MOLECULAR CHARACTERIZATION OF PUTATIVE VIRULENCE DETERMINANTS AND IDENTIFICATION OF SPECIFIC IMMUNOGENIC POLYPEPTIDES FOR THE SEROLOGICAL DIAGNOSIS OF *BURKHOLDERIA PSEUDOMALLEI* INFECTIONS

PUAH SUAT MOI

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2014

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: **Puah Suat Moi** (I.C/Passport No: **810321-10-5036**)

Registration/Matric No: **MHA100017**

Name of Degree: **Doctor of Philosophy**

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

Molecular characterization of putative virulence determinants and identification of specific immunogenic polypeptides for the serological diagnosis of *Burkholderia pseudomallei* infections

Field of Study: **Molecular Microbiology**

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date **12 June 2014**

Subscribed and solemnly declared before,

Witness's Signature Date **12 June 2014**

Name: **Dr. Chua Kek Heng**
Designation: **Professor**

Abstract

The Gram-negative saprophyte *Burkholderia pseudomallei* is the causative agent of melioidosis, an infectious disease which is endemic in Southeast Asia and northern Australia. This bacterium possesses many virulence factors which are thought to contribute to its survival and pathogenicity. Six genes *BPSL2033*, *BP1026B_I2784*, *BP1026B_I2780*, *BURPS1106A_A0094*, *BURPS1106A_1131* and *BURPS1710A_1419* were identified earlier in a screen by PCR-based subtractive hybridization, using a virulent clinical isolate of *B. pseudomallei* and a laboratory-acquired attenuated strain of the same isolate of *B. pseudomallei.* Therefore, the first objective of this study was to extensively characterize these genes at the molecular level, as well as one additional gene *BPSL3147* identified by other investigators. Through a reverse genetic approach, single-gene knockout mutants were successfully constructed by using site-specific insertion mutagenesis and confirmed by PCR. Likewise, complemented strains were successfully obtained by reintroducing an intact copy of the defective gene into the corresponding mutant strains. *BPSL2033::Km* and *BURPS1710A_1419::Km* mutants showed reduced survival inside macrophage RAW 264.7 cells and also low attenuation level in the virulence of nematode infection model. However, *BPSL2033::Km* only demonstrated a weak statistical significance ($p=0.049$) of intracellular survival compared to the wild type at 8 hour post infection in macrophage infection study but *BURPS1710A_1419::Km* showed a p-value of 0.165. Nevertheless, complemented strains of both genes were able to partially restore the gene defect both *in vitro* and *in vivo* studies, thus suggesting that they individually play a minor role in the virulence of *B. pseudomallei*. Lack of a universally acceptable antigen for serodiagnosis of infections caused by *B. pseudomallei* is another clinical challenge. The second

objective of this study was to search for an immunogen via a shotgun expression library created from clinically confirmed local virulent isolates of *B. pseudomallei*. After 2 rounds of immunoscreening with sera from melioidosis patients, 6 sero-positive clones expressing immunogenic polypeptides were sequenced and their identities were: BPSS1904 (benzoate 1,2-dioxygenase beta subunit), BURPS1710b_0454 (a putative 200 kDa antigen p200), BPSS1856 (phosphotransferase enzyme family protein), BPSS0897 (short chain dehydrogenase), BPSL3130 and BPSS1757 (hypothetical proteins). These immunogenic polypeptides were then purified and transferred to an ELISA platform for further large scale screening. Experimental screening using 60 melioidosis positive and 123 non-melioidosis sera allowed the identification of 2 immunogenic polypeptides BPSS1904 and BPSL3130 with diagnostic potential, which demonstrated sensitivities of 75% and 90%, and specificities of 90.24% and 88.62%, respectively. The results suggest that both are potential candidate antigens for the serodiagnosis of infections caused by *B. pseudomallei*. In summary, the present study suggests that *BPSL2033* and *BURPS1710A_1419* genes to be associated with virulence of *B. pseudomallei*, while immunogenic polypeptides BPSS1904 and BPSL3130 were shown to be potential antigens for the serological diagnosis of its infection.

Abstrak

Burkholderia pseudomallei merupakan sejenis bakteria gram-negatif yang bersifat saprofit. Bakteria ini adalah agen penyebab melioidosis iaitu penyakit berjangkit yang berleluasa di Asia Tenggara dan utara Australia. Ia berupaya menghasilkan pelbagai faktor kevirulenan yang membantu dalam kemandirian dan kepatogenan. Enam gen *BPSL2033*, *BP1026B_I2784*, *BP1026B_I2780*, *BURPS1106A_A0094*, *BURPS1106A_1131* dan *BURPS1710A_1419* telah dikenal pasti terlebih dahulu oleh 'PCR-based subtractive hybridization' dalam saringan menggunakan pencilan klinikal virulen *B. pseudomallei* dan pencilan makmal yang teratenuasi. Oleh itu, objektif pertama penyelidikan ini merupakan pencirian gen-gen tersebut serta satu gen tambahan *BPSL3147* yang telah dikenal pasti oleh penyelidik lain pada peringkat molekul. Melalui cara genetik terbalik, mutan-mutan 'knockout' gen tunggal telah berjaya dibina dengan menggunakan mutagenesis kemasukan (insertion) spesifik dan disahkan oleh PCR. Di samping itu, komplemen bagi setiap mutant juga berjaya dibina dengan memperkenalkan semula satu salinan gen yang berkenaan ke dalam strain mutan masing-masing. Mutan *BPSL2033::Km* dan *BURPS1710A_1419::Km* telah menunjukkan pengurangan kemandirian dalam makrofaj RAW 264.7 dan tahap atenuasi yang rendah dalam model jangkitan nematod. Walau bagaimanapun, hanya *BPSL2033::Km* menunjukkan perbezaan statistik signifikan yang lemah (p=0.049) selepas 8 jam selepas infeksi dalam makrofaj berbanding dari strain jenis liar, manakala *BURPS1710A_1419::Km* menunjukkan nilai p=0.165. Walau bagaimanapun, komplemen bagi kedua-dua gen dapat memulihkan kecacatan gen sekurang-kurangnya sebahagiannya dalam kajian *in vitro* dan *in vivo*. Keputusan ini menunjukkan bahawa kedua-dua gen memainkan peranan minor dalam kevirulenan dalam *B. pseudomallei*

secara individu. Kekurangan antigen universal yang boleh diterima untuk serodiagnosis jangkitan disebabkan oleh *B. pseudomallei* adalah satu lagi cabaran klinikal. Oleh itu, objektif kedua penyelidikan ialah mencari immunogen melalui 'shotgun expression library' yang dibina daripada pencilan klinikal tempatan *B. pseudomallei*. Selepas 2 saringan dengan meggunakan sera daripada pesakit melioidosis, 6 klon positif mengekspresi polipeptida imunogenik telah didapati dan identiti adalah seperti berikut: BPSS1904 (benzoate 1,2-dioxygenase beta subunik), BURPS1710b_0454 ('putative'200 kDa antigen p200), BPSS1856 (enzim phosphotransferase keluarga protein), BPSS0897 (dehidrogenase rantaian pendek), dan BPSL3130 dan BPSS1757 (protein andaian). Kemudian, kesemua immunogen ditulen dan dipindahkan ke platform ELISA untuk saringan secara besar-besaran. Pemeriksaan yang menggunakan 60 melioidosis positif sera dan 123 sera bukan melioidosis telah membolehkan pengecaman 2 polipeptida iaitu BPSS1904 dan BPSL3130 yang berpotensi diagnostik, iaitu masing-masing mempunyai sensitiviti 75% dan 90%, dan spesifikasi 90.24% dan 88.62%. Keputusan ini menunjukkan bahawa kedua-dua adalah calon antigen yang berpotensi untuk digunakan dalam serodiagnosis jangkitan yang disebabkan oleh *B. pseudomallei.* Secara keseluruhannya, kajian ini telah menunjukkan bahawa gen *BPSL2033* dan *BURPS1710A_1419* adalah berkaitan dengan kevirulenan *B. pseudomallei*, manakala polipeptida imunogenik BPSS1904 dan BPSL3130 merupakan antigen potensi untuk diagnosis serologi bagi jangkitan ini.

Acknowledgments

I would like to express my sincere gratitude to all the people who have contributed to my research, helped and supported me during the course of my post-graduate study. In particular I would like to acknowledge;

Prof. Dr. Chua Kek Heng, my supervisor and an experienced mentor, for always having an open door and time for questions and advice, for encouragement and excellent guidance in the field of molecular research throughout this project, for always creating a generous and inspiring environment.

Prof. Emeritus Savithiri Devi Ampalan Puthucheary, my co-supervisor, for teaching me how to write papers and getting them published, for all your invaluable and detailed critiques of my thesis that resulted in significant improvement over my submitted draft version. It is with her trust and patience that I can conquer the mountain eventually.

Prof. Wang Jin-Town and his team from the Department of Microbiology, College of Medicine at National Taiwan University, Taipei for providing laboratory training in working with knock-out construction, vector and *E. coli* strains used in this study. Dr. Pan Yi-Jiun has been incredibly supportive and offered invaluable advice to me regarding knock-out experiment. I am very grateful to all members from Lab R739: Dr. Hsieh Pei-Fang, Dr. Lin Tzu-Lung, Dr. Hsu Chun-Ru, Dr. Wu Meng-Chuan, Pin-Shuan, Han-Ru, Yi-Xiang, Bo-An, Jin-Ting, Jun-Tang, Julie Tsai, Yi-Ying, Qing-Qing, Andrew Yang, Jack Wu for their hospitality and many joyful moments. There's always time for laughter!

My lab mates, Ching Hoong, Hwa Jia, Tze Pheng and Boon Pin for creating a pleasant atmosphere at the lab and frienship. It has been a great pleasure sharing my work and life in general with all of you.

My gangs and family, for their understanding, love and trust in me.

All patients and healthy volunteers for participating in this study.

I am also blessed to have financial support from University Malaya Research Grant RG409/12HTM, FP037-2013A, University Malaya Fellowship Scheme, and Bright Sparks University Malaya Scheme.

TABLE OF CONTENTS

Contents **Page**

ABSTRACT …………………………………………………………………..…. iii

ABSTRAK ……………………………………………………………………. v

ACKNOWLEDGEMENTS …………………………………………………. vii

LIST OF FIGURES ……………………………………………………………. xvi

LIST OF TABLES …………………………………………………………... xix

ABBREVIATIONS …………………………………………………………... xxi

LIST OF APPENDICES ……………………………………………………..… xxv

CHAPTER 1

Introduction …………………………………………………………………..… 1

CHAPTER 2

***Burkholderia pseudomallei* and melioidosis**

2.1 History ……………………………………………………………………….. 4

2.2 *Burkholderia pseudomallei*

2.2.1 Bacteriology ………………………………………………………. 4

2.2.2 Genome …………………………………………………………. 6

2.2.3 Epidemiology and prevalence ……………………………….……. 7

2.2.4 Transmission ……………………………………………………. 10

Contents **Page**

2.2.5 Risk factors …………………………………………………….. 12
2.2.6 Clinical manifestations of melioidosis patients……………….. 13

2.3 ***Burkholderia pseudomallei* and host interaction**
2.3.1 Intracellular lifecycle ……………………………………..….. 14
2.3.2 Virulence factors …………………………………………….. 19
2.3.3 *in vivo* and *in vitro* models to study pathogenesis ………… 25
2.3.3.1 Features of *Caenorhabditis elegans* system …….... 26

2.4 **Host defense against *Burkholderia pseudomallei*** …………………….. 28

2.5 **Laboratory diagnosis and detection** ………………………………….. 29
2.5.1 Culture-based methods ………………………………………. 30
2.5.2 Serodiagnosis ………………………………………………… 30
2.5.2.1 Antigen detection ……………………………….... 31
2.5.2.2 Antibody detection …………………………….... 34
2.5.3 Molecular identification techniques …………………………. 38
2.5.4 Commercial identification system …………………………..... 39
2.5.5 Medical imaging ……………………………………………... 40

2.6 **Treatment and vaccine development** ……………………………..... 41

2.7 **Shotgun expression library** ……………………………………………. 42

CHAPTER 3

General materials and methods

3.1 **Introduction** ………………………………………………………………. 43

Contents (continued) **Page**

3.2 Materials

3.2.1 Bacterial strains and plasmids ………………………….………. 43

3.2.2 Biochemicals and materials ……………………………………….. 43

3.2.3 Buffers and other solutions ………………………………………... 43

3.3 General methods ……………………………………………………… 43

3.3.1.1 Steam sterilization …………………………….... 44

3.3.1.2 Membrane sterilization ……………………….…. 44

3.3.2 Bacterial culture and maintenance conditions ……………….. 44

3.3.3 Polymerase chain reaction (PCR) amplification …………….. 44

3.3.4 PCR from a bacterial colony ……………………………….... 45

3.4 Concentration measurement

3.4.1 Quantitation of genomic DNA ………………………………. 46

3.4.2 Quantitation of protein sample …………………………….... 46

3.5 Electrophoresis

3.5.1 Agarose gel electrophoresis (AGE) ………………………….. 47

3.5.2 SDS-Polyacrylamide Gel Eletrophoresis (SDS-PAGE) ……... 47

3.5.3 Bio-Safe Coomassie Blue Staining ………………………….. 49

3.6 Purification methods

3.6.1 Isolation and purification of genomic DNA from bacterial culture …………………………………………………... 49

3.6.2 Isolation and purification of plasmids …………………...... 50

3.6.3 Isolation and purification of DNA bands from agarose gel ….. 51

3.6.4 Isolation and purification of dsDNA products …………..... 52

Contents (continued) **Page**

3.7 Cloning

3.7.1 Restriction enzyme digestion ……………………………….. 52

3.7.2 Calf intestinal alkaline phosphatase (CIP) treatment ………... 53

3.7.3 DNA ligation ………………………………………………… 53

3.7.4 Preparation of chemically competent cells ………………….. 53

3.7.5 Heat shock transformation ………………………………….... 54

3.8 Sequencing ……………………………………………………….... 54

CHAPTER 4

Characterization of putative virulence determinants in *B. pseudomallei*

4.1 Introduction ………………………………………………………. 55

4.2 Material and methods

4.2.1 Bacterial strains, plasmids and culture conditions …………... 60

4.2.2 Cell lines and culture conditions …………………………….. 60

4.2.3 Nematode strain and culture conditions ……………………... 61

4.2.4 Insertion mutagenesis

4.2.4.1 Primers designation ……………………………. 66

4.2.4.2 Construction of suicide plasmids for conjugation . 68

4.2.4.3 Filter conjugation ………………………………. 70

4.2.5 Construction of complemented plasmids for mutant strains 72

4.2.6 Bacterial growth curves …………………………………….... 74

4.2.7 Replication of bacteria in macrophage cells ………………... 74

Contents (continued) **Page**

4.2.8 *Caenorhabditis elegans* virulence assay
4.2.8.1 Worm synchronization …………………………. 76
4.2.8.2 Slow killing assay ……………………………….. 77
4.2.9 Statistical analysis ……………………………………….... 78

4.3 Results
4.3.1 Construction of *BPSL2033*::Km mutant …………………….. 80
4.3.2 Construction of *BP1026B_I2784*::Km mutant ……………… 82
4.3.3 Construction of *BP1026B_I2780*::Km mutant ……………… 84
4.3.4 Construction of *BURPS1106A_A0094*::Km mutant ………… 86
4.3.5 Construction of *BURPS1106A_1131*::Km mutant ………….. 88
4.3.6 Construction of *BURPS1710A_1419*::Km mutant …………. 90
4.3.7 Construction of *BPSL3147*::Km mutant ……………………. 92
4.3.8 Growth curve ………………………………………………..... 94
4.3.9 Construction of complemented strains …………………….... 95
4.3.10 Bacterial replication and survival in macrophage assay …….. 97
4.3.11 *Caenorhabditis elegans* killing assay ……………………….. 101

4.4 Discussion
4.4.1 Natural competence of Bp-CMS …………………………….. 106
4.4.2 Growth curve ………………………………………………..... 106
4.4.3 Insertion mutagenesis as a genetic tool for investigating gene function ………………………………………………………. 107
4.4.4 Assessment of virulence of the candidate genes …………….. 108
4.4.5 Structural basis of 2 novel virulence determinants
4.4.5.1 *BPSL2033* ……………………………………..….…. 113
4.4.5.2 *BURPS1710A_1419* …………………………..…. 115

Contents (continued) **Page**

4.5 Concluding discussion future prospects ……………………………… 117

4.6 Limitations of the study …………………………………………….. 118

CHAPTER 5

Identification of potential immunogenic polypeptides of *B. pseudomallei* and evaluation of their efficacy for serodiagnosis of melioidosis

5.1 Introduction ……………………………………………………. 119

5.2 Materials and methods

5.2.1 Bacterial strains and plasmids …………………………......… 123

5.2.2 Construction of genomic expression library ………………..... 124

5.2.3 Colony immunoscreening …………………………………... 125

5.2.4 Plasmid preparation and sequencing ……………………..... 126

5.2.5 Preparation of purified recombinant polypeptides …………... 126

5.2.6 SDS-PAGE and Western blot analysis ………………………. 127

5.2.7 Serum samples ……………………………………………….. 127

5.2.8 Evaluation of purified recombinant polypeptides …………..... 128

5.2.9 Statistical analysis …………………………………………… 129

5.3 Results

5.3.1 *DNase I* shotgun cleavage …………………………………… 131

5.3.2 Construction of genomic library and selection of immunogenic polypeptides ………………………………………………… 132

5.3.3 Confirmation of the identity of clones by sequencing ………… 134

Contents (continued) **Page**

5.3.4 SDS-PAGE and Western blot analysis of the 6 recombinant polypeptides 138
5.3.5 Investigation on the serodiagnostic potential of 6 recombinant polypeptides 140

5.4 Discussion
5.4.1 Shotgun genomic expression library as a tool for the screening of immunogens 143
5.4.2 Analysis of the identical potential immunogenic polypeptides 147
5.4.3 Evaluation of the efficacy of recombinant polypeptides for serodiagnosis of melioidosis 148

5.5 Concluding discussion and future prospects 153

5.6 Limitations of the study 154

REFERENCES 155

PUBLICATIONS 178

APPENDICES 179

LIST OF FIGURES

Figure **Page**

2.1 Seven unique *B. pseudomallei* colony morphotypes on Ashdown's agar …...…………………………………………………………….. 5

2.2 Global map showing the categories of distribution of melioidosis and *B. pseudomallei* …………………………………………………….… 9

2.3 Intracellular lifestyle of *B. pseudomallei* ……………………………... 16

2.4 Life cycle of *C. elegans* ………………………………………………. 27

4.1 Substractive hybridization of virulent (v) and attenuated (av) *B. pseudomallei* strains ………..…………………………………….. 57

4.2 Construction of plasmids for conjugation ……………………………. 68

4.3 Construction of the insertion mutants in Bp-CMS …………………... 71

4.4 Construction of a complemented plasmid …………………………… 73

4.5 Summary of the experiments carried out in this study ……………….. 79

4.6 Verification of the construction of *BSL2033*::Km mutant by PCR using specific alignment primers ……………………………………….... 81

Figure **Page**

4.7 Verification of the construction of *BP1026B_I2784*::Km mutant by PCR using specific alignment primers ……………………………..… 83

4.8 Verification of the construction of *BP1026B_I2780*::Km mutant by PCR using specific alignment primers ……………………………….. 85

4.9 Verification of the construction of *BURPS1106A_A0094*::Km mutant by PCR using specific alignment primers ……………………………. 87

4.10 Verification of the construction of *BURPS1106A_1131*::Km mutant by PCR using specific alignment primers ……………………………….. 89

4.11 Verification of the construction of *BURPS1710A_1419*::Km mutant by PCR using specific alignment primers ……………………………….. 91

4.12 Verification of the construction of *BPSL3147*::Km mutant by PCR using specific alignment primers ……………………………….…. 93

4.13 Growth of wild type and mutant strains of *B. pseudomallei* in liquid media at 37°C monitored by absorbance at 600 nm over 8 hours …… 94

4.14 Verification of the construction of complementary plasmid by PCR … 96

4.15 Bacterial survival ad replication within RAW264.7 macrophage-like cells infected at MOI of 100 with wild type and each mutant and its complemented strains ……………………………………………….. 98

Figure **Page**

4.16 Kaplan-Meier survival curves for *C. elegans* infected with different strains of *B. pseudomallei* 102

4.17 ClustalW alignments of amino acid sequences of *BPSL2033* 114

4.18 ClustalW alignments of amino acid sequences of *BURPS_1710A_1419* (putative lipoprotein) 116

5.1 EtBr-stained 2% (w/v) agarose gel of digested genomic DNA of *B. pseudomallei* 131

5.2 Representative clones (1-8) of colony blot assay in 2 different pooled sera 133

5.3 SDS-PAGE showing purified recombinant polypeptides 138

5.4 Western blot analysis of candidate polypeptide antigens 139

5.5 Summary of the experiments carried out in this study 146

5.6 Alignment of amino acid sequences 149

5.7 Sensitivities, specificities and diagnostic odds ratio 151

LIST OF TABLES

Tables **Page**

2.1 Mortality rates of melioidosis…..……………………………………….. 8

2.2 Examination of mutants of virulence determinants in different infection models ……………………………………………………………….. 21

2.3 Various antigen detection tests for the diagnosis of melioidosis ………. 32

2.4 Antibody detection tests for the diagnosis of melioidosis …………….. 35

3.1 Recipes for separating and stacking gels ………………………………. 48

4.1 Characterization of 7 candidate genes for their roles in *B. pseudomallei* infection ……………………………………………………………... 58

4.2 Bacterial strains and plasmids used in this study …………………..….. 62

4.3 Primers used for PCR and constructing mutants and complemented plasmids in this study …………………………………………………... 66

4.4 Survival proportion of *C.elegans* challenge with bacterial strains ……. 101

5.1 Bacterial strains and plasmids used in this study ………………………. 123

Tables **Page**

5.2 BlastX results of the 6 recombinant polypeptides of *B. pseudomallei* … 135

5.3 Characteristics of the 6 recombinant polypeptides of *B. pseudomallei* .. 137

5.4 ELISA screening for melioidosis and disease control sera using the 6 recombinant polypeptides …………………………………………... 141

5.5 Evaluation of the diagnostic performance of the 6 *B. pseudomallei* recombinant polypeptides ……………………………………………… 142

ABBREVIATIONS

°	degree
°C	degree Celsius
%	percentage
1X	one time
AGE	agarose gel electrophoresis
A_{260}	absorbance at wavelength 260 nm
A_{280}	absorbance at wavelength 280 nm
Abs	antibodies
Ags	antigen
Amp	ampicillin
ASA	Ashdown's agar
BPSA	*B. pseudomallei* selective agar
bp	base pair
CAT	chloramphenicol acetyltransferase cassette
CB	carbenicillin
CDS	coding sequence
CFU	colony forming unit
CI	confidence interval
CIP	calf intestinal alkaline phosphatase
Cm	chloramphenicol
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylenediamine tetraacetic acid
e.g.	for example

ELISA	enzyme-linked immunosorbent assay
et al.	et alia
EtBr	ethidium bromide
FITC	fluorescein isothiocyanate
FUDR	5-fluorodeoxyuridine
g	gram
GI	genomic islands
h	hour
H_2SO_4	acid sulfuric
ICT	immunochromatographic test
i.e.	that is
IF	Immunofluorescence
IFAT	indirect fluorescent antibody test
IFN	interferon
IHA	indirect hemagglutination assay
iNOS	inducible nitric oxide synthase
i.n.	intranasal
i.p.	intraperitoneal
IPTG	isopropyl-beta-thio galactopyranoside
IVET	*in vivo* expression technology
Kan	kanamycin
kb	kilobase
kDa	kiloDalton
L	liter
LAMP	loop-mediated isothermal DNA
LB	Luria-Bertani

LPS	lipopolysaccharide
M	molar
m	milli
MAb-LA	monoclonal antibody latex agglutination
mbp	megabase pairs
min	minute
mg	milligram
MAbs	monoclonal antibodies
MOI	multiplicity of infection
NGM	nematode growth medium
nm	nanometer
µl	micro
OD	optical density
OR	odds ratio
PAGE	polyacrylamide gel electrophoresis
PAMPs	pathogen-associated-molecular patterns
PBS	phosphate buffered saline
PBST	phosphate buffered saline 0.2% Tween-20
PCR	polymerase chain reaction
pNPP	p-Nitrophenyl Phosphate
R	resistant
RE	restriction enzyme
RNase A	ribonuclease A
rpm	revolution per minute
S	sensitive
SDS	sodium dodecyl sulphate

sdH_2O	sterilized distilled water
sec	second
SH	subtractive hybridization
STM	signature-tagged mutagenesis
Taq	*Thermus aquaticus*
TBE	tris-borate buffer
TBS	tris-buffered saline
TBST	tris-buffered saline tween-20 (0.05%)
TEMED	*N,N,N',N'*-tetramethlyenediamine
Tet	tetracycline
Tris	tris (hydroxymethyl) aminomethane
TSA	tryptic soy agar
TSS1	Type 3 Secretion System Cluster 1
µg	microgram
µl	microliter
µM	micromolar
U	unit
uv	ultraviolet
v/v	volume per volume
w/v	weight per volume
WT	wild type
YT	Yeast-Tryptone

LIST OF APPENDICES

I Sources of chemicals and reagents …………………………………. 179

II Commonly used stock solutions …………………………………….. 181

III *Caenorhabditis elegans* killing assay ………………………………... 184

IV Solutions used in agarose gel electrophoresis (AGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) ….. 186

V Solutions used in Colony-blot detection ……………………………... 188

VI Solutions used in Western blot analysis ……………………………... 189

VII Solutions used in ELISA assay ………………………………………. 191

VIII Example of sequencing result ……………………………………….. 192

IX Raw data for replication of bacteria in macrophage cells …………….. 193

Chapter 1

Introduction

Burkholderia pseudomallei is a gram negative bacillus, motile, facultative anaerobe and an environmental saprophyte. It is readily recovered from the soil and surface waters in endemic areas, i.e., South-east Asia and Northern Australia (Wiersinga et al., 2012).

This bacterium possesses a remarkable capacity to infect humans and animals, causing melioidosis which is an important cause of sepsis in the tropics. Infections occur after contact with contaminated soil and water through cutaneous lesions, by inhalation of contaminated dust, or ingestion of contaminated water (Cheng and Currie, 2005). The clinical presentation of the disease is protean, which range from acute to chronic pneumonia, localized infection involving only one organ, disseminated septicemia disease involving multiple organs, and septic shock (Puthucheary, 2009).

It has a huge genome, 7.25 megabase pairs (Mbp), represented by two chromosomes, one 4.07 Mbp and the other 3.17 Mbp (Holden et al., 2004). The sequenced genomic data was reported in a study by Holden et al (2004) previously and has been published in databases. The availability of this allows researchers to carry out comparative genomics study for identification of gene(s) encoding virulence determinants, in order to understand pathogenesis and provide further insights into microbial diversity and evolution.

Our previous study had used subtractive hybridization technique to distinguish and determine possible differences between a virulent clinical isolate *B. pseudomallei* and its attenuated strain of *B. pseudomallei* (Puthucheary et al., 2012a). Interestingly, there were 6 strain-specific DNA fragments in the "virulent" clinical isolate of *B. pseudomallei (v)* which were absent in the "attenuated" strain of the same isolate *B. pseudomallei* (av) and could therefore be considered as potential virulence determinants, however, their roles in virulence are still unknown.

The first objective of this study was to extensively characterize these putative virulence determinants using methods combining gene knockout, cell line and nematode models infection studies. Apart from the candidate genes identified from our previous study, one additional gene, *BPSL3147* predicted as a putative lipoprotein detected by other researchers (Cuccui et al., 2007) was also included into our candidate list due to its potential as lipoprotein. Insertion inactivation mutants were constructed in the parent *B. pseudomallei* strain (Bp-CMS) involved integration of a plasmid into the chromosome via homologous recombination through single crossover event. This method required an internal fragment homologous to the gene of interest cloned into a suicide vector pUT-Km carrying a kanamycin resistance cassette. Murine macrophage cell line RAW264.7 and *Caenorhabditis elegans* were used as *in vitro* and *in vivo* models, respectively, for elucidating pathogenic mechanisms involved in *B. pseudomallei* infections.

Challenges in the clinical setting are associated with the fact that melioidosis is difficult to diagnose clinically due to the wide range of presenting symptoms. Culture still represents the gold standard for the confirmation of the disease melioidosis, but it is slow and it takes a minimum of 24-48 hours. The development of a rapid and accurate

serodiagnostic test is therefore needed for the detection of *B. pseudomallei* infection and would complement currently available diagnostic methods. However, the lack of a well-established universally acceptable antigen for serologic diagnosis of melioidosis infection is a major obstacle. It is of paramount importance that the ideal antigen consists of all or most of the relevant immunogens that are recognized by a patient infected with *B. pseudomallei.*

So, a second objective of this study sought to identify immunogenic polypeptides of *B. pseudomallei* and evaluation of their diagnostic potential. A shotgun genomic expression library was constructed using the NovaTope system from clinically confirmed local virulent isolates of *B. pseudomallei*, each of which expresses a small peptide derived from the protein under study. The library is screened by colony blot detection assay using pooled sera from melioidosis positive patients as the probe and immunodominant polypeptides that gave the best signals were selected for further experiments. ELISA detection assay using individual samples was then performed in order to find the most significant candidate that could improve the accuracy and reliability of serologic diagnostic methods for infections with *B. pseudomallei.*

Thereby, the new knowledge generated in my study is expected to advance our understanding of the molecular mechanism of action of newly identified putative virulence determinants in *B. pseudomallei* infections and add new candidate(s) for serodiagnostic test that could support the diagnosis of melioidosis.

Chapter 2

Burkholderia pseudomallei and melioidosis

2.1 History

Burkholderia pseudomallei, a gram-negative soil saprophyte was first described by Whitmore in 1911 when he and his assistant isolated this organism from morphine addicts in Rangoon, Burma (Stanton and Fletcher, 1932). It caused a glanders-like disease, now termed melioidosis. It was named from the Greek words "melis" meaning "a distemper of assess" and "eidos" meaning resemblance in 1921 by Staton and Fletcher (Stanton and Fletcher, 1921). Since its discovery, it has been described using a variety of names, generally well known as *Pseudomonas pseudomallei,* but until 1992, it was reclassified into a new genus, *Burkholderia* (Yabuuchi et al., 1992).

2.2 *Burkholderia pseudomallei*

2.2.1 Bacteriology

B. pseudomallei is a motile, small (1 to 5 μm in length and 0.5 to 1 μm in diameter), facultatively anaerobic, non-spore forming bacterium that having a safety-pin like appearance (Cheng and Currie, 2005). Its natural habitats are wet soils, agricultural soils, stream, pools, stagnant water, and paddy fields in endemic areas (Bruce, 2002).

B. pseudomallei can be cultured on many microbial media in the laboratory, but Ashdown's selective medium is commonly used and the organism shows differing colonial morphology, with mostly small, smooth, glistening purple colonies initially (24

h), changing to rough, dry or wrinkled colonies on further incubation (48 h) (Cheng and Currie, 2005). Interestingly, the organism develops seven unique colony morphotypes (type I to VII) (Figure 2.1) which are associated with phenotypes that alter the ability of *B. pseudomallei* to survive in adverse environmental conditions (Chantratita et al., 2007; Tandhavanant et al., 2010).

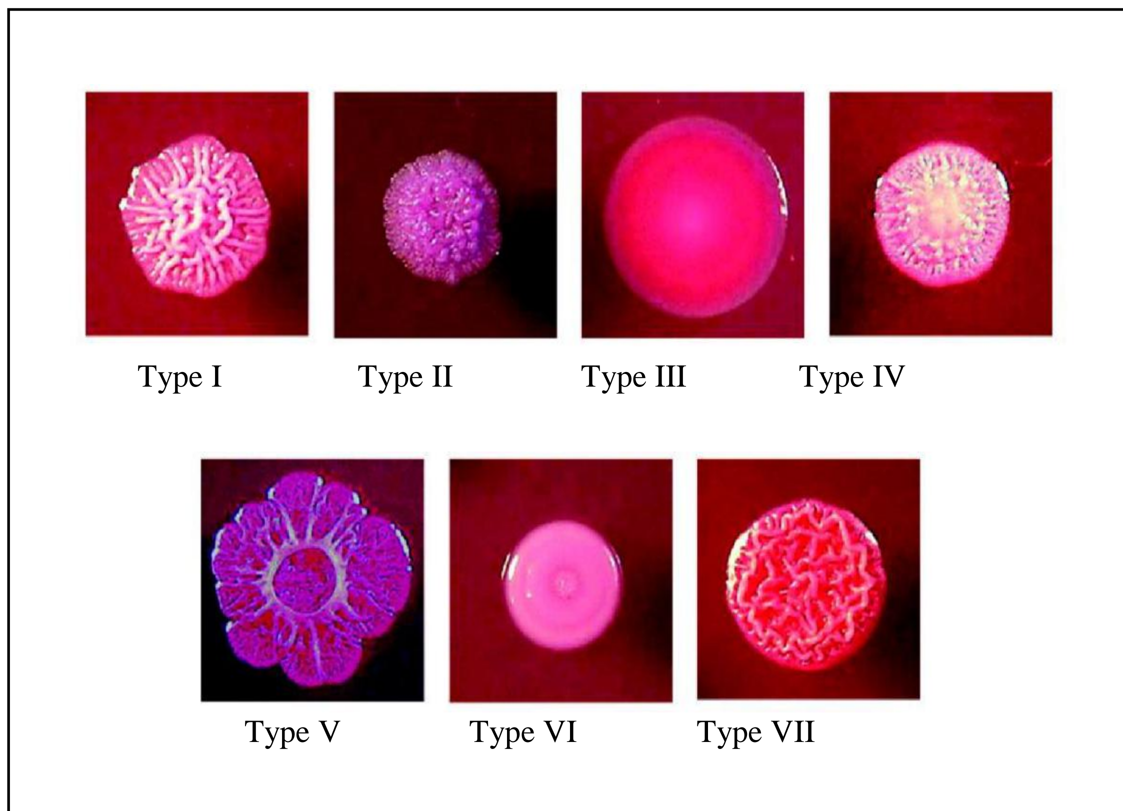


Figure 2.1. Seven unique *B. pseudomallei* colony morphotypes on Ashdown's agar. (Chantratita et al., 2007)

B. pseudomallei is oxidase positive. It utilizes glucose via oxidative pathway, and does not assimilate arabinose. It can survive in a variety of conditions, including niches of varied temperature (24ºC to 32ºC), acidic environments (pH 4.5 for up to 70 days) (Cheng and Currie, 2005), detergents solutions (Gal et al., 2004), and even in nutrient-deficient liquid environments for period of up to 28 days (Robertson et al., 2010).

2.2.2 Genome

In 2004, the genome of *B. pseudomallei* strain K96243 had been fully sequenced, annotated, and published (Holden et al., 2004). This bacterium has a huge genome, 7.25 megabase pairs (Mbp), represented by two chromosomes, one 4.07 Mbp and the other 3.17 Mbp (Holden et al., 2004). A large chromosome encodes 3,460 coding sequences (CDSs) required for housekeeping functions including metabolism, motility, cell wall and protein biosynthesis. On the other hand, the second small chromosome encodes 2,395 CDSs associated with adaptation and survival in different niches (Holden et al., 2004; Wiersinga et al., 2006).

Intriguingly, approximately 6.1% of the genome is made up of 16 putative genomic islands (GIs). GIs are referred to as regions having distinct G+C content compared to general genome backbone and usually contain mobility genes (e.g. insertion sequence elements), and/or genes known to be involved in facilitating genetic recombination (e.g. inegrase, transposase, and recombinase genes) (Nandi and Tan, 2012; Tuanyok, 2012). These GIs have probably been acquired via horizontal gene transfer and they are absent in the closely related species, genome of the *B. thailandensis* and *B. mallei* (Holden et al., 2004). Tuanyok and co-workers further expanded this information as 71 distinct GIs have been identified by comparing genomic sequences of *B. pseudomallei* K96243 to other 4 *B. pseudomallei* strains, 1710b, 1106a, MSHR668, and MSHR305 (Tuanyok

et al., 2008). These genome analyses, provide a scaffold for researchers to understand its microbial diversity and evolution, as well as bacterial pathogenesis.

2.2.3 Epidemiology and prevalence

B. pseudomallei is widely distributed in the tropics, mainly between the latitudes of 20º North and 20º South (Cheng and Currie, 2005). Endemic region for melioidosis include northern Australia, Southeast Asia, Papua New Guinea, most of the Indian subcontinent and southern China, Hong Kong and Taiwan (Figure 2.2), and most prominently in northeast Thailand, northern Australia, Singapore and parts of Malaysia (Currie et al., 2008; Wiersinga et al., 2012). In additional, sporadic cases of melioidosis have recently reported in Brazil (Rolim et al., 2005), Belgium (Ezzedine al., 2007), Indian Ocean (Currie et al., 2008) and Denmark (Badran et al., 2010), underscores an emerging global problem.

In northeast Thailand, the annual incidence of disease is estimated to be 12.7 cases per 100,000 population between 1997 and 2006 (Limmathurotsakul et al., 2010). While in the Top End of the Northern Territory, the annual incidence of 19.6 cases per 100,000 population was reported over a 14-year period from 1989 to 2003, a peak rate at 41.7 cases per 100,000 individuals in 1998 associated with high annual rainfall and two severe weather events: tropical cyclone Les (January) and tropical cyclone Thelma (December) (Currie et al., 2004). The annual incidence in Singapore was defined as 1.7 cases per 10,000 between 1989 and 1996 (Heng et al., 1998).

In Malaysia, melioidosis was first described in animals and human from Kuala Lumpur in 1913 and 1917 (Stanton and Fletcher, 1921). The disease were subsequently reported in several states, i.e., Kuala Lumpur (Puthucheary et al., 1992), Pahang (How et al.,

2005), Johor Bahru (Pagalavan, 2005), Kedah (Hassan et al., 2010) and Kelantan (Deris et al., 2010) but the true incidence of melioidosis in Malaysia is relatively unknown. Most recently, a remarkably high annual incidence was reported in the state of Kedah with a report of 16.35 cases per 100,000 individuals and a case-fatality rate of 33.8% (Hassan et al., 2010). Based on the analysis of 145 cases reported between 2005 to 2008, the authors reported that the age of patients ranged from seven days to 83 years with the male to female ratio of 3:1 and Malays had the highest incidence compared to other ethnic groups, namely, Chinese and Indian (Hassan et al., 2010).

Overall, the mortality of melioidosis varies widely the endemic area as demonstrated in Table 2.1.

Table 2.1 Mortality rates of melioidosis.

No	Country	Period	Mortality rate	References
1	Malaysia	1976 to 1991	65%	Puthucheary et al., 1992
2	Northern Territory	1989 to 1999	19%	Currie et al., 2000a
3	North Queensland	1996 to 2004	25%	Malczewski et al., 2005
4	Thailand	1986 to 2004	49%	Limmathurotsakul et al., 2006
5	Singapore	1989 to 2007	16%	Lo et al., 2009

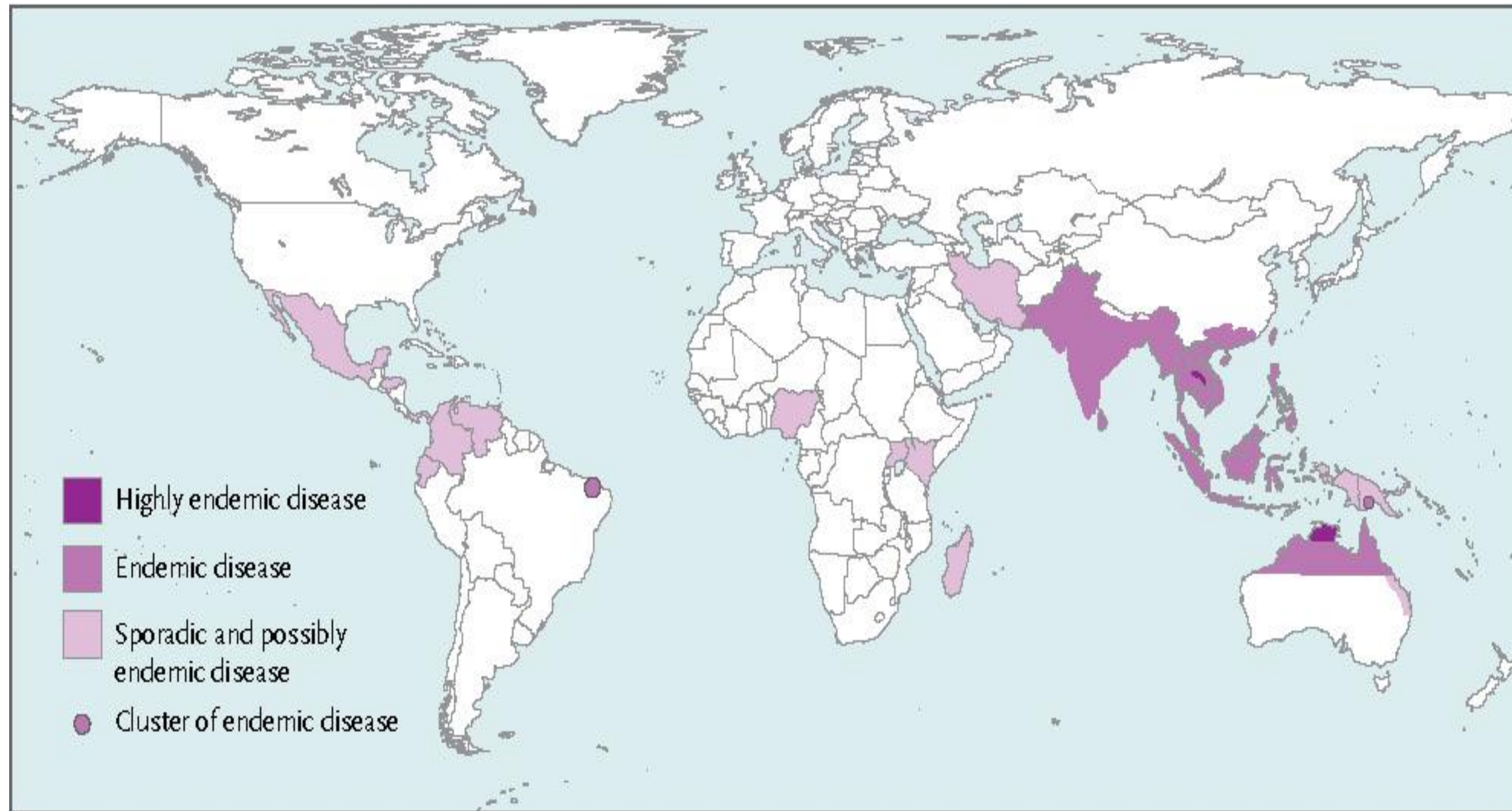


Figure 2.2. Global map showing the categories of distribution of melioidosis and *B. pseudomallei*.

(Wiersinga et al., 2012)

2.2.4 Transmission

B. pseudomallei infection can be acquired by inhalation, entry through cutaneous lesions and by ingestion; however, the relative contribution of each route remains unclear (Cheng and Currie, 2005).

Inhalation is reported a primarily route of acquisition, based on the finding that helicopter crews in the Vietnam War had increased incidence of disease, likely due to inhalation of soil and dust containing *B. pseudomallei* following aerosolization by circulating rotor blades (Howe et al., 1971). Another example was from Malaysia as a case report demonstrated a 24-year-old Malaysian female with acute non-fatal septicemia melioidosis after inhaling infective dust during a blast injury (Wang et al., 1993). Moreover, Currie and Jacups (2003) described that inhalation transmission in endemic areas during heavy rainfall not only contribute to melioidosis, but also a shift to the pneumonic presentation of disease, and this was further supported by the recent observation from northern Australia (Cheng et al., 2008) and Singapore (Lo et al., 2009).

Inoculation is the commonest route of disease transmission by direct contact of contaminated soil and water through skin abrasions or wounds. *B. pseudomallei* can be readily isolated from moist soil and still water in endemic areas such as paddy fields, palm fields, cleared fields as well as drains and ditches (Wiersinga et al., 2006). A high incidence of melioidosis was observed in rice paddies workers and their working families, who rarely wear protective clothing (Wiersinga et al., 2006). The organism is believed to be drawn to the surface during the monsoonal rains leading to an increased number of melioidosis cases which has been reported in Thailand (Suputtamongkol et al., 1994) and Australia (Currie and Jacups, 2003), however, a weaker or no correlation

was found in Malaysia and Singapore, respectively (Hassan et al., 2010; Heng et al., 1998; Sam and Puthucheary, 2007).

Another route of infection is through ingestion. In animals such as sheeps and pigs, this route has been implicated by findings of ingestion of contaminated food (Stanton & Fletcher, 1921) and contaminated water (Choy et al., 2000; Millan et al., 2007). In the context of human melioidosis, ingestion of contaminated water is considered as a potential risk (Currie et al., 2001; Inglis et al., 2000a). An example of ingestion transmission involved victims of the Asian Tsunami in 2004, where melioidosis was seen as a life threatening infection to those ingested to *B. pseudomallei*-contaminated water (Kongsaengdao et al., 2005).

Person-to-person transmission has been reported but is extremely rare. These cases occurred via sexual contact (McCormick et al., 1975), mother-to-child spread (Abbink et al., 2001), breast milk transmission (Ralph et al., 2004), perinatal transmission (Halder et al., 1998) and another report involved two siblings, one of which had cystic fibrosis (Holden et al., 2004). In addition, neonatal cases of melioidosis have been reported from United States of America, Thailand, Malaysia, United Kingdom and India (Noyal et al., 2009).

2.2.5 Risk factors

Numerous studies have shown that patients with particular underlying conditions are at risk of *B. pseudomallei* infection: 88% in Australia (Currie et al., 1993), 77.4% in Singapore (Heng et al., 1998), 76% in Malaysia (Puthucheary et al., 1992), and 53% in Thailand (Suputtamongkol et al., 1994).

The risk factors for melioidosis include diabetes mellitus, renal dysfunction, alcoholism, chronic lung disease, steroid therapy, occupational exposure, trauma and parental drug abuse (Cheng and Currie, 2005; Puthucheary, 2009; Wiersinga et al., 2006). The contribution of each underlying conditions to melioidosis remains unclear, but these conditions are suggested lead to immune deficits such as phagocytic defects, diminished humoral and cellular response and diminished cytokine production, therefore, enhancing the ability of *B. pseudomallei* to survive within the host (Puthucheary, 2009).

The most notable risk factor was diabetes mellitus and the case rates have been recorded 57% in Thailand (Limmathurotsakul et al., 2006), 56.6% in Malaysia (Hassan et al., 2010), 48% in Singapore (Lo et al., 2009), and 42% in Australia (Malczewski et al., 2005). The reason for the association between diabetes and risk of developing melioidosis are unclear. It was recently demonstrated that polymorphonuclear neutrophil from diabetic subjects displayed impaired phagocytic function, migration and apoptosis when compared to those of healthy subjects (Chanchamroen et al., 2009).

2.2.6 Clinical manifestations of melioidosis patients

The clinical presentation of melioidosis is difficult as the broad spectrum of its clinical symptoms can range from benign skin and soft tissue infection to a rapidly fulminant and septicaemia (Puthucheary, 2009). Due to this wide array of clinical signs and symptoms, *B. pseudomallei* is dubbed as “the great mimicker” (Wiersinga et al., 2006).

The incubation period of melioidosis may range from 1 day (Currie et al., 2000b) to 62 years, thus indicating the remarkable capability of the bacterium to remain latent in the host (Ngauy et al, 2005). Recently, a very short incubation period of melioidosis for a

high inoculum in near-drowning event in Phang Nga, a region in southern Thailand was documented by Chierakul and co-workers (2005).

In general, the broad clinical spectrum of melioidosis can be divided into to: acute, subacute, chronic and latent melioidosis (Limmathurotsakul et al., 2012). Acute and subacute melioidosis are characterized by the presence of symptoms for less than 2 weeks, and 2 weeks to 2 months, respectively, prior to presentation. The most common acute clinical presentation of melioidosis is pneumonia. Chronic melioidosis is often localized, with an incubation period longer than 2 months that may mimic cancer or tuberculosis. Latent melioidosis is an asymptomatic infection that the organism can remain dormant for years, but which may become active at some indefinite point in future (Limmathurotsakul et al., 2012).

However, there exists a clinical continuum as the less acute or localized forms may rapidly progress to the septicaemia form, so Puthucheary and Vadivelu (2002) suggested another more functional and useful clinical classification into septicemia and non-septicemia (localized) melioidosis. The presentation of septicemia melioidosis include simple to disseminated bacteremia while localized melioidosis include: pneumonia, acute suppurative lesions, chronic granulomatous, superficial abscess, cellulitis, sepsis following trauma, cervical lymphadenophy, septic arthritis, osteomyelitis, parotitis, chronic otitis media, prostatitis, peritonitis and mycotic aneurysms (Puthucheary and Vadivelu, 2002).

The type of clinical presentation can vary largely based on geographical location, inoculating dose, strain virulence, route of exposure, risk factors in the host, and the host immune response (Wiersinga et al., 2012). For instance, there is a high incidence

of genitourinary infection with prostatic abscesses in Australia, but these cases rare in Thailand (White, 2003). On the contrary, suppurative parotitis is a common presentation in Thai children with melioidosis, but it is uncommon in Australia (Dance et al., 1989).

2.3 *Burkholderia pseudomallei* and host interaction

2.3.1 Intracellular lifecycle

B. pseudomallei is a facultative intracellular pathogen that can efficiently invade and replicate inside non-phagocytic cells including HeLa, CHO, A549 and VERO (Harley et al., 1998a; Jones et al., 1996; Kespichayawattana et al., 2000), phagocytic cells such as polymorphonuclear leukocytes, macrophages and human monocyte-derived dendritic cells (Charoensap et al., 2009; Jones et al., 1996; Utaisincharoen et al., 2001), and *Acanthamoeba astronyxis* (Inglis et al., 2000b; Inglis et al., 2003).

Figure 2.3 is a simple overview of the bacterium utilizing diverse survival strategies to persist in the intracellular environment. It is likely that there is no single cause but rather a combination of a complicated and multifactorial interaction of various virulence factors. The attachment of *B. pseudomallei* to host cells represents an essential early step in the pathogenesis. In particular, it was reported that adhesion of *B. pseudomallei* to lung carcinoma A549 cell line appeared to be temperature dependent, as the bacteria grown at 30°C were more adherent than bacteria grown at 37°C (Brown et al., 2002). The GM1-GM2 glycosphingolipid ganglioside had been identified as host receptors for *B. pseudomallei* based on attachment inhibition studies (Gori et al., 1999; Kanai et al., 1997). Other candidate virulence factors include capsule, flagellum *fliC*, pilus, two adhensins, *BoaA* and *BoaB* have been reported to mediate attachment of the bacterium to epithelial cells (Figure 2.3).

