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

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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 biofilm telah baru-baru ini dikaitkan dengan kevirulenan bakteria ini. Walaupun lektin (protin pengikatan gula) telah dilaporkan penting untuk pembentukan biofilm beberapa bakteria Gram-negatif termasuk Pseudomonas aeruginosa dan Burkholderia cenocepacia, tiada kajian mengenai lektin B. pseudomallei dilaporkan. Kajian ini menyiasat pembentukan biofilm untuk 76 isolat klinikal B. pseudomallei dengan menggunakan kaedah pewarnaan kristal ungu. Keputusan yang diperolehi dihubungkaitkan dengan morfotip koloni masing-masing pada Burkholderia pseudomallei “selective agar”. Oleh kerana lektin telah dilaporkan berperanan untuk memulakan pembentukan biofilm 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 spesis 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 biofilm tinggi (X> 11.01), manakala 37 (48.7%) adalah penghasil biofilm sederhana (3.45 <X <11.01) dan 19 (25.0%) adalah pengeluar biofilm rendah (X <3.45) berbanding dengan strain rujukan B. thailandensis ATCC 700388. Walau bagaimanapun, tidak ada korelasi antara morfotip B. pseudomallei dengan kebolehan pembentukan biofilm (p> 0.05).
ACKNOWLEDGEMENTS

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

DMMB  1,9-dimethyl methylene blue

DMSO  dimethyl sulfoxide

DNA  deoxyribonucleic acid

dH₂O  sterile distilled water

dNTP  deoxyribonucleoside triphosphate

EDTA  ethylenediamine tetraacetic acid

e.g.  for example

EMBL-EBI  European Molecular Biology Laboratory – European Bioinformatics Institute

*et al.*  et alia

FDA  Fluorescein-di-acetate

*g*  gram

*hsdSB*  host specific determinant *Salmonella typhimurium*

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

LecA  Gene encoding *Pseudomonas aeruginosa* lectin I

LecB  Gene encoding *Pseudomonas aeruginosa* 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

OmpT  outer membrane protease

ORF  open reading frame

PA-IL  *Pseudomonas aeruginosa* lectin I gene

PA-IIL  *Pseudomonas aeruginosa* lectin II gene

PBS  Phosphate Buffered Saline

PCR  polymerase chain reaction

pg  picogram

PIA  polysaccharide adhesin

PVDF  Polyvinylidene fluoride

rpm  Revolutions per minute

RSL  *Ralstonia solanacearum* lectin gene

s  seconds

SDS-PAGE  Sodium dodecyl sulphate – polyacrylamide gel electrophoresis

SOC  Super Optimal broth with Catabolite repression

Taq  *Thermus aquaticus*

TBE  TRIS-borate-EDTA
<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>Tris</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>U</td>
<td>unit</td>
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<tr>
<td>UniParc</td>
<td>UniProt Archive</td>
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<tr>
<td>UniProt</td>
<td>The Universal Protein Resource Database</td>
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<td>UniProtKB</td>
<td>UniProt Knowledgebase</td>
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<tr>
<td>UniRef</td>
<td>UniProt Reference Clusters</td>
</tr>
<tr>
<td>UPI</td>
<td>unique identifier</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
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<tr>
<td>V</td>
<td>volt</td>
</tr>
<tr>
<td>(v/v)</td>
<td>volume per volume</td>
</tr>
<tr>
<td>(w/v)</td>
<td>weight per volume</td>
</tr>
<tr>
<td>XTT</td>
<td>2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazodium-5-Carboxanilide</td>
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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 (Ngauy *et al.*, 2005). A prolonged period of dormancy may occur between exposure to *B. pseudomallei* and clinical manifestation of infection (Puthucheary, 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 (Puthucheary, 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 melioidiosis 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 & Puthucheary, 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 (Puthucheary, 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 (Puthucheary, 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 20th 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 (Puthucheary, 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 (Puthucheary &
Due to the wide array of clinical signs and symptoms, *B. pseudomallei* is also called “the great mimicker” (Puthucheary, 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 (Puthucheary, 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 (Puthucheary & Vadivelu, 2002). Once it is inside the human host, the bacteria can stay dormant for many years. According to review by Puthucheary (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 & Puthucheary, 2005); iii) slow and inefficient formation of phagolysosome which enables *B. pseudomallei* to overcome host immune response (Puthucheary & 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 (Puthucheary, 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.

