai penyakit pada haiwan domestik. Tanda-tanda jangkitan penyakit ini bermula secara tiba-tiba dan membawa kematian dalam tempoh masa 24 jam. Di Malaysia, wabak HS telah menyebabkan kematian purata 360 ekor lembu dan kerbau dalam masa setahun. Penggunaan vaksin konvensional seperti alum-precipitated or oil-adjuvant broth bacterins disuntik subkutaneous untuk memberi perlindungan terhadap HS. Walau bagaimanapun, imunitinya hanya untuk jangka masa pendek dan perlu disuntik kerap. Dalam kajian sebelum ini, gen fragmen yang singkat dari *P. multocida* serotype B diperolehi melalui teknik "shortgun" pengklonan dan kemudian telah diklon ke dalam sistem aspirasi bakteria. pQE32-ABA392 didapati mempunyai aktiviti imunogenik terhadap HS apabila diuji secara vivo dalam model tikus. Dalam kajian ini, gen sasaran iaitu ABA392 diklon ke dalam DNA vektor pVAX1 dan dinamakan sebagai pVAX1-ABA392. Vaksin rekombinan baru ini stabil dan diaspirasikan melalui suntikan intraotot. Sampel serum yang dikumpul daripada semua kategori tikus untuk ujian ELISA. Antibodi dalam tikus imunisasi menunjukkan titer yang tinggi dan boleh diuji sebagai vaksin imunasi dalam kajian kajian seterusnya. Jumlah kiraan darah putih menunjukkan leukositosis. RFT dan LFT berada dalam nilai normal dalam kumpulan yang divaksin. Vaksin rekombinan ini adalah imunogenik dan berpotensi digunakan sebagai vaksin pada masa akan datang untuk menangani penyakit HS.

Kata kunci: penyakit septisemia berdarah; *Pasteurella multocida*; DNA vektor pVAX1; rekombinan vaksin.

**ACKNOWLEDGEMENTS**

First and foremost, my sincere gratitude to Prof Dr Salmah Binti Ismail, my supervisor at Institute of Biological Sciences, Faculty of Science in University of Malaya giving me the opportunity to work with full support and invaluable guidance in completing my research project successfully. I want to acknowledge my co-supervisor Dr Nurshamimi Nor Rashid, Faculty of Medicine, University of Malaya for her advice, assistance and patience supported me throughout my thesis work. Heartiest gratitude to University Malaya for UM Postgraduate Research Grant (PPP) PG237-2014B offered to perform this study.

Special thanks go to Prof Dr Nurul Kabir and Dr Hussin Alwan Rothan for their great help in histopathology and molecular work. I gratefully acknowledge all colleagues from the Molecular Bacteriology and Toxicology Lab (MBT) for assistance in the laboratory and also bench work. Not to forget, all lecturers and staffs from the Molecular Medicine Department and Biomedical Science for providing some of the laboratory equipment for bench work.

I am indebted to my fellow lab mates, Ms Rita Devi Vellapan, Ms Kang Tzin Lim, Mr Chuan Wei Kelvin Swee, Dr Arezoo and Ms Iddzan for their highly qualified help and discussions. I thank them for willing to tolerate, co-operate and share their knowledge and wisdom with me. My deep appreciation also goes to my friends Ms Jamunaa, Ms Sivasangkary and Ms Thamilvaani who gave encouragement and invaluable guidance in finishing this research.

Finally, I must express my very profound gratitude to my beloved parents, sisters and life partner for providing me the continuous support, encouragement and understanding throughout my years of study. This accomplishment would not have been possible without them.

*-SHAMINI CHELLIAH-*

## TABLE OF CONTENTS

**CHAPTER 3: MATERIAL AND METHOD**

Section A

**CHAPTER 4: RESULTS**



**CHAPTER 5: DISCUSSION**

## LIST OF FIGURES

## LIST OF TABLES

## LIST OF SYMBOLS AND ABBREVIATION

| | | |
|---|---|---|
| µg | : | microliter |
| µl | : | microliter |
| µm | : | micrometer |
| ºC | : | degree Celcius |
| ~ | : | approximately |
| > | : | strict inequality |
| < | : | Strict inequality |
| % | : | percent |
| ± | : | plusminus |
| w/v | : | weight per volume |
| - | : | Sub-cloned into (e.g. ABA392-pVAX1 stands for ABA392 gene clone into pVAX1 DNA vector |
| ATP | : | Adenosine Triphosphate |
| BGH | : | Bovine Growth Hormone |
| BHI | : | Brain Heart Infusion |
| BLAST | : | Basic local alignment search tool |
| bp | : | base pair |
| BSA | : | Bovine serum Albumin |
| $CaCI_2$ | : | Calcium Chloride |
| CMV | : | Human Cytomegalovirus |
| dATP | : | Deoxyadenosine Triphosphate |
| dCTP | : | Deoxycytidine Triphosphate |
| dGTP | : | Deoxyguanine Triphosphate |
| $dH_2O$ | : | distilled water |

| | | |
|---|---|---|
| DNA | : | Deoxyribonucleic acid |
| dTTP | : | Deoxythymidine Triphophate |
| F | : | Forward |
| g | : | gram |
| HCI | : | Hydrogen Chloride |
| H&E | : | Haemotoxylin & Eosin |
| HRP | : | Hydrogen peroxidase |
| IACUC | : | Institute Animal Care and Use Committee |
| Ig | : | Immunoglobulin |
| kb | : | kilobase pair |
| KCL | : | Potassium chloride |
| kDa | : | kilo dalton |
| LB | : | Luria Bertani |
| LFT | : | Liver Function test |
| M | : | Molar |
| MBT | : | Molecular and Bacteriology Toxicology |
| mM | : | millimolar |
| mg | : | milligram |
| $MgCl_2$ | : | Magnesium Chloride |
| ml | : | milliliter |
| NaCl | : | Sodium chloride |
| NaOH | : | Sodium hydroxide |
| NBF | : | Neutral Buffer Formalin |
| NCBI | : | National Center Biotechnology |
| nm | : | nanometer |
| OAV | : | Oil adjuvant vaccine |

| | | |
|---|---|---|
| OIE | : | Office International des Zooties |
| Omp | : | Outer membrane protein |
| PB | : | Phosphate buffer |
| PBS | : | Phosphate buffer saline |
| PCR | : | Polymerase chain reaction |
| PMB | : | *Pasteruella multocida* serotype B strain |
| PMPT | : | Passive mouse protection test |
| PPUM | : | Pusat Perubatan Universiti Malaya |
| psi | : | Per square inch |
| R | : | Reverse |
| RE | : | Restriction enzyme |
| RFT | : | Renal Function Test |
| RNA | : | Ribonucleic acid |
| RNASE | : | Ribonuclase |
| rpm | : | Revolution per minute |
| SE | : | Standard error |
| SDS | : | Sodium Dodecyl Sulphate |
| $T_m$ | : | Melting temperature |
| TMB | : | Tetramethylbenzidine |
| Tris | : | Tris(hydroxymethyl) methylamine |
| UV | : | Ultraviolet |
| V | : | Volt |
| WBC | : | White Blood Count |

## LIST OF APPENDICES

# CHAPTER 1: INTRODUCTION

## 1.1 Background of the study

Haemorrhagic septicaemia (HS) is a lethal, deadly, septicaemia disease caused by fastidious serotype of *Pasteurella multocida* in cattle and buffaloes. This bacterial pathogen is a Gram-negative, *Pasteurellaceae* family related with a variety of diseases in livestock (Wijewardana, 1992; Tabatabaei *et al.,* 2007). *P. multocida* is a normal flora in the nasopharynx of many domestic. The infection starts from healthy carriers, or possibly from ticks and biting insects (Radostits *et al.,* 2003). In Asia, *P. multocida* serotype B:2 is the most significant reason of HS (Abubakar & Zamri-Saad, 2011), some part of Africa and also in India with high mortality and morbidity leading to huge economic loss in these countries (De Alwis, 1999; FAO, 2005; OIE, 2009). Besides in Asia, HS outbreak also were confirmed in West Bengal and Egypt. Through post mortem among buffaloes and cattle, *P. multocida* was isolated from nasopharyngeal and lungs (Karimkhani *et al*., 2011).

In Malaysia, HS was demonstrated from tonsil of healthy buffalo by isolation of *P. multocida* (Omar *et al.,* 1962) and was subsequently detected in states of Melaka, Negeri Sembilan, Perak, Pahang and Kedah. The losses were estimated to be at RM1.5 million in 1966, West Malaysia (Thomas, 1972) with an average annual lose estimated at RM200,000 from 1967 until 1976 (Joseph, 1979). It was then in the year of 2005 it was confirmed that the HS diseases in Malaysia were almost caused by *P. multocida* serotype B strain (*P. multocida* B:2) (Kamarudin, 2005). According to the report published by Saad in year 2013, HS outbreak in Malaysia causes an average loss of 360 heads of buffaloes and cattle. This loss should take into account which effect variety of factors, such as loss of productivity; meat, milk, draught power, and impairment of the reproductive potential of animals. Therefore, the production of quality livestock is

always a major problem in Malaysia, since the animals are constantly fraught with diseases, especially HS. The most possible route for HS infection to cattle and buffaloes could be due utilization of river water which contaminated with infected carcasses during monsoon season (Khaleel *et al*., 2013).

## 1.2 Problem statement

Various antibiotics have been extensively used for treatment and prevention of *P. multocida*. *P. multocida* is highly resistant to penicillin G, followed by streptomycin, oxytetracycline, ampicillin, and thiamphenicol. However, all these antibiotics are only effective when administered in early phase of infection. Therefore, prevention would be a better choice than treatment. Although antibiotics are the main ways to control such microbial incidences, their drawback highlight the need to find new potent ways to manage infections among animals. Furthermore, the remains of those antibiotics in the animal products indirectly could affect human health. Multiple antibiotic resistances have been accounted for some strains of *P. multocida* except for the B:2 serotypes. Hence, the principal means of prevention is by vaccination. Vaccination has become the principal method of controlling HS in many countries. The bacteriophage based on marker vaccine (PL-Vacl) had a more effective and longer immunity response in mice and rabbit against HS compared to widely used alum precipitated HS vaccine (HS-Vacl) (Qureshi & Saxena, 2016). Thus, the development of recombinant IROMP based indirect ELISA could serve an effective tool to differentiate between infected and vaccinated cattle and buffaloes against HS (Qureshi & Saxena, 2017). In India, P-52 strain (*P. multocida* serotype B:2) is highly virulent field isolated from a disease outbreak and used for HS vaccination. This seed culture of HS vaccine P-52 strain are tested for its efficiency in mice before and after passage in natural host by using an endpoint dilution assay for calculation of $LD_{50}$ ( Gowrakkal *et al.,* 2014). However, it

causes low protection, high virulence and unsafe for primary vaccination in young calves.

In Malaysia, vaccination is considered the most common and cheapest way of controlling outbreaks of HS. The most commonly used vaccines in Malaysia are the alum-precipitated vaccine (APV) and oil adjuvant vaccine (OAV). Repeated vaccination is required to give sufficient immunity to the host. These vaccines makes an impact of increasing the immunity of animals against HS but poor efficacy, safety and other side effects (Rahman *et al.,* 2016; Ray & Singh, 2015). The effective and safe vaccine against HS are still lacking (Hussaini *et al.,* 2012a). This may be due to the facts that those vaccines only provides short-term immunity and requires repeated administration for effectiveness (Chandrasekaran *et al*., 1994). A lot of research has been done in order to produce a better vaccine for this disease. For example, immunogenicity study of soluble protein are tested for immunity against HS and as potential vaccine development in future (Jumahat *et al.,* 2015). It is generally perceived that a prefect vaccine should possess certain characteristics, for example, economical to produce, easy to administer, stable for use, no adverse reactions, easy to handle in field and with high level of immunity to control of the disease.

### 1.3 Significance of the study

Immunogenicity and pathogenicity studies of various derivatives, components, and clones has been tested in order to find a suitable immunization for *P. multocida* (Montserrat *et al*., 2004; Tabatabaei *et al*., 2007; Keith *et al*., 2007). The DNA insert was derived from serotype B:2 strain (PMB202) isolated specifically occur in buffalo haemorrhagic septicaemia outbreak in 1991 located at Negeri Sembilan. It was isolated, purified and kindly provided in 1993 by Veterinary Diagnostic Laboratory (VDL), Petaling Jaya, Selangor (Salmah, 1997). Among the selected recombinant plasmids,

ABA392 gene was found toxigenic in rats where it showed mortality in mice and therefore it was further characterised (Salmah Ismail, 1997). The potentials ABA392 gene as a vaccine candidate has been described and carried out in mice model to against haemorrhagic septicaemia (HS). The result from the various vaccinations demonstrated that the gene ABA392 was immunogenic with the 3 dose schedule of both live and killed vaccines can protect the mice when it was challenged with *P. multocida*. The doses of vaccination provided 66% immunity and showed a high titre of antibody production with ELISA test. Stability testing of recombinant plasmid DNA of ABA392/pVAX1 was performed in order to confirm the plasmid was stable and could be reproduced by subculturing them up to 20 generations as described previously (Salmah Ismail, 2000). The potential of the recombinant clone ABA392 derived serotype B:2 strain (PMB202) to protect laboratory animal against HS was determined (Hussaini *et al*., 2012b). Hence, pVAX1 vector is specifically designed for use in the development of DNA vaccine. Currently, there is no specific DNA vaccine for HS in cattle and buffaloes in global. Therefore, the recombinant clone ABA392-pVAX1 as DNA vaccine is immunogenic against HS and this could be used as potential vaccine in future.

## 1.4 RESEARCH OBJECTIVES

### 1.4.1 General objective:

To evaluate the immunogenic potential and protective efficacy of DNA vaccine from pVAX1-ABA392 recombinant clone in rats against HS.

### 1.4.2 Specific objectives:

1. To prepare and analyse DNA insert stability of pVAX1-ABA392 recombinant clone.

2. To access the immunogenicity of DNA recombinant clone pVAX1-ABA392 in vaccinated rats against HS.

3. To evaluate and determinate the histopathological effect in vaccinated rats against HS.

# CHAPTER 2: LITERATURE REVIEW

## 2.1 Haemorrhagic Septicaemia (HS)

The disease of man and animals caused by *Pasteurellae* is termed as Pasteurellosis, which have five types of capsular serotypes A, B, D, E and F. Pasteurellosis has been termed differently in different animal species; haemorrhagic septicaemia (HS) in cattle and buffaloes, fowl cholera in poultry, duck plague in ducks, atrophic rhinitis in pigs, snuffles in rabbits, pneumonic pasteurellosis in sheep and goats, as well as in American bison, yak, deer, hippopotamus, elephants, camels, mink, monkey, cat, horse, elk, tortoise, snow leopard, lion and bubonic plague in humans (De Alwis, 1992). This versatile pathogen has multiple mysteries deeply buried in its genome (Hunt *et al.,* 2000; Harper *et al.,* 2006). Hueppe in 1886 has given a collective name of HS to the infection by *P. multocida* in cattle and buffaloes (Bain *et al.,* 1982).

HS occurs in Southern Europe, Africa, near and middle east countries and throughout Southeast Asia (Joseph, 1979; Bain *et al.,* 1982; De Alwis, 1992). There is two specific serotypes of B:2 (Asian serotype) and E:2 (African serotype) which causes HS disease (De Alwis, 1999). In countries of South and Southeast Asia, HS is caused by serotypes B:2 leading to a fatal systemic disease of cattle and buffaloes (De Alwis, 1995). List B classified as a HS disease by the International Office des Epizooties (OIE), gives 100% mortality to infected animals in endemic areas of Africa and Asia. HS has a high global index as an animal health control to poor farmers (Perry *et al.,* 2002) and the most economically important bacterial disease in these countries (De Alwis, 1999; FAO, 2005; OIE, 2009). Even though there is effective control or eradication rinderpest and continued low mortality from foot-and-mouth disease (FMD), but HS emerged as a major disease of livestock (Benkirane & De Alwis, 2002).

Frequency and distribution of HS fluctuate significantly from a few cases to high numbers upon the type of husbandry practices, geographical area and agro-climatic conditions prevailing in a specific region (De Alwis, 1999; Benkirane & De Alwis, 2002; Perry *et al*., 2002).

## 2.2 Mechanism of Haemorrhagic Septicaemia in animals

The disease occurs in 3 types of form; acute, sub-acute and chronic. The initial phase of temperature elevation are usually unnoticed, the phase of respiratory and the terminal phase of septicaemia involvement which leading to sudden onset and death within 24 hours (De Alwis, 1999). The acute form, the rise of high body temperature (41–42$^{o}$C) and with a rapid thin pulse, shallow respiration and cyanosis of visible mucus membranes in infected animal (De Alwis, 1999; Benkirane & De Alwis, 2002; OIE, 2009). During sub-acute form, the infected are restlessness with mild colic pain, muscular tremors, lacrimation, nasal discharge and diarrhea are also observed. In the chronic form, the course of disease is longer with signs of short, rapid painful respiration with mucopurulent or blood-stained nasal discharge. Besides that, other lesions include fibrinous pneumonia, petechial-to-echymotic hemorrhages, congestion and/or parenchyma of the lungs (Appendix A), pleurisy and pericarditis (De Alwis, 1999; OIE, 2009; Khaleel *et al*., 2014). In addition, swelling of the head, neck, and brisket found in nearly all infected animal. This oedema comprises of blood-stained fluid or coagulated serofibrinous mass with straw colour. The incubation period is usually 1–3 days, with no visible clinical signs and prolonged course extending up to 5 days before sudden death (Carter & De Alwis, 1989; De Alwis, 1999).

### 2.2.1 Diagnosis of the disease in animals

The specimens for diagnosis, blood samples and nasal secretions to be collected from infected live animals and from dead animals are heart blood, liver, spleen, bone marrow and lung (OIE, 2009). For standard techniques, bacterial isolation are isolated by using Brain-heart infusion (BHI) broth and sheep blood agar culture for identification on colony morphology (Holt *et al*., 1994; OIE, 2009); subsequent tests may include inoculation, pathogenicity testing, biotyping and also antibiogram determination. Post-mortem hemorrhage, oedema and hyperaemia are also observed.

This worst outbreak takes place in animal with poor physical condition occurred during the monsoon rains of the year (Benkirane & Alwis, 2002). Control of the natural disease is transmitted by ingestion or inhalation. Various stressful condition such as transportation, climate change and housing management contribute significantly reduces the defense mechanism of the respiratory tract, leading to outbreak of HS in cattle and buffaloes (Zamri-Saad, 2005). The consumption of river water contaminated with infected HS carcasses could be due to the outbreak of HS among cattle and buffaloes (Abdullah *et al.,* 2013). The cases fatality rate of buffaloes is nearly three times higher than cattle. An appropriate precautions should be taken because many of serotypes *P. multocida* have the high potential to infect humans through animal bite or scratch especially cats as healthy carriers (Thais *et al.,* 2015).

## 2.3 Haemorrhagic Septicaemia in Malaysia

In Malaysia, HS continues to be the major cause of mortality in cattle and buffaloes although pigs, sheep and goats are also susceptible to the bacteria (Murty & Kaushik, 1965). Government reports showed that buffaloes were more susceptible to HS than cattle (Chandrasekaran & Yeap, 1978). Losses were estimated at about RM 1.5 million in 1966 in West Malaysia (Thomas, 1972) with an average annual lose estimated at RM

200, 000 for 1967-1976 (Joseph, 1979). The HS outbreaks were at the highest during Nipah virus in the year of 2000. In the year 2005, HS was reported in Perak and all isolations were found to be *P. multocida* serotype B: 2 (Kamarudin, 2005). The quality production of livestock is always a major problem since the animal is constantly fraught with disease, especially HS which threatens the quality of products (Saharee, 2000). This situation is hardening the government's objective for livestock production to reach approximately RM 8 billion by 2010. In the last 12 years, about 8 to 11 outbreaks were reported in the states of Pahang, Terengganu, Kelantan and Perak. However, states of Perlis, Selangor and Johor were free of any HS outbreaks. In February 2006 in Pasir Mas, Kelantan, HS disease was reported with 77 buffaloes were found dead (Lim, 2006). HS outbreak in Rantau Panjang was also reported in *Mingguan Malaysia* newspaper in May 2008. The HS outbreak in Malaysia has caused an average of 360 heads of cattle and buffaloes killed in a year, while approximately 1,000 heads in the Philippines, Myanmar and Cambodia, 600 heads in India and 285 heads in Sri Lanka (Saad, 2013).

According to Terengganu Department of Veterinary Services director, Dr Mohd Termizi Ghazali, the disease had hit three districts in Terengganu since the end of May, with 354 deaths of reared animals, comprising 298 cows and 56 buffaloes with losses of about RM1.8 million (Appendix B), with an average loss of RM5,000 for each animal which died. In addition, 18 animal rearers had been affected by the epidemic, comprising nine in Marang, eight in Kuala Terengganu and one in Kuala Nerus (Bernama, 2017). There is no doubt that outbreak of haemorrhagic septicaemia leads to a devastating effect in livestock industry.

