EVALUATE THE IMMUNOGENICITY OF PLASMID ENCODING pscC PROTEIN OF *PSEUDOMONAS AERUGINOSA* IN RATS

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BIOTECHNOLOGY RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH
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THIS THESIS IS SUBMITTED TO FULFILL THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE (BIOTECHNOLOGY)

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IJAZAH: MASTER OF SCIENCE (BIOTECHNOLOGY)

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DECLARATION

I hereby declared that the material in this thesis is my own except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

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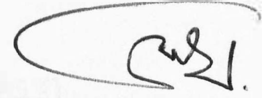

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In the name of Allah, all praise is to Allah the Almighty. Had it not been due to His will, this thesis will not be completed. This thesis is the result of two and half years of work whereby I have been accompanied and supported by many people. It is pleasant aspect and I have now the opportunity to express my gratitude for all of them.

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Md. Safiul Alam Bhuiyan,
February, 2015.

ABSTRACT

Pseudomonas aeruginosa is a non-sporulating Gram negative, aerobic bacillus universally distributed in natural environment such as soil and water, which causes serious lethal infections. Its main targets are immunocompromised patients with attenuated host defense function which include burn victims, organ transplant and cancer patient's long term therapy involves the use of antibiotics. Currently, there is no commercially available vaccine that can confer immunity to *P. aeruginosa*; therefore this study was conducted to determine the antigenic potential of a protein associated with the cell surface, which could subsequently be translated into a DNA based vaccine. The complete genome of *P. aeruginosa* strain PA01 was screened to determine an outer membrane surface coat Type III secretion protein (pscC) which is an important virulence determinant of type III secretion system (T3SS). Specific primers used were designed based on *P. aeruginosa* PA01 genome sequence available at the GenBank (NP250407) and subsequently cloned into two different *Escherichia coli* expression vector pGS-21a and pET-22b. The recombinant protein was analyzed by MALDI TOF-TOF mass spectrometer followed by purification using size exclusion chromatography. Finally, pscC gene was cloned onto a mammalian expression vector for plasmid immunization. The pMC2.1-pscC recombinant plasmid was directly injected intramuscularly in laboratory Sprague Dawley rats. Recombinant pscC antigen induced a specific humoral immune response against the antigen which was validated by agglutination and ELISA tests. The results clearly demonstrated that anti-pscC antibody was elicited using the animal model. The antibody level increased in 3 weeks of post immunization of all experimental doses compared with control group. The surface virulence Type III secretion protein (pscC) which is encoded by the outer membrane of T3SS genes will lead to the development of commercial plasmid vaccines to induce protective immunity against virulent *Pseudomonas* infection.

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ABSTRAK

MENILAI KEIMUNOGENAN PENGEKODAN PLASMID pscC PROTEIN PSEUDOMONAS AERUGINOSA DALAM TIKUS

Pseudomonas aeruginosa adalah bakteria aerobic bacillus gram negative yang tidak menghasilkan spora, bakteria ini boleh didapati secara meluas di alam sekitar seperti tanah dan air yang boleh menyebabkan maut jika dijangkiti. Sasaran utama bakteria ini adalah pesakit yang mempunyai antibody yang rendah seperti mangsa kebakaran, pesakit yang mengadakan pemindahan organ dan pesakit kanser. Pada masa ini, belum ada vaksin penawar yang boleh meningkatkan imuniti terhadap bakteria P. aeruginosa ditemui. Oleh itu, kajian ini dilakukan untuk menentukan protein antigen yang berpotensi terhadap permukaan sel di mana protin tersebut akan diterjemahkan kepada vaksin dengan menggunakan E.coli sebagai paparan. Keseluruhan informasi genetic (genom) P. aeruginosa PA01 di skrin untuk menentukan lapisan luar permukaan membrane Type III rembesan pscC protien di mana ia adalah ciri penting untuk menentukan tahap P. aeruginosa sebagai Type III secretion system (T3SS). Primer khas yang digunakan adalah berdasarkan rantaian genom pseudomonas PA01 yang diperolehi daripada GenBank (NP250407) di mana seterusnya di klon untuk menghasilkan dua E. coli ekspresi vector pGS-21a dan pET22b. Protein rekombinan dianalisis dengan MALDITOF-TOF mass spektrometer diikuti dengan penulinan menggunakan kromatografi penyisihan saiz. Akhirnya, pscC gen telah diklon ke ekspresi vector mamalia untuk proses imunisasi menggunakan plasmid. Plasmid rekombinan pMC2.1-pscC telah secara langsung disuntik otot di dalam makmal Sprague Dawley tikus. Penghasilan antigen rekombinan pscC yang disebabkan tindak balas tertentu humoral imun terhadap antigen yang telah disahkan dengan kit aglutinasi dan ELISA. Keputusan jelas menunjukkan bahawa antibodi anti-pscC telah dihasilkan dengan menggunakan model haiwan. Tahap antibodi meningkat dalam 3 minggu selepas imunisasi, semua dos eksperimen dibandingkan dengan kumpulan kawalan. Permukaan Jenis protein III rembesan pscC yang dikodkan oleh membran luar gen T3SS akan membawa kepada pembangunan vaksin plasmid komersial dan berpotensi mendorong imuniti perlindungan terhadap jangkitan Pseudomonas getir.