<table>
<thead>
<tr>
<th>Target</th>
<th>Method &amp; References</th>
<th>Target organism</th>
<th>Results of evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>23S rDNA</td>
<td>PCR and hybridization (Lew and Desmarchelier, 1994)</td>
<td>Detect <em>B. pseudomallei</em> and <em>B. mallei</em></td>
<td>Not able to differentiate <em>B. pseudomallei</em> and <em>B. mallei</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Produced non-specific products with some <em>B. cepacia</em> strains (Brook et al., 1997)</td>
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<td></td>
<td></td>
<td></td>
<td>Failed to detect environmental strains, low sensitivity for detection of clinical isolates (Haase et al., 1998)</td>
</tr>
<tr>
<td>16S rDNA</td>
<td>Nested PCR (Dharakul et al., 1996)</td>
<td>Detect <em>B. pseudomallei</em></td>
<td>Not ideal for clinical application due to possible carry over contamination (Rattanathongkom et al., 1997)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>False positive in patients (Haase et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>PCR (Brook et al., 1997)</td>
<td>Identify <em>B. pseudomallei</em></td>
<td>Low specificity and sensitivity (Kunakorn et al., 2000)</td>
</tr>
<tr>
<td>Unidentified</td>
<td>PCR (Ratanathongkom et al., 1997)</td>
<td>Detect <em>B. pseudomallei</em> in blood</td>
<td>Higher sensitivity compared to those reported by Lew and Desmarchelier (1994)</td>
</tr>
<tr>
<td>gene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unidentified</td>
<td>PCR and probe hybridization (Sura et al., 1997)</td>
<td>Detect <em>B. pseudomallei</em></td>
<td>Not evaluated for clinical diagnostic use (Sura et al., 1997)</td>
</tr>
<tr>
<td>gene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23S rDNA</td>
<td>PCR (Bauernfeind et al., 1998)</td>
<td>Detect <em>B. mallei</em> and discriminate <em>B. pseudomallei</em></td>
<td>Not evaluated for clinical diagnostic use</td>
</tr>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>rpsU, fliC</td>
<td>PCR (Hagen et al., 2002)</td>
<td>Detect <em>B. pseudomallei</em></td>
<td>Requires sequencing for differentiation of *B. pseudomallei, B. mallei, B. thailandensis</td>
</tr>
<tr>
<td>Flagellin</td>
<td>Duplex PCR (Sonthayanon et al., 2002), modified from Wajananugarn et al. (1999)</td>
<td>Detect and differentiate <em>B. pseudomallei</em> and <em>B. thailandensis</em></td>
<td><em>B. mallei</em> was not included, not evaluated for clinical diagnostic use.</td>
</tr>
<tr>
<td>Flagellin</td>
<td>PCR-RFLP (Sprague et al., 2002)</td>
<td>Differentiate <em>B. mallei</em> and <em>B. pseudomallei</em></td>
<td>Only for detection of *B. thailandensis, B. mallei, B. pseudomallei but not for differentiation of <em>B. pseudomallei</em> and <em>B. mallei</em> (Sprague et al., 2002; Tanpiboonsak et al., 2004)</td>
</tr>
<tr>
<td>Flagellin (fliC)</td>
<td>PCR-RFLP (Sprague et al., 2002)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Technique</td>
<td>Method</td>
<td>Application</td>
<td>Notes</td>
</tr>
<tr>
<td>-----------</td>
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</tr>
<tr>
<td>16S rDNA PCR</td>
<td>(Gee et al., 2003)</td>
<td>Differentiate B. pseudomallei and B. mallei</td>
<td>Requires sequence comparison. Not evaluated for routine clinical diagnostic use.</td>
</tr>
<tr>
<td>TTS1 PCR</td>
<td>(Smith-Vaughan et al., 2003)</td>
<td>Diagnostic and identify B. pseudomallei</td>
<td>Good specificity, low sensitivity (Gal et al., 2005)</td>
</tr>
<tr>
<td>Unidentified gene PCR-RFLP</td>
<td>(Tangiboonsak et al., 2004)</td>
<td>Differentiation of B. mallei and B. pseudomallei</td>
<td>Not performed on B. thailandensis.</td>
</tr>
<tr>
<td>TTS1, TTS2 Real-time PCR</td>
<td>(Thibault et al., 2004)</td>
