

**COLONY MORPHOTYPE, BIOFILM FORMATION AND  
IDENTIFICATION OF LECTINS OF  
*Burkholderia pseudomallei***

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**FACULTY OF MEDICINE  
UNIVERSITY OF MALAYA  
KUALA LUMPUR**

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AND IDENTIFICATION OF LECTINS OF  
*Burkholderia pseudomallei***

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## ABSTRACT

*Burkholderia pseudomallei*, the causative agent of melioidosis, is an important bacterial pathogen in the tropical regions. Melioidosis, the disease caused by *B. pseudomallei*, has been reported with high mortality and morbidity rates in the endemic regions. Although lectins (sugar binding proteins) had been reported to be important for biofilm formation of several Gram-negative bacteria including *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*, there is yet any study on the lectins of *B. pseudomallei*. This study investigated biofilm production of 76 clinical isolates of *B. pseudomallei* using a standard biofilm crystal violet staining assay. The results obtained were correlated with their respective colony morphotypes on *Burkholderia pseudomallei* selective agar medium. As lectin has been reported to initiate bacterial biofilm formation, this study aims to identify, clone and express hypothetical lectin genes in *B. pseudomallei*. The hypothetical genes were also explored for development of a multiplex polymerase chain reaction (PCR) assay for identification of *B. pseudomallei* and other closely related species. Based on the colonial morphology of *B. pseudomallei* on *B. pseudomallei* selective agar medium, seven distinct colony morphotypes were identified in this study. Most isolates (40.8 %) were identified as colony morphotype group 1 which displayed a rough centre with irregular circumference on the agar medium. Of the 76 *B. pseudomallei* isolates investigated, 20 (26.3 %) were identified as high biofilm producer ( $X > 11.01$ ), while 37 (48.7%) isolates were medium ( $3.45 < X < 11.01$ ), and 19 (25.0 %) were low biofilm ( $X < 3.45$ ) producers, when compared to *B. thailandensis* ATCC 700388 strain. No correlation was found between *B. pseudomallei* morphotypes with biofilm forming abilities ( $p > 0.05$ ). Seven genes encoding hypothetical lectin (*BPSS0713*, *BPSS0767*, *BPSS1124*, *BPSS1488*, *BPSS1649*, *BPSS2022*, and *BPSL2056*) were retrieved from the genome sequence of *B.*

*pseudomallei* K96243 reference strain. By inclusion of primers targeting 16S rRNA gene and two hypothetical lectin genes (*BPSS2022* and *BPSS1649*), a multiplex PCR assay was successfully developed and evaluated for rapid differentiation of *B. pseudomallei*, *B. mallei*, *B. thailandensis* and *B. cepacia* complex. The PCR assay was specific and was able to detect up to 109, 60, 23, and 9 ng of the DNA of *B. pseudomallei*, *B. mallei*, *B. thailandensis* and *B. cepacia* complex, respectively. Four hypothetical genes (*BPSS0713*, *BPSS0767*, *BPSS1124*, and *BPSS1488*) were successfully cloned and expressed as recombinant proteins in this study. However, none of the recombinant proteins demonstrated positive findings for the hemagglutination assays. Thus, the functions of four hypothetical lectin genes of *B. pseudomallei* were not confirmed. Many factors including post-translational modification, protein denaturation, and absence of co-factors might affect the expression of lectin activity. For future investigation, glycan array, isothermal titration calorimetry or surface plasmon resonance could be explored to identify lectin in *B. pseudomallei*.

## ABSTRAK

*Burkholderia pseudomallei*, agen penyebab melioidosis, ialah patogen bakteria penting di kawasan tropika. Melioidosis, penyakit yang disebabkan oleh *B. pseudomallei*, telah dilaporkan dengan kadar kematian dan morbiditi yang tinggi di kawasan endemik. Keupayaan *B. pseudomallei* untuk membentuk pelbagai morfotip dan biofilem telah baru-baru ini dikaitkan dengan kevirulenan bakteria ini. Walaupun lektin (protin pengikatan gula) telah dilaporkan penting untuk pembentukan biofilem beberapa bakteria Gram-negatif termasuk *Pseudomonas aeruginosa* dan *Burkholderia cenocepacia*, tiada kajian mengenai lektin *B. pseudomallei* dilaporkan. Kajian ini menyiasat pembentukan biofilem untuk 76 isolat klinikal *B. pseudomallei* dengan menggunakan kaedah pewarnaan kristal ungu. Keputusan yang diperolehi dihubungkan dengan morfotip koloni masing-masing pada *Burkholderia pseudomallei* "selective agar". Oleh kerana lektin telah dilaporkan berperanan untuk memulakan pembentukan biofilem bakteria, kajian ini bertujuan untuk mengenalpasti, klon dan mengekspres gen lektin andaian *B. pseudomallei*. Gen-gen andaian ini juga ditinjau untuk pembangunan satu esai multipleks PCR untuk pengenalpastian *B. pseudomallei* dan spesies yang berkait rapat. Berdasarkan morfotip koloni bakteria pada *B. pseudomallei* selective agar, tujuh morfotip berbeza telah dikenalpasti. Kebanyakan isolat telah dikenalpasti sebagai kumpulan morfotip 1 yang memaparkan koloni dengan pusat bergelora dan lilitan yang tidak teratur pada media agar. Antara 76 isolat *B. pseudomallei* yang dikaji, 20 (26.3%) telah dikenalpasti sebagai pengeluar biofilem tinggi ( $X > 11.01$ ), manakala 37 (48.7%) adalah penghasil biofilem sederhana ( $3.45 < X < 11.01$ ) dan 19 (25.0%) adalah pengeluar biofilem rendah ( $X < 3.45$ ) berbanding dengan strain rujukan *B. thailandensis* ATCC 700388. Walau bagaimanapun, tidak ada korelasi antara morfotip *B. pseudomallei* dengan kebolehan pembentukan biofilem ( $p > 0.05$ ).

Tujuh gen pengkodan lektin andaian (*BPSS0713*, *BPSS0767*, *BPSS1124*, *BPSS1488*, *BPSS1649*, *BPSS2022*, dan *BPSL2056*) telah diperolehi dari urutan genom strain rujukan *B. pseudomallei* K96243. Dengan menggunakan primer yang menyasarkan gen 16S rRNA dan dua gen hipotetikal lectin (*BPSS2022* dan *BPSS1649*), satu esai multipleks PCR telah dibangunkan dan dinilai untuk pembezaan cepat antara *B. pseudomallei*, *B. mallei*, *B. thailandensis* dan *B. cepacia* kompleks. Esai PCR assay ini spesifik dan dapat mengesan sekurang-kurangnya 109, 60, 23, and 9 ng DNA *B. pseudomallei*, *B. mallei*, *B. thailandensis* dan kompleks *B. cepacia*. Empat protein andaian (*BPSS0713*, *BPSS0767*, *BPSS1124*, dan *BPSS1488*) telah berjaya diekspreskan. Walau bagaimanapun, tiada protein rekombinan yang menunjukkan reaksi positif dalam esai “hemagglutination”. Maka, fungsi gen hipotetikal lektin *B. pseudomallei* tidak dapat dikenalpastikan. Banyak faktor termasuk modifikasi protein selepas translasi, denaturasi protein dan kekurangan kofaktor mungkin mempengaruhi aktiviti lektin. Untuk siasatan masa depan, penggunaan teknologi baru seperti “glycan array”, “isothermal titration calorimetry” atau “surface plasmon resonance” boleh diterokai untuk mengenalpasti lektin dalam *B. pseudomallei*.

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***Vast learning, perfect handicraft, a highly trained discipline, and always speaking pleasant. This is the highest blessing. --- The Discourse of Blessings (Mangala Sutta)***



## TABLE OF CONTENTS

<b>ABSTRACT</b> .....	<b>iii</b>
<b>ABSTRAK</b> .....	<b>v</b>
<b>ACKNOWLEDGEMENTS</b> .....	<b>vii</b>
<b>TABLE OF CONTENTS</b> .....	<b>viii</b>
<b>LIST OF FIGURES</b> .....	<b>xiii</b>
<b>LIST OF TABLES</b> .....	<b>xv</b>
<b>LIST OF SYMBOLS AND ABBREVIATIONS</b> .....	<b>xvii</b>
<b>LIST OF APPENDICES</b> .....	<b>xxii</b>
<b>CHAPTER 1: INTRODUCTION</b> .....	<b>1</b>
1.1. General introduction .....	1
1.2. Objectives of the study .....	3
<b>CHAPTER 2: LITERATURE REVIEW</b> .....	<b>4</b>
2.1. Melioidosis: historical background.....	4
2.2. <i>Burkholderia pseudomallei</i> .....	5
2.3. Other <i>Burkholderial</i> species .....	6
2.4. Clinical manifestations of melioidosis.....	6
2.5. Transmission and pathogenesis .....	7

2.6. Laboratory diagnosis of melioidosis .....	9
2.7. Molecular tests for melioidosis .....	11
2.8. Treatment of melioidosis .....	14
2.9. Bacterial biofilm.....	15
2.10. Techniques for biofilm study .....	16
2.11. Lectins.....	17
2.12. Strategy for identification and recognition of bacterial lectins .....	22
2.13. Molecular cloning and recombinant protein expression.....	23
<b>CHAPTER 3: MATERIAL AND METHODS.....</b>	<b>26</b>
Objective 1: To determine colony morphotype, biofilm forming ability, and hemagglutination of clinical isolates of <i>B. pseudomallei</i> .....	26
3.1.1. Culture and maintenance of test and reference strains .....	26
3.1.2. Biofilm quantitation of clinical isolates of <i>B. pseudomallei</i> .....	26
3.1.3. Determination of colony morphotypes of <i>B. pseudomallei</i> .....	27
3.1.4. Hemagglutination assay .....	27
3.1.5. Statistical analysis.....	28
Objective 2: To identify potential lectin genes in <i>B. pseudomallei</i> using bioinformatic approach .....	28
3.2. Bioinformatical studies of potential lectins of <i>B. pseudomallei</i> .....	28
3.2.1. Data mining .....	28
3.2.2. Sequence similarity search against non <i>B. pseudomallei</i> species .....	29
3.2.3. Sequential alignment and annotation.....	29
Objective 3: To develop multiplex PCR assay for identification of species closely related with <i>B. pseudomallei</i> based on hypothetical lectin genes .....	29
3.3.1. Bacterial strains.....	29
3.3.2. Culture conditions.....	30

3.3.3. Preparation of DNA template for PCR assays .....	30
3.3.3.1. Nucleic acid purification .....	30
3.3.3.2. Boiling method.....	32
3.3.3.3. Alcohol inactivation sample preparation method .....	32
3.3.4. Development of a multiplex PCR assay for identification of <i>B. pseudomallei</i> , <i>B. mallei</i> , <i>B. thailandensis</i> and <i>B. cepacia</i> complex based on putative lectin genes .....	32
3.3.4.1. Primer design.....	32
3.3.4.2. Construction of an internal amplification control (IAC) for multiplex PCR assay.....	35
3.3.4.3. Multiplex PCR assay .....	36
3.3.4.4. Confirmation of the presence of hypothetical lectin genes .....	37
3.3.4.5. Analysis of PCR products.....	37
3.3.4.6. Validation of multiplex PCR assay .....	38
Objective 4: To clone and express hypothetical lectin genes, and to assess the hemagglutination activity of the recombinant protein .....	38
3.4.1. Cloning of hypothetical lectin genes .....	38
3.4.1.1. Primers for amplification of hypothetical lectin genes .....	38
3.4.1.2. PCR for hypothetical lectin genes.....	39
3.4.1.3. Purification of PCR products .....	40
3.4.1.4. Ligation independent cloning (LIC) .....	41
3.4.1.5. Preparation of competent cells .....	42
3.4.1.6. Transformation of vector .....	43
3.4.1.7. Plasmid preparation .....	43
3.4.2. Expression and purification of recombinant hypothetical lectins .....	44
3.4.2.1. Expression of recombinant protein .....	44

3.4.2.2. Protein purification .....	45
3.4.2.3. SDS-PAGE .....	45
3.4.2.4. Western blotting .....	45
3.4.2.5. Detection of recombinant protein .....	46
3.4.2.6. Protein quantitation .....	47
3.4.2.7. Silver staining of SDS-PAGE gel .....	47
3.4.3. Screening for lectin activity of recombinant proteins.....	48
3.4.3.1. Hemagglutination assays (for recombinant proteins) .....	48
<b>CHAPTER 4: RESULTS .....</b>	<b>49</b>
4.1. Determination of colony morphotype, biofilm forming ability, and hemagglutination of clinical isolates of <i>B. pseudomallei</i> .....	49
4.1.1. Determination of the colony morphotypes of <i>B. pseudomallei</i> .....	49
4.1.2. Biofilm forming ability of clinical isolates of <i>B. pseudomallei</i> .....	49
4.1.3. Determination of the association between colony morphotype groups and biofilm formation.....	52
4.1.4. Hemagglutination assay using whole bacteria cells .....	55
4.2. Identification of sugar binding proteins (lectins) in <i>B. pseudomallei</i> using bioinformatical approach. ....	56
4.3. Use of hypothetical lectin genes for development of a multiplex PCR for rapid identification of <i>B. pseudomallei</i> , <i>B. thailandensis</i> , <i>B. mallei</i> and <i>B. cepacia</i> complex .....	56
4.3.1. Assessment of multiplex PCR assay for target and non-target organisms..	59
4.3.2. Image analysis of the multiplex PCR profile .....	59
4.3.3. Safety assessment of alcohol inactivation sample preparation method.....	59
4.4. Molecular probing of lectin genes in <i>B. pseudomallei</i> isolates.....	62
4.5. Cloning and expressions of hypothetical lectin proteins .....	62

4.6. Hemagglutination assay using recombinant proteins .....	65
<b>CHAPTER 5: DISCUSSION.....</b>	<b>66</b>
5.1. Colonial morphotype, biofilm forming ability and hemagglutination of <i>B. pseudomallei</i> clinical isolates .....	66
5.2. Identification of lectin in <i>B. pseudomallei</i> using bioinformatic approach .....	68
5.3. Development of a multiplex PCR assay for identification of species closely related with <i>B. pseudomallei</i> based on genes encoding hypothetical lectin .....	69
5.4. Determination hemagglutination/lectin activity of recombinant proteins.....	71
<b>CHAPTER 6: CONCLUSIONS AND FUTURE PROSPECTS.....</b>	<b>74</b>
<b>REFERENCES .....</b>	<b>75</b>
<b>LIST OF PUBLICATIONS AND PAPERS PRESENTED.....</b>	<b>93</b>
<b>APPENDICES .....</b>	<b>109</b>

## LIST OF FIGURES

Figure 2.1:	Natural history of infection with <i>Burkholderia pseudomallei</i> .....	8
Figure 2.2:	Biofilm developmental process in <i>Pseudomonas aeruginosa</i> .....	16
Figure 3.1:	Sequential alignment of <i>B. pseudomallei</i> K96243 (BPSS2022) and <i>B. thailandensis</i> ATCC 700388 (CP000085.1: 379615-380514).....	33
Figure 3.2:	Sequential alignment of <i>B. pseudomallei</i> K96243 (BPSS1958), <i>B. mallei</i> ATCC 23343 (CP000010.1: 1155075-1156148).....	33
Figure 3.3:	Sequential alignment of <i>B. pseudomallei</i> K96243 (BPSS1649), <i>B. mallei</i> ATCC 23344 (CP000011.2: 1814489-1816909 and <i>B. thailandensis</i> ATCC 700388 (CP000085.1: 858567-860987) .....	34
Figure 3.4:	The sequential alignment of each generated consensus region of 16S ribosomal RNA gene for <i>B. cepacia</i> complex, <i>B. mallei</i> , <i>B. pseudomallei</i> and <i>B. thailandensis</i> .....	34
Figure 3.5:	Graphical illustration on recombinant protein cloning strategy using pET-46 Ek/LIC expression vector .....	41
Figure 4.1:	Representative pictures (Group 1 - 7) of the distinct seven colonial morphotypes of <i>B. pseudomallei</i> on BPSA .....	50
Figure 4.2:	Bar chart showing overall biofilm formation of <i>B. pseudomallei</i> clinical isolates.....	51
Figure 4.3:	Relative biofilm formation with descriptive statistics for <i>B. pseudomallei</i> isolates.....	52

Figure 4.4:	Boxplot analysis of biofilm formation of different morphotype groups of <i>B. pseudomallei</i> compared to that of <i>B. thailandensis</i> ATCC 700388...	53
Figure 4.5:	Hemagglutinations of whole bacterial cells.....	55
Figure 4.6:	Representative agarose gel electrophoretic analysis of amplified fragments generated from multiplex PCR assay .....	58
Figure 4.7:	Gel image analysis using PyElph software.....	61
Figure 4.8:	Clustering analysis of the tested species based on the fragments obtained from agarose gel electrophoresis using PyElph software .....	61
Figure 4.9:	Representative gel showing the presence of hypothetical sugar binding protein genes in <i>B. pseudomallei</i> K96243 reference strains.....	62
Figure 4.10:	Western blot showing the expression of crude recombinant proteins ...	64
Figure 4.11:	Western blot showing the purified recombinant proteins.....	65
Figure 4.12:	Hemagglutination of rabbit erythrocytes with <i>B. pseudomallei</i> recombinant proteins.....	65
Figure AF.1:	Schematic construction of internal amplification control .....	118
Figure AF.2:	Agarose gel electrophoresis of amplified fragments using DNA template prepared from boiling method and alcohol inactivation method .....	118

## LIST OF TABLES

Table 2.1:	Molecular methods for detection and identification of <i>Burkholderia pseudomallei</i> and closely related species.....	12
Table 2.2:	<i>In vitro</i> analytical techniques with respective methods for microbial biofilm study.....	18
Table 3.1:	Bacterial strains used in the development and validation of multiplex PCR assay .....	31
Table 3.2:	Primers for detection of hypothetical lectin genes.....	35
Table 3.3:	Primer sequences used for cloning of hypothetical and reference lectins with amplicon size.....	39
Table 4.1:	Descriptive statistics for biofilm formation of different morphotypes group .....	54
Table 4.2:	ANOVA for biofilm formation of different groups .....	54
Table 4.3:	The search results from UniProt for lectin based proteins in the <i>B. pseudomallei</i> K96243 genome .....	57
Table 4.4:	Molecular detection of various hypothetical lectin genes in <i>Burkholderia</i> species (based on BLAST results) .....	57
Table 4.5:	Assessment of multiplex PCR assay for target and non-target organisms .....	60
Table 4.6:	Details of the hypothetical proteins expressed in this study.....	63



Table AF.1: Composition of PCR reagents for multiplex PCR assay .....117

Table AF.2: Composition of PCR reagents for cloning of hypothetical lectins.....117

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## LIST OF SYMBOLS AND ABBREVIATIONS

°C	degree Celsius
%	Percentage
1 X	one time
ANOVA	analysis of variance
ASA	Ashdown's medium
ATCC	American Type Culture Collection
bp	base pair
<i>BC2L</i>	<i>Burkholderia cenocepacia</i> lectin
BCIP/NBT	5-bromo-4-chloro-3-indolyl-phosphate with nitro blue tetrazolium
BCSA	<i>Burkholderia cepacia</i> selective agar
BHI	Brain heart infusion
BLAST	Basic Local Alignment Search Tool
BPSA	<i>Burkholderia pseudomallei</i> selective agar
BTA	BioTimer assay
cDNA	complementary DNA
CV	crystal violet

<i>CV-III</i>	<i>Chromobacterium violaceum</i> fucose-binding lectin
DMMB	1,9-dimethyl methylene blue
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dH <sub>2</sub> O	sterile distilled water
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylenediamine tetraacetic acid
e.g.	for example
EMBL-EBI	European Molecular Biology Laboratory – European Bioinformatics Institute
<i>et al.</i>	et alia
FDA	Fluorescein-di-acetate
g	gram
<i>hsdSB</i>	host specific determinant <i>Salmonella typhimurium</i>
i.e.	that is
IMAC	immobilized metal affinity chromatography
INSDC	International Nucleotide Sequence Database Collaboration
IPTG	isopropyl-beta-thio galactopyranoside

kDA	kilodalton
l	liter
LB	Lysogeny Broth
<i>LecA</i>	Gene encoding <i>Pseudomonas aeruginosa</i> lectin I
<i>LecB</i>	Gene encoding <i>Pseudomonas aeruginosa</i> lectin II
LIC	Ligation independent cloning
M	Molar
Mb	mega base pair
mg	milligram
μg	microgram
min	minute
ml	milliliter
μl	microliter
μM	micromolar
mw	molecular weight
NCTC	National Collection of Type Cultures
ng	nanogram
nm	nanometer

OD	optical density
OE-PCR	overlap extension PCR
<i>OmpT</i>	outer membrane protease
ORF	open reading frame
<i>PA-IL</i>	<i>Pseudomonas aeruginosa</i> lectin I gene
<i>PA-IIIL</i>	<i>Pseudomonas aeruginosa</i> lectin II gene
PBS	Phosphate Buffered Saline
PCR	polymerase chain reaction
pg	picogram
PIA	polysaccharide adhesin
PVDF	Polyvinylidene fluoride
rpm	Revolutions per minute
<i>RSL</i>	<i>Ralstonia solanacearum</i> lectin gene
s	seconds
SDS-PAGE	Sodium dodecyl sulphate – polyacrylamide gel electrophoresis
SOC	Super Optimal broth with Catabolite repression
<i>Taq</i>	<i>Thermus aquaticus</i>
TBE	TRIS-borate-EDTA

Tris	Tris(hydroxymethyl)aminomethane
U	unit
UniParc	UniProt Archive
UniProt	The Universal Protein Resource Database
UniProtKB	UniProt Knowledgebase
UniRef	UniProt Reference Clusters
UPI	unique identifier
UV	ultra violet
V	volt
(v/v)	volume per volume
(w/v)	weight per volume
XTT	2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfohenyl)-2H-Tetrazodium-5-Carboxanilide

## LIST OF APPENDICES

Appendix A: Details of the isolates with morphotypes grouping and relative biofilm formation.....	109
Appendix B: Preparation of <i>Burkholderia pseudomallei</i> selective agar (BPSA).....	110
Appendix C: Preparation of various agar and broth .....	112
Appendix D: Preparation of buffers and solutions .....	114
Appendix E: Preparation of silver stain working solutions .....	115
Appendix F: Miscellaneous tables & figures .....	117
Appendix G: BLAST results with respective hypothetical lectin genes .....	119

## CHAPTER 1: INTRODUCTION

### 1.1. General introduction

*Burkholderia pseudomallei* is the causative agent of melioidosis, a life-threatening disease of man and animals in the tropics. The pathogen originates from soil and water, and is able to survive harsh environmental conditions. Once the bacterium is introduced into a suitable host, the clinical spectrum of melioidosis is extremely broad, ranging from an indolent local infection to septicaemia, particularly in the immunocompromised. The pathogen can stay in dormant in human host for many years (Ngaay *et al.*, 2005). A prolonged period of dormancy may occur between exposure to *B. pseudomallei* and clinical manifestation of infection (Puthuchery, 2009). The dormancy of *B. pseudomallei* has been linked to its ability to form biofilm and microcolonies (Kamjumphol *et al.*, 2013; Limmathurotsakul *et al.*, 2014; Vorachit *et al.*, 1995).