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LIST OF SYMBOLS AND ABBREVIATIONS

AHWLA	Assessing the Health and Welfare of Laboratory Animals
Amp	Ampicillin
APC	Antigen presenting cell
APS	Ammonium per sulfate
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CMV	Cytomegalo virus
COPD	Chronic obstructive pulmonary diseases
CTL	Cytotoxic T cell
DTT	Dithiothreiotol
DNA	Deoxyribonucleic acid
dNTP	Dioxynuclotides triphosphate
DSB	Diethanolamaine substrate buffer
EDTA	Ethylene diamine tetra acetic acid
GST	Glutathione –S-transferase
His (H)	Histidine
hr	Hour
IB	Inclusion body
Ig G	Immunoglobulin G
IPTG	Isopropyl thiogalactosidase
kb	Kilo base
kDa	Kilo dalton
LB	Luria-Bertani
LPS	Lipopolysaccharides
MALDI-TOF	Matrix-Assisted-Laser Desorption-Time-of-Flight
MCS	Molecular cloning site
MDR	Multi drug resistance

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LIST OF SYMBOLS AND ABBREVIATIONS

AHWLA	Assessing the Health and Welfare of Laboratory Animals
Amp	Ampicillin
APC	Antigen presenting cell
APS	Ammonium per sulfate
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CMV	Cytomegalo virus
COPD	Chronic obstructive pulmonary diseases
CTL	Cytotoxic T cell
DTT	Dithiothreiotol
DNA	Deoxyribonucleic acid
dNTP	Dioxynuclotides triphosphate
DSB	Diethanolamaine substrate buffer
EDTA	Ethylene diamine tetra acetic acid
GST	Glutathione –S-transferase
His (H)	Histidine
hr	Hour
IB	Inclusion body
Ig G	Immunoglobulin G
IPTG	Isopropyl thiogalactosidase
kb	Kilo base
kDa	Kilo dalton
LB	Luria-Bertani
LPS	Lipopolysaccharides
MALDI-TOF	Matrix-Assisted-Laser Desorption-Time-of-Flight
MCS	Molecular cloning site
MDR	Multi drug resistance

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min	Minute
mM	Milli molar
mRNA	Messenger ribonucleic acid
MS	Mass spectophotography
NCFB	Non-cystic fibrosis bronchiectasis
Nm	Nanometer
NF	Non flageller
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
pmol	Picomol
PRRs	Pattern recognition receptors
RE	Restriction enzyme
RNase	Ribonuclease
RND	Resistance nodulation of cell division
Rpm	Rotations per min
RT	Room temperature
SDS	Sodium dodecyl sulphate
sec	Second
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TEMED	N, N, N′, N′-tetramethylethylenediamin
TLR4	Toll-like receptor 4
T_m	Melting temperature
TMHMM	Transmembrane based on a hidden Markov Model
TNFα	Tumor necrosis factor Alpha
TPR's	Tetratricopeptide repeats
tRNA	Transfer RNA
Xg	Rotation per min