<td>Identify and discriminate B. pseudomallei, B. mallei and B. thailandensis</td>
<td>Not evaluated for routine clinical diagnostic use.</td>
</tr>
<tr>
<td>Repetitive DNA element Multiplex PCR</td>
<td>(Lee et al., 2005)</td>
<td>Detect and differentiate B. pseudomallei, B. mallei and B. thailandensis</td>
<td>Useful in epidemiology study and strain typing only for B. mallei and B. pseudomallei. Not evaluated for routine clinical diagnostic use.</td>
</tr>
<tr>
<td>16S rDNA, rpsU, fliC Real-time PCR</td>
<td>(Tomaso et al., 2005)</td>
<td>Detect B. mallei and B. pseudomallei</td>
<td>Low sensitivity with clinical samples (Chantratita et al. 2007). Unable to differentiate B. mallei and B. pseudomallei</td>
</tr>
<tr>
<td>SNP Real-time PCR</td>
<td>(U'Ren et al., 2005)</td>
<td>Detect and discriminate B. mallei and B. pseudomallei</td>
<td>B. thailandensis not included. Clinical evaluation is still pending.</td>
</tr>
<tr>
<td>lpxO, phaC PCR (with sequencing) and Real-time PCR</td>
<td>(Merritt et al., 2006)</td>
<td>Identify B. pseudomallei</td>
<td>High specificity, results could be obtained in 2 hours and 30 minutes (Merritt et al. 2006).</td>
</tr>
<tr>
<td>orf2 of TTS1 Real-time PCR</td>
<td>(Novak et al., 2006)</td>
<td>Identify B. pseudomallei</td>
<td>Rapid and high specificity. Results could be obtained in 3 hours (Novak et al., 2006) 91% sensitivity for detection of confirmed melioidosis cases (Meumann et al., 2006).</td>
</tr>
<tr>
<td>Flagellin P gene (fliP) Real-time PCR</td>
<td>(Tomaso et al., 2006)</td>
<td>Identify B. mallei</td>
<td>Only for specific detection of B. mallei.</td>
</tr>
<tr>
<td>Burkholderia intracellular motility A gene (bimAma) Real-time PCR</td>
<td>(Ulrich et al., 2006a; Ulrich et al. 2006b)</td>
<td>Identify B. mallei</td>
<td>Only for specific detection of B. mallei.</td>
</tr>
<tr>
<td>Metalloprotease Gene (mprA) PCR</td>
<td>(Neubauer et al., 2007)</td>
<td>Identify B. pseudomallei</td>
<td>Only for specific detection of B. pseudomallei</td>
</tr>
<tr>
<td>Hypothetical protein: <strong>BPSS1187</strong> (Assay 8653) &amp; <strong>BPSS2089</strong> (Assay 9438)</td>
<td>Real-time PCR (Supaprom et al., 2007)</td>
<td>Identify <strong>B. pseudomallei</strong></td>
<td>71% sensitivity and 82% specificity for Assay 8653; 54% sensitivity and 88% specificity for Assay 9438</td>
</tr>
<tr>
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</tr>
<tr>
<td><strong>TTS1</strong> gene clusters</td>
<td>Loop-mediated isothermal amplification (LAMP) (Chantratita et al. 2008)</td>
<td>Detection and identification of <strong>B. pseudomallei</strong></td>
<td>Does not need thermal cycler however the assay is of low sensitivity and not suitable for clinical diagnostic use. (Chantratita et al. 2008)</td>
</tr>
<tr>
<td><strong>BipD</strong> (BPSS1529), <strong>BopE</strong> (BPSS1525), putative oxidoreductase (BPSL2748)</td>
<td>Aptamers (Gnanam et al., 2008)</td>
<td><strong>Diagnostic B. pseudomallei</strong></td>
<td>Not evaluated or application in clinical settings</td>
</tr>
<tr>
<td><strong>lpxO</strong></td>
<td>PCR (Inglis et al., 2008)</td>
<td>Identify <strong>B. pseudomallei</strong></td>
<td>Requires Agilent Bioanalyzer due to low molecular weight range, laboratory chip is not reusable and requires 12 samples per run.</td>
</tr>
<tr>
<td>Multiple locus variable number of tandem repeat analysis (MLVA)</td>
<td>PCR (Michelle Wong Su et al., 2009)</td>
<td><strong>Typing of B. pseudomallei</strong></td>
<td>Not applicable in routine clinical diagnostic.</td>
</tr>
<tr>
<td>Various gene targets</td>
<td>Microarray (Schmooick et al., 2009)</td>
<td>Identify 12 <strong>Burkholderia</strong> species.</td>
<td>Requires highly purified genomic DNA. Not evaluated for routine clinical diagnostic use.</td>
</tr>
</tbody>
</table>