In the laboratory, the colonial morphology of *B. pseudomallei* varies both within and between clinical isolates (Chantratita *et al.*, 2007). It is postulated that *B. pseudomallei* undergoes a process of adaptation involving altered expression of surface determinants which facilitates bacterial survival *in vivo* (Ulett, 2001). This gives rise to the notion that colony morphology of *B. pseudomallei* may possibly provide some indications of the virulence of a particular strain.

A major feature of melioidosis is that bacterial eradication is difficult to achieve. The clinical response to antimicrobials is slow and recurrent disease is common, despite appropriate therapy for 12 to 20 weeks (Puthuchery, 2009). The ability to form biofilms is likely to contribute to the occurrence of persistent bacterial infection in the host and may account for the greater likelihood of asymptomatic infections as in melioidosis. Bacterial biofilm infections are particularly problematic, because sessile



bacteria can withstand host immune defense mechanisms and are extremely resistant to antimicrobials. In addition, the fact that levels of humoral antibodies in patients who have had melioidosis remain high and seldom come down to basal levels even years after recovery from acute infections supports the notion of persistence (Vasu *et al.*, 2003). It is clear that *B. pseudomallei* can become adapted for survival *in vivo* (Chantratita *et al.*, 2007), but the mechanisms by which this occurs in humans have yet to be demonstrated.

Lectins are carbohydrate-binding proteins or sugar binding proteins (Adam *et al.*, 2007) which have been reported in *Pseudomonas aeruginosa*, *Chromobacterium violaceum* and *Ralstonia solanacearum* (reviewed by Gilboa-Garber *et al.*, 2011). Lectins have been reported to play important role for host cell attachment and biofilm initiation of *P. aeruginosa* (Gilboa-Garber & Garber, 1992). Although the ability of *B. pseudomallei* to form biofilm has been recently highlighted as one of the possible virulence factors, there is no information whether lectins are present.

In the first phase of this study, colony morphotypes of *B. pseudomallei* isolates on *B. pseudomallei* selective agar (BPSA) were determined. The biofilm production of 76 clinical isolates of *B. pseudomallei* was investigated using a crystal violet staining assay. The results obtained were correlated with the colony morphotypes. A conventional hemagglutination assay was used to investigate the presence of lectins in *B. pseudomallei*. In the second phase of the study, a bioinformatical approach was used to search for potential lectin genes in *B. pseudomallei*, by referring to the whole genome sequence of *B. pseudomallei* K96243 (Genbank accession no. BX571965 and BX571966). On parallel, a multiplex PCR was designed based on the unique sequences of the hypothetical lectin genes for differentiation of closely related *Burkholderia*

species. In the third phase of the study, the hypothetical lectin genes were cloned in *E. coli* and the protein were expressed for evaluation of lectin activities.

## **1.2. Objectives of the study**

The objectives of this study are:

- a) to determine colony morphotype, biofilm forming ability, and hemagglutination of clinical isolates of *B. pseudomallei*
- b) to identify potential lectin genes in *B. pseudomallei* using bioinformatic approach
- c) to develop multiplex PCR assay for identification of species closely related with *B. pseudomallei* based on hypothetical lectin genes
- d) to clone and express hypothetical lectin genes, and to assess the hemagglutination activity of the recombinant proteins

## CHAPTER 2: LITERATURE REVIEW

### 2.1. Melioidosis: Historical Background

Melioidosis is a life threatening disease of both humans and animals in Southeast Asia and Northern Australia (White, 2003). The disease is increasingly being recognised around the world due to the heightened awareness and improved diagnostic tests (Peacock, 2006). The term “melioidosis” coined by Stanton and Fletcher in 1921, is derived from the Greek word “melis,” which means “a distemper of asses” and “eidos” which refers to its resemblance to glanders, a disease caused by *B. mallei* (Ip *et al.*, 1995). Mortality rates for melioidosis in the endemic areas remains high despite several decades of intensive clinical research (Peacock, 2006).

The disease was first reported in 1912 by Whitmore and Krishnaswami in Rangoon, Burma. According to Alfred Whitmore’s own account (Whitmore, 1913), he had performed numerous animal studies using the isolated “Bacillus” like bacteria and the disease manifestation on the animals closely resembled glanders infection. However, bacteriological investigation did not confirm the provisional diagnosis. As there was no literature describing the disease at that time, melioidosis was recognized as an entirely new disease. The bacteria was initially named as *Bacillus pseudomallei*, and later, recognized as a different organism from *Bacillus mallei* (currently known as *Burkholderia mallei*), the causative agent of glanders (Whitmore, 1913). The bacterium had undergone several taxonomic reclassifications over the past 100 years, and had been given names such as *Bacillus whitmorii*, *Malleomyces pseudomallei* and *Pseudomonas pseudomallei* (Cheng & Currie, 2005). In 1992, the bacterium has been classified in the genus *Burkholderia*, a member of the order *Burkholderiaceae* by Yabuuchi *et al.* (1992), and it is now officially known as *Burkholderia pseudomallei*.

The endemic region of melioidosis is in the tropical area (between the latitudes 20°N and 20°S) (Dance, 1991). Thailand has the highest incidence of melioidosis, with 21.3 cases of melioidosis being reported per 100,000 people annually (Limmathurotsakul *et al.*, 2010). New melioidosis cases have also been reported in Africa, Brazil, France, New Caledonia, the Middle East, and Americas (Currie *et al.*, 2008; Cheng & Currie, 2005). The mortality rate for melioidosis can be as high as 40 % in Northeast Thailand (35 % in children) and 14 % in Australia (Peacock, 2006). In Malaysia, melioidosis cases have been reported in different regions of the country, including Kuala Lumpur (Sam & Puthuchear, 2006), Johor Bahru (Pagalavan, 2005), Selangor (Strauss *et al.*, 1969), Kelantan (Deris *et al.*, 2010) and Pahang (How *et al.*, 2005). The calculated annual incidence of melioidosis in Pahang was 6.07 / 100,000 population per year (How *et al.*, 2005). Infection in animals such as buffalo, crocodile, sheep, deer, monkey, parrot, zebra and hamster have also been reported (Puthuchear, 2009).

## **2.2. *Burkholderia pseudomallei***

*B. pseudomallei* is a soil saprophyte which is a nonmotile, aerobic, straight or slightly curved, Gram-negative bacillus with a “safety pin appearance” (Cheng & Currie, 2005). The bacterium grows on most agar media and produces a mouldy odour. Visible colonies are clearly observed on agar within 24-48 hours at 37°C (Puthuchear, 2009). The organism can survive hostile environmental conditions and may pose a potential risk especially to rural communities and rice farmers (Wuthiekanun *et al.*, 1995). Together with *B. mallei*, *B. pseudomallei* has been recognized as a potential biological weapon of the 20<sup>th</sup> century (Mahenthiralingam *et al.*, 2005; Wiersinga *et al.*, 2006). The genome (G+C content of 68 %) is relatively large and has been divided into two chromosomes of 4.07 Mb and 3.17 Mb (Holden *et al.*, 2004).

### **2.3. Other *Burkholderial* species**

*Burkholderia* is a genus of proteobacteria which include environmental and medically important human and animal pathogens (Estrada-de los Santos *et al.*, 2013). *B. pseudomallei*, *B. mallei*, *B. thailandensis* and *B. cepacia* complex are closely related species of Gram-negative bacteria with significant distinctive features in their pathogenicity and ecological niches.

*B. mallei* is primarily responsible for causing glanders *disease* in horses and other animals. *B. cepacia* complex are opportunistic pathogens giving rise to infections in patients with cystic fibrosis and other chronic granulomatous disease, while *B. thailandensis* is generally avirulent and has been reported only in Southeast Asia (Glass & Popovic, 2005).

Apart from *B. mallei* which mainly causes disease in animals, the other three *burkholderial* species give considerable problems for identification in the clinical microbiology laboratory, particularly in the differentiation between *B. pseudomallei* and *B. cepacia* complex (Chantratita *et al.*, 2008; Wongtrakoongate *et al.*, 2007).

### **2.4. Clinical manifestations of melioidosis**

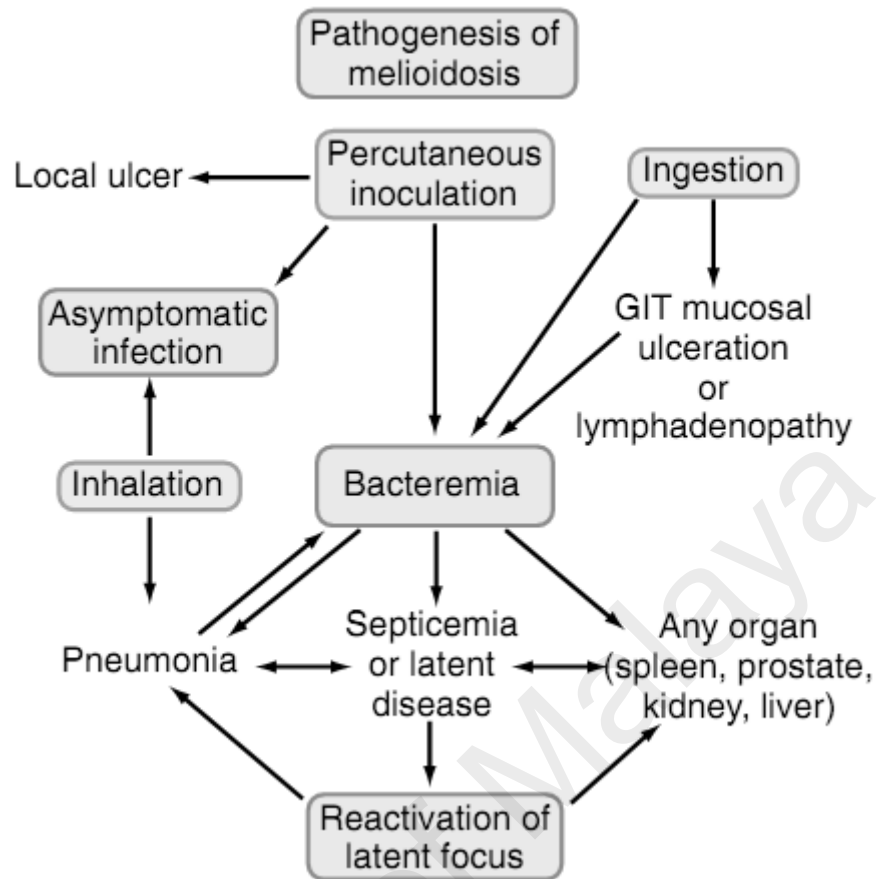
Melioidosis is usually perceived as an acute pulmonary illness; however it has also been recognized to give rise to inapparent infections, transient bacteraemia, asymptomatic pulmonary infiltration, acute localized suppurative lesion, acute pulmonary infection, disseminated septicaemic or non-septicaemic infection or chronic suppurative infection (Puthuchery, 2009). Since the symptoms are non-specific, the clinical classification for melioidosis is controversial. The mortality rate due to melioidosis is affected by the development of septicaemia in the patients, with lower mortality rate observed in cases with non-septicaemic melioidosis (Puthuchery &

Vadivelu, 2002). Due to the wide array of clinical signs and symptoms, *B. pseudomallei* is also called “the great mimicker” (Puthuchery, 2009).

Reinfection of melioidosis were often reported in immunocompromised host, in either relapse or recurrence episode. Reappearances of clinical signs and symptoms during antimicrobial treatment is known as “relapse”, whereas a reinfection via the same organism after a complete recovery is known as “recurrence”. Such reinfections were mostly due to the same original infecting strain. The inability of the host to eliminate the organism during the initial infection can cause problems in melioidosis survivors at the later part of their life. It is interesting to note that relapse of melioidosis is common for children (Puthuchery, 2009). The prolonged latency and recurrence of melioidosis may be caused by: i) *B. pseudomallei* is able to survive within phagocytic cell and evade host immune response (Jones *et al.*, 1996); ii) formation of glycocalyx, biofilms and microcolonies, where such barrier is impenetrable by antimicrobials agents (Sawasdidoln *et al.*, 2010)

## **2.5. Transmission and pathogenesis**

The natural history of infection with *B. pseudomallei* is summarized in Figure 2.1. There are three modes of acquisition, i.e., inoculation, ingestion and inhalation. Inoculation is the major mode of acquisition, and the severity of the disease is dependent on the size of inoculum into the wounds (Cheng & Currie, 2005). High incidence of melioidosis had been reported amongst United States of America (USA) helicopter crews in Vietnam due to inhalation of dusts initiated by helicopter rotor (Howe *et al.*, 1971). As a result, this has earned the disease a nickname, “the Vietnamese time bomb” (Clayton *et al.*, 1973). Ingestion is suggested as an uncommon mode of transmission while human cases in the endemic region could be resulted from skin penetration after exposure to muddy soil or contaminated water (Currie, 2010).



**Figure 2.1:** Natural history of infection with *B. pseudomallei*. (GIT : gastrointestinal tract). Three modes of acquisition, i.e., inhalation, inoculation and ingestion and their subsequent clinical presentation. Adapted from Currie (2010).

*B. pseudomallei* demonstrated high adaptation ability in various changing environmental conditions (Puthuchery & Vadivelu, 2002). Once it is inside the human host, the bacteria can stay dormant for many years. According to review by Puthuchery (2009), four aspects contributing to the virulence of *B. pseudomallei* are: i) extracellular mucoidic polysaccharide layer which enables forming of microcolonies and protection of the organism from antibiotic penetration (Currie *et al.*, 2000); ii) survival of *B. pseudomallei* inside human macrophages (Nathan & Puthuchery, 2005); iii) slow and inefficient formation of phagolysosome which enables *B. pseudomallei* to overcome host immune response (Puthuchery & Nathan, 2006); and iv) low level of nitric oxide

produced by host cell for microbicidal mechanism (Ismail *et al.*, 1988) . Additionally, Sarovich *et al.* (2014) reported several virulence factors in *B. pseudomallei*: cytotoxin *Burkholderia* lethal factor 1, capsular polysaccharide I, the cluster I type VI secretion system, and the Bsa type III secretion system cluster 3 (Burtnick *et al.*, 2011; Cruz-Migoni *et al.*, 2011; Reckseidler *et al.*, 2001; Wiersinga *et al.*, 2006). Sarovich *et al.* (2014) had also proposed a model framework for assessing virulence factors and their association with pathogenesis, by studying two virulence factors i.e., *Burkholderia mallei*-like actin polymerization (*bimA<sub>Bm</sub>*) gene and filamentous hemagglutinin (*fhaB3*) gene.

## **2.6. Laboratory diagnosis of melioidosis**

Clinical diagnosis for melioidosis is hardly possible as the clinical manifestations of melioidosis in patients are nonspecific in nature. Isolation and identification of *B. pseudomallei* from body fluids of patients remains the “gold standard” for definitive diagnosis but it is time consuming and may be problematic (Cheng & Currie, 2005). Different colony morphologies have been observed when the organism is first isolated from clinical samples. Large and small colony variants are observed on primary agar plates especially when blood samples are cultured (Puthuchery, 2009). Variations in colonial morphology of *B. pseudomallei* often pose difficulties to untrained personnel in the clinical diagnostic laboratory. Three selective agar media, i.e. Ashdown’s medium (ASA), *Burkholderia cepacia* selective agar (BCSA) and *B. pseudomallei* selective agar (BPSA) (Chantratita *et al.*, 2007; Chen *et al.*, 2009; Henry *et al.*, 1999) have been used with equivalent sensitivity (Peacock *et al.*, 2005). A colony morphotyping scheme has been developed based on Ashdown’s medium (Ashdown, 1979; Wuthiekanun *et al.*, 1990).



In the routine laboratory practice, suspected bacterial cultures are usually identified using biochemical testing system such as API20NE (bioMerieux), VITEK 2 GN card system etc. However, the process may take time as the doubling time for *B. pseudomallei* ranges from 1.5 to 2.3 h (Lee *et al.*, 2007). Identification of bacterial cultures by API20NE may need 24 or 48 hours; hence, this hampers effort for rapid response (Glass & Popovic, 2005). Paradoxically, many authors gave inconsistent opinions about the reliability of biochemical tests including API20NE. The accuracy of API20NE identification has been reported to range from 97.5 % to 99 % in several studies (Amornchai *et al.*, 2007; Dance *et al.*, 1989; Lowe *et al.*, 2002). However, in one study, the kit showed poor performance as only 60 % isolates were identified correctly (Glass & Popovic, 2005).

Additionally, a low identification rate (78.3%) of VITEK 2 GN card system compared with API20NE (86.7 %) had been reported (Deepak *et al.*, 2008). *B. pseudomallei* was identified as either *B. cepacia*, *Burkholderia* spp., *Ralstonia* spp, *Pseudomonas putida*, *Stenotrophomonas maltophilia*, etc. (Koh *et al.*, 2003) as the Phoenix (BD) automated identification and susceptibility testing system did not include *B. pseudomallei* in its database.

Serological diagnosis is still widely used in the endemic region of melioidosis. Many antigen detection methods have been developed for melioidosis but none are commercially available except for a monoclonal antibody latex agglutination test which is widely used in Thailand (Anuntagool *et al.*, 2000). However, it should be noted that antigen detection system requires antibodies that bind specifically to *B. pseudomallei* antigen, and most hospitals do not have the system ready especially those outside of the endemic region. Furthermore past exposure may result with seroconversion which can

lead to false positive finding using the serological methods (Cheng & Currie, 2005; White, 2003).

## **2.7. Molecular tests for melioidosis**

In view of the seriousness, severity and high mortality rates of melioidosis, and the difficulty in making a clinical diagnosis, a number of molecular diagnostic tests have been developed to reduce the time for identification of *B. pseudomallei*. Most applications require purified genomic DNA sample, sequencing, and sophisticated real-time PCR system, and have not been extensively validated in the field (Cheng & Currie, 2005). Methods for rapid identification and differentiation of *B. pseudomallei* from closely related organisms, i.e., *B. mallei*, *B. thailandensis* and *B. cepacia* complex, are critically needed in the clinical settings in order to initiate appropriate antimicrobial therapy. Most of the detection methods have low sensitivity for detection of *B. pseudomallei* in the blood as the viable bacteria count in septicaemia human is usually low (Peacock, 2006). Table 2.1 summarizes the molecular detection methods which have been developed in the past.

A multiplex PCR assay is useful for rapid identification and differentiation of closely-related *burkholderial* species. Two multiplex PCR assays have been developed for such purpose. One amplifies a region flanking variable copies of the bacterial repetitive element for identification of *B. pseudomallei*, *B. mallei* and *B. thailandensis* (Lee *et al.*, 2005), and the other targets a *Tat* domain protein, for both 70-kDa and a 12-kDa protein for amplification of *B. pseudomallei*, *B. thailandensis*, and the *B. cepacia* complex, respectively (Ho *et al.*, 2011).

**Table 2.1:** Molecular methods for detection and identification of *Burkholderia pseudomallei* and closely related species.

