UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF AN AVIRULENT *PASTEURELLA MULTOCIDA* B:2 BY DISRUPTION OF THE ABA392 DNA FRAGMENT

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

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MASTER OF SCIENCE
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2008

Specially dedicated to:

Abah and Mak,

ENCIK MOHAMED ZULPERI ZAKARIA
PUAN SAMIRAH ISMAIL

Adik,

ZARIRAH MOHAMED ZULPERI

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the degree of Master of Science

DEVELOPMENT OF AN AVIRULENT *PASTEURELLA MULTOCIDA* B:2 BY DISRUPTION OF THE ABA392 DNA FRAGMENT

By

DZARIFAH BINTI MOHAMED ZULPERI

Chairman: Siti Khairani Bejo, PhD

Faculty: Veterinary Medicine

Haemorrhagic septicaemia (HS) is an acute disease infecting cattle and buffaloes caused by *Pasteurella multocida* serotype B:2 which leads to great economic losses in countries with expanded animal industries such as Malaysia. This study was conducted to construct a mutant derived from the 921bp ABA392 virulence gene in order to make avirulent *P. multocida* serotype B:2, thus making this *P. multocida* B:2 mutant a potential candidate for a live-attenuated vaccine against HS.

The detection of a fragment which is related to *P. multocida* B:2 pathogenicity, the 921bp ABA392 virulence gene was carried out using Polymerase Chain Reaction (PCR) assay. Two *P. multocida* isolates, namely PMTB and 3030, isolated from a HS outbreak were used for PCR amplification. The particular 921bp DNA fragment was found to be present in *P. multocida* B:2 genome as 803bp in size. Sequencing of the recombinant plasmids confirmed that the 803bp gene was 98% homologous to the reference sequence, the virulence 921bp ABA392 DNA fragment.

Southern hybridization analysis revealed the 804bp of ABA392 gene was located at approximately 6kb position in the *P. multocida* B:2 genome. PCR performed towards the approximately 6kb DNA fragment produced an 803bp band which was confirmed to be the ABA392 virulence gene. The amplified PCR product was cloned and sequenced. Nucleotide sequences obtained were 98% identical to the reference strain, the 921bp ABA392 virulence gene. Pathogenicity test on the recombinant plasmids proved that the 804bp gene inserted within these plasmids still possessed the virulence properties of the 921bp ABA392 gene which may lead to HS disease in cattle and buffaloes.

In an attempt to produce *P. multocida* B:2 mutants through allelic exchange, the 804bp of ABA392 gene which was disrupted with kanamycin cassette was cloned inside suicide plasmid pAKA19 through shotgun ligation technique. The desired 7kb product was transformed into several different *E. coli* (TOP 10, JM109, AS11Yλ and DH5α) hosts for preservation. Verification of the 7kb ligation product with digestion by *Pst*I, *Hind*III and *Xho*I restriction enzymes revealed the exact sizes of kanamycin cassette (1.2kb), pAKA19 (5.0kb) and the disrupted 804bp of ABA392 virulence gene (804bp). An antibiotic sensitivity test on *P. multocida* B:2 and the respective *E. coli* strains were performed in order to select the donor and recipient strains for conjugation process. This test revealed that *E. coli* DH5α was suitable for the donor strain since it showed low resistance to streptomycin and *P. multocida* B:2 as the recipient strain for its high resistance towards streptomycin. Conjugation between donor and recipient strains was then achieved by plate-mating method. About 20 single colonies of

positive transconjugants were picked and subcultured on BHI blood agar containing kanamycin for 5 days to encourage loss of pAKA19 plasmid and to enhance the allelic exchange between the *ABA392*::Km^R insert with the native *ABA392* gene on the recipient chromosome. No plasmid was observed which indicated the loss of suicide plasmid. Direct colony PCR was performed to detect the changes in the *ABA392* gene of the parent strain, where a 2kb band was observed signifying that allelic exchange has taken place and the organism is now a *P. multocida* B:2 mutants.