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 (Puthucheary, 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 mimic the *in vivo* situation to assess the infectivity and tissue damage on human cells, however, such assessment 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 19th 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 (Gabius 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).

<table>
<thead>
<tr>
<th>Methods</th>
<th>Mode of action</th>
<th>Benefits</th>
<th>Limitation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Staining Assays</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crystal violet (CV) assay</td>
<td>Quantitation of crystal violet stained extracellular polymeric substances</td>
<td>Amount of dye is proportional to biofilm sizes</td>
<td>Low reproducibility with high standard deviation value</td>
</tr>
<tr>
<td>(Stepanović <em>et al.</em>, 2000)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1,9-dimethyl ethylene blue (DMMB) assay (Toté <em>et al.</em>, 2008)</td>
<td>Quantitation of DMMB bound onto intracellular polysaccharide adhesin (PIA)</td>
<td>PIA is considered a logical target to be detected</td>
<td>Limited to few bacterial species possessing PIA-related biofilm matrix</td>
</tr>
<tr>
<td><strong>Fluorescein-di-acetate (FDA) assay</strong> (Tawakoli <em>et al.</em>, 2013)</td>
<td>Detection of the presence of fluorescein through hydrolyses of FDA</td>
<td>Inexpensive and easy to perform</td>
<td>Requires fluorescence microplate reader. Not suitable for matured biofilm due to thickness of biofilm and limited field of view</td>
</tr>
<tr>
<td><strong>Live/Dead BacLight Assay</strong></td>
<td>Two nucleic acid binding stains, for fluorescence microscopy examination of live and dead bacteria populations</td>
<td>Observation of live and dead cells</td>
<td>Requirement of observation of statically significant portion to represent total population</td>
</tr>
<tr>
<td>Metabolic Assays</td>
<td>Resazurin assay (Punithavathy et al., 2012)</td>
<td>Reduction of Resazurin into pink-fluorescent resorufin by cellular metabolic activity to quantify viable cells in biofilm</td>
<td>Detect and quantify the actual viable number of microorganism</td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------------------------------------</td>
<td>-----------------------------------------------------------------------------------------------------------------</td>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>XTT assay (Adam et al., 2002)</td>
<td>Reduction of XTT to water soluble formazan by metabolic activity to enumerate viable cells in biofilm</td>
<td>Absorbance reading of metabolic reduction of XTT to deduce number of viable bacteria in biofilm</td>
<td>Different metabolism gradient due to complexity and heterogeneity of biofilm structure and composition</td>
</tr>
<tr>
<td>BioTimer assay (BTA) (Pantanella et al., 2008)</td>
<td>Switching of colour from red to yellow due to fermentative metabolism, switching rate is dependent on initial bacterial concentration</td>
<td>Low cost and easily perform to count living bacteria in biofilm</td>
<td>Difficult in applying BioTimer assay for evaluation of multispecies biofilm</td>
</tr>
</tbody>
</table>

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-IIL*), *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, Tickel 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-IIL (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-IIL*) 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), 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* (*hsdSB*) (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).
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$_{540}$ 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 µl 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 µl of water and the wells were air dried. The crystal violet stain was eluted by adding 200 µl 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$_{595}$ 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 µl of 2 % erythrocytes and 50 µl 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., “lectin” 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

<table>
<thead>
<tr>
<th>Organism</th>
<th>Source</th>
<th>No. isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Burkholderia spp.</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. pseudomallei</em> (n=46)</td>
<td>Clinical</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Animal</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Environmental</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>ATCC 23343</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>NCTC 13178</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>K96243</td>
<td>1</td>
</tr>
<tr>
<td><em>B. mallei</em> (n=4)</td>
<td>EY100</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>EY2235</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>EY2236</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>EY2237</td>
<td>1</td>
</tr>
<tr>
<td><em>B. thailandensis</em> (n=6)</td>
<td>ATCC 700388</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Environmental</td>
<td>5</td>
</tr>
<tr>
<td><em>B. cepacia</em> (n=22)</td>
<td>Clinical</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>Environmental</td>
<td>3</td>
</tr>
</tbody>
</table>

