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ADHERENCE, INVASION AND INTRACELLULAR SURVIVAL BETWEEN Pasteurella multocida B:2 AND ITS DERIVATIVES TOWARDS BOVINE AORTIC ENDOTHELIAL CELL

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Pasteurella multocida B:2 causes bovine haemorrhagic septicaemia leading to acute fatality in cattle and buffaloes. This bacterium spread rapidly from the respiratory tract into the bloodstream causing death within 24 hours. Countries where the disease is endemic resort to routine prophylactic vaccination. However, it failed to contain and eradicate the disease. Live-attenuated vaccines have the advantage of a natural route of entry to the host which allows targeting of immunomodulatory factors to the same sites of the immune system as occurs in the natural infection and achieves longer lasting immunity.*Pasteurella multocida* B:2 GDH7 is an attenuated derivative of the wild-type *P. multocida* B:2 isolated from a previous outbreak in Malaysia, that upon intranasal administration is an efficient vaccine for HS. This strain was genetically modified by the disruption of the wild-type *gdhA* gene with the insertion of a kanamycin cassette. This resulted in an interference of bacterial metabolism hence arresting its pathogenicity. This study primarily aims to investigate the potential of *P. multocida* B:2 GDH7 strain as a delivery vehicle for DNA vaccine applications. Following this, an investigation on the adherence, invasion and intracellular survival of the bacterial strains within the bovine aortic endothelial cell line (BAEC) were carried out. The parent strain and another mutant strain from Sri Lanka, *P. multocida* B:2 JRMT12 were used as control. The potential vaccine strain, *P. multocida* B:2 GDH7, was significantly better at adhering to and invading BAEC ($p \leq 0.05$) compared to the wild-type. Moreover, this strain was observed to survive intracellularly 7 hours post-treatment, although a steady decline in viability was noted with time.

A dual reporter plasmid, pSRGM that expresses red fluorescent protein (RFP) from a constitutive prokaryotic promoter within *P. multocida* B:2 and green fluorescent protein (GFP) from a constitutive eukaryotic promoter within mammalian cells was subsequently transformed into *P. multocida* B:2 GDH7. This construct was used to colocalize the bacteria when moving from the extracellular environment into the intracellular compartment of the mammalian cells. Intracellular trafficking of the vaccine strain, *P. multocida* B:2 GDH7 was visualized by tracking the reporter proteins via confocal laser scanning microscopy (CLSM). *Pasteurella multocida* B:2 GDH7 was found intracellularly of the mammalian cells and manage to release the reporter plasmid into the cytoplasm and allows GFP expression from the mammalian host at 3 h post-treatment. The ability of *P. multocida* B:2 GDH7 to model a bactofection represents the possibility for this potential vaccine strain to be used as a delivery vehicle for DNA vaccine. From this study, *P. multocida* B:2 GDH7, showed to be a promising candidate as a potential delivery vehicle for DNA vaccine.

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

INTERAKSI ANTARA *Pasteurella multocida* B:2 DAN DERIVATIFNYA DENGAN BOVINE AORTIC ENDOTHELIAL CELL

Oleh

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Hawar berdarah (HS) adalah penyakit yang disebabkan oleh bakteria *Pasteurella multocida* B:2 yang menyebabkan kematian kerbau dan lembu. Bakteria ini merebak cepat ke saluran penafasan dan salur darah haiwan dan seterusnya mengakibatkan kematian dalam masa 24 jam. Negara di mana penyakit ini sering berlaku menjalankan vaksinasi secara berterusan. Walau bagaimanapun, ia gagal untuk membendung dan membasmi penyakit ini. Vaksin hidup-atenuat mempunyai kelebihan menggunakan laluan semulajadi untuk masuk ke dalam hos yang membolehkan penyasaran faktor imun pada sistem imun sama seperti yang berlaku dalam jangkitan semula jadi dan bakal mencapai imuniti yang lebih bertahan lama.