A two-component response regulator, *irlRS* which is involved in regulating resistance to heavy-metals was reported to regulate the invasion of *B. pseudomallei* to epithelial cells, but not macrophages (Jones et al., 1997). *B. pseudomallei* contains 3 Type 3 Secretion Systems (T3SSs); however, only *Burkholderia* secretion apparatus (*Bsa*) which is homologous to the *inv/spa/prg* of *Salmonella typhimurium* and the *ipa/mxi/spa* of *Shigella flexneri* (Attree and Attree, 2001), are important for pathogenesis (Muangsombut et al., 2008; Stevens et al., 2003). Several Bsa proteins including BpoE (Stevens et al., 2003), BipD (Stevens et al., 2003) and BsaQ (Muangsombut et al., 2008) have been reported to facilitate bacterial invasion in non-phagocytic cells. The effector protein BpoE resembles *Salmonella enterica* SopE/SopE2, which promotes invasion by acting as a guanine nucleotide exchange factor for RhoGTPases that induces cytoskeletal rearrangements (Stevens et al., 2003).

Following internalization of the bacterium into cells, *B. pseudomallei* have been shown to reside inside plasma-membrane derived phagosomes (Puthucheary and Nathan, 2006). Using transmission electron microscopy, the authors demonstrated that phagolysosome fusion occurred slowly in *B. pseudomallei*-infected macrophages in patients with melioidosis compared to normal subjects with a concomitant increase in the intracellular bacteria (Puthucheary and Nathan, 2006).

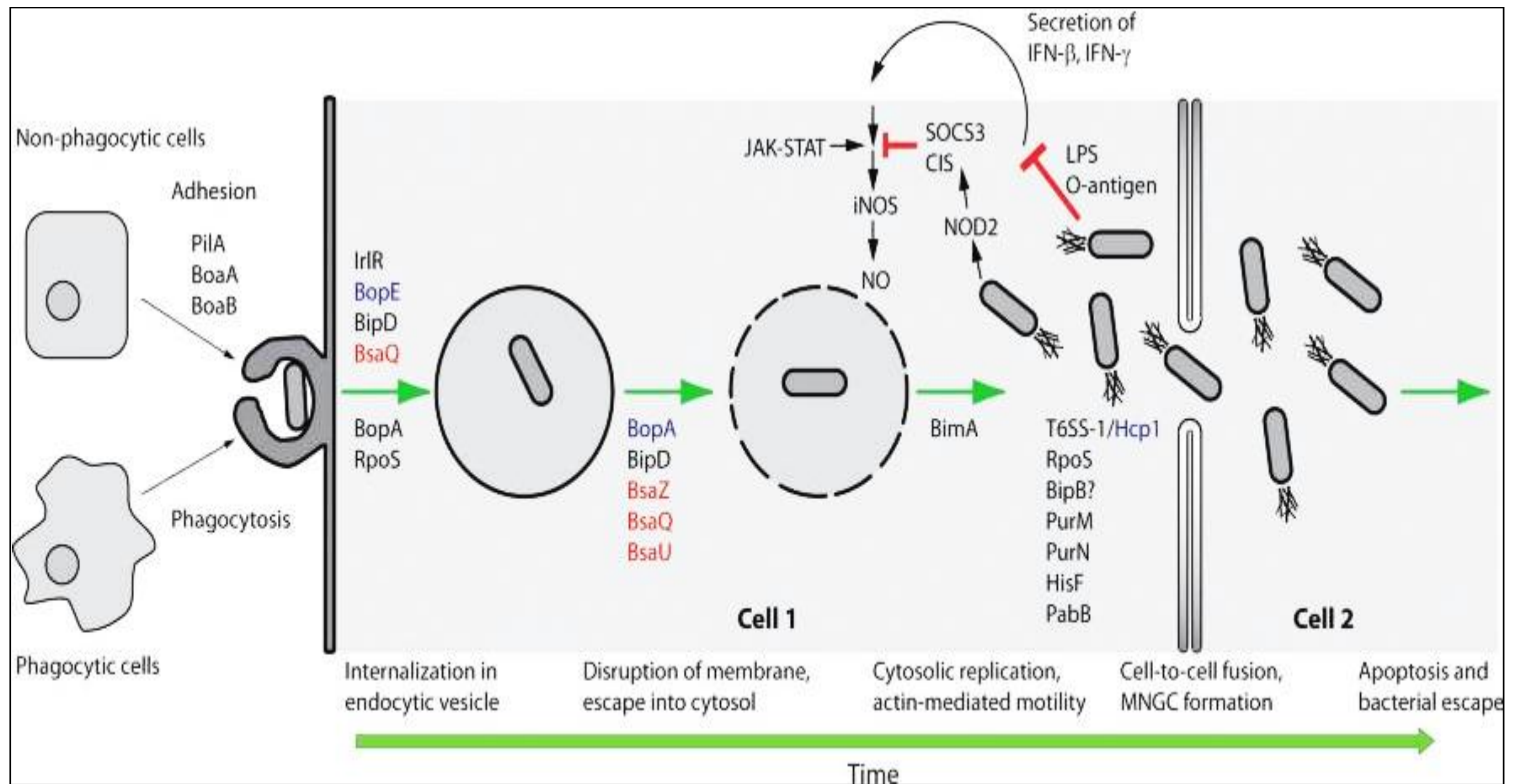


Figure 2.3. **Intracellular lifestyle of *B. pseudomallei*. (Allwood et al., 2011)**

The ability of *B. pseudomallei* to survive intracellularly is crucial for its pathogenesis. It can inhibit the inducible nitric oxide synthase (iNOS), an antimicrobial molecule needed to generate reactive nitrogen intermediate to control the intracellular growth of this bacterium in macrophage (Utaisincharoen et al., 2001). Inhibition of inducible iNOS expression was reported to be associated with the defective production of interferon (IFN)-β, leading to reduced interferon regulatory factor 1 and subsequently iNOS expression (Utaisincharoen et al., 2003). Besides, low level expression of iNOS also might be partly modulated by the O-antigenic polysaccharide moiety of *B. pseudomallei* (Arjcharoen et al., 2007; Utaisincharoen et al., 2000). On the other hand, the expression of two negative regulators, a suppressor of cytokine signaling 3 and cytokine-inducible Src homology 2-containing protein that demonstrated interference of cytokine production via a STAT-1 pathway, could result in depression of iNOS expression, thus facilitating its intracellular survival (Utaisincharoen et al., 2005).

As early as 15 minutes of internalization, the bacteria can escape from endocytic vesicles into the cytoplasm by destroying phagosome membrane (Harley et al., 1998b). Defects in *bsa* T3SSs in mutants lacking *BopA* (Cullinane et al., 2008; Gong et al., 2011), *BipD* (Gong et al., 2011), *BsaZ* (Stevens et al., 2002) and *BsaQ* (Muangsombut et al., 2008) resulted in delayed or no escape from phagosome. Furthermore, *B. pseudomallei* mutants lacking either *BipD* or *BsaZ* demonstrated unable to escape into cytoplasm at 6 h after infection in J774.2 cells (Stevens et al., 2002) while they also displayed decreased bacteria escape in RAW 264.7 cells (Burtnick et al., 2008; Gong et al., 2011).

B. pseudomallei actively evades autophagy and enhances its survival by the secretion of Bsa T3SS effector protein, BopA (Cullinane et al., 2008; Gong et al., 2011). Once in

the cytoplasm, it is able to form actin-based membrane protrusions into neighboring cells, which allows the organism to spread directly from cell-to-cell without exposure to host innate and adaptive immune responses (Kespichayawattana et al., 2000; Stevens et al., 2006). The actin polymerization of *B. pseudomallei* is mediated by BimA, a bacterial protein with an actin-binding domain which is essential for binding to host actin, located at the site of actin nucleation (Stevens et al., 2005a). This notion is supported by subsequent studies by Stevens et al. (2005b) and Sitthidet et al. (2011), who showed that *bimA* mutant loses ability to form membrane protrusions in J774.2 cells and did not form plaques in A549 monolayers.

The spread of bacteria from cell-to-cell appears to cause cell fusion and this result in the formation of multinucleated giant cells (MNGC) (Kespichayawattana et al., 2000). Inactivation of *BipB* (*bsa* T3SS), *rpoS* or *hcp* genes showed impaired ability of *B. pseudomallei* to induce MNGC formation in J774A.1 macrophage-like cells at 6 hours post infection (Suparak et al., 2005) and RAW 264.7 cell monolayers (Burtnick et al., 2011; Utaisincharoen et al., 2006). These findings indicate that by the stimulation of cell fusion, MNGC support the growth of *B. pseudomallei* within its hosts while protecting the bacterium from host immune responses. In addition, the detection of giant cells in lung of humans had also been reported under histopathological study of 5 autopsy cases (Wong et al., 1995).

2.3.2 Virulence factors

Discovery of virulence factors is very important for the understanding of host-pathogen interactions in melioidosis, which may also serve as novel targets in drug and vaccine development in combating bacterial disease. The virulence factors in this context are referred as proteins or other molecules that are involved in the infection, which enable a pathogen to enter, survive, replicate within host cells and maintain the disease state.

Several approaches, including subtractive hybridization (SH) (Puthucheary et al., 2012a; Reckseidler et al., 2001), comparative genomics (Yu et al., 2006), signature-tagged mutagenesis (STM) (Cuccui et al., 2007), transposon mutagenesis (Rholl et al., 2008), *in vivo* expression technology (IVET) (Shalom et al., 2007), and microarray (Chieng et al., 2012) have accelerated the virulence factor discovery over the past decades. The availability of the genomic sequences of several *B. pseudomallei* strains (Section 2.2.2) has rapidly add candidate virulence genes to databases.

With the recent development in the construction of gene-targeted knock-out mutants using transposons, integrative vectors, allelic exchange vectors, and broad-host-range plasmids (Choi et al., 2008; Galyov et al., 2010; Kang et al., 2011), these tools can assess the importance of candidate virulence factors at the molecular level. The methods used to generate *B. pseudomallei* mutants involved random insertional mutagenesis using transposon Tn5-OT182 (DeShazeret al., 1997; Reckseidler et al., 2001) and *Himar 1*-based elements (Rholl et al., 2008). Site-specific mutagenesis requires integration of a suicide plasmid carrying resistance cassetets into the chromosome by a single crossover recombination (Burtnick et al., 2008; Sarkar-Tyson et al., 2007; Song et al., 2005).

Gene replacement is a way to create mutants by replacing the relevant gene with an antibiotic resistance marker such as kanamycin from pUT-Km (Chua et al., 2003), *tet* cassette from pFRTT1 or zeocin cassette from pCLOXZ1 (Tan et al., 2010), allowing easy selection of mutant. Another method is unmarked deletion involving allelic exchange and the use of counter-selectable markers such as *sac*B (Yuen et al., 2012), I-*Sce*I (Kvitko et al., 2012) and *pheS* (Norris et al., 2011).

After obtaining mutant strains, their role can be studied using available models for investigating the disease and virulence as shown in Table 2.1. However, no single animal and/or cultured cell monolayer model can answer all biological questions, thus various different *in vivo* infection models such as mice, diabetic rat, hamsters, *Caenorhabditis elegans* as well as *in vitro* cell lines including A549, BEAS-2B, RPMI-2650, Hep2, A549, NHBE, J774A.1, HeLa, CHO, and RAW 264.7 have been extensively used in *B. pseudomallei* research (Allwood et al., 2011).

As shown in Table 2.1, previous efforts to identify potential virulence factors in *B. pseudomallei*, have been met with varied results using *in vivo* and/or *in vitro* models. The most notable candidates for virulence factors were Type IV pili (*pilA*), *bpscN, Bsa* T3SS and T6SS (*hcp1, virAG, bprC, tssAB*). Studies by Essex-Lopresti et al. (2005) showed that unmarked in-frame deletion mutant of *pilA* of *B. pseudomallei* reduced bacterial adherence to human respiratory cell lines as well as significantly attenuated virulence in *C. elegans* and BALB/c mice. This result was further supported by Boddey and colleagues (2006) who reported the *pilA* gene, putatively encoding a type IV pilin, enhanced association with eukaryotic cells by mediating microcolony formation.

Table 2.2. Examination of mutants of virulence determinants in different infection models.

Virulent determinant(s)		Mutant phenotype		References
Product	**Gene**	**Vertebrate/ invertebrate models**	**Eukaryotic cell culture models**	
Quorum sensing	*luxI* and *luxR*	Decreased virulence in mice (i.n) and hamster (i.p); Less lung colonization in mice (i.n)	ND	Ulrich et al., 2004
	PmII-PmIR	Full virulence in mouse model	ND	Valade et al., 2004
Type IV pili (TFP)	*pilA*	Slightly decreased virulence in mice (i.n but not i.p)	Reduced adherence to A549, BEAS-2B and RPMI-2650	Boddey et al., (2006); Essex-Lopresti et al., (2005)
Adhesin	*boaA/ boaB*	ND	Reduced adherence to Hep2, A549 and NHBE and decreased growth in J774A.1.	Balder et al., 2010
Flagelum	*fliC*	Reduced virulence in mice (i.n and i.p), but no in *C. elegans,* diabetic rats and hamsters (i.p)	Same as wild type	Chua et al., 2003; Chuaygud et al., 2008; DeShazer et al., 1997
Two-component response regulator	*irlR*	No virulence in diabetic rats and hamsters	Less invasion in A549, HeLa and CHO, but not in macrophages	Jones et al., 1997
TTSS1	*bpscN*	Reduced virulence in mice (i.n)	Diminished survival and replication in RAW264.7	D'Cruze et al., 2011
Bsa T3SS	*bpoE*	Same as wild type in mice (i.p and i.n)	Reduced invasion in HeLa, but same as wild type in J774A.1	Steven et al., 2002; Steven et al., 2003; Steven et al., 2004
	bipD	Reduced virulence in mice (i.p and i.n)	Reduced invasion in J774A.1 and RAW264.7, but no HeLa.	Gong et al., 2011; Steven et al., 2002; Steven et al., 2003; Steven et al., 2004
	bsaQ	ND	Reduced invasion in A549.	Muangsombut et al., 2008; Sun et al., 2005

Table 2.1. (Continued)

Virulent determinant(s)		Mutant phenotype		References
Product	**Gene**	**Vertebrate/ invertebrate models**	**Eukaryotic cell culture models**	
Bsa T3SS	*bsaZ*	Reduced virulence in mice (i.n) and hamster (i.p)	Reduced invasion in J774A.1 and can survive in RAW264.7.	Burtnick et al., 2008; Stevens et al., 2002; Stevens et al., 2004; Warawa and Woods, 2005;
	sctU1/sctU2/bsaZ	Reduced virulence in hamster (i.p)	Same as wild type in RAW264.7.	Burtnick et al., 2008; Warawa and Woods, 2005
	bsaU	Reduced virulence in mice (i.n)	Same as wild type in HeLa and RAW264.7.	Pilatz et al., 2006
	bopA	Same as wild type in mice (i.p) and hamster (i.p)	Impaired survival in RAW264.7.	Cullinane et al., 2008; Gong et al., 2011; Stevens et al., 2004; Warawa and Woods et al., 2005
	bipB	Reduced virulence in mice (i.p)	Reduced invasion in HeLa	Suparak et al., 2005
	bpoC	ND	Reduced invasion in A549.	Muangman et al., 2011
T6SS	*tssH-5, bhuT*	ND	Same as wild type in RAW264.7.	Shalom et al., 2007
	hcp1	Reduced virulence in hamster (i.p)	Impaired growth in RAW264.7.	Burtnick et al., 2011
	virAG, bprC, tssAB	Reduced virulence in mice (i.n)	Impaired growth in RAW264.7.	Chen et al., 2011
	BcaA, BcaB	Same as wild type in mice (i.n)	Same as wild type in A549.	Campos et al., 2013
Phosphoribosyl-formyl-glycinamidine	*purM*	Reduced virulence in mice (i.n) and hamster (i.n)	Same as wild type in HeLa.	Pilatz et al., 2006; Propst et al., 2010
Phosphoribosyl-glycinamide	*purN*	Reduced virulence in mice (i.n)	Same as wild type in HeLa.	Pilatz et al., 2006

Table 2.2. (Continued)

Virulent determinant(s)		Mutant phenotype		References
Product	**Gene**	**Vertebrate/ invertebrate models**	**Eukaryotic cell culture models**	
Imidazole glycerol phosphate	*hisF*	Reduced virulence in mice (i.n)	Same as wild type in HeLa.	Pilatz et al., 2006
Para-aminobenzoate synthetase	*pabB*	Reduced virulence in mice (i.n)	Same as wild type in HeLa.	Pilatz et al., 2006
Putative exported protein	*Bpsl1528*	ND	Same as wild type in HeLa.	Pilatz et al., 2006
Autosecreted Protein	bimA	ND	Abolished actin-based motility in J774.2.	Stevens et al., 2005a
Lactonase family protein A	*lfpA*	ND	Same as wild type in RAW264.7	Boddey et al., 2007
Phospholipase C	*plc-1, plc-2*	ND	Reduced plaque formation	Korbsrisate et al., 2007
	plc-3	Reduced virulence in hamster (i.p)	ND	Tuanyok et al., 2006
Capsule	*wcb*	Reduced virulence in hamster (i.p)	Increased deposition of C3b	Reckseidler-Zenteno et al., 2005
Oxidative regulatory factor	*rpoS*	ND	Reduced invasion in Raw264.7	Utaisincharoen et al., 2006
	oxyR	ND	Hypersensitive to H2O2	Loprasert et al., 2002
Siderophores	*mbaJ, mbaF, mbaL, fmtA, mbaA*	ND	Defect growth in iron-limiting conditions (Chelax-treated M9)	Alice et al., 2006
Superoxide dismutases	*sodC*	Reduced virulence in mice (i.p)	Impaired survival in J774A.1	Vanaporn et al., 2011

ND – Not done; i.n – intranasal; i.p - intraperitoneal

The Type 3 Secretion System Cluster 1 (TSS1) gene *bpscN* contains 63.5% amino acid that is identical to a *HrcN* in *R. solanacearum,* which is a predicted TTSS-associated ATPase. Mutants lacking gene *bpscN* demonstrated less virulence than wild type in BALB/c mice and diminished survival and replicative capacity in RAW264.7 cells (D'Cruze et al., 2011). The *Bsa* T3SS is important for virulence as demonstrated by numerous studies (Burtnick et al., 2008; Cullinane et al., 2008; Gong et al., 2011; Jones et al., 1997; Muangman et al., 2011; Muangsombut et al., 2008; Pilatz et al., 2006; Stevens et al., 2002; Stevens et al., 2003; Stevens et al., 2004; Suparak et al., 2005; Warawa and Woods, 2005). Warawa and co-workers (2005) have shown that *Bsa*/TTSS3 was required for optimal virulence via intraperitoneal (i.p) route in Syrian Golden hamsters.

B. pseudomallei possess 6 clusters of genes associated with Type Six Secretion System (T6SS) and T6SS-1 cluster was first identified in the *B. pseudomallei* infected macrophages through IVET (Shalom et al., 2007). The mutation in the T6SS-1 cluster, *hcp1* gene significantly attenuated virulence in the hamster model. The mutant showed impaired growth in RAW264.7 cells and it also failed to stimulate MNGC formation (Burtnick et al., 2011). The expression of T6SS-1 cluster is dependent on *BprC* and *VirAG* inside the host cells (Chen et al., 2011).

2.3.3 *in vivo* and *in vitro* models to study pathogenesis

The use of animal models in the study of *B. pseudomallei* infection provides valuable insights into the host and bacterial factors involved in melioidosis as it reflects the natural infection process in humans. Thus far, a wide spectrum of, models, ranging from non-mammalian *C. elegans* to mammalian, i.e., small animal such as mice and Syrian hamster to large animals models such as goats, pigs and non-human primates are available (Sivalingam et al., 2012). It is not yet clear which animal model of melioidosis best reflects the disease pattern in humans.

The mouse model system is probably the most adaptable and popular for studying the pathogenesis of melioidosis. For instance, BALB/c and C57BL/6 mouse models exhibited the acute and chronic forms of human melioidosis, respectively (Sivalingam et al., 2012). However, long reproductive cycles, the difficulties and cost associated with obtaining and maintaining sufficient number of animals for experiments is discouraging. Hence, one of the simplest inverterbrate model, the nematode worm, *C. elegans* has gained significant attention as a model organism since Sidney Brenner first promoted its use in the study of neurobiology in 1960s (Brenner, 1974) while Tan and colleagues first reported its use in the study of *Pseudomonas aeruginosa* pathogenesis. It has since been used extensively to study host-pathogen interaction for various pathogens such as *B. cenocepacia* (Kothe et al., 2003), *Staphylococcus aureus* (Sifri et al., 2003) as well as *Drechmeria coniospora* fungus (Jansson et al., 1994) by simply replacing its food, *E. coli* OP50 with pathogens and measuring the survival of worms.

In the context of *B. pseudomallei*, previous investigators have supported the use of *C. elegans* as a surrogate host for infection study although they have conflicting opinion- whether nematode killing by *B. pseudomallei* is due to toxin (Gan et al., 2002; Ooi et

al., 2012) or direct prolonged contact with organism (Gan et al., 2002; Lee et al., 2011; O'Quinn et al., 2001). Overall, the *C elegans* model offers several advantages as a host model including simple growth conditions, short generation time and being cost-effective. Therefore, in this study, *C elegans* has been chosen as an infection model and details of this system will be further elaborated in Section 2.3.3.1.

2.3.3.1 Features of *Caenorhabditis elegans* system

C. elegans is a small body sized, non-parasitic free-living nematode (roundworm) that lives in soil, where it feeds on microorganisms. The standard *C. elegans* laboratory strain is Bristol N2 and it is typically propagated on agar plates containing a lawn of slow-growing uracil auxotrophic *Escherichia coli* strain of OP50.

The life cycle of *C. elegans* is simple and fast: approximately two to three days at room-temperature (from fertilized egg to an adult). As illustrated in Figure 2.4, it has four larval stages (L1 to L4) and the larva will go into dauer stage under unfavorable life conditions for up to four months. Once food becomes available, dauer larva feed and continue development to the adult stage. After worms have reached adulthood, they survive for about 2 to 3 weeks (Altun and Hall, 2005).

C. elegans population consists of two sexes, hermaphrodites and males. Hermaphrodites produce both oocytes and sperm and are capable of self-fertilization, where a single hermaphrodite will produce approximately 300 eggs. Males occur spontaneously non-disjunction in the hermaphrodite germline at low frequency (0.1%). It produces only sperm and must mate with a hermaphrodite in order to reproduce (Altun and Hall, 2005).

Importantly, it is the first multicellular organism to have its whole genome sequenced to enable the study of an array of molecular genetics and signaling pathways (The *C. elegans* Sequencing Consortium, 1998). The body of *C. elegans* is transparent and its entire cell lineage from egg to adult is visible under the light microscope. The transparency also permits the direct observation of host-pathogen interactions at the cellular level when bacterial pathogens are tagged with fluorescent proteins (Luhachack et al., 2012).

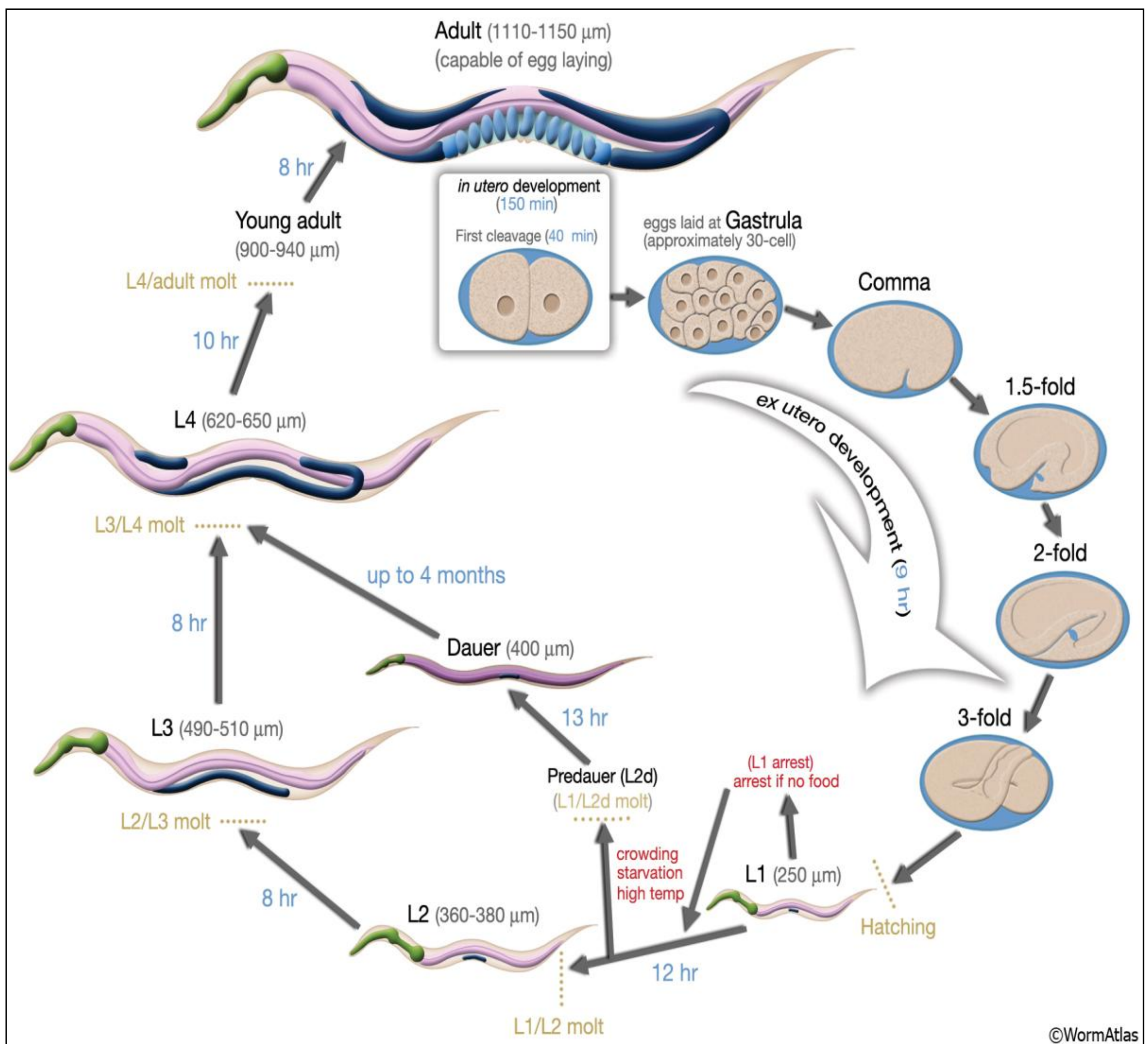


Figure 2.4. Life cycle of *C. elegans*. (Altun and Hall, 2005)

2.4 Host defense against *Burkholderia pseudomallei*

Similar to other bacterial infections, the innate and adaptive immune systems are important in controlling infection by *B. pseudomallei.* Toll-like receptors (TLR) play a vital role in initiating innate immune cells via pattern recognition receptors, which recognize pathogen-associated-molecular patterns (PAMPs) of *B. pseudomallei* and subsequently signal the production of inflammatory cytokines and chemokines. Supporting this, an upregulation of multiple TLRs, i.e., TLR1, TLR2 and TLR4 are seen in melioidosis patients with septic shock (Wiersinga et al., 2007). It is worthwhile to note that TLR2 knockout mice demonstrated significantly improved host defenses as reflected by an improvement in survival with decreased bacterial loads and distant organ injury as well as reduced lung inflammation. A recent study by West et al. (2012) also demonstrated a significant association between TLR polymorphisms and melioidosis patients.

The production of proinflammatory cytokines IFN-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-8 and IL-12 are elevated in severe melioidosis patients (Lauw et al., 1999). A study using mouse model demonstrated direct inhibition of IFN-γ with the neutralizing monoclonal antibody increased the bacterial burden in organs greater than 4,000-fold and reduced the median lethal dose from > 5×10^5 to ca 2 CFU (Santanirand et al., 1999). Besides, the use of CpG oligodeoxynucleotides to enhance innate immune response in mice prior to challenge with *B. pseudomallei*, has been reported (Wongratanacheewin et al., 2004).

Apart from cell-mediated immune response, the humoral immune response also plays a role in limiting disease progression (Chenthamarakshan et al., 2001a; Chenthamarakshan et al., 2001b). Not surprisingly, melioidosis patients have detectable

antibody titers, with the dominant isotype IgG and consisting of subclasses IgG1 and IgG2 (Chenthamarakshan et al., 2001a ; Ho et al., 1997). These 2 subisotypes are thought to activate the classical complement pathway and IgG1, has high opsonic potential, binds to mononuclear cells (Chenthamarakshan et al., 2001a). Another 2 immunoglobulins, IgM and IgA were elevated in melioidosis patients with localised, soft tissue infections (Chenthamarakshan et al., 2001a ; Ho et al., 1997). This may reflect continuous B cell priming due to sequestration of intracellular bacterial antigens, perhaps necessitating continued antimicrobial therapy (Vasu et al., 2003).

2.5 Laboratory diagnosis and detection

Melioidosis is difficult to diagnose clinically due to the wide range of presenting and associated symptoms (Section 2.2.6). The mortality is high among patients with underlying diseases especially diabetes and immunosuppression (Section 2.2.5). Definitive diagnosis of melioidosis has historically been based on the isolation of the bacterium from specimens such as blood, urine, sputum or pus using culture techniques. Bacterial culture may take up to 4 days and reportedly can have associated problems such as inter-strain and medium dependent variability in colony morphology. Apart from this “gold standard”, various serological assays, molecular techniques and commercial identification system are also being developed, aiming to enhance diagnostic performance. Besides, appropriate imaging techniques are utilized to assist in detecting infection sites. With this brief background, details of each diagnostic method will be further described in Sections 2.5.1 to 2.5.5.

2.5.1 Culture-based methods

Ashdown's agar (ASA) is currently the favored selective medium for the isolation of *B. pseudomallei*, which contains neutral red (differentiation of this organism from others), glycerol (enhances the formation of wrinkled colonies), crystal violet and gentamicin (inhibition of other organisms) (Ashdown, 1979). The presumptive identification of *B. pseudomallei* can be identified on the basis of colonial color, i.e., purple color with dry and wrinkled appearance when grown on this selective media after 24 to 48 h of incubation (Figure 2.1).

A second medium, *B. pseudomallei* selective agar (BPSA) improves recovery of the mucoid colonies and provides large wrinkled colonies faster than ASA (Howard et al., 2003). A third medium, *B. cepacia* medium, is widely available and offers a suitable alternative in non-endemic areas for the isolation of *B. pseudomallei* from patients with suspected melioidosis. These 3 media have been evaluated for their ability to grow *B. pseudomallei*. The sensitivity of each medium was comparable, but the BPSA showed the lowest selectivity (Peacock et al., 2005).

2.5.2 Serodiagnosis

A major drawback of using the culture method is time-consuming. Therefore, attempts to identify the organism more rapidly than culture method such as, immunological assays have been developed for either detection of antigens (Ags) or antibodies (Abs) against the organism in clinical specimens.

2.5.2.1 Antigen detection

Antigen detection assays are based on the use of specific antibody that reacts with *B. pseudomallei* in clinical specimens through different types of tests as shown in Table 2.2. This approach was first undertaken by Ismail et al. (1987) when they developed a monoclonal antibody-based ELISA assay for quantitation of exotoxin (> 16 ng/mL) of *B. pseudomallei* in culture supernatant fluid. Monoclonal antibodies (MAbs) for cell wall components, including lipopolysaccharide (LPS) (Dharakul et al., 1999; Sirisinha et al., 2000), 200-kDA protein (Anuntagool et al., 2000; Rudgech et al., 1995; Samosornsuk et al., 1999; Sirisinha et al., 2000), exopolysaccharide (EPS) (Steinmetz et al., 1999) and 30-kDA protein (Ekpo et al., 2007; Pongsunk et al., 1999), were developed for the detection of *B. pseudomallei* antigen present in blood, sputum, pus and urine, however, none are yet commercially available.

Of these assays, Dharakul et al. (1999) described a highly satisfactory test based on the use of a monoclonal antibody latex agglutination (MAb-LA) against LPS (Bsp1-L1) in 88 blood cultures, which gave both sensitivity and specificity of 100% (Dharakul et al., 1999). Another 2 MAb-LA tests specific to 30 and 200-kDa proteins had a high sensitivity and specificity overall after evaluation in 11 and 12 hospitals in north eastern part of Thailand, respectively. It was shown that MAb specific to the 30-kDA protein could directly agglutinate with all 243 clinical isolates of *B. pseudomallei* but not with other Gram-negative bacteria, except for one strain of *B. mallei*; however, the cross-reaction with some gram-positive bacteria was observed (Ekpo et al., 2007; Pongsunk et al., 1999).

Table 2.3. Various antigen detection tests for the diagnosis of melioidosis.

Methods	Antibody used	References	Sensitivity (%)	Specificity (%)
Indirect ELISA	Monoclonal anti-exotoxin	Ismail et al., 1987	NR	NR
	MAb 5F8 (MAb anti 200-kDA protein)	Rudgech et al., 1995	NR	NR
		Anuntagool et al., 1996	75	98
		Sirisinha et al., 2000	59	56
Direct agglutination	5F8	Rudgech et al., 1995	NR	NR
	anti 30-kDA protein	Pongsunk et al., 1999	94.1	98.3
MAb-LA	Bsp1-L1(anti-LPS)	Dharakul et al., 1999	100	100
	anti-EPS	Steinmetz et al, 1999	NR	NR
	Bsp L2 (anti-LPS)	Sirisinha et al., 2000	23	100
	5F8,4B11,6G9-LA (anti 200-kDA protein)	Samosornsuk et al., 1999	95	100
		Anuntagool et al., 2000	95.1	99.7
	anti 30-kDA protein	Pongsunk et al., 1999	100 [a] 100 [b]	96.5 [a] 85.9 [b]
		Ekpo et al., 2007	96.8	99.6
Direct IF	Whole-cell specific antibodies of *B. pseudomallei* conjugated FITC	Walsh et al., 1994	73	99
		Wuthiekanun et al., 2005	66	99.5

NR- Not reported; a-Blood culture; b-Brain Heart Infusion Broth specimens

The other investigators used a MAb mixture consisting of 5FB, 4B11 and 6G9 reactive with a 200-kDa protein antigen highlighting that the serodiagnostic potential of this assay was due to its ability to distinguish *B. pseudomallei* from *B. thailandensis* and the ability to react with *B. pseudomallei* strains with atypical LPS patterns (Anuntagool et al., 2000; Samosornsuk et al., 1999).

Immunofluorescence (IF) can be used to detect *B. pseudomallei* directly from sputum, urine and pus, using whole-cell specific antibodies conjugated to fluorescein isothiocyanate (FITC). Standard IF is relatively labor intensive and takes more than 2 hours to complete (Walsh et al., 1994) whereas a modified rapid IF can be completed in 10 minutes (Wuthiekanun et al., 2005). A standard and rapid IF had sensitivities of 73% and 66%, and specificities of 99% and 99.5%, respectively. However, the use of this test may be limited due to the requirement of specialized microscopy facilities.

2.5.2.2 Antibody detection

An antibody detection test measures the presence of humoral antibodies against immunodominant antigens of *B. pseudomallei*. Efforts to develop antibody detection tests for the diagnosis of melioidosis have been under way for decades, and the performance of these tests has been well described in Table 2.3. Basically, these tests are developed into formats depending on (a) antigen source either native or recombinant protein (b) class of immunoglobulin detection, e.g., IgG, IgM or total antibodies (c) on the test design. Overall, the most common types of tests included indirect hemagglutination assay (IHA), indirect immunofluorescent test (IFAT), enzyme-linked immunosorbent assay (ELISA) and immunochromatographic test (ICT).

The IHA is used to detect serum antibodies of patients, based on agglutination of sheep red cells sensitized with *B. pseudomallei* antigens (Cheng et al., 2006; Gilmore et al., 2007; Vadivelu et al., 1995). The antibody titer is recorded as the highest dilution that shows agglutination. However, IHA assay is poorly standardized, with variation in *B. pseudomallei* strains, antigen preparation protocol and difference of detection threshold. The diagnostic cut-off for IHA in Thailand is ≥ 1:160 (Wuthiekanun et al., 2006a) while in Australia, it is ≥ 1:40 to 1:160 (Cheng et al., 2006). Strikingly, high background titers in healthy individuals were reported and led to difficulty in interpretation (Chantratita et al., 2007; Wuthiekanun et al., 2006a; Wuthiekanun et al., 2006b). For an example, 53 out of 200 healthy blood donors in Malaysia demonstrated positive antibody titer (≥ 1:40) against *B. pseudomallei* (Norazah et al., 1996).

Table 2.4. Antibody detection tests for diagnosis of melioidosis.

Methods	Antigen	References	Sensitivity (%)	Specificity (%)
IHA	Meliodin	Kunakorn et al., 1990	88	97.4
		Petkanjanapong et al.,1992	80.9	91.4
		Vadivelu et al., 1995	45	100
		Dharukul et al., 1997	71	74.7
		Sermswan et al., 2000	61.9	79.8
		O'Brien et al., 2004	80	NR
		Wuthiekanun et al., 2004	72	68
		Chuah et al., 2005	76	99
IFAT	Whole cell Ag of *B. pseudomallei*	Vadivelu et al., 1995	91	100
		Vadivelu et al., 2000	100	72
		Mathai et al., 2003	36	99
	Whole cell Ag of *B. thailandensis*	Puthucheary et al., 2010	98.5	92.3
Western blot	16-kDa recombinant Bps antigen	Sirisinha et al., 2000	70	91
Dot immunoassay	CF 40-kDa	Sirisinha et al., 2000	72	64
		Sermswan et al., 2000	85.7	85.3
Gold blot	Whole cell Ag	Kunakorn et al., 1990 (IgM)	87.5	88
		Kunakorn et al., 1990 (Protein A)	100	91
Immunoblot assay	19.5-kDa	Anuntagool et al., 1993	92	91
	39-kDa	Anuntagool et al., 1993	92	80
	Crude cell extract	Anuntagool et al., 1993	92	79
	Veronal extract	Anuntagool et al., 1993	87	79
ELISA	Whole cell Ag of *B. pseudomallei*	Kunakorn et al., 1990	88	92.2
		Vadivelu et al., 1995	91	96 (IgM)
	Affinity purified antigen	Dharukul et al., 1997	85.7	82.5 (IgG)
		Dharukul et al., 1997	63.5	81.8 (IgM)
	Immunoaffinity purified antigen	Sermswan et al., 2000	71.4	86.2 (IgG)
	Culture filtrate antigen	Chenthamarakshan et al., 2001	96	94 (IgG)
		Chenthamarakshan et al., 2001	74	99 (IgM)

Table 2.4. (Continued)

Methods	Antigen	References	Sensitivity	Specificity
ELISA	Recombinant truncated flagellin	Chen et al., 2003	93.8	96.3 (IgG)
	Crude LPS	Thepthai et al., 2005	96	100
	Affinity purified Ag	Chantratita et al., 2007	82	70
	Crude extract	Chantratita et al., 2007	81	70
	EPS	Chantratita et al., 2007	64	74
	LPS	Chantratita et al., 2007	66	81
	EPS + LPS	Chantratita et al., 2007	77	74
	BipB	Duar et al., 2008	68	61
	BipD	Duar et al., 2008	63	61
	BPSL0972	Allwood et al., 2008	95	98
	BipD	Allwood et al., 2008	42	100
	OmpA	Allwood et al., 2008	51	100
	EPS	Splettstoesser et al., 2008	83.3	98 (IgG)
	Recombinant flagellin from *B. thailandensis*	Wajanarogana et al., 2012	82.7	94.6
	TssD-5	Hara et al., 2013	71	96
	Omp3	Hara et al., 2013	59	90
	smBpF4	Hara et al., 2013	41	89
	Omp85	Hara et al., 2013	19	96
	Cocktails	Hara et al., 2013	65	99
	Multiple antigens	Hara et al., 2013	88.2	96
ICT	Culture filtrate antigen	Cuzzubbo et al., 2000 (IgG)	100	95
		Cuzzubbo et al., 2000 (IgM)	93	95
		O'Brien et al., 2004 (IgG)	77	90
		O'Brien et al., 2004 (IgM)	88	69
		Chuah et al., 2005 (IgG)	50.6	97.4
		Chuah et al., 2005(IgM)	72	71.5
		Cheng et al., 2006 (IgG)	86	47
		Cheng et al., 2006 (IgM)	83	47
		Splettstoesser et al., 2008 (IgG)	90	98.5
		Splettstoesser et al., 2008 (IgM)	84	99.5

NR- Not reported

IFAT uses whole-cell antigen labeled with fluorescent dye for the detection of total antibodies to *B. pseudomallei* and view under fluorescent microscope. Each test serum is serially diluted at various dilutions in PBS and cut-off values differ between laboratories. The diagnostic cut-off for Malaysia has been set at titer $\geq$ 1:10 for IgG and $\geq$1:40 for IgM (Vadivelu et al., 1995) while the threshold cut-off in India is $\geq$ 1:32 for both IgG and IgM antibodies (Mathai et al., 2003). As shown in Table 2.3, the reported sensitivities varied between 36% and 100% but specificities remained high at between 72% and 100% (Mathai et al., 2003; Vadivelu et al., 1995; Vadivelu et al., 2000). Interestingly, a modified IFAT using *B. thailandensis* as antigen was developed and this resulted in good sensitivity of 98.5% and satisfactory specificity of 92.3% (Puthucheary et al., 2010). However, the major drawback of both IFAT assays using either *B. pseudomallei* or *B. thailandensis* is subjective scoring test which is based on the judge decision of laboratory personnel.

An ELISA utilizing either native or recombinant antigens to capture antibodies (IgG/IgM or both) specific to *B. pseudomallei*, have included an affinity purified antigen, recombinant truncated flagellin, crude lipopolysaccharide (LPS), exopolysaccharide (EPS), BipB, BipD, BPSL0972, OmpA, TssD-5, Omp3, smBpF4 and Omp85 (Allwood et al., 2008; Chantratita et al., 2007; Chen et al., 2003; Chenthamarakshan et al., 2001; Duar et al., 2008; ; Hara et al., 2013; Sermswan et al., 2000; Splettstoesser et al., 2008; Thepthai et al., 2005; Wajanarogana et al., 2012). However sensitivities and specificities of these assays were highly variable as shown in Table 2.3. Thus far, an ICT (PanBio Ltd, Australia) is the only test that has been marketed (Cuzzubbo et al., 2000). The strip test used pre-coated culture filtrate antigen for the detection of IgG and IgM antibodies, and the results could be obtained in 10 minutes (Chuah et al., 2005; Cuzzubbo et al., 2000; Wuthiekanun et al., 2004). The test

showed varied performance of sensitivity (range 50.6% to 100%) and specificity (range 47% to 99.5%). Given the unsatisfactory performance, it was not further developed and the kit is not currently commercially available.

2.5.3 Molecular identification techniques

With the advances in molecular technology, various molecular detection platforms for *B. pseudomallei* have been developed, but few have been evaluated in the field. PCR-based diagnostic assays targeting 16S rRNA gene (Dharakul et al., 1996), 23S rRNA gene (Lew and Desmarchelier, 1994), 16S-23S rRNA intergenic region (Kunakorn et al., 1995), LPS (Rattanathongkom et al., 1997), and flagellin C *(fliC)* and ribosomal protein subunit S21 *rpsU* (Tomaso et al., 2005) have been evaluated for detecting *B. pseudomallei* DNA. Generally, these PCR assays demonstrated satisfactory sensitivities and specificities when tested on bacterial cultures however direct testing on blood specimens remain problematic.

Despite of the conventional end-point detection platform, a quantitative real-time PCR targeting various region such as *fliC* (Tomaso et al., 2005); TTS1-orf1 (Meumann et al., 2006; Supaprom et al., 2007), TTSS1-orf 2 (Kaestli et al, 2012), TTSS3-orf2 (Novak et al., 2006) also had been developed for specific and rapid identification of the bacterium. A high sensitivity with low limit of detection but also high specificity was documented from these reports. For instance, Supaprom and co-workers (2007) reported that their qPCR was able to detect the presence of *B. pseudomallei* at a low threshold of 50 femtogram of DNA and a specificity of 100%. Recently, another new method of real time PCR, BurkDiff was introduced by Bowers and co-workers (2010) and this test is targeted on single nucleotide polymorphisms that differentiate *B. pseudomallei* and *B. mallei.*

Generally, the high sensitivity of both conventional and real time PCR-based assays is due to the ability to utilize minute bacterial DNA to produce a high yield of the amplified targeted DNA and high specificity is based on the unique DNA sequence of the marker used. However, these methods are relatively expensive and not easily available in regions where melioidosis is endemic. Therefore, an inexpensive and easy system, loop-mediated isothermal DNA (LAMP) was developed wherein the gene amplification could be completed in 90 minutes under isothermal conditions employing a set of four specially designed primers, by incubating all the reagents in a single tube. The detection limit of LAMP was 38 genomic copies per reaction. Further work to improve the sensitivity of the test is required as the overall sensitivity of 30.2% was too low for detection of *B. pseudomallei* (Chantratita et al., 2008).

2.5.4 Commercial identification system

Many commercial systems are available for the identification of *B. pseudomallei*, which can be divided into two groups, manual and automated system. The manual systems include test panels of API 20E, API 20NE, API 50CH (bioMérieux, France), Microbact 24E (MetVet, Australia) and RapID NF Plus (Remel, USA). Lowe and colleagues (2002) reported that both API 20E and 20NE correctly identified 99% and 98% of 103 clinical isolates of *B. pseudomallei,* respectively. On the contrary, Inglis et al. (2005) demonstrated the poor perfomance of API 20NE which only identified 37% of 71 *B. pseudomallei* siolates obtained from a diverse range of sources. The reasons for the inconsistency in the results are not clear but API 20NE is still commonly used in diagnostic laboratories (Amornchai et al., 2007; Glass and Popovic, 2005; Inglis et al., 1998; Inglis et al., 2005; Lowe et al., 2002).

Comparison of clinical and soil isolates of *B. pseudomallei* was performed by Wuthiekanun et al. (1996) by using API 50CH. The most significant differences were that soil isolates (Ara+ isolates) assimilate L-arabinose (100%), adonitol (100%), 5-keto-gluconate (90%) and D-xylose (84%), but do not assimilate dulcitol (0%), erythritol (0%) and trehalose (10%). Another test, Microbact 24E was considered to be reliable for the identification of *B. pseudomallei* as it correctly identified 46 of 50 isolates, but the system has not yet been definitively evaluated. RapID NF plus system was found not suitable for the identification of *B. pseudomallei* as a preliminary report showed that it did not correctly identify any of the 58 *B. pseudomallei* isolates from diverse geographically areas (Glass and Popovic, 2005).

Various automated systems such as VITEK (bioMériuex), BD phoenix system (Becton Dickinson) and MicroScan WalkAway-96 (Dade Behring) have been used in microbiology laboratories to ensure that patient management is more cost effective. Lowe et al. (2002) reported a good result with the use of Vitek 1, which demonstrating 99% accuracy for 103 clinical isolates. However, the performance of Vitek 2, BD phoenix, and MicroScan WalkAway-96 systems have been shown to misidentify *B. pseudomallei* as *B. cepacia* (Kiratisin et al., 2007; Koh et al., 2003; Lowe et al., 2002; Weissert et al., 2009; Zong et al., 2012).

2.5.5 Medical imaging

As described in Section 2.2.6, melioidosis can involve any organ system and may present with varying clinical manifestations. Hence, medical imaging including X-ray, computerized axial tomography, ultrasound and magnetic resonance imaging are very helpful in confirming sites of infection and supporting decision making for

management, medical imaging is also important in assessing patients whose clinical condition deteriorates or do not respond to treatment (Ramsay, 2012).

2.6 Treatment and vaccine development

B. pseudomallei is characteristically resistant to a wide range of antimicrobial agents including penicillin, amino-penicillins, first-, second- and third-generation cephalosporins, most amino-glycosides, most macrolides and rifampicin whilst sensitive to newer β-lactam antibiotics, such as ceftazidime, imipenem, meropenem, piperacillin, amoxicillin-clavulanate, ceftriaxone, and cefotaxime (Cheng and Currie, 2005). Recently, however, response to ceftazidime treatment is slow due to the extensive pathology of melioidosis and the immunocomprised state of the host (Puthucheary and Sam, 2012b). Hence, imipenem or meropenem is considered as an alternative to ceftazidime.

The disease therefore can be treated with the use of appropriate medication. An initial intravenous therapy with ceftazidime or meropenem is given for 10-14 days followed by oral maintenance therapy for 12 to 20 weeks. Oral treatment is with three drug combination of doxycycline and trimethoprime-sulfamethoxazole (co-trimoxazole) (Peacock et al., 2008; Wiersinga et al., 2012). The maintenance therapy is important to prevent relapse or recurrence. For instance, Puthucheary and Vadivelu (2002) reported that the rate of relapse or recurrence in Malaysia was found to be approximately 13% over a period of 5 years, however, this is an underestimate due to cases being lost to follow-up.

Currently, there is no effective vaccine to protect against melioidosis but efforts to develop vaccine have been underway for many years. Mouse monoclonal antibodies

specific to the capsular polysaccharide, lipopolysaccharide, or proteins of *B. pseudomallei* were developed and have been shown able to passively protect against *B. pseudomallei* infection using a mouse model (Jones et al., 2002). Another DNA-based vaccine encoding the flagellin gene *fli*C has been tested with promising results (Chen et al., 2006). However, BipD has also been evaluated as a protein subunit vaccine but conferred no protection in a mouse model (Stevens et al., 2004). The development of mouse scFv antibodies against *B. pseudomallei* exotoxin (Nathan et al., 2002) and protease (Chan et al., 2004) had been published, but these antibodies have not been tested in animal models.

2.7 Shotgun expression library

A shotgun expression library is a genomic library that represents the entire genome of an organism. It is useful for analysis since whole genome of an organism is too large to be analysed directly. Basically, the genomic DNA of an organism is randomly digested into small pieces and then cloned into a suitable expression vector, for example pSCREEN-T in this study. The clones express a small peptide or protein and mapping can be performed by using an antibody of interest as the probe. Positive clones can be further analyzed by DNA sequencing to identify the amino acid sequence of interest (Novatope System, USA).

Chapter 3

General materials and methods

3.1 Introduction

All materials and methods used in this study are described in detail in Chapters 4 and 5; only those general methods and preparations are described below.

3.2 Materials

3.2.1 Bacterial strains and plasmids

Several local clinical isolates of *B. pseudomallei* were obtained from Department of Medical Microbiology, Faculty of Medicine, University of Malaya (UM), Kuala Lumpur. The details of strains and plasmids used in study are described in Chapters 4 and 5.