## 2.4 The Organism

### 2.4.1 Genus *Pasteurella*

The genus was named after Louis Pasteur. Genus *Pasteurella* belongs to the family Pasteurellaceae (Bergey *et al.,* 1984), formed by three closely related genera. Therefore, they are grouped together as the *Haemophilus-Actinobacillus-Pasteurella* group (Mannheim, 1981; Kilian & Frederikson, 1981; Bergey *et al.,* 1984). *Pasteurella* is a genus consists of biochemically inactive, nonmotile, gram negative bacilli which often show bipolar staining, particularly with Leishman or methylene blue stain. Most species are catalase-positive and oxidase-positive (Breed *et al.,* 1948). The group is relatively homogeneous, with the exception the tularaemia bacillus, which is set apart by its fastidious nutritive requirement and certain other characteristics, and is differentiable by physiological properties. These bacteria are primarily pathogen of lower animals. The etiological agent of fowl cholera was among the first of them to be described, and it was also the bacterium used by Pasteur in his early studies on immunity.

Members of the bacterial genus *Pasteurella* usually are regarded as opportunistic, secondary invaders in vertebrates. *P. multocida* is the only representative of the genus regarded as a major pathogen. The species of genus *Pasteurella* can be divided into three subgroups on the basis of host relationship and type of disease. The members of the first group cause HS, are found in lower animals. There are two species in this group; *Pasteurella multocida* and *Pasteurella haemolytica.* The members of the second group cause acute, subacute to chronic infections in rodents and are transmissible to man. There are two species in the group; *Pasteurella pestis* and *Pasteurella tularense.* The one species in the third category cause a chronic, focalised caseation necroses in rodents. It is *Pasteurella pseudotuberculosis.*

### 2.4.2 *Pasteurella multocida*

*Micrococcus gallicidus* was first named for this organism by Burril in 1883. It was renamed as *Micrococcus cholerae gallinarum* by Zopf (1885). Trevisan (1885) changed the name to *Bacterium cholerea-gallinarum.* Lignieries (1900) in his 'Zoological Classification' used epithets such as aviseptica, boviseptica and oviseptica to denote the organisms were isolated from birds, cattle and sheep, respectively. This classification was adopted for several years, although not universally. In 1929, the epithet *septica* was introduced by Topley and Wilson, and the name *Pasteurella septica* became widely used in United Kingdom. The combination *Pasteurella multocida* was finally introduced in 1939 by Rosenbusch and Mechant. This nomenclature was universally accepted. A summary of the historical evolution of the nomenclature of *Pasteurella multocida* is shown in Table 1.

*Pasteurella multocida* is a facultative pathogenic bacterium that can infect a wide range of wild and domesticated mammals and avian species. The pathogenic potential of *P. multocida* in vertebrate animals was recognized over a century ago. *P. multocida* can colonize mucous membranes of the upper respiratory and urogenital tract without any symptoms. The organism can produce primary pasteurellosis such as haemorrhagic septicaemia of cattle and buffalo, fowl cholera in various avian species, and atrophic rhinitis in swine. Secondary infection with *P. multocida* may conduce to complicated pneumonia in ruminants, horses and pigs and snuffles in rabbits. Sporadic human infections are associated mostly with cat and dog bites and scratches and with licking of either intact or injured skin by pet animals. Inhalation of the microorganism is a rare route of infection in humans.

Diseases caused by *P. multocida* contribute to a great economic loss on the livestock industry. The organism is transmitted by contact and by the consumption of

contaminated food and water. In some cases, droplet infection is of significance. This has led to intensive research to understand host-adaptation mechanisms and virulence factors in order to develop effective vaccines. Additionally, the desire for rapid diagnostic tests to either complement or substitute traditional methods and gather more information over general features of this bacterium (identification of species, capsular type, somatic serotype, toxicity) is present.

**Table 1:** History of the nomenclature of *Pasteurella multocida* (Namioka, 1978)

| ***Author*** | ***Year*** | ***Name*** |
|---|---|---|
| Bollinger | 1879 | - |
| Pasteur | 1880 | - |
| Burril | 1883 | *Micrococcus gallicidus* |
| Zopf | 1885 | *Micrococcus cholerae-gallinarum* |
| Kitt | 1885 | *Bacterium bipolare multocidium* |
| Oreste and Armani | 1887 | *Bacillus septicaemiae* |
| Trevisan | 1887 | *Pasteurella cholerae-gallinarum* |
| Lehmann and Neumann | 1889 | *Bacterium multocidium* |
| Sternberg | 1893 | *Bacterium septicaemiae haemorrgicae* |
| Lignieres | 1900 | According to host species: *Pasteurella aviseptica* *Pasteurella boviseptica* *Pasteurella suiseptica etc* |
| Topley and Wilson | 1929 | *Pasteurella septica* |
| Rosenbach and Merchant | 1939 | *Pasteurella multocida* |

### 2.4.3 Pathogenicity of *Pasteurella multocida*

The term 'pathogenicity' in general means ability to produce disease. Outbreaks of HS occur during periods of environmental stress, when the changes in weather causes immunosuppression to the hosts. During the intervening periods the causative organism persists in the tonsil and naso-pharyngeal regions and such animals serve as 'carriers' of the disease (Mustafa *et al.,* 1978; Hiramune & De Alwis, 1982; De Alwis *et al.,* 1990; Annas *et al.,* 2014). Spread of the disease occurs by the ingestion of the contaminated foodstuffs and inhalation of infected aerosol which may originate from clinically normal 'carriers' or clinical cases.

The initial site of multiplication is the tonsil region, upon entry of the *Pasteurella* organism into the animal. The interaction between the virulence of the organism and its rate of multiplication *in vivo* depends on the outcome of this infection (De Alwis *et al.,* 1986). Thus, the dose of infection is a important factor and clinical disease will occur if the organism overcomes the host's defence mechanisms. If the defence mechanisms dominate over the organism, what is described as an "arrested infection" occur, and the animals become an immune animals and contribute to the "herd immunity" (De Alwis *et al.,* 1986). It was established that the highly-virulent *Pasteurella multocida* strain was capable of intracellular reproduction in the cells of the macrophages (Moshkin, 1975) and the protective course of infection was largely determined by its high rate of reproduction and its capacity to paralyze the protective function of leukocytes of the host (Moshkin, 1975).

It is believed that bacterial adhesion to host cells is a crucial step in colonization and eventually the pathogenesis of the disease (Esslinger *et al.,* 1993). Adherence is a complex interaction between the bacterium and the target cell in which enables colonization to occur and allows the bacterium to exert its pathogenic and immunogenic

effects on the host. Many bacteria produce proteins, known as exotoxins that modify, by enzymatic action, or otherwise destroy certain cellular structures. Examples proteases, collagenase and hyaluronidases, which act extracellularly. *Clostridium perfringens* produces potent collagenase, whilst *Staphylococcus aureus* produces a hyaluronidase. Damages to the connective tissue matrix by hyaluronidase and collagenase can "loosen up" the tissue fibers allowing the organism to spread through the tissues more easily. The main component among the structures of *Pasteurella multocida* that is found to be involved in adhesion to the host cells is the capsule, although in 1993 Jacques *et al* has revealed that the capsule is a virulent factor of *Pasteurella multocida* to the cells of the respiratory tract cells and mucus.

It has been observed that *Pasteurella multocida* B:2 produced hyaluronidase, although the role of this enzyme in pathogenesis of haemorrhagic septicaemia was not been thoroughly studied. However, the role of *Pasteurella multocida* B:2 in pathogenesis is thought to be dissimilar as those of *Staphylococcus aureus* that damages the connective tissue to allow better spreads of the organism (De Alwis, 1999). The mechanisms whereby pathogenic bacteria adhere to the mucosal surfaces of animal tissues, prior to colonisation, have contributed greatly to the understanding of the pathogenesis of important bacterial infections. This pathogenesis, which could have important implications for vaccination, has received little attention in *Pasteurella* infections in general and none to date in HS.

## 2.5 Bacteriology of *Pasteurella multocida*

### 2.5.1 Growth characteristics and identification

*Pasteurella multocida* easily grows in most common laboratory media such as nutrient agar. Special media such as dextrose-starch agar and casein-sucrose-yeast (CSY) medium support an abundant growth of the bacteria. Besides that, blood agar and

CSY agar with 5% blood (bovine, sheep) are a convenient media for routine laboratory culture. The optimum growth temperature is 35-37ºC. In enriched media at 37ºC, colonies 1-3 mm in diameter are produced after 18-24 hours culture. *P. multocida* will not growth on MacConkey agar but can grow poorly on some Cystine lactose electrolyte deficient (CLED) agars and sensitive to penicillin. *Pasteurella* species have both an oxidative and fermentative metabolism. Glucose and other cardohydrates are catabolised with the production of acid but no gas. Most species are catalase-positive and oxidase-positive; nitrates are reduced to nitrites by almost all species. Typical strains of *P. multocida* are not difficult to identify. Although there are now fewer unidentifiable *Pasteurella*-like organisms than there were before the recognition of *Pasteurella pneumotropica* (Jawetz, 1950) and *Pasteurella gallinarum* (Hall *et al.,* 1955), strains are encountered that do not altogether confirm to the accepted criteria for *Pasteurella multocida.* These are usually referred to as *Pasteurella*-like organisms.

### 2.5.2 Staining

In fresh cultures and animal tissues, *P. multocida* grown *in vivo* gives typical bipolar appearance when stained with Leishman or methylene blue stains. The effect of bipolar staining is best seen in more mature bacteria, e.g. in twenty four-hour cultures of recently isolates or in animals which had died of pasteurellosis. Younger bacteria in living animals or in cultures may have a more uniform solid appearance. The bipolar appearance is no longer seen after a few sub-cultivations. The capsules are best demonstrated with green light in the phase-contrast microscope by wet India ink method; the ink should be bacteria-free and the film should be made thin by pressing on the cover slip with blotting paper. The Congo red-serum-HCL method, followed by a counterstain of alkaline crystal violet, is useful in experienced hands. It is difficult to stain the capsules positively except with alcian blue of related dyes, using saturated solutions and prolonged staining (overnight).

### 2.5.3 Morphologic Features

*P. multocida* is a nonmotile, pleormorphic, nonspore-forming, bipolar Gram-negative coccobacillus short rod, 0.2 - 0.5 μm by 0.5 – 1.0 μm in size which grows aerobically on ordinary nutrient agar (Namioka, 1978; Carter, 1967). *P. multocida* does not cause haemolysis on blood agar. Repeated laboratory subcultures of old cultures or cultures grown under unfavourable conditions tend to be pleomorphic and longer rods and filamentous forms. In tissues, exudates and recently isolated cultures, the organism shows the typical coccobacillary forms. The organism shows different types of colonies, which are related to the capsular type. The largest colonies produce by capsular type A, which are translucent, greyish in colour, and mucoid in consistency.

### 2.5.4 Serologic Classification

Various studies have been carried out to identify the different serotypes of *P. multocida.* Indirect hemagglutination (IHA) test, the most widely used for identification of *P. multocida* (Table 2).

**Table 2:** Serologic classification of *Pasteurella multocida* (Salmah, 2009)

| Author identified | Basic | Types |
|---|---|---|
| **Capsular typing** | | |
| Carter (1955) | Indirect haemagglutination (IHA) | A,B,C,D |
| Carter (1961) | IHA | E |
| Carter (1963) | IHA and passive mouse protection | Excluded |
| Type C | | |
| Namioka and Murata (1961a) | Slide agglutination of fresh cultures | A,B,D,E |
| Rimler and Rhoades (1986) | IHA | F |
| **Somatic typing** | | |
| Namioka and Murata (1961b) | Agglutination of HCI-treated cells | I-II |
| Namioka and Bruner (1963) | | |
| Namioka and Murata (1964) | | |
| Heddleston *et al.* (1972) | Agar gel precipitation test using 1-hour boiled supernatant | 1-16 |

## 2.6 Vaccination for prevention and controlling of HS

Haemorrhagic septicaemia is primarily a bacterial disease. Therefore it should theoretically lend itself to effective antibiotic therapy. However, treatment is constrained by a number of factors. The acute nature of most cases of the disease limits the efficacy of antimicrobial therapy of sick animals, but it can be effective if they are detected and treated in the early stages of the disease. As the disease occurs in place with sub-standard husbandry practices, most cases will escape early detection. For treatment and prevention, various sulfonamides, tetracyclines, penicillin, and chloramphenicol are an effective antibiotics if administered early. Although multiple antibiotic resistance has been reported for some strains of *P. multocida*, it has not been described for the HS serotypes yet. A live vaccine prepared from a B:3, 4 serotype a fallow deer strain of *P. multocida* has shown considerable promise with protection for as long as a year. This strain, serotype B:3, 4, is closely related immunologically to serotype B:2 but is less virulent.

Effective treatment for HS cases is difficult due to its sudden death in the infected animals. Field experienced has shown that treatment of infected animals must begin before the appearance of clinical signs, to expect a good response (Singh, 1966; Thomas, 1972; Bain *et al.,* 1982). If the animals are depressed, weak and demonstrate labored breathing, the disease is often so advanced that the response to treatment would be unsatisfactory.

Vaccination appears to be an alternative effective control option. A solid, long-lasting immunity is conferred on animals that recover from the natural disease, which persists longer than that induced by vaccination (De Alwis, 1999). HS is preventable using vaccines containing the causative bacterial agent. Since *Pasteurella* is a poor

immunogen, a large amount of antigen has to be administered. This procedure leads to endotoxic shock (De Alwis, 1999). At the moment, three types of vaccine are widely used: formalinised bacterin, alum-precipitated-type vaccine, and oil-adjuvanted vaccine (Appendix C). An oil-adjuvant vaccine for HS was first developed in the 1950s (Bain and Jones, 1955). In Asian countries, some significant success in the control of HS has been achieved by the immunization of buffaloes and cattle with alum-precipitated and oil-adjuvant bacterins (Carter & De Alwis, 1989). For the duration of immunity, the vaccine is lasting from six to nine months on primary vaccination and 12 months after secondary vaccination. An outbreak of HS in Zambia in 1979 was largely controlled by using formalinised bacterin obtained from Sudan (Francis *et al.,* 1980). Alum-precipitated vaccine is the most common vaccine has been used since it is the easiest vaccine to inject. The most effective vaccine is the oil-adjuvant—one dose provides protection for 9-12 months and it should be administered annually. Whereas the alum-precipitated-type bacterin is given at 6-months intervals. The oil-adjuvant bacterin has the advantage of requiring only one dose annually, but it has the disadvantages of being difficulty in syringing of their high viscosity and occasional adverse tissue reactions. In the past decade, an amount of research has been done in South Asia which aimed in producing oil-adjuvant vaccines with low viscosity. It is known by one of the authors (Lubroth *et al.,* 2007) that Sri Lanka and Indonesia have successfully used lower levels of lanoline as the emulsifying agent to reduce viscosity. In India, at least one vaccine producer is marketing a combined FMD-HS-Blackquarter oil-adjuvant vaccine.

### 2.6.1 Vaccination for HS in Malaysia

In Malaysia, the first report of prophylactic measures taken was in 1929 when antisera and vaccine imported from Imperial Veterinary Research Institute, Mukteshwar, India, were used to immunize cattle and buffaloes in Kedah (Cheah, 1960). This broth vaccine contains a suspension of formalin-killed bacteria (broth

bacterin). It is adsorbed fairly rapidly and provides quick protection by the fifth day. Later, the Institute of Medical Research (IMR), Kuala Lumpur, and the Veterinary Research Institute (VRI), Ipoh, also produced the vaccines using local strains. The oil-adjuvant vaccine (OAV) production in Malaysia was started in 1966 at the VRI, Ipoh. The procedure used was similar to that described by Bain and Jones (1955). The use of the alum-precipitation technique has been modified to concentrate broth cultures in order to reduce the dose volume of the oil-adjuvant in the vaccine formulation in which they believe it will facilitate injection (unpublished data). An ideal control measure can only be formulated based on a good knowledge of the disease in the local area. In Malaysia, apart from the vaccination carried out annually and disease occurrences reported by veterinarians, a live vaccine prepared from B:3,4 serotype a fallow deer strain of *P. multocida* has shown a promising protection for as long as a year. This strain serotype is closely related immunologically to serotype B:2 but is less virulent.

### 2.6.2 DNA vaccine for Haemorrhagic Septicemia

Multiple antibiotic resistances strains of *P. multocida* have also been accounted. Yet, prevention would be a better choice than treatment. Vaccination has been the best key to against HS outbreaks in countries (Dabo *et al*., 2008). As early as 1928, HS vaccines have been in use. In 1978, the first effective vaccine was reportedly produced in Malaysia, for its ability as immunogen and lipopolysaccharide (Chandrasekaran & Yeap, 1978). However, it only provides partial immunity against HS caused by *P. multocida* serotype B:2 when experimented on mice (Adler *et al*.,1996). But, lipopolysaccharide-protein complex isolated from a potassium thiocyanate extract, provide 100% protection in mice (Ryu & Kim, 2000). Several studies had been carried out in several outer membrane proteins (OMP) of *P. multocida* serotype B:2 and was found that the 37kDa OMP had the potentials to be a candidate vaccine (Tomer *et al*., 2002). It was found that OMP provided 67% survival rate among vaccinated mice

(Srivastava, 1998). Vaccine using inactivated *P. multocida* serotype B:2 cells are well-studied by demonstrating to be safe and protective when calves were challenged after a year of vaccination with virulent strain of *P. multocida*. A percentage of 100% protection was achieved in vaccinated animals while in unvaccinated control groups, all signs and symptoms of HS were seen (Shah *et al*., 2001). Live attenuated vaccines are considered to render better immunity compared to killed vaccines to the host, as they contain live organism in attenuated form or reduced virulence and the other factor is the natural route of entry (Hodgson *et al*., 2005). This vaccine provided 83% protection to calves when challenged after six months with virulent strain and only 33% protection was achieved one year post vaccination (Myint *et al*., 1987). In spite of live vaccines providing protection against *P. multocida* serotype B:2, there have been reports on wide range of protection between 33% to 88%. It was found that intramuscular vaccination with live vaccine did not protect the animal following a subcutaneous challenge (Carter *et al*., 1991). *P. multocida* cexA mutant use as vaccine proved to be immunogenic and provided significant protection against HS (Boyce & Adler, 2001). Mutation in the *aro*A gene is another modified avirulent *P. multocida* type (Rafidah *et al.,* 2011; Saleem *et al.,* 2014). The *aro*A gene is involved in the conversion of shikimic acid to chorismic acid, a common intermediate in the biosynthesis of aromatic amino acids. The mutated type will not be able to grow in the host since the mammalian cells lacks the pathway in synthesis of aromatic compounds.

Gene cloning and expression resulted in the generation of various recombinant proteins from *P. multocida*, which represent only the part of an organism yet stimulate relatively strong immune responses (Hatfaludi *et al*., 2010). Several genes involved in virulence and immunity in *P. multocida* were localized and targeted as having potential in the development of subunit vaccines (Fuller *et al*., 2000; May *et al*., 2001; Hatfaludi *et al*., 2010). Consequently, DNA vaccination in other species particularly in outbreed

large animals of important economic interest, such as cattle, still remains unpredictable. Infectious diseases remain one of the leading causes of morbidity and mortality of humans and livestock worldwide. There is considerable promise that current frontier areas of research involving new technology on the development of effective new age HS vaccines may prove effective in control of HS (Shivachandra *et al.*, 2011).

### 2.6.3 Recombinant Clone ABA392 (Accession No. DQ334273)

Plasmid has been used in several studies in order to learn more about the pathogenicity and virulence mechanism (Shivashankara, 2000) of the *P. multocida.* Originally, the recombinant clone ABA392 was constructed in *E.* coli system via genomic shotgun cloning and the DNA insert was further characterised (Salmah, 1997). The DNA insert was derived from *Pasteurella multocida* serotype B strain (PMB202) isolated specifically from haemorrhagic septicaemia outbreak buffalo in 1991 located at Negeri Sembilan. It was isolated, purified and kindly provided in 1993 by Veterinary Diagnostic Laboratory (VDL), Petaling Jaya, Selangor (Salmah, 1997). Briefly, the extracted PMB202 genomic DNA was partially digested with the restriction endonuclease *Sau*3A1, generating DNA fragments of approximately 500 to 1000 bp before inserted at the unique *Bam*HI site of plasmid DNA pUC18 (Salmah, 2004). Among the selected recombinant plasmids studied, ABA392 was found toxigenic in rats where it showed mortality in mice and therefore it was further characterised (Salmah, 1997). The recombinant clone ABA392 size was estimated at 3.2kb with the insert DNA of 921bp (Salmah, 2004). The potentials of the clone ABA392 as a vaccine candidate against haemorrhagic septicaemia (HS) has been described and explored in mice model where the result from the various vaccinations showed that the clone ABA392 was immunogenic with the 3 dose schedule of both lived and killed vaccines can protect the mice when challenged with *P. multocida*. The 3 dose vaccination provided 66% protection and ELISA results showed a high titre of antibody production.

Stability testing of recombinant plasmid DNA of ABA392/pVAX1 was performed in order to make sure the plasmid was stable and could be reproduced by subculturing them up to 20 generations as described previously (Salmah Ismail, 2000). The potential of the previously contracted recombinant clone ABA392 derived from *Pasteurella multocida* serotype B to protect laboratory animal against haemorrhagic septicaemia (HS) was determined (Hussaini *et al*., 2012b). It was found that the recombinant clone ABA392 is immunogenic and could be used as vaccine in future.