Target	Method & References	Target organism	Results of evaluation
23S rDNA	PCR and hybridization (Lew and Desmarchelier, 1994)	Detect <i>B. pseudomallei</i> and <i>B. mallei</i>	Not able to differentiate <i>B. pseudomallei</i> and <i>B. mallei</i>
			Produced non-specific products with some <i>B. cepacia</i> strains (Brook <i>et al.</i> , 1997)
			Failed to detect environmental strains, low sensitivity for detection of clinical isolates (Haase <i>et al.</i> , 1998)
16S rDNA	Nested PCR (Dharakul <i>et al.</i> , 1996)	Detect <i>B. pseudomallei</i>	Not ideal for clinical application due to possible carry over contamination (Rattanathongkom <i>et al.</i> , 1997)
			False positive in patients (Haase <i>et al.</i> , 1998)
			Low specificity and sensitivity (Kunakorn <i>et al.</i> , 2000)
16S rDNA	PCR (Brook <i>et al.</i> , 1997)	Identify <i>B. pseudomallei</i>	Higher sensitivity compared to those reported by Lew and Desmarchelier (1994)
Unidentified gene	PCR (Rattanathongkom <i>et al.</i> , 1997)	Detect <i>B. pseudomallei</i> in blood	High sensitivity and rapid (Kunakorn <i>et al.</i> , 2000)
Unidentified gene	PCR and probe hybridization (Sura <i>et al.</i> , 1997)	Detect <i>B. pseudomallei</i>	Not evaluated for clinical diagnostic use (Sura <i>et al.</i> , 1997)
23S rDNA	PCR (Bauernfeind <i>et al.</i> , 1998)	Detect <i>B. mallei</i> and discriminate <i>B. pseudomallei</i>	Not evaluated for clinical diagnostic use
<i>rpsU</i> , <i>fliC</i>	PCR (Hagen <i>et al.</i> , 2002)	Detect <i>B. pseudomallei</i>	Requires sequencing for differentiation of <i>B. pseudomallei</i> , <i>B. mallei</i> , <i>B. thailandensis</i>
Flagellin	Duplex PCR (Sonthayanon <i>et al.</i> , 2002), modified from Wajanarogana <i>et al.</i> (1999)	Detect and differentiate <i>B. pseudomallei</i> and <i>B. thailandensis</i>	<i>B. mallei</i> was not included, not evaluated for clinical diagnostic use.
Flagellin ( <i>fliC</i> )	PCR-RFLP (Sprague <i>et al.</i> , 2002)	Differentiate <i>B. mallei</i> and <i>B. pseudomallei</i>	Only for detection of <i>B. thailandensis</i> , <i>B. mallei</i> , <i>B. pseudomallei</i> but not for differentiation of <i>B. pseudomallei</i> and <i>B. mallei</i> (Sprague <i>et al.</i> , 2002; Tanpiboonsak <i>et al.</i> , 2004)

16S rDNA	PCR (Gee <i>et al.</i> , 2003)	Differentiate <i>B. pseudomallei</i> and <i>B. mallei</i>	Requires sequence comparison. Not evaluated for routine clinical diagnostic use.
TTS1	PCR (Smith-Vaughan <i>et al.</i> , 2003)	Diagnostic and identify <i>B. pseudomallei</i>	Good specificity, low sensitivity (Gal <i>et al.</i> , 2005) Not evaluated for <i>B. mallei</i> .
Unidentified gene	PCR-RFLP (Tanpiboonsak <i>et al.</i> , 2004)	Differentiation of <i>B. mallei</i> and <i>B. pseudomallei</i>	Not performed on <i>B. thailandensis</i> . Not evaluated for routine clinical diagnostic use.
TTS1, TTS2	Real-time PCR (Thibault <i>et al.</i> , 2004)	Identify and discriminate <i>B. pseudomallei</i> , <i>B. mallei</i> and <i>B. thailandensis</i>	Not evaluated for routine clinical diagnostic use.
Repetitive DNA element	Multiplex PCR (Lee <i>et al.</i> , 2005)	Detect and differentiate <i>B. pseudomallei</i> , <i>B. mallei</i> and <i>B. thailandensis</i>	Useful in epidemiology study and strain typing only for <i>B. mallei</i> and <i>B. pseudomallei</i> . Not evaluated for routine clinical diagnostic use.
16S rDNA, <i>rpsU</i> , <i>fliC</i>	Real-time PCR (Tomaso <i>et al.</i> , 2005)	Detect <i>B. mallei</i> and <i>B. pseudomallei</i>	Low sensitivity with clinical samples (Chantratita <i>et al.</i> 2007) Unable to differentiate <i>B. mallei</i> and <i>B. pseudomallei</i>
SNP	Real-time PCR (U'Ren <i>et al.</i> , 2005)	Detect and discriminate <i>B. mallei</i> and <i>B. pseudomallei</i>	<i>B. thailandensis</i> not included. Clinical evaluation is still pending.
<i>lpxO</i> , <i>phaC</i>	PCR (with sequencing) and Real-time PCR (Merritt <i>et al.</i> , 2006)	Identify <i>B. pseudomallei</i>	High specificity, results could be obtained in 2 hours and 30 minutes (Merritt <i>et al.</i> 2006).
<i>orf2</i> of TTS1	Real-time PCR (Novak <i>et al.</i> , 2006)	Identify <i>B. pseudomallei</i>	Rapid and high specificity. Results could be obtained in 3 hours (Novak <i>et al.</i> , 2006) 91 % sensitivity for detection of confirmed melioidosis cases (Meumann <i>et al.</i> , 2006)
Flagellin P gene ( <i>fliP</i> )	Real-time PCR (Tomaso <i>et al.</i> , 2006)	Identify <i>B. mallei</i>	Only for specific detection of <i>B. mallei</i> .
<i>Burkholderia</i> intracellular motility A gene ( <i>bimA<sub>ma</sub></i> )	Real-time PCR (Ulrich <i>et al.</i> 2006a; Ulrich <i>et al.</i> 2006b)	Identify <i>B. mallei</i>	Only for specific detection of <i>B. mallei</i> .
Metalloprotease Gene ( <i>mprA</i> )	PCR (Neubauer <i>et al.</i> , 2007)	Identify <i>B. pseudomallei</i>	Only for specific detection of <i>B. pseudomallei</i>

Hypothetical protein: <i>BPSS1187</i> (Assay 8653) & <i>BPSS2089</i> (Assay 9438)	Real-time PCR (Supaprom <i>et al.</i> , 2007)	Identify <i>B. pseudomallei</i>	71% sensitivity and 82% specificity for Assay 8653; 54% sensitivity and 88% specificity for Assay 9438
TTS1 gene clusters	Loop-mediated isothermal amplification (LAMP) (Chantratita <i>et al.</i> 2008)	Detection and identification of <i>B. pseudomallei</i>	Does not need thermal cycler however the assay is of low sensitivity and not suitable for clinical diagnostic use. (Chantratita <i>et al.</i> 2008)
<i>BipD</i> ( <i>BPSS1529</i> ), <i>BopE</i> ( <i>BPSS1525</i> ), putative oxidoreductase ( <i>BPSL2748</i> )	Aptamers (Gnanam <i>et al.</i> , 2008)	Diagnostic <i>B. pseudomallei</i>	Not evaluated or application in clinical settings
<i>lpxO</i>	PCR (Inglis <i>et al.</i> , 2008)	Identify <i>B. pseudomallei</i>	Requires Agilent Bioanalyzer due to low molecular weight range, laboratory chip is not reusable and requires 12 samples per run.
Multiple locus variable number of tandem repeat analysis (MLVA)	PCR (Michelle Wong Su <i>et al.</i> , 2009)	Typing of <i>B. pseudomallei</i>	Not applicable in routine clinical diagnostic.
Various gene targets	Microarray (Schmooock <i>et al.</i> , 2009)	Identify 12 <i>Burkholderia</i> species.	Requires highly purified genomic DNA. Not evaluated for routine clinical diagnostic use.

A gene from *B. pseudomallei*, *BPSL1958* has been reported as a specific genetic marker for *B. pseudomallei* in previous studies (Kim *et al.*, 2005; Wongtrakoongate *et al.*, 2007). Since the 16S rRNA gene of *B. cepacia* complex is significantly different from those of *B. pseudomallei*, *B. mallei* and *B. thailandensis*, primers can be designed targeting the conserved region in this gene to cover most subspecies in the complex.

## 2.8. Treatment of melioidosis

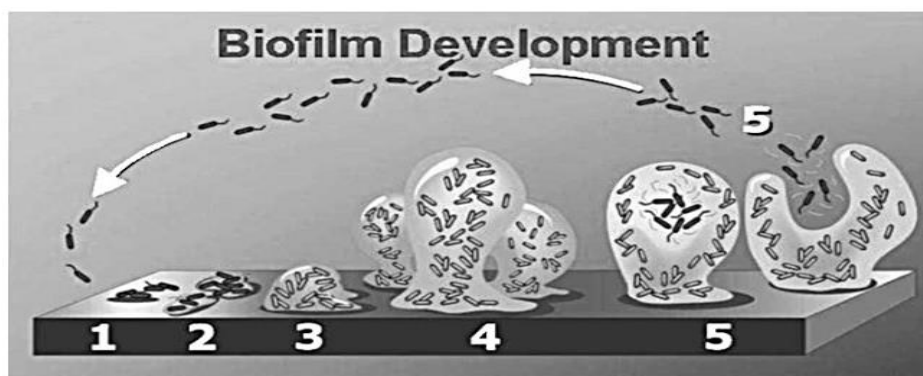
*B. pseudomallei* exhibits resistance to a wide variety of antibiotics, including third generation cephalosporins, penicillins, rifamycins, aminoglycosides, quinolones and macrolides (Puthuchear, 2009). As a result, the therapeutic option for the disease is limited (Cheng & Currie, 2005). Standard treatment of melioidosis requires 2–4 weeks

of parenteral therapy e.g. with ceftazidime as initial intensive therapy, followed by 3–6 months of oral eradication therapy e.g. with trimethoprim/sulfamethoxazole, doxycycline, chloramphenicol or a combination therapy (Sawasdidoln *et al.*, 2010).

## **2.9. Bacterial biofilm**

Biofilm, according to Flemming and Wingender (2010), is defined as microbial aggregates that usually accumulate at a solid–liquid interface, and are encased in a matrix of highly hydrated extracellular polymeric substances. Biofilm has been described as a structured, dynamic and complex biological system (Hall-Stoodley *et al.*, 2004) which allows microcolonies formation in a protective environment. The glycocalyx structures of biofilm cause significant resistance to the penetration of antibiotics (Vorachit, *et al.*, 1993), antimicrobial peptides (AMPs) (Burtnick & Woods, 1999), and enables evasion of host defences (Govan & Deretic, 1996; Korbsrisate, *et al.*, 2005). Additionally, bacteria develop biofilms to provide them a shelter against environmental fluctuations. Such collaborative cross-feeding and protective shielding provides them competitive advantages in the fierce competition for nutrients (Moons *et al.*, 2009).

*P. aeruginosa*, a closely related bacterial species of *B. pseudomallei*, is known to produce biofilm. *P. aeruginosa* synthesizes two types of lectins: *LecA* (responsible for *P. aeruginosa* lectin I [*PA-IL*]) and *LecB* (responsible for *P. aeruginosa* lectin II [*PA-IIL*]) respectively. These two lectins have been reported to be important for bacterial colonization and biofilm formation (Tielker *et al.*, 2005). Figure 2.2 illustrates the biofilm developmental process in *P. aeruginosa*. There are five stages involved: (i) initial reversible cell attachment, (ii) irreversible attachment, (iii) microcolony formation, (iv) maturation of biofilm, and (v) biofilm dispersion (Stoodley *et al.*, 2002; Wei & Ma, 2013).



**Figure 2.2:** Biofilm developmental process in *P. aeruginosa*. Stage (1) initial reversible cell attachment, (2) irreversible attachment, (3) microcolony formation, (4) maturation of biofilm, and (5) biofilm dispersion. Image taken and modified from Stoodley *et al.* (2002).

*B. pseudomallei* has been reported to form biofilms and microcolonies (Vorachit *et al.*, 1995). However, no correlation between biofilm production and source of isolation, including the virulence of bacteria has been reported (Taweechaisupapong *et al.*, 2005).

## 2.10. Techniques for biofilm study

Several approaches have been applied to study biofilm biology (Coenye & Nelis, 2010). Human cell line models are sometimes used to mimick the *in vivo* situation to assess the infectivity and tissue damage on human cells, however, such assesment is performed without consideration of the influence of the host immune system. Flow displacement system involves the continuous flow of nutrients and continuous removal of waste products, and is mainly used for assessment of oral biofilms and water system disinfections. Microfluidic devices have been used as a study model for biofilm formation and eradication, where the microfluidic channels are constructed using photosensitive polymers, which allows simultaneous analysis of multiple biofilm studies (Coenye & Nelis, 2010). For *in vivo* biofilm model systems, experiments can be performed by inducing infection on worms or animals model. Objects such as

extracellular matrix, beads, plastics tubes or biomaterials will be firstly grown with biofilm forming bacteria, and used as subcutaneous body infection model for implantation into animal models. The purpose of this method is to investigate the dissemination of the biofilm forming organism into various organs, or to study the effect of antibiotics on preventing bacteria to form biofilm onto those objects.

For *in vitro* model system, organism is induced to form biofilm on laboratory environment such as microtiter plate, within cell culture models, flow displacement biofilm systems or on microfluidic devices. Two types of analytical techniques for microbial biofilm study are summarized in Table 2.2 (adapted from Pantanella *et al.*, 2013). Of all the methods mentioned, microtiter plate-based system is most commonly used as this method is straightforward, cheap and allows mass screening.

## **2.11. Lectins**

Lectins were described as early as in the 19<sup>th</sup> century when a German scientist, Peter Hermann Stillmark observed agglutination of the seed extracts of *Ricinus communis* (castor bean) with animal erythrocytes. He named this agglutinin as “ricin” (Cummings & Etzler, 2009). Blood group specific agglutinins were discovered later in seeds or certain parts of plants, and based on this observation, Boyd and Shapleigh proposed the term “lectin” which is derived from the Latin word, *lego* (Boyd use the word *legere* in later publication), meaning “to choose or to pickout” (Boyd, 1970; Boyd & Shapleigh, 1954). Lectins are found in most organisms, including microorganisms, plants and animals (Ghazarian *et al.*, 2011). These carbohydrate-binding proteins (or known as sugar binding protein) are non-immune in origin and have high specificity and binding affinity to saccharide structures (Adam *et al.*, 2007). Lectins are involved in cell to cell interactions (Gabijs *et al.*, 2002), pilus genesis and proteolytic activity (Sonawane *et al.*, 2006).



**Table 2.2:** *In vitro* analytical techniques with respective methods for microbial biofilm study (adapted from Pantanella *et al.*, 2013).

	<b>Methods</b>	<b>Mode of action</b>	<b>Benefits</b>	<b>Limitation</b>
Staining Assays	Crystal violet (CV) assay (Stepanović <i>et al.</i> , 2000)	Quantitation of crystal violet stained extracellular polymeric substances	Amount of dye is proportional to biofilm sizes	Low reproducibility with high standard deviation value
	1,9-dimethyl ethylene blue (DMMB) assay (Toté <i>et al.</i> , 2008)	Quantitation of DMMB bound onto intracellular polysaccharide adhesin (PIA)	PIA is considered a logical target to be detected	Limited to few bacterial species possessing PIA-related biofilm matrix
	Fluorescein-di-acetate (FDA) assay (Tawakoli <i>et al.</i> , 2013)	Detection of the presence of fluorescein through hydrolyses of FDA	Inexpensive and easy to perform	Requires fluorescence microplate reader. Not suitable for matured biofilm due to thickness of biofilm and limited field of view
	Live/Dead BacLight Assay (Jin <i>et al.</i> , 2005)	Two nucleic acid binding stains, for fluorescence microscopy examination of live and dead bacteria populations	Observation of live and dead cells	Requirement of observation of statically significant portion to represent total population

Metabolic Assays	Resazurin assay (Punithavathy <i>et al.</i> , 2012)	Reduction of Resazurin to pink-fluorescent resorufin by cellular metabolic activity to quantify viable cells in biofilm	Detect and quantify the actual viable number of microorganism	Limitation on bacterial growth phase, biofilm thickness, reduction rate due to species dependent
	XTT assay (Adam <i>et al.</i> , 2002)	Reduction of XTT to water soluble formazan by metabolic activity to enumerate viable cells in biofilm	Absorbance reading of metabolic reduction of XTT to deduce number of viable bacteria in biofilm	Different metabolism gradient due to complexity and heterogeneity of biofilm structure and composition
	BioTimer assay (BTA) (Pantanella <i>et al.</i> , 2008)	Switching of colour from red to yellow due to fermentative metabolism, switching rate is dependent on initial bacterial concentration	Low cost and easily perform to count living bacteria in biofilm	Difficult in applying BioTimer assay for evaluation of multispecies biofilm

In the past, lectins have been defined as proteins which possessed the ability to agglutinate erythrocytes (Sharon, 2008). In recent studies, lectins have been defined as a group of sugar or carbohydrate binding proteins even without the hemagglutination effect of erythrocytes (Komath *et al.*, 2006; Sharon & Lis, 2004). Although lectins demonstrate similar binding preferential to carbohydrates, there is no similar protein homology between plant and animal lectins (Ghazarian *et al.*, 2011). As such, a new definition for lectins were proposed by Komath *et al.* (2006) to define lectins as group of proteins having topological similarities rather than the classifications based on carbohydrate recognitions.

Bacteria produce lectins to initiate infections through binding to complementary carbohydrates on the surface of the host tissues (Gupta, 2012). Among the known bacterial lectins, two have been isolated from *P. aeruginosa* (*LecA* and *LecB*), one each from *Chromobacterium violaceum* (*CV-III*), *Ralstonia solanacearum* (*RSL*) (Gilboa-Garber *et al.*, 2011) and *B. cenocepacia* (*BC2L*) (Lameignere *et al.*, 2010). The lectins have been reported to involve in anchoring glycosylated cell surface receptors to the host cells (Gilboa-Garber, *et al.*, 2011). According to a review written by Gilboa-Garber *et al.* (2011), although lectins are highly expressed in bacterial cells, its concentrations on bacterial cell surfaces are relatively low, as biofilm initiation and host cells interaction do not require a lot of lectins on the cell surface.

The *LecA* gene (366 bp) of *P. aeruginosa* encodes for a tetrameric protein (PA-IL) consisting of four 12.75 kDa subunits which are known to bind D-galactose and its derivatives (Diggle *et al.*, 2006). *LecA* is mainly located within the cytoplasm of the cell and only small fractions are present on the cell surface (Glick & Garber, 1983). It has been shown to cause cytotoxic effects on respiratory epithelial cells and contribute to respiratory injury, as well as induce permeability defects in intestinal epithelium (Bajolet-Laudinat *et al.*, 1994; Laughlin *et al.*, 2000). *LecA* contributes to biofilm development in *P. aeruginosa*. A *LecA* mutant of *P. aeruginosa* was reported to be incapable of forming parental-type biofilms (Diggle *et al.*, 2006). *LecA* also serves as vaccine for lethal infections in mice against *P. aeruginosa* (Avichezer *et al.*, 1989; Gilboa-Garber & Sudakevitz, 1982). The cloning and sequencing of the PA-IL with high antigenicity enable the production of synthetic peptides to be used as a vaccine for protection against *P. aeruginosa* infections (Avichezer *et al.*, 1992). PA-IL also assists bacterial adherence to surface epithelia and fibronectin (Rebiere-Huet *et al.*, 2004) and causes defects in the cellular barrier and allows toxin penetration (Wu *et al.*, 2003). There are not many publications regarding *LecB*. *LecB* encodes a tetrameric protein

(PA-11L) consisting of four 11.73 kDa subunits and each subunit shows high specificity for L-fructose and derivatives as well as a low affinity for D-mannose (Gilboa-Garber, Katcoff, & Garber, 2000). *LecB*, located mainly in the cytoplasm of planktonic cells (Glick & Garber, 1983), is exposed on the surface of sessile *Pseudomonas* cells (Morimoto *et al.*, 2001). It contributes significantly to the development of chronic respiratory infections (Scanlin & Glick, 2001). *LecB* also inhibits important defence mechanisms of the human lung as shown *in vitro* by decreasing the ciliary beat frequency of the airway epithelium (Adam *et al.*, 1997). In addition, Tiekkel *et al.* (2005) reported that a *LecB*-deficient *P. aeruginosa* mutant had impaired biofilm formation. The findings of their study suggest that *LecB* also plays an important role in the process of biofilm formation and is associated with the bacterial cell surface via binding to carbohydrate ligands.

The usage of both lectins (*LecA* and *LecB*) as vaccines was shown to provide full protection against *P. aeruginosa* infection in mice (Gilboa-Garber & Sudakevitz, 1982). The antibodies produced were able to agglutinate the intact lectin-bearing bacterial cells and compete efficiently with the adhesion of pathogens to host cells. Deguise *et al.* (2007) synthesized hetero-bifunctional glycol-dendrimers as new therapeutic antiadhesin agents against *P. aeruginosa* by binding both lectins *PA-IL* and *PA-IIL*. Additionally, mutagenesis of amino acids has enabled the identification of amino acid responsible for the lectin sugar preference, and the approach has been used in the drug design for treatment of *Pseudomonas* infection (Adam *et al.*, 2007).

Lectins from *Chromobacterium violaceum* (*CV-IIL*) (Zinger-Yosovich *et al.*, 2006) and *Ralstonia solanacearum* (*RS-IIL*) (Sudakevitz *et al.*, 2004) resemble lectin from *P. aeruginosa* (*PA-IIL*). Both lectins agglutinate human erythrocytes regardless of their A, B or O type. On top, the molecular weights for both lectins are almost similar: *CV-IIL*

(11.86 kDa) and RS-III (11.60 kDa). Both lectins bind preferentially to L-fucose and L-galactose (Sudakevitz *et al.*, 2002; Zinger-Yosovich, *et al.*, 2006).