3.2.2 Biochemicals and other materials

All chemicals and reagents used were of Analar® grade or the highest grade available commercially, and obtained from various companies (Appendix I).

3.2.3 Buffers and other solutions

Buffers and solutions used were made as described in methods (Appendix II-VII) and obtained from Sambrook and Russell (2001), and Stiernagle (2006).

3.3 General methods

All methods used were referred or modified from the protocols as described by Sambrook and Russell (2001).

3.3.1 Sterilization

3.3.1.1 Steam sterilization

Media, solutions, buffers, universal bottles, and other apparatus were steam sterilized at 121ºC (15 psi) for 15 min in an autoclave (HVE-50, Hirayama, Japan). Micropipette tips, microcentrifuge tubes, canisters containing pipettes, bottles, and other apparatus were sterilized in the same manner and dried in an oven (Memmert, Germany) at 65ºC.

3.3.1.2 Membrane sterilization

Thermo-labile solutions were filter-sterilized via disposable sterile membrane filters with 0.22 µM (Millipore, USA).

3.3.2 Bacterial culture and maintenance conditions

In general, *B. pseudomallei* or *E. coli* cultures were grown overnight in LB broth at 37ºC. An appropriate antibiotic solution consisting of ampicillin (50 µg/mL), carbenicillin (50 µg/mL), chloramphenicol (100 µg/mL), gentamicin (100 µg/mL), streptomycin (50 µg/mL), and tetracycline (15 µg/mL) was added to LB broths when required. Sterile glycerol was added to the overnight culture to a final concentration of 20% (v/v) for storage at -80ºC for future work.

3.3.3 Polymerase chain reaction (PCR) amplification

All PCRs were carried out using appropriate primers, on a Mastercycler (ABI, USA). Generally, a master mix consisting of 10X LA PCR buffer II, 25 mM $MgCl_2$, 2.5 mM dNTPs mixtures, 5 U/µL LA *Taq* DNA polymerase (Takara, Japan), 20 pmol/µL upstream and downstream primers, were usually prepared and dispensed into individual 0.2 mL thin-walled tubes (Axygen, USA) which contained DNA templates and nucleases-free water, respectively. A negative control (using sdH_2O) was included in

every PCR assay to ensure there was no DNA contamination during reaction mixture preparation. The mixtures were subjected to PCR using an appropriate thermal cycling program for the different reactions. The common PCR program used was 96°C – 3 min (1 cycle); 96°C – 30 sec, 52°C – 15 sec, 72°C – 1 min (30 cycles); 72°C – 10 min (1 cycle). As a general rule, extension time of 1 min per 1 kb was used in the extension step.

3.3.4 PCR from a bacterial colony

Amplification of the target genes was carried out using the same protocol as described in Section 3.2.3 but using a crude DNA instead of purified DNA and different PCR reagents. Firstly, a colony from an agar plate was picked using a sterile 200 µL pipette tip and transferred to a 1.5 mL tube containing 100 µL LB broth with an appropriate antibiotic. The culture was then incubated for 3 h at 37°C with shaking at 150 rpm.

Next, 3 µL of culture was transferred to a 0.2 mL PCR tube and boiled in a heat block at 95°C for 5 min to lyse the cells and denature DNases. Colony PCR was then carried out in a volume of 20 µL containing 1X ThermoPol buffer (New England Biolabs, UK), 0.10 mM of each dNTPs, 1.00 mM $MgCl_2$, 20 pmol of each primers, 0.07 U of *Taq* DNA polymerase (New England Biolabs, UK), and 3 µL of crude DNA.

3.4 Concentration measurement

The concentration of extracted DNA or protein was determined by using a spectrophotometer (Implen, Germany).

3.4.1 Quantitation of genomic DNA

The LabelGuard^{TM} Microliter cell (Implen, Germany) was inserted into the cell holder with the cell windows facing the light beam. Three microliter of DNA sample was pipetted onto the centre of the measuring window.

An optical density (OD) ratio of 1 corresponds to approximately 50 µg of double-stranded DNA/mL. The DNA concentration was calculated with this following formula - A_{260} x 50 µg/mL x Dilution factor. The A_{260}/A_{280} ratio provides an estimate for the purity of the nucleic acid. While pure DNA preparation demonstrated A_{260}/A_{280} of 1.8, a range of 1.8 to 2 was acceptable.

3.4.2 Quantitation of protein sample

The estimation of protein concentration was performed by using quick Start^{TM} Bradford Protein Assay (Bio-Rad, USA) according to the manufacturer's protocol. Twenty microliter of sample was mixed with 1 mL of 1X dye reagent and incubated at room temperature for 5 min. Later, the mixture was transferred to a disposable polystyrene microcuvette and the OD was measured at 595 nm. The concentrations of unknown samples were assayed in duplicate and determined by comparison with the standard curve, working range of 0.125 to 2 mg/mL.

3.5 Electrophoresis

3.5.1 Agarose gel electrophoresis (AGE)

Based on the percentage of the gel needed, an appropriate amount of agarose powder (Promega, USA) was mixed with 1X TBE [90 mM Tris-borate, 1 mM EDTA (pH 8.0)] in a Schott bottle and dissolved by melting the agarose in a microwave oven (Sanyo, Japan) for at least 1 min. Ethidium bromide (0.5mg/mL) was then incorporated into the gel solution. The homogenous molten agarose was cooled down to about 60ºC before it was poured onto a gel tray. A comb was inserted at one side of the tray and the gel was allowed to be solidified. After that, the gel surface was flooded with 1X TBE and the comb was removed.

The PCR products, each mixed with an appropriate amount of 6X loading dye [0.25% (w/v) Bromophenol blue, 0.25% (w/v) Xylene cyanol, 50% (v/v) Glycerol, 1 mM Tris (pH 8.0)] mixture, were loaded into the sample wells of the gel. AGE was then carried out at room temperature at a constant voltage of 130 using a power pack PAC 300 (BioRad, U.S.A.). Upon completion of the AGE, the gel was viewed and photographed by using a MS Compact 2 Megapixel CCD Image System (Major Science, Taiwan).

3.5.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The glass plates were assembled vertically according to the instructions of the manufacturer (Bio-Rad, USA).

The glass plates were then cleaned thoroughly with deionized water and wiped with ethanol to ensure that the glass plates were free of grease spots in order to prevent air bubbles from forming in the gel. The recipes of 12% PAGE were as below:

Table 3.1. Recipes for separating and stacking gels.

Stock solution	Separating gel	Stacking gel
30% acrylamide:bisacrylamide (29:1) ready-mix gel solution	6 mL	650 µL
4X Tris.Cl/SDS	3 mL 750 µL (pH 8.8)	1 mL 250 µL (pH 6.8)
10% APS	5 mL 250 µL	3 mL 50 µL
TEMED	50 µL	25 µL
Distilled water	10 µL	5 µL

During preparation of the gel solution, APS and TEMED were added at the last steps and the mixture stirred gently to avoid the formation of air bubbles. APS is used as a catalyst for the copolymerization of acrylamide and bisacrylamide gels while TEMED, acts as a free-radical catalyst.

In order to prepare the gel, separating gel solution was dispersed quickly into the assembled gel cassette before the acrylamide polymerized. Even flow was maintained so that the solution was filled from the bottom without trapping air bubbles. After the gel solution reached the height around 6 cm of the plates, H_2O-saturated isobutyl alcohol was added slowly to cover the top of the gel. The gel was then allowed to polymerize for at least 45 min at room temperature.

Upon completion of polymerization, H_2O-saturated isobutyl alcohol was poured off and rinsed with 1X Tris.Cl/SDS buffer. Later, stacking gel solution (Table 3.1) was prepared and dispensed till it reached the top of the plates, and a clean comb was immediately inserted into the gel. The stacking gel was then allowed to polymerize for at least 45 min at room temperature. After that, the comb was gently removed. The gel cassette assemblies were removed from the casting stand and placed into the electrophoresis apparatus with the shorter plates facing inwards.

The inner chamber was filled with 1X SDS running buffer [25 mM Tris, 192 mM Tricine, 0.1% (w/v) SDS] until the level reached halfway between the tops of the taller and shorter glass plates of the gel cassettes. The Mini tank was also filled using the same buffer. After the samples were loaded into the respective wells, the SDS-PAGE was conducted using a constant voltage of 150. The power supply PAC 300 (BioRad, USA) was switched off when bromophenol blue dye migrated to the bottom of the gels. The gel was then stained with coomassie blue or used for western blot analysis.

3.5.3 Bio-Safe Coomassie Blue Staining

After separating protein samples by SDS-PAGE, the mini gels were rinsed 3 times with distilled water and stained with Bio-Safe Coomassie Blue (Bio-Rad, USA) for 30 min. Destaining of gels using distilled water was then performed until the stained protein bands could be clearly seen.

3.6 Purification methods

3.6.1 Isolation and purification of genomic DNA from bacterial culture

The bacterial genomic DNA was prepared by using Bacterial Genomic DNA Isolation Kit (Norgen Biotek, Canada) according to the manufacturer's protocol. One milliliter of overnight bacterial culture was transferred into a microcentrifuge tube and centrifuged at 14,000 x g for 30 sec. The supernatant was carefully removed and discarded as not to disturb the cell pellet. The pellet was then resuspended in 250 µL of Resuspension Solution by gently vortexing. To the content, 250 µL of the Lysis Solution and 12 µL of Proteinase K were added, mixed well and incubated at 55ºC for 30 min. Following that, 50 µL of Binding Solution was added to the lysate and mixed thoroughly to obtain a homogenous mixture. The mixture was passed through a column and centrifuged for 4 min at 5,200 x g. After that, 500 µL of Wash Solution I was added to the column to

remove impurities and centrifuged at 14,000 x g for 1 min. After the flow-through was discarded, 500 µL of Wash Solution II was added and the column was re-centrifuged at 14,000 x g for 2 min. An additional centrifugation at 14,000 x g for 1 min was performed in order to completely dry the column. In order to elute the DNA, the waste collection tube was replaced with an elution tube and 100 µL of Elution Buffer was added to the column. Centrifugation at 3,000 x g for 1 min was applied to allow for hydration of the DNA to occur. Finally, centrifugation at 14,000 x g for an additional 2 min was done and the eluted DNA was collected. The extracted DNA was subjected to spectrophotometric measurement and stored at -20ºC for further use.

3.6.2 Isolation and purification of plasmids

The HiYield^{TM} Plasmid Mini Kit (RBC, Taiwan) was used for plasmid isolation according to the manufacturer's protocol. The bacteria that carry the desired clone was inoculated into 3 mL of LB medium containing appropriate antibiotic and incubated overnight at 37ºC with vigorous shaking. The bacterial culture was harvested using centrifugation at 14,000 x g for 1 min. The supernatant was discarded and the pellet was resuspended in 200 µL of PD1 buffer containing RNase A by vortexing. Next, 200 µL of PD2 buffer was added to the suspension, gently mixed by inverting the tube 10 times and incubated at room temperature for 2 min until the lysate was clear. After that, 300 µL of PD3 buffer was added to the mixture and mixed immediately by inverting the tube 10 times, and centrifuged at 14,000 x g for 3 min. After centrifugation, the supernatant was transferred to a PD column in a collection tube. The flow-through was discarded after centrifugation at 6,000 x g for 30 sec. The column was washed by adding 400 µL of W1 Buffer and centrifuged at 6,000 x g for 30 sec. After that, the flow-through was removed and 600 µL of Wash Buffer was added, and the column was re-centrifuged at 6,000 x g for 30 sec. An additional centrifugation step at 14,000 x g

for 2 min was performed in order to dry the column completely. The column was placed in a clean microcentrifuge tube and 50 µL of water was added onto the center of the column, left for 2 min to allow hydration of the plasmid to occur. Finally, centrifugation at 14,000 x g for 2 min was done and the eluted plasmid was collected.

3.6.3 Isolation and purification of DNA bands from agarose gel

The QIAEX II Gel Extraction Kit (QIAGEN, Germany) was used for DNA fragment recovery according to the manufacturer's protocol. The expected DNA band was excised from the agarose gel and transferred to a microcentrifuge tube. Three volumes of Buffer QX1 were added to 1 volume of the gel (100 mg of gel ~ 300 µL of Buffer QX1), 10 µL of QIAEX II was then added to the mixture and incubated at 50ºC for 10 min. The mixture was mixed by vortexing every 2 min to keep QIAEX II in suspension. The sample was centrifuged at 14,000 x g for 30 sec and supernatant were carefully removed by using a micropipette. After that, 500 µL of Buffer QX1 was added to the pellet in order to remove agarose residuals and centrifuged at 14,000 x g for 30 sec. The pellet was then washed twice with 500 µL of Buffer PE to remove salt residuals and centrifuged at 14,000 x g for 30 sec. Next, the pellet was air-dried for 15 min and resuspended in 20 µL of H_2O. The suspension was incubated at room temperature for 5 min followed by centrifugation at 14,000 x g for 30 sec. The supernatant containing the purified DNA was transferred to a new microcentrifuge tube and stored at -20ºC for further use.

3.6.4 Isolation and purification of dsDNA products

The QIAquick PCR purification kit (QIAGEN, Germany) was used for recovery of dsDNA from enzymatic reaction according to the manufacturer's protocol. To 1 volume of sample, 5 volumes of Buffer PB was added and mixed by inverting the microcentrifuge tube. The mixture was transferred to a QIAquick spin column and centrifuged at 10,000 x g for 30 sec and the flow-through was carefully discarded. After that, 750 µL of Buffer PE was added to the spin column for washing purpose and centrifuged at 10,000 x g for 30 sec. After the flow-through was discarded, the spin column was re-centrifuged for 1 min to remove the wash buffer residual. The column was placed in a new microcentrifuge tube and 50 µL of water was added into the center of the column and left for 1 min. Finally, centrifugation at 10,000 x g for 1 min was carried out and elution was collected.

3.7 Cloning

3.7.1 Restriction enzyme digestion

Digestion of the amplified products was carried out in a final volume of 100 µL with each containing 20 to 40 U of appropriate restriction endonuclease (New England Biolabs, UK) and corresponding 1X of restriction buffer and then incubated at 37°C for 3 h. The digested products obtained were then purified using QIAEX II Gel Extraction Kit (QIAGEN, Germany) before continuing with next experiment.

3.7.2 Calf intestinal alkaline phosphatase (CIP) treatment

One microgram of the restricted digested vector was treated with 10 U of calf intestinal alkaline phosphatase (CIP) (New England Biolabs, UK) and 1X NEB buffer 3 [100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT] at 37°C for 1 h. The treated products were then purified using QIAquick PCR Purification Kit (Section 3.6.4) prior to the ligation process.

3.7.3 DNA ligation

A ligation reaction consisted of purified DNA to be cloned into purified restriction enzyme digested plasmid vector using a 3:1 or 5:1 molar ratio, 1 µL of T4 DNA ligase (New England Biolabs, UK), 1X ligation buffer [50 mM KCl, 10 mM Tris-HCl pH 7.4, 0.1 mM EDTA, 1 mM DTT and 50% (v/v) glycerol] and appropriate amount of distilled water to a final volume of 20 µL. The mixture was incubated overnight at 16°C followed by transformation into chemically-treated competent cells.

3.7.4 Preparation of chemically competent cells

A single bacterial colony was used to inoculate 3 mL of LB broth and incubated overnight at 37ºC with agitation at 200 rpm. On the following day, 200 µL of overnight culture was added to 20 mL prewarmed LB broth. This was incubated at 37ºC with agitation until an absorbance reading of 0.5 was reached. The bacterial culture was centrifuged at 3,850 x g for 10 min at 4ºC. The supernatant was then carefully removed and discarded into a container of Clorox bleach. Later, 8 mL of ice-cold $CaCl_2$ was added to the cell pellet, followed by incubation on ice for 1 h. Centrifugation was performed once again, and pellets were resuspended in 1 mL ice-cold $CaCl_2$ and 10% glycerol. The mixture was aliquoted (100 µL each) into labeled 1.5 mL microcentrifuge tubes and kept at -80ºC.

3.7.5 Heat shock transformation

Two microliter of ligation reaction was added to 50 µL competent cells and mixed by gentle stirring with the pipette tip. Following that, the reaction was incubated on ice for 30 min, then heat-shocked at 42ºC for 1 min followed by 2 min on ice. To each transformation, 1 mL of prewarmed LB broth (with no antibiotic added) was added and incubated at 37ºC for 1 h to allow the recovery of the bacteria and the expression of the antibiotic resistance genes. Transformed cells were then collected by centrifugation at 13,000 x g for 1 min and resuspended in 200 µL LB broth. One hundred microliter of the bacterial suspension was plated onto LB agar plates supplemented with appropriate antibiotic and grown at 37ºC overnight.

3.8 Sequencing

All the samples to be sequenced in this study were sent to 1st Base Laboratories (Malaysia) and ABI 3730xl automated sequencer was used for DNA sequencing.

Chapter 4

Characterization of putative virulence determinants in *B. pseudomallei*

4.1 Introduction

B. pseudomallei has been considered as a potential bioweapon and virulence factors which associated with the bacterial pathogenesis are being intensively studied at an increasing rate. The availability of a complete genome sequence database of the organism further allows researchers to discover many molecules and mechanisms that may be involved in the virulence of the organism (Holden et al., 2004; Tuanyok et al., 2008) via comparative analysis approach either between species or within isolates.

Past studies have focused on genomic differences between species, i.e., the virulent *B. pseudomallei* and a closely related but avirulent family member *B. thailandensis* (Reckseidler et al., 2001; Yu et al., 2006). On the contrary, PCR-based subtractive hybridization was recently undertaken in our laboratory using a virulent clinical isolate *B. pseudomallei (v)* and an attenuated strain of the same *B. pseudomallei* isolate *(av)* (Puthucheary et al., 2012a).

The virulent strain of *B. pseudomallei* (*v*) was isolated from the blood culture of a 44 year old male diabetic patient in 1994, who subsequently died after developing the infection. This virulent strain was used for antigen preparation for the purpose of in-house serological tests. However, in 2003, the freshly prepared antigen failed to give a positive reaction and the organism also did not agglutinate with our in-house polyvalent

antiserum. The loss of virulence in this strain was further confirmed by challenge experiment using Balb/C mice. The survival rate of mice, in days, was twice longer compared to a virulent strain. Henceforth this strain is referred to as the "attenuated" *B. pseudomallei* (*av*) strain. PCR-based subtractive hybridization demonstrated 6 subtracted DNA fragments that were unique to the virulent strain of *B. pseudomallei* (*v*), whereas these DNA fragments were not seen in its "attenuated" strain (a*v*) as illustrated in Figure 4.1 (Puthucheary et al., 2012a). Subtracted DNA fragments were sequenced and revealed 6 unique genes with unknown functions as stated in Table 4.1. However much is still unknown regarding the bacterial factors contributing to the virulence.

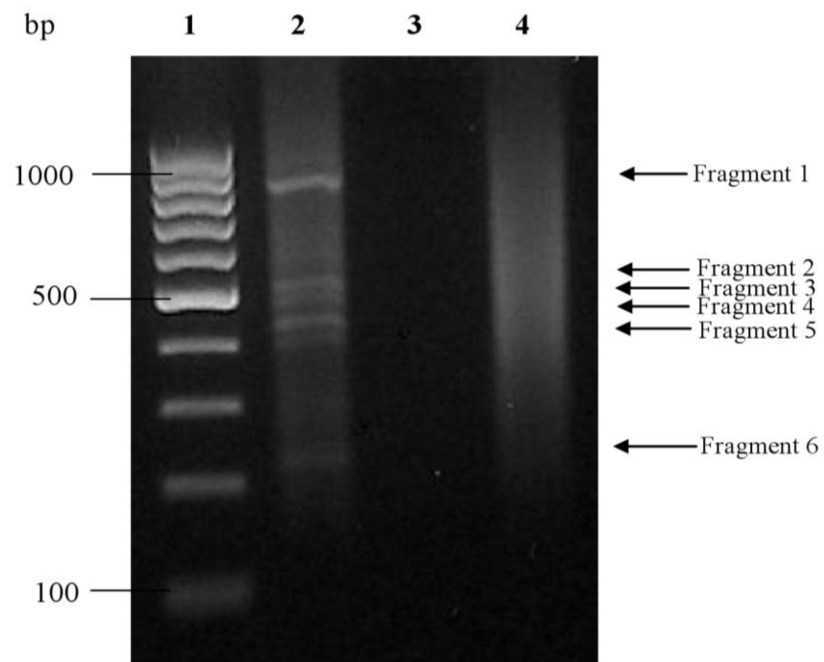


Figure 4.1. Subtractive hybridization of virulent (v) and attenuated (av) *B. pseudomallei* strains

Lane (s) 1 – 100bp DNA ladder (Fermentas, USA)

2 – virulent *B. pseudomallei (v)* hybridized with attenuated *B. pseudomallei (av)*

3 – *B. pseudomallei (av)* hybridized with virulent *B. pseudomallei (v)*

4 – control (unsubstracted genomic DNA from virulent *B. pseudomallei (v)*

(Puthucheary et al., 2012a)

Table 4.1. Characterization of 7 candidate genes for their roles in *B. pseudomallei* infection.

No	Locus tag	Nucleotide sequence	Amino acid	Location	Annotation (RefSeq: NCBI)	Orthologs/ comparative genomics	References
1	Fragment 2: *BPSL2033*	1287	428	Chr I: 2428723..2430009	YP_108630.1	BG003741	Current study
2	Fragment 3: *BP1026B_I2784*	927	308	Chr I: complement 3091068..3091994	NC_017831.1	-	
3	Fragment 4: *BP1026B_I2780*	870	289	Chr I: 3085731..3086600	NC_017831.1	BG015169	
4	Fragment 5: *BURPS1106A_A0094*	636	211	Chr II: complement 82340..82975	YP_001074137.1	BG005228	
5	Fragment 6: *BURPS1106A_1131*	342	113	Chr I: complement (1112960..1113301)	YP_001065411.1	BG011052	
6	Fragment 1: *BURPS1710A_1419*	225	74	Chr I: 1354038-1354262	NZ_CM000832.1	BG013631	
7	*BPSL3147*	1011	336	Chr I: complement 3746560..3747570	YP_109740.1	BG000325	Cuccui et al., 2007

Besides, another candidate *BPSL3147* encoding a putative lipoprotein was identified by signature-tagged mutagenesis (STM) (Cuccui et al., 2007). Interestingly, this putative lipoprotein contains 39.16% amino acid sequence that is identical to a *VacJ* lipoprotein in *Shigella flexneri*. The Tn10 mutant of *S. flexneri* YSH6000T *VacJ* lipoprotein was found unable to spread from cell to cell, suggesting *VacJ* is important for intercellular spread of the organism (Suzuki et al., 1994). There remains much opportunity for further investigation of *BPSL3147* contributing to the pathogenesis of *B. pseudomallei* infection.

Hence, the aim of this study was to characterize the 7 putative virulence determinants using a gene knockout approach, *in vitro* and *in vivo* assays. Six of these genes were absent in the "attenuated" strain (*av*) that is believed to have reduced virulence after several subcultures and long-term storage in the laboratory; and an additional candidate, *BPSL3147*, was chosen as it might be important for cell to cell spreading of the bacterium.

The specific objectives for the work in this chapter are:

1. to construct 7 insertion mutant strains of *B. pseudomallei.*
2. to construct complemented strains for the 7 insertion mutants.
3. to examine the survival of these mutants as well as complemented strains in murine RAW246.7 macrophage cell line.
4. to investigate the possible virulence of these mutants as well as complemented strains using *C. elegans* model.

4.2 Materials and methods

4.2.1 Bacterial strains, plasmids and culture conditions

The virulent *B. pseudomallei* strain was isolated from patient CMS, at the University Hospital, University of Malaya (UM), who died from melioidosis (Puthucheary et al., 2012a) and this strain was used throughout the study (henceforth referred to as Bp-CMS). The *Escherichia coli* strains DH10B, CC118λpir, S17-1λpir and vector pUT-Km were kind gifts from Prof. Dr. Wang Jin-Town (National Taiwan University, Taiwan).

The *E. coli* DH10B was mainly used for propagation of CC118λpir plasmid and maintaining the delivery plasmids; S17-1λpir which was used as the donor strain in conjugation experiments to allow mobilization of the λpir-dependent plasmid pUT-Km into *B. pseudomallei* CMS (Table 4.1). All strains were grown in LB broth at 37°C overnight with 150 rpm agitation. Antibiotics were used at the following final concentrations: ampicillin 100 µg/mL; kanamycin 50 µg/mL; chloramphenicol 100 µg/mL and streptomycin 100 µg/mL.

4.2.2 Cell lines and culture conditions

RAW264.7, the mouse leukaemic monocyte macrophage cell line was obtained from American Type Culture Collection (USA). It was cultured and maintained in 25 or 75 cm^2 flask (Corning, USA) with DMEM (Gifco, USA) supplemented with 10% (v/v) fetal bovine serum (Gifco, USA), 4 mM L-glutamine and an antibiotic mixture containing 100 U/mL penicillin and 0.1 mg/mL streptomycin at 37°C in 5% CO_2 atmosphere.

4.2.3 Nematode strain and culture conditions

The wild type *C. elegans* N2 was obtained from Carolina Biological Supply Company (USA). The nematode was propagated on nematode growth medium (NGM) plate and fed on the normal food source *E. coli* OP50-1, which was a kind gift from Prof. Dr. Sheila Nathan (National University of Malaysia, Malaysia).

Table 4.2. Bacterial strains and plasmids used in this study.

No	Strains/plasmids	Relevant characteristic(s)	Reference
B. pseudomallei **strains**			
1	Bp-CMS	Clinical isolate from blood; Wild type parental strain for generation of insertion mutants	Current study
2	*BPSL2033*::Km	*BPSL2033*::Km derivative of CMS; Kan^R	
3	*BPSL2033*::Km (pC-*BPSL2033*)	*BPSL2033*::Km complemented in trans with pC-*BPSL2033*; Kan^R Cm^R	
4	*BP1026B_I2784*::Km	*BP1026B_I2784*::Km derivative of CMS; Kan^R	
5	*BP1026B_I2784*::Km (*pC-P1026B_I2784)*	*BP1026B_I2784*::Km complemented in trans with pC-*BP1026B_I2784*; Kan^R Cm^R	
6	*BP1026B_I2780*::Km	*BP1026B_I2780*::Km derivative of CMS; Kan^R	
7	*BP1026B_I2780*::Km (*pC-BP1026B_I2780*)	*BP1026B_I2780*::Km complemented in trans with pC-*BP1026B_I2780*; Kan^R Cm^R	
8	*BURPS1106A_A0094*::Km	*BURPS1106A_A0094*:: Km derivative of CMS; Kan^R	
9	*BURPS1106A_A0094*::Km (pC-*BURPS1106A_A0094*)	*BURPS1106A_A0094*::Km complemented in trans with pC-*BURPS1106A_A0094*; Kan^R Cm^R	
10	*BURPS1106A_1131*::Km	*BURPS 1106A_1131*::Km derivative of CMS; Kan^R	
11	*BURPS1106A_1131*::Km (pC-*BURPS1106A_1131*)	*BURPS 1106A_1131*::Km complemented in trans with pC- *BURPS1710A_1419*; Kan^R Cm^R	
12	*BURPS1710A_1419*::Km	*BURPS1710A_1419*::Km derivative of CMS; Kan^R	
13	*BURPS1710A_1419*::Km (pC-*BURPS1710A_1419*)	*BURPS1710A_1419*::Km complemented in trans with pC-*BURPS1710A_1419*; Kan^R Cm^R	
15	*BPSL3147*::Km	*BPSL3147*::Km derivative of CMS; Kan^R	
16	*BPSL3147*::Km (pC-*BPSL3147*)	*BPSL3147*::Km complemented in trans with pC-*BPSL314*7; Kan^R Cm^R	

Table 4.2. (Continued)

No	Strains/plasmids	Relevant characteristic(s)	Reference
***E. coli* strains**			
1	DH10B	A general cloning strain; F_ *mcrA* D*(mrr-hsdRMS-mcrBC)* f80*lacZ*D*M15* D*lacX74 recA1 endA1 araD139* D*(ara, leu)7697 galU galK* l_ *rpsL nupG*	Gifts from Prof. Dr. Wang Jin-Town, National Taiwan University, Taiwan.
2	CC118λpir	Strain used for maintenance of suicide plasmid; D*(ara-leu) araD* D*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE(Am) recA1* l*pir* phage lysogen	
3	S17-1λpir	Strain used for conjugational transfer of suicide plasmid from *E.coli* to *B. pseudomallei*; hsdR recA pro RP4-2 (Tc::Mu; Km::Tn7)(lλpir)	
4	OP50-1	A streptomycin-resistant derivative of *E.coli* OP50 used as food source in *C. elegans* cultivation and uninfected control	Gift from Prof. Dr. Sheila Nathan, National University Malaysia, Malaysia.

Table 4.2. (Continued)

No	*Strains/plasmids*	Relevant characteristic(s)	Reference
Plasmid			
1	pUT-Km	Source of kanamycin resistance cassette; *ori*R6K *mob*RP4 Kan^R Amp^R	(Chuang et al., 2006)
2	pGEM-T easy	Cloning vector for PCR cloning; Amp^R	Promega, USA
3	pUT-*BURPS1710A_1419*	pUT-Km carrying 196 bp amplified *BURPS1710A_1419* DNA fragment from Bp-CMS	Current study
4	pUT-*BPSL2033*	pUT-Km carrying 500 bp amplified *BPSL2033* DNA fragment from Bp-CMS	Current study
5	pUT-*BP1026B_I2784*	pUT-Km carrying 346 bp amplified *BP1026B_I2784* DNA fragment from Bp-CMS	Current study
6	pUT-*BP1026B_I2780*	pUT-Km carrying 323 bp amplified *BP1026B_I2780* DNA fragment from Bp-CMS	Current study
7	pUT-*BURPS1106A_A0094*	pUT-Km carrying 205 bp amplified *BURPS1106A_A0094* DNA fragment from Bp-CMS	Current study
8	pUT-*BURPS1106A_1131*	pUT-Km carrying 247 bp amplified *BURPS1106A_1131* DNA fragment from Bp-CMS	Current study
9	pUT-*BPSL3147*	pUT-Km carrying 492 bp amplified *BPSL3147* DNA fragment from Bp-CMS	Current study
10	pC-*BPSL2033*	A modified pGEM-T carrying 1703 bp *BPSL2033* PCR product containing *BPSL2033* promoter, Cm^R	Current study
11	pC-*BP1026B_I2784*	A modified pGEM-T carrying 1213 bp *BP1026B_I2784* PCR product containing *BP1026B_I2784* promoter, Cm^R	Current study

Table 4.2. (Continued)

No	*Strains/plasmids*	Relevant characteristic(s)	Reference
Plasmid			
12	pC-*BP1026B_I2780*	A modified pGEM-T carrying 1395 bp *BP1026B_I2780* PCR product containing *BP1026B_I2780* promoter, Cm^R	Current study
13	pC-*BURPS1106A_A0094*	A modified pGEM-T carrying 1119 bp *BURPS1106A_A0094* PCR product containing *BURPS1106A_A0094* promoter, Cm^R	Current study
14	pC-*BURPS1106A_1131*	A modified pGEM-T carrying 861 bp *BURPS1106A_1131* PCR product containing *BURPS1106A_1131* promoter, Cm^R	Current study
15	pC-*BPSL3147*	A modified pGEM-T carrying 1705 bp *BPSL3147* PCR product containing *BPSL3147* promoter, Cm^R	Current study
16	pC-*BURPS1710A_1419*	A modified pGEM-T carrying 699 bp *BURPS1710A_1419* PCR product containing *BURPS1710A_1419* promoter, Cm^R	Current study

Kan – kanamycin; Amp - ampicillin; Cm - chloramphenicol; R - resistant

4.2.4 Insertion mutagenesis

4.2.4.1 Primers designation

Primer pairs designed to amplify particular regions of target genes are shown below:

Table 4.3 Primers used for PCR and construction of mutants and complemented plasmids in this study.

Name	Primer sequence (5' ⟶ 3')	Fragment size (bp)
P1 (pUT-R)	TTTGAGTGACACAGGAACAC	-
P2 (KmF)	ATGAGCCATATTCAACGGGA	-
P3 P4	AGAACTTCGAGCAATTGCTG GAGAGATGACGTTCGGTCTT	500
P5 P6	GTCGAGAGTACGGTGTGTTC CCTGCGAAATCCTTATCAC	346
P7 P8	AAACCAGAAGGGCGATTTC GCGTTCTTTTAAGAATTGGGTAG	323
P9 P10	AAGGAGTTGGGTACGTCG CCCTTGTGCCATTGATAG	247
P11 P12	GCGATAGCGATTGGAAAACT GAATCCAGACCCATTCCGT	196
P13 P14	GACCAGTACGCGCTCAAG GAACGAATACTTGTCGATCG	492
P15 P16	GTGAACTGGTACAAAGAAATATCG CACGTTTCTCGGATAGAGC	6256
P17 P18	CTGTTTTCTAAGCGTCAGAAG AAATATGCAGGAAATAGCCCG	5771
P19 P20	CAGGTACGATTCATGGAACG GGTATTCCGTGACCTGAATGT	5648
P21	ACGCATGCTCAAGAAATG	-
P22 P23	GTGAATGTCGATCTTGCG TCAATCTCCAGCGAGCTT	5541

Table 4.3 (Continued)

Name	Primer sequence (5’ ⟶ 3’)	Fragment size (bp)
P24 P25	GATGTACACGTTCAACGACAAG CTCTTCCGGCATCTCGTA	5751
P26 P27	CTAGAGCGCGGCCTCGCG CATCACTCGGCGCAATGAGACTG	1703
P28 P29	CCTTCGCGCTGATTTGGT CTACTTCGTAGCTTGATGCGCC	1213
P30 P31	AGGCTCGGAGTAGTAACACTT CTACTGCCTATGCTGGGGTAT	1395
P32 P33	TTCTACCAGCGACTTGGC TCAATCTCCAGCGAGCTT	1119
P34 P35	GTGGTTCAGCCAGGCACG GTATGTGTCGTCCGCATTTG	861
P36 P37	TGCACAAGCTGTTCAAATG TTAAGGCTTGGGTGCAAG	695
P38 P39	TTCAGGGTTACGAAGCGAAG TCAGTGCAGCCGGATGCTCG	1705

4.2.4.2 Construction of suicide plasmids for conjugation

The strategy to construct all 7 plasmids targeting genes *BPSL2033, BP1026B_I2784, BP1026B_I2780, BURPS1106A_A0094, BURPS1106A_1131, BURPS1710A_1419* and *BPSL3147* for conjugation purpose is outlined in Figure 4.2.

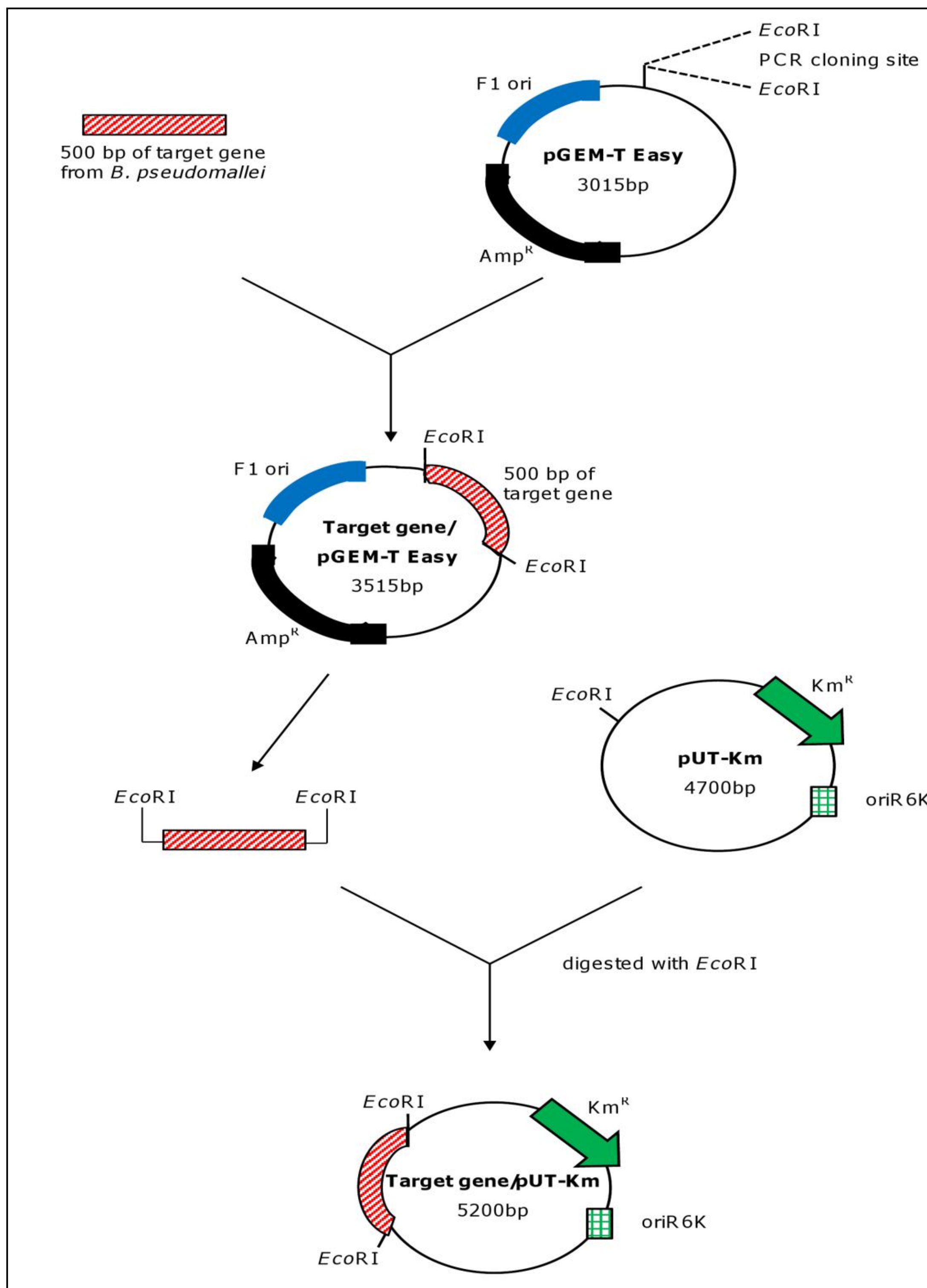


Figure 4.2. Construction of plasmids for conjugation.

A partial region of the gene to be inactivated (serving as a significant site of gene exchange through conjugation and recombinant events) was amplified by PCR (Section 3.3.3) using primer pairs as listed in Table 4.3. Two microliter of the amplicon was subcloned into pGEM-T Easy (Promega, USA) and then transformed into 100 µL of competent *E. coli* DH10B cells (Section 3.7.4). Selection of transformants by plating onto LB agar containing 50 µg/mL ampicillin/X-Gal/IPTG and rapid screening for desired inserts by colony PCR was performed (Section 3.3.4).

The transformed *E. coli* carrying the desired insert (positive clone) was inoculated into 3 mL of LB broth with 100 µg/mL of ampicillin and incubated overnight at 37ºC with agitation at 150 rpm. The next day, DNA plasmid was isolated as described previously (Section 3.6.2) and subjected to *Eco*RI digestion (Section 3.7.1). The digested products were then gel purified and ligated into *EcoR*I–restricted, calf intestinal phosphatase treated pUT-Km vector (Section 3.7.2). The pUT-Km is a suicide vector having the R6K origin of replication and can be maintained only in host strains producing particular protein, i.e., CC118λpir. Therefore, 2 µL of ligation products were transformed into 100 µL chemically competent *E. coli* CC118λpir and followed by transformant selection by plating the bacteria onto LB agar plates containing 50 µg/mL kanamycin. Positive clones with correct orientation were confirmed by colony PCR (Section 3.3.4).

4.2.4.3 Filter conjugation

The resulting pUT-Km plasmids containing the flanking regions of gene of interest of *B. pseudomallei* are suicide vectors and they cannot replicate in the recipient cells which do not have the π protein. Hence, 2 μL of each recombinant plasmid was transformed into 100 μL of *E. coli* S17-1λpir and used for chromosomal integration into the wild type *B. pseudomallei* (Bp-CMS) by conjugation.

A single colony of donor (*E. coli* S17-1λpir harboring suicide plasmid) and recipient (wild type Bp-CMS) were separately grown in 3 mL of LB broth with 50 μg/mL kanamycin and without antibiotic, respectively. The culture was then incubated overnight at 37°C with agitation at 150 rpm. The next day, to the 3 mL of LB broth, 30 μL of the overnight culture was inoculated and incubated for 4 h at 37°C with shaking at 150 rpm until the OD600 reached 0.6 - 0.8. An equal amount of donor and recipient were mixed together into 2 mL of 10 mM $MgSO_4$. The mixture was spotted on 0.2 μM sterile mixed cellulose ester membrane (Fioroni, France). The filter paper was transferred onto LB agar without antibiotic and incubated overnight for mating purpose at 37°C.

After that, filter paper was transferred into a sterile 50 mL falcon tube containing 5 mL of 10 mM $MgSO_4$. The bacterial lawn on each filter was then harvested after 3 min of vigorous vortexing. Serial dilutions of the culture were plated on minimal medium supplemented with 50 μg/mL kanamycin to counter select donors and untransformed recipient cells and these plates were incubated overnight at 37°C.

As illustrated in Figure 4.3, transconjugants with pUT-Km vector carrying a partial homology DNA sequence integrated into the target gene on wild type *B. pseudomallei*

chromosome via single cross recombination were confirmed for their insertion by PCR using alignment specific primers and sequencing. The primers pUT-R and KmF are located in pUT-Km plasmid that annealed only to the mutated sequences when paired with corresponding chromosomal primers. In other words, primers pUT-R and KmF were used to detect the presence or absence of the pUT-Km vector sequence in the *B. pseudomallei* choromosome. This suggests that integration of pUT-Km plasmid has taken place in the intended location on the *B. pseudomallei* chromosome.

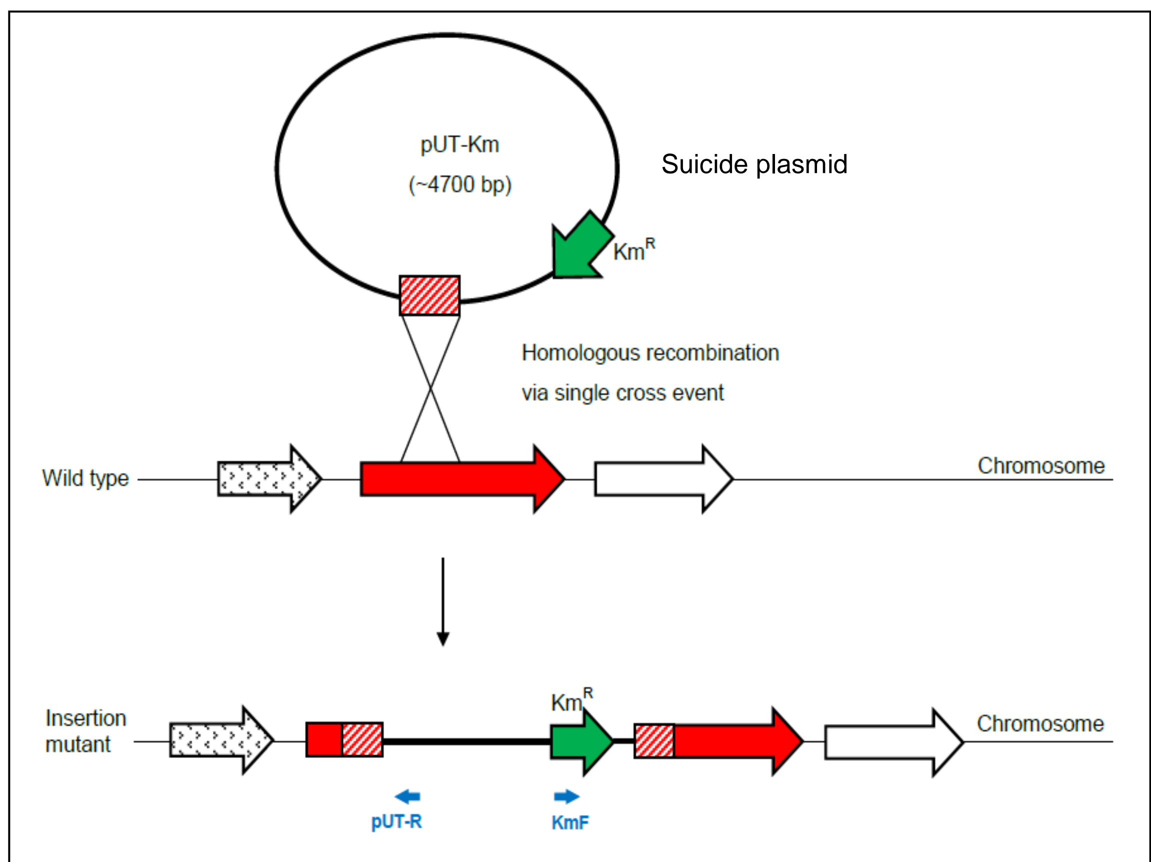


Figure 4.3. Construction of the insertion mutants in Bp-CMS.

4.2.5 Construction of complemented plasmids for mutant strains

Kanamycin was used to select for an insertion mutant as described in the previous section (Section 4.2.4.3). To construct a complemented plasmid, a second antibiotic resistance marker such as chloramphenicol was chosen. A chloramphenicol acetyltransferase (CAT) cassette with approximately size of 900 bp (a gift from Prof. Dr. Wang Jin-Town, National Taiwan University, Taiwan) was inserted into a pGEM-T Easy at *Nco*I site.

Next, the intact *BURPS1710A_1419, BPSL2033, BP1026B_I2784, BP1026B_I2780, BURPS1106A_A0094, BURPS1106A_1131*, and *BPSL3147* genes and their promoter were amplified from the genomic DNA of Bp-CMS by using primer pairs as shown in Table 4.3. Their promoters were identified using Softberry (BPROM) software. Two microliter of amplicon was cloned into a modified pGEM-T easy vector, which contains CAT, followed by transforming into 100 µL of *E. coli* DH10B. Positive transformants were selected by plating onto LB agar containing 100 µg/mL of chloramphenicol.

The confirmation of the positive clones and their insert orientation was then performed by colony PCR using each forward primer paired with a T7 primer (Section 3.3.4). A simplified diagram to describe the location of CAT and PCR fragment at pGEM-T easy is shown in Figure 4.4.

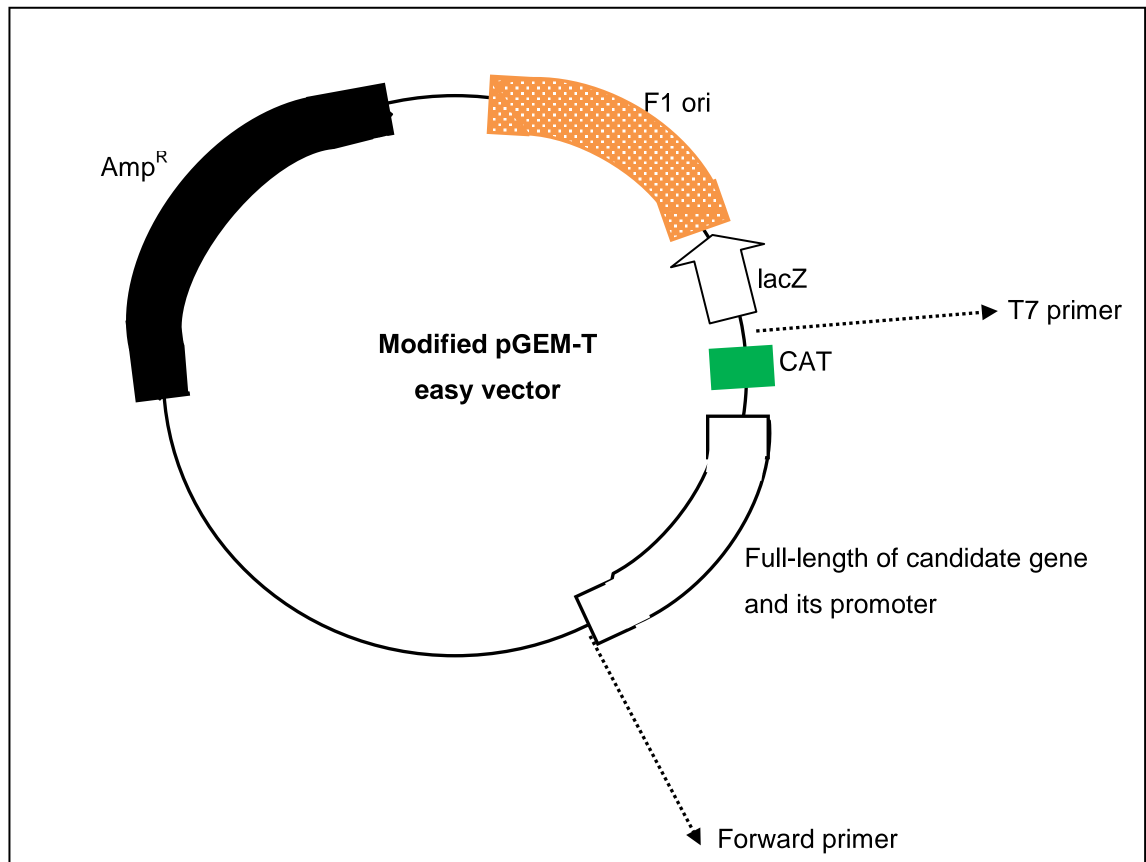


Figure 4.4. Construction of a complemented plasmid.

4.2.6 Bacterial growth curves

A single bacterial colony was inoculated into 3 mL of LB broth with or without antibiotic and incubated overnight at 37°C with agitation at 150 rpm. The next day, 200 µL of an overnight culture was added into 20 mL of prewarmed LB broth containing the appropriate antibiotics and incubated 37°C with agitation at 150 rpm. The growth was monitored over 8 h and 1 mL of culture broth was taken every hour to perform OD600 readings. *B. pseudomallei* wild type Bp-CMS was used as a positive control.

4.2.7 Replication of bacteria in macrophage cells

Mouse leukaemic monocyte macrophage cells (RAW264.7) were cultured in DMEM containing antibiotic to confluence, scraped from cell culture flasks, and transferred into 24 well culture plates (Corning, USA) at a density of 1 X 10^5 cells/well and incubated overnight at 37°C with 5% CO_2. A single colony of wild-type, insertion mutant and complemented strains were incubated overnight in 3 mL LB with or without antibiotics at 37°C.

On the following day, an overnight culture was diluted 1:100 and inoculated into a fresh LB followed by incubation at 37°C for 3 h with shaking at 250 rpm until the OD600 reached 0.4 to 0.6. The bacterial culture was transferred to a microcentrifuge tube and was spun briefly at 13,000 x g for 5 min. The pellet was washed once with pre-warmed DMEM without antibiotic and resuspended in 1 mL of DMEM without antibiotic. The bacterial suspension was adjusted to 1 X 10^7 cfu/mL in DMEM without antibiotic by measuring the OD600 using a spectrophotometer (Implen, Germany). Cell monolayers were washed twice with PBS and incubated in fresh DMEM without antibiotic for 1 h prior to infection with bacteria.