# CHAPTER 3: MATERIAL AND METHODS

## (A) Material

### 3.0 Bacteria strain, plasmids and clones

The bacteria strains used in this research was the virulence gene recombinant clone ABA392 previously produced (Salmah, 1997 & 2004), was derived from parental strain, which is *Pasteurella multocida* Serotype B:2 (PMB202), ABA392 were found toxigenic to mice. These bacteria strains was provided by Prof Dr. Salmah Ismail, Molecular Bacteriology and Toxicology Laboratory (MBT Lab), Faculty Science, University Malaya.

### 3.1 Polymerase Chain Reaction (PCR)

#### 3.1.1 Primers

Set of primers used in this research project. The primer was synthesized from MyTACG Bioscience Enterprise. The primers were specially designed for DNA sequencing analysis in which the primers were used to sequence the gene of interest, ABA392 (804bp) designed based on parental PMB202 strain. This primer was use during PCR amplification, colony screening and gene insert analysis. The DNA sequence below shows the designed primers:

**ABA392/PMB202 for pVAX1 (*Hin*dIII and *Bam*HI)**

**Forward: F-5′-ATGAGTCTTTTGTTCTGTCGCTTG-3′**

**Reverse : R-5′-CTAGCGGCGGTGGAAACCCGCCAT-3′**

Note: All the primers used in this research were kept in fridge -20°C.

### 3.1.2 Mastermix

Econotaq Plus Green 2x mastermix with total volume of PCR mix 25µl have been used in this research. The master mix consists of 0.1units/µl of Econotaq DNA polymerase, reaction buffer (pH 9.0) dATP, 400µmdGTP, 400µm dCTP, 400µm dTTP, 3mm $MgCI_2$ and PCR enhancer/stabilizer and blue/yellow tracking dyes. The PCR mix composition method were described in Section 3.14. The mastermix stored at -20°C.

### 3.1.3 Electrophoresis

### 3.1.4 Agarose Gel

The materials involved in preparing agarose gel for gel electrophoresis is purchased from Promega (USA), 1x TBE buffer (Tris/Acetic Acid/ EDTA buffer, pH8.0) which dilute from 50x TBE buffer (Bio-Rad). The concentration of agarose gel was depending on the amount of agarose powder used. There were two types of gel concentration used that were 0.7% and 1% agarose.

#### 3.1.4.1 Agarose (0.7%)

The mass of agarose powder used in this gel was about 0.7% w/v of 1X TBE buffer volume. This concentration allows better separation of bands for PCR samples compare with plasmid samples. The standard amounts used for preparing the gel were as below.

Agarose powder : 0.35 g

1x TBE buffer : 50 ml

To prepare the agarose gel, 0.35g of agarose powder were diluted in 50ml 1x TBE buffer and heated in a microwave oven for a minute at medium high temperature. The solution was poured into gel loader and was let to solidify for 20 minutes after the well comb was inserted. The well comb was removed and the gel loader was ready to be used for gel electrophoresis.

### 3.1.4.2 Agarose (1.0%)

For 1.0 % agarose gel, the mass of agarose powder used was 1% w/v of 1x TBE buffer and the gel was used in band separation of plasmid samples. This is because PCR samples separates slower in higher agarose concentration compared to 0.7% agarose gel.

### 3.1.4.3 50x TBE Tris/Acetic Acid/EDTA buffer

The solution was diluted to 1x (100ml) strength as a working solution into 4900ml $dH_2O$. The buffer was used for agarose gel electrophoresis and stored at room temperature.

### 3.1.4.4 DNA markers and loading dye

There are three types of DNA marker, 1kb Plus DNA Ladder (0.1µg/µL), Gene ruler 1kb plus DNA ladder (0.5µg/µL) and supercoiled DNA ladder (500µg/ml) purchased from Invitrogen used in the research. The volume used is about 1 µl to 5 µl. Meanwhile, the gel loading dye purple (6x) and volume that used about 1µl (1 volume of the dye solution, 5 volumes of the DNA sample) purchased from Promega (USA). The DNA marker and loading dye used in the process were kept in fridge at -20°C.

### 3.1.4.5 Gel Red™ Staining

Gel is stained in gel red staining once the gel electrophoresis is done. The Gel Red™ 10000x stock solution is diluted 3x of staining solution in distilled water. The staining preparation as below:

Gel red : 30 µl

Sterile distilled water : 100 ml

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

## 3.2 DNA cloning

### 3.2.1 Vectors

The vector used in the research were pVAX1© mammalian DNA vaccine vector (pVAX1 without *LacZ* gene which has size of 2999bp) which provided MBT Lab and kept in fridge at -20 ºC.

### 3.2.2 Restriction endonuclease (RE)

The RE used in the research project was *Hin*dIII and *Bam*H1 enzymes. These enzymes were used together with 10x restriction enzyme buffer during digestion process. These solutions are also purchased from New England BioLabs. The RE for both enzymes are shown as stated:

*Hin*dIII : AA GCT T

*Bam*H1 : GG ATC C

Note: All the RE used in this research was kept in fridge at -20°C.

### 3.2.3 DNA ligation solutions

The DNA ligation solutions used in this research were 10x T4 DNA Ligase Reaction buffer (consists of Tris-HCI, $MgCI_2$, ATP and DTT) to provide optimum environment, vector system, PCR product (which is the desired gene, ABA392), and also T4 DNA ligase whereby the enzyme is used to ligate sticky ends of the DNA fragments. All of these solutions were kept in fridge at -20 ºC. The proper volume used is further described in the method section.

## 3.3 Apparatus

The machines involved in the research were such as the incubator (manufactured by Binder, Germany), weighting balance (manufactured by A&D Company Limited, Japan), volt power supply machine (manufactured by Hoefer® Inc., USA) and Super Bright UV trans-illuminator (manufactured by Vilbert-Lourmat, USA) for gel electrophoresis, hot plate or magnetic stirrer (manufactured by Sigma Corp., UK), oven, Polymerase chain reaction 96 well thermal cycler, Sorvall ST 40R Centrifuge (4°C), incubator shaker is from Thermo Scientific, U.S.A meanwhile gel doc (EZ) imager is from Bio-Rad, U.S.A. Mini-spin (eppendorf), Fisher Scientific, U.S.A. Centrifuge machine (manufactured by Hettich Zentrifugen, Germany), microcentrifuge machine (manufactured by Sigma, UK), vortex Maxi Mixed ii machine (manufactured by Barnstead, USA), autoclave machine (manufactured by Hirayama, Japan), spectrophotometer (manufactured by Secomam, USA), water bath (manufactured by Memmert, Germany), -20°C/4°C freezer (manufactured by Thermo Electron Corp., USA). Bench top professional pH meter-BP3001 is from Transinstrument, Singapore. Magnetic stirrer-Cimarec 2 is from Thermolyne, Canada. Water bath-Grant is from sub-aqua, pro instrument, UK. Orbital shaker 110V is from Komabiotech, Korea.

Meanwhile, other apparatus needed are such as the pipettes, tips, tips container and micropipette (manufactured by Eppendorf Research, Germany). Others are including Petri dishes, Bunsen burner, inoculate loops, felcon tube, universal bottles, ice box and floater.

### 3.4 Media and broth

The main ingredients for media and broth preparation in the research were Luria-Bertani (LB) and Brain Heart Infusion (BHI). However, most of the preparation was done in LB broth, agar and slant instead of BHI. BHI was only used to increase the yield of positive colonies. Both BHI (BBL$^{TM}$) and LB (Difco $^{TM}$) powders were purchased from Becton, Dickson, and Company which is manufactured at USA.

#### 3.4.1 Brain Heart Infusion (BHI) broth

BHI broth powder : 37.0 g

Distilled water : 1000.0 ml

The broth was mixed and about 10ml distributed into universal bottles and autoclaved on liquid cycle at 121ºC for 2 hours. This broth was used to culture *P. multocida* isolates for plasmid isolation.

#### 3.4.2 LB/Ampicillin/Kanamycin broth

Standard LB broth powder : 20.0 g

Distilled water : 1000 ml

Ampicilin (100µg/ml) : 1000 µl

Kanamycin (30µg/ml) : 1000 µl

The standard LB broth was dissolved and stirred in 1000ml $dH_2O$ before 10ml of the mixture aliquot in each universal bottle. It was autoclaved on liquid cycle at 121 ºC for 2 hours before it can be directly used or added with antibiotics. Ampicillin and kanamycin were then added under sterile condition. This broth was used for clone selection.

### 3.4.3 LB/Ampicillin/Kanamycin agar

Standard LB agar powder : 35.0 g

Distilled water : 1000 ml

Ampicillin (100µg/ml) : 1000 µl

Kanamycin (30µg/ml) : 1000 µl

The LB broth was prepared, autoclaved on liquid cycle at 121 ºC for 2 hours and cooled to 45-45 ºC. Ampicillin and kanamycin were then added under sterile condition. This medium was used for clone selection.

## 3.5 Antibiotic stock solutions

Antibiotic stock solution for ampillicin (100 mg/ml) and kanamycin (100 mg/ml) were prepared as 1000 times working concentrations and whenever needed were sterilized by filtration through sterile membrane filters (type PES, 0.22 µm pore size, Millipore Corp. USA) Millex-GP 33mm Syringe Driven Filter Unit. The stock solutions were then dispensed in 1 ml aliquots into sterile 1.5 ml microcentrifuge tubes for storage at -20 ºC. Upon removal from the cold, working stocks were allowed to come to room temperature prior to use. Appropriate volume was then added aseptically to sterile medium to give the final antibiotic concentration required. The antibiotic stock solution was prepared especially for cloning and recombinant clone cultivation.

## 3.6 DNA Purification and Extraction kit

Plasmid DNA purification and PCR clean-up Gel extraction kits have been used in this research.

### 3.6.1 Plasmid DNA Purification Kit NucleoSpin® (MACHEREY-NAGEL)

Components:

| | |
|---|---|
| Cell Resuspension Solution (Buffer A1) | 15.0 ml |
| (50 mM Tris-HCI,pH 7.5; 10 MM EDTA; 100µg/ml RNase A | |
| Cell Lysis Solution (Buffer A2) | 15.0 ml |
| (0.2 MNaOH and 1% SDS) | |
| Neutralization Solution Buffer A3 | 20.0 ml |
| (4.09 Mguanidine hydrochloride; 0.759 M potassium acetate) | |
| Column Wash Solution | 20.0 ml |
| (35 ml of 95% ethanol added before use) | |
| Elution Buffer AE | 15.0 ml |
| RNase A (lyophilized) | 6.0 mg |
| NucleoSpin® Plasmid QuickPure Columns | 50 pc |
| Collection Tubes (2ml) | 50 pc |

### 3.6.2 Buffer A1

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

### 3.6.3 Buffer A4

Buffer A4 added with 24ml of 96% ethanol and mixed thoroughly.

## 3.7 DNA Gel extraction

The DNA extraction kit manufactured from Macherey-Nagel to extract gene of interest, ABA392 from the agarose gel. The kit known as PCR clean-up Gel Extraction Kit (NucleoSpin® Gel and PCR Clean-Up).

Components:

| | |
|---|---|
| Binding Buffer NT1 | 2x25.0 ml |
| Wash Buffer NT3 | 20.0 ml |
| Elution Buffer NE | 15.0 ml |
| NucleoSpin® Gel Columns | 50 pcs |
| Collection Tubes (2ml) | 50 pcs |

### 3.7.1 Wash Buffer NT3

80mL of 96% ethanol is added into wash buffer NT3 and mixed thoroughly.

## 3.8 Competent cells

### 3.8.1 Type of cells

The host competent cells used were from the *Escherichia coli* (*E.coli*) strains which TOP10 strain was used in the pVAX1 transformation.

### 3.8.2 Solutions for preparing competent cells

The concentration and volume for the solutions needed in preparing competent cells were as below. Generally, the solutions are bought from Sigma which is based at UK.

Autoclaved glycerol : 600 µl

Two LB broth : 2 x 10 ml

A single colony was incubated into 10ml LB broth overnight at 37 ºC and shaking 250 rpm. Then, 600µl of the overnight culture was added into 600µl of autoclaved glycerol, and was mixed and kept in -80 ºC as glycerol stock. Meanwhile, 5 ml of the overnight culture was taken and inoculated into another 50ml of LB broth. The sub-culture was incubated for 2 hours at 37 ºC and shaking 250rpm to promote colony growth until the spectrophotometer showed the value of optical density of ~0.5 at 650nm (or incubated about 2 ½ hours or until the "log phase" of growth was achieved).

Once achieved, the culture was separated into two felcon tube, 25 ml each and culture was then centrifuged at 4 ºC, 2400 X G for 1 hour. The supernatant was discarded and the cells were resuspended in 1000µl of 1x TSS solution. The resuspended cells were placed on ice for 5 minutes. After that, the cells were aliquot into microcentrifuge tube, with each containing 100µl of competent cells. The aliquot cells were kept in freezer at -80 ºC.

## 3.9 Enzyme-Linked Immunosorbent Assay (ELISA)

### 3.9.1 Coating Buffer, pH 9.6

| | |
|---|---|
| Sodium Carbonate | 5.3 g |
| Sodium Bicarbonate | 4.2 g |
| NaOH/HCI | Adjust pH |
| Distilled water | 1000 ml |

### 3.9.2 Coating Antigen

About 30 uniform single colonies of *P. multocida* B: 2 were 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 phosphate buffered saline (PBS) and centrifuge at 4000rpm for 30minutes respectively. The pellet was resuspend with 200ml of coating buffer (Section 3.9.1) and boiled at 97ºC for 20 minutes. The inoculum was left to cool and was allocated into 1.5ml microcentrifuge tubes. Stored at -20ºc till prior to use.

### 3.9.3 Washing Buffer, pH 7.4

| | |
|---|---|
| Sodium chloride, NaCl (58.44) | : 8.0 g |
| Potassium dihydrogen phosphate, $KH_2PO_4$ (136.09) | : 0.2 g |
| Sodium phosphate dibasic, $HNa_2O_4P.2H_2O$ (141.96) | : 2.9 g |
| Potassium chloride, KCl (74.55) | : 0.2 |
| Tween 20(added after autoclave) | : 0.5 ml |
| Distilled Water | : 1000 ml |
| 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 till prior to use.

### 3.9.4 Blocking Buffer, pH7.4

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

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

### 3.9.5 Tetramethylbenzidine (TMB)

This solution was purchased commercially stored at 4ºC.

### 3.9.6 Stopping Buffer

| | |
|---|---|
| Acid sulphuric, $H_2SO_4$ (98.00) | :146.8 ml |
| Distilled Water | : 1000 ml |

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

### 3.9.7 Secondary antibody

Ig G antibody (HRP) conjugate were bought commercially and stored at 4ºC.

## 3.10 Histopathology Analysis

### 3.10.1 Mono Basic Phosphate (0.1M)

Mono Basic Phosphate $NaH_2PO_4$ (Mol. Wt 156) : 15.6 g

Distilled Water : 1000 ml

15.6g is dissolved in 1000mL distilled water to make 0.1M solution. This stock solution is stored at room temperature.

### 3.10.2 Dibasic Phosphate (0.1M)

Dibasic Phosphate $Na_2HPO_4.2H_2O$ (Mol Wt. 178) :17.8 g

Distilled Water : 1000 ml

17.8g is dissolved in 1000mL distilled water to make 0.1M solution. 1000mL of distilled water is filled in beaker and start to stirring. Small amount of dibasic phosphate is added. Dibasic phosphate is added gradually until the whole amount is dissolved. This stock solution is stored in room temperature.

### 3.10.3 Basic Phosphate (0.1M PB)

0.1M Mono, $NaH_2PO_4$ (Section 3.10.1) : 500 ml

0.1M Di Basic Phosphate, $Na_2HPO_4.2H_2O$ (Section 3.10.2) : Adjust pH

500ml of 0.1M Mono Basic Phosphate added into Scott bottles. pH is adjust using 0.1M Di Basic Phosphate until pH reach 7.3. This solution is stored at room temperature.

### 3.10.4 Neutral Buffer Formalin (10%), pH 7.3

0.1M PB (Section 3.10.3) : 500 ml

Formalin (37%) :100 ml

Distilled Water : 400 ml

All the solution was added together to obtain 1000ml of 10% buffered formalin. This solution is stored at room temperature.

### 3.10.5 Mayer's Albumin

Fresh egg white : 50 ml

Glycerol : 50 ml

Sodium Salicylate, $C_7H_5NaO_3$ (160.11) : 1 g

All the solution is mixed together and stored at room temperature or 4ºC.

### 3.10.6 Histopathology Staining

#### 3.10.6.1 Harris Hematoxylin

This solution was purchased commercially and stored at room temperature. Harris hematoxylin is filtered and 50ml is aliquot in falcon tube prior to use.

#### 3.10.6.2 Eosin Y

Eosin Y : 100 ml

Glacial Acetic Acid, $CH_3COOH$ (60.05) : 0.5 ml

Acidified Eosin Y solution, Aqueous is prepared by slowly adding 0.5mL of glacial acetic acid per 100mL. 50mL of Eosin Y is aliquot in falcon tube. This solution is stored at room temperature.

## (B) Methods

### 3.11 Storage and maintenance of bacterial cultures

For long term storage, overnight broth cultures of *P. multocida* and *E.coli* (TOP10 strain) are mixed with equal volume of sterile 60% glycerol and stored at -20ºC in sterile tubes. The frozen stock cultures were plated on BHI agar medium supplemented with 5% (v/v) Ox-blood and incubated at 37 ºC. Working stock cultures were sub-cultured every two weeks. Bacterial cultures were also maintained on agar slants for a period of 3 to 5 months.

### 3.12 Washing procedures

All glassware and items were soaked overnight in a diluted teepol solution and scrubbed with suitable brushes and were rinse thoroughly with tap water followed by distilled water. Then, they were allowed to dry in oven (60ºC).

### 3.13 Sterilization

Sterile conditions were applied throughout the experiment, all media, reagents, chemicals, distilled water and all plastic ware such as pipettes tips and microfuge tubes were sterilized in autoclave on liquid cycle at 121 ºC (50psi) for 45 minutes. Glassware such as conical flasks, beakers, measuring cylinders, bottles (universal and bijou) was sterilized in a hot air oven at 180ºC for 2 hours. Antibiotic and other solutions were sterilized using Millipore filters.

### 3.14 Polymerase Chain Reaction (PCR) screening

The solutions used in PCR were independent of the concentration of DNA template used in each PCR reaction. Similar to the ligation solution, these PCR solutions were bought under EconoTaq® Plus Green 2x Master Mix (Lucigen). Table 3 shows the volumes of each solution and samples used to prepare one portion of master mix that is in each PCR tube:

**Table 3:** Volume of solutions and samples involved in PCR master mix for each PCR tube.

| Required Solutions | Volume (µl) per PCR tube |
|---|---|
| $dH_2O$ | 7.5 |
| PCR Buffer (Master Mix) | 12.5 |
| F primer | 0.5 |
| R primer | 0.5 |
| DNA template | 4.0 |
| **Total master mix volume per tube** | **25.0** |

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 3.1) 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 3.1.4.1).

**Table 3.1:** The PCR was run for 30 Cycles

| | |
|---|---|
| 95ºC | 2 minutes |
| ---------- | |
| 95ºC | 0.30 seconds |
| 64ºC | 0.30 seconds |
| 72ºC | 0.30 seconds |
| ---------- | |
| 72ºC | 10 minutes |

### 3.15 ABA392/pUC57 Purification using Plasmid DNA purification

Purification ABA392/pUC57 isolated using NucleoSpin® Plasmid DNA purification Kit (Section 3.6.1). LB broth added ampicillin with concentration of 100µg are used to grow a single colony of ABA392/pUC57 at overnight in 37°C shaking incubator. Preparation of 600µl glycerol in different microcentrifuge tube as Section 3.11 for long term storage. Overnight 5ml of LB culture harvested in a microcentrifuge for 1 mintue at 11,000 x g. After supernatant was discarded, the harvested cell was suspended in 250µl buffer A1 by pipetting up and down slowly until there were no clumps remained to lysis the cells. Then, 250µl buffer A2 was added and mixed gently by inverting the tube 5 to 8 times until lytase appeared clear and incubated for 5 min in room temperature. Buffer A3 (350µl) added, mixed thoroughly and centrifuge for 10 minutes at 11,000 x g. After centrifuge, 750µl of supernatant collected using pipette into column and centrifuged for 1 min at 11,000 x g. The flow through are discarded and nucleospin

plasmid column is placed back into collection tube. Buffer AW, preheated to 50°C and 500µl are added into column to wash cells. Collection tube was centrifuge for 1 min at 11,000 x g and followed by washing step Buffer A4. Buffer 600µl of A4 added and centrifuged for 1 min. The flow is discard and to dry the silica membrane, the column was centrifuge twice for 2 min at 11,000 x g and the remaining collection tube is discard. Nucleospin plasmid column is placed on clean 1.5ml centrifuge tube and 50ml buffer AE is added to elute DNA. The tube was incubate at room temperature for 1min and centrifuge for 1 min at 11,000 x g. All the DNA purity sample is stored at -20°C to prior use.