Recently, *B. cenocepacia* has been reported to contain three soluble carbohydrate-binding proteins (*BC2L-A*, *BC2L-B* and *BC2L-C*) related to the fucose-binding lectin (*PA-III*) of *P. aeruginosa* (Lameignere *et al.*, 2010). At present, there are no reports or publications on *B. pseudomallei* lectins.

## **2.12. Strategy for identification and recognition of bacterial lectins**

Traditionally, hemagglutination (agglutination of red blood cells) is used as an indicator to determine the presence of lectins (Glick & Garber, 1983). With the current definition of lectin, which is also referred as sugar binding protein (Komath, *et al.*, 2006; Sharon & Lis, 2004), sugar binding mechanism can be detected by using surface plasmon resonance and isothermal titration calorimetry (Lameignere *et al.*, 2008). There is also proposal to define lectins as having topological similarities rather than carbohydrate recognitions (Komath, *et al.*, 2006). In this case, a lectin can be recognized by comparison with the crystal structure of another known lectin.

Additionally, the reference strain for *B. pseudomallei* strain K96243 has been fully sequenced, annotated and deposited in the European Molecular Biology Laboratory - European Bioinformatics Institute (EMBL-EBI) database (Holden *et al.*, 2004), as a part of International Nucleotide Sequence Database Collaboration (INSDC). The Universal Protein Resource (UniProt) database obtains protein sequences data by translation of the genetic coding sequences submitted to INSDC ([http://www.uniprot.org/help/sequence\\_origin](http://www.uniprot.org/help/sequence_origin)), and provides protein sequences and annotation data in an easily retrievable format. UniProt is a collaboration between the European Bioinformatics Institute (EMBL-EBI) (<http://www.ebi.ac.uk/>), the Swiss Institute of Bioinformatics (SIB) (<http://www.isb-sib.ch/>) and the Protein Information

Resource (PIR) (<http://pir.georgetown.edu/>), which provides extensive protein sequence and annotation data resources (Jain *et al.*, 2006; Consortium, 2011). The UniProt database is consisted of 3 components: UniProt Knowledgebase (UniProtKB), UniProt Reference Clusters (UniRef), and UniProt Archive (UniParc) (Consortium, 2011). UniProtKB is the main data collection for functional protein information and has two sections: i) "UniProtKB/Swiss-Prot" for annotated protein entries, and ii) "UniProtKB/TrEMBL" for unreviewed and automatically annotated protein information entries through automatic data processing from available protein sequences information, whereby these proteins will be labelled as putative (hypothetical) or uncharacterized proteins (Consortium, 2011). UniParc is a comprehensive and non-redundant database which only stores each unique protein sequence for once and each protein will be assigned a stable and unique identifier (UPI) (Leinonen *et al.*, 2004). The UniRef provides clustered sets of sequences from the UniProt Knowledgebase (UniProtKB) and selected UniParc records to obtain complete coverage of sequence space at several different resolutions to assist in managing and organize all sequence datasets, reduces sampling bias and sequence over-representation (Suzek *et al.*, 2007). With such database information available, the use of bioinformatic approach in searching for potential lectins or sugar binding protein is possible.

### **2.13. Molecular cloning and recombinant protein expression**

Molecular cloning and recombinant protein expression is a common approach in molecular biology experiments. It is performed by PCR amplification of the DNA fragment of the target DNA, followed by restriction enzyme digestion and ligation into an appropriate protein expression vector. The constructed vector will be inserted and propagated in an appropriate protein expression host, and the protein will only be conditionally expressed upon induction by relevant inducers. There are many vectors

which are commercially available, and the proteins are generally expressed with various affinity tags for the ease of recombinant protein purification for subsequent study.

The choice of the host for recombinant protein synthesis is the main decisive factor for designing the recombinant protein expression process (Rosano & Ceccarelli, 2014). While there are a wide varieties of microorganisms and eukaryotic cell lines which are capable in becoming an expression host, the protein expressed can be affected by the post translational modification, glycosylation or other protein stability factors (Rosano & Ceccarelli, 2014).

*Escherichia coli* has been widely used as a host organism for protein expression. Some of the advantages of using *E. coli* as the host for protein expression is that it is fast growing, and can achieve high cell densities. The growing and expression media for *E. coli* are readily available and cheap. The transformation of exogenous DNA is rapid and easy (Rosano & Ceccarelli, 2014). By incorporating antibiotic resistance genes as selection markers, it is able to deter plasmid free cells and other contaminants from growing in antibiotics selection agar plate or media (Rosano & Ceccarelli, 2014).

For protein expression, *E. coli* host, BL21(DE3) and derivative strains are the most widely used (Rosano & Ceccarelli, 2014). BL21 cells lack proteases such as outer membrane protease (*OmpT*) and Lon protease which degrade proteins. Furthermore, mutation of the host specificity determinant *Salmonella typhimurium* (*hdsB*) (Fuller-Pace *et al.*, 1984) gene in BL21 strains prevents DNA methylation and degradation, thus making it an ideal expression host (Rosano & Ceccarelli, 2014).

In order to purify and to detect the presence of a recombinant protein, an affinity tag will be expressed in fusion with the targeted protein. Expression vectors allow the positioning of the tag on either N-terminal or C-terminal of the recombinant protein.

The hexa-histidine tag (6 x Histidine amino acids tag) is one of the most commonly used tag for recombinant protein expression, with the advantage of being short and usable under denaturing conditions (Singh & Jain, 2013). Furthermore, commercial detection and purification kits for hexa-histidine tagged recombinant protein are widely available (Rosano & Ceccarelli, 2014). Western blot can be performed to detect the presence of the hexa-histidine tagged protein during a protein expression trial, and immobilized metal ion affinity chromatography (IMAC) using Nickel and Cobalt ions can be used to recover the his-tagged recombinant protein (Rosano & Ceccarelli, 2014).

University of Malaya



## CHAPTER 3: MATERIAL AND METHODS

**Objective 1: To determine colony morphotype, biofilm forming ability, and hemagglutination of clinical isolates of *B. pseudomallei***

### **3.1.1. Culture and maintenance of test and reference strains**

A total of 76 clinical isolates of *B. pseudomallei* obtained from the Department of Medical Microbiology, Faculty of Medicine, University of Malaya, were used in this study. The isolates were collected from several Malaysian hospitals from 1990 to 2008. Majority of the isolate were from blood cultures ( $n=43$ ) and pus specimens ( $n=12$ ). The origin and details of the bacterial strains are shown in Appendix A. Three *B. pseudomallei* reference strains (ATCC 23343, NCTC 13178, and ATCC 700388) and *P. aeruginosa* ATCC 27852 were also included in the study. *P. aeruginosa* ATCC 27852 was a well studied biofilm producer (Tielker *et al.*, 2005). Working cultures were maintained at 37°C aerobically on Lysogeny Broth (LB) agar. All the chemicals and formulae for preparing the reagents used in this study are shown in Appendix B - E.

### **3.1.2. Biofilm quantitation of clinical isolates of *B. pseudomallei***

Biofilm formation of *B. pseudomallei* was determined using a modified protocol from Taweechaisupapong *et al.* (2005). *B. pseudomallei* was subcultured on LB agar and incubated overnight at 37°C. A pure colony was picked and suspended in 1 ml BHI broth (Appendix C). The bacterial density was standardized to OD<sub>540</sub> between 1.000-1.100 using a GENESYS™ 20 Visible Spectrophotometer (Thermo Scientific, USA). This was followed by inoculation of 200 µl of the bacterial suspension into 8 wells of a 96-well flat bottom microtiter plate. The plate was incubated at 37°C for 48 hours. After incubation, the medium was discarded from each well. The wells were washed 3 times with 200 µl of PBS buffer (Appendix D). The biofilm in each well was fixed with 200 µl of 100% methanol for 15 minutes. The methanol was then removed and the wells

were air dried for 1 hour inside a biosafety hood. The staining of biofilm was performed by using 200  $\mu$ l of 2% filtered crystal violet solution (Appendix D) for 5 minutes. The crystal violet solution was then removed, and each well was washed 3 times with 200  $\mu$ l of water and the wells were air dried. The crystal violet stain was eluted by adding 200  $\mu$ l of 95% (v/v) ethanol into each well and left for 10 minutes. The eluted crystal violet solution was transferred to a round bottom 96-well microtiter plate and the absorbance at OD<sub>595</sub> was measured using GENESYS™ 20 Visible Spectrophotometer (Thermo Scientific, USA). Eight replicates were performed for each isolate and the average OD reading was taken. The degree of biofilm formation was assessed based on the relative fold difference as compared with that of *B. thailandensis* ATCC 700388. Uninoculated BHI broth was used as a negative control.

### **3.1.3. Determination of colony morphotypes of *B. pseudomallei***

A single colony from an overnight culture of *B. pseudomallei* on LB agar was stabbed onto a freshly-prepared *Burkholderia pseudomallei* selective agar (BPSA) plate (Howard and Inglis, 2003) (Appendix B) with a straight wire loop. The plate was then incubated for 5 days at 37°C. The morphological features of each isolate were recorded individually and the images of the morphotypes were captured using a digital camera (Canon Cybershot) with macro shot mode.

### **3.1.4. Hemagglutination assay**

Hemagglutination assay was performed to screen for bacterial lectin activity as described by Zinger-Yosovich *et al.* (2006). Rabbit erythrocytes collected in Alsevers solution (Appendix D) were washed with PBS buffer (Appendix D) for 3 times each by centrifugation at 1000 g for 10 minutes at 4°C. A stock solution of 2 % (v/v) erythrocytes in PBS buffer was prepared for all hemagglutination assays. For assessment of hemagglutination, 50  $\mu$ l of 2 % erythrocytes and 50  $\mu$ l of bacterial whole

cells (standardized at McFarland turbidity of No. 2) were mixed on a 96-well U bottom microtiter plate (resulted with 1 % erythrocytes) and incubated at 37°C for 1 hour. Hemagglutination was indicated by the observation of visible agglutination of the erythrocytes with *Chromobacterium violaceum* CV2A. PBS buffer was used as a negative control.

### **3.1.5. Statistical analysis**

Biofilm forming ability of *B. pseudomallei* was determined by comparing the OD readings of the eluted crystal violet from each isolate with that of *B. thailandensis* ATCC 700388. Microsoft Excel 2007 and the statistical software MINITAB 14 (USA) was used to compute descriptive statistics and box plot analysis. Based on the box plot analysis, all the isolates were grouped as high ( $X > Q3$ ), medium ( $Q1 < X < Q3$ ) and low ( $X < Q1$ ) biofilm producers, as well as outliers, where X was the relative fold difference of biofilms formed as compared with that of *B. thailandensis* ATCC 700388. The association of the morphotypes with biofilm formation was determined using ANOVA statistical analysis where P value of  $>0.05$  was considered as significant.

**Objective 2: To identify potential lectin genes in *B. pseudomallei* using bioinformatic approach**

## **3.2. Bioinformatical studies of potential lectins of *B. pseudomallei***

### **3.2.1. Data mining**

Two keywords, i.e., “lectins” and “K96243” (indicates the fully sequenced *B. pseudomallei* K96243 reference strain) were used for searching of lectin genes in the UniProt online database (<http://www.uniprot.org/>). All the search results were manually retrieved in fasta format.

### **3.2.2. Sequence similarity search against non *B. pseudomallei* species**

Sequences of the potential lectin genes of *B. pseudomallei* K96243 retrieved from UniProt database were subjected to BLAST analysis against NCBI database. The parameters used were: standard nucleotide blast (blastn) algorithm against nucleotide collection database, program selection mode optimized for highly similar sequences (megablast) (Zhang *et al.*, 2000). The results were retrieved and manually assessed. The sequences which matched with query coverage of more than 90 % and identities of more than 80 % were considered as having high similarity with the potential lectin gene of *B. pseudomallei*.

### **3.2.3. Sequential alignment and annotation**

Both DNA and protein sequential alignment and annotation were performed by Unipro UGENE v1.12.0 bioinformatics software with ClustalW 1.83 (Larkin *et al.*, 2007) default settings.

### **Objective 3: To develop multiplex PCR assay for identification of species closely related with *B. pseudomallei* based on hypothetical lectin genes**

A multiplex PCR assay was designed in this study to distinguish between closely related *Burkholderia* species. A rapid sample preparation method known as alcohol inactivation method was used for fast, easy and safe PCR template preparation. On top, a PCR internal amplification control was constructed to determine the presence of PCR inhibitors and to rule out false negative results.

### **3.3.1. Bacterial strains**

Most of the bacterial strains used in this study were obtained from a culture collection kept at the Department of Medical Microbiology, University of Malaya. Five of each *Burkholderia pseudomallei* and *Burkholderia thailandensis* strains were obtained from Professor Surasakdi Wongratanacheewin from Melioidosis Research

Center, Khon Kaen University, Thailand. The origin and details of the strains are shown in Table 3.1. *B. mallei* DNA was provided by Professor Sumalee Tungpradabkul and Professor Eiko Yabuuchi (Tanpiboonsak *et al.*, 2004).

### **3.3.2. Culture conditions**

Stock cultures were maintained in LB broth supplemented with 20% (v/v) glycerol in cryogenic vials and stored at -80°C.

### **3.3.3. Preparation of DNA template for PCR assays**

Several colonies from an overnight culture of *B. pseudomallei* were suspended in 2 ml distilled water to the McFarland standard of 2.0 prior to PCR template preparation. Bacterial DNA was prepared using three methods mentioned below:-

#### **3.3.3.1. Nucleic acid purification**

DNA was extracted from bacterial cultures using Wizard® Genomic DNA purification kit (Promega, Madison, USA, Lot #268023) in accordance to the manufacturer's protocol. For each sample, 1 ml of overnight culture was centrifuged at 13000 rpm for 2 minutes in a benchtop centrifuge (Sigma 1-14, Sigma Laborzentrifugen GmbH, Germany). The pellet was added with 600 µl Nuclei Lysis Solution and the cells were resuspended by pipetting up and down for several times. The cell suspension was incubated at 80°C for 5 minutes to lyse the cells. The cell lysate was cooled to room temperature and added with 3 µl of RNase Solution, followed by incubation at 37°C for 30 minutes. A 200 µl of Protein Precipitation Solution was added to the cell lysate and mixed by vortexing for 20 seconds. The sample was then incubated on ice for 5 minutes to enhance protein precipitation. After centrifugation at 13000 rpm for 3 minutes to pellet the protein, the supernatant was then transferred to a 1.5 ml centrifuge tube containing 600 µl of room temperature pure isopropanol, and mixed gently by inversion. The DNA was then precipitated by centrifugation at 13000 rpm for 5

minutes. To wash the DNA pellet, a 600 µl of 70 % ethanol was added with gentle inversion. The sample was then subjected to centrifugation again at 13000 rpm for 5 minutes. The excess ethanol was discarded and the pellet was air-dried for 20 minutes. Finally, the DNA was resuspended in 50 µl of DNA Rehydration Solution and incubated at 65°C for 1 hour. The DNA solution was stored at -20°C prior to use.

**Table 3.1:** Bacterial strains used in the development and validation of multiplex PCR assay

Organism	Source	No. isolate
<i>Burkholderia spp.</i>		
<i>B. pseudomallei</i> (n=46)	Clinical	34
	Animal	4
	Environmental	5
	ATCC 23343	1
	NCTC 13178	1
	K96243	1
<i>B. mallei</i> (n=4)	EY100	1
	EY2235	1
	EY2236	1
	EY2237	1
<i>B. thailandensis</i> (n=6)	ATCC 700388	1
	Environmental	5
<i>B. cepacia</i> (n=22)	Clinical	19
	Environmental	3
<i>Non-Burkholderia spp. (n=37)</i>		
<i>Acinetobacter spp.</i>	Clinical	10
<i>Aeromonas hydrophila</i>	Clinical	2
<i>Alcaligenes xylosoxidans</i>	Clinical	1
<i>Klebsiella pneumoniae</i>	Clinical	1
<i>Chromobacterium violaceum</i>	Clinical	1
<i>Chromobacterium violaceum</i>	Environmental	6
<i>Ochrobactrum anthropi</i>	Clinical	2
<i>Proteus mirabilis</i>	Clinical	2
<i>Ralstonia pickettii</i>	Clinical	1
<i>Salmonella enteritidis</i>	Clinical	1
<i>Salmonella typhi</i>	Clinical	1
<i>Stenotrophomonas maltophilia</i>	Clinical	2
<i>Pseudomonas aeruginosa</i>	Clinical	1
<i>Pseudomonas aeruginosa</i>	ATCC 27853	1
<i>Pseudomonas stutzeri</i>	Clinical	1
MRSA	Clinical	1
<i>Staphylococcus aureus</i>	ATCC 25923	1
<i>E. coli</i>	ATCC 25922	1
<i>E. coli</i>	JM107	1

\* EY, Eiko Yabuuchi; Department of Bacteriology, Osaka City University Medical School, Osaka, Japan (Tanpiboonsak *et al.* 2004)

### **3.3.3.2. Boiling method**

The boiling method was modified from the procedures described by Barletta *et al.*, (2009). DNA was prepared by boiling a bacterial culture in 50 µl of distilled water for 15 minutes using a hot water bath. The lysate was cooled down at room temperature for 15 minutes before centrifugation at 13,000 rpm for 15 min. The clear supernatant was collected in a new Eppendorf tube and stored at -20°C prior to use.

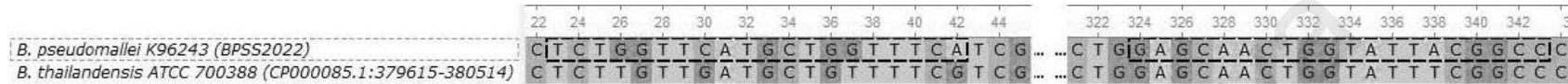
### **3.3.3.3. Alcohol inactivation sample preparation method**

The alcohol inactivation sample preparation method was designed to prepare DNA template for highly infectious bacteria for PCR assays. A loopful suspended colony of 50 µl bacterial culture was added with 200 µl of pure isopropyl alcohol (AMRESCO, USA) in a 1.5 ml Eppendorf tube. The bacterial suspension was mixed by pipetting up and down for 5 times and vortexed for 5 seconds, before centrifugation at 16000 rpm for 1 minute. The residual alcohol was removed by pipetting, and the tube was air-dried by leaving it on the perforated grill of a biological safety hood for 5 minutes. The dried pellet was resuspended in 100 µl of nuclease-free water prior to use in PCR assay.

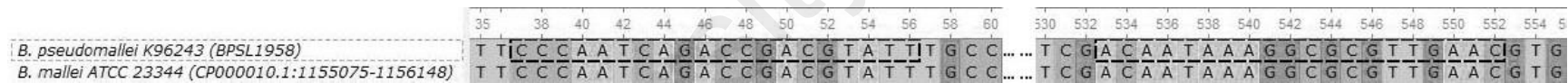
### **3.3.4. Development of a multiplex PCR assay for identification of *B. pseudomallei*, *B. mallei*, *B. thailandensis* and *B. cepacia* complex based on putative lectin genes**

#### **3.3.4.1. Primer design**

Primers were designed manually using Primer3 software (<http://frodo.wi.mit.edu>) (Rozen and Skaletsky 2000) based on the DNA sequences obtained from GenBank database (Figure 3.1 – 3.4, Table 3.2). The designed primers were subjected to Primer-BLAST analysis to ensure the primers specificity. All the primers were commercially synthesized by Bioneer Corporation, Korea.

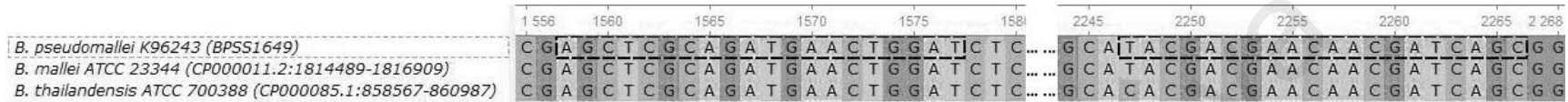


**Figure 3.1:** Sequential alignment of *B. pseudomallei* K96243 (BPSS2022) and *B. thailandensis* ATCC 700388 (CP000085.1: 379615-380514). The boxed region is the forward (32F) and reverse (32R) primer designed to detect *B. pseudomallei* isolates where it spans a region with 4 nucleotides difference with *B. thailandensis*, yield amplicon of 321 bp with reverse primer (32R) in *B. pseudomallei* but not in *B. thailandensis*. Similar DNA sequences were not found in *B. cepacia* complex and *B. mallei*.

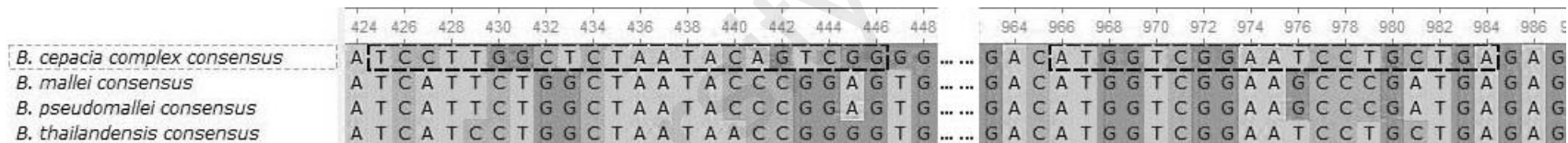


**Figure 3.2:** Sequential alignment of *B. pseudomallei* K96243 (BPSL1958), *B. mallei* ATCC 23344 (CP000010.1: 1155075-1156148). The boxed regions are where the forward (51F) and reverse (51R) primers were designed, yield amplicon of 516 bp for both *B. pseudomallei* and *B. mallei*. No similar DNA sequences were found in *B. thailandensis* and *B. cepacia* complex.