Parent strains of *P*. *multocida* B:2 were highly virulent and killed mice within 24 hours. Mice inoculated with *P. multocida* B:2 mutant survived. Direct smear from mice's heart blood inoculated with the mutant showed the existence of bipolar organism which indicated the presence of *P. multocida* B:2. This result firmly suggests that the mutant, named as PMTBK was greatly attenuated and is thus a potential candidate organism for a live attenuated vaccine against HS.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGHASILAN *PASTEURELLA MULTOCIDA* B:2 AVIRULEN MELALUI TINDAKAN GANGGUAN TERHADAP FRAGMEN DNA ABA392

Oleh

DZARIFAH BINTI MOHAMED ZULPERI

Pengerusi: Siti Khairani Bejo, PhD

Fakulti: Perubatan Veterinar

Hawar berdarah (HS) merupakan penyakit akut yang menyerang lembu dan kerbau berpunca daripada bakteria *Pasteurella multocida* serotaip B:2 yang menyebabkan kemerosotan ekonomi bagi negara yang mempunyai industri penternakan berkembang maju seperti Malaysia. Kajian ini telah dijalankan untuk menghasilkan mutan avirulen daripada bakteria *P. multocida* B:2 terbitan daripada gen virulen ABA392 bersaiz 921bp, seterusnya menjadikan mutan ini calon yang berpotensi bagi penghasilan vaksin hidup teratenuat terhadap hawar berdarah.

Pengesanan fragmen DNA yang berkait rapat dengan kepatogenan *P. multocida* B:2 iaitu gen virulen ABA392 bersaiz 921bp telah dijalankan melalui assai amplifikasi Tindakbalas Berantai Polymerase (PCR). Dua isolat *P. multocida* iaitu PMTB dan 3030 yang telah dipencilkan dari kawasan wabak penyakit hawar berdarah digunakan dalam amplifikasi PCR. Fragmen DNA bersaiz 921bp telah disahkan hadir dalam genom *P. multocida* B:2 dengan saiz 803bp. Penjujukan terhadap plasmid rekombinan membuktikan bahawa gen

803bp adalah 98% homologous dengan jujukan rujukan iaitu fragmen DNA ABA392 yang virulen.

Analisis penghibridan Southern menunjukkan gen ABA392 bersaiz 804bp terletak pada anggaran posisi 6kb dalam genom *P. multocida* B:2. Amplifikasi PCR ke atas fragmen DNA tersebut menghasilkan produk bersaiz 803bp lalu mengesahkan kehadiran gen virulen ABA392. Produk PCR ini kemudian diklon dan dijujuk di mana jujukan-jujukan nukleotida yang diperoleh adalah 98% seiras dengan jujukan rujukan iaitu gen virulen ABA392 bersaiz 921bp. Ujian kepatogenan ke atas plasmid rekombinan membuktikan gen 804bp yang telah disisipkan ke dalam plasmid tersebut masih mengekalkan ciri-ciri virulen dalam gen ABA392 bersaiz 921bp yang menyebabkan penyakit hawar berdarah pada lembu dan kerbau.

Percubaan untuk menghasilkan mutan *P. multocida* B:2 melalui penukaran allel dijalankan melalui penyisipan kaset kanamycin ke dalam gen ABA392 bersaiz 804bp dan diklon ke dalam plasmid pengorban pAKA19 menerusi teknik ligasi 'shotgun'. Produk bersaiz 7kb yang diperoleh dipindahkan kepada beberapa perumah *E. coli* yang berbeza (TOP 10, JM109, AS11Yλ dan DH5α) untuk tujuan penyimpanan. Verifikasi ke atas produk ligasi bersaiz 7kb melalui tindakan pembatasan oleh enzim-enzim pembatas *Pst*I, *Hin*dIII dan *Xho*I mengesahkan kehadiran kaset kanamycin (1.2kb), pAKA19 (5.0kb) dan gen virulen gangguan ABA392 (804bp). Ujian sensitiviti terhadap antibiotik telah dijalankan ke atas *P. multocida* B: 2 dan strain-strain *E. coli* dalam usaha