### 3.16 Reconfirmation of the ABA392 gene

The DNA sample, ABA392/pUC57 was isolate using NucleoSpin® Plasmid DNA purification Kit (Section 3.6.1). The designed primer with restriction endonuclease (RE) side were amplified against PMB202 and ABA392 strains via the PCR reaction. RE side was carried out to obtain the ABA392 fragment size. The insert was prepared by treating the ABA392 clone carrying the insert with RE side *Hin*dIII and *Bam*HI. About 4µl of DNA sample was used and pipetted with the PCR master mix (as mentioned earlier). The PCR cycle was set in 30 cycles with extension of 5 minutes. Meanwhile, the denaturing, annealing, and elongation temperatures were set to 95 ºC, 55 ºC and 72ºC respectively. The amplified samples confirmed by 1% agarose gel electrophoresis running on 60V with 1kb Plus DNA Ladder, and PCR control to detect bands with size of 829bp.

The ABA392 of with the band size of interest was excised from agarose gel using Gel extraction (NucleoSpin® Gel and PCR hindClean-up). DNA was carefully transferred into new fresh tube and ready to ligate into vector or stored at -20 ºC for long term usage.

### 3.17 Isolation and purification of pVAX1 vector

A single colony of pVAX1 from LB/kanamycin agar 30µg (Section 3.4.3) was grown in LB broth (Section 3.4.2) which contain kanamycin with final concentration of 30µg at overnight in 37ºC shaking incubator. Before proceeding with purification step, 600 µl is aliquot in different microcentrifuge tube for glycerol stock preparation Section 3.11 and followed by purification step Section 3.6.1. All the DNA purify sample is stored at -20ºC to prior use.

### 3.18 Agarose gel electrophoresis for purify ABA392/pUC57 and pVAX1 vector

The purify ABA392/pUC57 and pVAX1 were analyzed by 1% gel electrophoresis which prepared Section 3.1.4.2. Loading dye 1µl and 4µl of purified DNA sample was mixed and pipetted into the gel wells. Supercoiled ladder of 5µl, mixed up with 1µl loading dye as the size reference. The gel were run for at 70V for 60 to 75 minutes. The detection was made by using UV light and was photographed.

### 3.19 Restriction Endonuclase Digestion of ABA392 gene and pVAX1

Restriction endonuclease digestion was carried out to obtain the insert ABA392 and pVAX1 fragment size confirmation. The insert was prepared by treating ABA392 and pVAX1 vector with restriction digestion enzyme *Hin*dIII and *Bam*HI. Both gene and vector were digested using the ingredients and condition as below.

| | |
|---|---|
| Cut Smart® Buffer | 2.5µl |
| Restriction enzyme: | |
| *Hin*dIII | 1.0µl |
| *Bam*HI | 1.0µl |
| DNA fragment | 20.5µl |
| Total | 25.0µl |

The solution was mixed and incubated at 37ºC for 2 hours. The digestion was confirmed by agarose gel electrophoresis.

### 3.20 Gel Extraction and PCR clean up

The DNA fragment was cut with the band size from the agarose gel using a clean sharp scalpel. The gel slice was placed into clean microcentrifuge tube. For each 100mg of agarose gel, 200µl buffer NTI was added. For 5 to 10 minutes, the tube was incubated at 50ºC until the gel slice completely dissolved and sample were vortex every 2-3 minutes until the gel dissolve completely. Nucleospin gel and PCR Clean-up Column was placed in collection tube (2ml) to bind the DNA. All the dissolved sample is loaded into the tube and centrifuge for 1 min at 11, 000 x g. The flow through is discarded and the column is placed back into the collection tube. To wash the silica membrane, 700µl buffer NT3 is added into the nucleospin gel and PCR Clean-up Column, centrifuge for 1 min at 11,000 x g. The flow through discarded and the column is placed back into the collection tube. This washing step are repeated to minimize chatropic salt carry over and centrifuge for 1 min at 11, 000 x g to remove buffer NT3. To elute the DNA, the nucleospin gel and PCR Clean-up Column is placed on microcentrifuge tube. 30µl buffer NE is added and incubated for 1 min at room temperature. The column was centrifuged for 1 min at 11, 000 x g. The extracted gel were analyzed by 1% gel electrophoresis which prepared as Section 3.1.4.2. Loading dye of 1µl was mixed with 4µl of purified DNA sample and pipetted into the gel wells. DNA ladder of 3µl are mixed up with 1µl loading dye was used as the size reference. The gel were run for at 70V for 60 to 75 minutes. The detection was made by using UV light and was photographed.

### 3.21 Ligation of ABA392 into pVAX1 cloning system

The pVAX1 vector and digested insert of ABA392 was used for ligation process. The vector increases the yield of ABA392 and for long term storage. The standard volumes used for ligation process. The standard volumes used in this process were shown below:

| | |
|---|---|
| 2x Rapid Ligation Buffer, T4 DNA Ligase | 5.0 µl |
| Vector (digested pVAX1 with *Bam*HI and *Hin*dIII) | 1.0 µl |
| Insert (digested ABA382 with *Bam*HI and *Hin*dIII) | 3.0 µl |
| T4 DNA Ligase (3 Weiss unit/µl) | 1.0 µl |
| **Total** | **10.0µl** |

The solution incubated for 16 hours at 16°C before transformed into *E.coli* TOP10 strain.

### 3.22 Plasmid DNA Transformation into *Escherichia coli* (TOP10)

#### 3.22.1 Competent cell preparation

TOP10 from stock culture was steaked on LB agar and incubated at 37°C overnight. A single colony was inoculated into 10ml LB broth and incubated overnight. 1ml of the overnight culture was added into 50ml LB broth and was shaken at 220 r.p.m for 2 hours 37°C until $OD_{600}$ 0.5 at 600nm. The culture were centrifuged at 3500 r.p.m at 4°C for 1 hour. The supernatant was discarded and the pellet resuspended in chilled 5ml 0.1M calcium chloride and place on ice for 30 minutes.

### 3.22.2 pVAX1-ABA392 transformation into *E.coli* TOP10

From section 3.21, 5µl of the ligation reaction was transformed into 100µl of *E.coli* TOP10 competent cells in the ligation tube. The ligation tube is placed in the ice for 30 minutes and heating block at 42º for 2 minutes. The ligation tube placed back again on ice for 5 minutes. LB broth of 0.9 ml was added and were incubate at 37ºC in a shaking incubator for 90 minutes. After incubation, the tube is centrifuge for 1 min at 13,000 x g. Supernatant is discarded and LB broth of 50µl was added and mix well. The 50µl was spread on LB agar kanamycin (Section 3.4.3) for screening. The transformed *E.coli* TOP10 were incubated at 37ºC overnight and only transformed cultures which is resistance to kanamycin/chloramphenicol will survive on this plate. The transformed colonies were analysed using direct colony PCR. Positive colonies were grown in kanamycin/chloramphenicol broth to amplify the construct and for storage as glycerol stock for future usage.

### 3.23 Colony library and PCR colony screening

Through PCR colony screening, the colonies were screened on resistance towards kanamycin/chloramphenicol. Grids were drawn in a new LB kanamycin/ chloramphenicol agar plate before proceeding with PCR colony screening. Single colony from the overnight transformation library plate were touched using clean and sterilized toothpick. Then plate was incubated overnight at 37º C. The next day, PCR was performed without DNA purification. Each colony is picked from each grid using sterilized inoculate loop from overnight plate to detect the positive colonies that carrying the gene of insert and mixed by pipetting with the PCR solution as in the Table 3.1. The sample were subject to electrophoresis using 1% agarose gel (Section 3.1.4.2).

### 3.24 DNA sequencing

The purified plasmid was sent for DNA sequencing at MyTacg Bioscience Enterprise for confirmation gene of interest ABA392 in pVAX1 vector. The chromatogram raw data with the nucleotide sequences was analysed through sequence scanner software known Bioedit to retrieve nucleotide sequence.

#### 3.24.1 Basic Local Alignment Search Tool (BLAST)

The nucleotide sequence from Bioedit were confirmed using BLAST search from National Center for Biotechnology Information (NCBI) website www.ncbi.nih.gov.

### 3.25 DNA stability testing and insert analysis of pVAX1-ABA392

The recombinant clones of pVAX1-ABA392 are subjected to plasmid stability testing prior to begin the project. The clones are grown overnight at 37$^{o}$C in BHI broth and this step are repeated for 8-10 times. The cultures are subjected to DNA extraction for presence of the recombinant plasmid DNA of PVAX1-ABA392 after each inoculation. The plasmid DNA of PVAX1-ABA392 is isolated by using large scale plasmid extraction (Close and Rodriquez, 1982). Single colonies were chosen from transformation and cultures were grown in 1.5ml of LB broth with kanamycin added by shaking 220 r.p.m overnight at 37$^{o}$C until reached an $OD_{600}$ of 0.6. For rat immunization experiment, plasmid DNA is isolated by using endotoxin free Plasmid DNA Purification Kit NucleoSpin®.

### 3.26 Plasmid Analysis

The overnight culture is extracted as mentioned in the (Section 3.15). 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 3.19) using the same enzyme, *Hin*dIII and *Bam*HI. Both extracted plasmid and digested sample were subject to electrophoresis using 1% agarose gel (Section 3.1.4.2).

### 3.27 Immunogenicity Test

#### 3.27.1 Experimental Animals

Six to eight weeks old pathogen free, twelve white Spargue Dawly's female rats weighing between 150-200g were used for this part of experiment. Rats were obtained from Animal Experimental Unit (AEU), Faculty of Medicine, University of Malaya. The animal were housed in clean individual cage at the experimental room, Animal House, Faculty Medical, University of Malaya and were fed 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) ISB/18/11/2015, University Malaya prior to the research.

#### 3.27.2 Bacterial suspension

The bacterial suspension preparation for vaccine was adapted from Hussaini *et al*. (2009) with modification. A single pure bacterium was grown overnight at 37ºC in 10 ml of BHI broth medium. The cultures were then centrifuged for 5 minutes at 14000 r.p.m to pellet the cells and washed three times with sterile PBS to remove all traces of the growth medium. The cells were resuspended in 5 ml of PBS pH 7.2 to achieve the concentration of colony forming units CFU and formalin concentration 0.2% was added. The cell suspensions were left to stand for overnight and centrifuged at 3500 r.p.m 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 was then allocated into 1.5ml microcentrifuge tube and stored at -20ºC prior to use. This formalin killed vaccine serve as positive control.

### 3.27.3 Immunization of Animals

Rats were divided into 4 different groups where 3 rats in each groups. All the rats were inoculated subcutaneously with the proper amount according to the body weight (Diehl, 2001). To determine the immunogenicity of purified plasmid DNA of pVAX1-ABA392, Group 1 rats was treated with pVAX1-ABA392 (100µg/ml) and group 2 rats as positive control which given formalin killed vaccine (50µg/ml). Group 3 given purified vector of pVAX1 (50µg/ml) rats and meanwhile group 4 was the negative control were given sterile PBS (100µl). Two dose vaccination schedule was carried out. The rats were immunized on day first and followed by 2nd booster were given two weeks later via intramuscular route. Figure 3.0 shows, blood was collected on day 1st, 7th, 14th, 21st, 28th and 35$^{th}$ and at the end of the immunization, rats were sacrificed by overdose anaesthesia following the animal ethic requirement. Lungs, liver, kidney and heart were collected and fixed in 10% formalin. Serum were separated by centrifuging at 13,000 x g for 20 minutes, pooled and stored at -20ºC until it was used.

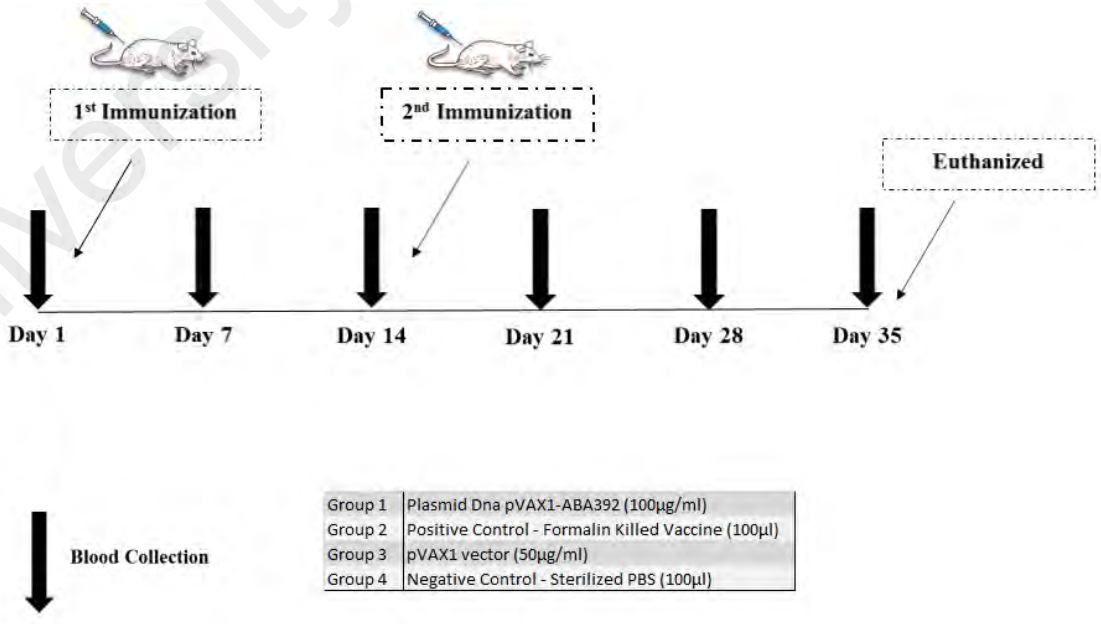



**Figure 3.0:** Animal Immunization Studies Timeline

### 3.27.4 Hematological Studies

Haematological studies, 1ml blood was collected via tail and rats were bled on day 1, 7th, 14th, 21st, 28th and 35th. Total white blood cell (WBC) and differential counts were performed. From 1ml, 10µl of blood were added to 190µl of Turks solution (1:20 dilution) mixed well. Then it loaded into a haemocytometer for total WBC counted under microscope using cell counter (Hussaini, 2009). Blood smear performed immediately after blood collection and stained with Wright-Giemsa Stain for differential WBC count. The cells were counter under microscope. For biochemical analysis, blood was collected via cardiac puncture and serum were separated. Serum was send to Biochemistry Laboratory, University Malaya Medical Centre for liver function test (LFT) and renal function test (RFT).

### 3.27.5 Organ Collection

Autopsy was performed and the organs, kidney liver, heart and lung of the animals were collected upon dissection. All the organs were placed on 10% NBF (Section 3.10.4) buffer fixation prevents acidity that would promote autolysis and cause precipitation of formol-heme pigment in the tissues.

### 3.27.6 Histopathological analysis

All the tissue samples were processed manually. The organs were trimmed into smaller size before they were processed. The steps involving tissues processing protocol are shown in the Table 3.2. After overnight fixation, the solution is discard into waste bottle. The tissue passed through a series of baths of increasing concentration of alcohol ( 70% > 85% > 95% > 100% ) for 30-45 minutes, culminating in several baths of 100% alcohol in order to remove all the water remained in tissue. This process was done in room temperature. Following dehydration, the ethanol are cleared from tissue by using xylene. The tissue was immersed in xylene for 3 hours. Once done, the tissue infiltrated

and embedded in paraffin kept in oven 60°C for 2 hours. After 2 hours, the paraffin is changed, the tissue is kept again in an oven 60°C for 2 hours overnight. Once the tissue is paraffin infiltrated, it is embedded in a block of paraffin using tissue embedding machine (Histo Embedder Leica, Japan). The tissue sample is placed in a small mold that is then filled with melted paraffin. Once the paraffin has cooled and hardened, the mold is removed, the solid block was trimmed before can be sectioned. The blocks were section at 6µm using Leica RM2035 microtome. The paraffin section, were gently placed on clean glass slide which smear with Mayer's albumin (Section 3.10.5) and water in order to adhere the paraffin section. The slides placed on 42°C temperature slide warmer for 20 minutes for placing into oven for overnight. This helps the section adhere completely and melt the paraffin.

**Table 3.2:** Tissue processing protocol

| Tissue Processing Step | Duration | Temperature ° C |
|---|---|---|
| 10% Neutral Buffer Formalin | overnight | 35 |
| 70% Alcohol | 30 minutes | 35 |
| 85% Alcohol | 30 minutes | 35 |
| 95% Alcohol | 30 minutes | 35 |
| 100% Alcohol | 45 minutes | 35 |
| 100% Alcohol | 45 minutes | 35 |
| Xylene | 3 hours | 35 |
| Paraffin Wax | 2 hours | 60 |
| Paraffin Wax | overnight | 60 |

### 3.27.7 Histopathology Staining

Once the tissue section placed on clean glass slide, histological staining steps were carry out. In this research, Harris Haematoxylin (Section 3.10.6.1) and Eosin Y (Section 3.10.6.2) staining (H & E) were used. The slides placed into xylene as de-paraffin step and followed by ethanol for hydration to remove the excess of xylene. The slides were rinsed with distilled water before stain with haematoxylin. The rinsing step is repeated before the slides were stained with Eosin. After rinsed, the slides were went through quick dip in alcohol for dehydration and finally the slides were place into xylene for clearing process followed by DPX mounting. The H&E steps followed as Table 3.3.

**Table 3.3:** H&E Staining Guideline

| Steps | Solutions | Duration |
|---|---|---|
| De-paraffin | Xylene | 1 hour |
| Hydration | 100% Alcohol | 5 min |
| | 90% Alcohol | 5 min |
| | 70% Alcohol | 5 min |
| Rinse | Distilled water | 1 min |
| Stain Nuclei | Haematoxylin | 35 |
| Rinse | Distilled water | 35 |
| Stain Cytoplasm | Eosin Y | 60 |
| Rinse | Distilled water | 60 |
| Dehydration | 70% Alcohol | Quick dips |
| | 90% Alcohol | Quick dips |
| | 100% Alcohol | Quick dips |
| Clearing | Xylene | 1 min |
| Mounting | DPX | |

### 3.28 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA was developed on the procedure by Cheng *et al*. (2010) with slight modification. ELISA method for detecting presence of antibody in immunized rat. The optimization of the antibody for Ig G was optimize. ELISA, 96-well plate 96-well plate was coated with 50µL per well of coating antigen (Section 3.9.2) and incubated at 4ºC overnight. After incubation period, the coating antigen was discarded and washed twice with 50µL per well with sterile washing buffer-PBS-Tween 20, pH7.4 (Section 3.9.3). Once the step is done, 200µL per well of sterile blocking buffer-PBS-Tween 20-BSA, pH7.4 (Section 3.9.4) were added and incubated at 37ºC for 1 hour. After the incubation, the blocking buffer was discarded gently and washing step was repeated as previously. Serum dilution was diluted in ratio 1:50 and about 50µL per well of serum (primary antibody) was added to the plate was incubated at 37ºC for 1 hour. After incubation period, the serum was discarded gently and washing step repeat as previously. 50µL of Ig G antibody conjugate (Section 3.9.7) was added and incubated for 1 hour at 37ºC. Antibody conjugate was discarded gently and washing step repeated as previously. Tetramethylbenzidine (TMB) light sensitive substrate (Section 3.9.5) 100µL was added and incubated at 37ºC for 15 minutes. After incubation, 50µL of stopping buffer (Section 3.9.6) was added and mixed by tapping the side of the plate to ensure the mixing properly. Confirmed there is no bubble on the surface of the liquid. The microplate reader is run and the measurement is conducted at 450nm immediately.

# CHAPTER 4: RESULT

## 4.0 Reconfirmation of Clone

## 4.1 ABA392 gene analysis

ABA392 in pUC57 competent cells was purified (Section 3.15) and RE digestion of ABA392/pUC57 (Section 3.19). For primer design, the primers were designed specifically with *Hin*dIII and *Bam*H1 RE sites. Figure 4.0 shows the result of PCR product with a size of 804bp.

**ABA392/PMB202 for pVAX1 (*Hin*dIII *and Bam*HI)**

**Forward: F-5′-ATGAGTCTTTTGTTCTGTCGCTTG-3′**

**Reverse : R-5′-CTAGCGGCGGTGGAAACCCGCCAT-3′**

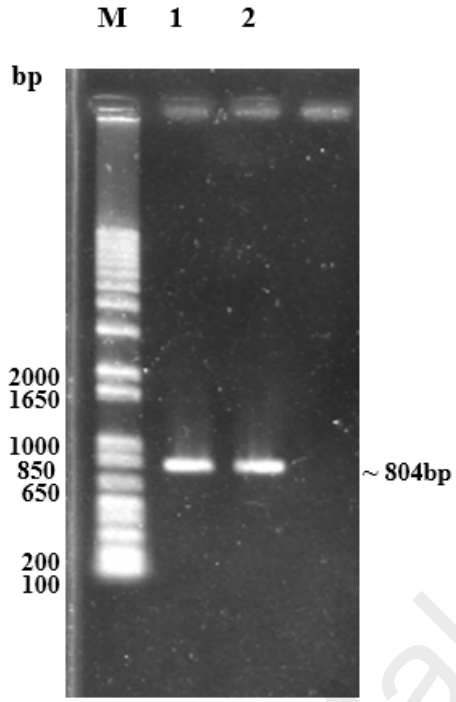



**Figure 4.0:** Agarose (1%) gel electrophoresis showing purified ABA392 gene.

Lane M Invitrogen Supercoiled DNA ladder and Lane 1 and 2 purified ABA392 gene. Determination of insert size approximately ~804bp as a result of ABA392 gene.