Twenty five microliter of bacterial suspension was added at a MOI of 100:1 and the co-culture was immediately centrifuged at room temperature with 170 x g for 5 min to bring the bacteria in direct contact with the host cells. Tissue culture plates were incubated at 37°C for 1 h to allow for the uptake of the bacteria by the cell. After that, cells were washed twice with PBS and then incubated in fresh DMEM containing 300 μg/mL of tetracycline to suppress the growth of residual extracellular bacteria. This point was taken as time zero, and cells were further incubated for 2, 4 and 8 h.

At each time point, infected monolayers were washed twice with PBS and lysed with 150 μL of 0.1% (v/v) Triton X-100 for 15 min. Serial dilutions of the released bacteria (expressed as colony forming unit, CFU) were plated on Tryptic Soy Agar (TSA) plates to enumerate bacterial loads by direct colony counts. The number of internalized bacteria obtained at 2 h post infection represented the initial entry of bacteria whereas 4 and 8 h post infection represented intracellular bacterial replication. Bacterial survival was normalized to counts obtained at 2 h post infection and the data presented as relative survival in percentage. The assays were performed in triplicate for each (2, 4, and 8 h) post infection and this process was repeated 3 times on different days.

4.2.8 *Caenorhabditis elegans* killing assay

4.2.8.1 Worm synchronization

All nematodes were age synchronized by bleaching procedure prior to the killing assay (Powell and Ausubel, 2008; Stiernagle, 1999). Gravid hermaphrodites were harvested from a Nematode Growth Medium (NGM) plate by washing with 1 mL M9 buffer across the plate several times to loosen worms and eggs that were stuck in the *E. coli* OP50-1 lawn. The suspension was aseptically collected into a 15 mL falcon tube and M9 buffer was added to total 3.5 mL. To the suspension, 0.5 mL of 5N NaOH and 1 mL of 5% bleach were added. The mixture was mixed by shaking the tubes for about 5 to 10 min until the mixture became clear and no worms visible, and then spun for 30 sec at 1,500 x g to collect the released eggs. The supernatant was carefully removed and 5 mL M9 buffer was added to wash the eggs. This washing step was repeated twice to remove all bleach solution in order to make sure the eggs would survive. After 2 washes, the eggs were resuspended in 5 mL of M9 buffer and allowed to hatch overnight at room temperature.

Next day, the hatched L1 larvae in the falcon tube were collected by centrifugation for 30 sec at 1,500 x g. The supernatant was discarded leaving behind the pellet in the remaining 100 µL of liquid and transferred to NGM plates seeded with an *E. coli* OP50-1 lawn. The plates were incubated for 2 days at room temperature until maturation. The killing assay was begun with L4 or young adult stage nematodes.

4.2.8.2 Slow killing assay

A single colony of *B. pseudomallei* derived-strains (wild type Bp-CMS, 7 insertion mutants and 7 complemented strains) and *E. coli* OP50-1 were inoculated into 3 mL of LB broth with or without antibiotic and incubated at 37°C overnight with agitation at 150 rpm. The overnight culture was then standardized to an OD600 of 2.0 and 40 µL of culture was plated on a modified NGM containing 50 µg/mL 5-fluorodeoxyuridine (FUDR) (Merck, USA) to inhibit the eggs from hatching. Plates were incubated at 37°C for 24 h and then allowed to equilibrate to room temperature for 24 h before co-culturing with the host worms.

The worms were washed with M9 buffer and pelleted by centrifugation for 30 sec at 1,500 x g. The same procedure was repeated 3 times to get rid of contaminating *E. coli* OP50-1 bacteria, and the worms were then transferred to killing agar plates. In each assay, a total of 30 age-matched hermaphrodites were individually transferred to freshly lawned plates by using the flattened tip of a worm pick (platinum wire). The plates were incubated at room temperature and virulence was tracked by counting the number of live and dead worms every 24 h for 3 days. A worm was considered dead on failure to respond to gentle touch by worm pick and any worms that died as a result of getting stuck to the wall of the plate were censored from the analysis. *E. coli* OP50-1 was used as a negative control. Three independent experiments were carried out for each strain, and each test was performed in triplicates (with a total of 270 worms) to yield consistent results.

4.2.9 Statistical analysis

For macrophage infection assay, percentage of survival was calculated using the formula as shown below and a graph was plotted using an excel database. Student's t-test was used to determine if there was statistical significance (P<0.05) of the mean value.

$$\text{Relative survival} = \frac{\text{number of intracellular bacteria post infection}}{\text{number of initial entry of bacteria}} \times 100$$

For killing assay, worm survival was analyzed using GraphPad Prism 5 software (GraphPad Software Inc, la Jolla, CA) and a Kaplan-Meier survival plot was constructed. The differences of the curves were compared using a log rank test, which generates a P value testing the null hypothesis that the survival curves are identical. Survival curves are considered significantly different from the control when P< 0.05.

The flow chart for the entire experimental procedures in this study is shown in Figure 4.5.

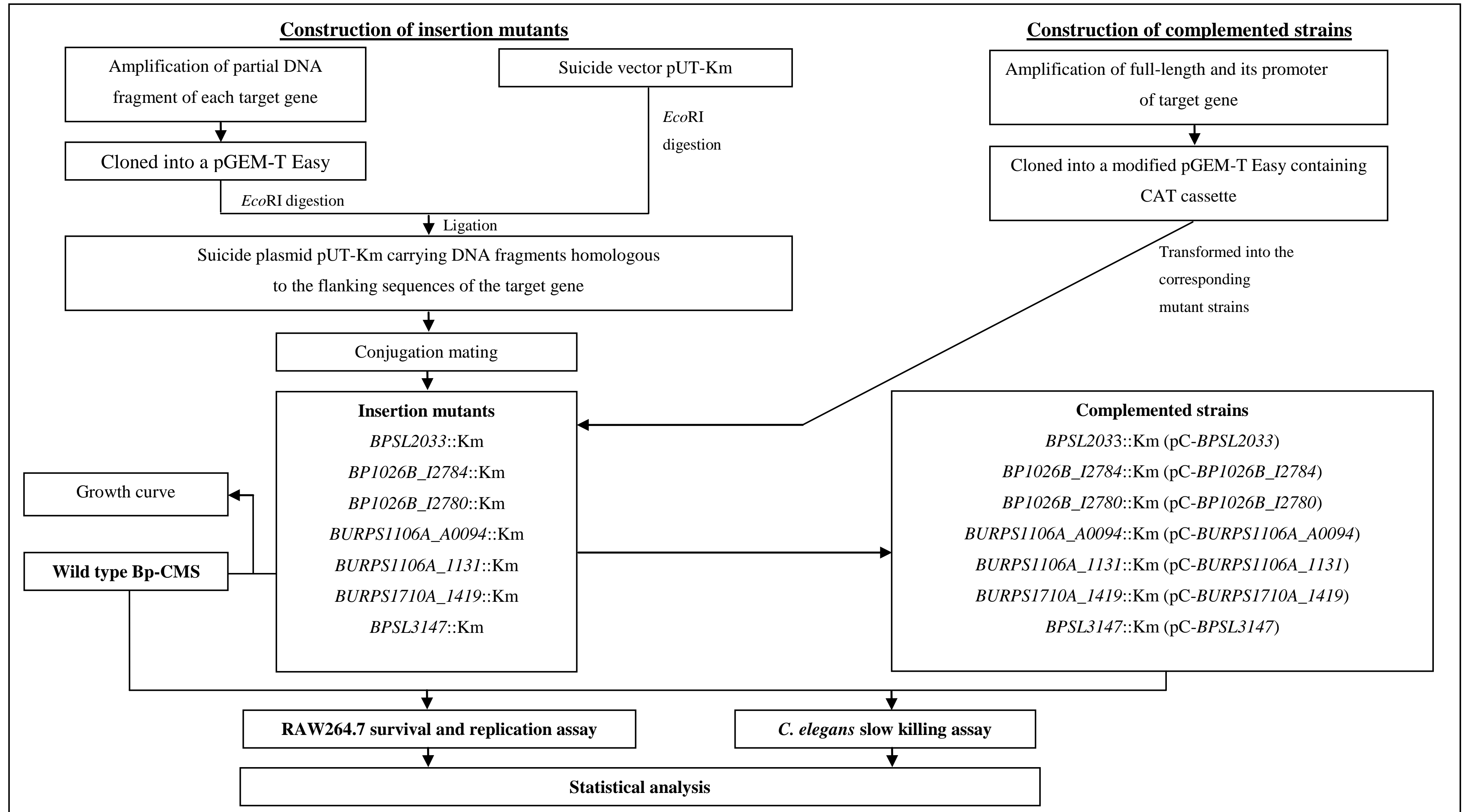


Figure 4.5. Summary of the experiments carried out in this study.

4.3 Results

4.3.1 Construction of *BPSL2033*::Km mutant

The putative 1287 bp *BPSL2033* gene encodes the predicted transport-related membrane protein of 428 amino acids. Primers P3 & pUT-R and P4 & KmF yielded amplicons of 599 bp and 1700 bp in the mutant but not in the wild type as shown in Figure 4.6. Later, the integration of pUT-*BPSL2033* (Table 4.2) into the *BPSL2033* gene on the *B. pseudomallei* chromosome was confirmed by using 2 chromosomal primers (P15 & P16) flanking the homologous region. The insertion mutant gave approximately the expected 6256 bp PCR product after amplification while wild type showed a band about 1056 bp. One of the resulting strains designated as *BPSL2033*::Km was used for further study.

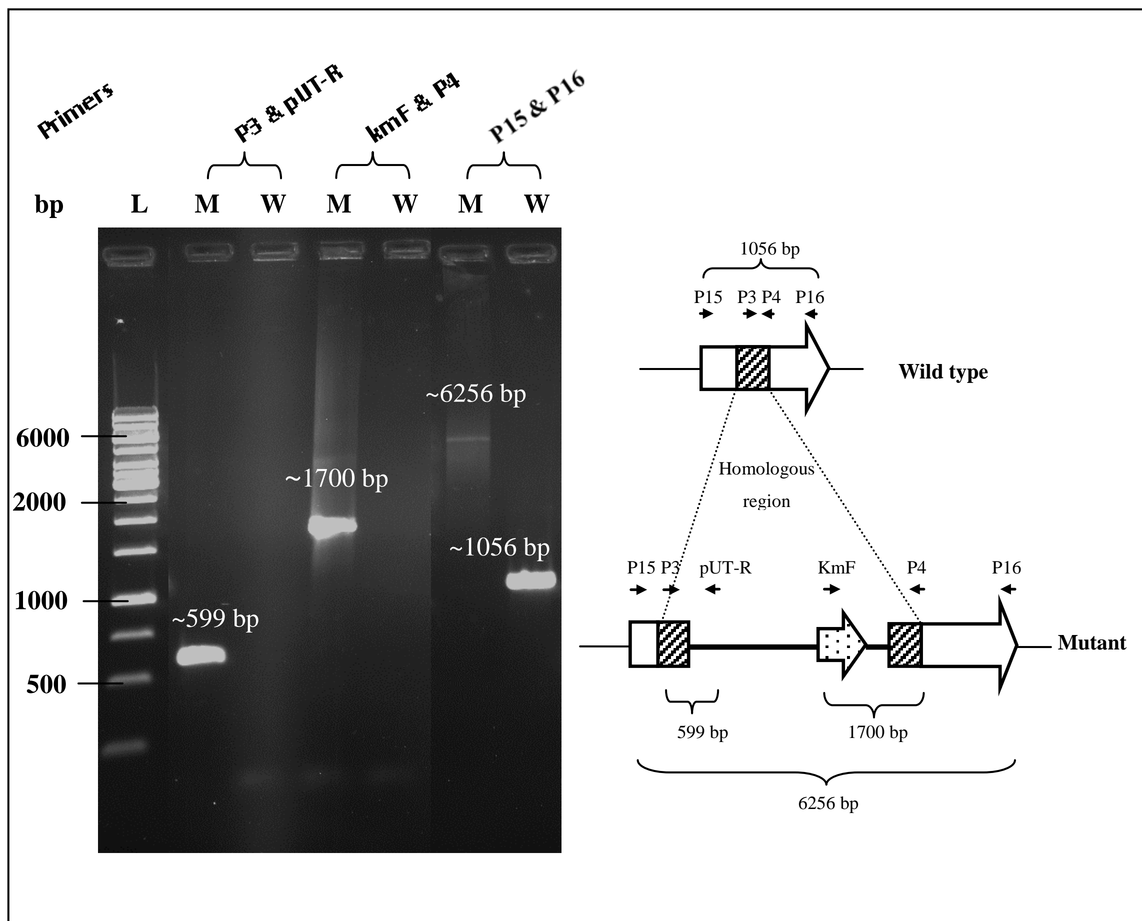


Figure 4.6. Verification of the construction of *BSL2033*::Km mutant by PCR using specific alignment primers. M and W represent mutant and wild type strains respectively, while L indicates 1 kb DNA ladder from Fermentas (USA).

4.3.2 Construction of *BP1026B_I2784*::Km mutant

The putative 927 bp *BP1026B_I2784* gene encodes for the hypothetical protein of 308 amino acids. PCR using primers P5 & pUT-R and KmF & P6 yielded DNA fragments of 497 bp and 1743 bp, respectively in the mutant but not in the wild type, suggesting the presence pUT-*BP1026B_I2784* in the intended location on the *B. pseudomallei* chromosome, which was verified by sequencing. Next, both upstream and downstream of flanking sequences of this homologous region were PCR amplified using primers P17 and P18 to confirm the integration of the entire pUT-*BP1026B_I2784* into the bacterial chromosome. PCR results demonstrated a band of 725 bp in the wild type and 5771 bp in the mutant (Figure 4.7). One mutant designated *BP1026B_I2784*::Km was then chosen for further study.

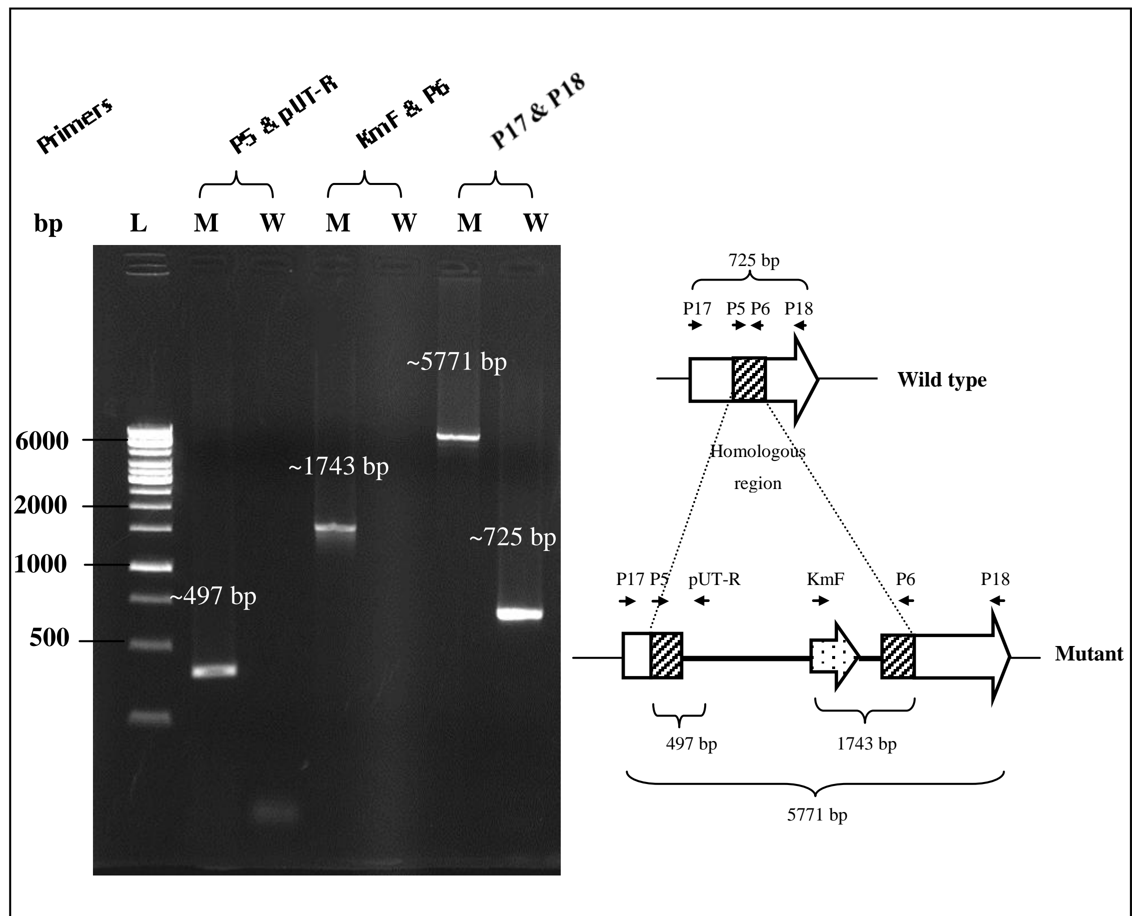


Figure 4.7. Verification of the construction of *BP1026B_I2784*::Km mutant by PCR using specific alignment primers. M and W represent mutant and wild type strains respectively, while L indicates 1 kb DNA ladder from Fermentas (USA).

4.3.3 Construction of *BP1026B_I2780*::Km mutant

The putative 870 bp *BP1026B_I2780* gene encodes for the hypothetical protein of 289 amino acids. The 422 bp and 1683 bp fragments were amplified by primers P7 & pUT-R and KmF & P8, respectively, in the mutant but not in the wild type. The amplicons were clearly visible on a 1% (w/v) agarose gel (Figure 4.8). On the contrary, no amplification product was observed in the wild type tested. Next, PCR analysis using chromosomal primers (P19 & P20) amplified 5648 bp and 625 bp in the mutant and wild type strains, respectively. The mutant designated as *BP1026B_I2780*::Km was used for further study.

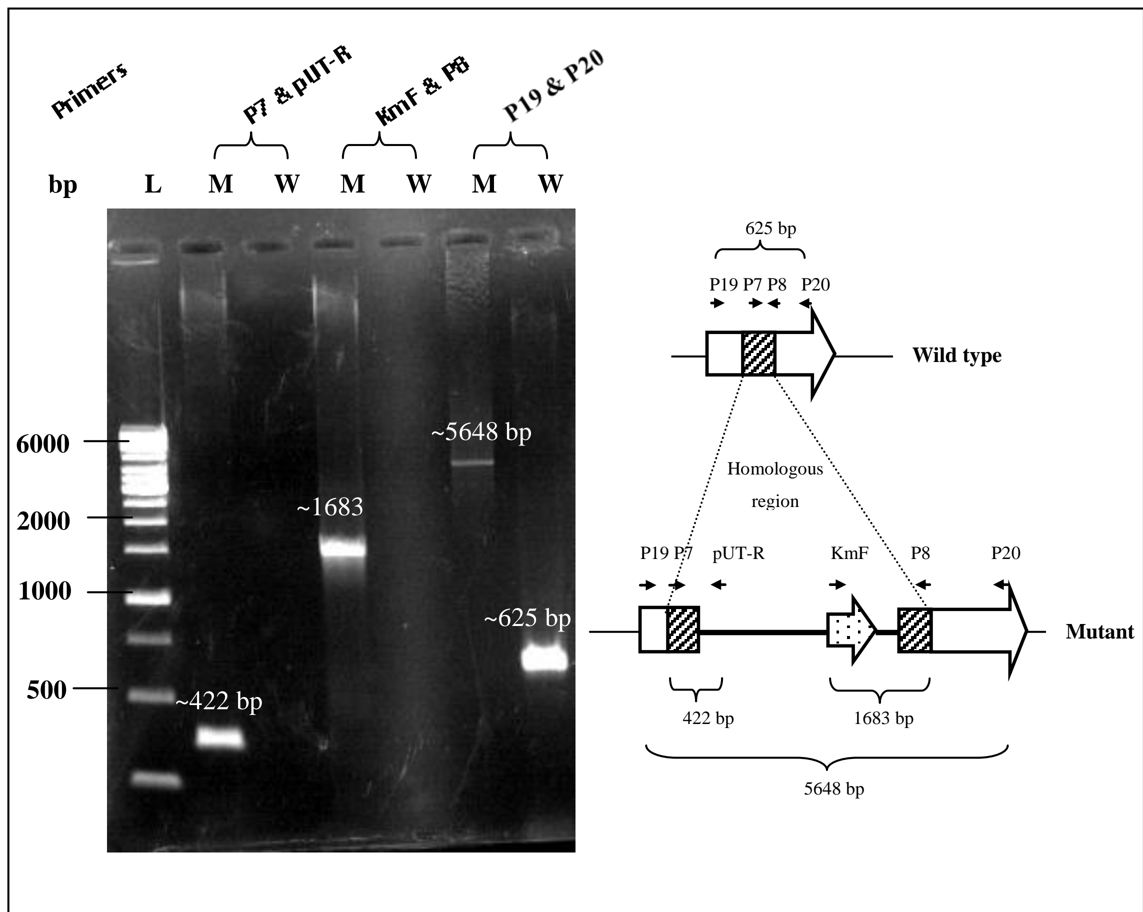


Figure 4.8. Verification of the construction of *BP1026B_I2780*::Km mutant by PCR using specific alignment primers. M and W represent mutant and wild type strains respectively, while L indicates 1 kb DNA ladder from Fermentas (USA).

4.3.4 Construction of *BURPS1106A_A0094*::Km mutant

The putative 636 bp *BURPS1106A_A0094* gene encodes for the regulatory protein of 211 amino acids. As shown in Figure 4.9, PCR using primers P21 & pUT-R and KmF & P23 amplified products of 540 bp and 1710 bp that were observed in the mutant but not in the wild type, suggesting the presence of pUT-Km vector sequence on *B. pesudomallei* chromosome as previously described (Section 4.2.4.3). Next, the mutant was confirmed by using chromosomal primers P22 & P23 to amplify full-length of the target gene and this generated a longer PCR fragment 5541 bp compared to the wild type, 636 bp. The mutant was designated as *BURPS1106A_A0094*::Km and used for further study.

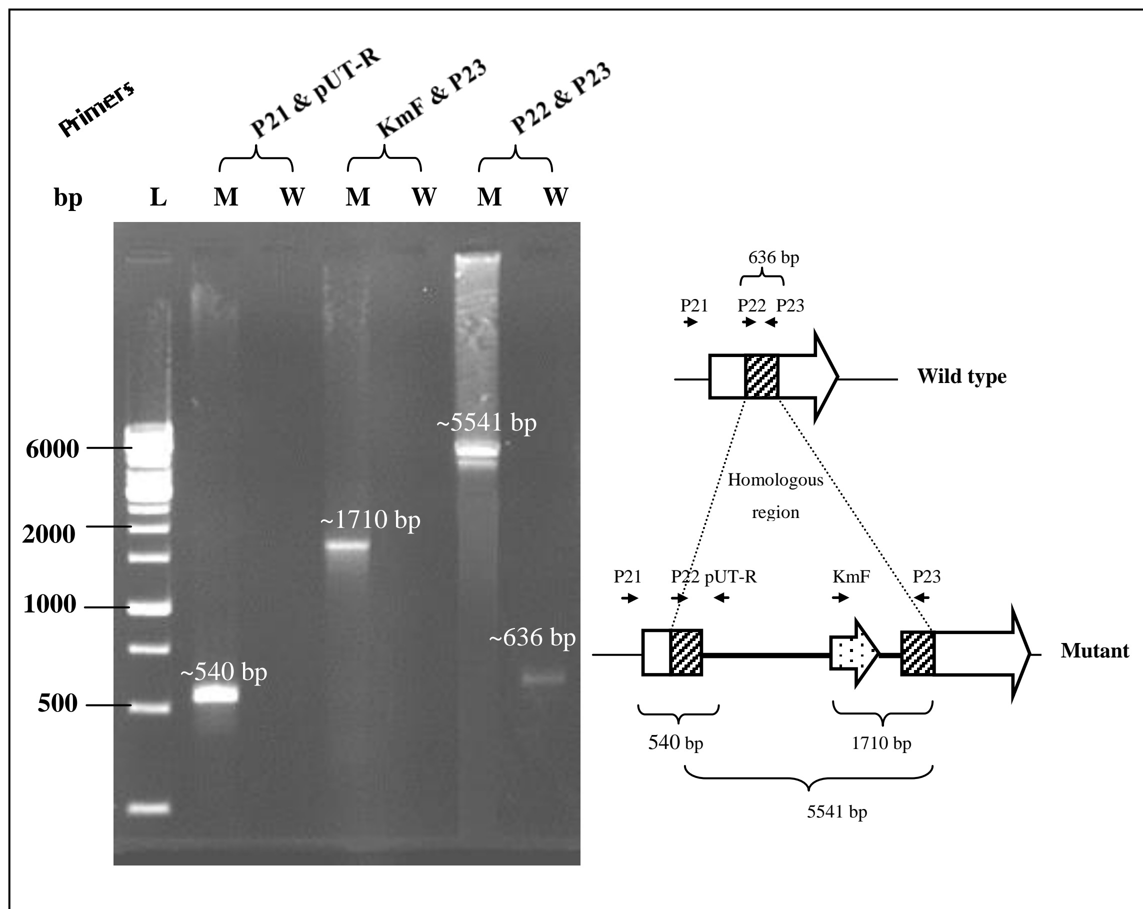


Figure 4.9. Verification of the construction of *BURPS1106A_A0094*::Km mutant by PCR using specific alignment primers. M and W represent mutant and wild type strains respectively, while L indicates 1 kb DNA ladder from Fermentas (USA).

4.3.5 Construction of *BURPS1106A_1131*::Km mutant

The putative 342 bp *BURPS1106A_1131* gene encodes the hypothetical protein of 113 amino acids. Figure 4.10 shows that the mutant yielded a DNA fragment of 346 bp, using P9 & pUT-R and 1347 bp fragment using KmF & P10. No amplification product was observed in the wild type by using primers containing plasmid backbone. Both results confirmed the presence of the pUT vector sequence. Furthermore, the mutant should be appeared to be legitimate single-crossover recombinant based on the principle of homologous recombination-mediated integration (Section 4.2.4.3). It was expected to give rise to 2 PCR products, small (247 bp) and large (4947 bp) fragments using primers P9 & P10 whereas wild type only amplified a small fragment (247 bp). One of the mutants was designated *BURPS1106A_1131*::Km and used for further study.

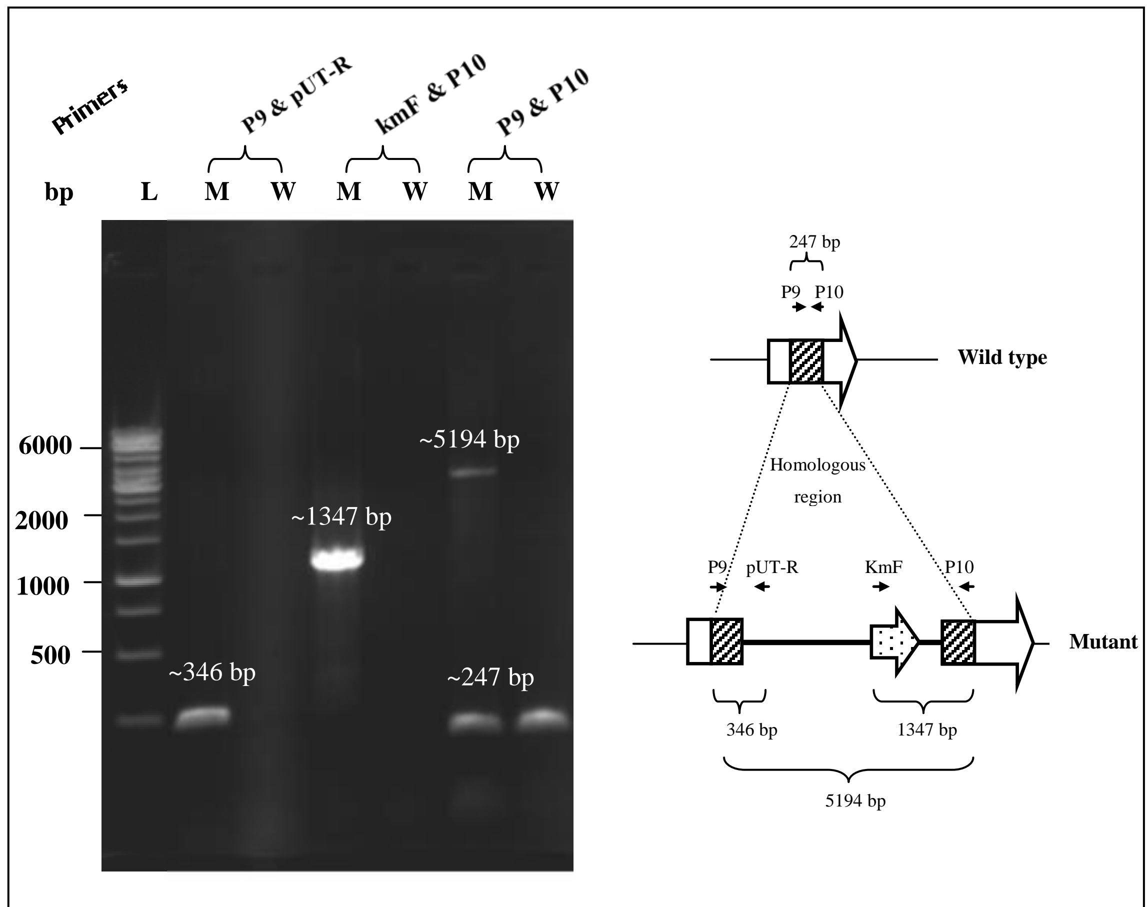


Figure 4.10. Verification of the construction of *BURPS1106A_1131*::Km mutant by PCR using specific alignment primers. M and W represent mutant and wild type strains respectively, while L indicates 1 kb DNA ladder from Fermentas (USA).

4.3.6 Construction of *BURPS1710A_1419*::Km mutant

The 225 bp *BURPS1710A_1419* gene encodes the putative lipoprotein of 74 amino acids. As illustrated in Figure 4.11, Primers P11 & pUT-R and KmF & P12 amplified 214 bp and 1296 bp, respectively in the mutant, but not in the wild type. Based on the principle of homologous recombination-mediated integration (Section 4.2.4.3), the mutant should be appeared to be legitimate single-crossover recombinant, the confirmation was then performed using primers P11 & P12. As expected, the mutant yielded 2 PCR products, small (196 bp) and large (5092 bp) fragments whereas the wild type only amplified a small fragment (196 bp). One of the mutants was designated *BURPS1710A_1419*::Km and used for further study.

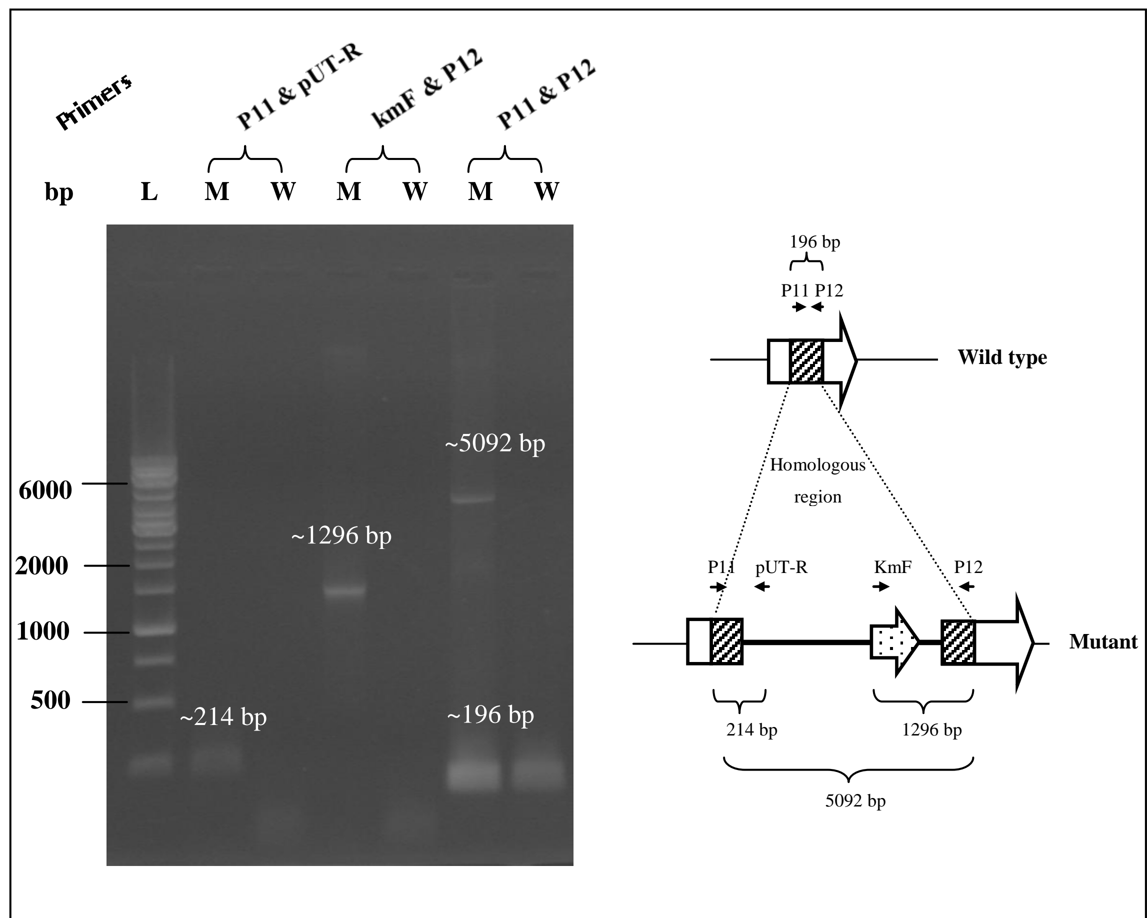


Figure 4.11. Verification of the construction of *BURPS1710A_1419*::Km mutant by PCR using specific alignment primers. M and W represent mutant and wild type strains respectively, while L indicates 1 kb DNA ladder from Fermentas (USA).

4.3.7 Construction of *BPSL3147*::Km mutant

The putative 1011 bp *BPSL3147* gene encodes the lipoprotein of 336 amino acids. As illustrated in Figure 4.12, amplicons of 591 bp and 1592 bp were seen in the mutant, but not in the wild type when using primer pairs, pUT-R and KmF, respectively. The insertion mutant was confirmed using PCR with primers flanking the expected site of mutagenesis (P24 & P25). The mutant yielded a longer expected PCR product, i.e., 5751 bp compared to the wild type with 637 bp. Both PCR results showed that the mutation of *BPSL3147* gene on *B. pseudomallei* chromosome was constructed and one strain named *BPSL3147*::Km was selected for further study.

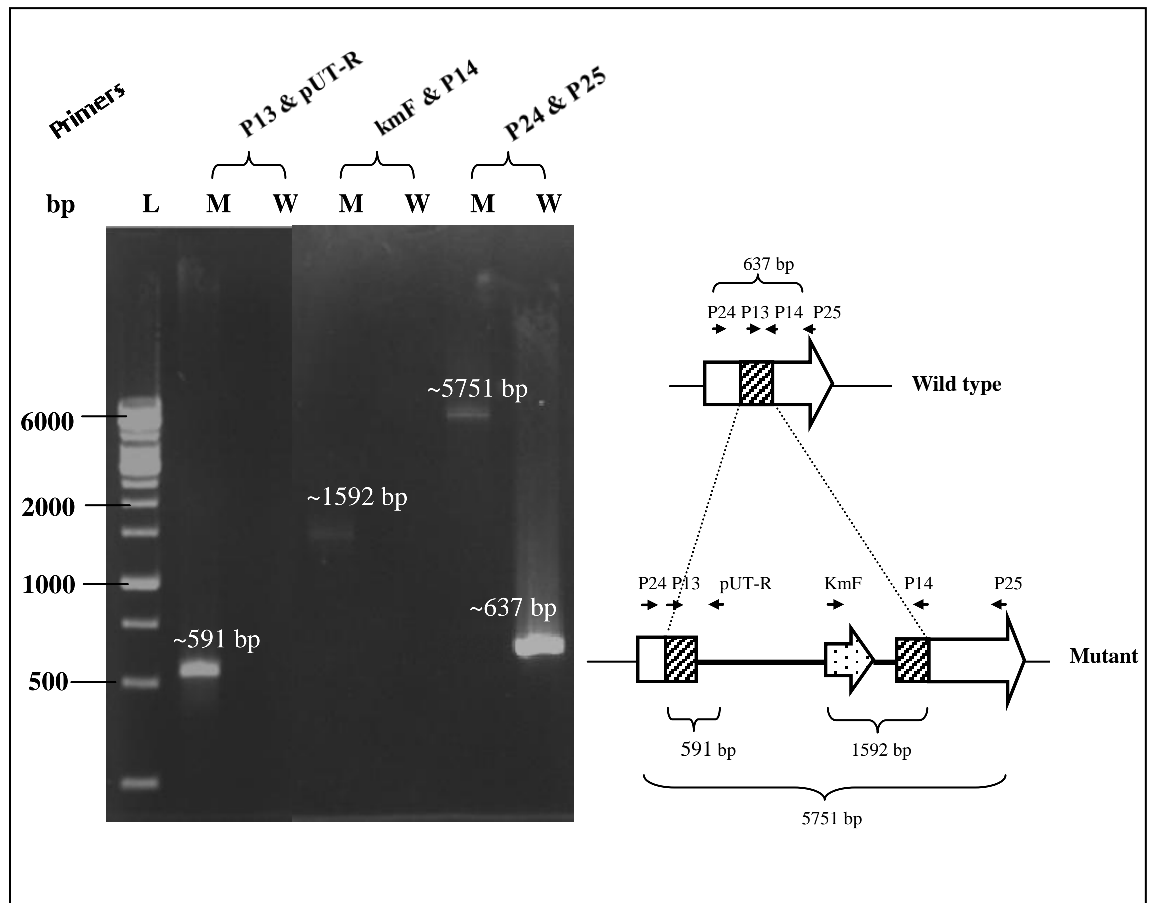
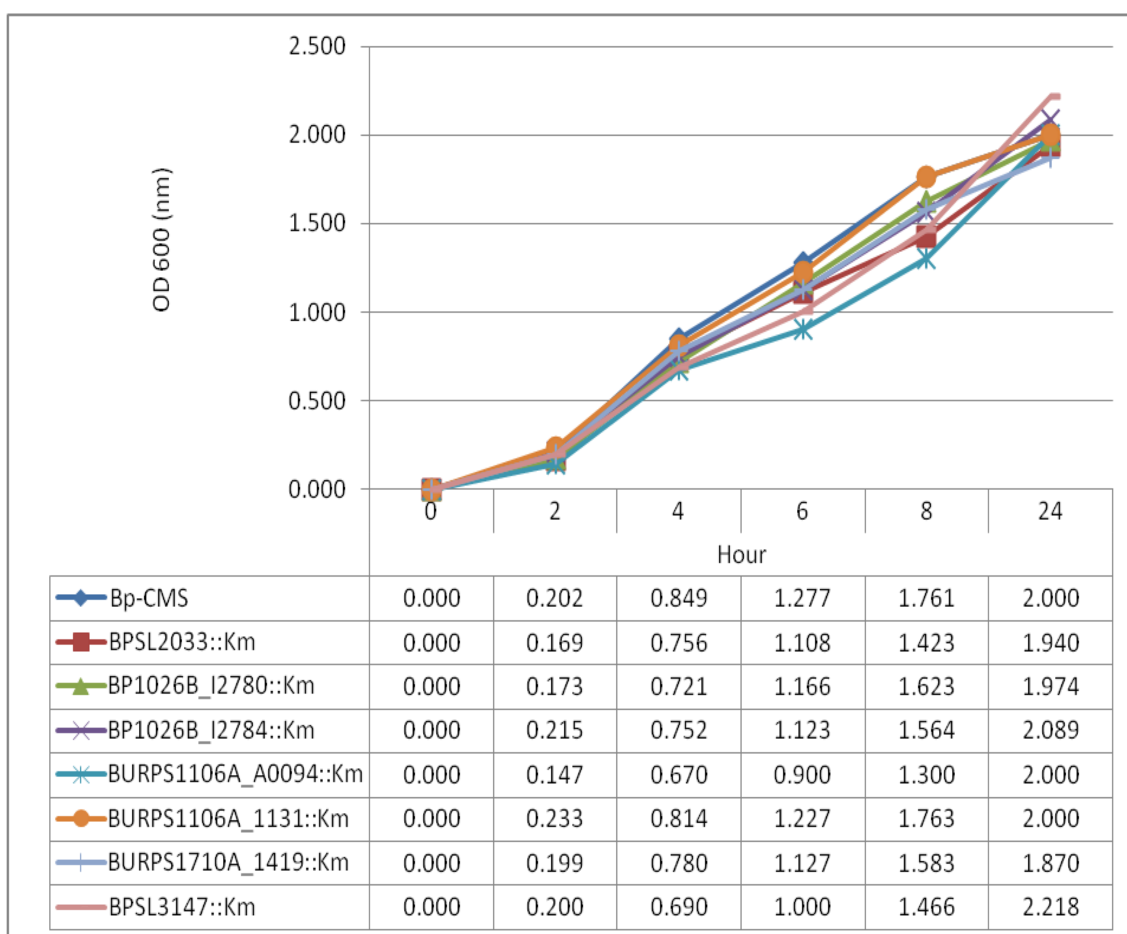


Figure 4.12. Verification of the construction of *BPSL3147*::Km mutant by PCR using specific alignment primers. M and W represent mutant and wild type strains respectively, while L indicates 1 kb DNA ladder from Fermentas (USA).

4.3.8 Growth curve

The growth rate of the parental *B. pseudomallei* (Bp-CMS) and the insertion derivatives were measured (OD reading) in liquid media over 8 h. All 7 mutant strains demonstrated similar growth rate to the parental strain (Figure 4.13). Hence, all 7 mutants were used for construction of complemented strains and further characterized for virulence *in vitro* and *in vivo*.



	0	2	4	6	8	24
Bp-CMS	0.000	0.202	0.849	1.277	1.761	2.000
BPSL2033::Km	0.000	0.169	0.756	1.108	1.423	1.940
BP1026B_I2780::Km	0.000	0.173	0.721	1.166	1.623	1.974
BP1026B_I2784::Km	0.000	0.215	0.752	1.123	1.564	2.089
BURPS1106A_A0094::Km	0.000	0.147	0.670	0.900	1.300	2.000
BURPS1106A_1131::Km	0.000	0.233	0.814	1.227	1.763	2.000
BURPS1710A_1419::Km	0.000	0.199	0.780	1.127	1.583	1.870
BPSL3147::Km	0.000	0.200	0.690	1.000	1.466	2.218

Figure 4.13. Growth of wild type and mutant strains of *B. pseudomallei* in liquid media at 37°C monitored by absorbance at 600 nm over 8 hours. (Mean from 2 independent experiments).

4.3.9 Construction of complemented strains

A complemented strain was constructed in following methods: a) a complemented plasmid was constructed by cloning an intact copy of the native gene containing its own promoter into the modified pGEM-T vector (Figure 4.4) b) reintroducing a complemented plasmid into the corresponding insertion mutant by transformation.

Figure 4.14 shows the expected fragments demonstrated by complemented plasmids. All PCR products from lane 2 were found to posses a longer DNA fragments compared to that in lane 1 due to the presence of CAT cassette. All of these complemented plasmids were transformed into each corespsonding mutant and the resulting complemented strains were designated as: *BPSL2033*::Km (pC-*BPSL2033*), *BP1026B_I2784*::Km (pC-*BP1026B_I2784*), *BP1026B_I2780*::Km (pC-*BP1026B_I2780*), *BURPS1106A_A0094*::Km (pC-*BURPS1106A_A0094*), *BURPS1106A_1131*::Km (pC-*BURPS1106A_1131*), *BURPS1710A_1419*::Km (pC-*BURPS1710A_1419*) and *BPSL3147*::Km (pC-*BPSL3147*) (Table 4.2).

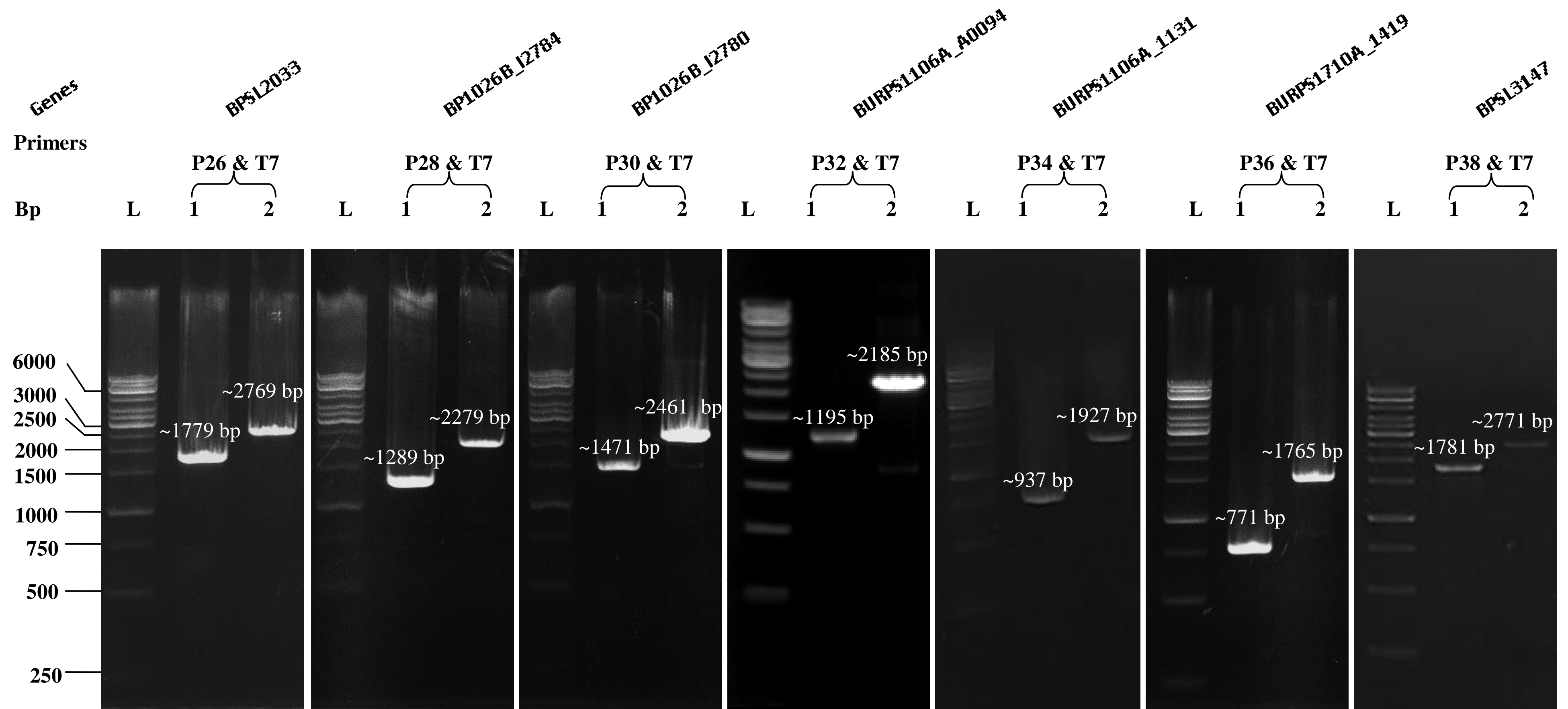


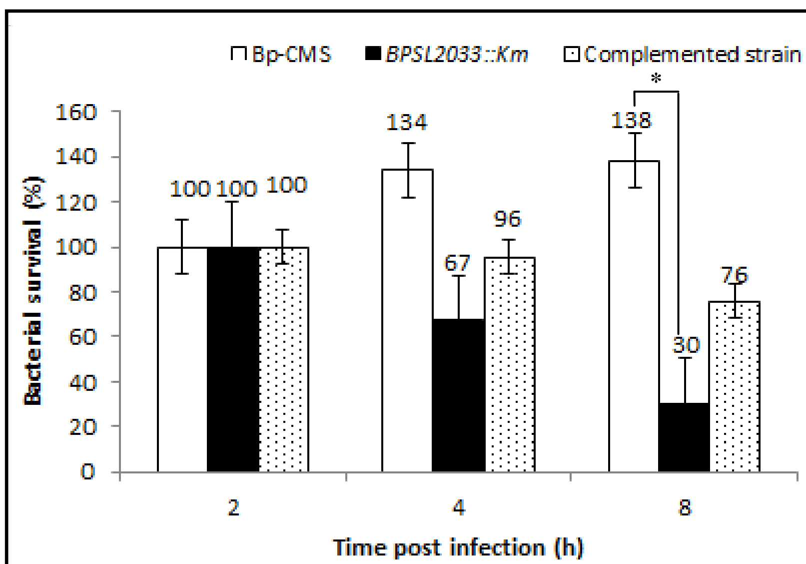
Figure 4.14. Verification of construction of complemented plasmids by PCR. Lanes 1 and 2 represent DNA fragments amplified from plasmids with and without CAT cassette by using specific primers for each gene. Lane L indicates 1 kb DNA ladder from Fermentas (USA).

4.3.10 Bacterial replication and survival in macrophage assay

The murine macrophages were infected with *B. pseudomallei* Bp-CMS and the mutants, and their intracellular survival ability was determined for up to 8 h post infection. Bacterial survival was presented as relative survival (%) after normalizing to CFU counts that were recovered at 2 h post infection.

As can be seen in Figures 4.15 (A) to (G), wild-type bacteria were able to survive and replicate in macrophage cells over the course of the experiment. In contrast, the 5 mutant strains *BPSL2033*::Km, *BP1026B_I2780*::Km, *BURPS1106A_A0094*::Km, *BURPS1710A_1419*::Km and *BPSL3147*::Km showed reduced intracellular survival inside RAW264.7 cells at 4 and 8 h post infection. However, only the differences of survival rate of *BPSL2033*::Km reached statistical significance (p=0.049) at 8 h post infection when compared to the wild type. On the other hand, the differences of survival rate in macrophages of another 2 mutant strains *BP1026B_I2784*::Km and *BURPS1106A_1131*::Km were not significant at 4 and 8 h post infection.