memilih penderma dan penerima yang sesuai bagi proses konjugatan. *E. coli* DH5α telah didapati sesuai sebagai penderma kerana mempunyai kerintangan rendah terhadap streptomycin manakala *P. multocida* B:2 sebagai penerima kerana kerintangan tinggi terhadap streptomycin. Proses konjugatan antara penderma dan penerima dijalankan melalui kaedah plat pengawanan. Kira-kira 20 koloni tunggal transkonjugan positif telah dipilih untuk disubkultur selama 5 hari berturut-turut pada agar darah BHI bagi menggalakkan pelenyapan plasmid pAKA19, di samping menggalakkan penukaran allel di antara gen sisipan *ABA392::Km*^{R} dan gen induk *ABA392* pada kromosom penerima. Ketidakhadiran plasmid pAKA19 melalui elektroforesis agarose gel membuktikan plasmid tersebut telah berjaya dilenyapkan daripada gen gangguan 804bp. Aplikasi PCR secara langsung bagi mengesan perubahan dalam gen induk strain *ABA392* menghasilkan jalur bersaiz 2kb menandakan berlakunya perubahan allel dan organisma tersebut kini adalah mutan *P. multocida* B:2.

Strain induk *P. multocida* B:2 didapati amat virulen kerana strain ini membawa maut kepada tikus dalam tempoh 24 jam. Walau bagaimanapun, tikus yang telah diinokulat dengan mutan *P. multocida* B:2 didapati hidup. Saput terus daripada jantung tikus yang diinokulat dengan mutan menunjukkan kewujudan organisma dwikutub yang menandakan kehadiran *P. multocida* B:2. Keputusan ini dengan kuat mencadangkan bahawa mutan iaitu PMTBK adalah avirulen dan merupakan calon yang berpotensi untuk penghasilan vaksin teratenuat hidup terhadap hawar berdarah.

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I certify that an Examination Committee has met on 11 August 2008 to conduct the final examination of Dzarifah binti Mohamed Zulperi on her Master of Science thesis entitled "Development of Avirulent *Pasteurella multocida* B:2 by Disruption of the ABA392 DNA Fragment" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Master of Science.

Members of the Examination Committee were as follows:

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Date: 26 September 2008

DECLARATION

I hereby declare that the thesis is based on my original work for quotation and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any degree at UPM or other institutions.

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DZARIFAH MOHAMED ZULPERI

Date: 26 September 2008

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This thesis was submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfillment of the requirements for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

%	- percent
°C	- degree celcius
µg	- microgram
µl	- microliter
µm	- micronmeter
µM	- micromolar
Amp^R	- ampicillin resistance
BHI	- brain heart infusion
bp	- base pair
BRENDA	- Bacterial Restriction Endonuclease Analysis
$CaCl_2$	- calcium chloride
CIAP	- calf intestine alkaline phosphatase
DIG	- digoxigenin
DNA	- deoxyribonucleic acid
dNTP	- deoxynucleotide triphosphate
ds	- double-stranded
E. coli	- *Escherichia coli*
EDTA	- ethylene-diamine-tetraacetic acid
ELISA	- enzyme linked immunosorbent assay
F	- fertility
G	- gram
HCl	- hydrochloric acid
H_2O	- water
HS	- haemorrhagic septicaemia
i.p.	- intraperitoneal
IPTG	- isopropyl-β-D-thiogalacosidase
kb	- kilobase pair
Km^R	- kanamycin resistance
LB	- Luria-Bertani
LPS	- lipopolysaccharide
L	- liter
M	- molar
$MgCl_2$	- magnesium chloride
min	- minutes
ml	- milliliter
MM	- milimeter
mM	- milimolar
mob	- mobilization
NaCl	- natrium chloride
NADH	- nicotinamide adenine dinucleotide hydrogenase
NaOH	- natrium hydroxide
NBT/BCIP	- nitro blue tetrazolium/5-bromo-4-Chloro-3'-indolyphosphate p-toluidine salt
ng	- nanogram