## 4.2 ABA392 gene cloning into pVAX1 vector and colony PCR screening

The mammalian vector, pVAX1 and ABA392 was digested by the similar protocol restriction enzymes *Hin*dIII and *Bam*HI with the presence of cut smart buffer and DNA ligation was carried out (Section 3.2.3) to produce the recombinant clone pVAX1-ABA392 (Appendix D). The clones were then transformed into TOP10 and grown in concentrated LB agar with kanamycin for DNA vaccine production. The colony library (Figure 4.1) was made and positive colonies were identified via colony screening 0.7% gel electrophoresis. Figure 4.2, shows colony screening of ABA392 gene (804bp) via PCR.

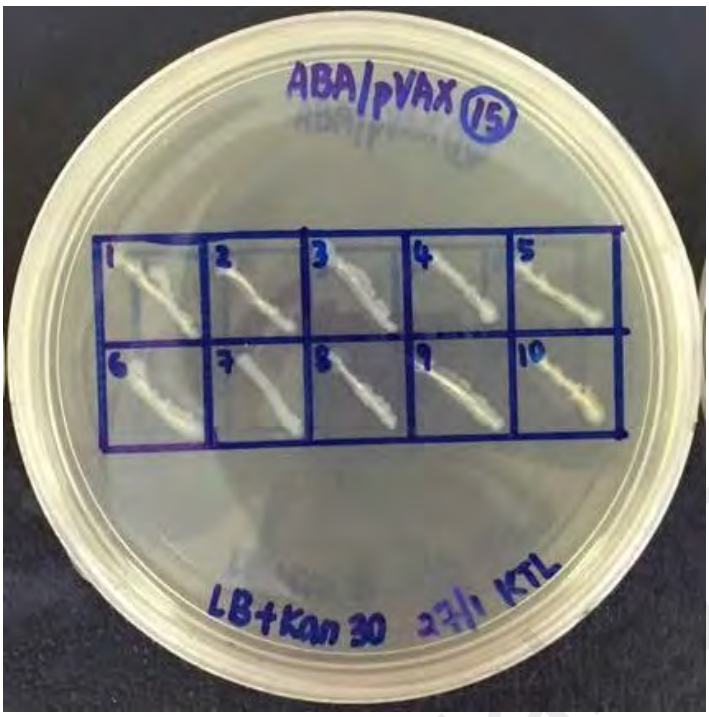



**Figure 4.1:** Colony library plate of pVAX1-ABA392

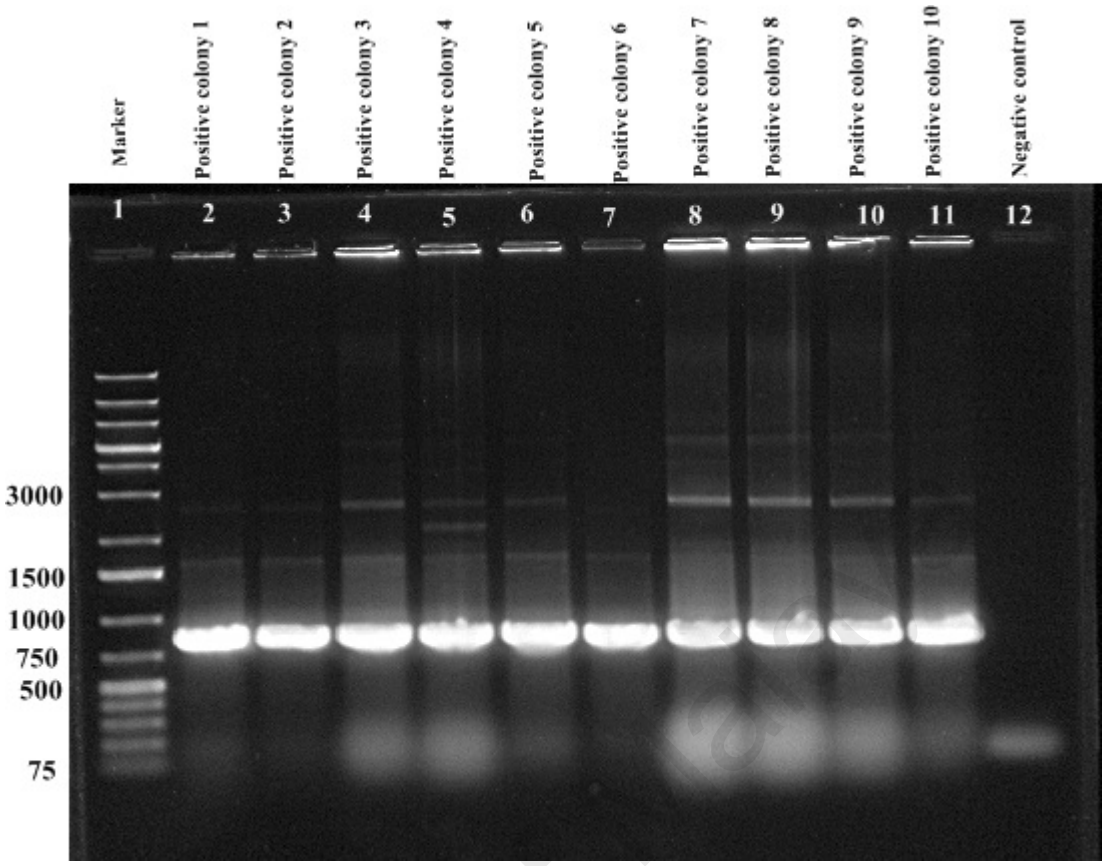



**Figure 4.2:** Agarose (1%) gel electrophoresis shows colony screening of ABA392-pVAX1 through colony PCR.

Lane 1, GeneRuler$^{TM}$ 1kb plus ladder; Lane 2 to 11 PCR product positive colony ABA392 gene; Lane 12 negative control. Positive colony contain ABA392 gene at size 804bp via PCR.

## 4.3 Confirmation of Recombinant clone

The recombinant clone isolated and digested of ABA392 gene and pVAX1 as reconfirmation the presence of insert. Figure 4.3 shows pVAX1-ABA392 recombination clone isolation, lane 1 is marker, lane 2 to 3 digested pVAX1 vector ~3.0kb and lane 4 to 5 purified pVAX1-ABA392 of ~3804bp. Lane 7 to 8 shows ~804bp digested gene ABA392 while lane 10 shows ABA392 gene (insert) digested from pVAX1 vector.

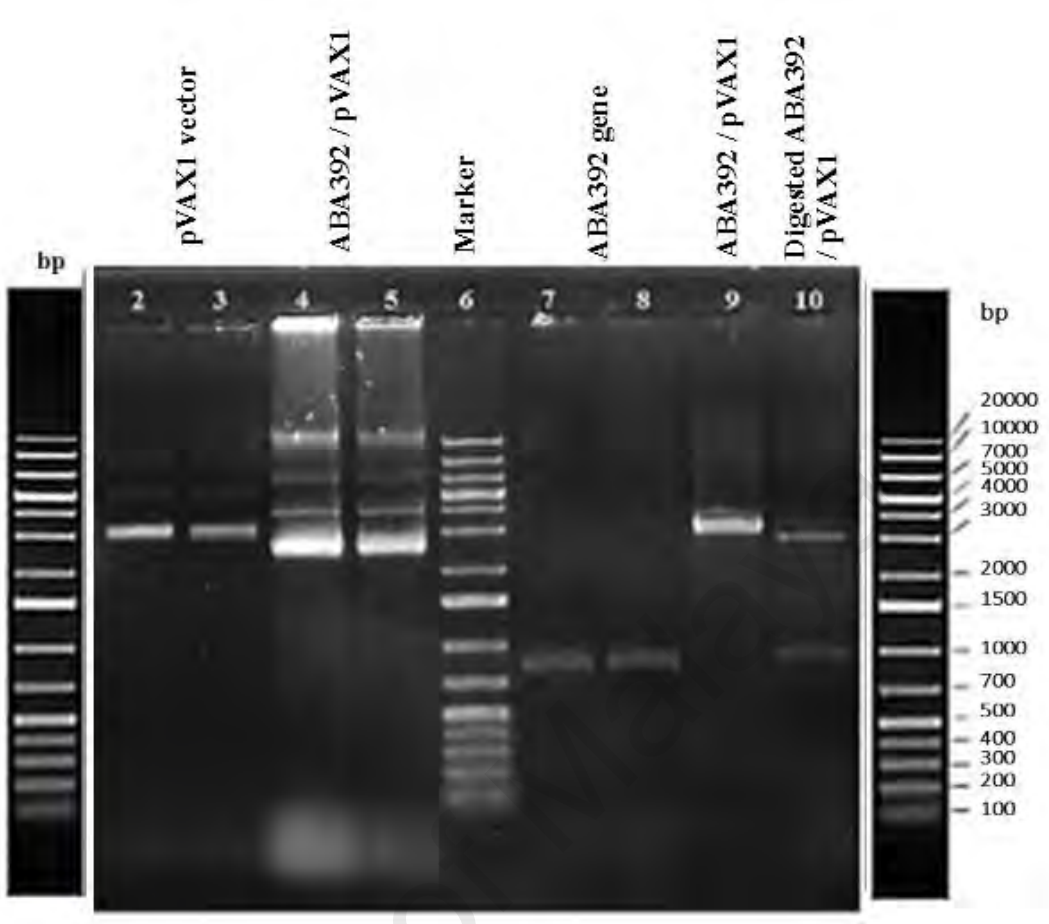



**Figure 4.3:** Agarose (1%) gel electrophoresis shows isolation of recombinant clone of ABA392-pVAX1 and digested of ABA392/pET-30a through restriction enzyme analysis.

Lane 1, GeneRuler$^{TM}$ 1kb plus ladder, Lane 2 to 3 pVAX1 vector; Lane 4 to 5 and 9 shows purified ABA392-pVAX1; Lane 6 Marker; Lane 7 to 8 PCR product positve colony contain ABA392 gene; Lane 10 digested ABA392-pVAX1 through restriction enzyme analysis and digested ABA392 gene shows size approximately ~804bp.

## 4.4 DNA stability testing and insert analysis of pVAX1-ABA392

The recombinant clones of pVAX1-ABA392 are subjected to plasmid stability, based on pET System Manual (Novagen, 1999) to make sure the clone was stable. The clone was cultured for several generation and every generation of the clone pVAX1-ABA392 was subjected to large scale of plasmid stability testing (Section 3.25) (Figure 4.4). The recombinant of pVAX1-ABA392 were grown overnight in a 10ml BHI broth medium

containing 10µl kanamycin at 37°C. For rat immunization experiment, plasmid DNA is isolated by using endotoxin free Plasmid Purification Kit NucleoSpin® (Section 3.6.1). The clone samples were sent for DNA sequencing and BLAST application as confirmation of interest gene.

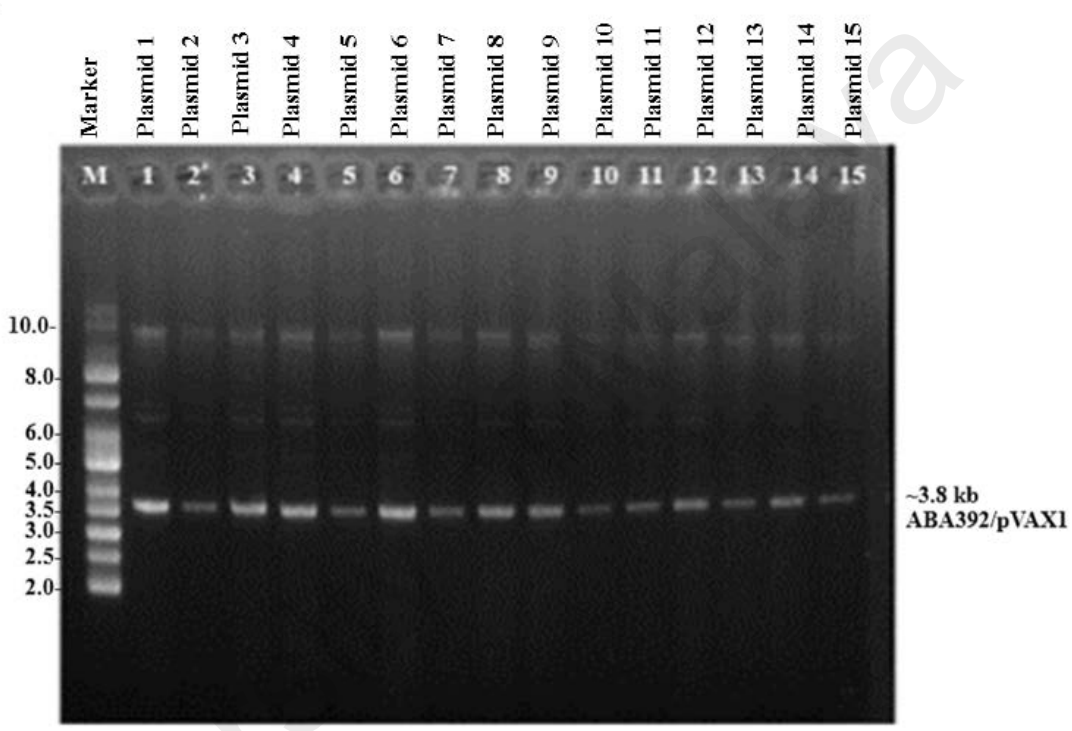
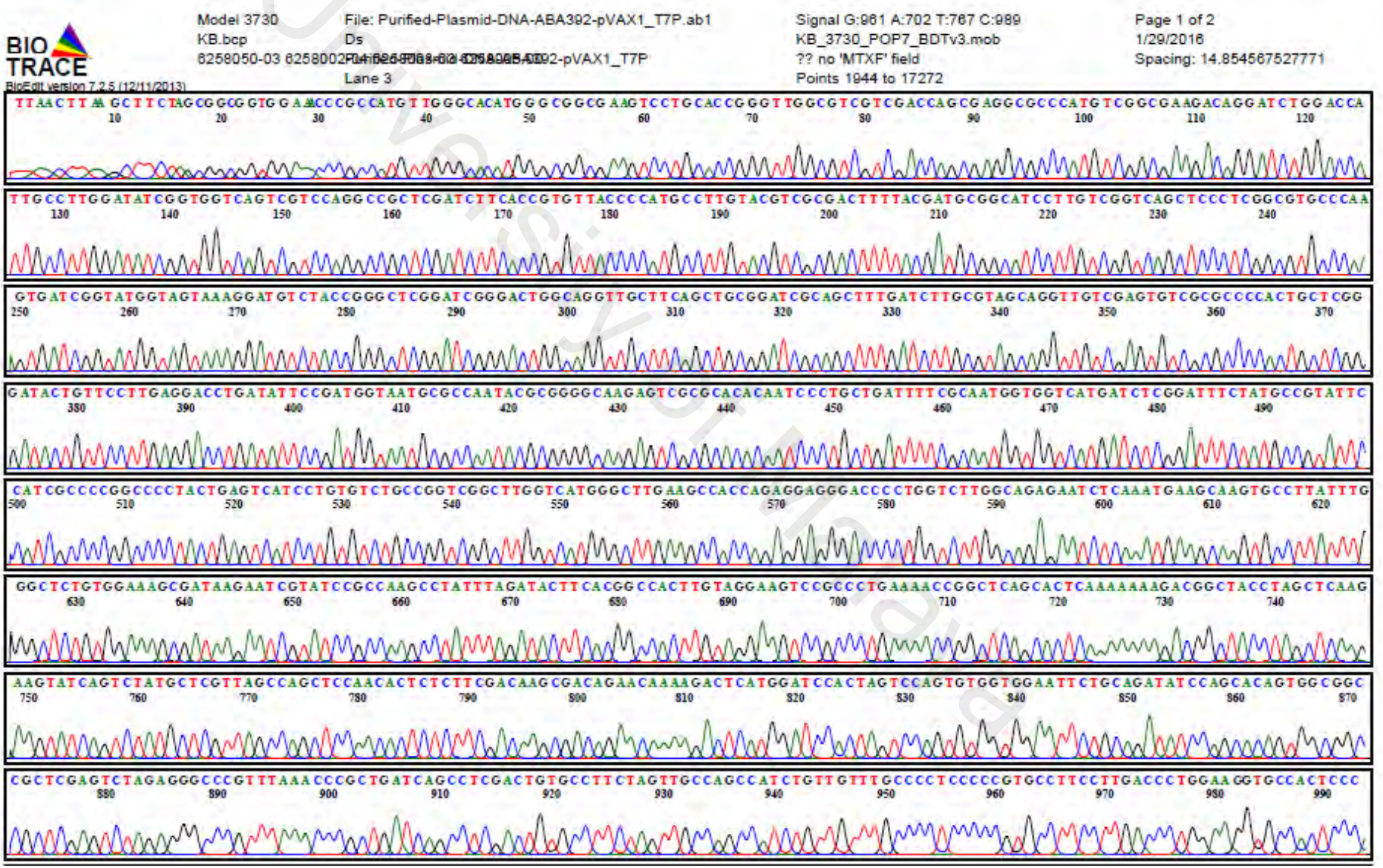



**Figure 4.4:** Gel eletrophoresis 1.0% agarose shows large scale of plasmid stability ABA392-pVAX1.

Lane Marker, GeneRulerTM 1kb plus ladder; Lane 1 to 15 purified ABA392-pVAX1 with size approximately ~3.8kb.

## 4.5 DNA sequencing of pVAX1-ABA392

Confirmation of interest gene, the clone samples were sent for DNA sequencing and BLAST application. The sequence were analysed using bioedit software. BLAST (Basic Allignment Search Tool) tool was used to compare regions of similarity between biological sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance. BLAST was accessed from National Centre for Biotechnology Information (NCBI) compare with native ABA392 gene. Figure 4.5 (a) shows chromatogram raw data from the sequencing result. Figure 4.5 (b) Chromatogram raw data of ABA392 gene sequences and underlined is the alignment of nucleotide of recombinant clone of pVAX1-ABA392 has the same identical sequence as PMB202 using BLAST application in NCBI (Accession No. DQ334273.1). Figure 4.5 (c) Shows the BLAST result of recombinant clone ABA392 in pVAX1. The sequence match 100% identity with FJ001839.1 and figure 4.5 (d) shows the alignment of nucleotide of recombinant plasmid ABA392-pVAX1 with reference sequence from NCBI, FJ001839.1 in BLAST.



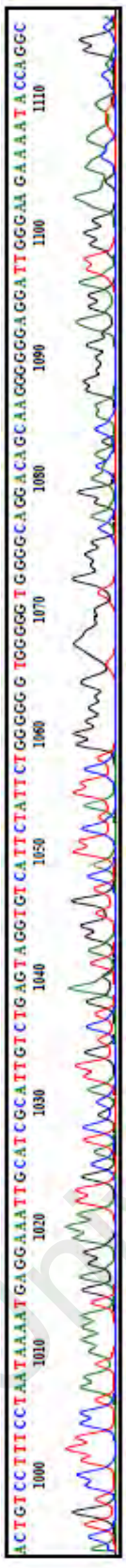


**Figure 4.5 (a):** Chromatogram raw data of ABA392 gene sequences.

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

ATA AGC CAG TAA GCA GTG GGT TCT CTA GTT AGC CAG AGA GCT CTG CTT ATA TAG ACC TCC CAC CGT ACA CGC CTA CCG CCC ATT TGC GTC AAT GGG GCG GAG TTG TTA CGA CAT TTT GGA AAG TCC CGT TGA TTT TGG TGC CAA AAC AAA CTC CCA TTG ACG TCA ATG GGG GTG GAG ACT TGG AAA TCC CCG TGA GTC AAA CCG CTA TCC ACG CCC ATT GAT GGA CTG GCC AAA ACC GCA TCA C

**Figure 4.5 (b):** Chromatogram of ABA392 sequences highlighted in bold (RE site).

Underlined is alignment of nucleotide of recombinant clone of pVAX1-ABA392 has the same identical sequence as PMB202 using BLAST application in NCBI (Accession No.DQ334273.1).