**Figure 3.3:** Sequential alignment of *B. pseudomallei* K96243 (BPSS1649), *B. mallei* ATCC 23344 (CP000011.2: 1814489-1816909) and *B. thailandensis* ATCC 700388 (CP000085.1: 858567-860987). The boxed regions are where the forward (71F) and reverse (71R) primers were designed, yield amplicon of 709 bp for all the 3 mentioned species. No similar DNA sequences were found in *B. cepacia* complex.



**Figure 3.4:** The sequential alignment of each generated consensus region of 16s Ribosomal RNA gene for *B. cepacia* complex, *B. mallei*, *B. pseudomallei* and *B. thailandensis*. The boxed regions are the forward (Bc16F) and reverse (Bc16R) primer designed for detection of *B. cepacia* complex, yield amplicon of 560 bp only in *B. cepacia* complex.

**Table 3.2:** Primers for detection of hypothetical lectin genes.

<b>Single-plex PCR</b>			
<b>Primer Name</b>	<b>Sequences</b>	<b>Target</b>	<b>Size</b>
2056F_702	cgggtactggcagttcgat	<i>BPSL2056</i>	702bp
2056R_702	acgagccacatgtgattgtc		
1124F_378	gtcacgaacctgaataccg	<i>BPSS1124</i>	378bp
1124R_378	gacgtattcctcgacgttgc		
1488F_224	aatgggtcgcgacgattcaac	<i>BPSS1488</i>	224bp
1488R_224	tcatggtcgatctccagaaa		
0713F_646	ctgacacctgacggacatcct	<i>BPSS0713</i>	646bp
0713R_646	tgaactggcgttgattcgc		
0767F_226	tcaaaaattcgtcggtagcgc	<i>BPSS0767</i>	226bp
0767R_226	gttgaccgtgaagtcggtct		

<b>Multiplex PCR</b>			
<b>Primer Name</b>	<b>Sequences</b>	<b>Target</b>	<b>Size</b>
71F	agctcgcagatgaactggat	<i>BPSS1649</i>	709bp
71R	gctgatcgttgctcgcgta		
32F	tctggtcatgctggttca	<i>BPSS2022</i>	321bp
32R	ggccgtaataccagttgctc		
51F	cccaatcagaccgacgtatt	<i>BPSL1958</i>	516bp
51R	gttcaacgcgcctttattgt		
Bc16F	tccttggtcctaatacagtcgg	16s RNA	560bp
Bc16R	tcagcaggattccgacct	(Bcc)	
xynB128F	agtgcgcaggacatcaccta	xynB	128bp
xynB128R	gggtttagtcgccgtaaga		

#### 3.3.4.2. Construction of an internal amplification control (IAC) for multiplex PCR assay

*Aspergillus niger* is a mould species which is commonly isolated from contaminated food. The fungus expresses xylanases in the presence of D-xylose and xylan as inducers (van Peij *et al.* 1998; de Vries *et al.* 1999). The full-length of endo- $\beta$ -1,4-xylanase gene (*xynB*) [GeneBank: XM\_001388485.1] contains 745 nucleotides and includes an intron of 67 nucleotides (Deng *et al.*, 2006). Based on the fact that cDNA does not exist in nature; an internal amplification control was designed with an intron spanning primer, where the primer spans through the intron region to avoid unspecific amplifying

potential unspecific binding in the genomic DNA of *A. niger*. Using an *A. niger* strain isolated from contaminated food source as a template, the partial double stranded cDNA sequence with expected amplicon size of 128 bp was generated in this study using a modified version of overlap extension polymerase chain reaction (OE-PCR) by lowering down the annealing temperature to 50°C for 15 seconds, using primers xynB128F and xynB128R (Table 3.2). The fragment was subsequently recovered using gel excision, followed by cloning into a cloning plasmid vector pJET1.2/Blunt (Fermentas, Lithuania) in accordance to the manufacturer's procedure. The sequence was verified by sequencing. The new recombinant plasmid (designated as pJXN128) was transferred and propagated in *E. coli* GigaSingles™ Competent Cells (Novagen, USA). Figure AF.1 in Appendix F illustrates the steps involving in the construction of the internal control. The purified plasmid was used as the internal amplification control in the multiplex PCR assay.

#### **3.3.4.3. Multiplex PCR assay**

The multiplex PCR assay was performed in a total volume of 15 µl containing 5 µl of crude sample or purified genomic DNA, 5 % of dimethyl sulfoxide (DMSO), 0.2 mM of each dNTP, 1 X Pol Buffer C, 2 mM of MgCl<sub>2</sub>, 0.75 unit Perpetual *Taq* DNA polymerase (EURx, Gdansk, Poland), 0.2 µM primers Bc16F, Bc16R, 32F, 32R, 71F and 71R, 0.3 µM primers 51F and 51R, and 0.1 µM primers xynB128F and xynB128R, and 2.7 ng internal amplification control plasmid (pJXN128) (Appendix F: Table AF.1). The amplification condition was initiated with a denaturation step at 95°C for 3 min, followed by 30 cycles at 95°C for 20 s, 59°C for 35 s, and 72°C for 30 s. The whole amplification process took approximately one hour and 20 minutes on a SensoQuest LabCycler (SensoQuest GmbH, Gottingen, Germany). The PCR products were electrophoresed on 1.5 % agarose in 0.5 X TBE buffer at 140 V for 20 min and the image was captured using InGenius gel documentation system (Syngene, Cambridge,

England). An open source gel image analyzer, PyElph (Pavel & Vasile, 2012) was used to perform gel analysis, clustering analysis and to generate dendrogram.

#### **3.3.4.4. Confirmation of the presence of hypothetical lectin genes**

The presence of two hypothetical lectin genes, i.e., *BPSS2022* and *BPSS1649* in all *B. pseudomallei* isolates were confirmed using the multiplex PCR assay as described in section 3.3.4.3. To determine the presence of the remaining of the hypothetical lectin genes, i.e., *BPSL2056*, *BPSS0713*, *BPSS0767*, *BPSS1124* and *BPSS1488*, five single-plex PCR assays were designed using primers as shown in Table 3.2. All PCR assays were performed in a total volume of 15  $\mu$ l containing 5  $\mu$ l of crude sample or purified genomic DNA, 0.2 mM of each dNTP, 5 % of DMSO, 1x Pol Buffer C, 2 mM of  $MgCl_2$ , 0.75 unit Perpetual *Taq* DNA polymerase (EURx, Gdansk, Poland), and 0.2  $\mu$ M of respective forward and reverse primers of the particular targeted gene. The thermocycling process was similar to that of the multiplex PCR mentioned in section 3.3.4.3.

#### **3.3.4.5. Analysis of PCR products**

The PCR products were analyzed by electrophoresis on a 1.5% agarose gel (wt/vol) at 140 V for 20 min. The gel was stained with ethidium bromide, and the image was captured using an InGenius gel documentation system (Syngene, England). The multiplex PCR assay was interpreted based on the presence of amplified products on agarose gel: three fragments for *B. pseudomallei* (321 bp, 516 bp, 709 bp), two for *B. mallei* (516 bp and 709 bp) and one each for *B. thailandensis* (709 bp), *B. cepacia* complex (560 bp), and the internal control plasmid (128 bp). The multiplex PCR was validated using DNA extracts of 115 isolates representing 19 bacterial species (Table 3.1). To determine the minimum detection level of DNA for *B. pseudomallei*, *B. cepacia*, *B. mallei* and *B. thailandensis*, a serial titration was done to determine the

minimum amount of DNA required for PCR detection. PyElph software (Pavel & Vasile, 2012) was used to identify if there were any nonspecific products generated from the assay. The acquired agarose gel image was first loaded into the software, followed by image analysis, clustering analysis and dendrogram building which were performed in accordance to the instruction of the software.

#### **3.3.4.6. Validation of multiplex PCR assay**

The multiplex PCR assay was validated using a total of 115 bacterial strains which had been alcohol-inactivated, as shown in Table 3.1. For sensitivity testing, DNA of *B. pseudomallei* (109.0 µg), *B. mallei* (60.0 µg), *B. thailandensis* (23.0 µg) and *B. cepacia* complex (90.0 µg) were 10-fold serially diluted. The minimum detection level was determined based on the results of amplification from the DNA solution with the lowest concentration.

**Objective 4: To clone and express hypothetical lectin genes, and to assess the hemagglutination activity of the recombinant proteins**

#### **3.4.1. Cloning of hypothetical lectin genes**

##### **3.4.1.1. Primers for amplification of hypothetical lectin genes**

Based on the search results from UniProt database, 7 hypothetical lectins (*BPSS1649*, *BPSS2022*, *BPSS0767*, *BPSL2056*, *BPSS1124*, *BPSS0713*, and *BPSS1488*) and 3 known bacterial lectins, i.e., two from *P. aeruginosa* (*LecA*, *LecB*) and one from *C. violaceum* (*CV-III*) were selected for cloning into pET-46EK/LIC vector (Novagen, USA) using ligation independent cloning (LIC) approach. Special LIC primers (Table 3.3) were manually designed in accordance to the recommendation of the manufacturer. All the recombinant proteins had an additional of 6 x histidine amino acids tag in the N-terminal of the proteins, is for protein isolation using affinity chromatography and detection using Western blot. A stop codon was designed and incorporated in the

reverse primer to avoid the expression of S-tag which was supposedly added at the C-terminal of the protein. All constructs were verified by PCR (data not shown) prior to DNA sequencing by First BASE DNA Sequencing Services (Malaysia).

**Table 3.3:** Primer sequences used for cloning of hypothetical and reference lectins with amplicon size.

Gene (UniProt accession no.)	Name	Sequence	Amplicon size (bp)
BPSL2056 (Q63TB1)	ADP-BPSL2056F	<u>GACGACGACAAGATG</u> CCGACGGCGCCGGAT	2568
	ADP-BPSL2056R	GAGGAGAAGCCCGGTCAGGCGCCGATCCGGAT	
BPSS0713 (Q63ME4)	ADP-BPSS0713F	<u>GACGACGACAAGATG</u> ACGAAGAACGAAGAATCG	1236
	ADP-BPSS0713R	GAGGAGAAGCCCGGTCAGAGATCTCTCGCAAGACG	
BPSS0767 (Q63M93)	ADPBP0767F	<u>GACGACGACAAGATG</u> ACTCAAAAATTCGTCGGTAC	324
	A2DP-BPSS0767R	GAGGAGAAGCCCGGTCACGCGGGCGTCTCGGCGA	
BPSS1124 (Q63L84)	ADP-BPSS1124F	<u>GACGACGACAAGATG</u> ACGCTCAAGCTGGCC	984
	ADP-BPSS1124R	GAGGAGAAGCCCGGTCATCGCGACTCCACGAG	
BPSS1488 (Q63K77)	ADP-BPSS1488F	<u>GACGACGACAAGATG</u> CACGCGCCGTCATTC	837
	ADP-BPSS1488R	GAGGAGAAGCCCGGTCATGGTCGATCTCCAGAAA	
BPSS1649 (Q63JR7)	ADPB1649SF	<u>GACGACGACAAGATG</u> CATTTCTTTTCGATTCGC	2421
	A2DP-BPSS1649R	GAGGAGAAGCCCGGTCATGAGATCCGGATCGATG	
BPSS2022 (Q63IP7)	ADPB2022SF	<u>GACGACGACAAGATG</u> AAAAAATACGCATTGGCG	906
	A2DP-BPSS2022R	GAGGAGAAGCCCGGTC AACC CGGCGCGTAGCA	
<i>CV-III</i> (Q7NX84)	CV2L345-F	<u>GACGACGACAAGATG</u> GCTCAGCAAGGCGTG	342
	CV2L345-R	GAGGAGAAGCCCGGTCAGCCCAGCGGCCAGTT	
<i>LecA</i> (Q05097)	PALECA361-F	<u>GACGACGACAAGATG</u> GCTTGAAAGGTGAGGT	366
	PALECA361-R	GAGGAGAAGCCCGGTCAGGACTGATCCTTTCCAATA	
<i>LecB</i> (Q9HYN5)	PALECB348-F	<u>GACGACGACAAGATG</u> GCAACACAAGGAGTGTTT	345
	PALECB348-R	GAGGAGAAGCCCGGTTAGCCGAGCGGCCAGTT	

#### 3.4.1.2. PCR for hypothetical lectin genes

Specially designed LIC primers were used to clone the full ORF of the hypothetical lectins, using purified genomic DNA of *B. pseudomallei* K96243 as a template. The KOD Hot Start DNA Polymerase (Novagen, USA) or KOD Xtreme™ Hot Start DNA Polymerase (Novagen, USA) was used for amplification of the gene fragments. For PCR using KOD Hot Start DNA Polymerase, the reactions were performed in a total volume of 50 µl, consisting of 1X KOD buffer for KOD Hot Start DNA Polymerase (Novagen, USA), 1.5 mM MgSO<sub>4</sub>, 0.2 mM of each dNTPs, 0.3 µM of each forward and

reverse primer, 1U of KOD Hot Start DNA Polymerase, and 5 µl of DNA template (Appendix F: Table AF.2). The amplification was initiated with a denaturation step at 95°C for 2 minutes, followed by 30 cycles of denaturation at 95°C for 20 seconds, annealing at 60°C for 10 seconds and extension at 70°C for 1 minute and 30 seconds.

The PCR products were electrophoresed on 1.0 % agarose in 0.5 X TBE buffer at 100V for 1 hour and the image was captured using InGenius gel documentation system (Syngene, Cambridge, England). The DNA fragment corresponding with the predicted sizes were excised for purification prior to cloning.

#### **3.4.1.3. Purification of PCR products**

The DNA fragment of interest was excised from the agarose gel using a scalpel blade and carefully weighted, before purification using a commercial kit (MEGAquick-spin™ PCR & Agarose Gel DNA Extraction System, iNtRON Biotechnology, Korea). For each 100 mg of agarose gel slice, 300 µl of supplied BNL buffer was added. This was followed by a brief vortex and the mixture was incubated at 55°C for 10 – 15 minutes until the gel was completely dissolved. The mixture was then transferred to a spin column and centrifuged for 12,000 g for 1 minute using a bench top centrifuge Sigma 1-14 (Sigma Laborzentrifugen GmbH, Germany). The flow through was discarded and 700 µl of the washing buffer (provided in the kit) was added to the column before centrifugation at 12,000 g for 1 minute. The flow through was then again discarded, and the column was centrifuged for 12,000 g for another 3 minutes to dry the column for complete removal of wash buffer. For DNA elution, 50 µl of elution buffer was added to the middle of the column. The column was incubated at room temperature for 5 minutes and centrifuged again for 12,000 g for 1 minute to elute the PCR product.





The reaction mixture (in a total volume of 20  $\mu$ l) was composed of 5  $\mu$ l of purified PCR products, 2  $\mu$ l of 10X T4 polymerase buffer, 2  $\mu$ l of 25 mM dATP, 1  $\mu$ l of 100 mM DTT, 9.6  $\mu$ l of nuclease free water and 0.4  $\mu$ l of 2.5 U/ $\mu$ l T4 DNA Polymerase (Novagen, USA). The mixture was incubated at 22°C for 30 minutes, followed by enzyme inactivation at 75°C for 20 minutes. For ligation of the PCR product into the vector, 2  $\mu$ l of the prepared insert was mixed with 1  $\mu$ l of the vector pET-46EK/LIC and incubated at 22°C for 5 minutes, followed by adding 1  $\mu$ l of 25 mM EDTA and further incubate for 5 minutes at 22°C. The ligated vector was then transformed into a cloning host, NovaBlue GigaSingles™ Competent Cells (Novagen, USA) and plated on LB agar containing 50  $\mu$ g/ml carbenicillin for positive colony selection. The selected positive colony was propagated in Lysogeny Broth (LB) containing 50  $\mu$ g/ml carbenicillin and supplemented with 1% (w/v) glucose. Plasmid extraction was performed by the method below, and the plasmid was transformed into *E. coli* expression host for expression using the method as described in section 3.4.1.6.

#### **3.4.1.5. Preparation of Competent Cells**

Competent cells (for *Escherichia coli* BL21 (DE3) pLysS strains and ROSETTA-GAMI 2 (DE3) pLysS strains (Novagen, USA) were prepared by using calcium chloride transformation protocol as described by Klock and Lesley (2009) with some modification. For each cloning or expression host, the strain was seeded in 10 ml LB Broth, incubated at 37°C with shaking at 250 rpm until the optical density at 600 nm ( $OD_{600}$ ) was between 0.5 to 0.6. The culture was then centrifuged at 2500 g for 20 minutes at 4°C in a Sigma 1-14 centrifuge (Sigma Laborzentrifugen GmbH, Germany). An ice-cold 1 ml of 200 mM  $MgCl_2$  solution was then added and the pellet was resuspended gently using a micropipettor. The cells were then incubated on ice for 30 minutes and centrifuged at 2500 g for 20 minutes. The pellet was then resuspended

gently in 200 µl ice-cold solution of 100 mM CaCl<sub>2</sub> with 15 % glycerol. The competent cells were then aliquoted into 20 µl per tube and stored at -80°C.

#### **3.4.1.6. Transformation of vector**

For transformation of vector into competent cells, vial containing the competent cells was thawed on ice for 15 minutes. The cells were then pipetted into a tube containing 2 µl of plasmids and incubated in ice for 15 minutes. The cells were subjected to “heat shock” in a 42°C water bath for 45 seconds and returned immediately to ice for further incubation of 2 minutes. A 250 µl of SOC medium (#15544-034, Invitrogen, USA) was added to the cells while the tube was still on ice. The cells were then incubated at 37°C in a rotary shaker at 180 rpm for 1 hour, before plating in an LB agar plate supplemented with 50 µg/ml carbenicillin (Appendix C). The cells were distributed evenly on the agar surface by shaking with sterile glass beads (approximately 5 mm in diameter). The plate was then incubated inversely overnight.

#### **3.4.1.7. Plasmid preparation**

Recombinant plasmids were prepared and isolated by using QIAprep® Miniprep (Qiagen, Germany) according to the manufacturer’s protocol with slight modification. Pelleted cells were suspended into 250 µl of the provided Buffer P1, and subsequently mixed thoroughly with 250 µl of Buffer P2. This was followed by adding 350 µl of Buffer N3 and mixing by tube inversion prior to centrifugation for 10 minutes at 12000 g. The supernatant was then transferred to a QIAprep spin column and centrifuged for another 1 minute. A volume of 500 µl Buffer PB was then added and the spin column was centrifuged for another 1 minute. The flow through was discarded. The column was washed with 750 µl of PE buffer by centrifugation for 1 minute and another 5 minutes to remove residual wash buffer which contained ethanol. The QIAprep column was then placed in a new microcentrifuge tube and 50 µl of pre-warmed (at 40°C) Buffer EB was

added directly into the center of the spin column. The column was incubated at room temperature for 2 minutes prior to centrifugation for 5 minutes to elute the plasmid DNA.

### **3.4.2. Expression and purification of recombinant hypothetical lectins**

#### **3.4.2.1. Expression of recombinant protein**

The recombinant proteins (as mentioned in Table 3.3 above) were expressed in *E. coli* BL21 (DE3) pLysS strains and ROSETTA-GAMI 2 (DE3) pLysS strains (Novagen, USA). A single colony of the expression host containing the recombinant vector was picked from a freshly streaked plate and inoculated into 25 ml Lysogeny Broth (LB) containing 50 µg/ml carbenicillin and supplemented with 1% (w/v) glucose (Appendix C). The culture was incubated at 30°C with 150 rpm rotary shaking until the culture density (OD<sub>600</sub>) was more than 0.5. The cells were pelleted by centrifugation (6,000 g, 10 min, 4°C) and the medium was replaced by a fresh LB broth containing 50 µg/ml carbenicillin and supplemented with 1% (w/v) glucose (Appendix C) as well as 0.5 mM IPTG (Fermentas, Lithuania # R0393) (Appendix C) as inducer for recombinant protein expression. The protein expression was performed at 37°C (for 4 hours) or at 30°C (for 6 hours) during the initial attempt. *E. coli* cells were harvested by centrifugation (6,000 g, 10 min, 4°C). The cells were then resuspended in 5 ml Lysis-Equilibration-Wash (LEW) buffer (50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, pH 8.0), and subsequently disrupted in a Branson Sonifier 250 sonicator (Branson Ultrasonic, USA) using 5 x 30 seconds burst followed by 30 seconds with cooling on ice. The crude lysate protein was centrifuged at 10,000 g for 30 minutes at 4°C and the supernatant were syringed filtered using a 0.45 µm pore size syringe filter (Sartorius Minisart® High Flow #109-16537K).

#### **3.4.2.2. Protein purification**

Each recombinant protein was purified by gravity flow using Protino® Ni-TED 1000 Packed Columns (Macherey-Nagel, Germany) in native condition. The column was first equilibrated with 2 ml LEW buffer (provided by the kit) followed by loading with 3 ml of filtered crude protein, and washing with 4 ml of LEW buffer to remove unbound and unrelated protein. The protein was eluted by 1.5 ml of elution buffer (250 mM imidazole in LEW buffer) for 3 times, and the presence of protein was verified by western blot.