Derivatif dilemahkan *P. multocida* B: 2 GDH7 telah dihasilkan daripada *P. multocida* B:2 jenis-liar yang diperoleh daripada wabak sebelumnya di Malaysia, apabila diberikan secara intranasal yang merupakan vaksin yang berkesan untuk HS. Strain ini telah diubahsuai secara genetik oleh gangguan daripada jenis-liar *gdhA* gen dengan memasukkan kaset kanamycin. Ini menyebabkan gangguan metabolisma bakteria itu yang menghalang kepatogenan. Tujuan utama kajian ini adalah untuk mengkaji potensi *P. multocida* B: 2 GDH7 sebagai kaedah penghantaran untuk aplikasi vaksin DNA. Berikutan itu, perlekatan, penaklukan dan kelangsungan intraselular *Pasteurella multocida* B: 2 GDH7 terhadap sel Bovine Aortic Endothelial (BAEC) telah dijalankan. *P. multocida* B:2 jenis-liar dan strain mutan dari Sri Lanka, *P. multocida* B:2 JRMT12 telah digunakan sebagai kawalan positif. Vaksin yang berpotensi, *P. multocida* B: 2 GDH7 menunjukkan kadar perlekatan dan penaklukan yang lebih baik ($p \leq 0.05$) berbanding dengan *P. multocida* B:2 jenis-liar dan *P. multocida* B:2 JRMT12. Selain itu, strain ini diperhatikan untuk terus hidup secara intraselular 7 jam selepas rawatan,

walaupun berlaku kemerosotan dalam kebolehidupan dengan peningkatan masa.

Plasmid pSRGM adalah plasmid yang mengandungi dua gen pelapor yang mengekspreskan protein pendarfluor merah (RFP) daripada penggalak prokariot konstitutif dalam *P. multocida* B: 2 dan protein pendarfluor hijau (GFP) daripada penggalak eukariot konstitutif dalam sel mamalia telah ditransformasikan ke dalam *P. multocida* B: 2 GDH7. Konstruk ini telah digunakan untuk memerhatikan pergerakan bakteria apabila bergerak dari luar ke dalam ruang intraselular titisan sel mamalia. Pergerakan intraselular strain vaksin yang berpotensi ini telah digambarkan melalui reporter protein menggunakan confocal laser scanning microscope (CLSM). *Pasteurella multocida* B:2 GDH7 ditemui secara intraselular dan berjaya melepaskan plasmid ke dalam sitoplasma dan membenarkan ekspresi GFP dari hos mamalia. Keupayaan *P. multocida* B: 2 GDH7 untuk memodelkan sistem baktofeksi menunjukkan berkemungkinan ia akan digunakan sebagai kaedah penghantaran untuk vaksin DNA. Daripada kajian ini, *P. multocida* B:2 GDH7 telah menunjukkan kebolehan sebagai calon yang berpotensi untuk dijadikan sebagai kaedah penghantaran vaksin DNA.

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I certify that a Thesis Examination Committee has met on 26 May 2017 to conduct the final examination of Nur Iqmaliza Akmal bt Mohd Kamal on her thesis entitled "Adherence, Invasion and Intracellular Survival between *Pasteurella multocida* B:2 and its Derivatives Towards Bovine Aortic Endothelial Cell" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

A:1	*Pasteurella multocida* biotype A capsular serotype 1
APC	Antigen presenting cell
APV	Alum precipitated vaccine
Amp	Ampicillin
*aro*A	Aromatic amino acid metabolism gene
ATCC	American type culture collection
B:2	*Pasteurella multocida* biotype B capsular serotype 2
BAEC	Bovine aortic endothelial cell
BHI	Brain heart infusion
bp	Base pair
BL-3	Bovine lymphoma-3
˚C	Degree Celsius
CD	Cytochalasin D
CFU	Colony forming unit
cm/mm	centimeter / millimeter
CMV	Cytomegalovirus
DIC	Differential interference contrast
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleocide triphosphates
E:2	*Pasteurella multocida* biotype E capsular serotype 2
EBL	Embryonic bovine lung
ECM	Extracellular matrix

EMS	Emission
Exc	Excitation
FBS	Fetal bovine serum
FFL	Filtered fluorescent light
FI	Fluorescence intensity
g / mg / µg	Grams / milligrams / micrograms
GFP	Green fluorescence protein
GM	Gentamicin
h	Hour (s)
HS	Haemorrhagic septicaemia
Ig	Immunoglobulin
i.m.	Intramuscular
i.n.	Intranasal
kb	Kilobase pair
kDa	Kilo Dalton
kg	Kilogram
Km	Kanamycin
kV	Kilo Volts
l / ml / µl	Litres / millilitres / microliters
lac	Lactose operon gene
LB	Luria-Bertani
LPS	Lipopolysacharicle
MHC	Major histocompatibility complex
M / mM / µM	Molar / millimolar / micromolar

min	Minute (s)
MOI	Multiplicity of infection
mw	Molecular weight
ng	Nanogram
OAV	Oil adjuvant vaccine
OD_x nm	Optical density at wavelength X nm
OMP	Outer-membrane protein
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
pH	Hydrogen ion concentration
P. m	*Pasteurella multocida*
P	Polymyxin B
RFP	Red fluorescent protein
rfu	Relative fluorescence unit
RNA	Ribonucleic acid
rpm	Revolutions per minute
rt	Room temperature
s.c.	Subcutaneous
sec	Second (s)
Sm	Streptomycin
TAE	Tris acetate EDTA
Tm	Melting temperature
U	Units