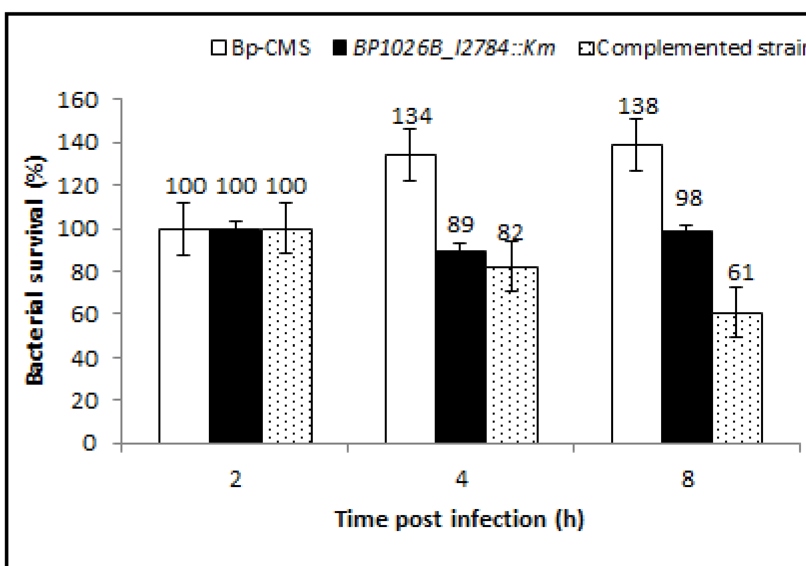
Importantly, most of these plasmid-complemented strains partially restored intracellular survival and replication except for *BP1026B_I2780*::Km, *BURPS1106A_A0094*::Km and *BURPS1106A_1131*::Km at 8 h post infection. The results postulated that 3 genes, *BPSL2033*::Km (p value=0.049), *BURPS1710A_1419*::Km (p value=0.165) and *BPSL3147*::Km (p value=0.076) individually had little effect on the survival of *B. pseudomallei* growing intracellularly in phagocytic cells.



(A)

BPSL2033::Km

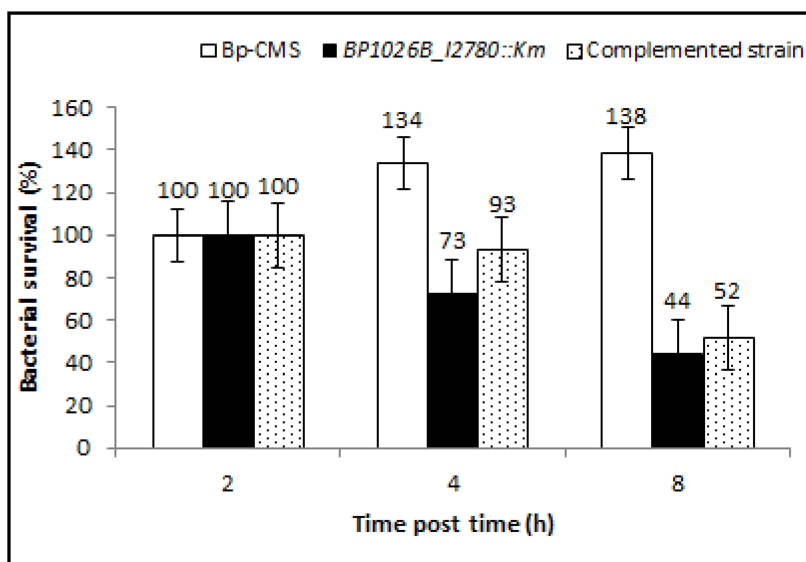
& complemented strain.



(B)

BP1026B_I2784::Km

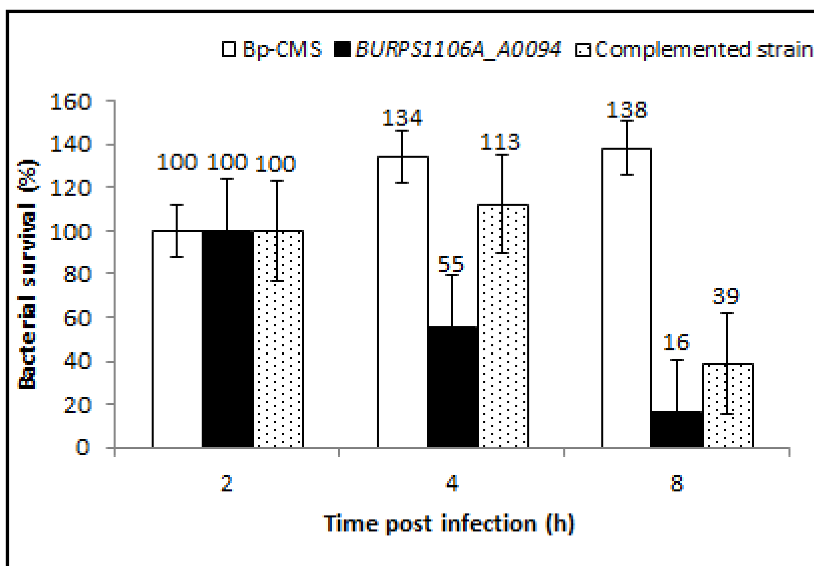
& complemented strain.



(C)

BP1026B_I2780::Km

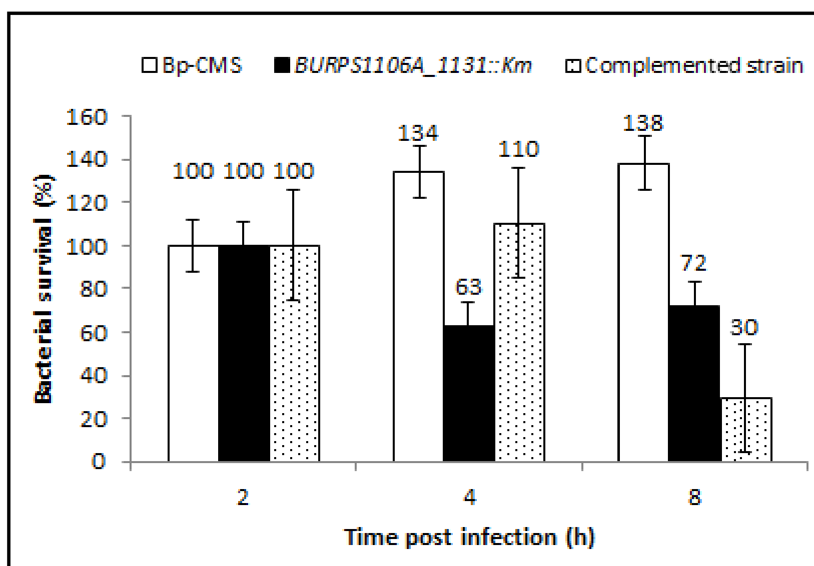
& complemented strain.



(D)

BURPS1106A_A0094::Km

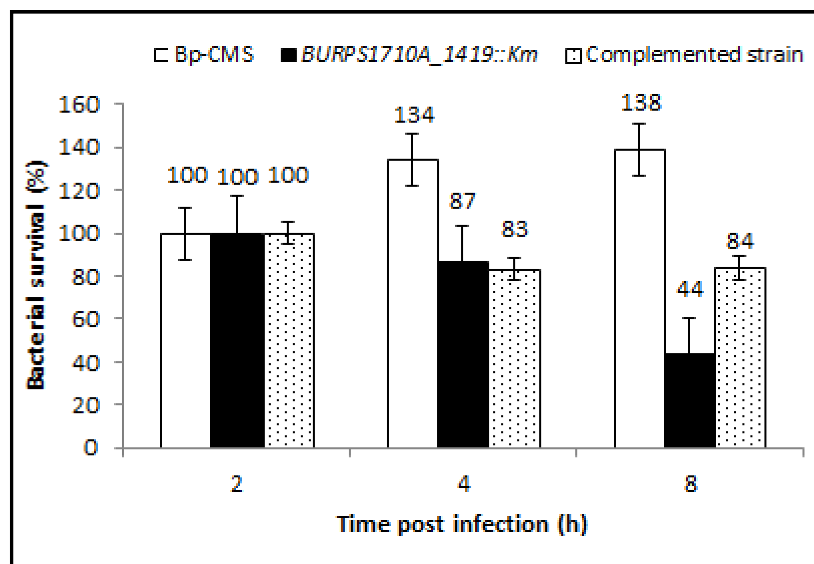
& complemented strain.



(E)

BURPS1106A_1131::Km &

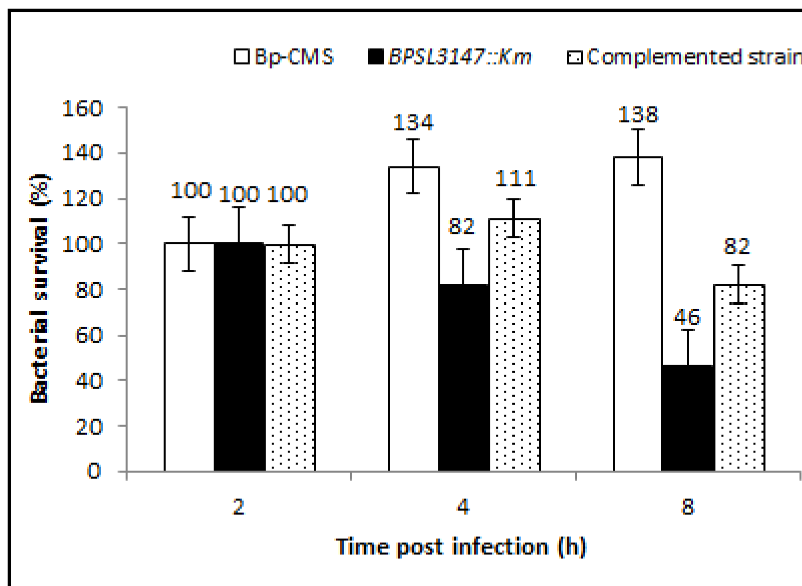
complemented strain.



(F)

BURPS1710A_1419::Km

& complemented strain.



(G)

BPSL3147::Km

& complemented strain.

Figure 4.15. Bacterial survival and replication within RAW264.7 macrophage-like cells infected at MOI of 100 using wild type strain and the 7 insertion mutants as well as their complemented strains. Data represents means and standard errors of 3 separate experiments; each experiment was carried out in 3 technical replicates for each time point. Asterisk indicates significant differences ($p<0.05$) relative to the wild type strain Bp-CMS at each time point.

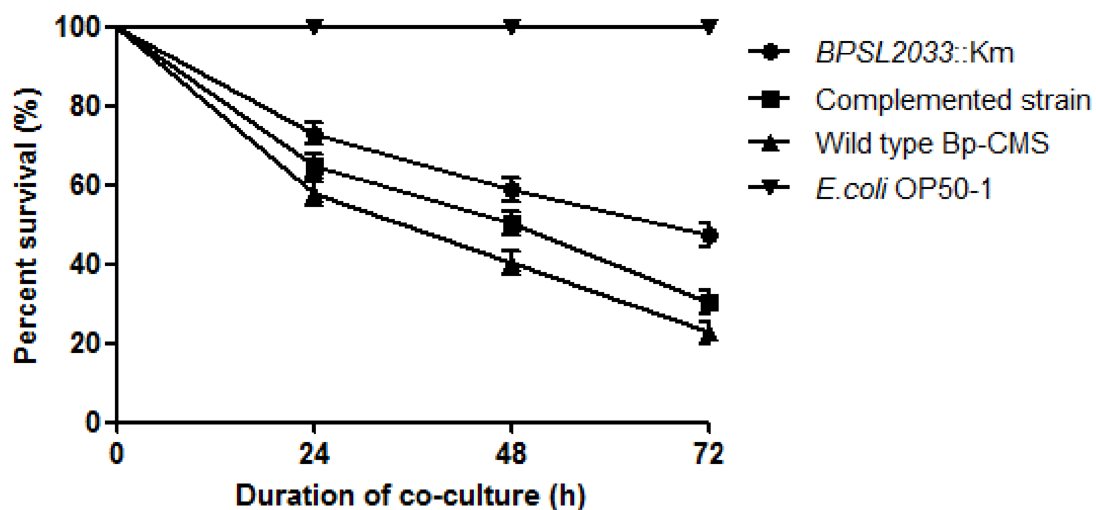
4.3.11 *Caenorhabditis elegans* killing assay

To further investigate the virulence of these mutants in *C. elegans,* Bristol N2 wild-type strains were infected on NGM plates containing FUDR. The results of survival proportions of *C. elegans* are shown in Table 4.4, while the comparison of survival curve for each mutant and its complemented strain against wild type are illustrated in Figure 4.16. The negative control was *E. coli* OP50-1.

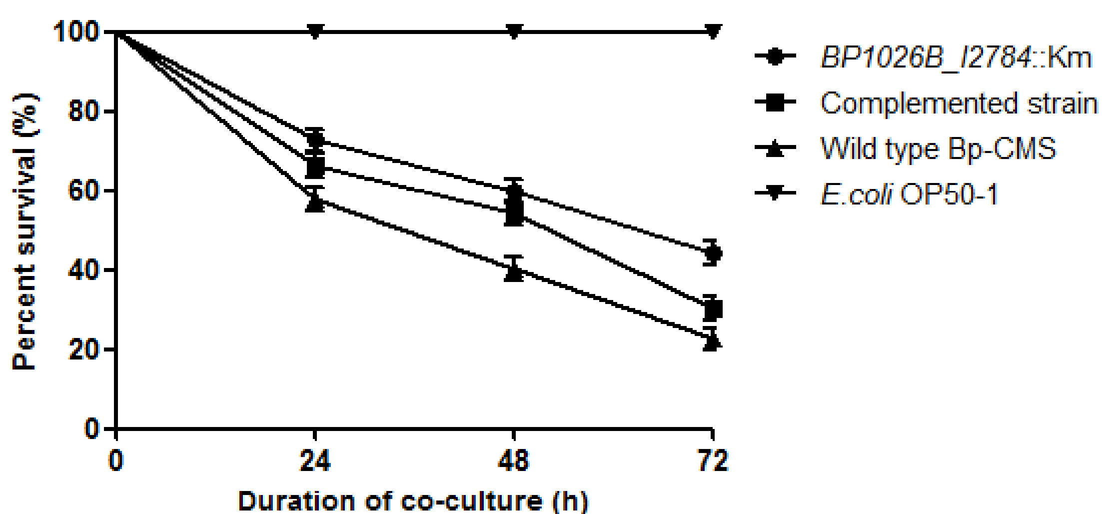
Table 4.4. Survival proportion of *C.elegans* challenged with bacterial strains.

Strains	**Survival proportions (%) in hours**			
	0	**24**	**48**	**72**
E. coli OP50-1	100	100	100	100
Wild type Bp-CMs	100	58	40	26
BPSL2033::Km	100	73	59	47
Complemented strain of *BPSL2033*::Km	100	65	45	30
BP1026B_I2784::Km	100	73	60	44
Complemented strain of *BP1026B_I2784*::Km	100	66	54	30
BP1026B_I2780::Km	100	74	64	45
Complemented strain of *BP1026B_I2780*::Km	100	73	64	36
BURPS1106A_A0094::Km	100	77	65	39
Complemented strain n of *BURPS1106A_A0094*::Km	100	73	54	32
BURPS1106A_1131::Km	100	70	62	50
Complemented strain of *BURPS1106A_1131*::Km	100	63	61	42
BURPS1710A_1419::Km	100	71	60	51
Complemented strain of *BURPS1710A_1419*::Km	100	60	49	28
BPSL3147::Km	100	79	67	44
Complemented strain of *BPSL3147*::Km	100	70	58	36

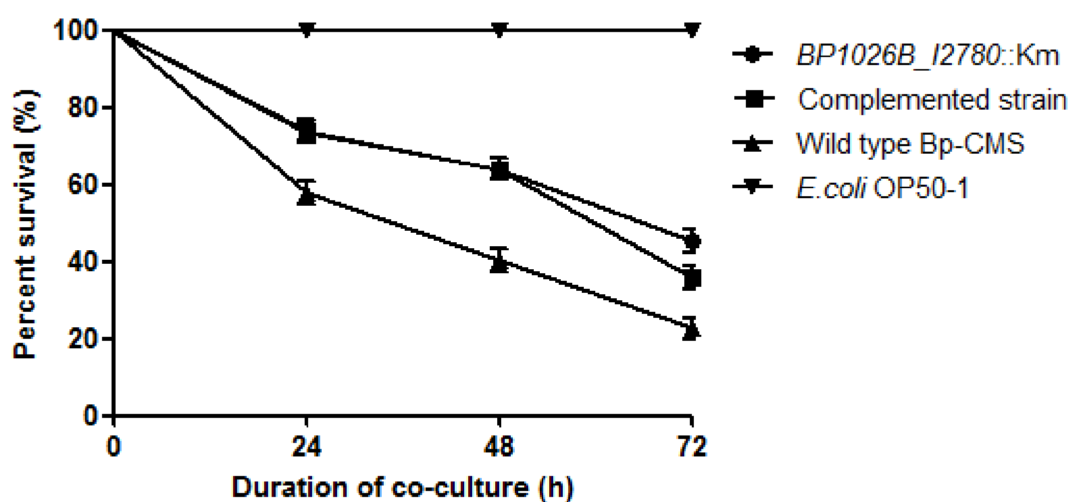
A) *BPSL2033*::Km & its complemented strain



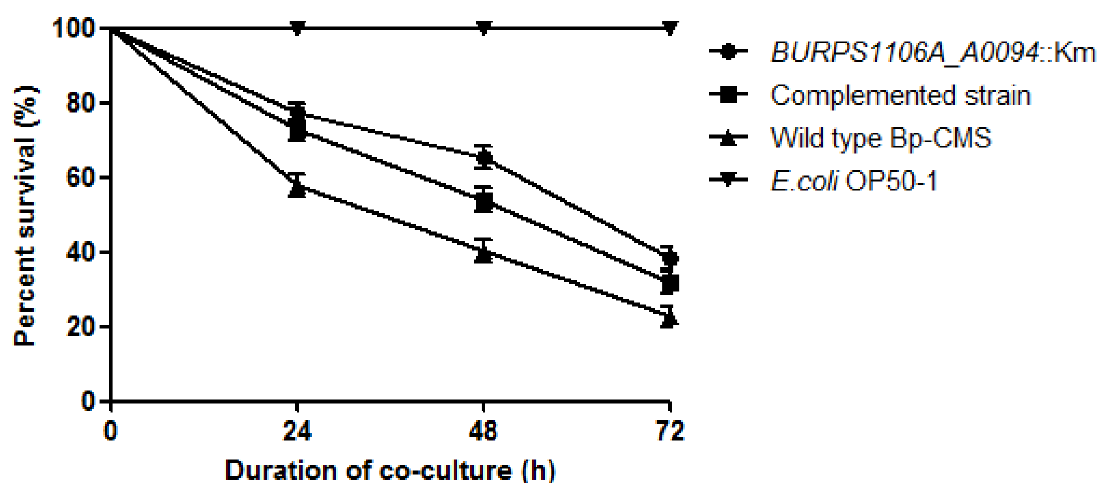
B) *BP1026B_I2784*::Km & its complemented strain



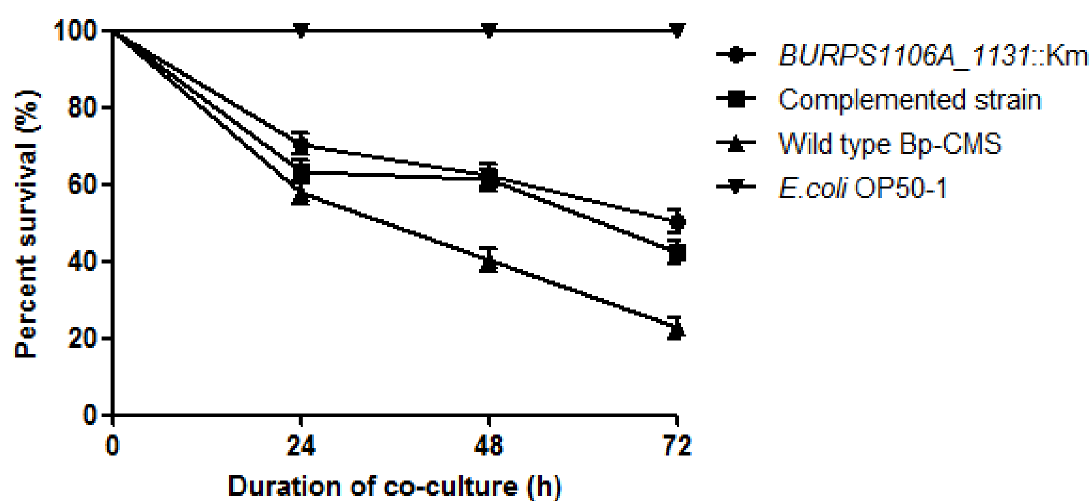
C) *BP1026B_I2780*::Km & its complemented strain



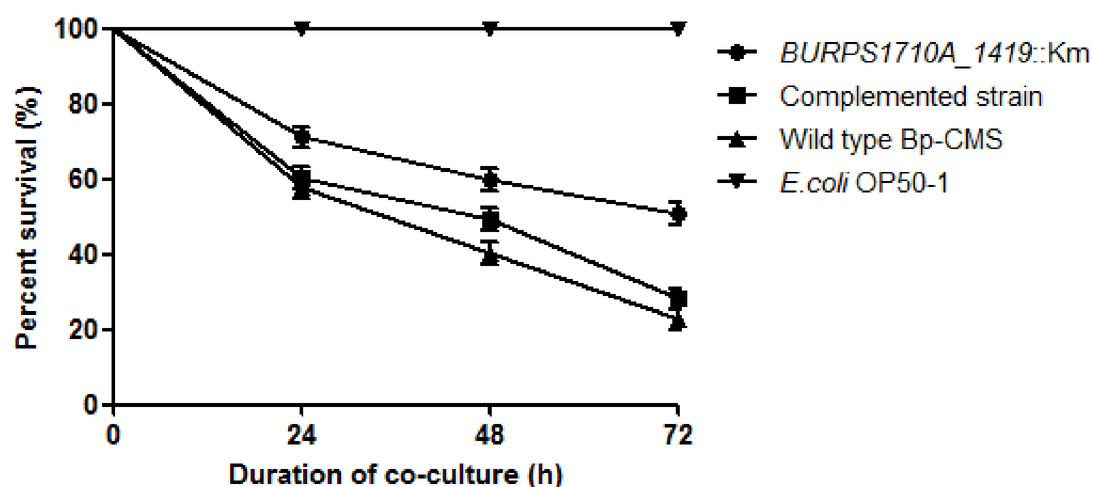
D) *BURPS1106A_A0094*::Km & its complemented strain



E) *BURPS1106A_1131*::Km & its complemented strain



F) *BURPS1710A_1419*::Km & its complemented strain



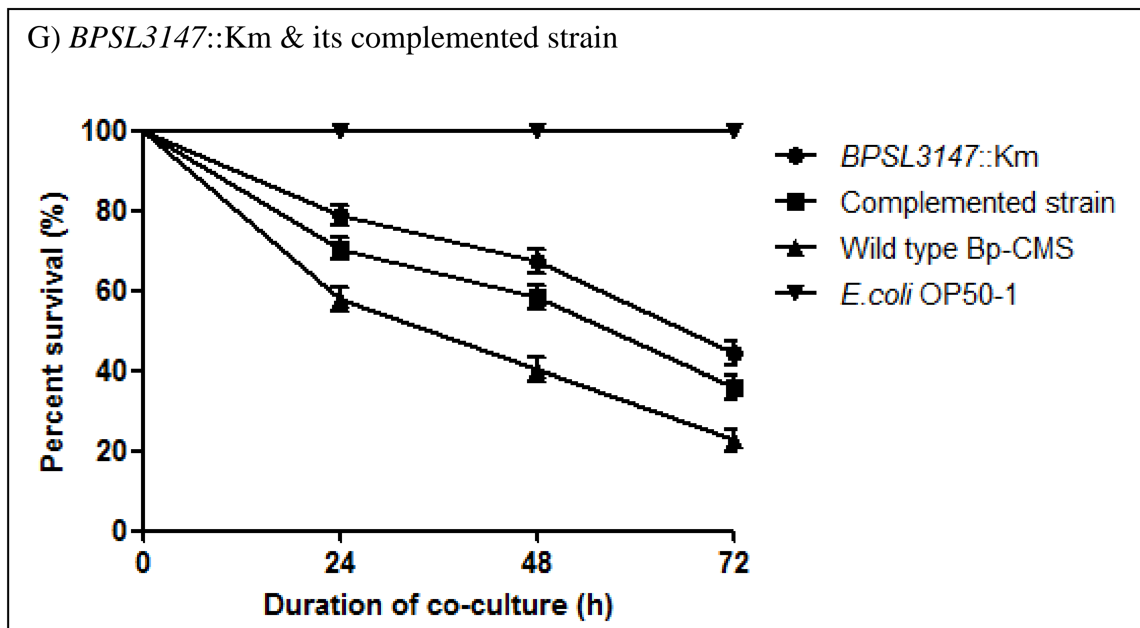


Figure 4.16. Kaplan-Meier survival curves for *C. elegans* infected with different strains of *B. pseudomallei*. Values are the pooled data from triplicate of 3 separate experiments (N=270) (*E. coli OP50-1* was the negative control).

The virulence of the wild type Bp-CMS is reflected in reduced survival as the strain killed approximately 42% and 74% of the worms after 1 and 3 days, respectively. No killing of *C. elegans* was observed under slow-killing conditions when they were fed *E. coli* OP50-1. By comparing all the graphs, the 6 mutants (*BPSL2033*::Km, *BP1026B_I2784*::Km, *BP1026B_I2780*::Km, *BURPS1106A_1131*::Km, *BURPS1710A_1419*::Km and *BPSL3147*::Km) exhibited low level of virulence attenuation in *C. elegans*, where survival rates of the worms were only twice higher than that of the wild type. Likewise, the complemented strains of mutants demonstrated approximately partial restoration of virulence for *BPSL2033*::Km, *BP1026B_I2784*::Km and *BURPS1710A_1419*::Km.

Among them, *BURPS1710A_1419*::Km was highly attenuated as 51% worms able to survive and followed by *BPSL2033*::Km (47%) than the wild-type parental strain (23%) after 3 days. From the results, both genes *BURPS1710A_1419*::Km and *BPSL2033*::Km seem to contribute a minor role to virulence in nematode infection model.

4.4 Discussion

4.4.1 Natural competence of Bp-CMS

The targeted mutagenesis procedure described here (Section 4.2.4) is based on natural transformation, i.e., naturally competent *B. pseudomallei* strains are able to acquire DNA and integrate this genetic material into their genomes. However, not all *B. pseudomallei* strains are naturally transformable, i.e., unable to facilitate mutant strain construction (Thongdee et al., 2008).

The parental wild type *B. pseudomallei* strain, named Bp-CMS that used to construct mutants was the virulent strain used earlier in mice challenge studies using male and female mice. This strain (Bp-CMS) was found to be virulent in a mouse model for both groups, male (p value = 6.9×10^{-8}) and female (p value = 3×10^{-7}) at 3 different doses, i.e., 10, 100 and 1000 cfu/mL (Puthucheary et al., 2012a). Therefore, natural transformation competence in Bp-CMS strain would allow the construction of all 7 insertion mutants (Sections 4.3.1 to 4.3.7). Positive chromosomal integration mutants were successfully constructed for the 7 candidate genes, on the first or second attempt.

4.4.2 Growth curve

The growth of the all 7 mutants, assayed in rich media showed no difference in growth rates compared to that of the wild type, ruling out the possibility that these genes are not affect the growth but involved in other aspects such as pathogenicity.

4.4.3 Insertion mutagenesis as a genetic tool for investigating gene function

Reverse genetic analysis offers a logical approach that enables investigation of the function of unknown genes, where a specific gene is disrupted to study the resulting phenotypic characteristic(s) of the microorganism (Reyrat et al., 1998). As mentioned in Section 2.3.2, a number of methods can be used to inactivate bacterial chromosomal genes and a site-specific insertion mutagenesis was chosen for this study, because this technique had been used successfully to generate mutants in *B. pseudomallei* (Lumjiaktase et al., 2006; Song et al., 2005; Stevens et al., 2002; Stevens et al., 2004; Tuanyok et al., 2006) as well as it has become a common procedure in knockout studies (Ortiz-Martin et al., 2006).

Thus far, only a few approved antibiotic selection markers for *B. pseudomallei* such as genes encoding resistance to kanamycin, zeocin and gentamicin have been described (Galyov et al., 2010; Schweizer and Peacock, 2008). A suicide vector pUT-Km that contains kanamycin resistance genes was chosen in this study. This vector is a valuable tool for constructing mutants by insertion mutagenesis. It is a straight-forward method as the mutants grow directly on agar plates containing appropriate antibiotics and can be confirmed by simple PCR assay (Chuang et al., 2006; Lin et al., 2008). Moreover, its utility to generate insertion mutant of quorum sensing genes in *B. pseudomallei* has already been successfully demonstrated by Song et al. (2005).

4.4.4 Assessment of virulence of the candidate genes

The infection assay on macrophage cells showed that without gene *BPSL2033* (putative transport-related membrane protein), the mutant demonstrated reduced ability to replicate and survive over 8 h. The result was supported by a plasmid-encoded complemented strain, which demonstrated partial restoration for intracellular survival when compared to wild type Bp-CMS. Similar outcomes have been noted with *BURPS1710A_1419* (putative lipoprotein) and *BPSL3147* (lipoprotein).

At 8 h post infection, loss of gene *BPSL2033* showed a survival reduction of 5-times in RAW264.7 cells while both *BURPS1710A_1419* and *BPSL3147* resulted in 3-times reduction. Our observations indicate that these genes may be involved in intracellular survival. There was a weak but still significant difference (p=0.049) of intracellular survival exhibited by *BPSL2033*::Km compared to the Bp-CMS. Thus, this suggests that *BPSL2033* may act in concert with other genes to play an essential role in virulence.

Subtractive hybridization as illustrated in Figure 4.1 revealed that Bp-CMS contained 6 DNA sequences that were not found in the attenuated strain, indicated evidence that maximal virulence probably requires multiple genes (Puthucheary et al ., 2012a). This hypothesis is further supported by the present study in which *B. pseudomallei* virulence in the phagocytic cell line model was not critically dependent on any single putative gene tested.

Several studies have suggested that a double mutant but not a single mutant of *B. pseudomallei* contribute significantly to the growth inside murine macrophage (Balder et al., 2010; Muller et al., 2012). Future experiment involved in the use of double mutant (i.e., *BPSL2033* and *BURPS1710A_1419*) will probably assess this possibility.

In the context of *BPSL3147*, there may be other unidentified gene(s) acting together for full virulence in *B. pseudomallei* infections.

It is unclear why these genes, i.e., *BPSL2033*, *BURPS1710A_1419* and *BPSL3147* with their corresponding complemented plasmids, only restored intracellular survival and replication to approximately 50% of wild-type levels. One possible explanation for this might be due to the gene being present on multiple copies of the plasmid. It is also possible that other genes present on adjacent loci may be contributing to the virulence. Another possibility is that a polar effect on the expression of downstream genes, co-transcribed with the target gene, may be responsible for the observed phenotype. This phenomenon has been reported by Muangsombut et al. (2008) in which a mutant lacking *bsa*Q of the type III secretion system, showed defects in secretion of BopE and BipD proteins due to the polar effect on the downstream genes (*bsaR/S/T*) expression.

C. elegans has been used as a simple surrogate host for modeling bacterial disease (Gravato-Nobre & Hodgkin, 2005). It has previously been shown that on a low nutrient, nematode growth medium (NGM), the *B. pseudomallei* kills *C. elegans* strain N2 within 3 days and this type of killing is referred to as "slow killing" (Chua et al., 2003; Gan et al., 2002; Song et al., 2005).

In this study, wild-type strain Bp-CMS killed 74% of the nematode population at 72 h time point. Our results are in agreement with previous studies reporting that differences in killing efficiency of *C. elegans* between wild type of *B. pseudomallei* (Gan et al., 2002; Lee et al., 2011; O'Quinn et al., 2001). As an example, the percentage of killing of worms by various *B. pseudomallei* strains, i.e., ATCC23343, EY4, #40 and KHW were approximately 50, 60, 75 and 90% at 72 h time point (Gan et al., 2002). The

virulence of *B. pseudomallei* into nematode is diverse and it is likely due to the different genetic determinants in the strains.

In the present study, all the constructed mutants were less effective in killing *C. elegans* under slow-killing conditions, i.e., twice as many worms survived when fed on mutant strains compared to those worms fed on wild type Bp-CMS after 72 h of co-culture. However, there was no significant difference of *C. elegans*-killing between the mutants and wild type. Two complemented strains of *BPSL2033*::Km and *BURPS1710A_1419*::Km achieved at least partial restoration of virulence at 72 h co-culture, thus suggesting that both genes are most probably involved in bacterial virulence.

The low level of attenuation in the mutants, may possibly due to;

(i) a single gene was insufficient to mediate full killing in the animal model. Probably at least 2 genes are required to act together in controlling virulence in *B. pseudomallei.* A double mutant Δ*relA*Δ*spoT* was reported to exhibit significant and severe attenuation in larva of the wax moth *Galleria mellonella* and C57BL/6 mice models, which were not seen in the single mutant (Muller et al., 2012).

(ii) *C. elegans* may not be sensitive enough to reveal phenotypes for all factors that contribute to the virulence in *B. pseudomallei*. A flagella mutant of *B. pseudomallei*, KHWΔ*fli*CKm was found to be equally lethal to *C. elegans* when compared to wild type but it had low virulence in Balb/c mice via intranasal infection (Chua et al., 2003). Another example was demonstrated by the isolate of *B. pseudomallei*, Sheep 4523, which showed high killing rate when exposed to *C. elegans* but had a low virulence in Balb/c mice while the reverse situation was observed for the isolate Ostrich 9166 (Lee

at al., 2011). Collectively, previous studies suggested that pathogenicity in nematodes may not correlate to mammals directly (Chua et al., 2003; O'Quinn et al., 2001).

(iii) *C. elegans* possess mechanisms to avoid or move away from pathogenic bacteria like *B. pseudomallei*. It has a simple nervous system that consists of 302 neurons that facilitate the identification of molecules, neurons and circuits involved in their behavior (White et al., 1986). It uses chemotaxis to find food on the plate and able to discriminate food both physically, based on size, and chemically, based on taste and olfaction (Kiyama et al., 2012). Interestingly, worms can modify their olfactory perference via neurotransmitter serotonin to avoid odours from pathogen and this learning occurs with exposures as short as 4 h (Zhang et al., 2005).

Zhang et al (2005) demonstrated that in the 4 choice maze assay, *C. elegans* were given a choice between non-pathogenic bacteria (*E. coli* OP50) and pathogenic bacteria (*Pseusomonas aeruginosa* PA14) that the worms had experienced earlier, and 2 novel bacterial strains that worms not experienced previously, i.e., non-pathogenic bacteria (*Pseudomonas fluorescens*) and pathogenic bacteria (*Serratia marcesces* ATCC13880). Their results showed that worms strongly preferred the known healthy bacteria *E. coli* OP50 and *Pseudomonas fluorescens* while attraction to the known pathogenic bacteria decreased compared to naïve worms.

So, olfactory learning enables worms to differentiate among natural food sources on the basis of experience. In our study, *C. elegans* was cultivated on *E. coli* OP50-1 that best supports growth, so the worms have already experienced good food, which might have increased their exploratory bahaviour when the switch to very bad food such as *B. pseudomallei,* especially in appetitive and leaving bahaviours. It is impossible to raise

worms on *B. pseudomallei* alone because of the virulence. Consequently, worm starvation over several hours before being transffered into a plate containing pathogen, could be considered in future experiments because hungry worms tend to accept any food they first encounter.

Working with *C. elegans* possesses 2 challenges. First is proper staging of L4 larval stage worms (Figure 2.4) is important in order to obtain reproducible data. The L4 or young adult stage of *C. elegans* is commonly used in most virulence experiments with bacteria, because the worms have maximum number of germ line nuclei (Tan et al., 1999). It is therefore important to start the experiment with synchronized culture with eggs as described in Section 4.2.8.1.

Second, it is difficult to detect and enumerate surviving worms because of phenotype "bagging of worms" in which the progeny has hatched internally. This was observed in nearly all of our preliminary experiments. *C. elegans* continued laying eggs and producing larva as the killing assay extended over its generation time and this phenomenon has been reported from elsewhere (O'Quinn, et al., 2001; Tan, et al., 1999). Addition of this drug FUDR will prevent growth of progeny subsequently prevent interference when conducting the killing assay (Irazoqui et al., 2008; Shivers et al., 2010). An alternative method had been suggested is the use of sterile germ line proliferation-deficient animals generated by *cdc-25.1* gene knock-down (Ashcroft and Golden, 2002).

4.4.5 Structural basis of 2 novel virulence determinants

To better understand the function of the two novel virulence determinants that had not been described previously, i.e., *BPSL 2033* and *BURPS1710A_1419*, the prediction of protein structures and functions from their amino acid sequence was performed by using bioinformatics database.

4.4.5.1 *BPSL2033*

BPSL2033 is a 428 amino acid protein with molecular mass of 46 kDa as a transport-related membrane protein. As seen in Figure 4.17, the predicted amino acid sequences of *BPSL2033* from strain K96243 were identical to other species in genus level with percentage of similarity as: *B. pseudomallei* strains 1710 (99.8%), MSHR305 (99.8%), BURPS1106A (99.5%), MSHR346 (99.5%), 1026b (99.5%) and 668 (99.3%); *B. thailandensis* strains E264 (93.2%) and MSMB121 (93.2%) as well as *B. mallei* NCTC 1022 (99.7%), indicating a high level of conservation.

Further bioinformatics analysis suggests that amino acids 23-324 of *BPSL2033* encode a domain belonging to major facilitator superfamily (MFS). MFS transporters are ubiquitous in all classes of organisms and several pathogens such as *Francisella tularensis* (Marohn et al., 2012) and *Legionella pneumophila* (Sauer et al., 2005). Chatfiled and colleagues (2011) have shown MFS protein play a role in virulence in promoting bacterial iron-siderophore import.

BPSL2033 is a member of the MFS of transporters but has no assigned function yet. Subsequently, homology modeling analysis was carried out by Phyre 2 server to obtain its structural information. The Phyre 2 is used for the alignment of combination hidden

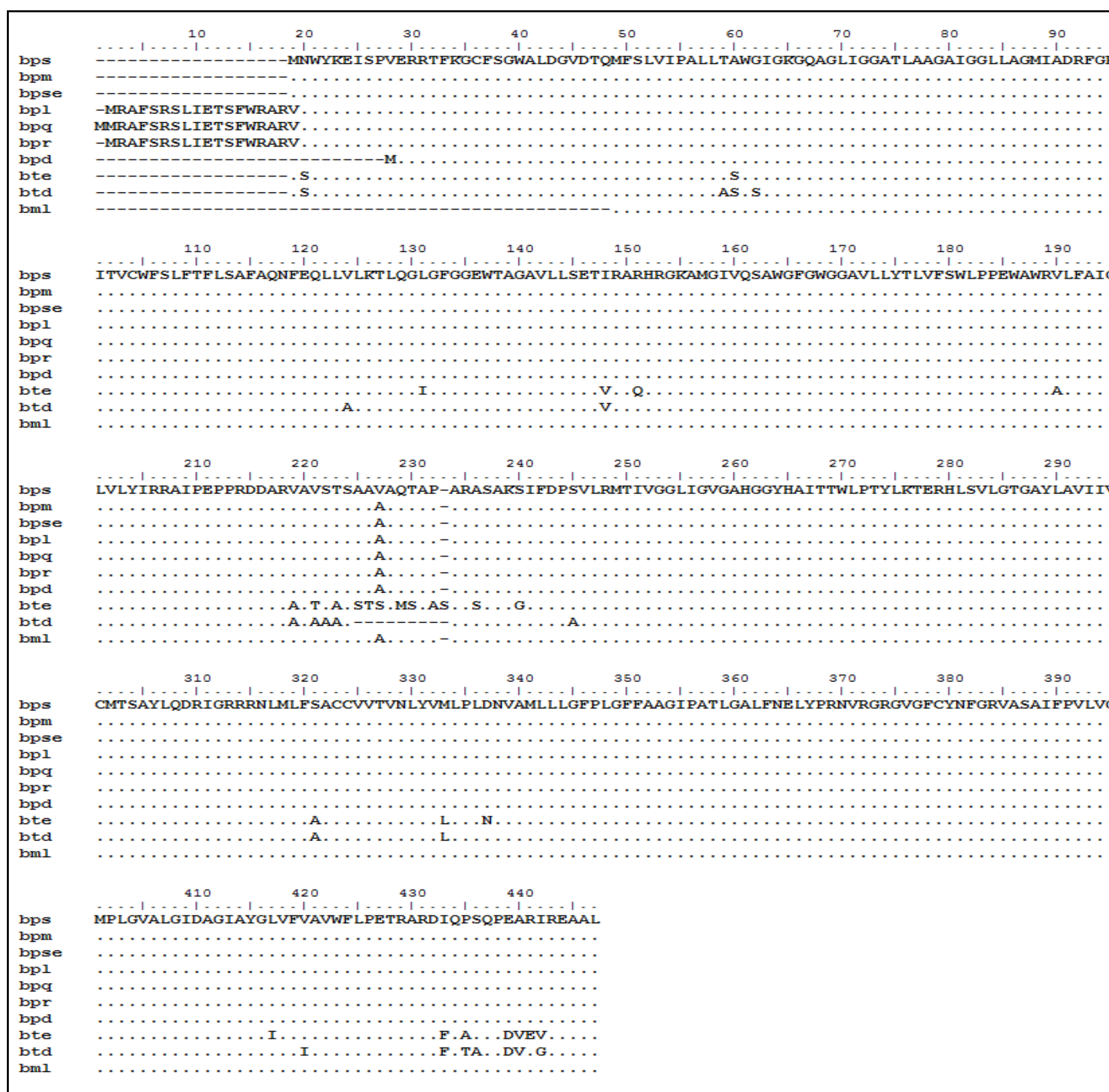


Figure 4.17. ClustalW alignments of amino acid sequences of *BPSL2033*. Species abbreviations: bps, *B. pseudomallei* K96243 (YP_333187.1); bpm, *B. pseudomallei* 1710b (YP_108630.1); bpse, *B. pseudomallei* MSHR305 (YP_008340740.1); bpl, *B. pseudomallei* BURPS1106A_1625 (YP_001065896.1); bpq, *B. pseudomallei* BPC006 (YP_006652457.1); bpr, *B. pseudomallei* MSHR346 (YP_002896364.1); bpd, *B. pseudomallei* 668 (YP_001058645.1); bte, *B. thailandensis* E264 (YP_443199.1); btd, *B. thailandensis* MSMB121 (YP_007918904.1); bml, *B. mallei* NCTC 10229 (YP_001026485.1). Dot indicates sequence identical to a standard Bps.

Markov models and *ab initio* folding simulation that could create the accurate model at very low sequence identity (15-25%) (Kelley and Sternberg, 2009).

BPSL2033 is a member of the MFS of transporters but has no assigned function yet. Subsequently, homology modeling analysis was carried out by Phyre 2 server to obtain its structural information. The Phyre 2 is used for the alignment of combination hidden Markov models and *ab initio* folding simulation that could create the accurate model at very low sequence identity (15-25%) (Kelley and Sternberg, 2009). The first step in the Phyre analysis was to do a secondary structure led to the template 1pw4A (the structure of the E. coli glycerol-3-phosphate transporter protein) even though it has 16% identity in terms of sequence alignment. The results imply that *BPSL2033* might exhibit new structural and/or functional characteristicsand further X-ray crystallography analysis may provide intense investigation. At present, it is postulated that *BPSL2033* transports nutrients (probably glycerol-3-phosphate) that are essential for the replication of *B. pseudomallei*.

4.4.5.2 *BURPS1710A_1419*

BURPS1710A_1419 is a 74 amino acid protein with a calculated molecular mass of 8 kDa is a putative lipoprotein. Database searches with the NCBI blastp tool identified this putative lipoprotein within genus level is diverged from 37 to 82%. As illustrated in Figure 4.18, the putative lipoprotein exhibited 82, 73, 70, 50, 47, 38 and 37% sequence identity to *B. glumae* BGR1, *B. gladioli* BSP3, *B. phymatum* STM815, *B. oklahomensis, B. lata*, *B. phytofirmans* PsJN and *B. ambifaria* MC40-6, respectively. No conserved domain was identified as well as a lack of three-dimensional structural information, possibly suggesting that *BURPS1710A_1419* gene product has new or different functional characteristics.

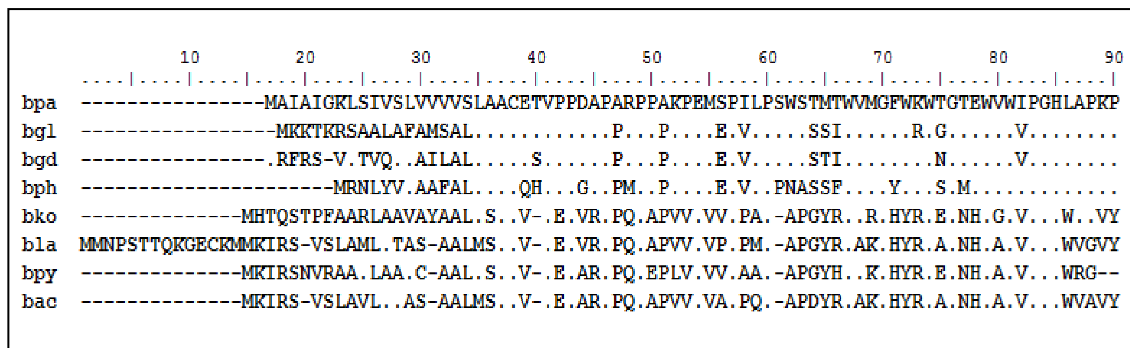


Figure 4.18. ClustalW alignments of *BURPS_1710A_1419* gene products (putative lipoprotein). Species abbreviations: bpa, *B. pseudomallei* BURPS_1710a (WP_004526362.1); bgl, *B. glumae* BGR1 (YP_002909360); bgd, *B. gladioli* BSP3 (YP_004358893); bph, *B. phymatum* STM815 (YP_001860254.1); bko, *B. oklahomensis* (WP_010111058.1); bla, *B. lata* (YP_372144.1); bpy, *B. phytofirmans* PsJN (YP_0018888760); bac, *B. ambifaria* MC40-6 (YP_001811047.1). Dot indicates sequence identical to a standard bpa.

4.5 Concluding discussion and future prospects

In this study, the insertional inactivation of 7 putative virulence determinants and their virulence assessment in 2 different infection models - murine macrophages and a nematode model has facilitated the identification of novel virulence determinants in *B. pseudomallei*.

BPSL2033::Km and *BURPS1710A_1419*::Km showed a reduced survival inside macrophage cell lines. Both complemented strains partial restored the defect in the macrophage cell lines as well as in *C. elegans* model. It is thus hypothesized that both gene, *BPSL2033* and *BURPS1710A_1419* are individually likely to contribute a minor role in virulence and provide a basis for further characterization of their role in pathogenesis.

It is speculated that the combination effect of both genes can provide a clear virulence role in *B. pseudomallei*. However, a major drawback of insertion mutagenesis is inability to construct a double mutant due to the limitation of the difficulty of drug selection. It is suggested that construction of a markerless double-gene deletion mutant in Bp-CMS via an unmarked deletion method warrants further investigation.

For the infection model, mammalian host such as Balb/c mouse is proposed for future experiment. Quantitation of bacterial burden in different organs such as liver, lung and spleen should be examined for tissue pathology in order to obtain additional information related to severity and bacterial load.

4.6 Limitations of the study

A drawback of the method is that no further manipulation of the mutant strains can be performed due to restriction of drug selection, also the same vector cannot be used, and consequently construction of multiple gene knockout mutants is not possible.

Chapter 5

Identification of potential immunogenic polypeptides of *B. pseudomallei* and evaluation of their efficacy for serodiagnosis of melioidosis

5.1 Introduction

B. pseudomallei has emerged as an important human pathogen causing a fatal infectious disease, called melioidosis. It is a gram-negative bacterium found as a saprophyte in soil and commonly present in melioidosis-endemic areas, i.e., northeast Thailand, Malaysia and Northern Australia and increasingly being recognized around the world in recent decades (Wiersinga et al., 2012). In addition to being a public health hazard it is also a potential Class B biological warfare agent (Rotz et al., 2002).

The clinical signs and symptoms of melioidosis are very varied and often seem to mimic other diseases (Section 2.2.6). In addition, it exhibits resistance to diverse groups of antimicrobials including third-generation cephalosporins (Wiersinga et al., 2012). These factors make it difficult to establish a diagnosis and the reported mortality is high among patients with underlying risk factors especially diabetes and immunosuppression (Puthucheary and Vadivelu, 2002).

Definitive identification of *B. pseudomallei* relies exclusively on the gold standard culture of a specimen. Isolation of the organism requires several days (may take up to 4 days) to produce colonies and reportedly can have associated problems such as inter-

strain and medium dependent variability in colony morphology (Puthucheary et al., 2010).

Most recently, quantitative real time PCR targeting various specific regions such as Type Three Secretion 1 (TTS1)-open reading frame 1 (Supaprom et al., 2007), TTS1-opening reading frame 2 (Kaestli et al., 2012) and asparty/asparaginly beta-hydroxylase (*lp*xO) (Kaestli et al., 2012), and loop-mediated isothermal DNA amplification (Chantratita et al., 2008) have been developed for identification of *B. pseudomallei.* However, these assays demonstrated only moderate sensitivities and direct testing on blood specimens remain problematic.

Thus serological assays appear to be a useful tool for the presumptive diagnosis of the melioidosis. In a broader context, serodiagnostic tests can have multiple uses, including: screening for febrile illness (in any patient who had visited endemic areas); patient management (when infections are deep-seated and no specimens are available); monitoring disease activity (investigating the persistence of antibody levels) (Vadivelu and Puthucheary, 2000).

The development of serological diagnostic tests for the detection of antibodies and antigens has been attempted for decades (Sections 2.5.2.1 and 2.5.2.2), but have yielded disappointing results due to lack of specific immunogenic antigen. The most common of these tests has focused on measuring the presence of antibodies against antigens of *B. pseudomallei* including IHA, ICT, IFAT and ELISA.

Among these assays, IHA is most commonly used in disease-endemic countries (Cheng et al., 2006; Chuah et al., 2005; O'Brien et al., 2004), but high background titers raise

problems in differentiating past and current infections (Vadivelu et al., 1995; Wuthiekanun et al., 2006). In addition, the IHA has been reported to have low sensitivity in sera from patients with acute septicemia (Appassakij et al. 1990; Dharakul et al., 1997).

In Malaysia, Vadivelu and coworkers have previously developed an IFAT using a whole cell antigen derived from *B. pseudomallei* for detection of total antibodies (IgG and IgM) in patients with possible melioidosis (Vadivelu et al., 1995). This IFAT test performed better than IHA and had satisfactory sensitivity and specificity compared to culture approach (Vadivelu et al., 1995). However, the drawback is preparation of *B. pseudomallei* as antigen will require biosafety laboratory level 3 (BSL-3) facilities, which are not commonly available in developing countries. A modification of the test by using *B. thailandensis* as antigen was developed and yielded satisfactory results in term of sensitivity (98.8%) and specificity (92.3%) (Puthucheary et al., 2010). However, both IFAT assays using either *B. pseudomallei* or *B. thailandensis* may be influenced by the subjective judgment and they are not readily adaptable to large scale investigations.