#### **3.4.2.3. SDS-PAGE**

SDS-PAGE was performed by XCell SureLock® Mini-cell (Invitrogen, Carlsbad, CA, USA) using pre-cast polyacrylamide NuPAGE® 4–12% Bis-Tris Gel (Invitrogen, Carlsbad, CA, USA). Electrophoresis was performed at 200 V for 60 minutes using NuPAGE® 1X MES SDS running buffer with antioxidant (Invitrogen, Carlsbad, CA, USA). Each well was loaded with 6.5 µl of protein sample added with 2.5 µl of NuPAGE® LDS Sample Buffer and 1 µl of NuPAGE® Reducing Agent. The SDS-PAGE gel was silver-stained or subjected to western blotting.

#### **3.4.2.4. Western blotting**

The process from a SDS-PAGE gel were transferred to a Amersham Hybond-P PVDF membrane (GE Life Sciences, USA) using the following protocol. The transfer buffer was prepared by adding 50 ml of NuPAGE® Transfer buffer (20X), 1 ml of NuPAGE® Antioxidant, 100 ml of methanol and the volume was topped up by distilled water to a final volume of 1000 ml. The blotting pads were soaked in the transfer buffer until saturated and air bubbles were removed by squeezing the blotting pads while submerging in the buffer. PVDF membrane was activated by soaking in 100 % methanol for 30 seconds, followed by rinsing in distilled water for 1 minute and

equilibration in transfer buffer for 5 minutes. Chromatography filter papers (FT-2-527-460570K, Sartorius Stedim Botech GmbH, Gottingen, Germany) were used as supporting filter paper and were soaked in transfer buffer immediately before using as a support to the PVDF membrane and the SDS-PAGE gel. To assemble the western blotting sandwich, two blotting pads were laid on top of the cathode core of the XCell II™ Blot Module (Invitrogen, Carlsbad, CA, USA), followed by one piece of supporting filter paper. The SDS-PAGE gel was carefully overlaid on top of the supporting filter paper and air bubbles were removed. This was followed by laying another piece of supporting filter paper and two blotting pads, before assembling with the anode core. Transfer buffer was added to cover the whole western blotting sandwich. The outer chamber was filled with “ice-cold” distilled water for heat dissipation. Western blot was performed at 30 V for 1 hour. The transfer was considered complete when the pre-stained protein markers had been completely blotted onto the membrane.

#### **3.4.2.5. Detection of recombinant protein**

The recombinant protein with 6x histidine-tag on Western blots was detected using HisDetector™ Western Blot Kit AP Colorimetric kit (KPL, Gaithersburg, Maryland, USA). The membrane was blocked by immersing in 20 ml of 1 X Detector Block Solution for 1 hour at room temperature with gentle rocking. This was followed by the addition of 1 µl of HisDetector Nickel-AP into the block solutions (to achieve 1/2000 dilutions), and further incubation for 1 hour at room temperature with gentle rocking. The membrane was then washed in 1X TBST buffer (Appendix D) 3 times for 5 minutes each with gentle rocking. The recombinant protein was detected by incubating the membrane in 10 ml of the provided BCIP/NBT solutions for 5 to 10 minutes, before stopping the reaction by immersion into distilled water. Any detected histidine-tagged protein band will be visibly shown as deep purple colour on membrane.

#### **3.4.2.6. Protein quantitation**

Protein quantitation was determined using SMART™ BCA Protein Assay Kit (iNtRON Biotechnology, Seoul, Korea). A panel of albumin standards was used to plot the protein standard curve. Three technical replicates were performed for both standards and sample. The working solution was prepared by mixing the provided solution A and solution B in 50:1 ratio prior to use. For each sample, 25 µl was added into a 96-microplate containing 200 µl working solution. The plate was mixed thoroughly on a plate shaker for 30 seconds and incubated at 37°C. The plate was then cooled to room temperature and the absorbance was read at 560 nm on a plate reader. The protein amount is compared with the standard curved calculated.

#### **3.4.2.7. Silver staining of SDS-PAGE gel**

Silver staining of SDS-PAGE gel was performed by using PageSilver™ Silver Staining Kit (#K0681, Fermentas, Vilnius, Lithuania), and all the working solutions (Appendix E) were prepared as in accordance to the instruction manual provided. After SDS-PAGE, the gel was placed in a staining tray and washed briefly with distilled water. The gel was then added with 100 ml Gel fixing solution 1 (Appendix E) and rocked gently for 60 minutes. The solution was then replaced by 100 ml gel fixing solution 2 and the gel was rocked gently for 20 minutes. The procedure was repeated for three times, followed by washing twice using 100 ml deionised water with gentle rocking for 20 seconds each. The gel was sensitized by adding 100 ml of sensitizing solutions. The gel was rocked gently for 1 minute. This was then followed by washing with 100 ml deionised water twice with gentle rocking for 20 seconds each. The solution was replaced by 100 ml staining solution and gently rocked for 20 minutes. The gel was then washed in 100 ml deionised water twice for 20 seconds each. Finally, the solution was replaced by 100 ml developing solutions. The gel was rocked gently for

approximate 3 minutes until all bands were well developed. The development on the gel was stopped by adding and mixing with 100 ml of stop solution for 10 minutes.

### **3.4.3. Screening for lectin activity of recombinant proteins**

#### **3.4.3.1. Hemagglutination assays (for recombinant proteins)**

Hemagglutination was performed to screen for lectins activity as described by Zinger-Yosovich *et al.* (2006). Rabbit erythrocytes collected in Alsevers solution (Appendix D), were washed with PBS buffer (Appendix D) for 3 times each with 2 settled volumes of the erythrocytes. A stock of 2 % (v/v) erythrocytes in PBS buffer was prepared for all standard hemagglutination assays. For assessment of hemagglutination, 50  $\mu$ l of 2 % erythrocytes and 50  $\mu$ l of bacteria whole cell (standardized at McFarland turbidity of No. 2) or expressed recombinant protein (standardized at 15  $\mu$ g/ml) were mixed on 96-well U bottom plates (resulted with 1 % erythrocytes) and incubated at 37°C for 1 hour. The hemagglutination activity was assessed by observing the visible agglutination of the erythrocytes with the recombinant proteins of *P. aeruginosa*, (*LecA* and *LecB*) while the negative control was PBS buffer.

## CHAPTER 4: RESULTS

### 4.1. Determination of colony morphotype, biofilm forming ability, and hemagglutination of clinical isolates of *B. pseudomallei*

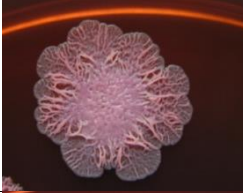






#### 4.1.1. Determination of the colony morphotypes of *B. pseudomallei*

Seven different groups or colonial morphotypes of *B. pseudomallei* on BPSA agar were recorded (Figure 4.1). The isolates could be differentiated into 2 major groups based on the colonial morphology: one where the surface texture had a mixed appearance and another where the surface texture was uniform. The group with mixed surface texture could be divided into 4 subgroups, i.e. (i) central rough surface with radiating wrinkling up to the edge, (ii) mixture of rough, wrinkled and smooth surface with irregular edges, (iii) central rough surface with smooth circumference and (iv) wrinkled central area with smooth circumference. The group with uniform texture was consisted of 3 subgroups, i.e. (i) convex, mucoid, with smooth colony surface, (ii) rough texture of entire colony with irregular edges, and (iii) wrinkled surface of entire colony. The most predominant morphotype was group 1 (40.8%), next was group 2 (18.4%), and followed by group 3 (15.8%) (Figure 4.1).

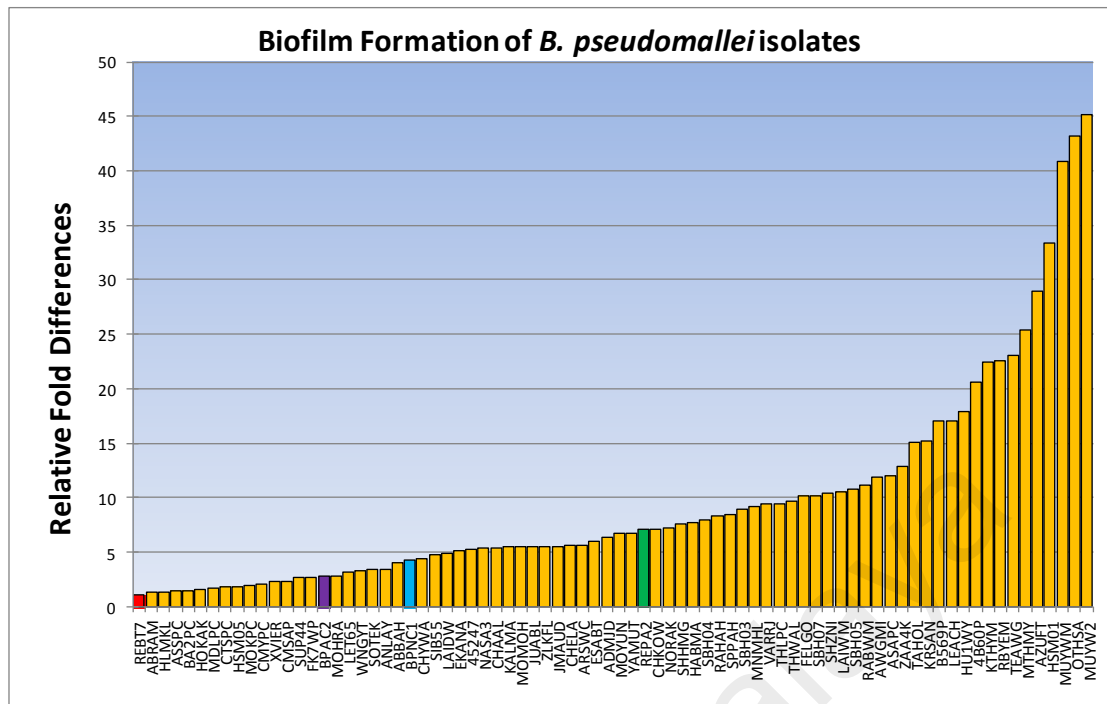
#### 4.1.2. Biofilm forming ability of clinical isolates of *B. pseudomallei*

In order to compare the biofilm formation between *B. pseudomallei* isolates, the relative fold differences of *B. pseudomallei* when compared to *B. thailandensis* ATCC 700388 was determined. *B. thailandensis* showed the lowest reading in this biofilm study and therefore, was used as the assay reference. Figure 4.2 shows the ranking of the *B. pseudomallei* isolates based on the relative fold differences. *P. aeruginosa* ATCC 27853 demonstrated 7.06 times higher OD<sub>540</sub> reading than *B. thailandensis* ATCC 700388.



Mixed Texture		
Group	Image	Description; <i>No. of isolates</i> (%)
1		Central rough surface with radiating wrinkling up to edge; <u>31</u> (40.8)
2		Mixture of rough, wrinkled and smooth surface with irregular edge; <u>14</u> (18.4)
3		Central rough surface with smooth circumference and edge; <u>12</u> (15.8)
4		Wrinkled central area with smooth circumference and edge; <u>5</u> (6.6)
Uniform Texture		
Group	Image	Description; <i>No. of isolates</i> (%)
5		Convex, mucoid, with smooth colony surface; <u>5</u> (6.6)
6		Rough texture of entire colony with irregular edges; <u>5</u> (6.6)
7		Wrinkled surface of entire colony; <u>4</u> (5.2)

**Figure 4.1:** Representative pictures (Group 1 - 7) of the distinct seven colonial morphotypes of *B. pseudomallei* on BPSA.

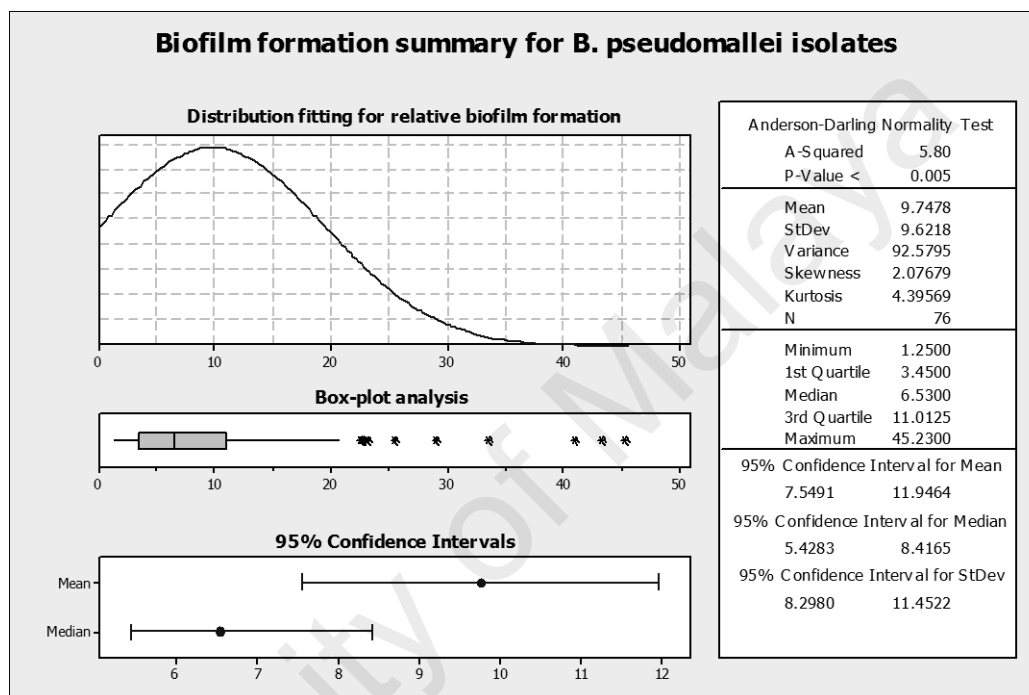


**Figure 4.2:** Bar chart showing overall biofilm formation of *B. pseudomallei* clinical isolates = orange; green = *P. aeruginosa* ATCC 27852; blue = *B. pseudomallei* NCTC 13178; purple = *B. pseudomallei* ATCC 23343; red = *B. thailandensis* ATCC 700388

Based on the results obtained from the relative fold difference, descriptive statistics and box plot analysis were performed to categorize the isolates into high, medium and low biofilm producers. A total of 20 *B. pseudomallei* isolates were identified as high biofilm producer ( $X > 11.01$ ), while 37 isolates were medium ( $3.45 < X < 11.01$ ) and 19 were low biofilm ( $X < 3.45$ ) producers. The high biofilm producing isolates demonstrated as high as 45.23 fold differences while the low biofilm producers showed at least 1.25 fold difference when compared to *B. thailandensis* ATCC 700388 strain (Figure 4.2).

Anderson-Darling Normality test were performed to detect data departures from normality. As the P value was  $< 0.005$ , the results suggested that the biofilm formation of overall distributions was not following a normal distribution. The positive skewness (2.077) indicated that the data for biofilm formed were skewed right and most values

were concentrated to the left of the mean, with some extreme values to the right, suggesting that most isolates were low biofilm producers. The positive Kurtosis (4.396) value follows leptokurtic distribution, indicates most of the values were concentrated around the mean, with some extreme values at the far end, as shown in the distribution fitting and box-plot in Figure 4.3.



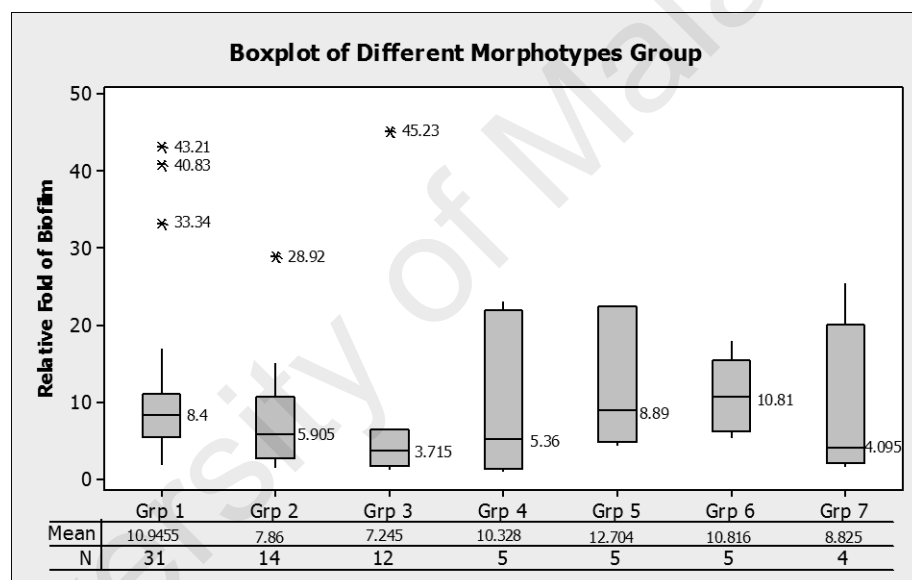
**Figure 4.3:** Relative biofilm formation with descriptive statistics for *B. pseudomallei* isolates.

#### 4.1.3. Determination of the association between colony morphotype groups and biofilm formation

Different colonial morphotype groups and correlation with biofilm formation had been analysed. Each and different morphotypes group had been individually analysed using descriptive statistics (Table 4.1) and box-plot (Figure 4.4) to present an overview of the data distribution among morphotypes group. All of the groups have positive skewness indicated that majority of the isolates were low biofilm formers. Group 4, 5 and 6 show negative kurtosis (platykurtic distribution), where the data distributions

were wider spread within own group's mean without extreme values. For the other groups (Group 1, 2, 3 & 7) positive kurtosis (leptokurtic distribution) was noted, where the data distributions were concentrated around the mean with some extreme values, especially for Group 1, 2 and 3 (Figure 4.3).

In order to investigate the correlation between different morphotypes group and biofilm formation, single factor ANOVA was employed to perform the analysis. As P-value resulted from the analysis was  $> 0.05$  (Table 4.2), no significant difference was noted in the biofilm formation of different morphotypes groups.



**Figure 4.4:** Boxplot analysis of biofilm formation of different morphotype groups of *B. pseudomallei* compared to that of *B. thailandensis* ATCC 700388. The star \* indicates outliers from each morphotype group. Values shown in the middle of the box plots were the median of the relative fold difference of the biofilms. The means of the relative fold difference of biofilms for each group is indicated below each plot.

**Table 4.1:** Descriptive statistics for biofilm formation of different morphotypes group

Variable	N	Mean	StDev	Variance	Minimum	Q1	Median	Q3	Maximum	Range	IQR	Skewness	Kurtosis
Grp1	31	10.95	10.25	105.1	1.96	5.38	8.4	11.08	43.21	41.25	5.7	2.24	4.67
Grp2	14	7.86	7.3	53.26	1.49	2.64	5.91	10.8	28.92	27.43	8.16	2.05	5.01
Grp3	12	7.25	12.13	147.15	1.4	1.78	3.72	6.53	45.23	43.83	4.75	3.3	11.15
Grp4	5	10.33	10.7	114.45	1.25	1.28	5.36	21.87	23.09	21.84	20.59	0.53	-3.05
Grp5	5	12.7	9.11	83	4.4	4.81	8.89	22.51	22.57	18.17	17.71	0.46	-3.17
Grp6	5	10.82	4.93	24.28	5.45	6.27	10.81	15.37	17.91	12.46	9.11	0.55	-0.43
Grp7	4	8.83	11.08	122.82	1.77	2.16	4.1	20.22	25.34	23.57	18.07	1.92	3.74

**Table 4.2:** ANOVA for biofilm formation of different groups

SUMMARY

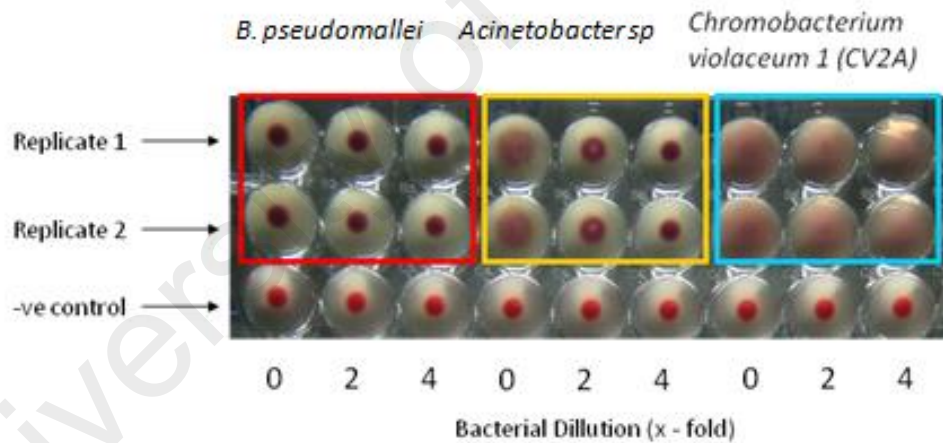
Groups	Count	Sum	Average	Variance
Column 1	31	339.31	10.94548	105.1027
Column 2	14	110.04	7.86	53.25728
Column 3	12	86.94	7.245	147.1487
Column 4	5	51.64	10.328	114.4505
Column 5	5	63.52	12.704	82.99638
Column 6	5	54.08	10.816	24.28318
Column 7	4	35.3	8.825	122.8218

## ANOVA

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	224.0192	6	37.33654	0.383398	0.887234	2.233171
Within Groups	6719.446	69	97.38328			
Total	6943.465	75				

#### 4.1.4. Hemagglutination assay using whole bacteria cells

*B. pseudomallei* K96243 (and another 25 clinical isolates, Appendix A: strain No 1-25), together with *Acinetobacter* spp (A9EMR) and *Chromobacterium violaceum* (CV2A) were assessed for hemagglutination. Figure 4.5 shows the results obtained from the hemagglutination assay for representative isolates of *B. pseudomallei* and the control strains. Strong hemagglutination was only observed for *C. violaceum*, while *Acinetobacter* spp demonstrated weak hemagglutination. All *B. pseudomallei* isolates did not agglutinate with the rabbit erythrocytes.