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CHAPTER 1

INTRODUCTION

Psudomonas aeruginosa is a gram-negative versatile bacterium found in a wide range of environmental habitats. This opportunistic pathogen causes both acute and chronic infections in patients with hospital-acquired pneumonia (Driscoll, 2007). It has been classified as the fourth leading cause of nosocomial infection and is associated with cystic fibrosis; burn wound infection, and pneumonic septicemia (Daniel Sel, 2002; Bernhardt *et al.*, 2002). *Pseudomonas* infections have become more complex and life-threating due to recurrent causes of nosocomial infection, as standard treatment are becoming ultimately ineffective. This organism displays intense signs of antibiotic resistance to wide variety of anti-microbiological agent, including β-lactam, chloramphenicol and fluoroquinolone (Zhang *et al.*, 2001). As a result, effective immune therapy is more desirable than conventional antibiotic therapy (Crazy *et al.*, 1984). It is vital to implement therapeutic vaccination schemes against *Pseudomonas* infections. *P. aeruginosa* PA01 strain may produce virulence factors such as extra or intra-cellular enzyme and toxin containing pili, flagella, protease, esterase, alginate, lipopolysaccharides, pyocyanin and rhamnolipid, all of which are known to contribute to the pathogenicity of *Pseudomonas* infections (Lui *et al.*, 1973; Stanysalvisky *et al.*, 1997). These virulence factors may cause tissue damage through induction of free radicals within host cells, resulting in the inhibition of host's protective immune cells (Lau *et al.*, 2004; Palmer *et al.*, 2005).

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The physical factor which governs the virulence of *P. aeruginosa* is the Type III secretion system (T3SS). *P. aeruginosa* relies on the T3SS pathway, which forms a channel for the translocation of bacterial effectors into the host cell and plays a great role in pathogenesis of the murine acute pneumonic infection model (Hauser, 2009; Diaz *et al.*, 2008). T3SS virulences factors are mainly considered during the interaction of the infective pathogen within the host cells and bacterial surface localization. T3SS is a complex protein secretory pathway which plays an important

role in *Pseudomonas* pathogenesis (Moreas *et al.*, 2008). However, a number of secretory proteins are encoded by the T3SS operon and these exhibits a wide-range of functions which include proteolysis, haemolysis, cytotoxicity, and protein phosphorylation, which are all toxic to host cells. T3SS pathways used a powerful needle complex which injects a virulence protein to be transported from the bacterial cytoplasm to the outer membrane of the host cell envelope. This secretion system is largely associated with sec-depended, auto transporter and flagellar system to release their toxin and convey virulent proteins into the host cell. However, T3SS relies on the sec secretory system mostly to transport the protein from the inner to the outer membrane (Hahn *et al.*, 2003; Henderson *et al.*, 2004). The effector proteins are virulent proteins that affect the host's immune defense mechanism. At the same time, the translocons are other secretory proteins that allow the relocation of effector proteins via the needle apparatus (Stein, 2003). The virulent pscC protein is the domain of the outer membrane needle apparatus, which acts as a secretory precursor of T3SS. Moreover, the pscC protein regulates the secretion of translocation to attachment the cell membrane of the host, intoxication of translocation and signaling movement during pathogenesis. This study has attempted to produce plasmid vaccine against *Pseudomonas* infection using the pscC antigen.

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Effective vaccines are designed to stimulate the innate immune response, as well as deliver antigens to specific subcellular sites for the elicitation of antigen-specific cytotoxic T cells. Physical delivery to specific locations within a cell is one of the major challenges when developing a suitable T3SS antigen candidate as a vaccine. Secretory needle antigens of T3SS are easily processed through T3SS pathways. This T3SS complexes form a host-pathogen interaction to identify the molecular pathogenesis to develop an effective vaccine. As a result, the secretory T3SS antigen directly stimulates antigen-specific cytotoxic T cells through the delivery of antigens to the antigen presenting cells (APC), causing a humoral immune response (Amanna *et al.*, 2011; Rossmann *et al.*, 1998; Chen *et al.*, 2006).