ELISA studies using either crude or purified antigen preparations for the detection of antibodies to *B. pseudomallei* demonstrated varying results of sensitivities and specificities (Allwood et al., 2008; Chenthamarakshan et al., 2001b; Druar et al., 2008; Sirisinha et al., 2000). However, this test offer advantages that enable many serum samples to be tested in parallel and the workflow can be completely automated to generate quantitative results.

In summary, a serological test detecting antibody response to recombinant antigen of *B. pseudomallei* would be less complex and less expensive than molecular detection techniques, and more rapid than traditional bacterial culture.

Hence, the general aim of this work was to identify immunogen(s) for the serological diagnosis of infections due to *B. pseudomallei.*

The specific objectives are:

1. to construct shotgun expression library from clinically confirmed local virulent isolates of *B. pseudomallei.*
2. to identify immunogenic polypeptides of *B. pseudomallei* recognized by the sera of patients with IFAT (serologically) proven melioidosis.
3. to evaluate these immunogens for potential use in the serological diagnosis of melioidosis.

5.2 Materials and methods

5.2.1 Bacterial strains and plasmids

Four clinical strains of *B. pseudomallei* were obtained from the University Hospital, University of Malaya (UM), Kuala Lumpur (details are shown in Table 5.1.). These strains had been isolated and identified by standard biochemical tests and the API 20NE system (bioMérieux, France). All 4 strains were then subjected to genomic DNA extraction as previously described (Section 3.6.1).

Table 5.1. Bacterial strains and plasmids used in this study.

Strains/plasmids	Relevant characteristic(s)	Sources of reference
Strains		
B. pseudomallei		
Bp C1	Clinical isolate from blood	Current study
Bp C2	Clinical isolate from swab	
Bp C3	Clinical isolate from sputum	
Bp C4	Clinical isolate from splenic biopsy	
E.coli		
NovaBlue (DE3)	endA1 hsdR17($r_{K12}^{-}m_{K12}^{+}$) supE44 thi-1 recA1 gyrA96 relA1 lac (DE3) F'[proA^{+}B^{+}lacI^{q}Z ΔM15::Tn10] (Tet^{R})	Novagen, USA
Plasmid		
pSCREEN T	Cloning vector	Novagen, USA

5.2.2 Construction of genomic expression library

The NovaTope System (Novagen, USA) was used for genome expression library construction according to the manufacturer's protocol. Ten microgram of pooled DNA of the 4 clinical strains were randomly cleaved with 1 µL of *DNase*I. Each was diluted 1:133, 1:200, 1:300 and 1:450, and incubated for 10 min at room temperature in the presence of 10 mM Mn^{2+}. The digested DNA fragments of 50 to 300 bp were recovered from a 2% (w/v) agarose gel with QIAEX II Gel Extraction Kit (Qiagen, Germany).

One microgram of purified DNA fragments were flush ended with T4 DNA polymerase together with 1X Flush buffer [0.5 M Tris-HCl pH 8.0, 50 mM $MgCl_2$, 1 mg/mL BSA], 1X dNTP mix [1 mM each dCTP, dGTP, dTTP, 10 mM dATP], 5 mM DTT for 20 min at 11ºC, and the reaction was stopped by heating for 10 min at 75ºC. The entire flushing reaction was then added with a single deoxyribosylalanine (dA) residue at 3' end using *Tth* polymerase in the presence of 1X dA Tailing buffer [100 mM Tris-HCl pH 9.0, 0.5 M KCl, 0.1% gelatin, 1% Triton X-100] for 15 min at 70ºC.

The A-tailed DNA fragments were then ligated into a linearized and T-tailed pSCREEN T-vector, designed for the expression of small peptides as a carboxy-terminal fusion to the T7 bacteriophage gene *10* capsid protein. One microliter of the ligation mixture was transformed into 20 µL NovaBlue (DE3) competent cells followed by selection of transformants by plating onto LB agar containing 50 µg/mL carbenicillin (CB) and 15 µg/mL tetracycline (Tet).

5.2.3 Colony immunoscreening

Transformed colonies were picked by sterilized toothpicks and re-streaked on fresh LB-agar containing CB and Tet and incubated overnight at 37ºC. Next day, bacterial colonies were transferred onto nitrocellulose filters (colony side up) and induced with 250 μM IPTG for 4 h at 37ºC on LB-agar plates containing CB and Tet. After induction, the filters were incubated sequentially in the petri dishes at room temperature as follows: 10% (w/v) SDS for 10 min to disrupt cell membranes; denaturing solution for 5 min; neutralization solution for 10 min and lastly 2X saline-sodium citrate (SSC) for 15 min for colony lysis and binding of protein to the filters.

The filters were then washed with 1X Tris-buffered saline (TBS) [10 mM Tris-HCl pH 8.0, 150 mM NaCl] for 10 min and blocked with 1% (v/v) fish gelatin (Amresco, USA) in 1X TBST [10 mM Tris-l pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween-20] for 1 h with gentle agitation at room temperature. Filters were again washed twice in 1X TBST for 10 min to remove bacterial debris and incubated with 2 different pools of sera, i.e., pooled melioidosis positive and melioidosis negative (1:1000 in blocking buffer) for 1 h at room temperature.

Next, filters were washed with 1X TBST and incubated for 1 h with AP-conjugated Immunopure® Protein A/G (1:10000 in blocking buffer) (Pierce, USA). The filters were washed with 1X TBST as described above, immunoreactive clones were then detected and visualized with BCIP/NBT phosphatase substrate (Pierce, USA). Reactions were stopped by immersing the filters in distilled water. Finally, mean signal intensity and background measurements were obtained for each clone on each filter using Image Scanner III (GE Healthcare, USA).

The dataset were analyzed using ImageQuant software (GE Healthcare, USA) and each filter was normalized using the signals of pSCREEN T-vector without insert. Calculation of the ratio of signal of intensities produced by melioidosis positive and negative groups was performed. Reactive clones were then recovered from the master plates and cryopreserved in 20% (v/v) glycerol at -80 ºC.

5.2.4 Plasmid preparation and sequencing

The HiYield™ Plasmid Mini Kit (RBC, Taiwan) was used for plasmid isolation from immunoreactive clones (Section 3.6.2). The plasmids were sent for sequencing (1st Base Laboratories, Malaysia) using the T7 gene *10* primer (5' TGAGGTTGTAGAAGTTCCG 3') and T7 terminator primer (5' GCTAGTTATTGCTCAGCGG 3'). Later, homology searches against known protein databases were performed using BlastX program at NCBI based on NCBI non-redundant protein database (*B. pseudomallei* strains K96243, 1710, 1106, MSHR668, and MSHR305).

5.2.5 Preparation of purified recombinant polypeptides

The selected colonies were cultured overnight at 37ºC in 10 mL of 2X YT broth containing both CB and Tet. The broth culture was diluted 1:20 with 100 mL fresh medium and incubated at 37ºC with shaking at 200rpm until the OD600 had reached the range from 0.5 to 0.7. The culture was induced by adding 250 µM IPTG and incubated for an additional of 4 h at 37ºC. The bacterial cells were collected by centrifugation and resuspended in 500 µL ice-cold 1X TES buffer [0.2 M Tris-HCl, 0.5 mM EDTA, 0.5 M sucrose]. A volume of 750 µL of 1/5X TES was added and the mixture incubated on ice for 30 min with agitation. The supernatant containing the soluble recombinant polypeptides was pelleted by centrifugation and stored at -20ºC for further use. The

concentration of recombinant polypeptides was determined by the Bradford assay (Section 3.4.2).

5.2.6 SDS-PAGE and Western blot analysis

The recombinant polypeptide samples were mixed with 2X Laemmli buffer (1:1) (Sigma-Aldrich, USA) and heated in boiling water for 5 min. The denatured samples were separated by electrophoresis on 12% SDS-PAGE at 170V for 1 h. The gel was transferred to a nitrocellulose membrane using TE 70 Semi-Dry Transfer Unit (GE, USA). Blocking, incubating and washing steps were performed as described in the section 5.2.3. Two pools of sera, i.e., melioidosis-positive and melioidosis-negative were used to confirm the presence of the recombinant polypeptides specific to *B. pseudomallei*. A negative control, pSCREEN T with no insert was included in the experiment.

5.2.7 Serum samples

The collected sera were serologically-confirmed melioidosis by the in-house IFAT test (Vadivelu, et al., 1995), at the University Malaya Medical Centre, Kuala Lumpur, from 2010 to 2012 (Ethical approval MEC: 260.1). Of 60 melioidosis-positive and 123 non-melioidosis sera i.e., IFAT-negative, 8 groups were identified as follows: Group A, sera from patients clinically and serologically (IFAT +ve) confirmed as melioidosis (n=60); Group B, sera from individuals negative (IFAT –ve) for melioidosis (n=60); Group C, healthy blood donors (n=10); Groups D to H, sera from patients who were diagnosed with *Legionella pneumoniae* (n=11); *Leptospirosis* (n=13); *Chlamydophila pneumoniae* (n=10); *Mycoplasma pneumoniae* (n=12) and Rickettsiosis (typhus group) (n=7).

5.2.8 Evaluation of purified recombinant polypeptides

An indirect ELISA was carried out to test the large number of samples in order to confirm the immunoreactivity of clones detected in the colony immunoblot assay. Optimization was performed using antigens (i.e., recombinant polypeptides) with the dilution ranging from 100 ng/µL to 20 µg/mL. The IFAT-confirmed melioidosis positive and negative sera were optimized using dilution ranging from 1:100 to 1:3,000. Antigen concentration of 20 µg/mL and dilution of sera of 1:500 were optimized by checkerboard titration.

ELISAs were performed in duplicate using Costar 96 well microliter plates (Corning, USA). Wells were coated and incubated overnight at 4ºC, with each antigen in 1X PBS (pH 8.4) in a final volume of 100 µL/well. Following the coating step, wells were washed 3 times with PBS containing 0.05% Tween 20 (1X PBST) to remove unbound antigen. Blocking step was then carried out by adding 100 µL of 1% fish gelatin (Amresco, USA) in PBST for 2 h at 37ºC. The plates were again washed using 1X PBST and the wells were incubated with 100 µL of sera at 37ºC for 2 h.

Following incubation, wells were washed 3 times with 1X PBST and adding of AP-conjugated Immunopure® Protein A/G (1:5,000 in blocking buffer) (Pierce, USA) at 37ºC for 2 h. Wells were washed 3 times with 1X PBST and the enzyme reaction was detected by the addition of 100 µL of p-Nitrophenyl Phosphate for 30 min at 37ºC in the dark. A solution of 100 µL of 2N H_2SO_4 was then added to stop the reaction. The optical density (OD) 415nm values were determined using an iMark reader (Biorad, USA).

5.2.9 Statistical analysis

The cutoff values for ELISA were determined by using the mean OD for the IFAT-confirmed melioidosis negative group (Group B) plus 3 standard deviations (M + 3SD). The sensitivity, specificity, positive and negative predictive values were calculated using the formula as below (Zhou et al., 2011):

Infection with *B. pseudomallei*

	Present	**Absent**
Positive result	**A**	**b**
Negative result	**C**	**d**

a: True positives (TP), number of diseased person with a positive test result

b: False positives (FP), number of non-diseased persons with a positive test result

c: False negatives (FN), number of diseased persons with a negative test result

d: True negatives (TN), numbered of non-diseased persons with a negative test result

i) Sensitivity = $\frac{a}{a + c}$

(proportion of people with the target disorder who have a positive test result)

ii) Specificity = $\frac{d}{b + d}$

(proportion of people without the target disorder who have a negative test result)

iii) Positive predictive value (PPV) = $\frac{a}{a + b}$

(the probability of disease among all persons with a positive test result)

iv) Negative predictive value (NPV) = $\frac{d}{c + d}$

(the probability of non-disease among all persons with a negative test result)

v) Positive likelihood ratio $= \dfrac{\text{sensitivity}}{1 - \text{specificity}}$

vi) Negative likelihood ratio $= \dfrac{1 - \text{sensitivity}}{\text{Specificity}}$

5.3 Results

5.3.1 *DNase* I shotgun cleavage

The starting genomic DNA was successfully cleaved into small fragments averaging 50 to 300 bp in size at random location by using different dilutions of *DNase* I. As illustrated in Figure 5.1, 2 dilutions of *DNase* I (1:133 to 1:200) demonstrated good digestion results. So, the same procedure (Section 5.2.2) was repeated and a pool of cleavage reactions containing fragments in the 50 to 300 bp size range was subjected to DNA recovery.

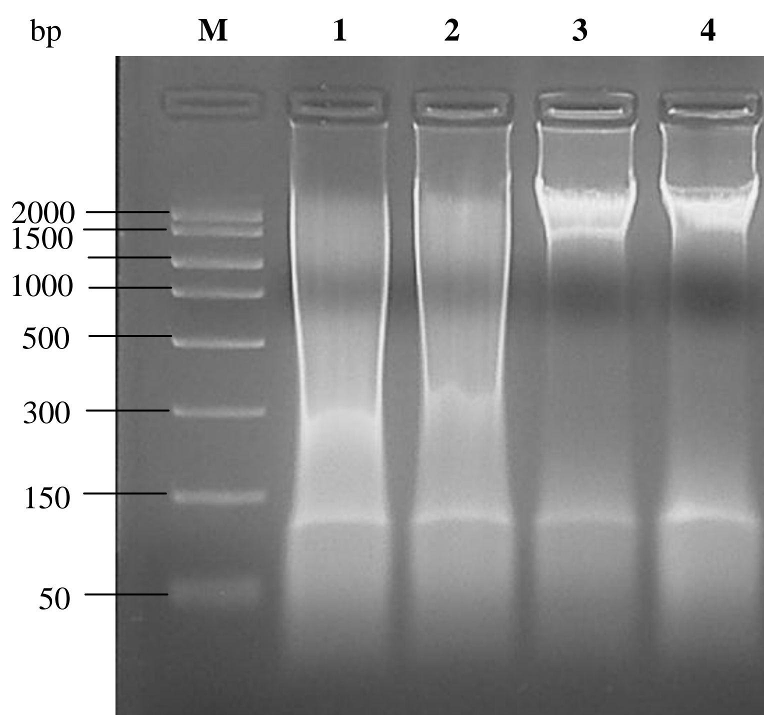


Figure 5.1. EtBr-stained 2% (w/v) agarose gel of digested genomic DNA of *B. pseudomallei.*

Lane (s) M – 100 bp DNA ladder (Fermentas, USA)
1 – 1:133 dilution of *DNase* I
2 – 1:200 dilution of *DNase* I
3 – 1:300 dilution of *DNase* I
4 – 1:450 dilution of *DNase* I

5.3.2 Construction of genomic library and selection of immunogenic polypeptides

A small diversely sized genomic expression library was successfully constructed using local clinical strains of *B. pseudomallei* carrying random fragmented inserts of 50-300 bp. From the resulting library, 480 transformants were chosen at random and subjected to colony blot detection assay using pooled positive (P: IFAT-confirmed positive) and pooled negative sera (N: IFAT-confirmed negative).

A total of 4 sets of pooled sera were prepared for screening purpose, i.e., Pos1, Neg1, Pos2 and Neg2 and each pool containing 10 samples. In initial screening step using sera Pos1 and Neg1, 79 clones with signal intensity ratios (P/N) of two-fold and above were observed. Subsequently, another set of pooled sera (Pos2 and Neg2) was used to retest for their reactivity and finally 20 clones with signal ratio intensities (P/N) of at least 3-fold and above were obtained.

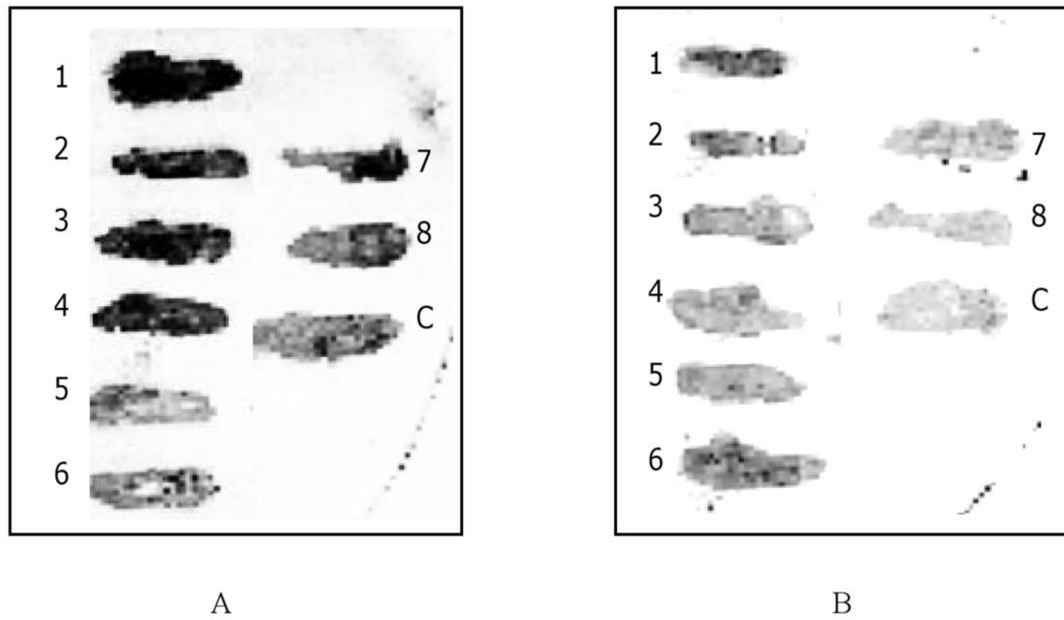


Figure 5.2. Representative clones (1-8) of colony blot assay in 2 different pooled sera.

A- melioidosis positive pooled sera

B- melioidosis negative pooled sera

C- control strain, *E. coli* NovaBlue (DE3) containing the pSCREEN T-vector with no insert

5.3.3 Confirmation of the identity of clones by sequencing

Twenty of the clones were subjected to confirmatory sequencing. As demonstrated in Table 5.2, a search performed using BlastX to identify homologous sequence showed that these clones represented 6 distinct polypeptides. These polypeptides including, BPSS1904, BPSL3130, BURPS1710b_0454, BPSS1856, BPSS0897, BPSS1757 encoded putative proteins corresponding to benzoate 1,2-dioxygenase beta subunit, a putative 200 kDa antigen p200, phosphotransferase enzyme family protein, short chain dehydrogenase and 2 hypothetical proteins as summarized in Table 5.3.

Table 5.2. BlastX results of the 6 recombinant polypeptides of *B. pseudomallei*.

Locus tag	Sequence alignment	Accession no.	Amino acid sequence		
			Identity (%)	Coverage (%)	E-value (%)
BPSS1904	Query 3 KYVVLKNDYINQLIDIYHI 59 KYVVLKNDYINQLIDIYHI Sbjct 145 KYVVLKNDYINQLIDIYHI 163	YP 111910	100	39.7	8.0E-5
BPSL3130	Query 43 MPESPNFPPSYOS 2 MPESPNFPPSYOS Sbjct 1 MPESPNFPPSYOS 14	YP_109723	100	33.9	0.00
BURPS1710b_0454	Query 2 ARFDACGGERRGQRDVQPLEALAVRLAV 84 ARFDACGGERRGQRDVQPLEALAVRLAV Sbjct 189 ARFDACGGERRGQRDVQPLEALAVRLAV 217 85 PAAQHADEIDGCLRARDETGELAHVEGG 168 PAAQHADEIDG LRARDETGELAHVEGG Sbjct 217 PAAQHADEIDGCLRARDETGELAHVEGG 245 169 QLDDVD 187 QLDDVD 245 QLDDVD 250	YP_331868	98	98	8E-31

Table 5.2 continued.

Locus tag	Sequence alignment	Accession No.	Amino acid sequence		
			Identity (%)	Coverage (%)	E-value (%)
BPSS1856	Query 114 IHNDFRFDNVVLDPADPLSIVGVLDWEM 31 IHNDFRFDNVVLDPADPLSIVGVLDWEM Sbjct 214 IHNDFRFDNVVLDPADPLSIVGVLDWEM 241 Query 30 ATLGDPLMDL 1 ATLGDPLMDL Sbjct 242 ATLGDPLMDL 251	YP 111860	100	99	3E-17
BPSS0897	Query 1 DYYLQGRALPRAQVPADVTGPVLFLLSD 84 DYYLQGRALPRAQVPADVTGPVLFLLSD Sbjct 206 DYYLQGRALPRAQVPADVTGPVLFLLSD 233 Query 85 AARFVTGQLLPVNGGF 132 AARFVTGQLLPVNGGF Sbjct 234 AARFVTGQLLPVNGGF 249	YP 110906	98	98	3E-21
BPSS1757	Query 206 MKAAMRAKESEr l a t I r l l l a aIKQREVD 121 MKAAMRAKESERLATIRLLLAAIKQREVD Sbjct 11 MKAAMRAKESERLATIRLLLAAIKQREVD 40 Query 120 ERVTLDDAGCTACCDKMIKQRKDSISQFE 33 ERVTLDDAGCTACCDKMIKQRKDSISQFE Sbjct 41 ERVTLDDAGCTACCDKMIKQRKDSISQFE 68 Query 32 AAGRTDLVEK 3 AAGRTDLVEK Sbjct 69 AAGRTDLVEK 78	YP 105144	100	98	3E-27

Table 5.3. Characteristics of the 6 recombinant polypeptides of *B. pseudomallei*.

Locus tag	Chromosome	Putative identity	Predicted function	Cellular location [a]
BPSS1904	2	benzoate 1,2-dioxygenase beta subunit	Secondary metabolites biosynthesis, transport, and catabolism	C
BPSL3130	1	hypothetical protein	Function unknown	CM
BURPS1710b_0454	1	200 kDa antigen p200, putative	Cell envelope biogenesis, outer membrane / carbohydrate transport and metabolism	C
BPSS1856	2	phosphotransferase enzyme family protein	Function unknown	C
BPSS0897	2	short chain dehydrogenase	Secondary metabolites biosynthesis, transport, and catabolism	C
BPSS1757	2	hypothetical protein	Function unknown	C

[a] As predicted by PSORTb V3.0 .Cytoplasm (C), cytoplasmic membrane (CM).

5.3.4 SDS-PAGE and Western blot analysis of the 6 recombinant polypeptides

Those selected 6 clones were induced to express a small peptide derived from the protein under study and the expressed polypeptides were separated under denaturing conditions on a 12% SDS-PAGE gel. Figure 5.3 shows a band of approximately 46 to 48 kDa (predicted calculated molecular weight). The band was clearly detected for all purified recombinant polypeptides indicating that protein under study was successfully expressed in the prokaryotic system.

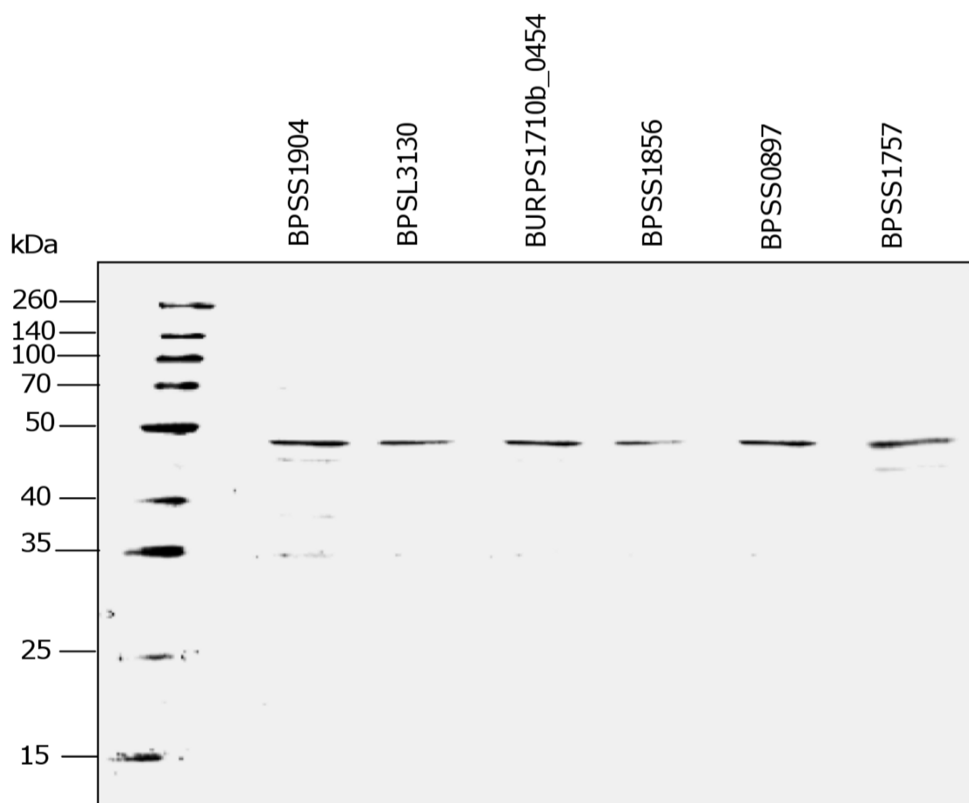


Figure 5.3. SDS-PAGE showing purified recombinant polypeptides.

The protein samples (~20µg) were fractionated and stained with Bio-Safe Coomassie Blue.

Subsequently, the expressed recombinant polypeptides were confirmed through western blotting using patients' sera. As shown in Figure 5.4, all 6 recombinant polypeptides demonstrated strong reactivity towards pooled melioidosis-positive sera (n=10) when compared to the pooled negative sera (n=10). No reactivity with either melioidosis-positive or negative sera was observed for the negative control, pSCREEN T vector with no insert.

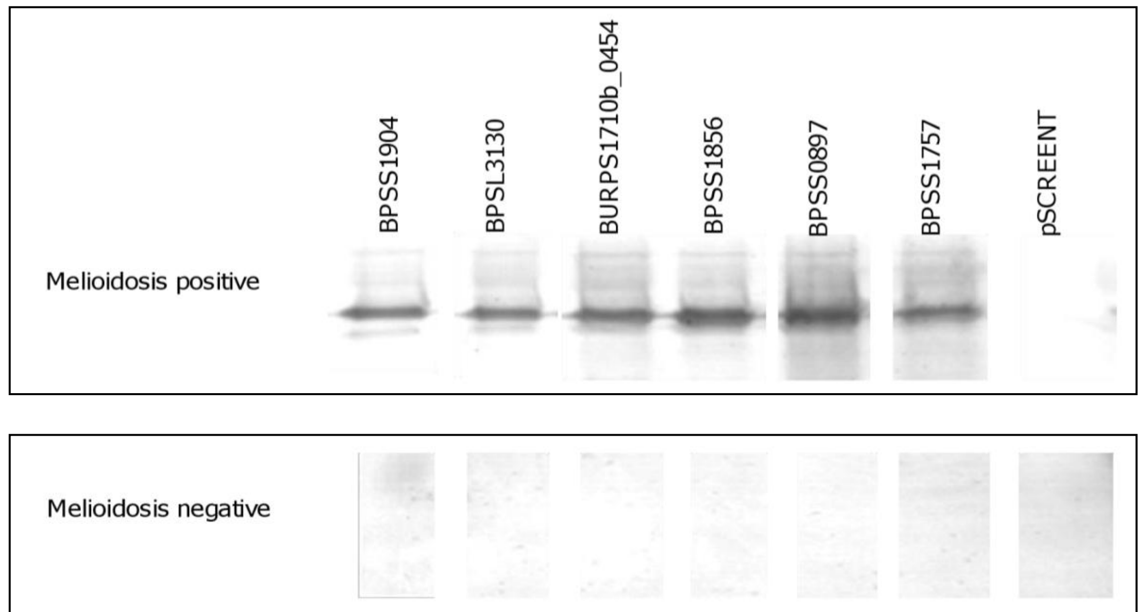


Figure 5.4. Western blot analysis of candidate polypeptide antigens.

Western blot with two different pool of sera obtained from melioidosis-positive and negative patients that were confirmed serologically by in-house IFAT assay. Each pooled sera was a mixture of 10 serum samples.

5.3.5 Investigation on the serodiagnostic potential of the 6 recombinant polypeptides

Based on our in-house IFAT as a reference test (sensitivity and specificity equal to 100%), the cutoff point was defined as the mean A_{415} + 3SD of the IFAT-confirmed melioidosis-negative sera. The cutoff values were 0.123, 0.133, 0.145, 0.136, 0.198 and 0.122 for BPSS1904, BPSL3130, BURPS1710b_0454, BPSS1856, BPSS0897 and BPSS1757, respectively. These values enabled us to identify positive and negative sera and the percentages of positive sera were calculated as demonstrated in Table 5.4. Furthermore, an estimation of the ELISA performance was also calculated and is presented in Table 5.5.

Overall, the 6 recombinant polypeptides showed widely variable performance in ELISA test, with sensitivities and specificities ranging from 33.3 to 97.6%. Two recombinant polypeptides, BPSS1904 and BPSL3130 recognized a large proportion of the melioidosis positive sera (54/60) and (45/60); however cross-reactions with some of the disease control sera were also observed (Table 5.4). Overall, both BPSS1904 and BPSL3130 demonstrated satisfactory performance, sensitivities of 75.0% and 90.0%, specificities of 90.2% and 88.6%, positive predictive value of 79.0% and 79.4%, negative predictive value of 88.1% and 94.8%, positive likelihood ratio of 7.79 and 7.91 and negative likelihood of 0.28 and 0.11, respectively (Table 5.5).

Table 5.4. ELISA screening for melioidosis and disease control sera using the 6 recombinant polypeptides.

Disease group	Percentage positivity					
	BPSS1904	**BPSL3130**	**BURPS1710b_0454**	**BPSS1856**	**BPSS0897**	**BPSS1757**
A Melioidosis-positive (n=60)	75.0 (45/60)	90.0 (54/60)	66.7 (40/60)	66.7 (40/60)	60.0 (36/60)	33.3 (20/60)
B Melioidosis-negative (n=60)	1.7 (1/60)	1.7 (1/60)	0/60	1.7 (1/60)	0/60	3.3 (2/60)
C Healthy control (n=10)	0/60	0/60	0/60	0/60	0/60	0/60
D *Legionella pneumoniae* (n=11)	36.4 (4/11)	36.4 (4/11)	27.3 (3/11)	36.4 (4/11)	27.3 (3/11)	0/11
E Leptospirosis (n=13)	15.4 (2/13)	7.7 (1/13)	7.7 (1/13)	15.4 (2/13)	7.7 (1/13)	0/13
F *Chlamydophila pneumoniae* (n=10)	20.0 (2/10)	20.0 (2/10)	0/10	20.0 (2/10)	0/10	10.0 (1/10)
G *Mycoplasma pneumoniae* (n=12)	16.7 (2/12)	33.3 (4/12)	25.0 (3/12)	25.0 (3/12)	25.0 (3/12)	0/12
H Typhus (rickettsiosis) (n=7)	14.3 (1/7)	28.6 (2/7)	0/7	0/7	0/7	0/7

Table 5.5. Evaluation of the performance of the 6 *B. pseudomallei* recombinant polypeptides.

Percentage (95% CI)	BPSS1904	BPSL3130	BURPS1710b_0454	BPSS1856	BPSS0897	BPSS1757
Sensitivity	75 (62.1-85.3)	90 (79.5-96.2)	66.7 (53.3-78.3)	66.7 (53.3-78.3)	60 (46.5-72.4)	33.3 (21.7-46.7)
Specificity	90.2 (83.6-94.9)	88.6 (81.6-93.6)	94.3 (88.6-97.7)	90.2 (83.6-94.9)	94.3 (88.6-97.7)	97.5 (93.0-99.5)
Positive predictive value	79.0 (66.1-88.6)	79.4 (67.9-88.3)	85.1 (71.7-93.8)	76.9 (63.2-87.5)	83.7 (69.3-93.2)	87.0 (66.4-97.1)
Negative predictive value	88.1 (81.1-93.2)	94.8 (89.0-98.1)	85.3 (78.2-90.8)	84.7 (77.5-90.5)	82.9 (75.6-88.7)	75.0 (67.6-81.5)
Positive likelihood ratio	7.69 (4.40-13.42)	7.91 (4.79-13.04)	11.71 (5.68-24.59)	6.83 (3.98-12.04)	10.54 (4.99-22.29)	13.67 (4.23-44.19)
Negative likelihood ratio	0.28 (0.18-043)	0.11 (0.05-0.24)	0.35 (0.25-0.51)	0.37 (0.26-0.53)	0.42 (0.31-0.58)	0.68 (0.57-0.82)

5.4 Discussion

5.4.1 Shotgun genomic expression library as a tool for the screening of immunogens

In the past, several attempts have been made to identify potential immunodominant antigens of *B. pseudomallei*, including proteomic-based biotinylation and immunoscreening approaches (Harding et al., 2007), bioinformatics prediction tools (Thompson et al., 2008) and protein array (Felgner et al., 2009; Suwannasaen et al., 2011). Although several immunogenic proteins in *B. pseudomallei* have been identified such as GroEL (Woo et al., 2001), OmpA (Hara et al., 2009) and Elongation factor-Tu (Nieves et al., 2010), these immunogens have not been further evaluated as potential serodiagnostic markers.

In the present study, shotgun genomic expression approach was chosen and utilized to facilitate the discovery of candidate antigens. This is due to the fact that shotgun expression method is more economical and less demanding compared to an array of platforms available commercially, for example, genome-wide proteome or immunogen arrays that allow the selection of candidates from a large number of proteins in *Mycobacterium tuberculosis* (Sartain et al., 2006) and *Francisella tularensis* (Sundaresh et al., 2007).

The introduction of recombinant expression, DNA vectors and purification of protein expressed in *E. coli* in NovaTope System provides a valuable tool for antigen discovery research. Most polypeptides are relatively stable and can be produced by inserting a desired sequence of interest into expression vector and producing large quantities of polypeptides, thus avoiding the problem of batch to batch variation.

This approach offered an effective and quicker strategy to thoroughly examine the short peptides or polypeptides expressed corresponding to a contiguous amino acid sequence fragment of a protein. In particular, the small foreign sequence of *B. pseudomallei* with an average insert size of 50 to 300 bp is stably expressed as part of a fusion containing the T7 gene *10* protein. This fusion method ensures that DNA fragment encoding protein will be efficiently transcribed and translated under the control of T7 RNA polymerase in *E. coli* NovaBlue DE3, which prevents the target sequence from breakdown by cellular proteases.

In addition, this system offers an advantage as the protein isolated will not be a full-length, but it will correspond only to a polypeptide representing the antibody-binding site involved in the recognition. The full-length of pure recombinant proteins are prone to degradation and misfolding, but the production of recombinant polypeptides is much easier and reproducible production with minimal batch-to-batch variation. This system allows the production of desired homogeneous antigenic polypeptides in large quantities from the bacterial recombinant and easily applied to other formats such as ELISA.

The polypeptide epitopes may allow for distinct specificities and subsequently avoiding unimportant and/or unwanted epitope present in whole protein. In other words, the use of essential sequences only on peptides is a straightforward approach to capture antigen-specific antibodies from serum samples. More recently, Casey et al. (2006) and Al-Khodari et al. (2011) provided evidence to suggest the potential for the use of peptide as an alternative to the complex antigens for serological detection of Epstein-Barr virus infection and tuberculosis, respectively. In contrast with the isolation of native antigen by handling a live infectious *B. pseudomallei* organism, production of recombinant

polypeptides without the need to use the entire pathogen, thus reducing the associated risk.

From a diagnostic perspective, relevant peptides are those that show significant ability to be recognized by any of the melioidosis-positive sera. In fact, the natural variation is expected to be occurred in immune response against *B. pseudomallei*. The reasons for heterogeneity of immune response are not known, but could be explained for example, by differences in the mode of acquisition, strain differences in the organisms, difference in the magnitude of the infecting bacterial load, as well as its immunological memory (previous exposure to different antigens). Therefore, the best search is to use of pooled sera from multiple patients as mentioned in Section 5.3.2 in panning procedures for minimizing individual patient variations in the immune response.

As a result, the effectiveness of the shotgun expression library with an average insert size of 50 to 300 bp expressing partial ORF, permitted the identification of immunogenic polypeptides that reacted with pooled positive sera from melioidosis patients (Section 5.3.4), see Figure 5.5 for a summary. This approach had been used successfully in the identification of useful immunodominant polypeptides of *Mycobacterium tuberculosis* (Bisen et al., 2003).

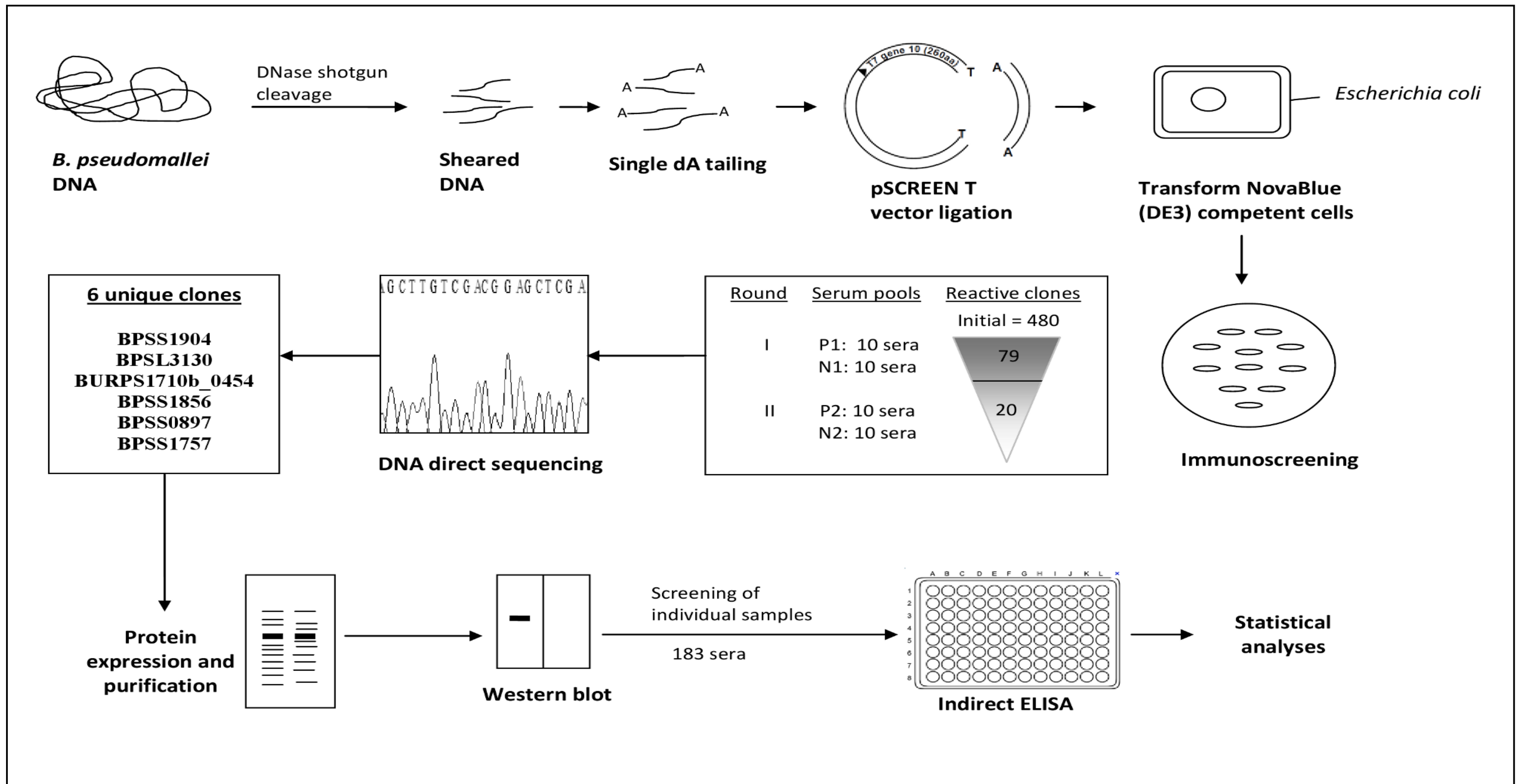


Figure 5.5. Summary of the experiments carried out in this study.

5.4.2 Analysis of the identified potential immunogenic polypeptides

By combining shotgun expression library and immunoscreening assay, 6 polypeptides that had not been described previously, i.e., BPSS1904, BPSL3130, BURPS1710b_0454, BPSS1856, BPSS0897 and BPSS1757 were successfully identified. These 6 new polypeptides reported here exhibited immunogenicity during human infection which was verified by western blot anlaysis (Section 5.3.4). So, it is tempting to hypothesize that these candidate polypeptides are probably displayed on the surface of the bacterium and might play an important role in the interaction with host cells during *in vivo* infection, and are thus referred to as immunogenic polypeptides. They also deserve further investigation as attractive immunodiagnostic candidates.

The hypothesis prompted us to predict subcellular location of these immunogenic polypeptides using PSORTb V3.0. Five of the polypeptides consist of cytoplasmic localized proteins while another one located in the cytoplasmic membrane. These results are in good agreement with previous study, Su and coworkers (2008) reported that a large portion of immunogenic proteins involved in human infection consists of cytoplasmic proteins (30%) and cytoplasmic membrane (24%). It is still unclear why a cytoplasmic protein generated a humoral response, but the authors propose that these proteins are released into surrounding during destruction of the bacterial cells by phagocytes, antimicrobial drugs or complement-mediated killing and then exposed to the immune system (Su et al., 2008).

Based on bioinformatics database, these 6 immunogenic polypeptides are predicted to be involved in secondary metabolite biosynthesis, cell envelope biogenesis as well as hypothetical proteins of unknown function(s) (Table 5.3). The majority of these immunogenic polypeptides were located on chromosome 2 (Table 5.3). So, the finding

was in concordance with those reported previously whereby chromosome 2 has been suggested to be associated with genes required for adaptation and survival in different niches (Holden et al., 2004) as mentioned in Section 2.2.2.

It has to be assumed that such polypeptides exhibit moonlighting activities (Henderson and Martin, 2011), i.e., have more than one biological action. Their detection may enhance our understanding of the pathogenicity of *B. pseudomallei*. Supporting this, 2 immunogens BPSS1904 and BPSS0897 have previously been described might be involved in the adaptation process during the early stationary phase of growth (Wongtrakoongate et al., 2011) and in response to salt stress associated with T3SS genes (Pumirat et al., 2010), respectively. BPSS1856 has homology to *Streptomyces coelicolor* putative phosphotransferase that might play a role in carbon metabolism (Kamionka et al., 2002) while BURPS1710b_0454 is predicted to regulate cell envelope biogenesis.

5.4.3 Evaluation of the efficacy of recombinant polypeptides for serodiagnosis of melioidosis

To the best of our knowledge, these 6 immunogenic polypeptides have not been previously evaluated as serodiagnostic antigens for melioidosis. Indeed, the cocktail of recombinant polypeptides may have contained contaminants with low impurities derived from *E. coli* NovaBlue (DE3) or pSCREEN T vector and could have influenced the specificity of the assay. Consequently, the 6 immunogens were purified and their diagnostic values were assessed by using ELISA format in this study. The main advantages of ELISA are their simplicity, speed (many serum samples can be tested in parallel) and generation of quantitative results in a short time frame compared to colony blot assay, which is cumbersome and poorly standardized.

In ELISA assay, our panel of polypeptides can detect IgG and IgM antibodies typically produced in human subjects after infection with *B. pseudomallei*. The best overall performance was achieved using BPSS1904 and BPSL3130 with sensitivities, specificities, positive and negative predictive values of above 75% (Table 5.5). It is therefore suggested that both polypeptides are potential candidate antigens. Still, cross-reactive responses were observed with disease-control sera in this study and this observation also has been reported by other studies (Chen et al., 2003; Hara et al., 2013). A possible reason for antibody response to these cross-reactions may be due to *B. pseudomallei* containing immunologic epitopes similar to other microorganisms. For example, BPSS1757 showed the identified antigenic cross-reactivity site to leptospira bacteria as indicated in Figure 5.6.

```
BPSL1757    LDDAGVTACCDKMIKQRKDS I S QFEAAGRTDLVEK
Leptospira  LT DT SVMQILKTNYKRRKDTALEYDKANRPDLS S K
            *  *   *         *  * * *       *  *  * *   *
```

Figure 5.6. Alignment of amino acid sequences.

The similarities among strains are marked as *.

Thus far, investigation of recombinant antigens for serodiagnosis of *B. pseudomallei* infection using ELISA format have only been analyzed in 3 studies, i.e., each from Taiwan (Chen et al., 2003); Australia (Allwood et al., 2008) and Malaysia (Hara et al., 2013). Different recombinant antigens were overexpressed, purified and evaluation have provided varying results with moderate to good sensitivities, specificities and diagnostic odd ratio (DOR) (Figure 5.7). DOR measures the diagnostic accuracy of a given diagnostic test by summarizing function of specificity and sensitivity (Glas et al., 2003). A DOR of 1 represent the test does not discriminate between patients with the disease and those without it.

The value of DOR ranged from 5.08 to 1067.50, with higher values indicating better discriminatory test performance. The recombinant OmpA ELISA assays demonstrated high sensitivity (95%), specificity (98%) and DOR (1067.50) (Allwood et al., 2008). However, a very weak positive response was reported by authors as the means ELISA readings obtained for tested samples were as low as 0.061 at dilution 1:125 and 0.041 at dilution 1:250. The observation herein may not constitute a true positive in a clinical situation and further investigation is needed. Another study from non-endemic area, Taiwan, also demonstrated good ELISA results in sensitivity (94%), specificity (96%) and DOR (385) by using recombinant truncated flagellin, but the assay was not evaluated using sera from endemic regions (Chen et al., 2003).

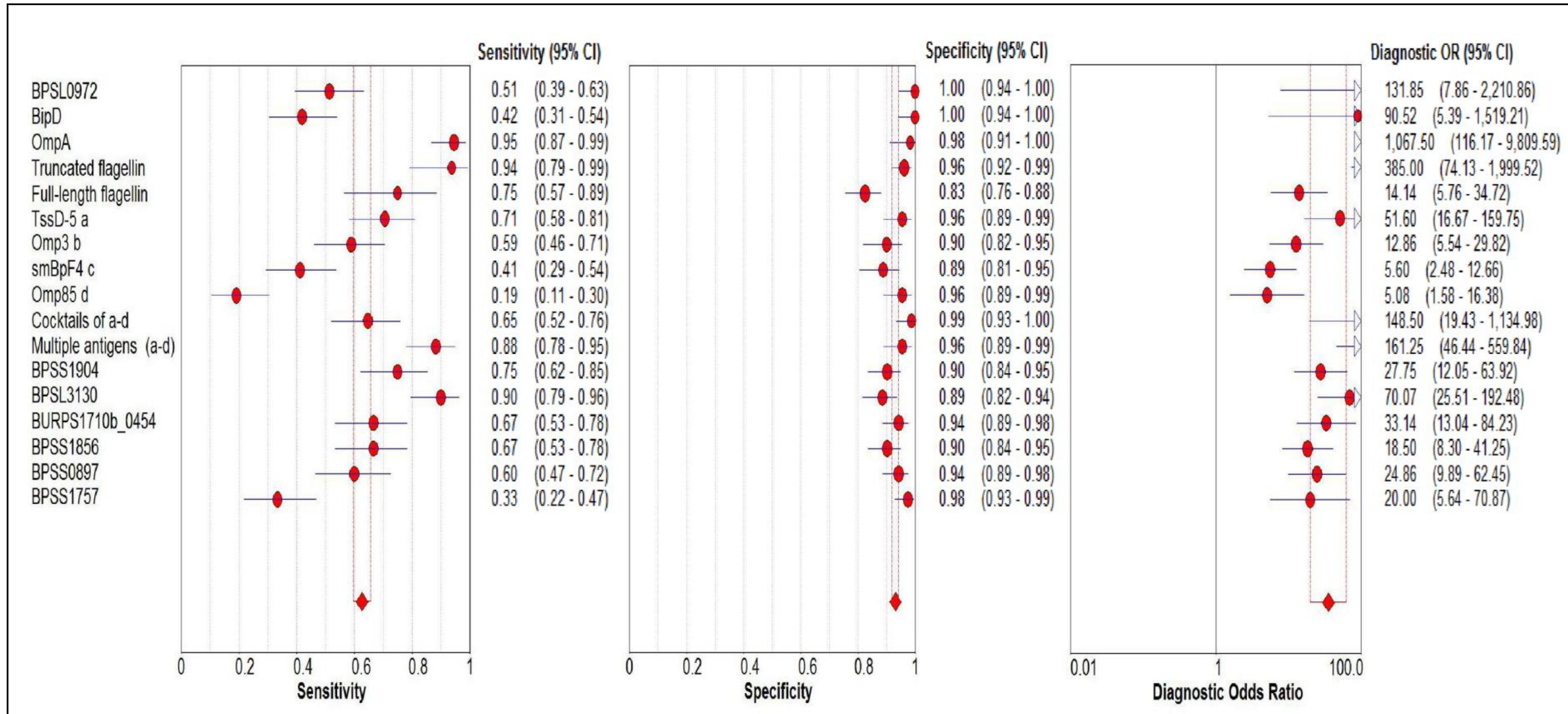


Figure 5.7. Sensitivities, specificities and diagnostic odds ratio.

Interestingly, another group of investigators have shown that using multiple antigens (TssD-5, Omp3, smBpF4 and Omp85) improved sensitivity of 88.2% and DOR of 161.25 while retaining good specificity compared to results when the antigens were used separately (Hara et al., 2013). In this study, serum samples from melioidosis patients reacted with, at least, one recombinant antigen, therefore it is tempting to hypothesize that the use of 2 (BPS1904 and BPSL3130) or all recombinant immunogenic polypeptides as a combination antigen could be able to detect anti-*B.pseudomallei* antibodies in all the serum samples used in this study. It is believed that the use of combination antigens will minimize individual variation and may further enhance sensitivity and DOR of assay.

5.5 Concluding discussion and future prospects

Overall, the systematic stepwise shotgun genomic expression library screening and ELISA assay enabled the identification of 2 potential immunodominant polypeptides, *B. pseudomallei,* i.e., BPSS1904 and BPSL3130, although their sensitivities were only moderate. Still, assays based on the detection of immune responses to melioidosis are attractive additional test for diagnosis of infection with *B. pseudomallei*.

Thus, it is suggested that further analysis using both BPSS1904 and BPSL3130 in combination, would probably be the superior potential serodiagnostic antigens for melioidosis. As single antigen tests did not have sufficient analytical sensitivity, the use of a combination of several polypeptides may minimize variations among individuals in their immune response and further enhance sensitivity and DOR of the assays.