**Figure 4.5:** Hemagglutinations of whole bacterial cells. The negative control was performed by using PBS buffer alone.

#### **4.2. Identification of sugar binding proteins (lectins) in *B. pseudomallei* using bioinformatical approach.**

By using the keywords “lectins” and “K96243”, 7 hypothetical proteins from UNIPROT online database were retrieved (Table 4.3). Two known lectins from *P. aeruginosa* (PA-I, a galactophilic lectin and PA-III, a fucose-binding lectin) and *C. violaceum* (CV-III, a fucose-binding lectin) were also retrieved for cloning and expression study (Table 4.3).

The sequences were manually retrieved and subjected to blastn search for highly similar DNA sequences in the GenBank database. Based on the retrieved blast results, the highly similar sequences (results sequences with query coverage of more than 90 % and sequences identity of more than 80 %) were summarized in Table 4.4. BPSL1985 were also listed as it has been used as a potential marker to distinguish between *B. pseudomallei* and *B. thailandensis* (Wongtrakoongate *et al.*, 2007). The gene was utilized as a target region for developing multiplex PCR assay in the subsequent study.

#### **4.3. Use of hypothetical lectin genes for development of a multiplex PCR for rapid identification of *B. pseudomallei*, *B. thailandensis*, *B. mallei* and *B. cepacia* complex**

A multiplex PCR has been developed for discrimination of *B. pseudomallei*, *B. thailandensis*, *B. mallei* and *B. cepacia* complex. The assay was interpreted based on the absence or presence of specific amplicons on agarose gel: three fragments for *B. pseudomallei* (321 bp, 516 bp, 709 bp), two for *B. mallei* (516 bp and 709 bp) and one each for *B. thailandensis* (709 bp) and *B. cepacia* complex (560 bp), in addition to a 128 bp fragment amplified from the internal control plasmid (Figure 4.6).

**Table 4.3:** The search results from UniProt for lectin based proteins in the *B. pseudomallei* K96243 genome.

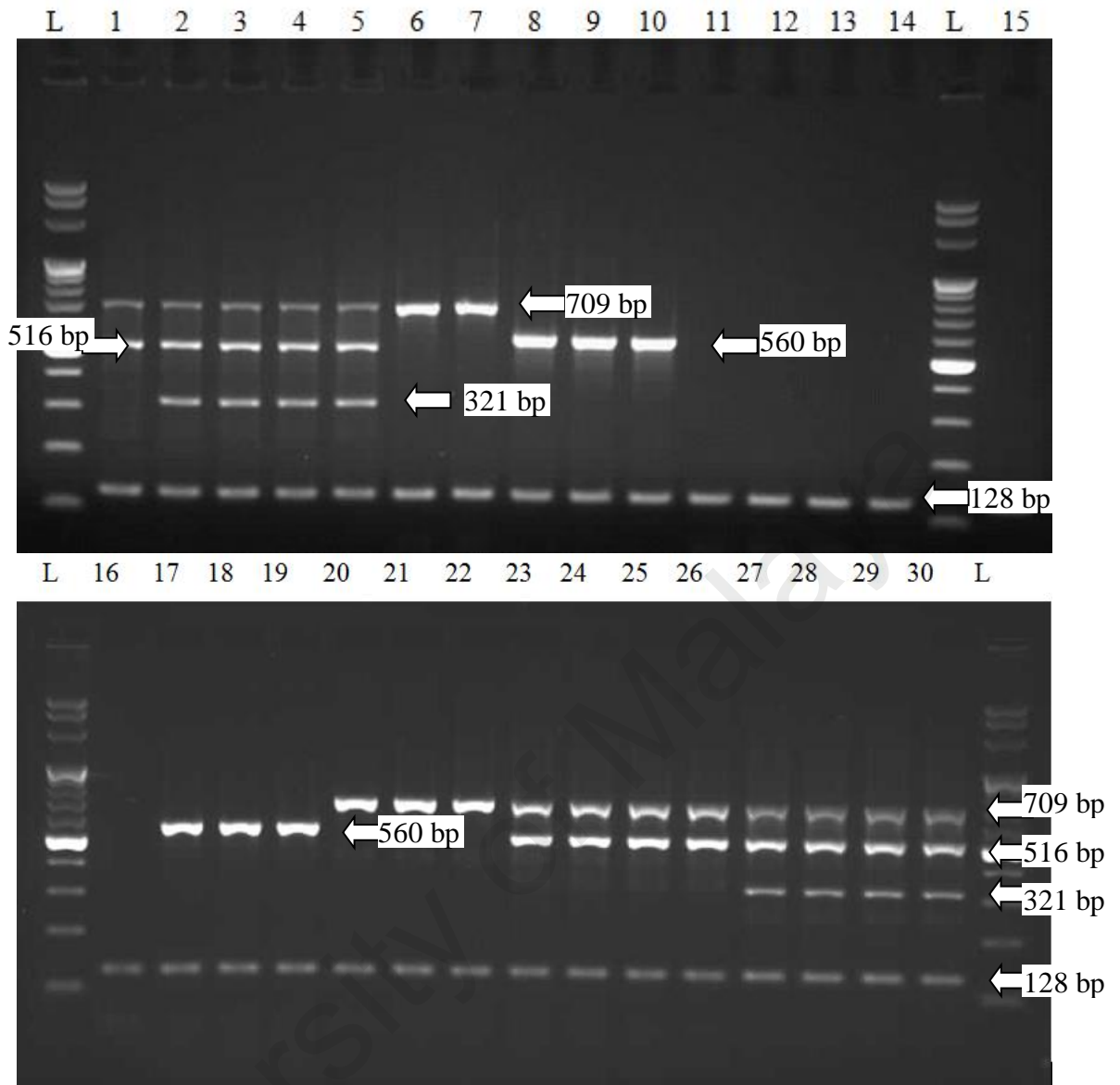
UniProt Accession	Protein names (in UniProt)	Gene names	Organism	Length of amino acid	Protein existence
Q63TB1	<b>Putative oxidase</b>	<i>BPSL2056</i>	<i>Burkholderia pseudomallei</i>	855	Predicted
Q63ME4	<b>Putative uncharacterized protein</b>	<i>BPSS0713</i>	<i>Burkholderia pseudomallei</i>	411	Predicted
Q63M93	<b>Putative uncharacterized protein</b>	<i>BPSS0767</i>	<i>Burkholderia pseudomallei</i>	107	Predicted
Q63L84	<b>Putative uncharacterized protein</b>	<i>BPSS1124</i>	<i>Burkholderia pseudomallei</i>	327	Predicted
Q63K77	<b>Putative exported protein</b>	<i>BPSS1488</i>	<i>Burkholderia pseudomallei</i>	278	Predicted
Q63JR7	<b>Putative sugar-binding protein</b>	<i>BPSS1649</i>	<i>Burkholderia pseudomallei</i>	806	Predicted
Q63IP7	<b>Putative outer membrane protein</b>	<i>BPSS2022</i>	<i>Burkholderia pseudomallei</i>	301	Predicted
Q7NX84	<b>Putative uncharacterized protein</b>	<i>CV_1744</i>	<i>Chromobacterium violaceum</i>	114	Proven (Zinger-Yosovich <i>et al.</i> , 2006)
Q9HYN5	<b>Fucose-binding lectin PA-III</b>	<i>lecB PA3361</i>	<i>Pseudomonas aeruginosa</i>	115	Proven (Gilboa-Garber, Katcoff, & Garber, 2000)
Q05097	<b>PA-I galactophilic lectin</b>	<i>lecA paII PA2570</i>	<i>Pseudomonas aeruginosa</i>	122	Proven (Gilboa-Garber & Sudakevitz, 1982)

**Table 4.4:** Molecular detection of various hypothetical lectin genes in *Burkholderia* species (based on BLAST results).

Hypothetical Protein	Highly similar sequences in respective species			
	<i>B. mallei</i>	<i>B. thailandensis</i>	<i>B. oklahomensis</i>	<i>B. cepacia complex</i>
<i>BPSL1958</i>	√	NA	NA	NA
<i>BPSL2056</i>	√	NA	NA	NA
<i>BPSS0713</i>	√	√	√	√
<i>BPSS0767</i>	√	√	√	NA
<i>BPSS1124</i>	NA	√	√	√
<i>BPSS1488</i>	√	√	√	NA
<i>BPSS1649</i>	√	√	√	NA
<i>BPSS2022</i>	NA	√	√	NA

NA, not available





**Figure 4.6:** Representative agarose gel electrophoretic analysis of amplified fragments generated from multiplex PCR assay. L, Perfect™ 100 bp DNA Ladder (E3134, Eurx, Poland); *B. mallei*, 1,23-26; *B. pseudomallei*, 2-5,27-30; *B. thailandensis*, 6-7,20-22; *B. cepacia* complex, 8-10, 17-19; *C. violaceum*, 11; *P. aeruginosa* ATCC 27853, 12, *S. aureus* ATCC 25923, 13; *Acinetobacter* spp., 14; *Salmonella typhi*, 16; sterile water (no template control), 15. The details of the strains assessed are shown in Table 4.5.

#### **4.3.1. Assessment of multiplex PCR assay for target and non-target organisms**

A panel of bacterial species (Table 4.5) was assessed for specificity of the multiplex PCR assay. The PCR assay was proven specific in identifying the target organisms. A representative agarose gel electrophoresis is shown in Figure 4.6. The minimum detection level of DNA in this assay for *B. pseudomallei* was 109 ng; 9 ng for *B. cepacia*; 60 ng for *B. mallei*; and 23 ng for *B. thailandensis*. This was determined by performing multiplex PCR using serial dilutions of known concentration genomic DNA template from the respective organisms (data not shown).

#### **4.3.2. Image analysis of the multiplex PCR profile**

The gel images were subjected to fragment analysis using PyElph software (Figure 4.7). All the fragments were detected based on the pixel intensity on the image, which was translated in a distinct digital graph for computation. Clustering analysis was performed and different bacterial species were shown as different group in the dendrogram (Figure 4.8) based on the fragments obtained from the multiplex PCR assay.

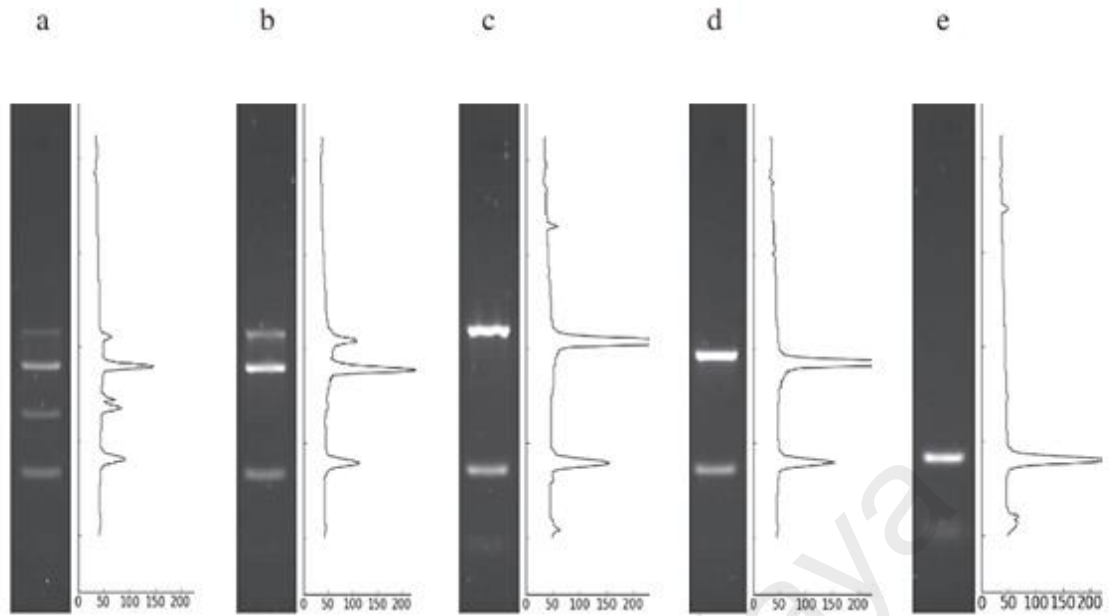
#### **4.3.3. Safety assessment of alcohol inactivation sample preparation method**

In a pilot study, *B. pseudomallei* was not able to be cultured from 20 alcohol-inactivated samples on LB plates (data not shown). The results showed that *B. pseudomallei* isolates were unable to be revived after inactivation by 70 % ethanol.

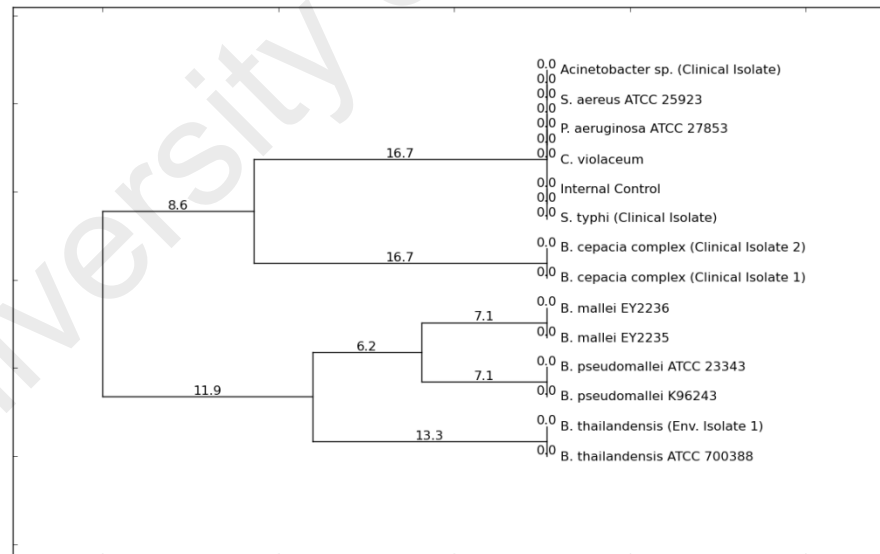
**Table 4.5:** Assessment of multiplex PCR assay for target and non-target organisms

Organism	Source	No. isolate	No. positive	Success rate
<u>Burkholderia spp.</u>				
<i>B. pseudomallei</i> (n=46)	Clinical	34	34	100 %
	Animal	4	4	(45/45)
	Environmental	5	5	
	ATCC 23343	1	1	
	NCTC 13178	1	1	
	K96243	1	1	
<i>B. mallei</i> (n=4) *	Horse (EY100)	1	1	100 %
	Horse (EY2235)	1	1	(4/4)
	Horse (EY2236)	1	1	
	Horse (EY2237)	1	1	
<i>B. thailandensis</i> (n=6)	ATCC 700388	1	1	100 %
	Environmental	5	5	(6/6)
<i>B. cepacia</i> (n=22)	Clinical	19	19	100 %
	Environmental	3	3	(22/22)
<u>Non-Burkholderia spp.</u>				
<i>Acinetobacter spp.</i>	Clinical	10	0	Not amplified
<i>Aeromonas hydrophila</i>	Clinical	2	0	(No False
<i>Alcaligenes</i>	Clinical	1	0	Positive
<i>Klebsiella pneumoniae</i>	Clinical	1	0	Detected)
<i>Chromobacterium</i>	Clinical	1	0	
<i>Chromobacterium</i>	Environmental	6	0	
<i>Ochrobactrum anthropi</i>	Clinical	2	0	
<i>Proteus mirabilis</i>	Clinical	2	0	
<i>Ralstonia pickettii</i>	Clinical	1	0	
<i>Salmonella enteritidis</i>	Clinical	1	0	
<i>Salmonella typhi</i>	Clinical	1	0	
<i>Stenotrophomonas</i>	Clinical	2	0	
<i>Pseudomonas</i>	Clinical	1	0	
<i>Pseudomonas</i>	ATCC 27853	1	0	
<i>Pseudomonas stutzeri</i>	Clinical	1	0	
MRSA	Clinical	1	0	
<i>Staphylococcus aureus</i>	ATCC 25923	1	0	
<i>E. coli</i>	ATCC 25922	1	0	
<i>E. coli</i>	JM107	1	0	

\* EY, Eiko Yabuuchi; Department of Bacteriology, Osaka City University Medical School, Osaka, Japan (Tanpiboonsak *et al.* 2004)



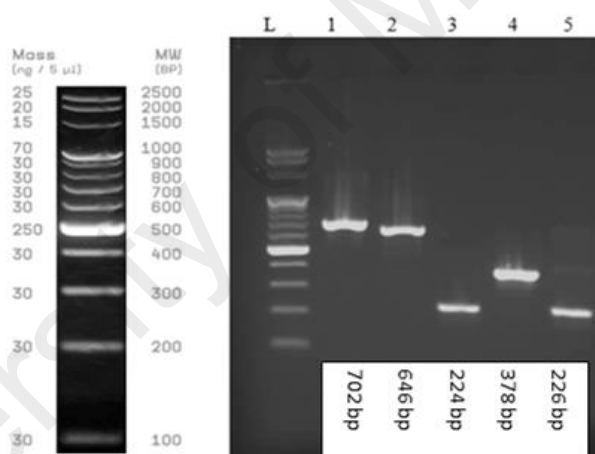
**Figure 4.7:** Gel image analysis using PyElph software. a: *B. pseudomallei*; b: *B. mallei*; c: *B. thailandensis*; d: *B. cepacia* complex; e: sterile water (negative control, showing amplicon from internal amplification control)



**Figure 4.8:** Clustering analysis of the bacterial species based on the fragments obtained from agarose gel electrophoresis using PyElph software. All the non-targeted species were clustered along with the internal control, showing negative results.

#### 4.4. Molecular probing of lectin genes in *B. pseudomallei* isolates

While confirmation of *BPSS1649* and *BPSS2022* was performed using multiplex PCR assay (section 4.3.1), the confirmation of the presence of hypothetical lectin genes obtained from UNIPROT database were carried out by using single-plex PCR assays. The genes i.e., *BPSL2056*, *BPSS0713*, *BPSS0767*, *BPSS1124* and *BPSS1488* were amplified using newly designed primers to generate amplicons of less than 1000 bp (Table 3.2). All the hypothetical lectin genes were confirmed to be present in all the *B. pseudomallei* isolates. Figure 4.9 shows an agarose gel image of the single-plex PCR assay used for detection *BPSL2056*, *BPSS0713*, *BPSS1488*, *BPSS1124* and *BPSS0767* from the *B. pseudomallei* K96243 reference strain.



**Figure 4.9:** Representative gel showing the presence of hypothetical sugar binding protein genes in *B. pseudomallei* K96243 reference strain. L: Ladder, 1: *BPSL2056*; 2: *BPSS0713*; 3: *BPSS1488*; 4: *BPSS1124*; 5: *BPSS0767*.

#### 4.5. Cloning and expressions of hypothetical lectin proteins

Seven *B. pseudomallei* hypothetical lectin genes were cloned into the expression vector pET-46EK/LIC and their predicted protein molecular weights are shown in Table 4.6.

