UNIVERSITI PUTRA MALAYSIA

CONSTRUCTION OF AN ATTENUATED *PASTEURELLA MULTOCIDA* B:2 BY MUTATION IN THE *GDHA* GENE

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

September 2007

"Things should be made as simple as possible, but not any simpler."

-Albert Einstein-

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

CONSTRUCTION OF AN ATTENUATED *PASTEURELLA MULTOCIDA* B:2 BY MUTATION IN THE *GDHA* GENE

By

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September 2007

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Pasteurella multocida B:2 is a Gram negative bacteria that has been associated with haemorrhagic septicaemia in cattle and buffaloes in Asia. It has been known to produce endotoxin that leads to haemorrhages and oedema, causing deaths due to either asphyxiation and dyspnoea or septicaemia. Vaccination has been used to control the disease but with little success due to the low vaccination coverage. Therefore, an alternative live vaccine should be considered.

In preparing an alternative live vaccine, an attenuated *P. multocida* B:2 is created by manipulating one of the housekeeping genes of the bacteria. The selected housekeeping gene, the glutamate dehyrogenase (*gdh*A) gene, was successfully isolated via PCR from wild type *P. multocida* B:2. The gene was then amplified using nested-PCR to determine its functional part. Both PCR products were cloned into plasmid pCR2.1, producing pSZ1 and pSZ2,

respectively before being sequenced. The whole sequence of the gene is 1108 bp while the functional part of the gene was 652 bp. The functional part was 99.8% identical to the model sequence, the PM70, which is a model genome sequence of *P. multocida* serotype A.

The pSZ1 was subsequently digested with a unique restriction enzyme, *Mun*I before the kanamycin cassette, isolated from plasmid pUC4K via PCR, was inserted at the centre of the housekeeping gene. The recombinant was named pSZ1K. After that, the *gdhA* gene that was disrupted by kanamycin cassette (GK) was isolated from the pSZ1K using restriction enzyme digestion, *Eco*RI. The suicide plasmid, pAKA19 was also digested with the same enzyme to achieve complimentary ligation sites. After ligation, the achieved recombinant plasmid was called pSZ19GK. All cloning products were transformed into *Escherichia coli* DH5*α*. En route for disruption of the gene in the host genome, both *E. coli* and *P. multocida* B:2 were subjected to spontaneous mutation towards streptomycin. After conveying the pSZ19GK into *P. multocida* B:2 via conjugation, the bacteria was incubated for five days to encourage allelic exchange to occur between disrupted gene and the host chromosome. Subsequently, PCR of the bacteria genome proved that allelic exchange has occurred and the mutant was called *P. multocida* B:2 (GK).

In order to verify the characteristic of the non-pathogenic *P. multocida* B:2 (GK) mutant, *in vitro* stability test and *in vivo* pathogenicity test were done. In *in vitro* stability test, 14 strains out of the 20 survived only up to 15 days of incubation. This proves that the mutants are unable to sustain life without

glutamate supplement and therefore having a short life-span. From there, several strains were picked to be tested *in vivo* using mouse experimental model. Mice infected intraperitoneally or subcutaneously with different concentrations of the mutant survived throughout the 5-day study period. They were compared to the mice that were infected intraperitoneally or subcutaneously with different concentrations of the wild type organism. None of the mice infected with the mutant died but all mice infected with the wild type did not survived and were dead in less than 24 hours. *P. multocida* B:2 were successfully isolated from organs of mice infected with both wild-type and mutant. This confirmed that the mutant, *P. multocida* B:2 (GK) became attenuated by the disruption of the *gdhA* gene and has a good potential to be used as an alternative live vaccine for HS.

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

PEMBENTUKAN BAKTERIA ATENUAT *PASTEURELLA MULTOCIDA* B:2 MELALUI PELAKUAN MUTASI DALAM GEN *GDHA*

Oleh

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Pasteurella multocida B:2 adalah bakteria Gram negatif yang dikaitkan dengan penyakit hawar berdarah di dalam lembu dan kerbau di Asia. Bakteria ini menghasilkan endotoksin yang menyebabkan hemoraj dan edema, kematian diakibatkan sama ada pengasfikasiaan dan dispnea atau septisemia. Vaksinasi telah digunakan untuk mengawal penyakit ini tetapi cara ini kurang efektif disebabkan kawasan liputan vaksinasi adalah rendah. Oleh itu, vaksin hidup alternatif perlu dihasilkan untuk menangani masalah ini.

Dalam percubaan menghasilkan vaksin hidup alternatif, *P. multocida* B:2 yang atenuat, salah satu daripada gen domestik dalam bakteria ini dimanipulasikan. Gen domestik ini, gen glutamate dehidrogenase, telah berjaya diasingkan menggunakan PCR daripada *P. multocida* B:2 jenis liar. Gen itu diamplifikasi sekali lagi menggunakan 'nested-PCR' untuk memperolehi bahagian fungsinya. Kedua-dua produk PCR ini telah diklonkan

ke dalam plasmid pCR2.1 menghasilkan pSZ1 dan pSZ2 dan kemudian ditentukan jujukannya. Keseluruhan jujukan gen tersebut mempunyai 1108 bp dan bahagian fungsinya mempunyai 652 bp. Bahagian fungsinya telah menghasilkan 99.8% komplimentari terhadap jujukan model, PM70 iaitu model keseluruhan jujukan genom bagi *P. multocida* serotip A.