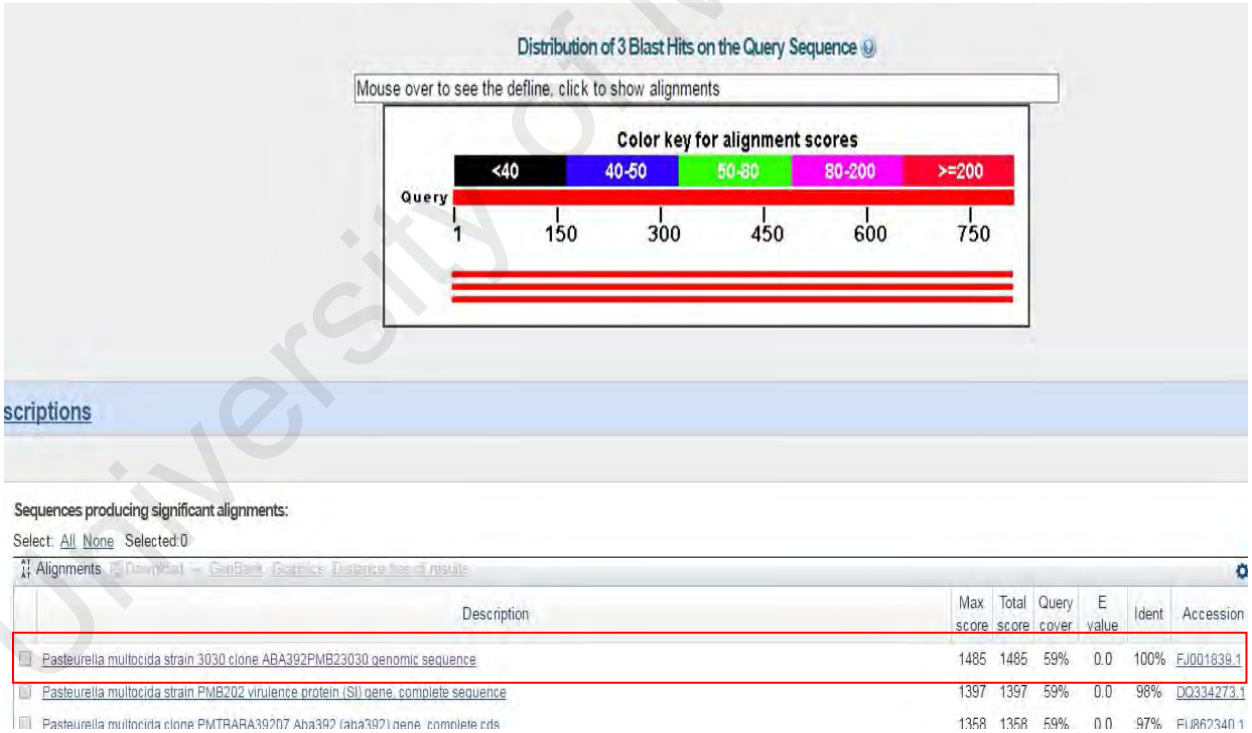



**Figure 4.5 (c):** Shows the BLAST result of recombinant clone ABA392 in pVAX1. The sequence match 100% identity with FJ001839.1

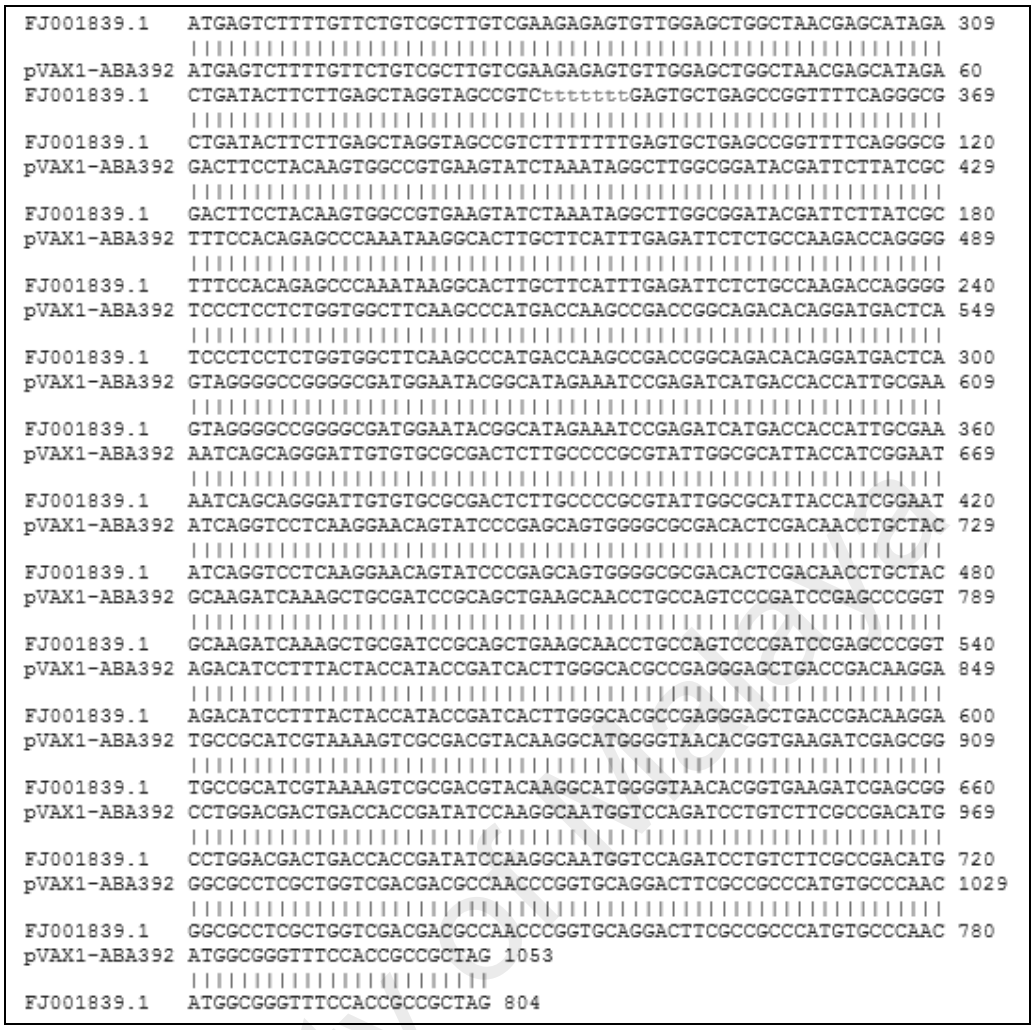



**Figure 4.5 (d):** Shows the alignment of nucleotide of recombinant plasmid ABA392-pVAX1 with reference sequence from NCBI, FJ001839.1 in BLAST.

## 4.6 Immunogenicity Test

### 4.6.1 Gross Pathological analysis

Gross pathological analysis were done on day 35. Organ heart, liver, kidney and lung collected from each group. Figure 4.6, shows there were no significant changes of hemorrhage septicaemia in tissue were observed in all organ from each groups. Table 4.0 shows total survival rate among groups of rats with various vaccination.

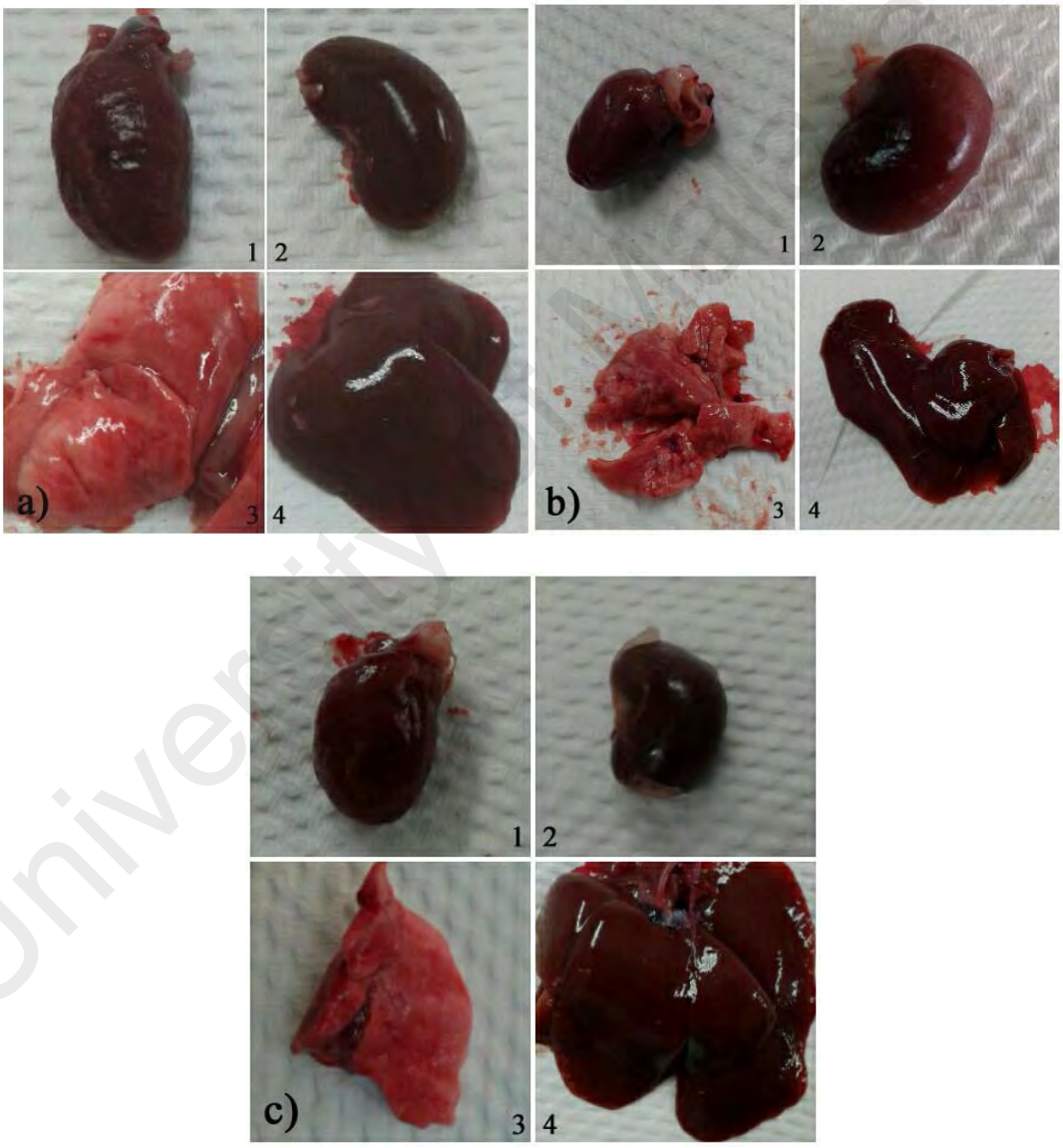



**Figure 4.6:** Gross pathological analysis for each organ, heart, kidney, lung and heart from (a) shows each organ from Group 1, immunized with pVAX1-ABA392 (100µg/ml), (b) shows Group 2 immunized with PBS (negative control) and (c) shows Group 4 with killed bacterin (50µg/ml) (positive control).

**Table 4.0:** Total survival rate among groups of rats with various vaccination.

| Type of Immunization | No. of Dosage | No. of Rats survived | Survived (%) |
|---|---|---|---|
| **Purified Clone pVAX1-ABA392 (100µg/ml)** | 2 | 3/3 | 100 |
| **Positive- Killed bacterin of *P. multocida* Serotype B (50µg/ml)** | 2 | 3/3 | 100 |
| **pVAX1 vector (50µg/ml)** | 2 | 3/3 | 100 |
| **Negative- Normal saline (PBS) (100µl)** | 2 | 3/3 | 100 |

### 4.6.2 Histopathological analysis

Histological slides were prepared from various organs collected from group 1, 2 and 4. Each slide was stained with hematoxylin and eosin (H&E). Sign of haemorrhage inflammation hemorrhage, inflammation and congestion was observed in tissues after vaccinated with pVAX1-ABA392.

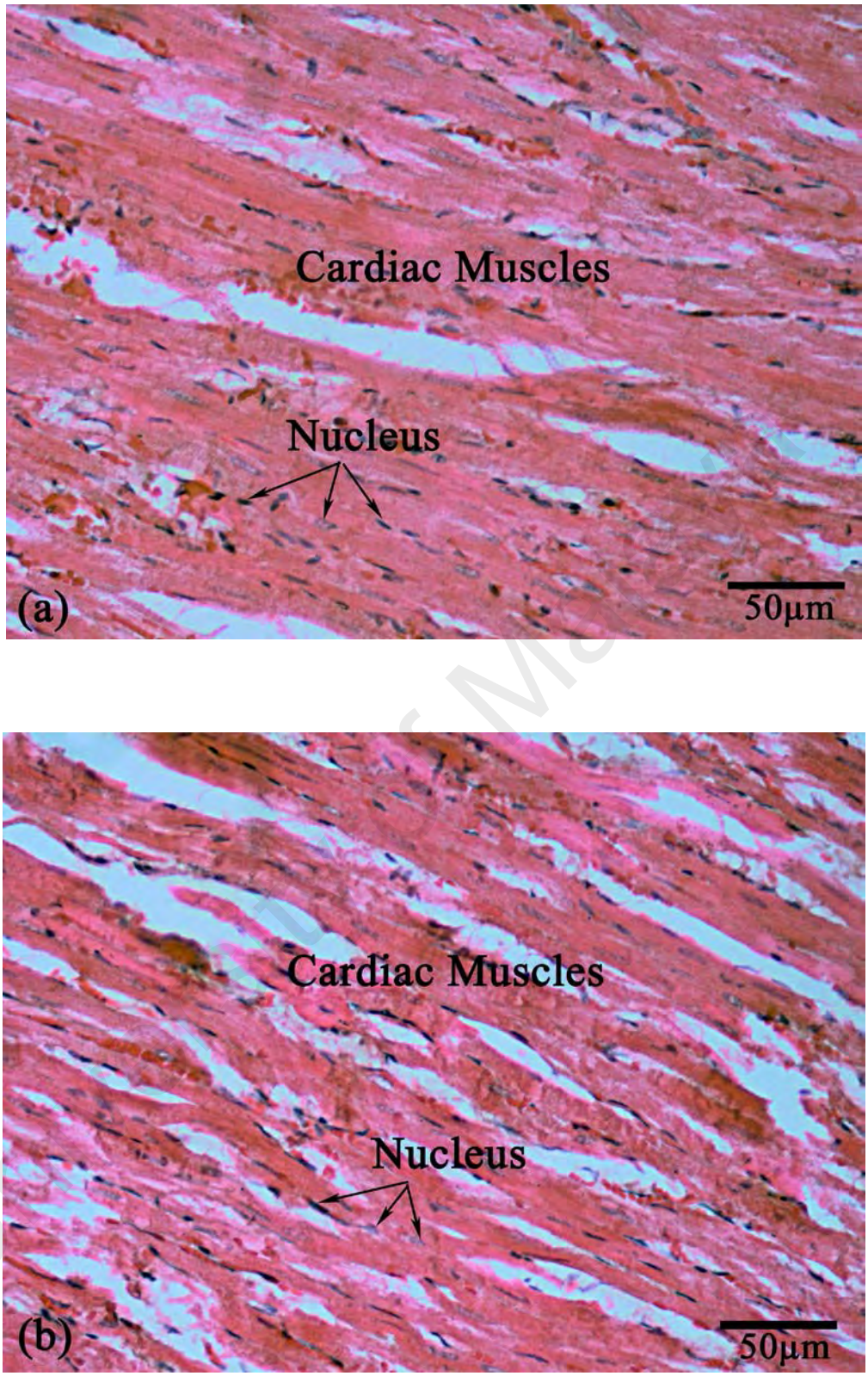



**Figure 4.7 (a):** Histopathological section of heart from rat vaccinated with pVAX1-ABA392 (100µg/ml); (b) PBS as negative control and (c) killed bacterin (50µg/ml) (positive control). H & E stain (20x).

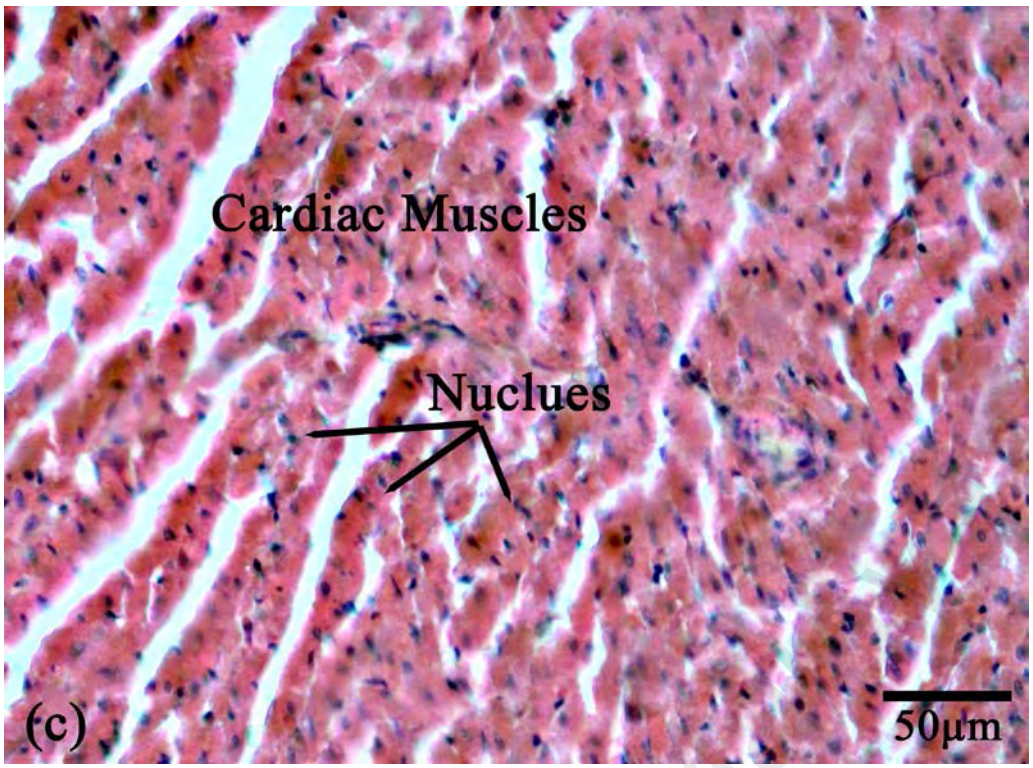



**Figure 4.7 (a):** continued.

Sections shows branches in cardiac muscle with normal nucleus cells. No abscess formation, no sign of inflammation and no sign of HS were observed.

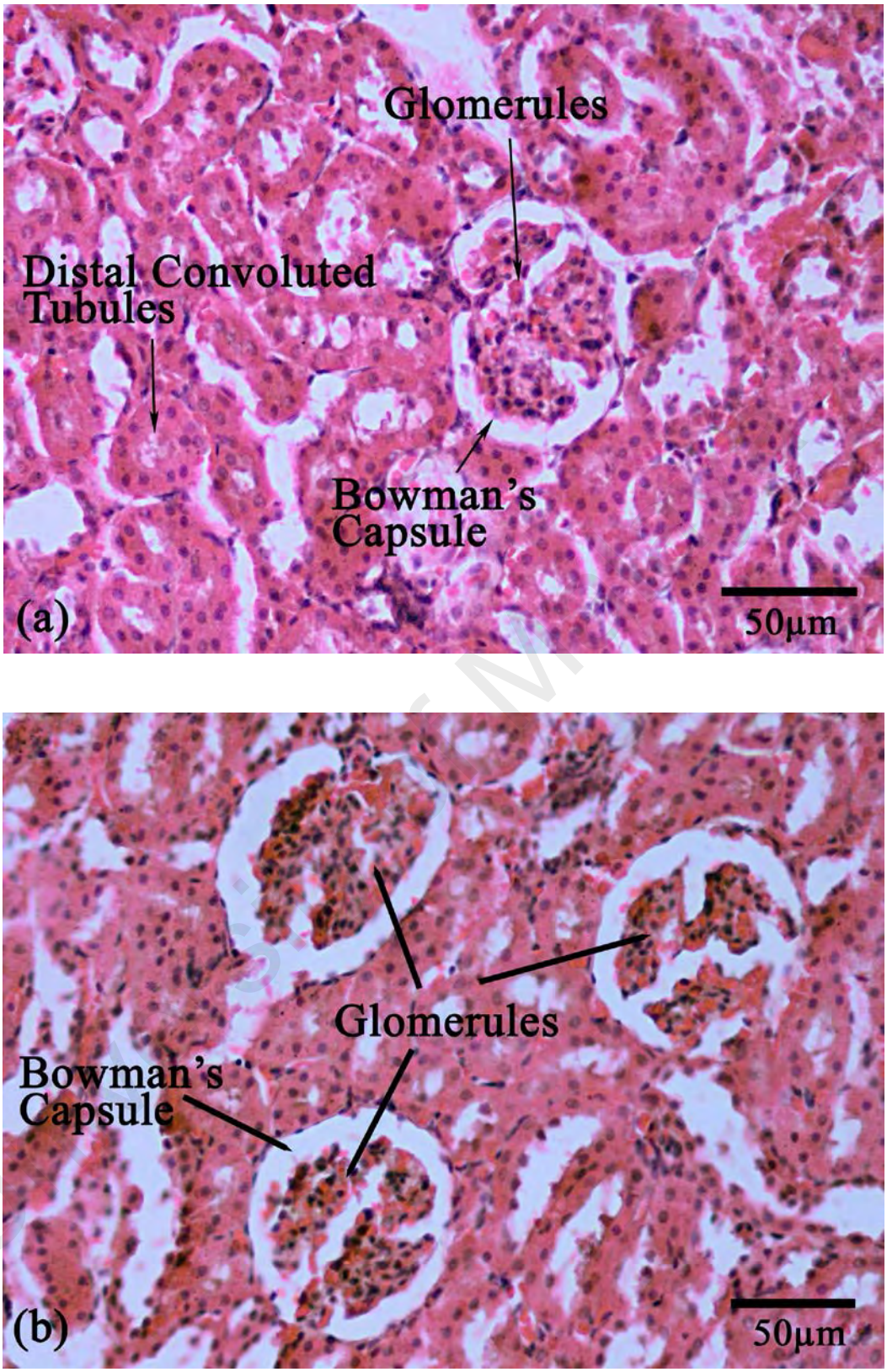



**Figure 4.8 (a):** Histopathological section of kidney from rat vaccinated with pVAX1-ABA392 (100µg/ml); (b) PBS as negative control and (c) killed bacterin (50µg/ml) (positive control). H & E stain (20x).