nm	- nanometer
OD	- optical density
OMP	- outer membrane protein
ORF	- open reading frame
ori	- origin
P. multocida	- *Pasteurella multocida*
PBS	- phosphate buffer saline
PCR	- polymerase chain reaction
rpm	- rotation per minute
R	- resistance
RE	- restriction enzyme
RNA	- ribonucleic acid
s	- seconds
SDS	- sodium dodecyl sulphate
SSC	- sodium chloride-sodium citrate
Str^R	- streptomycin resistance
TBE	- tris-boric EDTA
Taq	- *Thermus aquaticus*
T_M	- melting temperature
U	- unit
UV	- ultra-violet
V	- voltan/volt
v/v	- volume per volume
w/v	- weight per volume

Amino Acid	Single/Three Letter	Amino Acid Code
Alanine	A	Ala
Arginine	R	Arg
Asparagine	N	Asn
Aspartic Acid	D	Asp
Glutamine	Q	Gln
Glutamic acid	E	Glu
Glycine	G	Gly
Isoleucine	I	Ile
Leucine	L	Leu
Lycine	K	Lys
Methionine	M	Met
Phenylalanine	F	Phe
Proline	P	Pro
Serine	S	Ser
Threonine	T	Thr
Tryptophan	W	Trp
Valine	V	Val

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CHAPTER 1

INTRODUCTION

Haemorrhagic septicaemia (HS) is an acute disease, mainly infecting cattle and water buffaloes. This disease is caused by two major serotypes of *Pasteurella multocida*, serotype B: 2 and E: 2 according to Carter-Heddleston classification, or serotype 6: B or 6: E based on Namioka-Carter classification. The first detailed report of an acute pasteurellosis, which affected deer, cattle and swine, has been attributed to Bollinger in 1878 who then reproduced the disease in cattle (Carter and de Alwis, 1989).

The outbreaks of this disease are recorded in many countries and killed many cattle and buffaloes every year (Bain *et al*., 1982). This disease occurred in near and Middle East countries and in several parts in some regions of Africa (Carter and de Alwis, 1989). Moreover, it is also considered to be the most economically important disease of livestock in South East Asia and causes significant economic losses in India and Africa (Chandrasekaran *et al*., 1981). The latest occurrence of HS in Malaysia was reported in Perak in the year 2005 where the disease has been confirmed to be caused by *Pasteurella multocida* serotype B:2 (Kamarudin, 2005).

HS has emerged as a disease of great economic importance. The annual losses of North American cattle industry due to HS was reported being approximately $US800 million (Verma and Jaiswal, 1997). In Malaysia, the production of quality livestock is always a major problem since the animals are constantly fraught with diseases, especially HS which threatens the quality of products (Saharee, 2005). This situation is hardening the government's objective for livestock production to reach approximately RM 8 billion by 2010 (Mohd Nordin, 2000).

Vaccination has become the principal method of controlling HS in many countries. In Malaysia, vaccination is considered the most common and cheaper way of controlling outbreaks of HS (Zamri, 2005). Most commonly used vaccines in Malaysia are the alum-precipitated vaccine and the oil adjuvant vaccine. These vaccines did make an impact of increasing the immunity of animals against HS but still HS outbreaks and deaths remain (Dawkins *et al*., 1990). This may be due to the facts that those vaccines provide only short-term immunity and requires annual administration for effectiveness (Chandrasekaran *et al*., 1994).

A lot of research has been done in order to produce a better vaccine for this disease. It is widely recognized that an ideal vaccine should posses certain characteristics, such as; easy and economical to produce, stable for use in the tropics, easy to handle in the field with consistency that make it easy to administer, no adverse reactions, high level of immunity with a minimum delay