It is also true that secretion processes occur through unique pathways which are specific to a specific secretion protein (Pugsley *et al.*, 1993). Therefore, the current study has applied the *Pseudomonas* outer membrane protein (T3SS) coding

pscC gene immunized into the rat. The proteins designed to transport of T3SS pathway have distinct signals that direct them to the secretion machine to stimulate T-helper cells, conferring protection to a diversity of infectious diseases (Galan *et al.*, 2006). The primary aim of this study was to determine if the animal model could be applied to characterize the immune response elicited by the pscC antigen. It has been suggested in many studies that recombinant plasmid immunization may allow long-term persistence of immunogenic action in host cells without any risk of infection (Okada *et al.*, 2011; Frantz *et al.*, 2011). Plasmid DNA encoding a specific antigen is introduced into the muscle cell using mammalian plasmid expression vector. Further, the plasmid DNA is taken up by the host cells, expressed under the influence of a CMV promoter and subject to post-translation modification within the host (Robert-Guroff, 2007). Clinical trials have attested to the safety, efficiency and efficacy, as well as wide application of this immunization technique (James *et al.*, 2009).

Plasmid immunization represents the third generation vaccine, usually prepared by plasmids containing exogenous genetically engineered plasmid DNA for the purpose of creating antigens (Kaufmann *et al.*, 2011). Similarly, plasmid DNA vaccines may provoke a rapid and strong immunological response when highly active mammalian expression plasmids are applied and result in ideal protection for human bodies (Hawkridge *et al.*, 2011). First and second generation vaccine have several limitations, such as efficiency and non-specific immune reactions; plasmid immunization overcomes these limitations and there is need for an additional booster dose. The present study has been undertaken to describe a method to isolate the partial length of pscC gene from *P. aeruginosa* PA01 strain by PCR technique affording the rapid amplification of DNA fragments for cloning. The pGS-21a cloning vector has been used as an expression vector due to its compressive multiple cloning sites with appropriate antibiotics which contains an immuno-detectable oligo-peptide sequence fused to a 6X-HisTag and glutathione S-tranferase (GST) at the N-terminal sequences. The recombinant protein products, which are expressed when the plasmid is induced in *E. coli* (BL21) cells using IPTG, can be purified and concentrated using size exclusion chromatography. Concurrently a plasmid (pDream2.1/MCS vector) constructed with a pscC gene directly transferred to a muscle cell encoding an antigenic protein for the purpose of immunization and vaccine development. In

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an experimental study, Sprague Dawley rats were immunized pMC2.1-pscC, formulated with 0.25% Bupivacaine followed with the single dose application. It has been posited that bupivacaine acts as an immunomodulator. As a result, production of the protein within the cell occurs through biosynthetic processing and post-translational modifications induced by activation of the humoral cell response (Feltquate *et al.*, 1997). The serological screening was carried out using serum from the immunized rats, with recombinant protein as an antigen, and confirmed by agglutination and indirect ELISA. These tests are the most popular for confirming the immunogenic nature of a protein and antigen detection system.

The T3SS is the part of important virulence determinant of *P. aeruginosa*, in which the pscC protein is a fundamental part of the needle tip for *Pseudomonas* T3SS. The pscC protein has been established to be an important protective antigen of the bacterium. Once the bacterium interacts with host cell membranes, the T3SS system is activated and this in turn inhibits signal transduction resulting in cellular cytotoxicity or changes in the host immune response. This T3SS antigen may react with the host cell, whose maximum component should play a significant role in the host immunity. Therefore, we focused on the current understanding of the mechanisms involved in antigenic expression of pscC. Similarly, we highlighted the experiential development of alternative vaccination approach using a plasmid DNA immunization in animal model for the production of polyclonal antibody. Direct plasmid DNA immunization can lead to the expression of the recombinant protein within the muscle, thus reducing the time and effort required for production of the protein in alternative hosts. It has been proven that the recombinant antigens expressed from DNA vaccines can elicit a strong humoral immune response against antigens derived from bacterial secretory components. The cell surface display of a heterologous antigenic determinant is advantageous for the induction of an antibody against a specific antigen. The orientation of target antigens has been used to develop plasmid vaccines using recombinant DNA for immunization on rats for the capture and detection of an antibody from serum (Stober *et al.*, 2002). Therefore, it will be emphasized on display targeting and heterologous protein expression to outer surface of the bacteria in vivo after plasmid immunization. In conclusion a recombinant version of the *P. aeruginosa* pscC needle protein will be used as a

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