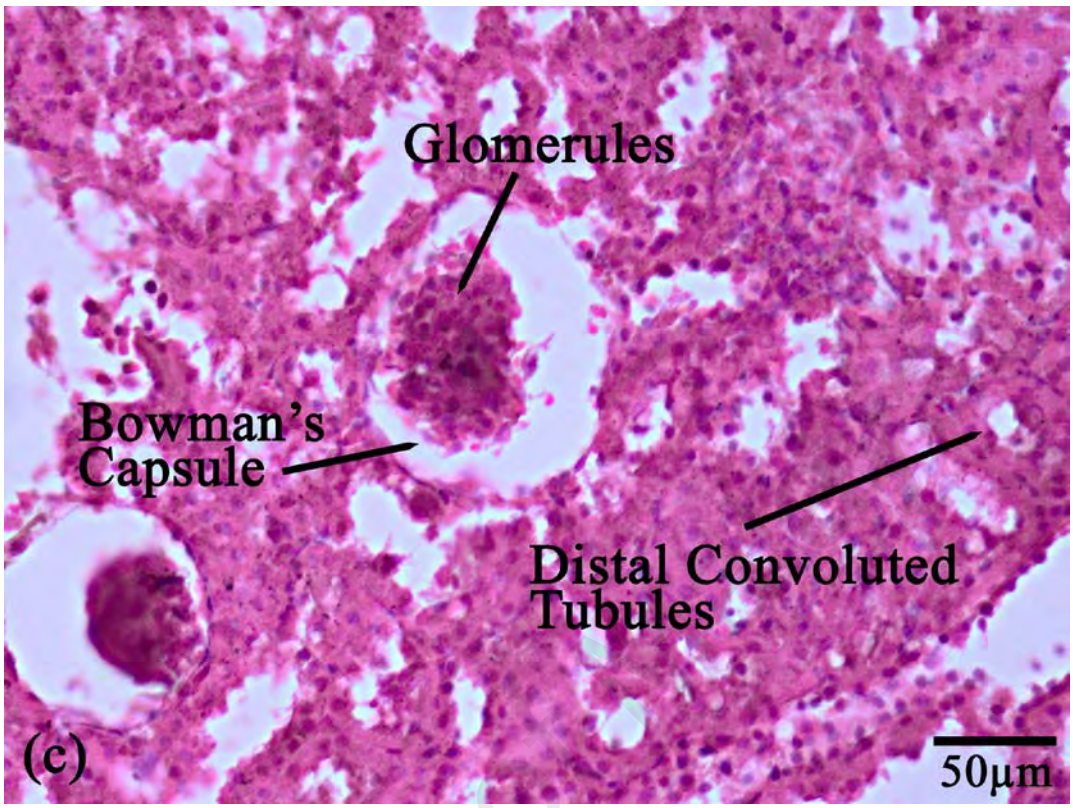



**Figure 4.8 (a):** continued

Section shows normal nucleus cells with normal glomerulus and distal convoluted tubules. There is no abscess formation, no sign of inflammation and no sign of HS were observed.

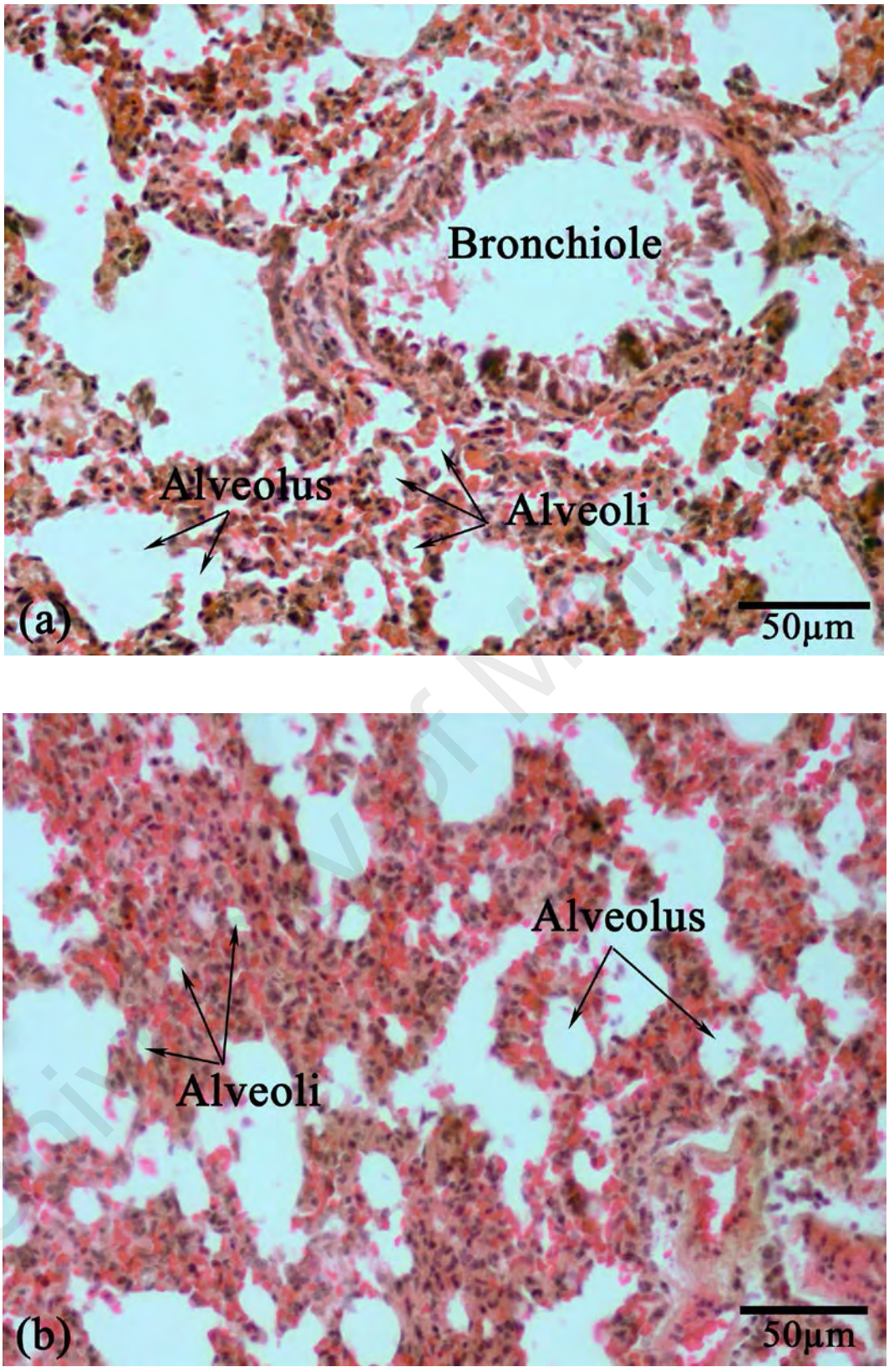



**Figure 4.9 (a):** Histopathological section of lung from rat vaccinated with pVAX1-ABA392 (100µg/ml); (b) PBS as negative control and (c) killed bacterin (50µg/ml) (positive control). H & E stain (20x).

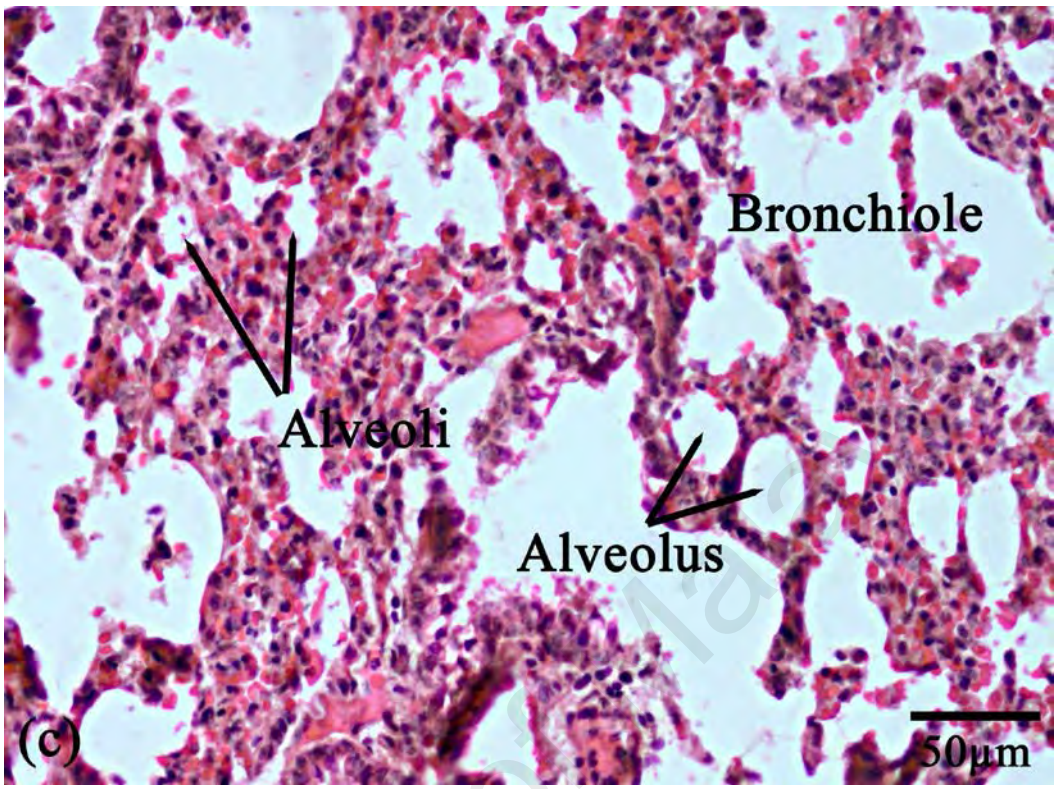



**Figure 4.9 (a):** continued

Nucleus cells with normal. There is slight thickened alveoli walls with red blood cell presents in positive control. No mucus or abscess formation, no sign of inflammation and no sign of HS were observed.

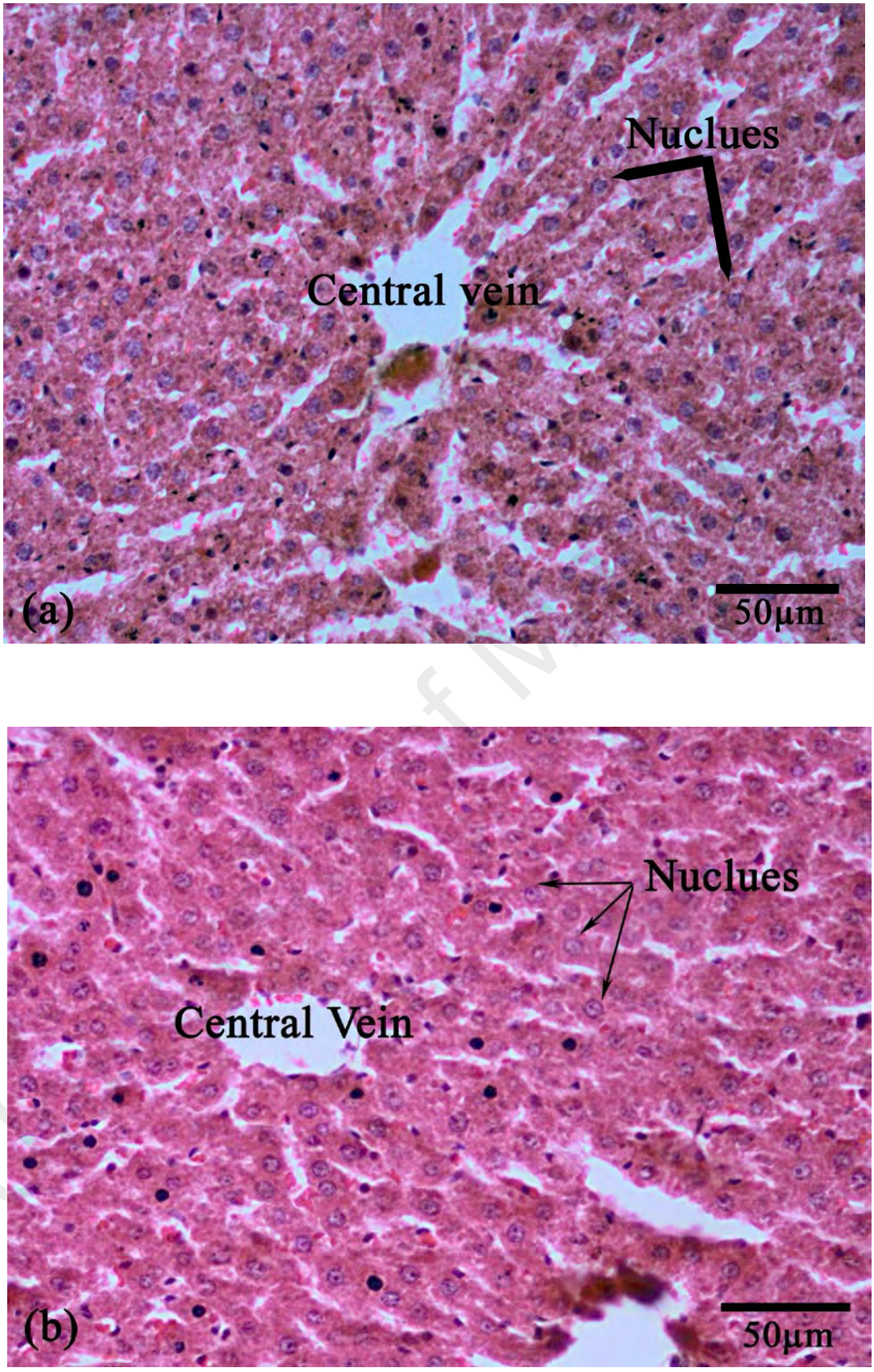



**Figure 4.10 (a)**: Histopathological section of liver from rat vaccinated with pVAX1-ABA392 (100µg/ml); (b) PBS as negative control and (c) killed bacterin (50µg/ml) (positive control). H & E stain (20x).

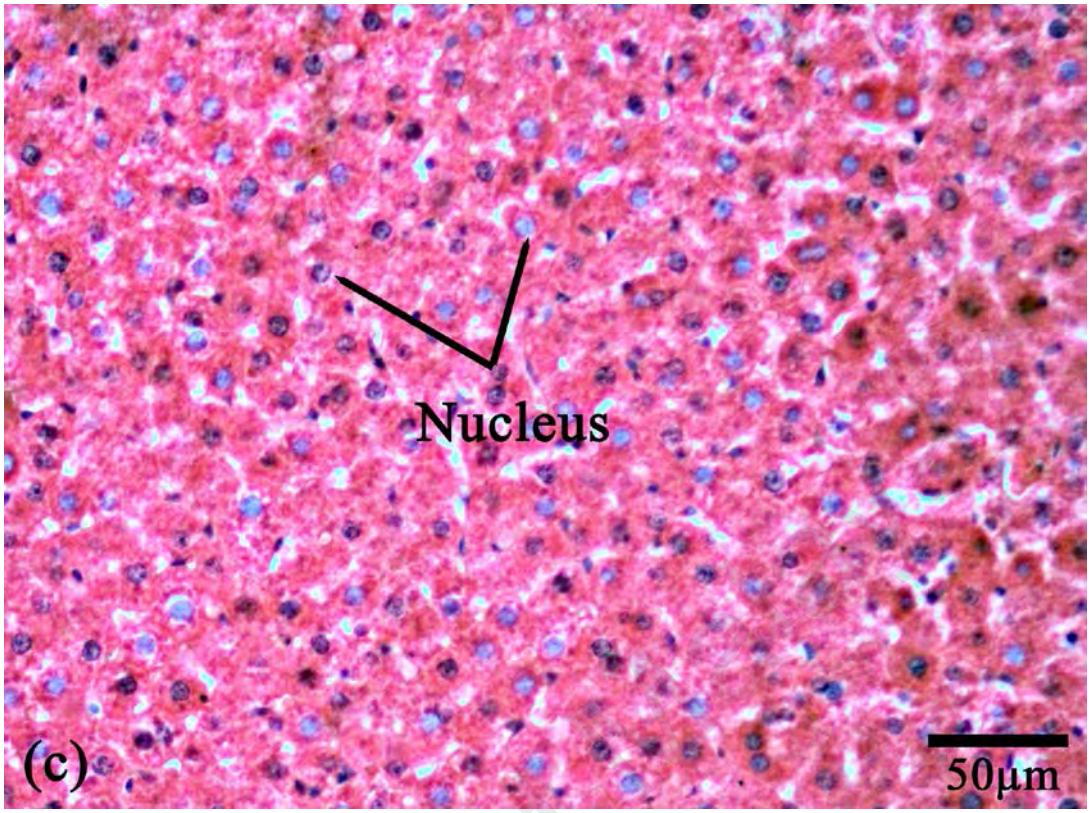



**Figure 4.10 (a):** continued.

Section shows central vein appear normal with normal nucleus cells. There is no abscess formation, no sign of inflammation and no sign of HS were observed.

### 4.6.3 Haematological Analysis

White blood count (WBC) count was performed on all group from day 1 of blood collected. Table 4.2 shows there is significance of value among rats vaccinated with Group 1 (pVAX1-ABA392), Group 2 killed bacterin (positive control), Group 3 (pVAX1 vector) and Group 4 PBS (negative control). Total white blood count of Group 1 (pVAX1-ABA392) and Group 2 (positive control) had slight increase of total WBC compare Group 3 (pVAX1 vector) and 4 PBS (negative control). First week of immunization, all group show no significant differences were observed till second booster immunization given. On 3$^{rd}$ week, the graph shows increase of leukocytosis from week 4 to 6 in group 1 and 4. There were statistically significance differences

($p<0.05$) between week 5 and 6. The rats in negative control and vector group did not show any significance increase in leucocyte count. All the collected data was analysed using T-independent test. From the Figure 4.11 show no significance different between vaccinated group with positive control meanwhile there were significance different between vaccinated group 1 and negative group.

**Table 4.1:** Total WBC count and significance of values among groups of rats.

| Animal Group | No. of animal | Type of Vaccination | T. White Blood Count (means ±S.E.M) Significance |
|---|---|---|---|
| Group 1 | 3 | pVAX1-ABA392 (100µg) | 11046 (1457)[a] |
| Group 2 | 3 | Positive control-Killed bacterin (50µg) | 9725 (880)[a] |
| Group 3 | 3 | pVAX1 vector (50µg) | 2294 (106)[b] |
| Group 4 | 3 | Negative control-PBS (100µl) | 2742 (122)[b] |

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

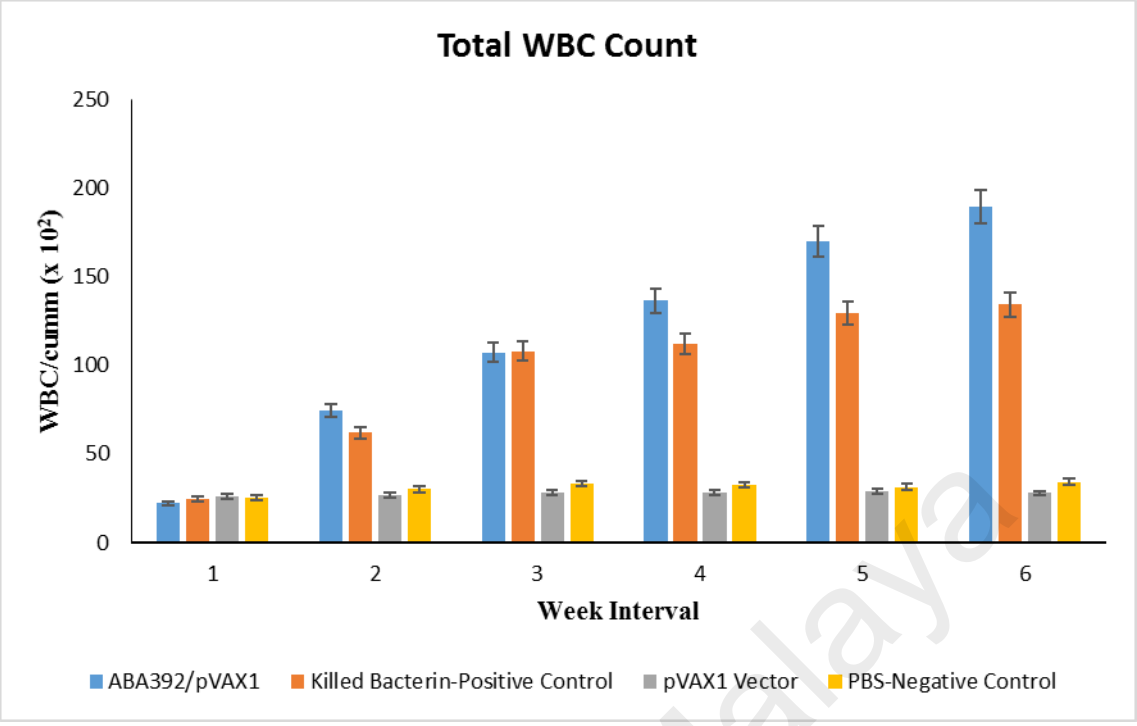



**Figure 4.11:** Graph representing the mean WBC count of various groups and treatment for 6 interval weeks.

Total WBC from vaccinated group ABA392-pVAX1 compared with normal group, pVAX1 vector and killed bacterin of P.multocida. Each points shows means of total WBC count of three pooled serum sample from three individual rats(n=3) and bar represents SE value. $p<0.05$.