Non-*Burkholderia* spp. (n=37)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Source</th>
<th>No. isolate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acinetobacter spp.</td>
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</tr>
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</tr>
<tr>
<td>Alcaligenes xylosoxidans</td>
<td>Clinical</td>
<td>1</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>Clinical</td>
<td>1</td>
</tr>
<tr>
<td>Chromobacterium violaceum</td>
<td>Clinical</td>
<td>1</td>
</tr>
<tr>
<td>Chromobacterium violaceum</td>
<td>Environmental</td>
<td>6</td>
</tr>
<tr>
<td>Ochrobactrum anthropi</td>
<td>Clinical</td>
<td>2</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>Clinical</td>
<td>2</td>
</tr>
<tr>
<td><em>Ralsonia pickettii</em></td>
<td>Clinical</td>
<td>1</td>
</tr>
<tr>
<td>Salmonella enteritidis</td>
<td>Clinical</td>
<td>1</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>Clinical</td>
<td>1</td>
</tr>
<tr>
<td>Stenotrophomonas maltophilia</td>
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<td>2</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
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<td>Pseudomonas aeruginosa</td>
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<tr>
<td>Pseudomonas stutzeri</td>
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</tr>
<tr>
<td><em>MRSA</em></td>
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</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
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</tr>
<tr>
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<td><em>E. coli</em></td>
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</tbody>
</table>

* EY, Eiko Yabuuchi; Department of Bacteriology, Osaka City University Medical School, Osaka, Japan (Tanpipoonsak 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 23343 (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.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Single-plex PCR Sequences</th>
<th>Target</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>2056F_702</td>
<td>cggtactggcagttcgtat</td>
<td>BPSL2056</td>
<td>702bp</td>
</tr>
<tr>
<td>2056R_702</td>
<td>aegagccacatggtgattgc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1124F_378</td>
<td>gtcacgaacctcgaattacgg</td>
<td>BPSS1124</td>
<td>378bp</td>
</tr>
<tr>
<td>1124R_378</td>
<td>gacgattccctcaggttgc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1488F_224</td>
<td>aattggcagcgatttcaac</td>
<td>BPSS1488</td>
<td>224bp</td>
</tr>
<tr>
<td>1488R_224</td>
<td>tcatggtcgatctcagaaa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0713F_646</td>
<td>ctgatctgcagcagacatct</td>
<td>BPSS0713</td>
<td>646bp</td>
</tr>
<tr>
<td>0713R_646</td>
<td>tgaacttgccgttgtattcg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0767F_226</td>
<td>tcaaaaattctgctgtagcc</td>
<td>BPSS0767</td>
<td>226bp</td>
</tr>
<tr>
<td>0767R_226</td>
<td>gttgacgctgaagtctgctt</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Multiplex PCR Sequences</th>
<th>Target</th>
<th>Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>71F</td>
<td>agctcgcagatgaactggat</td>
<td>BPSS1649</td>
<td>709bp</td>
</tr>
<tr>
<td>71R</td>
<td>gcgtatcgtgtgctgta</td>
<td></td>
<td></td>
</tr>
<tr>
<td>32F</td>
<td>tctgtctacgcttttca</td>
<td>BPSS2022</td>
<td>321bp</td>
</tr>
<tr>
<td>32R</td>
<td>gggcctttttaaccgctctc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>51F</td>
<td>cccaatcagacgacagctatt</td>
<td>BPSSL1958</td>
<td>516bp</td>
</tr>
<tr>
<td>51R</td>
<td>gttcaaccgcccccttttgt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bc16F</td>
<td>tccttgctcttaacagcctgg</td>
<td>16s RNA (Bcc)</td>
<td>560bp</td>
</tr>
<tr>
<td>Bc16R</td>
<td>tcagcaggattcgcaccat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>xynB128F</td>
<td>agtgcgcaggacatcactta</td>
<td>xynB</td>
<td>128bp</td>
</tr>
<tr>
<td>xynB128R</td>
<td>ggtgttagtcgctgtaaga</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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-β-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 MgCl₂, 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,
An open source gel image analyzer, PyElph (Pavel & Vasile, 2012) was used to perform gel analysis, clustering analysis and to generate dendogram.

### 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 µl containing 5 µl 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 µM 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, BPSS12056, BPSS1124, BPSS0713, and BPSS1488*) and 3 known bacterial lectins, i.e., two from *P. aeruginosa* (*LecA, LecB*) and one from *C. violaceum* (*CV-IIL*) 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.