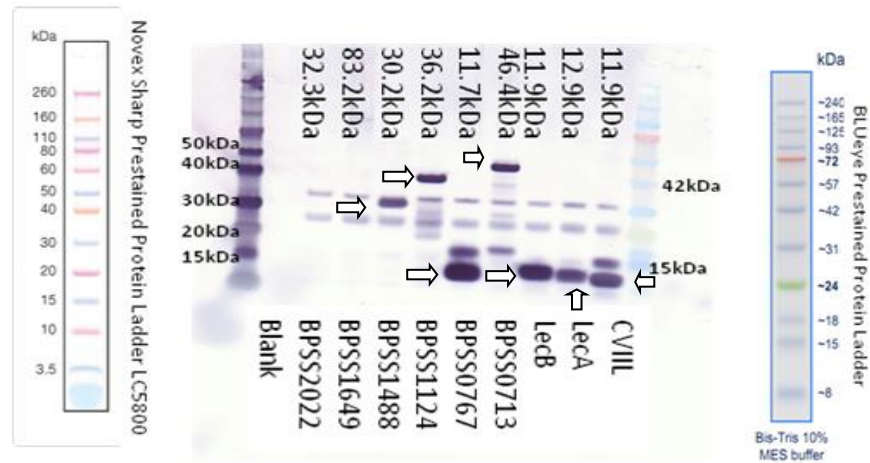
**Table 4.6:** Details of the hypothetical proteins expressed in this study

Hypothetical protein						
No.	UniProt Accession	Gene names	Organism	Predicted Molecular Weight (dalton)	Amino Acid Length	Protein Successfully Expressed (Yes / No)
1	Q63TB1	<i>BPSL2056</i>	<i>B. pseudomallei</i>	88,867	855	No
2	Q63ME4	<i>BPSS0713</i>	<i>B. pseudomallei</i>	46,443	411	Yes
3	Q63M93	<i>BPSS0767</i>	<i>B. pseudomallei</i>	11,689	107	Yes
4	Q63L84	<i>BPSS1124</i>	<i>B. pseudomallei</i>	36,249	327	Yes
5	Q63K77	<i>BPSS1488</i>	<i>B. pseudomallei</i>	30,182	278	Yes
6	Q63JR7	<i>BPSS1649</i>	<i>B. pseudomallei</i>	83,187	806	No
7	Q63IP7	<i>BPSS2022</i>	<i>B. pseudomallei</i>	32,343	301	No

Control protein						
No.	UniProt Accession	Gene names	Organism	Predicted Molecular Weight (dalton)	Amino Acid Length	Protein Successfully Expressed (Yes / No)
1	Q7NX84	<i>CV-III (CV_1744)</i>	<i>Chromobacterium violaceum</i>	11,972	114	Yes
2	Q9HYN5	<i>lecB</i>	<i>Pseudomonas aeruginosa</i>	11,863	115	Yes
3	Q05097	<i>lecA</i>	<i>Pseudomonas aeruginosa</i>	12,893	122	Yes

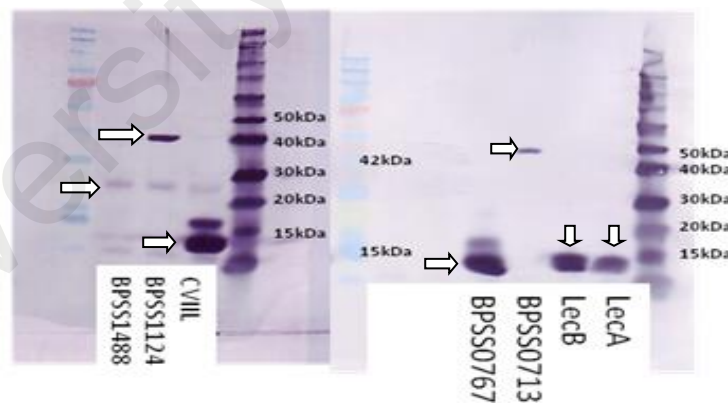
Six hypothetical proteins (*BPSS0713*, *BPSS0767*, *BPSS1124*, *BPSS1488*, *BPSS1649*, and *BPSS2022*) were successfully cloned and sequence verified, however; only 4 (*BPSS0713*, *BPSS0767*, *BPSS1124*, and *BPSS1488*) were expressed with the correct molecular weights (Table 4.6). Confirmation of the expressed protein using crude protein samples were detected by Western blot analysis using HisDetection which detects 6x his-tag region (Figure 4.10). The recombinant proteins from *C. violaceum* (*CV-III*) and *P. aeruginosa* (*LecA* and *LecB*) were also successfully cloned and expressed. Although the recombinant proteins were successfully detected, a proper validation can be done using a MALDI-TOF MS/MS approach or peptide sequencing for further confirmation.



**Figure 4.10:** Western blot showing the expression of crude recombinant proteins.

Background bands were obvious prior to column purification. The arrows show the position of the recombinant proteins.

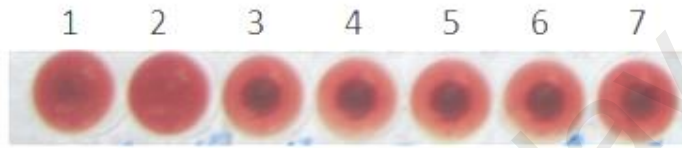
Column purification was performed to partially purify the recombinant proteins for removal of background proteins; however reduction in the protein yield for the expressed protein was observed (Figure 4.11).



**Figure 4.11:** Western blot showing the purified recombinant proteins. The background of each protein was significantly reduced after purification with Protino® Ni-TED 1000 Packed Columns. However, protein loss was observed for *BPSS1488*. The arrows show the position of the recombinant proteins.

#### 4.6. Hemagglutination assay using recombinant proteins

The four expressed and purified recombinant proteins (*BPSS0713*, *BPSS0767*, *BPSS1124*, and *BPSS1488*) were assessed for hemagglutination activity. Only two recombinant proteins from *P. aeruginosa* (*LecA* and *LecB*) showed hemagglutination activity. None of the recombinant proteins from *B. pseudomallei* showed any hemagglutination activity (Figure 4.12).



**Figure 4.12:** Hemagglutination of rabbit erythrocytes with *B. pseudomallei* recombinant proteins: 1) *LecA*, 2) *LecB*, 3) *BPSS0713*, 4) *BPSS0767*, 5) *BPSS1124*, 6) *BPSS1488*, 7) Blank (negative control). Only the recombinant proteins from *P. aeruginosa*, *LecA* and *LecB* showed hemagglutination activity.



## CHAPTER 5: DISCUSSION

### 5.1. Colonial morphotype, biofilm forming ability and hemagglutination of *B. pseudomallei* clinical isolates

Based on the growth morphology of 76 *B. pseudomallei* clinical isolates on BPSA, a colony morphotyping scheme was reported in this study. According to Howard & Inglis (2003), culturing of *B. pseudomallei* on BPSA agar provides more benefits than Ashdown's agar (ASA) medium. *B. pseudomallei* colonies grew faster in BPSA compare with ASA medium. Hence, this allows faster detection of *B. pseudomallei* from primary cultures. Besides, some mucoidal strains (as shown by Group 5 organism in this study) of *B. pseudomallei* were not inhibited by BPSA, in contrary to those reported by Howard & Inglis (2003) on ASA medium, hence; BPSA improves the recovery of *B. pseudomallei* isolates. Based on the above reasons, BPSA was chosen as the selective agar medium for colony morphotyping in this study.

A total of seven distinct colonial morphotypes (4 with mixed texture, 3 with uniform texture) of *B. pseudomallei* were identified on BPSA medium in this study. Variations in the colonial morphology of *B. pseudomallei* often pose difficulties to the untrained eye in the clinical diagnostic laboratory. As these morphotypes may be mistaken as mixed cultures, this may lead to unnecessary diagnostics workup and tests and results in delay in reporting. Additionally, the phenotypic plasticity of *B. pseudomallei* has important implications for treatment and vaccine development of melioidosis (Chantratita *et al.*, 2007). The development of a colony morphotyping scheme such as the one described in this study maybe the first step towards understanding the phenotypic switching of *B. pseudomallei* in response to changing environmental factors.

Attempts to correlate *B. pseudomallei* morphotypes with virulence in mice have been described in Chantratita *et al.* (2007), and the study was able to identify a *B.*

*pseudomallei* morphotype which favored enhanced survival and persistence of the bacterium. The finding in this study shows that *B. pseudomallei* isolates varied in their biofilm forming abilities (Figure 4.2), and thus, in agreement with the study by Taweechaisupapong *et al.* (2005). However, the attempt to correlate biofilm formation amongst the different morphotypes did not show any significant difference ( $p > 0.05$ ).

While most of the isolates were low biofilm producers, 9 isolates (labelled as KTHYM, RBYEM, TEAWG, MTHMY, AZUFT, HSM01, MUYWM, OTHSA & MUYW2) were extremely high biofilm producers, exhibiting 22.45 to 45.23 fold differences as compared to the reference strain (Figure 4.2). It is interesting to note that the extremely high biofilm producers did not dominate any of the colonial morphotypes, implying that biofilm formation of *B. pseudomallei* might not have any correlation with colonial morphotypes.

Attempts to correlate *B. pseudomallei* morphotypes with the virulence in mice have been described in two previous studies. Survival and persistence of different *B. pseudomallei* morphotypes have been assessed by Chantratita *et al.* (2007), and the authors suggested that changes in environmental conditions would cause *B. pseudomallei* to switch morphotypes reversibly. *B. pseudomallei* strains that tends to switch morphotype were found to be more invasive, had higher survival rate and more persistent (Chantratita *et al.*, 2007). In another study, Chen *et al.* (2009) reported that the survival of mice was affected by different bacterial colonial morphotypes, but the pathogenesis mechanism involved in different morphotypes was not clear. Hence, additional studies are needed to identify the internal and external factors which contribute to the high and low biofilm formation of *B. pseudomallei*.

None of the isolates in this study demonstrated agglutination with rabbit erythrocytes. (Figure 4.5). This could be due to the low level or absence of the lectins

on the cell surface of *B. pseudomallei*. Additionally, the expression of the lectin could be suppressed by some unknown reasons (for instance, culture condition, requirement for certain cofactors, etc.) which are yet to be explored.

## **5.2. Identification of lectin in *B. pseudomallei* using bioinformatic approach**

To verify whether lectin gene is present in *B. pseudomallei*, this study has performed a search in the UniProt database to identify the relevant hypothetical genes. Using keywords “K96243” (to indicate *B. pseudomallei* K96243 reference strain) and “lectin”, seven genes annotated as “putative lectin genes” were retrieved (Table 4.3). All the 7 hypothetical lectins were annotated as “Protein predicted”, and they were defined as protein sequence entries without evidence at protein (Mass spectrometry, X-ray crystal structure or NMR structure), transcript [cDNA(s)], or at homology (protein orthologs) levels.

The hypothetical genes were used for primer design and amplified for cloning and expression of recombinant proteins. The recombinant proteins were subsequently used to assess for lectin activity using hemagglutination assay. Additionally, three known lectin genes (1 lectin from *Chromobacterium violaceum*, 2 lectins from *Pseudomonas aeruginosa*) retrieved from the GenBank database were cloned and expressed as controls for hemagglutination assay (Table 4.3).

To investigate the similarities of the hypothetical lectin genes with other bacterial species, the hypothetical lectin gene sequences were searched for similar sequences in the NCBI database (Table 4.4). All the hypothetical lectin genes were uniquely conserved and similar to at least one or multiple *Burkholderia* species with more than 90 % query coverage and more than 80 % identities (Appendix G).

None of the hypothetical genes have any similarity with the known lectin gene sequences of *P. aeruginosa*, *C. violaceum* or *B. cenocepacia* through blast analysis, suggesting that *B. pseudomallei* is probably having different type of lectins or sugar binding proteins.

### **5.3. Development of a multiplex PCR assay for identification of species closely related with *B. pseudomallei* based on genes encoding hypothetical lectin**

*B. pseudomallei*, *B. mallei*, *B. thailandensis* and *B. cepacia* complex are closely related Gram-negative bacteria which are difficult to be differentiated morphologically. In clinical diagnostics setting, a rapid identification and highly discriminative assay is always useful to assist physicians to make precise and accurate treatment decision for patients. Molecular identification methods are always preferred in the medical diagnostic laboratories due to their rapidity, accuracy and specificity. In this study, a multiplex PCR assay was developed for rapid identification of species closely related with *B. pseudomallei* based on the nucleotide sequences of hypothetical lectin genes. Using the multiplex PCR, identification of *B. pseudomallei* can be accomplished within 2 hours starting from template preparation to the interpretation of results.

For preparation of DNA template for PCR, nucleic acid isolation and purification from bacterial cultures are usually performed using commercial available kits or manual extraction methods. Such DNA purification procedures will take approximately 1 to 2 hours even for a well-trained laboratory technologist. In order to shorten the time for nucleic acid purification, boiling method is widely used in many diagnostics laboratories for extraction of bacterial DNA. However, the method has a known risk of causing “tube popping”, due to the pressure built inside the microcentrifuge tubes. When samples are boiled at high temperature, a small amount of the sample will be

expelled to the environment through the aerosol generated from heating. While *B. pseudomallei* is known to be transmitted via inhalation through aerosol, such method is not recommended for use in a diagnostic laboratory.

In this study, a safe sample preparation method (referred as alcohol inactivation method) was introduced in section 3.3.3.3. An overnight grown bacterial colony suspended in sterile water was found to be sufficient to provide the genomic material for the PCR assay. In this method, a final volume of 80% ethanol was added to a bacterial cell suspension of 250 µl to inactivate the bacteria. The suspension was then centrifuged to harvest the cells. The pellet was air-dried, resuspended in distilled water, and used as a template for amplification. In an evaluation study to assess the safety aspect of the method, bacteria was not cultured from the pellets prepared from the alcohol inactivation method (data not shown), indicating that the alcohol inactivation method was safe. The method is rapid and able to shorten the time required for DNA extraction.

Additionally, comparison was made between boiling method and alcohol inactivation method. Figure AF.2 (Appendix F) describes the comparison between boiling method and alcohol inactivation method. There are no differences between both methods in terms of sensitivity. While both methods enable rapid sample preparation as compared with DNA purification using commercial kit, alcohol inactivation method is having advantage of being safer and more rapid.

The multiplex PCR assay developed in this study is easy to interpret based on the presence of amplified products on agarose gel: three fragments for *B. pseudomallei* (321 bp, 516 bp, 709 bp), two for *B. mallei* (516 bp and 709 bp) and one each for *B. thailandensis* (709 bp), *B. cepacia* complex (560 bp), on top of the band generated from the internal control plasmid (Figure 4.6). The result of the gel image analysis using PyElph software revealed the absence of nonspecific products (Figure 4.7). Validation

using 115 isolates consisting of 19 different bacterial species showed that the assay was specific (100%). The assay was able to detect up to 109 ng, 9 ng, 60 ng and 23 ng of the DNA *B. pseudomallei*, *B. cepacia*, *B. mallei* and *B. thailandensis*, respectively, and thus more than sufficient to detect DNA from a bacterial colony.

The assay has also included an internal amplification control which was constructed based on the cDNA gene sequence of an *Aspergillus niger* strain. This is important to rule out PCR inhibitory substances, and to eliminate false-negative results.

In the past, two multiplex PCR assays have been developed for identification of *burkholderial* species. The interpretation of the multiplex PCR assay by Lee *et al.* (2005) could be difficult as the results were interpreted based on highly polymorphic bacterial repetitive elements. The multiplex PCR assay by Ho *et al.* (2011) was able to differentiate *B. pseudomallei* from *B. thailandensis* and *B. cepacia* complex, but not on *B. mallei*. In addition, these two assays did not include internal amplification controls. As such, false-negative results can be generated due to the presence of PCR inhibitory substances in the samples.

As there is yet any diagnostics assay which is able to discriminate four *burkholderial* species simultaneously, the multiplex PCR assay developed in this study is a promising tool to facilitate rapid diagnosis of melioidosis and infections caused by other *burkholderial* species in the endemic regions.

#### **5.4. Determination hemagglutination/lectin activity of recombinant proteins**

Lectin activity can be determined based on conventional hemagglutination assay using animal or human erythrocytes. The initial screening of *B. pseudomallei* using whole bacterial cells did not exhibit any agglutination with rabbit red blood cells, as

opposed to the reported *P. aeruginosa* (Gilboa-Garber, 1982) and *C. violaceum* (Zinger-Yosovich *et al.*, 2006). The finding suggests that lectin is not present in *B. pseudomallei* or it is expressed in a very low amount, and hence, undetectable using conventional hemagglutination assay. In fact, very few bacterial lectins have been identified to date. The function of a lectin in *P. aeruginosa* is for host attachment and biofilm initiation. However, bacteria may also develop various other strategies for host colonization. For example, adhesin and pilin are proteins which mediate host colonization process for Gram-positive and Gram-negative bacteria (Telford *et al.*, 2006). It is possible that other strategies are more prevailing in *B. pseudomallei* for host attachment and biofilm initiation.

Since hypothetical lectin genes have been annotated in the *B. pseudomallei* genome, an attempt was made in this study to express the hypothetical lectin genes as recombinant proteins for assessment of lectin activity. To rule out potential technical errors, two lectins (LecA and LecB) from *P. aeruginosa*, and a lectin (CV-IIL) from *C. violaceum* were used as positive controls in this assay. Out of the 7 hypothetical proteins, only 4 *B. pseudomallei* proteins were successfully expressed and purified. The inefficacy of cloning and expression is likely due to GC-rich domains. *B. pseudomallei* is a GC-rich organism (Holden *et al.*, 2004). GC-rich domains will tend to form secondary structure, making the DNA less amenable to amplification by serving as pause or termination sites (McDowell, Burns, & Parkes, 1998). Furthermore, secondary structure is also known to affect protein expression (Bernstein *et al.*, 2007). However, none of the recombinant proteins demonstrated any lectin activity (as indicated by negative hemagglutination assay), while the control lectins from *P. aeruginosa* and *C. violaceum* showed hemagglutination (Figure 4.12). Some of the possible reasons for the absence of lectin activity in these recombinant proteins are as stated below:

- 1) Bacterial proteins are known to undergo endogenous post-translation modification (PTM) in host-pathogen interactions (Cain *et al.*, 2014); however during expression of the recombinant proteins in *E. coli*, the PTM that governs and determine protein structure, localization and specific activity (Wani *et al.*, 2015) might not occur correctly in order to activate the protein functionality. Some of the PTM process such as phosphorylation, acetylation and methylation required for lectin activity might be absent when the recombinant protein was produced in a non-native host.
- 2) Lost of lectin activity during sample preparation and protein purification process. As the recombinant protein was prepared through a sonication process, the heat generated during sonication might cause protein degradation and thus, reducing or destroying lectin activity (Ho *et al.*, 2008; Chisti & Moo-Young, 1986). Additionally, some of the buffers and chemical used in the protein purification (due to pH and salt concentration) might have also caused the lectin to lose its function.
- 3) Lectin may require some co-factors, including metal ions, or complexes with other protein monomers to exhibit carbohydrate-binding activity (Etzler *et al.*, 2009). These co-factors especially metal ions will affect carbohydrate binding activity.
- 4) The hypothetical lectin genes obtained from UniProtKB/TrEMBL database are based on unreviewed (uncurated and scientific conclusion) computationally generated annotation. As such, the hypothetical lectin genes of *B. pseudomallei* are in reality non-functional.

As there are many factors that can influence the expression of bacterial lectin activity, use of more sophisticated approach such as glycan array, isothermal titration calorimetry (ITC) or surface plasmon resonance (SPR) will be helpful for future investigation (Kletter *et al.*, 2009; Lameignere *et al.*, 2008).



## CHAPTER 6: CONCLUSIONS AND FUTURE PROSPECTS

This study demonstrated various mixed colonial morphology and different biofilm forming abilities of *B. pseudomallei* clinical isolates. No correlation was observed between colony morphology and biofilm forming abilities. Seven genes encoding hypothetical lectin (sugar binding protein) were retrieved from the genome sequence of *B. pseudomallei* K96243 reference strain. By inclusion of primers targeting 16Sr RNA gene and two hypothetical lectin genes (*BPSS2022* and *BPSS1649*), a multiplex PCR assay has been developed and evaluated for rapid differentiation of *B. pseudomallei*, *B. mallei*, *B. thailandensis* and *B. cepacia* complex. The seven hypothetical lectin genes were cloned, expressed and assessed for lectin activity using a conventional hemagglutination assay. Four hypothetical proteins were successfully expressed, however, none of the recombinant proteins showed hemagglutination activity. Thus, the lectin activity of these genes was not exhibited for their hypothetical functions as annotated in the UniProt database.

Further study is necessary to investigate phenotypic switching of *B. pseudomallei* in response to changing environmental factors, as well as to identify internal and external factors which contribute to the high and low biofilm formation of *B. pseudomallei*. It will be also worthwhile to understand the molecular genetics and biochemical pathway(s) involved in the biofilm formation. The information derived will provide insights on the survival and environmental adaptation of *B. pseudomallei*, and for development of more effective drug or vaccines for melioidosis. The use of glycan array, isothermal titration calorimetry (ITC) or surface plasmon resonance (SPR) will be helpful for future investigation of lectins in *B. pseudomallei*.

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## LIST OF PUBLICATIONS AND PAPERS PRESENTED

### PUBLICATIONS

Koh, S. F., Tay, S. T., Sermswan, R., Wongratanacheewin, S., Chua, K. H., & Puthucheary, S. D. (2012). Development of a multiplex PCR assay for rapid identification of *Burkholderia pseudomallei*, *Burkholderia thailandensis*, *Burkholderia mallei* and *Burkholderia cepacia* complex. *Journal of Microbiological Methods*, 90(3), 305-308.

Koh, S. F., Tay, S. T., & Puthucheary, S. D. (2013). Colonial morphotypes and biofilm forming ability of *Burkholderia pseudomallei*. *Tropical Biomedicine*, 30(3), 428-433.

### PATENT

Identification and differentiation of *Burkholderia pseudomallei* from other closely related gram negative bacteria.

Puthucheary, S. D., Tay, S. T., Koh, S. F., & Chua, K. H. (2010). *Malaysia patent No. PI2010004547*. Malaysia: The Patent Registration Office

## POSTER PRESENTATIONS

Koh, S. F., Tay, S. T., & Puthucheary, S. D. Biofilm Production by clinical isolates of *Burkholderia pseudomallei*. 18<sup>th</sup> Malaysian Society for Molecular Biology and Biotechnology (MSMBB) Scientific Meeting, The Saujana Kuala Lumpur, Malaysia, 18-20 August, 2009.

Koh, S. F., Tay, S. T., & Puthucheary, S. D. Morphotypes of *Burkholderia pseudomallei* on selective (BPSA) agar and possible correlation with biofilm production. 6<sup>th</sup> World Melioidosis Congress, Jupiter's, Townsville, Australia, 1st-3rd December, 2010.

Koh, S. F., Tay, S. T., & Puthucheary, S. D. Cloning, expression and hemagglutininations of putative sugar binding proteins (lectin) of *Burkholderia pseudomallei*. 7<sup>th</sup> World Melioidosis Congress, , Bangkok, Thailand, 18<sup>th</sup> -20<sup>th</sup> September 2013.