### 4.6.4 Liver Function Test (LFT)

Liver function test (LFT) was performed on all group on day 35. Table 4.3 shows, Group 1 (pVAX1-ABA392), Group 2 killed bacterin (positive control), Group 3 (pVAX1 vector) and Group 4 PBS (negative control). The serum level of albumin, alkaline Phosphatase, alanine aminotransferase, total bilirubin and G-glutamyl transferase. LFT in all groups were in normal value. There was no statistically significant differences were observed in albumin, alkaline phosphatase and alanine aminotransferase between vaccinated group 1 and negative control group ($p>0.05$). All the collected data were analysis using independent T-Test.

**Table 4.2:** Liver function test and significance of values among vaccinated group.

| | | 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 | pVAX1-ABA392 (100µg) | 39.67±0.607 [a] | 230.67±11.624[a] | 48.33±1.202 [a] | 2.00±0.00 | 6.00±0.00 |
| Group 2 | Positive control-Killed bacterin (50µg) | 37.33±0.333 [a] | 184.33±23.877[a] | 38.67±2.186 [a] | 2.00±0.00 | 6.00±0.00 |
| Group 3 | pVAX1 vector (50µg) | 36.67±0.667 [a] | 184.33±4.667 [a] | 38.67±4.702 [a] | 2.00±0.00 | 6.00±0.00 |
| Group 4 | Negative control-PBS (100µl) | 39.00±1.732 [a] | 150.33±9.262 [a] | 47.00±4.509 [a] | 2.00±0.00 | 6.00±0.00 |

Results are presented as mean±SD
SD value for both group total bilirubin and G-Glutamyl Transferase is 0.

### 4.6.5 Renal Function Test (RFT)

Renal function test (RFT) was performed on all group on day 35. Table 4.4 shows, Group 1 (pVAX1-ABA392), Group 2 killed bacterin (positive control), Group 3 (pVAX1 vector) and Group 4 PBS (negative control). The serum level of sodium, potassium, chloride, urea and creatinine. RFT in all groups were in normal value. There was no statistically significant differences were observed in sodium, potassium, chloride, urea, creatinine between vaccinated group 1 and negative control group ($p>0.05$). All the collected data were analysis using independent T-Test.

**Table 4.3:** Renal function test and significance of values among vaccinated group.

| | | Parameter | | | | |
|---|---|---|---|---|---|---|
| **Animal Group/ No. of animal** | **Type of Vaccination** | **Sodium (serum) mmol/L** | **Potassium (serum) mmol/L** | **Chloride (serum) mmol/L** | **Urea (serum) mmol/L** | **Creatinine (serum) mmol/L** |
| Group 1 | pVAX1-ABA392 (100µg) | 142±1.000 [a] | 6.4±0.458 [a] | 103.33±0.882 [a] | 7.13±0.384 [a] | 29.3±0.33 [a] |
| Group 2 | Positive control-Killed bacterin (50µg) | 140±1.528 [a] | 6.77±0.371 [a] | 101.0±1.528 [a] | 6.70±0.288 [a] | 28.6±0.880 [a] |
| Group 3 | pVAX1 vector (50µg) | 137±0.577 [a] | 5.43±0.581 [a] | 97.67±0.333 [a] | 6.56±0.417 [a] | 28.0±1.155 [a] |
| Group 4 | Negative control-PBS (100µl) | 137±0.577 [a] | 6.10±0.115 [a] | 99.33±0.882 [a] | 5.7±0.260 [a] | 27.3±1.200 [a] |

Result are presented as Mean±SD

### 4.6.6 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA were used to analysed the presence of IgG antibody response in rats. Table 4.5 ELISA OD reading using 450nm performed on serum from rat vaccinated pVAX1-ABA392 (100µg), positive control, pVAX1 vector and compared with vaccinated PBS group (negative control). Figure 4.12 graph shows vaccinated group pVAX1-ABA392 (100µg/ml) higher titre IgG compare to other groups. There were no significance differences after 1$^{st}$ immunization were given. Positive control (killed bacterin) and immunazation group shows constant presence of IgG after 2$^{nd}$ immunization on week 3 onwards. Level of IgG antibody increase and significant differences were observed from week 3 to week 6 ($p<0.05$). DNA vaccine induce cellular immunoresponse against recombinant clone. The figure demonstrates the levels of antibodies induced by the DNA vaccine, and it was found that the abilities of pVAX1-ABA392 to stimulate cellular immunoresponse is comparable to the attenuated killed bacterin. All the collected data was analysed using T-independent test.

**Table 4.4:** ELISA OD reading using 450nm performed on serum from rat vaccinated pVAX1-ABA392 (100µg), killed bacterin (positive control), pVAX1 vector (50µg) 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 | pVAX1-ABA392 (100µg) | 0.090(0.017)[a] |
| Group 2 | 3 | Positive control-Killed bacterin (50µg) | 0.080(0.005)[a] |
| Group 3 | 3 | pVAX1 vector (50µg) | 0.050(0.011)[a] |
| Group 4 | 3 | Negative control-PBS (100µl) | 0.044(0.008)[b] |

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

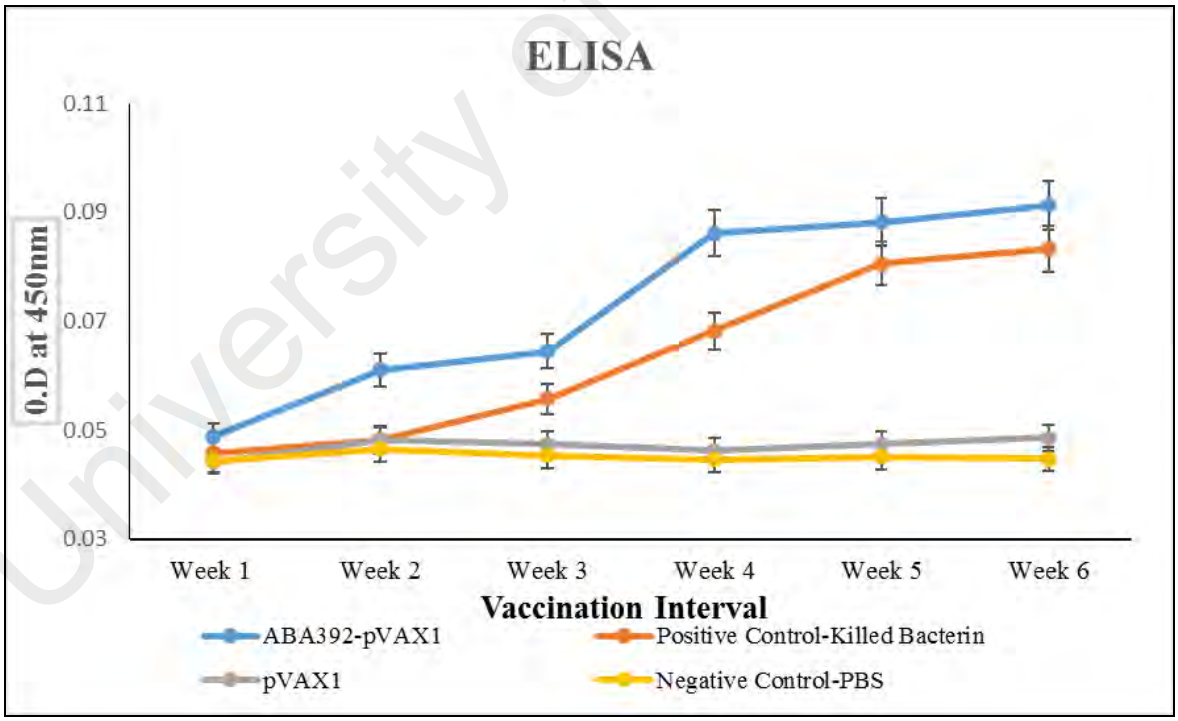



**Figure 4.12:** ELISA O.D Analysis using 450nm.

Scale shows, comparison on serum IgG from vaccinated rats with recombinant vaccine pVAX1-ABA392 with order groups, killed bacterin (positive) *P.multocida*

serotype B, pVAX1 vector and PBS (negative control). Each bar shows mean O.D ±standard error of four pooled serum sample where each contained sera from six individual rats. *$p \leq 0.05$.

# CHAPTER 5: DISCUSSIONS

## 5.1 Confirmation of findings

In this experiment, ABA392 in competent cells was purified (Figure 4.1). The primers were designed specifically with *Hin*dIII and *Bam*HI restriction enzyme (RE) sites. Through colony library (Figure 4.2), potential transformant were patch plated on LB added kanamycin 30µg and chloramphenicol 34µg to confirm the insert. Generally, ABA392 ligated into pVAX1 vector were successfully carried out (Figure 4.3). This gene stability maintained in *E. coli* TOP10 host (competent cells) and produced high copy number of replication in host cell. The recombinant clone, was extracted and characterised for plasmid analysis through PCR and gel electrophoresis and was sent for DNA sequencing for ABA392 gene and further analysed using BLAST, as well as by homology and nucleotide sequence analysis. The size of the insert was estimated about 804bp based on 1kb Plus DNA Ladder and the site of the recombinant clone pVAX1-ABA392 was at ~3.8kb base pair. The clone pVAX1-ABA392 shows promising result and large scale plasmid isolation (Figure 4.4) test carry out to reconfirm the clone stability although the clone has proven to stable up to 20 generations.

The purpose of analysis recombinant clone is to reconfirm the presence and the stability of ABA392 gene insert in the pVAX1 mammalian DNA vector, involving RE digestion method, DNA sequencing and BLAST. Through PCR analysis, detects the gene of interest in the recombinant mammalian vector and stability in vector system. Gel electrophoresis determine the size of the insert is expected to be 804bp. The primers were designed specifically with *HindIII* and *BamHI* restriction enzyme (RE) sites are used to allow ligated with digested vector as well to promoter with digested ABA392 gene by ligating through sticky ends. This positive amplification proved the primer designs could be used for future ABA392 gen amplification.

The pVAX1 mammalian vector is an expression vector mainly used for DNA vaccine and the size was found to be at ~3.0kb, kanamycin resistant and multiple cloning sites (Appendix E). This vector is produced and synthesized by Invitrogen (USA) and it contains human cytomegalovirus immediate-early (CMV) promoter for high-level expression in mammalian cells (Table 5.0). The designed-vector is created for immune respond.

**Table 5.0:** The features and benefits of pVAX1 mammalian vector.

| Features | Benefits |
|---|---|
| Human cytomegalovirus (CMV) | • Immediate-early promoter/enhancer<br>• Permits efficient, high-level expression on recombinant clone |
| T7 promoter/priming site | • *In vitro* transcription in sense orientation<br>• Allows sequencing through the insert |
| Multiple cloning site | • Allows insertion of gene and facilitates cloning |
| Bovine growth hormone (BGH) reverse priming site | • Allows sequencing through insert |
| BGH polyadenylation signal | • Efficient transcription termination<br>• Polyadenylation of mRNA |
| Kanamycin resistance gene | • Selection of vector in *E.coli* |
| pUC origin | • High-copy number replication and growth in *E.coli* |

The pVAX1 structure is similar to pUC vector with an AUG start codon, TAA, TAG or TGA stop codon, a cytomegalovirus (CMV) promoter and also a translation initiator known as the Kozak sequence (Montgomery & Prather, 2006). The only difference between both vectors is the absence of Intron A in the pVAX1 vector (William, 2014). The pVAX1 is a positive control vector for mammalian transfection

and expression. It is used as effective transfection conditions for cell line. The gene encoding β-galactosidase is expressed in mammalian cells under the control of the CMV promoter resulting in the β-galactosidase expression and can be easily assayed. pVAX1 vector was constructed to be consistent with the Food and Drug Administration (FDA) (Williams, JA. 2013).

### 5.2 Immunogenicity determination

Six to eight weeks old pathogen free, twelve white Spargue Dawly's female rats weighing between 150-200g were used and immunized with 2 doses of two weeks apart (100µg/ml) intramuscularly. The rats were monitored regularly and blood was collected via tail (1ml) for heamatological analysis. The result showed a promising finding in which 100% of total survival rate among groups of rats with various vaccination in the end of 35 days (Table 4.0). White blood count (WBC) count was performed on all group from day 1 of blood collected. On first week of immunization, all group show no significant differences were observed till second booster immunization given. On 3$^{rd}$ week, the graph shows increase of WBC from week 4 to 6 in vaccinated group and negative control group. Table 4.1 shows there is significance of value among vaccinated group and other groups. Total WBC count of killed bacterin group showed had slight increase of total WBC count (leukocytosis) compare pVAX1 vector and negative group. There were statistically significance differences ($p<0.05$) between week 5 and 6. The rats in negative control and vector group did not show any significance increase in leucocyte count.

On other hand, the serum obtained from vaccinated groups, positive control (killed bacterin), pVAX1 vector and negative control (normal saline) provided result from ELISA showed positive result. The presence of antibody Ig G from serum of rats immunized with recombinant clone pVAX1-ABA392 shows high titer antibody

compare to other groups. Significant difference was observed in serum from immunized pVAX1-ABA392 and negative group ($p<0.05$) which stimulate cellular immune response against recombinant clone (Salmah *et al.,* 2000). Positive control (killed bacterin) group sera showed, significance differences compare to negative control due to killed bacterin have homologus potential as inactivated vaccine (Tarek *et al*., 2014). The recombinant clone pVAX1-ABA392 vaccine produce high titer antibody compare positive group (killed bacterin) and pVAX1 vector. Figure 4.12, demonstrates the levels of antibodies induced by the DNA vaccine, and it was found that the abilities of pVAX1-ABA392 to stimulate cellular immunoresponse is comparable to the attenuated killed bacterin. All collected data were analysed using independent T-Test.

## 5.3 Pathogenicity Analysis

All the rats showed no signs and symptoms of HS was seen in the injected with recombinant clone pVAX1-ABA392 and killed bacterin. There is also no lesion or sign of abscess at injection site. The recombinant clone pVAX1-ABA392 signifies that, this vaccine does not cause any side effect or visible changes to the host. Gross pathology shows, there were no lesion and no sign of HS like hemorrhage, oedema were found in the heart, kidney, lung and liver (Figure 4.6) (Salmah *et al.,* 2010). Histopathological study was performed on both groups of vaccinated and negative control group. From histo-slides microscopic examination, both groups of pVAX1-ABA392 and negative control group shows there were no sign of hemorrhage on heart, kidney, lung and liver. This showed that recombinant clone pVAX1-ABA392 (DNA vaccine) are safe to use as vaccine since it shown no harm or any toxicity effect on the vaccinated rats especially on the site of administration.

## 5.4 Haematological Analysis

The hematological analysis shows increased in WBC count from week 1 to week 6. White blood count (WBC) count was performed on all group from day 1 of blood collected. Total WBC shows leukocytosis (Table 4.1) shows there is significance of value among vaccinated group when compared to negative group ($p<0.05$). The mammalian host depend on two types of immunity, the innate and adaptive. Innate immunity known as first line of protection which guard the host against various internal and external foreign texture. Meanwhile adaptive immunity when there is successive exposure to foreign texture there will be increased defensive response of the immune system (Kumar & Adrian, 2016). The biochemical analysis in this study being an effective parameters to reveal an impairment functional capacities of mammalian host.

Liver function test (LFT) was performed on all groups. This studies was carried out to see the effect of the vaccinated rats with pVAX1-ABA392 (Table 4.2), on liver function enzyme parameter such as albumin, alkaline phosphatase, alanine aminotransferase, total bilirubin and G-glutamyl transferase in order to assess the hepatotoxic potential of this recombinant DNA vaccine. The obtain result of ALP, ALT, total bilirubin, and GGT of treated with recombinant protein vaccine pVAX1-ABA392 (100µg) groups were in normal value. Meanwhile, histopathology examined of liver tissue also show no degenerative changes in both vaccinated groups Hepatic injury is often correlated with alteration in the serum and liver of some enzyme particularly alanine aminotransferase, alkaline phosphatase (Donepudi *et al.,* 2016).

Renal function test (RFT) are important to test the presence or absence lesion in the kidney. RFT in this study was conducted to see the impacts of the vaccinated rats with pVAX1-ABA392 (100µg) on the renal function test parameters of sodium, potassium, chloride, urea and creatinine (Table 4.3). Sodium, chloride, urea and creatinine level in

recombinant DNA vaccine group were in normal value. Histopathology examined of kidney tissue also show no degenerative changes in vaccinated groups.

# CHAPTER 6: CONCLUSION

## 6.1 Conclusion

*Pasteurella multocida* is the main cause of haemorrhagic septicaemia (HS) outbreak in livestock, such as cattle and buffaloes. HS disease cause severe losses in the animal husbandry industry. This respiratory disease which is acute, febrile and lethal capable of killing vulnerable animals in less than 36 to 48 hours (Dawkins *et al*., 1990). According to the report published by Saad (2013), HS outbreak in Malaysia causes an average loss of 360 heads of cattle and buffaloes in a year. Therefore, the production of quality livestock is always a major problem in Malaysia, since the animals are constantly fraught with diseases, especially HS. The most possible route for HS infection is buffaloes and cattle, and this could be due to the consumption of river water contaminated with HS infected carcasses during monsoon season (Jesse *et al*., 2013). Vaccination is the only available preventive measure for the control of disease, annual vaccination is recommended in the endemic areas. Since *Pasteurella* is a poor immunogen, a large amount of antigen has to be administered. This procedure leads to endotoxic shock to vaccinated animals (De Alwis, 1999).

This study was based on the previously created clone of ABA392 from *P. multocida* serotype B:2 (Salmah, 2000). The ABA392 gene causes HS sign and symptoms when injected into rats intraperitoneally. Mice died within 36 hours of injection. In order to further understand and study on the gene, this experiment was conducted. The ABA392 gene has shown promising results on previous study (Salmah *et al.*, 2010; Hussaini *et al*., 2012b) which could be potential in providing 83% immunity against HS in mice model and can be used as a vaccine candidate. The potential of the previously contracted recombinant clone ABA392 derived from *P. multocida* serotype B to protect laboratory animal against haemorrhagic septicaemia (HS) was determined (Hussaini *et al*., 2012b). Since HS determinant yet to be reported or discovered elsewhere, this study

conduct with great interest with an aim to evaluate the immunogenicity potential of recombinant DNA vaccine ABA392 using pVAX1 DNA vector. Hence, pVAX1 vector is specifically designed for use in the development of DNA vaccine. Currently, there is no specific DNA vaccine for HS in cattle and buffaloes in global.

The aim of this research was to evaluate the immunogenic potential and protective efficacy of DNA vaccine from pVAX1-ABA392 recombinant clone in rats against HS. The objective of this study divided into three phases; to prepare and analyse DNA insert stability of pVAX-1-ABA392 recombinant clone; access the immunogenecity of DNA recombinant clone pVAX1-ABA392 in vaccinated rats against HS and to evaluate and determinate the histopathological effect in vaccinated.

In phase one of this research it was important to reconfirm the clone stability of recombinant plasmid DNA (pVAX1-ABA392) in order to make sure the plasmid was stable and could be reproduced by subculturing them up to 20 generations as described previously (Salmah, 2000). The clone was found to be stable and the expected 3.8kb plasmid was recovered. Six to eight weeks old pathogen free, twelve white Spargue Dawly's female rats weighing between 150-200g were used as a possible animal models for HS studies.

Overall, this study shows the new recombinant vaccine pVAX1-ABA392 was successfully expressed and stably maintained in *E. coli* TOP10 host (competent cells). This recombinant vaccine were immunized into animal model via intramuscularly. Gross pathology and histo-slides microscopic examination shows, there were no lesion and no sign of HS like hemorrhage, oedema were found in the heart, kidney, lung and liver. There is no lesion or inflammation were observed in vaccinated rats. Serum sample collected from a group of vaccinated rats for ELISA test shows positive result, were the antibody in immunized rats were presence high titer compare with negative

group. Significant difference was observed in serum from immunized pVAX1-ABA392 and negative group ($p<0.05$). On the other hand, total WBC count of vaccinated group showed had slight increase of total WBC count (leukocytosis) compare pVAX1 vector and negative group. There were statistically significance differences ($p<0.05$) between week 5 and 6 which stimulate cellular immune response against recombinant clone.

We have demonstrated that, the DNA vaccine pVAX1-ABA392 has a capability to produce high titer of antibody against HS caused by *P. multocida* have a potential to be a vaccine candidate in this studies. Further challenge studies using *P. multocida* serotype B:2 to be used against this DNA vaccine to enhance the effectiveness of the recombinant clone and have positive potential to be use as vaccine in future.

In addition, we were not able to carry out protective properties of the vaccine candidate in the vaccinated animal which involving challenging with *P. multocida* serotype B: 2 as a positive control. 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. DNA vaccination has the high capability to provoke immune response, challenge studies using *P. multocida* serotype B: 2 against HS vaccination in future. Thus, this 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 vaccination in cattle or buffalo will be targeted as future study. Besides that, studies must be conducted on immune response from new vaccine and existing HS vaccine to compare the effectiveness.

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