UK	United Kingdom
US	United States
UV	Ultraviolet
V	Volts

CHAPTER 1

INTRODUCTION

Haemorrhagic septicemia (HS) is a major disease in cattle and buffaloes caused by the infection of *Pasteurella multocida* (Abubakar and Zamri-Saad, 2011). In Asia, the bacterial serotype responsible for this disease is *P. multocida* B:2 (Zamri-Saad and Annas, 2016). Transmission of HS can occur from diseased animals or carriers through intranasal and oral routes. Invasion of the bacteria through endothelial cells results in the rapid infiltration into the animals' bloodstream (Galdiero *et al.*, 2001). Conventional vaccination procedures against HS is usually conducted prior to rainy seasons using either an oil-adjuvant vaccine or alum-precipitated vaccine. Despite both vaccines containing the bacterin, only a short-termed protection was conferred in vaccinated animals (Saharee and Salim, 1991). This is due to the fact that they provide quick but lower level of immunity. (Chandrasekaran *et al.*, 1993). Therefore, it was suggested that utilization of a live-vaccine will be a better candidate for prolonged protection against HS in animals (Zamri-Saad and Annas, 2016).

Currently available live attenuated vaccines consist live organisms such as the attenuated strain of *P. multocida* B:2 that has been shown to possess reduced virulency (Chandrasekaran *et al.*, 1993). *Pasteuella multocida* B:2 GDH7 is an attenuated derivative of the wild-type strain previously isolated from an outbreak in Malaysia (Sarah *et al.*, 2006). The attenuation strategy for the wild-type involved the disruption of the *gdh*A gene by insertion of a kanamycin cassette. This resulted in an interference of pathogen metabolism hence disrupted its pathogenicity (Sarah *et al.*, 2006). It was subsequently shown that intranasal administration of this attenuated strain provides an efficient vaccine candidate for HS (Rafidah and Zamri-Saad, 2013). Similarly, *P. multocida* B:2 JRMT12 is an attenuated *aro*A mutant of strain *P. multocida* B:2 85020 isolated from an outbreak in Sri Lanka (Tabatabaei *et al.*, 2002). When administered intramuscularly, the attenuated strain of JRMT12 was shown to confer a high degree of protection in mouse and bovine models of HS (Dagleish *et al.*, 2007). Since currently available vaccines such as alum-precipitated vaccine and oil-adjuvant vaccine were discovered to be less effective, a new alternative is crucially needed. The aforementioned mutants of *P. multocida* B:2 (GDH7 and JRMT12) have been found to be a promising manipulation for a live-attenuated *P. multocida* B:2 vaccine development *in vivo*. In this study, therefore, the interaction capacity of both potential vaccine strains, *P. multocida* B:2 (GDH7 and JRMT12) and a local isolate of *P.multocida* B:2 wild-type from a previous outbreak of HS in Malaysia towards bovine aortic endothelial cells will be assessed.

This could aid on the long term aim of the present work which is to exploit the capacity of the live vaccine strains to invade mammalian cells in order to deliver plasmid DNA encoding a protective antigen for another disease of the target animal, thus providing heterologous protection against both HS and a secondary disease. A suitable *Pasteurella* dual-expression plasmid, pSRGM that enables protein expression in both prokaryotic and eukaryotic systems will be used in order to understand the fate of the plasmid DNA carrying the antigenic gene after being delivered by the live-vaccine strain *in vivo*. In this study, the constructed plasmid, pSRGM was manipulated to demonstrate the ability of this vaccine strains to transfer plasmid DNA intracellularly. Expressed proteins were tracked and recorded via confocal laser scanning microscopy (CLSM). This is to investigate the ability and efficiency of these strains to be in the intracellular environment of the host cells and its ability to transfer plasmid DNA intracellularly to the host cell. The ability of *P. multocida* B:2 GDH7 to model a bactofection represents the possibility for this vaccine strain to be a delivery vehicle for DNA vaccine (bactofection).

1.1 Objectives

The specific objectives for this study are :

1. To investigate the interaction capacity of the wild-type and vaccine strain of *P. multocida* B:2 to adhere, to invade and to survive intracellularly within Bovine Aortic Endothelial Cell (BAEC) line.
2. To track the localization of bacteria during the process of interaction using dual-expression plasmid, pSRGM.

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