Dalam pengklonan fasa kedua, pSZ1 telah dibatasi oleh enzim pembatasan yang unik, *Mun*I dan kaset kanamicin yang telah diasingkan daripada plasmid pUC4K melalui PCR dan telah dimasukkan di tengah-tengah gen domestik tersebut (GK). Rekombinan DNA itu dinamakan pSZ1K. Gen *gdhA* yang telah mutan diasingkan dengan menggunakan enzim pembatasan, *Eco*RI. Plasmid 'suicide', pAKA19 juga telah dibatasi dengan emzim pembatasan yang sama untuk menghasilkan hujung lekatan yang komplimentari dengan kaset GK. Selepas proses pelekatan, rekombinan plasmid itu dinamakan pSZ19GK. Kesemua produk pengklonan ditransformasi ke dalam *Escherichia coli* DH5*α*. Seterusnya untuk menukar gen yang telah dikonstruk pada genom hos, kedua-dua *E. coli* dan *P. multocida* B:2 telah melalui mutasi spontan terhadap antibiotik streptomicin. Setelah menghantar pSZ19GK ke dalam *P. multocida* B:2 melalui proses kojugasi, bakteria itu telah diinkubasi selama lima hari untuk menggalakkan proses pertukaran alel berlaku terhadap kaset GK dengan kromosom perumah. Selepas itu, PCR terhadap genom bakteria membuktikan bahawa pertukaran alel telah berlaku dan mutan yang terhasil dipanggil *P. multocida* B:2 (GK).

Seterusnya untuk membuktikan mutan *P. multocida* B:2 (GK) adalah tidak patogenik, ujian kestabilan secara *in vitro* dan ujian patogenik secara *in vivo* telah dijalankan. Dalam ujian kestabilan secara *in vitro,* 14 daripada 20 isolat telah berjaya dihidupkan selama 15 hari secara berterusan. Ini membuktikan adanya mekanisma kawalan dalam penghasilan bakteria yang tidak patogenik dengan jangka hayatnya yang pendek disebabkan oleh kekurangan glutamat. Dari situ, beberapa isolat telah dipilih untuk diuji dalam ujian patogenik secara *in vivo* menggunakan model eksperimen tikus. Mencit yang telah dijangkiti melalui ruang peritoneum dan dibawah lapisan kulit dengan *P. multocida* B:2 (GK) hidup yang dihasilkan dengan kepekatan yang berbeza, hidup sepanjang lima hari ujian. Berbanding dengan mencit yang dijangkiti oleh *P. multocida* B:2 pula, mati dalam masa kurang dari 24 jam. *P. multocida* B:2 berjaya diisolat dari organ mencit yang dijangkiti oleh kedua-dua bakteria. Ini membuktikan bakteria mutan itu, *P. multocida* B:2 (GK) telah atenuat dengan mengubah jujukan pada gen *gdhA* sekaligus berguna untuk penghasilan vaksin hidup alternatif bagi mengawal penyakit hawar berdarah ini.

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LIST OF ABBREVIATIONS

A_{260}	absorbance at 260 nm
$A_{450\ nm}$	absorbance at 450 nm
aa	amino acid
AcmA	*N*- acetylmuramidase
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
cDNA	complementary DNA
cfu	colony forming unit
CO_2	carbon dioxide
C-terminal	the carboxyl-terminal (-COOH) of a polypeptide
dH_2O	distilled water
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetate
EtBr	ethidium bromide
GMO	genetically modified organisms
h	hour
HCl	hydrochloric acid
His	histidine

i.p	intraperitoneally
i.v	intravenously
in vitro	in an experimental situation outside the organism
in vivo	in a living cell or organism
IPTG	isopropyl-D-galactopyranoside
kb	kilo base pair
kDa	kilo Daltons
LB	Luria Bertani
M	molarity
MCS	multiple cloning site
$MgCl_2$	magnesium chloride
min	minute
ml	millilitre
mM	milliMolar
NaCl	sodium chloride
NAG	*N*-acetylglucosamine
NAM	*N*-acetylmuramic acid
ng	nanogram
N-terminal	the amino-terminal (NH_2) of a polypeptide
^oC	degrees centigrade
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction

PEG	polyethylene glycol
pH	isoelectric point
POD	peroxidase
RE	restriction enzyme
RNase	ribonuclease
rpm	revolution per minute
RT	room temperature
s	second
TBE	tris-boric-EDTA
Taq	*Thermus aquaticus*
TCA	tri-carboxylic acid
TE	tris EDTA
U	unit
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume
%	percent/ pencentage
µl	microlitre
µg	microgram

CHAPTER 1

INTRODUCTION

Haemorrhagic septicaemia (HS) is a contagious bacterial disease caused by two serotypes of *Pasteurella multocida;* B2 and E2. It affects cattle (*Bos taurus* and *B. indicus*) and water buffaloes (*Bubalus bubalis*) with a high mortality rate in infected animals. It is irrefutably regarded as endemic in most parts of tropical Asia, Africa and India. Moreover, it is also considered to be the most economically important disease of livestock or large ruminants in South East Asia and causes significant economic losses in India and Africa. In Malaysia, the disease is enzootic in Kelantan, Terengganu, Kedah, Perak, Pahang and Negeri Sembilan due to the high population of cattle and buffaloes (Chadrasekeran 1981).

Outbreaks of haemorrhagic septicaemia have been observed during stressful conditions such as radical changes in weather, including the advent of monsoons, rainy seasons, debility caused by seasonal levels of low nutrition or changes in diet and the pressure of work (draft animals), such as increased activity during paddy cultivation. It is believed that the disease is spread by direct and indirect contact (fomites). The patterns of outbreaks revealed that carrier animals were subjected to stressful condition and start to shed the organisms and subsequently infect other susceptible animals.