In addition, synthetic peptides in their pure forms corresponded to the desired sequence of BPSS1904 and BPSL3130 could be used in future experiments in order to enhance sensitivity and specificity by lowering the background of the assay. On the other hand, recruitment of more samples for further evaluation is needed to obtain a thorough diagnostic profile.

5.6 Limitations of the study

Some limitations have been noted here although the expreimental design was carefully planned. It is unavoidable that in the shotgun library, the DNA sequence is not known from the start and the clones must be sequenced to obtain an identity. Another drawback was that only 1/6 inserts will be in the correct reading frame in the starting DNA by using DNase shotgun procedure.

References

ABBINK, F. C., ORENDI, J. M. & DE BEAUFORT, A. J. 2001. Mother-to-child transmission of *Burkholderia pseudomallei*. *N Engl J Med,* 344**,** 1171-2.

ALICE, A. F., LOPEZ, C. S., LOWE, C. A., LEDESMA, M. A. & CROSA, J. H. 2006. Genetic and transcriptional analysis of the siderophore malleobactin biosynthesis and transport genes in the human pathogen *Burkholderia pseudomallei* K96243. *J Bacteriol,* 188**,** 1551-66.

AL-KHODARI, N. Y., AL-ATTIYAH, R. & MUSTAFA, A. S. 2011. Identification, diagnostic potential, and natural expression of immunodominant seroreactive peptides encoded by five Mycobacterium tuberculosis-specific genomic regions. *Clin Vaccine Immunol,* 18**,** 477-82.

ALLWOOD, E. M., DEVENISH, R. J., PRESCOTT, M., ADLER, B. & BOYCE, J. D. 2011. Strategies for Intracellular Survival of *Burkholderia pseudomallei*. *Front Microbiol,* 2**,** 170.

ALLWOOD, E. M., LOGUE, C. A., HAFNER, G. J., KETHEESAN, N., NORTON, R. E., PEAK, I. R. & BEACHAM, I.R. 2008. Evaluation of recombinant antigens for diagnosis of melioidosis. *FEMS Immunol Med Microbiol,* 54**,** 144-53.

ALTUN, Z. F., & HALL, D.H. 2005. Hermaphrodite anatomy. In Altun, Z. F., & Hall, D.H. (Eds.)**,** WormAtlas.

AMORNCHAI, P., CHIERAKUL, W., WUTHIEKANUN, V., MAHAKHUNKIJCHAROEN, Y., PHETSOUVANH, R., CURRIE, B. J., NEWTON, P. N., VAN VINH CHAU, N., WONGRATANACHEEWIN, S., DAY, N. P. & PEACOCK, S. J. 2007. Accuracy of *Burkholderia pseudomallei* identification using the API 20NE system and a latex agglutination test. *J Clin Microbiol,* 45**,** 3774-6.

ANUNTAGOOL, A., INTACHOTE, P., NAIGOWIT, P. & SIRISINHA, S. 1996. Rapid antigen detection assay for identification of *Burkholderia (Pseudomonas) pseudomallei* infection. *J Clin Microbiol,* 34**,** 975-6.

ANUNTAGOOL, N., NAIGOWIT, P., PETKANCHANAPONG, V., ARAMSRI, P., PANICHAKUL, T. & SIRISINHA, S. 2000. Monoclonal antibody-based rapid identification of *Burkholderia pseudomallei* in blood culture fluid from patients with community-acquired septicaemia. *J Med Microbiol,* 49**,** 1075-8.

ANUNTAGOOL, N., RUGDECH, P. & SIRISINHA, S. 1993. Identification of specific antigens of *Pseudomonas pseudomallei* and evaluation of their efficacies for diagnosis of melioidosis. *J Clin Microbiol,* 31**,** 1232-6.

APPASSAKIJ, H., SILPAPOJAKUL, K. R., WANSIT, R. & PORNPATKUL, M. 1990. Diagnostic value of the indirect hemagglutination test for melioidosis in an endemic area. *Am J Trop Med Hyg,* 42**,** 248-53.

ARJCHAROEN, S., WIKRAIPHAT, C., PUDLA, M., LIMPOSUWAN, K., WOODS, D. E., SIRISINHA, S. & UTAISINCHAROEN, P. 2007. Fate of a *Burkholderia pseudomallei* lipopolysaccharide mutant in the mouse macrophage cell line RAW 264.7: possible role for the O-antigenic polysaccharide moiety of lipopolysaccharide in internalization and intracellular survival. *Infect Immun,* 75**,** 4298-304.

ASHCROFT, N. & GOLDEN, A. 2002. CDC-25.1 regulates germline proliferation in *Caenorhabditis elegans*. *Genesis,* 33**,** 1-7.

ASHDOWN, L. R. 1979. An improved screening technique for isolation of *Pseudomonas pseudomallei* from clinical specimens. *Pathology,* 11**,** 293-7.

ATTREE, O. & ATTREE, I. 2001. A second type III secretion system in *Burkholderia pseudomallei*: who is the real culprit? *Microbiology,* 147**,** 3197-9.

BADRAN, S., PEDERSEN, T. I., ROED, C., LUNDING, S., BIRK, N., VESTERGAARD, H., RODER, B., LILLELUND, H. K., KURTZHALS, J. A., KEMP, M. & CHRISTENSEN, J. J. 2010. Imported melioidosis in Danish travellers: a diagnostic challenge. *Scand J Infect Dis,* 42**,** 445-9.

BALDER, R., LIPSKI, S., LAZARUS, J. J., GROSE, W., WOOTEN, R. M., HOGAN, R. J., WOODS, D. E. & LAFONTAINE, E. R. 2010. Identification of *Burkholderia mallei* and *Burkholderia pseudomallei* adhesins for human respiratory epithelial cells. *BMC Microbiol,* 10**,** 250.

BISEN, P. S., GARG, S. K., TIWARI, R. P., TAGORE, P. R., CHANDRA, R., KARNIK, R., THAKER, N., DESAI, N., GHOSH, P.K., FRAZIANO, M. & COLIZZI, V. 2003. Analysis of the shotgun expression library of the *Mycobacterium tuberculosis* genome for immunodominant polypeptides: potential use in serodiagnosis. *Clin Diagn Lab Immunol,* 10**,** 1051-8.

BODDEY, J. A., DAY, C. J., FLEGG, C. P., ULRICH, R. L., STEPHENS, S. R., BEACHAM, I. R., MORRISON, N. A. & PEAK, I. R. 2007. The bacterial gene lfpA influences the potent induction of calcitonin receptor and osteoclast-related genes in *Burkholderia pseudomallei*-induced TRAP-positive multinucleated giant cells. *Cell Microbiol,* 9**,** 514-31.

BODDEY, J. A., FLEGG, C. P., DAY, C. J., BEACHAM, I. R. & PEAK, I. R. 2006. Temperature-regulated microcolony formation by *Burkholderia pseudomallei* requires pilA and enhances association with cultured human cells. *Infect Immun,* 74**,** 5374-81.

BOWERS, J. R., ENGELTHALER, D. M., GINTHER, J. L., PEARSON, T., PEACOCK, S. J., TUANYOK, A., WAGNER, D. M., CURRIE, B. J. & KEIM, P. S. 2010. BurkDiff: a real-time PCR allelic discrimination assay for *Burkholderia pseudomallei* and *B. mallei*. *PLoS One,* 5**,** e15413.

BRENNER, S. 1974. The genetics of *Caenorhabditis elegans*. *Genetics,* 77**,** 71-94.

BRUCE, H. S. 2002. Melioidosis: an important emerging infectious disease - a military problem? *ADF Health,* 3**,** 13-21.

BROWN, N. F., BODDEY, J. A., FLEGG, C. P. & BEACHAM, I. R. 2002. Adherence of *Burkholderia pseudomallei* cells to cultured human epithelial cell lines is regulated by growth temperature. *Infect Immun,* 70**,** 974-80.

BURTNICK, M. N., BRETT, P. J., HARDING, S. V., NGUGI, S. A., RIBOT, W. J., CHANTRATITA, N., SCORPIO, A., MILNE, T. S., DEAN, R. E., FRITZ, D. L., PEACOCK, S. J., PRIOR, J. L., ATKINS, T. P. & DESHAZER, D. 2011. The cluster 1 type VI secretion system is a major virulence determinant in *Burkholderia pseudomallei*. *Infect Immun,* 79**,** 1512-25.

BURTNICK, M. N., BRETT, P. J., NAIR, V., WARAWA, J. M., WOODS, D. E. & GHERARDINI, F. C. 2008. *Burkholderia pseudomallei* type III secretion system mutants exhibit delayed vacuolar escape phenotypes in RAW 264.7 murine macrophages. *Infect Immun,* 76**,** 2991-3000.

CAMPOS, C. G., BORST, L. & COTTER, P. A. 2013. Characterization of BcaA, a putative classical autotransporter protein in *Burkholderia pseudomallei*. *Infect Immun,* 81**,** 1121-8.

CASEY, J. L., COLEY, A. M., STREET, G., PARISI, K., DEVINE, P. L. & FOLEY, M. 2006. Peptide mimotopes selected from a random peptide library for diagnosis of Epstein-Barr virus infection. *J Clin Microbiol,* 44**,** 764-71.

CHAN, S. W., ONG, G. I. & NATHAN, S. 2004. Neutralizing chimeric mouse-human antibodies against *Burkholderia pseudomallei* protease: expression, purification and characterization. *J Biochem Mol Biol,* 37**,** 556-64.

CHANCHAMROEN, S., KEWCHAROENWONG, C., SUSAENGRAT, W., ATO, M. & LERTMEMONGKOLCHAI, G. 2009. Human polymorphonuclear neutrophil responses to *Burkholderia pseudomallei* in healthy and diabetic subjects. *Infect Immun,* 77**,** 456-63.

CHANTRATITA, N., MEUMANN, E., THANWISAI, A., LIMMATHUROTSAKUL, D., WUTHIEKANUN, V., WANNAPASNI, S., TUMAPA, S., DAY, N. P. & PEACOCK, S. J. 2008. Loop-mediated isothermal amplification method targeting the TTS1 gene cluster for detection of *Burkholderia pseudomallei* and diagnosis of melioidosis. *J Clin Microbiol,* 46**,** 568-73.

CHANTRATITA, N., WUTHIEKANUN, V., BOONBUMRUNG, K., TIYAWISUTSRI, R., VESARATCHAVEST, M., LIMMATHUROTSAKUL, D., CHIERAKUL, W., WONGRATANACHEEWIN, S., PUKRITIYAKAMEE, S., WHITE, N. J., DAY, N. P. & PEACOCK, S. J. 2007. Biological relevance of colony morphology and phenotypic switching by *Burkholderia pseudomallei*. *J Bacteriol,* 189**,** 807-17.

CHAROENSAP, J., UTAISINCHAROEN, P., ENGERING, A. & SIRISINHA, S. 2009. Differential intracellular fate of *Burkholderia pseudomallei* 844 and *Burkholderia thailandensis* UE5 in human monocyte-derived dendritic cells and macrophages. *BMC Immunol,* 10**,** 20.

CHATFIELD, C. H., MULHERN, B. J., BURNSIDE, D. M., & CIANCIOTTO, N. P. 2011. *Legionella pneumophila* LbtU acts as a novel, TonB-independent receptor for the legiobactin siderophore. *J Bacteriol,* 193**,** 1563-75.

CHEN, Y., WONG, J., SUN, G. W., LIU, Y., TAN, G. Y., & GAN, Y. H. 2011. Regulation of type VI secretion system during *Burkholderia pseudomallei* infection. *Infect Immun,* 79**,** 3064-73.

CHEN, Y. S., HSIAO, Y. S., LIN, H. H., YEN, C. M., CHEN, S. C. & CHEN, Y. L. 2006. Immunogenicity and anti-*Burkholderia pseudomallei* activity in Balb/c mice immunized with plasmid DNA encoding flagellin. *Vaccine,* 24**,** 750-8.

CHEN, Y. S., SHIUAN, D., CHEN, S. C., CHYE, S. M., & CHEN, Y. L. 2003. Recombinant truncated flagellin of *Burkholderia pseudomallei* as a molecular probe for diagnosis of melioidosis. *Clin Diagn Lab Immunol,* 10**,** 423-5.

CHENG, A. C. & CURRIE, B. J. 2005. Melioidosis: epidemiology, pathophysiology, and management. *Clin Microbiol Rev,* 18**,** 383-416.

CHENG, A. C., JACUPS, S. P., WARD, L. & CURRIE, B. J. 2008. Melioidosis and Aboriginal seasons in northern Australia. *Trans R Soc Trop Med Hyg,* 102 Suppl 1**,** S26-9.

CHENG, A. C., O'BRIEN, M., FREEMAN, K., LUM, G. & CURRIE, B. J. 2006. Indirect hemagglutination assay in patients with melioidosis in northern Australia. *Am J Trop Med Hyg,* 74**,** 330-4.

CHENTHAMARAKSHAN, V., KUMUTHA, M. V., VADIVELU, J. & PUTHUCHEARY, S. D. 2001a. Distribution of immunoglobulin classes and IgG subclasses against a culture filtrate antigen of *Burkholderia pseudomallei* in melioidosis patients. *J Med Microbiol,* 50**,** 55-61.

CHENTHAMARAKSHAN, V., VADIVELU, J., & PUTHUCHEARY, S. D. 2001b. Detection of immunoglobulins M and G using culture filtrate antigen of *Burkholderia pseudomallei*. *Diagn Microbiol Infect Dis,* 39**,** 1-7.

CHIENG, S., CARRETO, L. & NATHAN, S. 2012. *Burkholderia pseudomallei* transcriptional adaptation in macrophages. *BMC Genomics,* 13**,** 328.

CHIERAKUL, W., WINOTHAI, W., WATTANAWAITUNECHAI, C., WUTHIEKANUN, V., RUGTAENGAN, T., RATTANALERTNAVEE, J., JITPRATOOM, P., CHAOWAGUL, W., SINGHASIVANON, P., WHITE, N. J., DAY, N. P. & PEACOCK, S. J. 2005. Melioidosis in 6 tsunami survivors in southern Thailand. *Clin Infect Dis,* 41**,** 982-90.

CHOI, K. H., MIMA, T., CASART, Y., RHOLL, D., KUMAR, A., BEACHAM, I. R. & SCHWEIZER, H. P. 2008. Genetic tools for select-agent-compliant manipulation of *Burkholderia pseudomallei*. *Appl Environ Microbiol,* 74**,** 1064-75.

CHOY, J. L., MAYO, M., JANMAAT, A. & CURRIE, B. J. 2000. Animal melioidosis in Australia. *Acta Trop,* 74**,** 153-8.

CHUA, K. L., CHAN, Y. Y. & GAN, Y. H. 2003. Flagella are virulence determinants of *Burkholderia pseudomallei*. *Infect Immun,* 71**,** 1622-9.

CHUAH, S. C., GILMORE, G. & NORTON, R. E. 2005. Rapid serological diagnosis of melioidosis: an evaluation of a prototype immunochromatographic test. *Pathology,* 37**,** 169-71.

CHUANG, Y. P., FANG, C. T., LAI, S. Y., CHANG, S. C. & WANG, J. T. 2006. Genetic determinants of capsular serotype K1 of *Klebsiella pneumoniae* causing primary pyogenic liver abscess. *J Infect Dis,* 193**,** 645-54.

CHUAYGUD, T., TUNGPRADABKUL, S., SIRISINHA, S., CHUA, K. L. & UTAISINCHAROEN, P. 2008. A role of *Burkholderia pseudomallei* flagella as a virulent factor. *Trans R Soc Trop Med Hyg,* 102 Suppl 1**,** S140-4.

CUCCUI, J., EASTON, A., CHU, K. K., BANCROFT, G. J., OYSTON, P. C., TITBALL, R. W. & WREN, B. W. 2007. Development of signature-tagged mutagenesis in *Burkholderia pseudomallei* to identify genes important in survival and pathogenesis. *Infect Immun,* 75**,** 1186-95.

CULLINANE, M., GONG, L., LI, X., LAZAR-ADLER, N., TRA, T., WOLVETANG, E., PRESCOTT, M., BOYCE, J. D., DEVENISH, R. J. & ADLER, B. 2008. Stimulation of autophagy suppresses the intracellular survival of *Burkholderia pseudomallei* in mammalian cell lines. *Autophagy,* 4**,** 744-53.

CURRIE, B. J., DANCE, D. A. & CHENG, A. C. 2008. The global distribution of *Burkholderia pseudomallei* and melioidosis: an update. *Trans R Soc Trop Med Hyg,* 102 Suppl 1**,** S1-4.

CURRIE, B. J., FISHER, D. A., HOWARD, D. M., BURROW, J. N., LO, D., SELVA-NAYAGAM, S., ANSTEY, N. M., HUFFAM, S. E., SNELLING, P. L., MARKS, P. J., STEPHENS, D. P., LUM, G. D., JACUPS, S. P. & KRAUSE, V. L. 2000a. Endemic melioidosis in tropical northern Australia: a 10-year prospective study and review of the literature. *Clin Infect Dis,* 31**,** 981-6.

CURRIE, B., HOWARD, D., NGUYEN, V. T., WITHNALL, K. & MERIANOS, A. 1993. The 1990-1991 outbreak of melioidosis in the Northern Territory of Australia: clinical aspects. *Southeast Asian J Trop Med Public Health,* 24**,** 436-43.

CURRIE, B. J., FISHER, D. A., HOWARD, D. M., BURROW, J. N., SELVANAYAGAM, S., SNELLING, P. L., ANSTEY, N. M. & MAYO, M. J. 2000b. The epidemiology of melioidosis in Australia and Papua New Guinea. *Acta Trop,* 74**,** 121-7.

CURRIE, B. J. & JACUPS, S. P. 2003. Intensity of rainfall and severity of melioidosis, Australia. *Emerg Infect Dis,* 9**,** 1538-42.

CURRIE, B. J., JACUPS, S. P., CHENG, A. C., FISHER, D. A., ANSTEY, N. M., HUFFAM, S. E. & KRAUSE, V. L. 2004. Melioidosis epidemiology and risk factors from a prospective whole-population study in northern Australia. *Trop Med Int Health,* 9**,** 1167-74.

CURRIE, B. J., MAYO, M., ANSTEY, N. M., DONOHOE, P., HAASE, A. & KEMP, D. J. 2001. A cluster of melioidosis cases from an endemic region is clonal and is linked to the water supply using molecular typing of *Burkholderia pseudomallei* isolates. *Am J Trop Med Hyg,* 65**,** 177-9.

CUZZUBBO, A. J., CHENTHAMARAKSHAN, V., VADIVELU, J., PUTHUCHEARY, S. D., ROWLAND, D. & DEVINE, P. L. 2000. Evaluation of a new commercially available immunoglobulin M and immunoglobulin G immunochromatographic test for diagnosis of melioidosis infection. *J Clin Microbiol,* 38**,** 1670-1.

D'CRUZE, T., GONG, L., TREERAT, P., RAMM, G., BOYCE, J. D., PRESCOTT, M., ADLER, B. & DEVENISH, R. J. 2011. Role for the *Burkholderia pseudomallei* type three secretion system cluster 1 bpscN gene in virulence. *Infect Immun,* 79**,** 3659-64.

DANCE, D. A., DAVIS, T. M., WATTANAGOON, Y., CHAOWAGUL, W., SAIPHAN, P., LOOAREESUWAN, S., WUTHIEKANUN, V. & WHITE, N. J. 1989. Acute suppurative parotitis caused by *Pseudomonas pseudomallei* in children. *J Infect Dis,* 159**,** 654-60.

DERIS, Z. Z., HASAN, H. & SITI SURAIYA, M. N. 2010. Clinical characteristics and outcomes of bacteraemic melioidosis in a teaching hospital in a northeastern state of Malaysia: a five-year review. *J Infect Dev Ctries,* 4**,** 430-5.

DESHAZER, D., BRETT, P. J., CARLYON, R. & WOODS, D. E. 1997. Mutagenesis of *Burkholderia pseudomallei* with Tn5-OT182: isolation of motility mutants and molecular characterization of the flagellin structural gene. *J Bacteriol,* 179**,** 2116-25.

DHARAKUL, T., SONGSIVILAI, S., ANUNTAGOOL, N., CHAOWAGUL, W., WONGBUNNATE, S., INTACHOTE, P. & SIRISINHA, S. 1997. Diagnostic value of an antibody enzyme-linked immunosorbent assay using affinity-purified antigen in an area endemic for melioidosis. *Am J Trop Med Hyg,* 56**,** 418-23.

DHARAKUL, T., SONGSIVILAI, S., VIRIYACHITRA, S., LUANGWEDCHAKARN, V., TASSANEETRITAP, B. & CHAOWAGUL, W. 1996. Detection of *Burkholderia pseudomallei* DNA in patients with septicemic melioidosis. *J Clin Microbiol,* 34**,** 609-14.

DHARAKUL, T., SONGSIVILAI, S., SMITHIKARN, S., THEPTHAI, C. & LEELAPORN, A. 1999. Rapid identification of *Burkholderia pseudomallei* in blood cultures by latex agglutination using lipopolysaccharide-specific monoclonal antibody. *Am J Trop Med Hyg,* 61**,** 658-62.

DRUAR, C., YU, F., BARNES, J. L., OKINAKA, R. T., CHANTRATITA, N., BEG, S., STRATILO, C. W., OLIVE, A. J., SOLTES, G., RUSSELL, M. L., LIMMATHUROTSAKUL, D., NORTON, R. E., NI, S. X., PICKING, W. D., JACKSON, P. J., STEWART, D. I., TSVETNITSKY, V., PICKING, W. L., CHERWONOGRODZKY, J. W., KETHEESAN, N., PEACOCK, S. J. & WIERSMA, E. J. 2008. Evaluating *Burkholderia pseudomallei* Bip proteins as vaccines and Bip antibodies as detection agents. *FEMS Immunol Med Microbiol,* 52**,** 78-87.

EKPO, P., RUNGPANICH, U., PONGSUNK, S., NAIGOWIT, P. & PETKANCHANAPONG, V. 2007. Use of protein-specific monoclonal antibody-based latex agglutination for rapid diagnosis of *Burkholderia pseudomallei* infection in patients with community-acquired septicemia. *Clin Vaccine Immunol,* 14**,** 811-2.

ESSEX-LOPRESTI, A. E., BODDEY, J. A., THOMAS, R., SMITH, M. P., HARTLEY, M. G., ATKINS, T., BROWN, N. F., TSANG, C. H., PEAK, I. R., HILL, J., BEACHAM, I. R. & TITBALL, R. W. 2005. A type IV pilin, PilA, Contributes To Adherence of *Burkholderia pseudomallei* and virulence in vivo. *Infect Immun,* 73**,** 1260-4.

EZZEDINE, K., HEENEN, M. & MALVY, D. 2007. Imported cutaneous melioidosis in traveler, Belgium. *Emerg Infect Dis,* 13**,** 946-7.

FELGNER, P. L., KAYALA, M. A., VIGIL, A., BURK, C., NAKAJIMA-SASAKI, R., PABLO, J., MOLINA, D. M., HIRST, S., CHEW, J. S., WANG, D., TAN, G., DUFFIELD, M., YANG, R., NEEL, J., CHANTRATITA, N., BANCROFT, G., LERTMEMONGKOLCHAI, G., DAVIES, D. H., BALDI, P., PEACOCK, S. & TITBALL, R. W. 2009. A *Burkholderia pseudomallei* protein microarray reveals serodiagnostic and cross-reactive antigens. *Proc Natl Acad Sci U S A,* 106**,** 13499-504.

GAL, D., MAYO, M., SMITH-VAUGHAN, H., DASARI, P., MCKINNON, M., JACUPS, S. P., URQUHART, A.I., HASSELL, M. & CURRIE, B.J. 2004. Contamination of hand wash detergent linked to occupationally acquired melioidosis. *Am J Trop Med Hyg, 71***,** 360-362.

GALYOV, E. E., BRETT, P. J., & DESHAZER, D. 2010. Molecular insights into *Burkholderia pseudomallei* and *Burkholderia mallei* pathogenesis. *Annu Rev Microbiol,* 64**,** 495-517.

GAN, Y. H., CHUA, K. L., CHUA, H. H., LIU, B., HII, C. S., CHONG, H. L. & TAN, P. 2002. Characterization of *Burkholderia pseudomallei* infection and identification of novel virulence factors using a *Caenorhabditis elegans* host system. *Mol Microbiol,* 44**,** 1185-97.

GILMORE, G., BARNES, J., KETHEESAN, N. & NORTON, R. 2007. Use of antigens derived from *Burkholderia pseudomallei*, *B. thailandensis*, and *B. cepacia* in the indirect hemagglutination assay for melioidosis. *Clin Vaccine Immunol,* 14**,** 1529-31.

GLAS, A. S., LIJMER, J. G., PRINS, M. H., BONSEL, G. J., & BOSSUYT, P. M. 2003. The diagnostic odds ratio: a single indicator of test performance. *J Clin Epidemiol,* 56**,** 1129-35.

GLASS, M. B. & POPOVIC, T. 2005. Preliminary evaluation of the API 20NE and RapID NF plus systems for rapid identification of *Burkholderia pseudomallei* and B. mallei. *J Clin Microbiol,* 43**,** 479-83.

GONG, L., CULLINANE, M., TREERAT, P., RAMM, G., PRESCOTT, M., ADLER, B., BOYCE, J. D. & DEVENISH, R. J. 2011. The *Burkholderia pseudomallei* type III secretion system and BopA are required for evasion of LC3-associated phagocytosis. *PLoS One,* 6**,** e17852.

GORI, A. H., AHMED, K., MARTINEZ, G., MASAKI, H., WATANABE, K. & NAGATAKE, T. 1999. Mediation of attachment of *Burkholderia pseudomallei* to human pharyngeal epithelial cells by the asialoganglioside GM1-GM2 receptor complex. *Am J Trop Med Hyg,* 61**,** 473-5.

GRAVATO-NOBRE, M. J., & HODGKIN, J. 2005. *Caenorhabditis elegans* as a model for innate immunity to pathogens. *Cell Microbiol,* 7**,** 741-51.

HALDER, D., ZAINAL, N., WAH, C. M. & HAQ, J. A. 1998. Neonatal meningitis and septicaemia caused by *Burkholderia pseudomallei*. *Ann Trop Paediatr,* 18**,** 161-4.

HARA, Y., CHIN, C. Y., MOHAMED, R., PUTHUCHEARY, S. D., & NATHAN, S. 2013. Multiple-antigen ELISA for melioidosis - a novel approach to the improved serodiagnosis of melioidosis. *BMC Infect Dis,* 13**,** 165.

HARA, Y., MOHAMED, R. & NATHAN, S. 2009. Immunogenic *Burkholderia pseudomallei* outer membrane proteins as potential candidate vaccine targets. *PLoS One,* 4**,** e6496.

HARLEY, V. S., DANCE, D. A., DRASAR, B. S. & TOVEY, G. 1998a. Effects of *Burkholderia pseudomallei* and other *Burkholderia* species on eukaryotic cells in tissue culture. *Microbios,* 96**,** 71-93.

HARLEY, V. S., DANCE, D. A., TOVEY, G., MCCROSSAN, M. V. & DRASAR, B. S. 1998b. An ultrastructural study of the phagocytosis of *Burkholderia pseudomallei*. *Microbios,* 94**,** 35-45.

HARDING, S. V., SARKAR-TYSON, M., SMITHER, S. J., ATKINS, T. P., OYSTON, P. C., BROWN, K. A., LIU, Y., WAIT, R. & TITBALL, R.W. 2007. The identification of surface proteins of *Burkholderia pseudomallei*. *Vaccine,* 25**,** 2664-72.

HASSAN, M. R., PANI, S. P., PENG, N. P., VORALU, K., VIJAYALAKSHMI, N., MEHANDERKAR, R., AZIZ, N. A. & MICHAEL, E. 2010. Incidence, risk factors and clinical epidemiology of melioidosis: a complex socio-ecological emerging infectious disease in the Alor Setar region of Kedah, Malaysia. *BMC Infect Dis,* 10**,** 302.

HENDERSON, B., & MARTIN, A. 2011. Bacterial virulence in the moonlight: multitasking bacterial moonlighting proteins are virulence determinants in infectious disease. *Infect Immun,* 79**,** 3476-91.

HENG, B. H., GOH, K. T., YAP, E. H., LOH, H. & YEO, M. 1998. Epidemiological surveillance of melioidosis in Singapore. *Ann Acad Med Singapore,* 27**,** 478-84.

HO, M., SCHOLLAARDT, T., SMITH, M. D., PERRY, M. B., BRETT, P. J., CHAOWAGUL, W. & BRYAN, L. E. 1997. Specificity and functional activity of anti-*Burkholderia pseudomallei* polysaccharide antibodies. *Infect Immun,* 65**,** 3648-53.

HOLDEN, M. T., TITBALL, R. W., PEACOCK, S. J., CERDENO-TARRAGA, A. M., ATKINS, T., CROSSMAN, L. C., PITT, T., CHURCHER, C., MUNGALL, K., BENTLEY, S. D., SEBAIHIA, M., THOMSON, N. R., BASON, N., BEACHAM, I. R., BROOKS, K., BROWN, K. A., BROWN, N. F., CHALLIS, G. L., CHEREVACH, I., CHILLINGWORTH, T., CRONIN, A., CROSSETT, B., DAVIS, P., DESHAZER, D., FELTWELL, T., FRASER, A., HANCE, Z., HAUSER, H., HOLROYD, S., JAGELS, K., KEITH, K. E., MADDISON, M., MOULE, S., PRICE, C., QUAIL, M. A., RABBINOWITSCH, E., RUTHERFORD, K., SANDERS, M., SIMMONDS, M., SONGSIVILAI, S., STEVENS, K., TUMAPA, S., VESARATCHAVEST, M., WHITEHEAD, S., YEATS, C., BARRELL, B. G., OYSTON, P. C. & PARKHILL, J. 2004. Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. *Proc Natl Acad Sci U S A,* 101**,** 14240-5.

HOW, S. H., NG, K. H., JAMALLUDIN, A. R., SHAH, A. & RATHOR, Y. 2005. Melioidosis in Pahang, Malaysia. *Med J Malaysia,* 60**,** 606-13.

HOWARD, K. & INGLIS, T. J. 2003. Novel selective medium for isolation of *Burkholderia pseudomallei*. *J Clin Microbiol,* 41**,** 3312-6.

HOWE, C., SAMPATH, A. & SPOTNITZ, M. 1971. The *pseudomallei* group: a review. *J Infect Dis,* 124**,** 598-606.

INGLIS, T. J., CHIANG, D., LEE, G. S. & CHOR-KIANG, L. 1998. Potential misidentification of *Burkholderia pseudomallei* by API 20NE. *Pathology,* 30**,** 62-4.

INGLIS, T. J., GARROW, S. C., HENDERSON, M., CLAIR, A., SAMPSON, J., O'REILLY, L. & CAMERON, B. 2000a. *Burkholderia pseudomallei* traced to water treatment plant in Australia. *Emerg Infect Dis,* 6**,** 56-9.

INGLIS, T. J., MERRITT, A., CHIDLOW, G., ARAVENA-ROMAN, M. & HARNETT, G. 2005. Comparison of diagnostic laboratory methods for identification of *Burkholderia pseudomallei*. *J Clin Microbiol,* 43**,** 2201-6.

INGLIS, T. J., RIGBY, P., ROBERTSON, T. A., DUTTON, N. S., HENDERSON, M. & CHANG, B. J. 2000b. Interaction between *Burkholderia pseudomallei* and *Acanthamoeba* species results in coiling phagocytosis, endamebic bacterial survival, and escape. *Infect Immun,* 68**,** 1681-6.

INGLIS, T. J., ROBERTSON, T., WOODS, D. E., DUTTON, N. & CHANG, B. J. 2003. Flagellum-mediated adhesion by *Burkholderia pseudomallei* precedes invasion of *Acanthamoeba astronyxis*. *Infect Immun,* 71**,** 2280-2.

IRAZOQUI, J. E., NG, A., XAVIER, R. J. & AUSUBEL, F. M. 2008. Role for beta-catenin and HOX transcription factors in *Caenorhabditis elegans* and mammalian host epithelial-pathogen interactions. *Proc Natl Acad Sci U S A,* 105**,** 17469-74.

ISMAIL, G., NOOR EMBI, M., OMAR, O., ALLEN, J. C. & SMITH, C. J. 1987. A competitive immunosorbent assay for detection of *Pseudomonas pseudomallei* exotoxin. *J Med Microbiol,* 23**,** 353-7.

JANSSON, H. B. 1994. Adhesion of Conidia of *Drechmeria coniospora* to *Caenorhabditis elegans* Wild Type and Mutants. *J Nematol,* 26**,** 430-5.

JONES, A. L., BEVERIDGE, T. J. & WOODS, D. E. 1996. Intracellular survival of *Burkholderia pseudomallei*. *Infect Immun,* 64**,** 782-90.

JONES, A. L., DESHAZER, D. & WOODS, D. E. 1997. Identification and characterization of a two-component regulatory system involved in invasion of eukaryotic cells and heavy-metal resistance in *Burkholderia pseudomallei*. *Infect Immun,* 65**,** 4972-7.

JONES, S. M., ELLIS, J. F., RUSSELL, P., GRIFFIN, K. F. & OYSTON, P. C. 2002. Passive protection against *Burkholderia pseudomallei* infection in mice by monoclonal antibodies against capsular polysaccharide, lipopolysaccharide or proteins. *J Med Microbiol,* 51**,** 1055-62.

KAESTLI, M., RICHARDSON, L. J., COLMAN, R. E., TUANYOK, A., PRICE, E. P., BOWERS, J. R., MAYO, M., KELLEY, E., SEYMOUR, M. L., SAROVICH, D. S., PEARSON, T., ENGELTHALER, D. M., WAGNER, D. M., KEIM, P. S., SCHUPP, J. M. & CURRIE, B. J. 2012. Comparison of TaqMan PCR assays for detection of the melioidosis agent *Burkholderia pseudomallei* in clinical specimens. *J Clin Microbiol,* 50**,** 2059-62.

KAMIONKA, A., PARCHE, S., NOTHAFT, H., SIEPELMEYER, J., JAHREIS, K., & TITGEMEYER, F. 2002. The phosphotransferase system of *Streptomyces coelicolor*. *Eur J Biochem,* 269**,** 2143-50.

KANAI, K., SUZUKI, Y., KONDO, E., MAEJIMA, Y., MIYAMOTO, D., SUZUKI, T. & KURATA, T. 1997. Specific binding of *Burkholderia pseudomallei* cells and their cell-surface acid phosphatase to gangliotetraosylceramide (asialo GM1) and gangliotriaosylceramide (asialo GM2). *Southeast Asian J Trop Med Public Health,* 28**,** 781-90.

KANG, Y., NORRIS, M. H., WILCOX, B. A., TUANYOK, A., KEIM, P. S. & HOANG, T. T. 2011. Knockout and pullout recombineering for naturally transformable *Burkholderia thailandensis* and *Burkholderia pseudomallei*. *Nat Protoc,* 6**,** 1085-104.

KELLEY, L. A. & STERNBERG, M. J. 2009. Protein structure prediction on the Web: a case study using the Phyre server. *Nat Protoc,* 4**,** 363-71.

KESPICHAYAWATTANA, W., RATTANACHETKUL, S., WANUN, T., UTAISINCHAROEN, P. & SIRISINHA, S. 2000. *Burkholderia pseudomallei* induces cell fusion and actin-associated membrane protrusion: a possible mechanism for cell-to-cell spreading. *Infect Immun,* 68**,** 5377-84.

KIRATISIN, P., SANTANIRAND, P., CHANTRATITA, N. & KAEWDAENG, S. 2007. Accuracy of commercial systems for identification of *Burkholderia pseudomallei* versus *Burkholderia cepacia*. *Diagn Microbiol Infect Dis,* 59**,** 277-81.

KIYAMA, Y., MIYAHARA, K., & OHSHIMA, Y. 2012. Active uptake of artificial particles in the nematode *Caenorhabditis elegans. J Exp Biol,* 215**,** 1178-1183.

KOH, T. H., YONG NG, L. S., FOON HO, J. L., SNG, L. H., WANG, G. C. & TZER PIN LIN, R. V. 2003. Automated identification systems and *Burkholderia pseudomallei*. *J Clin Microbiol,* 41**,** 1809.

KONGSAENGDAO, S., BUNNAG, S. & SIRIWIWATTNAKUL, N. 2005. Treatment of survivors after the tsunami. *N Engl J Med,* 352**,** 2654-5.

KORBSRISATE, S., TOMARAS, A. P., DAMNIN, S., CKUMDEE, J., SRINON, V., LENGWEHASATIT, I., VASIL, M. L. & SUPARAK, S. 2007. Characterization of two distinct phospholipase C enzymes from *Burkholderia pseudomallei*. *Microbiology,* 153**,** 1907-15.

KOTHE, M., ANTL, M., HUBER, B., STOECKER, K., EBRECHT, D., STEINMETZ, I. & EBERL, L. 2003. Killing of *Caenorhabditis elegans* by *Burkholderia cepacia* is controlled by the cep quorum-sensing system. *Cell Microbiol,* 5**,** 343-51.

KUNAKORN, M., BOONMA, P., KHUPULSUP, K. & PETCHCLAI, B. 1990. Enzyme-linked immunosorbent assay for immunoglobulin M specific antibody for the diagnosis of melioidosis. *J Clin Microbiol,* 28**,** 1249-53.

KUNAKORN, M. & MARKHAM, R. B. 1995. Clinically practical seminested PCR for *Burkholderia pseudomallei* quantitated by enzyme immunoassay with and without solution hybridization. *J Clin Microbiol,* 33**,** 2131-5.

KVITKO, B. H., GOODYEAR, A., PROPST, K. L., DOW, S. W. & SCHWEIZER, H. P. 2012. *Burkholderia pseudomallei* known siderophores and hemin uptake are dispensable for lethal murine melioidosis. *PLoS Negl Trop Dis,* 6**,** e1715.

LAUW, F. N., SIMPSON, A. J., PRINS, J. M., SMITH, M. D., KURIMOTO, M., VAN DEVENTER, S. J., SPEELMAN, P., CHAOWAGUL, W., WHITE, N. J. & VAN DER POLL, T. 1999. Elevated plasma concentrations of interferon (IFN)-gamma and the IFN-gamma-inducing cytokines interleukin (IL)-18, IL-12, and IL-15 in severe melioidosis. *J Infect Dis,* 180**,** 1878-85.

LEE, S. H., OOI, S. K., MAHADI, N. M., TAN, M. W., & NATHAN, S. 2011. Complete killing of *Caenorhabditis elegans* by *Burkholderia pseudomallei* is dependent on prolonged direct association with the viable pathogen. *PLoS One,* 6**,** e16707.

LEW, A. E. & DESMARCHELIER, P. M. 1994. Detection of *Pseudomonas pseudomallei* by PCR and hybridization. *J Clin Microbiol,* 32**,** 1326-32.

LIMMATHUROTSAKUL, D., CHANTRATITA, N., TEERAWATTANASOOK, N., PIRIYAGITPAIBOON, K., THANWISAI, A., WUTHIEKANUN, V., DAY, N. P., COOPER, B. & PEACOCK, S. J. 2011. Enzyme-linked immunosorbent assay for the diagnosis of melioidosis: better than we thought. *Clin Infect Dis,* 52**,** 1024-8.

LIMMATHUROTSAKUL, D., CHAOWAGUL, W., CHIERAKUL, W., STEPNIEWSKA, K., MAHARJAN, B., WUTHIEKANUN, V., WHITE, N. J., DAY, N. P. & PEACOCK, S. J. 2006. Risk factors for recurrent melioidosis in northeast Thailand. *Clin Infect Dis,* 43**,** 979-86.

LIMMATHUROTSAKUL, D., KOH, G.C.K.W., PEACOCK. S.J. & CURRIE, B.J. 2012. Chronic melioidosis, relapse and latency. In Ketheesan, N. (Eds.), *Melioidosis: a century of observation and research* (pp.120-129). Amsterdam, Netherlands: Elsevier.

LIMMATHUROTSAKUL, D., WONGRATANACHEEWIN, S., TEERAWATTANASOOK, N., WONGSUVAN, G., CHAISUKSANT, S., CHETCHOTISAKD, P., CHAOWAGUL, W., DAY, N. P. & PEACOCK, S. J. 2010. Increasing incidence of human melioidosis in Northeast Thailand. *Am J Trop Med Hyg,* 82**,** 1113-7.

LIN, T. L., LEE, C. Z., HSIEH, P. F., TSAI, S. F. & WANG, J. T. 2008. Characterization of integrative and conjugative element ICEKp1-associated genomic heterogeneity in a *Klebsiella pneumoniae* strain isolated from a primary liver abscess. *J Bacteriol,* 190**,** 515-26.

LO, T. J., ANG, L. W., JAMES, L. & GOH, K. T. 2009. Melioidosis in a tropical city state, Singapore. *Emerg Infect Dis,* 15**,** 1645-7.

LOPRASERT, S., SALLABHAN, R., WHANGSUK, W. & MONGKOLSUK, S. 2002. The *Burkholderia pseudomallei* oxyR gene: expression analysis and mutant characterization. *Gene,* 296**,** 161-9.

LOWE, P., ENGLER, C. & NORTON, R. 2002. Comparison of automated and nonautomated systems for identification of *Burkholderia pseudomallei*. *J Clin Microbiol,* 40**,** 4625-7.

LUHACHACK, L. G., VISVIKIS, O., WOLLENBERG, A. C., LACY-HULBERT, A., STUART, L. M. & IRAZOQUI, J. E. 2012. EGL-9 controls *C. elegans* host defense specificity through prolyl hydroxylation-dependent and -independent HIF-1 pathways. *PLoS Pathog,* 8**,** e1002798.

LUMJIAKTASE, P., DIGGLE, S. P., LOPRASERT, S., TUNGPRADABKUL, S., DAYKIN, M., CAMARA, M., WILLIAMS, P. & KUNAKORN, M. 2006. Quorum sensing regulates dpsA and the oxidative stress response in *Burkholderia pseudomallei*. *Microbiology,* 152**,** 3651-9.

MALCZEWSKI, A. B., OMAN, K. M., NORTON, R. E. & KETHEESAN, N. 2005. Clinical presentation of melioidosis in Queensland, Australia. *Trans R Soc Trop Med Hyg,* 99**,** 856-60.

MAROHN, M. E., SANTIAGO, A. E., SHIREY, K. A., LIPSKY, M., VOGEL, S. N. & BARRY, E. M. 2012. Members of the *Francisella tularensis* phagosomal transporter subfamily of major facilitator superfamily transporters are critical for pathogenesis. *Infect Immun,* 80**,** 2390-401.

MATHAI, E., JESUDASON, M. V. & ANBARASU, A. 2003. Indirect immunofluorescent antibody test for the rapid diagnosis of melioidosis. *Indian J Med Res,* 118**,** 68-70.

MCCORMICK, J. B., SEXTON, D. J., MCMURRAY, J. G., CAREY, E., HAYES, P. & FELDMAN, R. A. 1975. Human-to-human transmission of *Pseudomonas pseudomallei*. *Ann Intern Med,* 83**,** 512-3.

MEUMANN, E. M., NOVAK, R. T., GAL, D., KAESTLI, M. E., MAYO, M., HANSON, J. P., SPENCER, E., GLASS, M. B., GEE, J. E., WILKINS, P. P. & CURRIE, B. J. 2006. Clinical evaluation of a type III secretion system real-time PCR assay for diagnosing melioidosis. *J Clin Microbiol,* 44**,** 3028-30.

MILLAN, J. M., MAYO, M., GAL, D., JANMAAT, A. & CURRIE, B. J. 2007. Clinical variation in melioidosis in pigs with clonal infection following possible environmental contamination from bore water. *Vet J,* 174**,** 200-2.

MUANGMAN, S., KORBSRISATE, S., MUANGSOMBUT, V., SRINON, V., ADLER, N. L., SCHROEDER, G. N., FRANKEL, G. & GALYOV, E. E. 2011. BopC is a type III secreted effector protein of *Burkholderia pseudomallei*. *FEMS Microbiol Lett,* 323**,** 75-82.

MUANGSOMBUT, V., SUPARAK, S., PUMIRAT, P., DAMNIN, S., VATTANAVIBOON, P., THONGBOONKERD, V. & KORBSRISATE, S. 2008. Inactivation of *Burkholderia pseudomallei* bsaQ results in decreased invasion efficiency and delayed escape of bacteria from endocytic vesicles. *Arch Microbiol,* 190**,** 623-31.

MULLER, C. M., CONEJERO, L., SPINK, N., WAND, M. E., BANCROFT, G. J. & TITBALL, R. W. 2012. Role of RelA and SpoT in *Burkholderia pseudomallei* virulence and immunity. *Infect Immun,* 80**,** 3247-55.

NAIGOWIT, P., KURATA, T., WANGROONGSUB, P., PETKANJANAPONG, V., KONDO, E. & KANAI, K. 1993. Application of indirect immunofluorescence microscopy to colony identification of *Pseudomonas pseudomallei*. *Asian Pac J Allergy Immunol,* 11**,** 149-54.

NANDI, T., & TAN, P. 2012. The *Burkholderia pseudomallei* genome – an emerging model for microbial complexicity and pathogen virulence. In Ketheesan, N. (Eds.), *Melioidosis: a century of observation and research* (pp.68-81). Amsterdam, Netherlands: Elsevier

NATHAN, S., LI, H., MOHAMED, R. & EMBI, N. 2002. Phage display of recombinant antibodies toward *Burkholderia pseudomallei* exotoxin. *J Biochem Mol Biol Biophys,* 6**,** 45-53.

NGAUY, V., LEMESHEV, Y., SADKOWSKI, L. & CRAWFORD, G. 2005. Cutaneous melioidosis in a man who was taken as a prisoner of war by the Japanese during World War II. *J Clin Microbiol,* 43**,** 970-2.

NIEVES, W., HEANG, J., ASAKRAH, S., HONER ZU BENTRUP, K., ROY, C. J. & MORICI, L. A. 2010. Immunospecific responses to bacterial elongation factor Tu during *Burkholderia* infection and immunization. *PLoS One,* 5**,** e14361.

NORAZAH, A., ROHANI, M. Y., CHANG, P. T. & KAMEL, A. G. 1996. Indirect hemagglutination antibodies against *Burkholderia pseudomallei* in normal blood donors and suspected cases of melioidosis in Malaysia. *Southeast Asian J Trop Med Public Health,* 27**,** 263-6.

NORRIS, M. H., PROPST, K. L., KANG, Y., DOW, S. W., SCHWEIZER, H. P. & HOANG, T. T. 2011. The *Burkholderia pseudomallei* Delta asd mutant exhibits attenuated intracellular infectivity and imparts protection against acute inhalation melioidosis in mice. *Infect Immun,* 79**,** 4010-8.

NOVAK, R. T., GLASS, M. B., GEE, J. E., GAL, D., MAYO, M. J., CURRIE, B. J. & WILKINS, P. P. 2006. Development and evaluation of a real-time PCR assay targeting the type III secretion system of *Burkholderia pseudomallei*. *J Clin Microbiol,* 44**,** 85-90.

NOYAL, M. J., HARISH, B. N., BHAT, V. & PARIJA, S. C. 2009. Neonatal melioidosis: a case report from India. *Indian J Med Microbiol,* 27**,** 260-3.

O'BRIEN, M., FREEMAN, K., LUM, G., CHENG, A. C., JACUPS, S. P., & CURRIE, B. J. 2004. Further evaluation of a rapid diagnostic test for melioidosis in an area of endemicity. *J Clin Microbiol,* 42, 2239-40.

O'QUINN, A. L., WIEGAND, E. M. & JEDDELOH, J. A. 2001. Burkholderia pseudomallei kills the nematode *Caenorhabditis elegans* using an endotoxin-mediated paralysis. *Cell Microbiol,* 3**,** 381-93.

OOI, S. K., LIM, T. Y., LEE, S. H. & NATHAN, S. 2012. *Burkholderia pseudomallei* kills *Caenorhabditis elegans* through virulence mechanisms distinct from intestinal lumen colonization. *Virulence,* 3**,** 485-96.

ORTIZ-MARTIN, I., MACHO, A. P., LAMBERSTEN, L., RAMOS, C., & BEUZON, C. R. 2006. Suicide vectors for antibiotic marker exchange and rapid generation of multiple knockout mutants by allelic exchange in Gram-negative bacteria. *J Microbiol Methods,* 67**,** 395-407.

PAGALAVAN, L. 2005. Melioidosis: the Johor Bahru experience. *Med J Malaysia,* 60**,** 599-605.

PEACOCK, S. J., CHIENG, G., CHENG, A. C., DANCE, D. A., AMORNCHAI, P., WONGSUVAN, G., TEERAWATTANASOOK, N., CHIERAKUL, W., DAY, N. P. & WUTHIEKANUN, V. 2005. Comparison of Ashdown's medium, *Burkholderia cepacia* medium, and *Burkholderia pseudomallei* selective agar for clinical isolation of *Burkholderia pseudomallei*. *J Clin Microbiol,* 43**,** 5359-61.

PEACOCK, S. J., SCHWEIZER, H. P., DANCE, D. A., SMITH, T. L., GEE, J. E., WUTHIEKANUN, V., DESHAZER, D., STEINMETZ, I., TAN, P. & CURRIE, B. J. 2008. Management of accidental laboratory exposure to *Burkholderia pseudomallei* and *B. mallei*. *Emerg Infect Dis,* 14**,** e2.

PETKANJANAPONG, V., NAIGOWIT, P., KONDO, E. & KANAI, K. 1992. Use of endotoxin antigens in enzyme-linked immunosorbent assay for the diagnosis of P. pseudomallei infections (melioidosis). *Asian Pac J Allergy Immunol,* 10**,** 145-50.

PILATZ, S., BREITBACH, K., HEIN, N., FEHLHABER, B., SCHULZE, J., BRENNEKE, B., EBERL, L. & STEINMETZ, I. 2006. Identification of *Burkholderia pseudomallei* genes required for the intracellular life cycle and in vivo virulence. *Infect Immun,* 74**,** 3576-86.

PONGSUNK, S., THIRAWATTANASUK, N., PIYASANGTHONG, N. & EKPO, P. 1999. Rapid identification of *Burkholderia pseudomallei* in blood cultures by a monoclonal antibody assay. *J Clin Microbiol,* 37**,** 3662-7.

POWELL, J. R., & AUSUBEL, F. M. 2008. Models of *Caenorhabditis elegans* infection by bacterial and fungal pathogens. *Methods Mol Biol,* 415**,** 403-27.

PROPST, K. L., MIMA, T., CHOI, K. H., DOW, S. W. & SCHWEIZER, H. P. 2010. A *Burkholderia pseudomallei* delta purM mutant is avirulent in immunocompetent and immunodeficient animals: candidate strain for exclusion from select-agent lists. *Infect Immun,* 78**,** 3136-43.

PUMIRAT, P., CUCCUI, J., STABLER, R. A., STEVENS, J. M., MUANGSOMBUT, V., SINGSUKSAWAT, E., STEVENS, M. P., WREN, B. W. & KORBSRISATE, S. 2010. Global transcriptional profiling of *Burkholderia pseudomallei* under salt stress reveals differential effects on the Bsa type III secretion system. *BMC Microbiol,* 10**,** 171.

PUTHUCHEARY, S. D. & NATHAN, S. A. 2006. Comparison by electron microscopy of intracellular events and survival of *Burkholderia pseudomallei* in monocytes from normal subjects and patients with melioidosis. *Singapore Med J,* 47**,** 697-703.

PUTHUCHEARY, S. D. 2009. Melioidosis in Malaysia. *Med J Malaysia,* 64**,** 266-74.

PUTHUCHEARY, S. D., ANUAR, A. S., & TEE, T. S. 2010. *Burkholderia thailandensis* whole cell antigen cross-reacts with *B. pseudomallei* antibodies from patients with melioidosis in an immunofluorescent assay. *Southeast Asian J Trop Med Public Health,* 41**,** 395-400.

PUTHUCHEARY, S. D., PARASAKTHI, N. & LEE, M. K. 1992. Septicaemic melioidosis: a review of 50 cases from Malaysia. *Trans R Soc Trop Med Hyg,* 86**,** 683-5.

PUTHUCHEARY, S. D., PUAH, S. M., CHAI, H. C., THONG, K. L. & CHUA, K. H. 2012a. Molecular investigation of virulence determinants between a virulent clinical strain and an attenuated strain of *Burkholderia pseudomallei*. *J Mol Microbiol Biotechnol,* 22**,** 198-204.

PUTHUCHEARY, S. D., & SAM, I. C. 2012b. Why is the response rate slow in ceftazidime therapy for melioidosis? *Expert Rev Anti Infect Ther, 10***,**5-7.

PUTHUCHEARY S. D., & VADIVELU J. 2002. Clinical Manifestations, In: Human Melioidosis Singapore University Press, Singapore pp:33-43

RALPH, A., MCBRIDE, J. & CURRIE, B. J. 2004. Transmission of *Burkholderia pseudomallei* via breast milk in northern Australia. *Pediatr Infect Dis J,* 23**,** 1169-71.

RAMSAY, S. 2012. Medical imaging in melioidosis. In Ketheesan, N. (Eds.), *Melioidosis: a century of observation and research* (pp.174-180). Amsterdam, Netherlands: Elsevier.

RATTANATHONGKOM, A., SERMSWAN, R. W. & WONGRATANACHEEWIN, S. 1997. Detection of *Burkholderia pseudomallei* in blood samples using polymerase chain reaction. *Mol Cell Probes,* 11**,** 25-31.

RECKSEIDLER, S. L., DESHAZER, D., SOKOL, P. A. & WOODS, D. E. 2001. Detection of bacterial virulence genes by subtractive hybridization: identification of capsular polysaccharide of *Burkholderia pseudomallei* as a major virulence determinant. *Infect Immun,* 69**,** 34-44.

RECKSEIDLER-ZENTENO, S. L., DEVINNEY, R. & WOODS, D. E. 2005. The capsular polysaccharide of *Burkholderia pseudomallei* contributes to survival in serum by reducing complement factor C3b deposition. *Infect Immun,* 73**,** 1106-15.

REYRAT, J. M., PELICIC, V., GICQUEL, B. & RAPPUOLI, R. 1998. Counterselectable markers: untapped tools for bacterial genetics and pathogenesis. *Infect Immun,* 66**,** 4011-7.

RHOLL, D. A., TRUNCK, L. A. & SCHWEIZER, H. P. 2008. In vivo Himar1 transposon mutagenesis of *Burkholderia pseudomallei*. *Appl Environ Microbiol,* 74**,** 7529-35.

ROBERTSON, J., LEVY, A., SAGRIPANTI, J. L. & INGLIS, T. J. 2010. The survival of *Burkholderia pseudomallei* in liquid media. *Am J Trop Med Hyg,* 82**,** 88-94.

ROLIM, D. B., VILAR, D. C., SOUSA, A. Q., MIRALLES, I. S., DE OLIVEIRA, D. C., HARNETT, G., O'REILLY, L., HOWARD, K., SAMPSON, I. & INGLIS, T. J. 2005. Melioidosis, northeastern Brazil. *Emerg Infect Dis,* 11**,** 1458-60.

ROTZ, L. D., KHAN, A. S., LILLIBRIDGE, S. R., OSTROFF, S. M., & HUGHES, J. M. 2002. Public health assessment of potential biological terrorism agents. *Emerg Infect Dis,* 8**,** 225-30.

RUGDECH, P., ANUNTAGOOL, N. & SIRISINHA, S. 1995. Monoclonal antibodies to *Pseudomonas pseudomallei* and their potential for diagnosis of melioidosis. *Am J Trop Med Hyg,* 52**,** 231-5.

SAM, I. C. & PUTHUCHEARY, S. D. 2007. Melioidosis and rainfall in Kuala Lumpur, Malaysia. *J Infect,* 54**,** 519-20.

SAMBROOK J. AND RUSSELL D.W. 2001. Molecular cloning: a laboratory manual (Volume 2). Cold Spring Harbor Laboratory Press.

SAMOSORNSUK, N., LULITANOND, A., SAENLA, N., ANUNTAGOOL, N., WONGRATANACHEEWIN, S. & SIRISINHA, S. 1999. Short report: evaluation of a monoclonal antibody-based latex agglutination test for rapid diagnosis of septicemic melioidosis. *Am J Trop Med Hyg,* 61**,** 735-7.

SANTANIRAND, P., HARLEY, V. S., DANCE, D. A., DRASAR, B. S. & BANCROFT, G. J. 1999. Obligatory role of gamma interferon for host survival in a murine model of infection with *Burkholderia pseudomallei*. *Infect Immun,* 67**,** 3593-600.

SARKAR-TYSON, M., THWAITE, J. E., HARDING, S. V., SMITHER, S. J., OYSTON, P. C., ATKINS, T. P. & TITBALL, R. W. 2007. Polysaccharides and virulence of *Burkholderia pseudomallei*. *J Med Microbiol,* 56**,** 1005-10.

SARTAIN, M. J., SLAYDEN, R. A., SINGH, K. K., LAAL, S. & BELISLE, J. T. 2006. Disease state differentiation and identification of tuberculosis biomarkers via native antigen array profiling. *Mol Cell Proteomics,* 5**,** 2102-13.

SAUER, J. D., BACHMAN, M. A. & SWANSON, M. S. 2005. The phagosomal transporter A couples threonine acquisition to differentiation and replication of *Legionella pneumophila* in macrophages. *Proc Natl Acad Sci U S A,* 102**,** 9924-9.

SCHWEIZER, H. P. & PEACOCK, S. J. 2008. Antimicrobial drug-selection markers for *Burkholderia pseudomallei* and *B. mallei*. *Emerg Infect Dis,* 14**,** 1689-92.

SERMSWAN, R. W., WONGRATANACHEEWIN, S., ANUNTAGOOL, N. & SIRISINHA, S. 2000. Comparison of the polymerase chain reaction and serologic tests for diagnosis of septicemic melioidosis. *Am J Trop Med Hyg,* 63**,** 146-9.

SHALOM, G., SHAW, J. G. & THOMAS, M. S. 2007. In vivo expression technology identifies a type VI secretion system locus in *Burkholderia pseudomallei* that is induced upon invasion of macrophages. *Microbiology,* 153**,** 2689-99.

SHIVERS, R. P., PAGANO, D. J., KOOISTRA, T., RICHARDSON, C. E., REDDY, K. C., WHITNEY, J. K., KAMANZI, O., MATSUMOTO, K., HISAMOTO, N. & KIM, D. H. 2010. Phosphorylation of the conserved transcription factor ATF-7 by PMK-1 p38 MAPK regulates innate immunity in *Caenorhabditis elegans*. *PLoS Genet,* 6**,** e1000892.

SIFRI, C. D., BEGUN, J., AUSUBEL, F. M. & CALDERWOOD, S. B. 2003. Caenorhabditis elegans as a model host for *Staphylococcus aureus* pathogenesis. *Infect Immun,* 71**,** 2208-17.

SIRISINHA, S., ANUNTAGOOL, N., DHARAKUL, T., EKPO, P., WONGRATANACHEEWIN, S., NAIGOWIT, P., PETCHCLAI, B., THAMLIKITKUL, V. & SUPUTTAMONGKOL, Y. 2000. Recent developments in laboratory diagnosis of melioidosis. *Acta Trop,* 74**,** 235-45.

SITTHIDET, C., KORBSRISATE, S., LAYTON, A. N., FIELD, T. R., STEVENS, M. P. & STEVENS, J. M. 2011. Identification of motifs of *Burkholderia pseudomallei* BimA required for intracellular motility, actin binding, and actin polymerization. *J Bacteriol,* 193**,** 1901-10.

SIVALINGAM, S.P., ULETT, G.C. & NELSON, M. 2012. Model of infection. In Ketheesan, N. (Eds.), Melioidosis: a century of observation and research (pp.242-256). Amsterdam, Netherlands: Elsevier.

SONG, Y., XIE, C., ONG, Y. M., GAN, Y. H. & CHUA, K. L. 2005. The BpsIR quorum-sensing system of *Burkholderia pseudomallei*. *J Bacteriol,* 187**,** 785-90.

SPLETTSTOESSER, W. D., FRANGOULIDIS, D. & PUTHUCHEARY, S. D. 2008. Validation and comparison of an extrapolysaccharide (EPS)-based in-house ELISA and the PanBio melioidosis rapid cassette test-kits for serodiagnosis of melioidosis in a non-endemic area. *Trans R Soc Trop Med Hyg,* 102 Suppl 1**,** S45-6.

STANTON, A. T. & FLETCHER, W. 1921. Melioidosis, a new disease of the tropics. Trans. Fourth Congr *Far East Assoc. Trop. Med.,* 2**,** 196-8.

STANTON, A. T. & FLETCHER, W. 1932. Melioidosis. In Studies from the Institute for Medical Research, Federated Malay States London: John Bale and Danielson Ltd. 21.

STEINMETZ, I., REGANZEROWSKI, A., BRENNEKE, B., HAUSSLER, S., SIMPSON, A. & WHITE, N. J. 1999. Rapid identification of *Burkholderia pseudomallei* by latex agglutination based on an exopolysaccharide-specific monoclonal antibody. *J Clin Microbiol,* 37**,** 225-8.

STEVENS, J. M., GALYOV, E. E. & STEVENS, M. P. 2006. Actin-dependent movement of bacterial pathogens. *Nat Rev Microbiol,* 4**,** 91-101.

STEVENS, J. M., ULRICH, R. L., TAYLOR, L. A., WOOD, M. W., DESHAZER, D., STEVENS, M. P. & GALYOV, E. E. 2005a. Actin-binding proteins from *Burkholderia mallei* and *Burkholderia thailandensis* can functionally compensate for the actin-based motility defect of a Burkholderia pseudomallei bimA mutant. *J Bacteriol,* 187**,** 7857-62.

STEVENS, M. P., FRIEBEL, A., TAYLOR, L. A., WOOD, M. W., BROWN, P. J., HARDT, W. D. & GALYOV, E. E. 2003. A *Burkholderia pseudomallei* type III secreted protein, BopE, facilitates bacterial invasion of epithelial cells and exhibits guanine nucleotide exchange factor activity. *J Bacteriol,* 185**,** 4992-6.

STEVENS, M. P., HAQUE, A., ATKINS, T., HILL, J., WOOD, M. W., EASTON, A., NELSON, M., UNDERWOOD-FOWLER, C., TITBALL, R. W., BANCROFT, G. J. & GALYOV, E. E. 2004. Attenuated virulence and protective efficacy of a *Burkholderia pseudomallei* bsa type III secretion mutant in murine models of melioidosis. *Microbiology,* 150**,** 2669-76.

STEVENS, M. P., STEVENS, J. M., JENG, R. L., TAYLOR, L. A., WOOD, M. W., HAWES, P., MONAGHAN, P., WELCH, M. D. & GALYOV, E. E. 2005b. Identification of a bacterial factor required for actin-based motility of *Burkholderia pseudomallei*. *Mol Microbiol,* 56**,** 40-53.

STEVENS, M. P., WOOD, M. W., TAYLOR, L. A., MONAGHAN, P., HAWES, P., JONES, P. W., WALLIS, T. S. & GALYOV, E. E. 2002. An Inv/Mxi-Spa-like type III protein secretion system in *Burkholderia pseudomallei* modulates intracellular behaviour of the pathogen. *Mol Microbiol,* 46**,** 649-59.

STIERNAGLE, T. (Ed.). (1999). *Maintenance of C. elegans*. New York: Oxford University Press.

SU, Y. C., WAN, K. L., MOHAMED, R., & NATHAN, S. 2008. A genome level survey of *Burkholderia pseudomallei* immunome expressed during human infection. *Microbes Infect,* 10**,** 1335-45.

SUN, G. W., LU, J., PERVAIZ, S., CAO, W. P. & GAN, Y. H. 2005. Caspase-1 dependent macrophage death induced by *Burkholderia pseudomallei*. *Cell Microbiol,* 7**,** 1447-58.

SUNDARESH, S., RANDALL, A., UNAL, B., PETERSEN, J. M., BELISLE, J. T., HARTLEY, M. G., DUFFIELD, M., TITBALL, R. W., DAVIES, D. H., FELGNER, P. L. & BALDI, P. 2007. From protein microarrays to diagnostic antigen discovery: a study of the pathogen *Francisella tularensis*. *Bioinformatics,* 23**,** i508-18.

SUPAPROM, C., WANG, D., LEELAYUWAT, C., THAEWPIA, W., SUSAENGRAT, W., KOH, V., OOI, E. E., LERTMEMONGKOLCHAI, G. & LIU, Y. 2007. Development of real-time PCR assays and evaluation of their potential use for rapid detection of *Burkholderia pseudomallei* in clinical blood specimens. *J Clin Microbiol,* 45**,** 2894-901.

SUPARAK, S., KESPICHAYAWATTANA, W., HAQUE, A., EASTON, A., DAMNIN, S., LERTMEMONGKOLCHAI, G., BANCROFT, G. J. & KORBSRISATE, S. 2005. Multinucleated giant cell formation and apoptosis in infected host cells is mediated by *Burkholderia pseudomallei* type III secretion protein BipB. *J Bacteriol,* 187**,** 6556-60.

SUPUTTAMONGKOL, Y., HALL, A. J., DANCE, D. A., CHAOWAGUL, W., RAJCHANUVONG, A., SMITH, M. D. & WHITE, N. J. 1994. The epidemiology of melioidosis in Ubon Ratchatani, northeast Thailand. *Int J Epidemiol,* 23**,** 1082-90.

SUWANNASAEN, D., MAHAWANTUNG, J., CHAOWAGUL, W., LIMMATHUROTSAKUL, D., FELGNER, P. L., DAVIES, H., BANCROFT, G. J., TITBALL, R. W. & LERTMEMONGKOLCHAI, G. 2011. Human immune responses to *Burkholderia pseudomallei* characterized by protein microarray analysis. *J Infect Dis,* 203**,** 1002-11.

SUZUKI, T., MURAI, T., FUKUDA, I., TOBE, T., YOSHIKAWA, M. & SASAKAWA, C. 1994. Identification and characterization of a chromosomal virulence gene, vacJ, required for intercellular spreading of *Shigella flexneri*. *Mol Microbiol,* 11**,** 31-41.

TAN, K. S., CHEN, Y., LIM, Y. C., TAN, G. Y., LIU, Y., LIM, Y. T., MACARY, P. & GAN, Y. H. 2010. Suppression of host innate immune response by *Burkholderia pseudomallei* through the virulence factor TssM. *J Immunol,* 184**,** 5160-71.

TAN, M. W., MAHAJAN-MIKLOS, S. & AUSUBEL, F. M. 1999. Killing of Caenorhabditis elegans by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. *Proc Natl Acad Sci U S A,* 96**,** 715-20.

TANDHAVANANT, S., THANWISAI, A., LIMMATHUROTSAKUL, D., KORBSRISATE, S., DAY, N. P., PEACOCK, S. J. & CHANTRATITA, N. 2010. Effect of colony morphology variation of *Burkholderia pseudomallei* on intracellular survival and resistance to antimicrobial environments in human macrophages in vitro. *BMC Microbiol,* 10**,** 303.

The *C. ELEGANS* SEQUENCING CONSORTIUM, 1998. Genome sequence of the nematode *C. elegans*: a platform for investigating biology. *Science,* 282**,** 2012-8.

THEPTHAI, C., SMITHTIKARN, S., SUKSUWAN, M., SONGSIVILAI, S. & DHARAKUL, T. 2005. Serodiagnosis of melioidosis by a competitive enzyme-linked immunosorbent assay using a lipopolysaccharide-specific monoclonal antibody. *Asian Pac J Allergy Immunol,* 23**,** 127-32.

THOMPSON, D. B., CRANDALL, K., HARDING, S. V., SMITHER, S. J., KITTO, G. B., TITBALL, R. W. & BROWN, K. A. 2008. In silico analysis of potential diagnostic targets from *Burkholderia pseudomallei*. *Trans R Soc Trop Med Hyg,* 102 Suppl 1**,** S61-5.

THONGDEE, M., GALLAGHER, L. A., SCHELL, M., DHARAKUL, T., SONGSIVILAI, S. & MANOIL, C. 2008. Targeted mutagenesis of *Burkholderia thailandensis* and *Burkholderia pseudomallei* through natural transformation of PCR fragments. *Appl Environ Microbiol,* 74**,** 2985-9.

TOMASO, H., PITT, T. L., LANDT, O., AL DAHOUK, S., SCHOLZ, H. C., REISINGER, E. C., SPRAGUE, L. D., RATHMANN, I. & NEUBAUER, H. 2005. Rapid presumptive identification of *Burkholderia pseudomallei* with real-time PCR assays using fluorescent hybridization probes. *Mol Cell Probes,* 19**,** 9-20.

TUANYOK, A. 2012. Genomic islands in *Burkholderia pseudomallei*. In Ketheesan, N. (Eds.), Melioidosis: a century of observation and research (pp.82-86). Amsterdam, Netherlands: Elsevier.

TUANYOK, A., LEADEM, B. R., AUERBACH, R. K., BECKSTROM-STERNBERG, S. M., BECKSTROM-STERNBERG, J. S., MAYO, M., WUTHIEKANUN, V., BRETTIN, T. S., NIERMAN, W. C., PEACOCK, S. J., CURRIE, B. J., WAGNER, D. M. & KEIM, P. 2008. Genomic islands from five strains of *Burkholderia pseudomallei*. *BMC Genomics,* 9**,** 566.

TUANYOK, A., TOM, M., DUNBAR, J. & WOODS, D. E. 2006. Genome-wide expression analysis of *Burkholderia pseudomallei* infection in a hamster model of acute melioidosis. *Infect Immun,* 74**,** 5465-76.

ULRICH, R. L., DESHAZER, D., BRUEGGEMANN, E. E., HINES, H. B., OYSTON, P. C. & JEDDELOH, J. A. 2004. Role of quorum sensing in the pathogenicity of *Burkholderia pseudomallei*. *J Med Microbiol,* 53**,** 1053-64.

UTAISINCHAROEN, P., ANUNTAGOOL, N., LIMPOSUWAN, K., CHAISURIYA, P. & SIRISINHA, S. 2003. Involvement of beta interferon in enhancing inducible nitric oxide synthase production and antimicrobial activity of *Burkholderia pseudomallei*-infected macrophages. *Infect Immun,* 71**,** 3053-7.

UTAISINCHAROEN, P., ARJCHAROEN, S., LENGWEHASATIT, I., LIMPOSUWAN, K. & SIRISINHA, S. 2005. *Burkholderia pseudomallei* invasion and activation of epithelial cells requires activation of p38 mitogen-activated protein kinase. *Microb Pathog,* 38**,** 107-12.

UTAISINCHAROEN, P., ARJCHAROEN, S., LIMPOSUWAN, K., TUNGPRADABKUL, S. & SIRISINHA, S. 2006. *Burkholderia pseudomallei* RpoS regulates multinucleated giant cell formation and inducible nitric oxide synthase expression in mouse macrophage cell line (RAW 264.7). *Microb Pathog,* 40**,** 184-9.

UTAISINCHAROEN, P., TANGTHAWORNCHAIKUL, N., KESPICHAYAWATTANA, W., ANUNTAGOOL, N., CHAISURIYA, P. & SIRISINHA, S. 2000. Kinetic studies of the production of nitric oxide (NO) and tumour necrosis factor-alpha (TNF-alpha) in macrophages stimulated with *Burkholderia pseudomallei* endotoxin. *Clin Exp Immunol,* 122**,** 324-9.

UTAISINCHAROEN, P., TANGTHAWORNCHAIKUL, N., KESPICHAYAWATTANA, W., CHAISURIYA, P. & SIRISINHA, S. 2001. *Burkholderia pseudomallei* interferes with inducible nitric oxide synthase (iNOS) production: a possible mechanism of evading macrophage killing. *Microbiol Immunol,* 45**,** 307-13.

VADIVELU, J. & PUTHUCHEARY, S. D. 2000. Diagnostic and prognostic value of an immunofluorescent assay for melioidosis. *Am J Trop Med Hyg,* 62**,** 297-300.

VADIVELU, J., PUTHUCHEARY, S. D., GENDEH, G. S. & PARASAKTHI, N. 1995. Serodiagnosis of melioidosis in Malaysia. *Singapore Med J,* 36**,** 299-302.

VALADE, E., THIBAULT, F. M., GAUTHIER, Y. P., PALENCIA, M., POPOFF, M. Y. & VIDAL, D. R. 2004. The PmlI-PmlR quorum-sensing system in *Burkholderia pseudomallei* plays a key role in virulence and modulates production of the MprA protease. *J Bacteriol,* 186**,** 2288-94.

VANAPORN, M., WAND, M., MICHELL, S. L., SARKAR-TYSON, M., IRELAND, P., GOLDMAN, S., KEWCHAROENWONG, C., RINCHAI, D., LERTMEMONGKOLCHAI, G. & TITBALL, R. W. 2011. Superoxide dismutase C is required for intracellular survival and virulence of *Burkholderia pseudomallei*. *Microbiology,* 157**,** 2392-400.

VASU, C., VADIVELU, J. & PUTHUCHEARY, S. D. 2003. The humoral immune response in melioidosis patients during therapy. *Infection,* 31**,** 24-30.

WAJANAROGANA, S., NIMNUCH, P., THONGMEE, A. & KRITSIRIWUTHINAN, K. 2013. Potential of recombinant flagellin fragment from *Burkholderia thailandensis* as an antigen for melioidosis antibody detection by indirect ELISA. *Mol Cell Probes,* 27**,** 98-102.

WALSH, A. L., SMITH, M. D., WUTHIEKANUN, V., SUPUTTAMONGKOL, Y., DESAKORN, V., CHAOWAGUL, W. & WHITE, N. J. 1994. Immunofluorescence microscopy for the rapid diagnosis of melioidosis. *J Clin Pathol,* 47**,** 377-9.

WANG, C. Y., YAP, B. H. & DELILKAN, A. E. 1993. Melioidosis pneumonia and blast injury. *Chest,* 103**,** 1897-9.

WARAWA, J., & WOODS, D. E. 2005. Type III secretion system cluster 3 is required for maximal virulence of *Burkholderia pseudomallei* in a hamster infection model. *FEMS Microbiol Lett,* 242**,** 101-8.

WEISSERT, C., DOLLENMAIER, G., RAFEINER, P., RIEHM, J. & SCHULTZE, D. 2009. *Burkholderia pseudomallei* misidentified by automated system. *Emerg Infect Dis,* 15**,** 1799-801.

WEST, T. E., CHIERAKUL, W., CHANTRATITA, N., LIMMATHUROTSAKUL, D., WUTHIEKANUN, V., EMOND, M. J., HAWN, T. R., PEACOCK, S. J. & SKERRETT, S. J. 2012. Toll-like receptor 4 region genetic variants are associated with susceptibility to melioidosis. *Genes Immun,* 13**,** 38-46.

WHITE, N. J. 2003. Melioidosis. *Lancet,* 361**,** 1715-22.

WHITE, J. G., SOUTHGATE, E., THOMSON, J. N., & BRENNER, S. 1986. The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Philos Trans R Soc Lond B Biol Sci,* 314**,** 1-340.

WIERSINGA, W. J., CURRIE, B. J. & PEACOCK, S. J. 2012. Melioidosis. *N Engl J Med,* 367**,** 1035-44.

WIERSINGA, W. J., VAN DER POLL, T., WHITE, N. J., DAY, N. P. & PEACOCK, S. J. 2006. Melioidosis: insights into the pathogenicity of *Burkholderia pseudomallei*. *Nat Rev Microbiol,* 4**,** 272-82.

WIERSINGA, W. J., WIELAND, C. W., DESSING, M. C., CHANTRATITA, N., CHENG, A. C., LIMMATHUROTSAKUL, D., CHIERAKUL, W., LEENDERTSE, M., FLORQUIN, S., DE VOS, A. F., WHITE, N., DONDORP, A. M., DAY, N. P., PEACOCK, S. J. & VAN DER POLL, T. 2007. Toll-like receptor 2 impairs host defense in gram-negative sepsis caused by *Burkholderia pseudomallei* (Melioidosis). *PLoS Med,* 4**,** e248.

WONG, K. T., PUTHUCHEARY, S. D. & VADIVELU, J. 1995. The histopathology of human melioidosis. *Histopathology,* 26**,** 51-5.

WONGRATANACHEEWIN, S., KESPICHAYAWATTANA, W., INTACHOTE, P., PICHYANGKUL, S., SERMSWAN, R. W., KRIEG, A. M. & SIRISINHA, S. 2004. Immunostimulatory CpG oligodeoxynucleotide confers protection in a murine model of infection with *Burkholderia pseudomallei*. *Infect Immun,* 72**,** 4494-502.

WONGTRAKOONGATE, P., ROYTRAKUL, S., YASOTHORNSRIKUL, S. & TUNGPRADABKUL, S. 2011. A proteome reference map of the causative agent of melioidosis *Burkholderia pseudomallei*. *J Biomed Biotechnol,* 2011**,** 530926.

WOO, P. C., LEUNG, P. K., WONG, S. S., HO, P. L. & YUEN, K. Y. 2001. groEL encodes a highly antigenic protein in *Burkholderia pseudomallei*. *Clin Diagn Lab Immunol,* 8**,** 832-6.

WUTHIEKANUN, V., AMORNCHAI, P., CHIERAKUL, W., CHENG, A. C., WHITE, N. J., PEACOCK, S. J. & DAY, N. P. 2004. Evaluation of immunoglobulin M (IgM) and IgG rapid cassette test kits for diagnosis of melioidosis in an area of endemicity. *J Clin Microbiol,* 42**,** 3435-7.

WUTHIEKANUN, V., CHIERAKUL, W., LANGA, S., CHAOWAGUL, W., PANPITPAT, C., SAIPAN, P., THOUJAIKONG, T., DAY, N. P. & PEACOCK, S. J. 2006a. Development of antibodies to *Burkholderia pseudomallei* during childhood in melioidosis-endemic northeast Thailand. *Am J Trop Med Hyg,* 74**,** 1074-5.

WUTHIEKANUN, V., CHIERAKUL, W., RATTANALERTNAVEE, J., LANGA, S., SIRODOM, D., WATTANAWAITUNECHAI, C., WINOTHAI, W., WHITE, N. J., DAY, N. & PEACOCK, S. J. 2006b. Serological evidence for increased human exposure to *Burkholderia pseudomallei* following the tsunami in southern Thailand. *J Clin Microbiol,* 44**,** 239-40.

WUTHIEKANUN, V., DESAKORN, V., WONGSUVAN, G., AMORNCHAI, P., CHENG, A. C., MAHARJAN, B., LIMMATHUROTSAKUL, D., CHIERAKUL, W., WHITE, N. J., DAY, N. P. & PEACOCK, S. J. 2005. Rapid immunofluorescence microscopy for diagnosis of melioidosis. *Clin Diagn Lab Immunol,* 12**,** 555-6.

WUTHIEKANUN, V., SMITH, M. D., DANCE, D. A., WALSH, A. L., PITT, T. L. & WHITE, N. J. 1996. Biochemical characteristics of clinical and environmental isolates of *Burkholderia pseudomallei*. *J Med Microbiol,* 45**,** 408-12.

YABUUCHI, E., KOSAKO, Y., OYAIZU, H., YANO, I., HOTTA, H., HASHIMOTO, Y., EZAKI, T. & ARAKAWA, M. 1992. Proposal of *Burkholderia* gen. nov. and transfer of seven species of the genus *Pseudomonas* homology group II to the new genus, with the type species *Burkholderia cepacia* (Palleroni and Holmes 1981) comb. nov. *Microbiol Immunol,* 36**,** 1251-75.

YU, Y., KIM, H. S., CHUA, H. H., LIN, C. H., SIM, S. H., LIN, D., DERR, A., ENGELS, R., DESHAZER, D., BIRREN, B., NIERMAN, W. C. & TAN, P. 2006. Genomic patterns of pathogen evolution revealed by comparison of *Burkholderia pseudomallei*, the causative agent of melioidosis, to avirulent *Burkholderia thailandensis*. *BMC Microbiol,* 6**,** 46.

YUEN, C. W., ONG, E. B., MOHAMAD, S., MANAF, U. A. & NAJIMUDIN, N. 2012. Construction and characterization of a *Burkholderia pseudomallei* wzm deletion mutant. *J Microbiol Biotechnol,* 22**,** 1336-42.

ZHANG, Y., LU, H., & BARGMANN, C. I. 2005. Pathogenic bacteria induce aversive olfactory learning in *Caenorhabditis elegans*. *Nature,* 438**,** 179-184.

ZHOU, X. H., OBUCHOWSKI, N.A. & MCCLISH D.K. 2011. Measure of diagnostic accuracy. In Statistical methods in diagnostic medicine (pp. 15-56). New Jersey, United Statess: John Wiley & Sons.

ZONG, Z., WANG, X., DENG, Y. & ZHOU, T. 2012. Misidentification of *Burkholderia pseudomallei* as *Burkholderia cepacia* by the VITEK 2 system. *J Med Microbiol,* 61**,** 1483-4.

Publications

1. PUTHUCHEARY, S. D., **PUAH, S. M.**, CHAI, H. C., THONG, K. L. & CHUA, K. H. 2012. Molecular investigation of virulence determinants between a virulent clinical strain and an attenuated strain of *Burkholderia pseudomallei. J Mol Microbiol Biotechnol,* 22**,** 198-204.

2. **PUAH, S. M.**, PUTHUCHEARY, S. D., & CHUA, K. H. 2013. Potential immunogenic polypeptides of *Burkholderia pseudomallei* identified by shotgun expression library and evaluation of their efficacy for serodiagnosis of melioidosis. *Int J Med Sci,* 10**,** 539-47.

3. Molecular characterization of putative virulent determinants of *Burkholderia pseudomallei.* (Submitted, 2014)

4. A systematic review for serological antibody detection tests in diagnosis of melioidosis. (Manuscript in preparation)

APPENDICES

Appendix I: Sources of chemicals and reagents

Company	Origin	Reagents
		▪ Bovine serum albumin (BSA) ▪ Boric acid (H_3BO_3) ▪ Ethylenediamine tetraacetate (EDTA) ▪ Fish gelatin ▪ Sodium chloride (NaCl) ▪ Sodium dodecyl sulfate (SDS) ▪ Tris-base ▪ Tris, Tris (Hydroxymethyl) aminomethane ▪ Triton X -100 ▪ Tween-20
BD	USA	▪ Bacto Agar ▪ Bacto Peptone ▪ LB agar Miller ▪ LB broth ▪ Tryptic soy agar
Bio-Rad laboratories Ltd.	USA	▪ Acrylamide:bisacrylamide (29:1) (30 % w/v) solution ▪ Ammonium persulfate (APS) ▪ *N,N,N',N'*-tetramethylethylenediamine (TEMED)
Carolina	USA	Caenorhabditis elegans N2 (wild type strain)
Fermentas Life Sciences	USA	▪ 50 bp and 100 bp DNA molecular weight markers ▪ DreamTaq DNA polymerase
GCC	UK	▪ Absolute ethanol (EtOH)
Life technologies	USA	▪ DMEM with L-glutamine ▪ Fetal Bovine Serum ▪ Gentamycin ▪ Penicillin-Streptomycin ▪ TrypLE

Appendix I (Continued)

Company	Origin	Reagents
Merck	Germany	▪ Ampicilin ▪ Carbenicillin ▪ Chloramphenicol ▪ Ethidium Bromide (EtBr) ▪ Glycine ▪ Potassium dihydrogen phosphate (KH_2PO_4) ▪ Magnesium sulphate ($MgSO_4$) ▪ Sodium hydroxide (NaOH) ▪ Disodium phosphate (Na_2HPO_4) ▪ Phosphate-buffered saline (PBS)
Nacalai Tesque	Japan	▪ Calcium chloride ($CaCl_2$) ▪ Cholesterol ▪ Glucose ▪ Sorbitol ▪ Sucrose ▪ Streptomycin ▪ Trisodium citrate dehydrate ($C_6H_5Na_3O_7.2H_2O$)
New England Biolabs (NEB) Inc.	UK	▪ CIP ▪ Restrictions enzymes *EcoRI, NcoI, Not I* ▪ T4 DNA Ligase
Pierce	USA	▪ AP-conjugated Immunopure® ProteinA/G ▪ BCIP/NBT phosphatase substrate
Promega	USA	▪ Agarose powder ▪ Isopropyl b-D-thiogalacpyranoside (IPTG) ▪ X-gal
Sigma-Aldrich	USA	▪ Kanamycin ▪ Laemmli buffer ▪ Tetracycline
Takara	Japan	▪ LA *taq* polymerase
VWR	USA	▪ Methanol ▪ Sulfuric acid

Appendix II: Commonly used stock solutions

50 mg/mL Ampicillin

Ampicillin	0.5 g
sdH_2O to	10 mL

The stock solution was sterilized by filtration and aliquoted into smaller working volumes of 1 ml, which were kept at -20ºC.

50 mg/mL Carbenicillin

Carbenicillin	0.5 g
sdH_2O to	10 mL

The stock solution was sterilized by filtration and aliquoted into smaller working volumes of 1 ml, which were kept at -20ºC.

25 mg/mL Chloramphenicol

Chloramphenicol	0.25 g
Ethanol (100%) to	10 mL

The powder was dissolved in absolute ethanol and sterilized by filtration. Next, the solution was aliquoted into smaller working volumes of 1 ml, which were kept at -20ºC.

25 mg/mL Isopropyl b-D-thiogalacpyranoside (IPTG)

IPTG	0.25 g
sdH_2O to	10 mL

The stock solution was sterilized by filtration and aliquoted into smaller working volumes of 1 ml, which were kept at -20ºC.

25 mg/mL Kanamycin

Kanamycin sulfate 0.25 g

sdH_2O to 10 mL

The stock solution was sterilized by filtration and aliquoted into smaller working volumes of 1 ml, which were kept at -20ºC.

50 mg/mL Streptomycin

Streptomycin 0.5 g

sdH_2O to 10 mL

The stock solution was sterilized by filtration and aliquoted into smaller working volumes of 1 ml, which were kept at -20ºC.

5 mg/mL Tetracycline

Tetracyline 0.25 g

sdH_2O to 50 mL

The stock solution was sterilized by filtration and aliquoted into smaller working volumes of 1 ml, which were kept at -20ºC.

20 mg/mL 5-bromo-4-chloro-3-indolyl b-D-galactopyranoside (X-gal)

X-gal 0.2 g

dimethylformamide 10 mL

The stock solution was mixed well and aliquoted into smaller working volumes of 1 ml in microcentrifuge tubes which were wrapped in foil to protect it from light and kept at -20ºC.

Ethanol (EtOH)

Ethanol was used in various concentrations (v/v), i.e., 70 and 100%. The stock of absolute ethanol was adjusted with sdH_2O accordingly.

LB agar

Difco ™ LB Agar, Miller	40 g
sdH_2O to	1000 mL

The powder was completely dissolved in 800 mL of sdH_2O by heating with frequent agitation. The volume was then adjusted to 1 liter and sterilized by autoclaving.

LB broth

Difco ™ LB Broth, Miller	25 g
sdH_2O to	1000 mL

The powder was completely dissolved in 800 mL of sdH_2O by heating with frequent agitation. The volume was then adjusted to 1 liter and sterilized by autoclaving.

Minimal medium plate

5 X M9 salts	200	mL
1 M $MgSO_4$	2	mL
20% glucose	20	mL
1 M $CaCl_2$	0.1	mL
Agar	15	g

The volume was then adjusted to 1 liter and sterilized by autoclaving.

Tryptic Soy Agar (TSA)

Difco ™ TSA agar	40 g
sdH_2O to	1000 mL

The powder was completely dissolved in 800 mL of sdH_2O by heating with frequent agitation. The volume was then adjusted to 1 liter and sterilized by autoclaving.

Appendix III: *Caenorhabditis elegans* killing assay

Nematode growth agar

Agar	17 g
NaCl	2.9 g
Peptone	2.5 g
1 M $CaCl_2$	1 ml
5 mg/mL Cholesterol	1 mL
1 M $MgSO_4$	1 mL
1 M KH_2PO_4	25 mL
sdH_2O to	1000 ml

The first 3 reagents were mixed and sterilized by autoclaving. Next, the last 4 reagents were added after the mixture was cooling to 55°C.

M9 buffer

KH_2PO4	3 g
Na_2HPO_4	6 g
NaCl	5 g
1 M $MgSO_4$	1 mL
sdH_2O to	1000 mL

The first 3 reagents were mixed and filter sterilized by using a 0.45 μm filter. Next, 1 mL of 1 M $MgSO_4$ was added when the solution was cooling to 55°C.

Freezing buffer

1 M NaCl	20 mL
1 M KH_2PO_4, pH 6	10 mL
Glycerol	60 mL
0.1 M $MgSO_4$	0.6 mL
sdH_2O to	200 mL

The first 3 reagents were mixed and sterilized by autoclaving. Next, 0.6 mL of 0.1 M $MgSO_4$ was added under sterile condition when the solution was cooling to 55°C.

Appendix IV: Solutions used in agarose gel electrophoresis (AGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

10X Tris-borate EDTA buffer (TBE), pH 8.3

Tris base	108 g
Boric acid	55 g
0.5 M EDTA	40 mL
sdH_2O to	1000 mL

A 10X TBE stock solution was prepared and stored at room temperature. It was used as soon as possible because precipitation occurred on long storage. The solution was diluted to appropriate strengths, i.e., 5X or 1X for use.

6X Loading Dye (LD)

Bromophenol blue	0.25 g
Xylene cyanol	0.25 g
Glycerol	50 mL
1 M Tris, pH 8.0	1 mL
sdH_2O to	100 mL

The 100 mL stock solution was prepared by mixing all of the above mentioned and stored at room temperature.

10 mg/mL Ethidium bromide (EtBr)

EtBr	0.1 g
sdH_2O to	10 mL

The stock solution was prepared and stored at room temperature in a bottle wrapped in aluminium foil. EtBr was used at a final concentration of 0.5 μg/mL.

10% (w/v) Ammonium persulphate (APS)

APS	0.02 g
sdH_2O	200 µL

The above solution was prepared freshly prior to use.

4X Tris.Cl/SDS (pH 6.8)

Tris base	6.05 g
sdH_2O	100 mL

Tris base was dissolved in 40 mL H_2O and pH adjusted to pH6.8 with 1 N HCl, later H_2O was added to 100 mL. The solution was then filtered through a 0.45 µM filter and 0.4 g of SDS was added into solution and stored at 4°C.

4X Tris.Cl/SDS (pH 8.8)

Tris base	91 g
sdH_2O	500 mL

Tris base was dissolved in 300 mL H_2O and pH adjusted to pH8.8 with 1 N HCl, later H_2O was added to 500 mL. The solution was then filtered through a 0.45 µM filter and 2 g of SDS was added into solution and stored at 4°C.

5X SDS running buffer

Tris base	15 g
Glycine	72 g
SDS	5 g
sdH_2O	1000 mL

The 1000 mL stock solution was prepared by mixing all of the above mentioned and stirred until all dissolved. The solution was diluted to 1X for use.

Appendix V: Solutions used in Colony-blot detection

10% Sodium Dodecyl Sulphate (SDS)

SDS	100 g
sdH_2O to	1000 mL

The solution was adjusted to pH7.2 with HCl.

Denaturing solution

NaOH	20 g
NaCl	87.7 g
sdH_2O to	1000 mL

NaOH and NaCl were slowly dissolved in sdH_2O and autoclaved at 121℃ for 15 minutes, and stored at room temperature.

Neutralization solution

NaCl	87.7 g
Tris base	60.6 g
sdH_2O to	1000 mL

NaCl and Tris base were slowly dissolved in sdH_2O, adjusted to pH7.4 with HCl and autoclaved at 121℃ for 15 minutes, and stored at room temperature.

20X Saline Sodium Citrate (SSC)

NaCl	175.3 g
Sodium citrate.2H_2O	88.2 g
sdH_2O to	1000 mL

NaCl and sodium citrate.2H_2O was slowly dissolved in sdH_2O. The pH of the solution was then adjusted to 7.0 with HCl. The stock solution was autoclaved at 121ºC for 15 minutes, and stored at room temperature.

Appendix VI: Solutions used in Western blot analysis

Towbin Transfer buffer

Tris	3 g
Glycine	14.4 g
SDS	1 g
Methanol	200 mL
sdH_2O to	800 mL

NaCl and sodium citrate.2H_2O was slowly dissolved in sdH_2O. The pH of the solution was then adjusted to 7.0 with HCl. The stock solution was autoclaved at 121ºC for 15 minutes, and stored at room temperature.

10X Tris Buffered Saline (TBS), pH 7.5

Tris HCl	24 g
Tris base	5.6 g
NaCl	88 g
sdH_2O to	1000 mL

All 3 materials were slowly dissolved in 800 mL sdH_2O. The pH of the solution was then adjusted to 7.5 and lastly sdH_2O was added to a final volume of 1 liter. The solution was diluted to 1X for use.

1X Tris Buffered Saline 0.05% Tween-20 (TBST), pH 7.5

One hundred mL of 10X TBS was diluted with 900 mL sdH_2O and 0.5 mL of Tween-20 was then added into the solution.

Blocking buffer

One percent of gelation was added into 1X PBS prior to the experiment and was used immediately.

1X TES

Tris-HCl	24.23 g
EDTA	0.19 g
Sucrose	171.2 g
sdH_2O to	1000 mL

The solution above was filtered by using a 0.45 µm filter and kept at 4ºC. The solution was diluted to 1/5X TES buffer by adding 1 volume of 1X TES buffer to 4 volumes of sdH_2O.

Appendix VII: Solutions used in ELISA assay

1X Phosphate Buffered Saline (PBS), pH 7.3-7.5

OmniPur PBS tablet	1 tablet
sdH_2O to	100 mL

The tablet was slowly dissolved in 100 mL sdH_2O and stored at room temperature.

1X Phosphate Buffered Saline 0.2% Tween-20 (PBST)

Of 100 mL of 1X PBS, 0.2 mL of Tween-20 was then added into the solution.

Blocking buffer

One gram of bovine serum albumin was added into 100 mL of 1X PBST prior to the experiment and was used immediately.

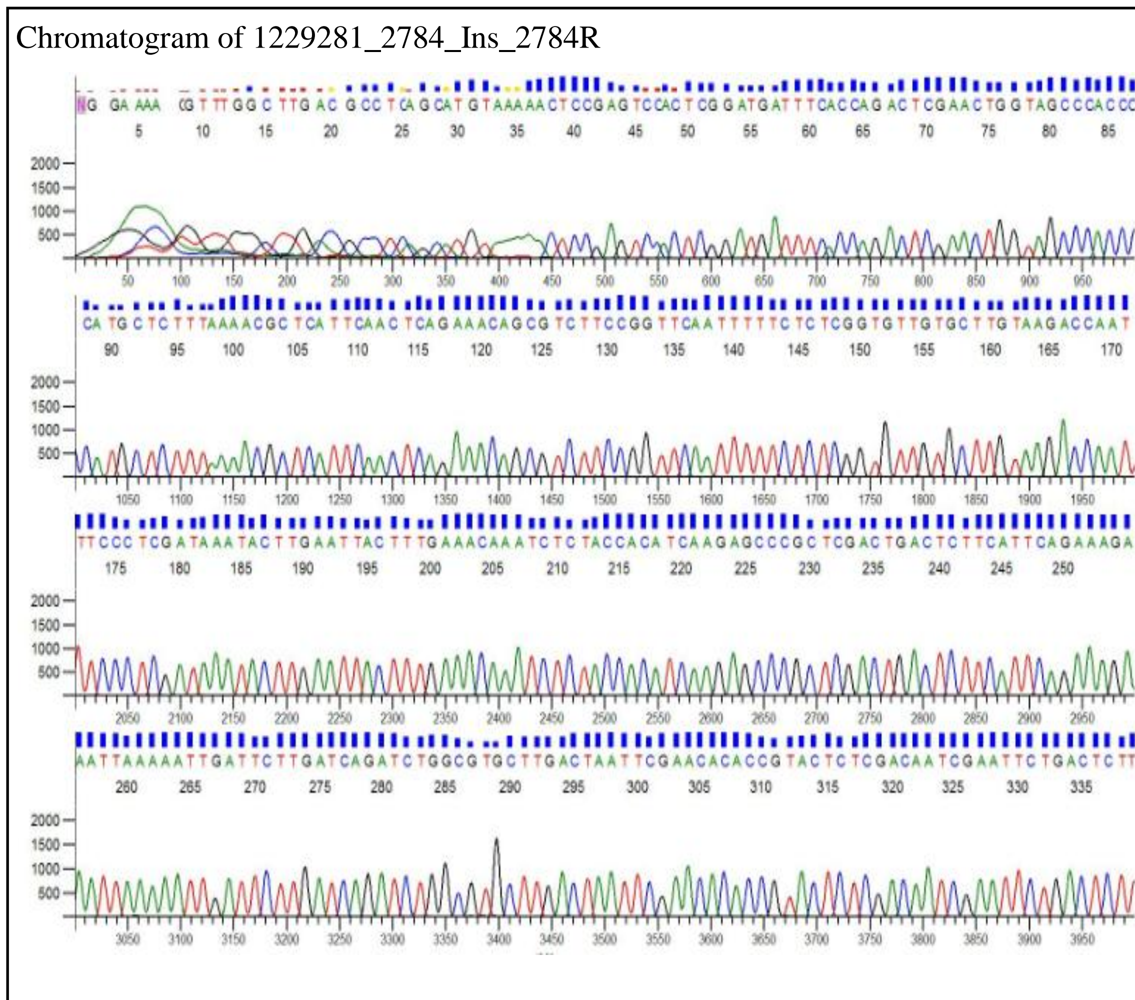
Stop buffer

0.1 M H_2SO_4

Sulfuric acid (H_2SO_4 1M)	5.6 mL
sdH_2O to	100 mL

Sulfuric acid was slowly added to 80 mL sdH_2O and mixing well, and was then adjusted to final volume of 100 mL. The solution was then diluted to 1/10X buffer by adding 1 volume of 1M H_2SO_4 to 9 volumes of sdH_2O.

Appendix VIII: Example of sequencing result



Sequence alignment using ClustalW

```
Reference              GCCTCAGCATGTAAAAACTCCGAGTCCACTCGGATGATTTCACCAGACTC 100
1229281_2784_Ins_2784R GCCTCAGCATGTAAAAACTCCGAGTCCACTCGGATGATTTCACCAGACTC 50
                       **************************************************

Reference              GAACTGGTAGCCCACCCCATGCTCTTTAAAACGCTCATTCAACTCAGAAA 150
1229281_2784_Ins_2784R GAACTGGTAGCCCACCCCATGCTCTTTAAAACGCTCATTCAACTCAGAAA 100
                       **************************************************

Reference              CAGCGTCTTCCGGTTCAATTTTTCTCTCGGTGTTGTGCTTGTAAGACCAA 200
1229281_2784_Ins_2784R CAGCGTCTTCCGGTTCAATTTTTCTCTCGGTGTTGTGCTTGTAAGACCAA 150
                       **************************************************

Reference              TTTCCCTCGATAAATACTTGAATTACTTTGAAACAAATCTCTACCACATC 250
1229281_2784_Ins_2784R TTTCCCTCGATAAATACTTGAATTACTTTGAAACAAATCTCTACCACATC 200
                       **************************************************

Reference              AAGAGCCCGCTCGACTGACTCTTCATTCAGAAAGAAATTAAAAATTGATT 300
1229281_2784_Ins_2784R AAGAGCCCGCTCGACTGACTCTTCATTCAGAAAGAAATTAAAAATTGATT 250
                       **************************************************

Reference              CTTGATCAGATCTGGCGTGCTTGACTAATTCGAACACACCGTACTCTCGA 350
1229281_2784_Ins_2784R CTTGATCAGATCTGGCGTGCTTGACTAATTCGAACACACCGTACTCTCGA 300
                       **************************************************
```

Appendix IX: Raw data for replication of bacteria in macrophage cells

No	Hours	Before normalization (cfu)			After normalization (%)		
		2	4	8	2	4	8
1	Bp-CMS	1.13E+07	1.52E+07	1.57E+07	100	134	138
2	*BPSL2033::Km*	6.89E+06	4.63E+06	2.08E+06	100	67	30
	Complemented-strains	1.30E+07	1.24E+07	9.84E+06	100	96	76
3	*BP1026B_I2780::Km*	2.56E+07	1.86E+07	1.14E+07	100	73	44
	Complemented-strains	2.87E+07	2.68E+07	1.49E+07	100	93	52
4	*BP1026B_I2784::Km*	8.24E+06	7.36E+06	8.11E+06	100	89	98
	Complemented-strains	1.26E+07	1.03E+07	7.64E+06	100	82	61
5	*BURPS1106A_A0094::Km*	1.91E+07	1.06E+07	3.12E+06	100	55	16
	Complemented-strains	1.72E+07	1.93E+07	6.64E+06	100	113	39
6	*BURPS1106A_1131::Km*	1.35E+07	8.49E+06	9.77E+06	100	63	72
	Complemented-strains	5.68E+06	6.27E+06	1.68E+06	100	110	30
7	*BURPS1710A_1419::Km*	1.31E+07	1.13E+07	5.73E+06	100	87	44
	Complemented-strains	3.48E+07	2.90E+07	2.92E+07	100	83	84
8	*BPSL3147::Km*	8.10E+06	6.62E+06	3.74E+06	100	82	46
	Complemented-strains	1.93E+07	2.14E+07	1.59E+07	100	111	82

*Raw data of before normalization represent the mean value obtained from 3 separate experiments and each experiment was performed in 3 technical replicates for each time point.