<table>
<thead>
<tr>
<th>Gene (UniProt accession no.)</th>
<th>Name</th>
<th>Sequence</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPSL2056 (Q63TB1)</td>
<td>ADP-BPSL2056F</td>
<td>GACGACGACAAGATGCCGACGGCAGCGGAT</td>
<td>2568</td>
</tr>
<tr>
<td></td>
<td>ADP-BPSL2056R</td>
<td>GAGGAGAAGGCCGGTCAGGGCCAGTCAGGGAT</td>
<td></td>
</tr>
<tr>
<td>BPS50713 (Q63ME4)</td>
<td>ADP-BPS50713F</td>
<td>GACGACGACAAGATGCCGACGGCAGCGGAT</td>
<td>1236</td>
</tr>
<tr>
<td></td>
<td>ADP-BPS50713R</td>
<td>GAGGAGAAGGCCGGTCAGGGCCAGTCAGGGAT</td>
<td></td>
</tr>
<tr>
<td>BPS50767 (Q63M93)</td>
<td>A2DP-BPS50767F</td>
<td>GACGACGACAAGATGCCGACGGCAGCGGAT</td>
<td>324</td>
</tr>
<tr>
<td></td>
<td>A2DP-BPS50767R</td>
<td>GAGGAGAAGGCCGGTCAGGGCCAGTCAGGGAT</td>
<td></td>
</tr>
<tr>
<td>BPS51124 (Q63L84)</td>
<td>ADP-BPS51124F</td>
<td>GACGACGACAAGATGCCGACGGCAGCGGAT</td>
<td>984</td>
</tr>
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<td></td>
<td>ADP-BPS51124R</td>
<td>GAGGAGAAGGCCGGTCAGGGCCAGTCAGGGAT</td>
<td></td>
</tr>
<tr>
<td>BPS51488 (Q63K77)</td>
<td>ADP-BPS51488F</td>
<td>GACGACGACAAGATGCCGACGGCAGCGGAT</td>
<td>837</td>
</tr>
<tr>
<td></td>
<td>ADP-BPS51488R</td>
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</tr>
<tr>
<td>BPS51649 (Q63JR7)</td>
<td>A2DP-BPS51649F</td>
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<td>A2DP-BPS51649R</td>
<td>GACGACGACAAGATGCCGACGGCAGCGGAT</td>
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</tr>
<tr>
<td>BPS52022 (Q63IP7)</td>
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<td></td>
<td>ADP-BPS52022R</td>
<td>GACGACGACAAGATGCCGACGGCAGCGGAT</td>
<td></td>
</tr>
<tr>
<td>CV-IIL CV2L345</td>
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<td>GACGACGACAAGATGCCGACGGCAGCGGAT</td>
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<tr>
<td>LecA PA</td>
<td>PALECA361-F</td>
<td>GACGACGACAAGATGCCGACGGCAGCGGAT</td>
<td>366</td>
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<tr>
<td>LecB PA</td>
<td>PALECB348-F</td>
<td>GACGACGACAAGATGCCGACGGCAGCGGAT</td>
<td>345</td>
</tr>
</tbody>
</table>

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 MgSO₄, 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.
3.4.1.4. **Ligation independent cloning (LIC)**

Directional cloning of PCR-generated fragments into vector pET-46EK/LIC (Novagen, USA) was done by using ligation independent cloning (LIC) approach according to the manufacturer’s recommendation (Figure 3.5). The compatible overhang of the PCR product was generated by treating the PCR product with T4 polymerase.

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**Figure 3.5**: Graphical illustration on recombinant protein cloning strategy using pET-46 Ek/LIC expression vector. Adapted from manufacturer provided manual (Novagen Ek/LIC cloning kits) and vector map (pET-46 Ek/LIC).
The reaction mixture (in a total volume of 20 µl) was composed of 5 µl of purified PCR products, 2 µl of 10X T4 polymerase buffer, 2 µl of 25 mM dATP, 1 µl of 100 mM DTT, 9.6 µl of nuclease free water and 0.4 µl of 2.5 U/µl 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 µl of the prepared insert was mixed with 1 µl of the vector pET-46EK/LIC and incubated at 22°C for 5 minutes, followed by adding 1 µl of 25 mM EDTA and further incubate for 5 minutes at 22°C. The ligated vector was then transformed into a cloning host, NovaBlue Giga Singles™ Competent Cells (Novagen, USA) and plated on LB agar containing 50 µg/ml carbenicillin for positive colony selection. The selected positive colony was propagated in Lysogeny Broth (LB) containing 50 µg/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 (OD₆₀₀) 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 MgCl₂ 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 CaCl₂ 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 $\mu$g/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$_{600}$) 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
$\mu$g/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$_2$PO$_4$, 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 $\mu$m 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 µl of 2 % erythrocytes and 50 µl of bacteria whole cell (standardized at McFarland turbidity of No. 2) or expressed recombinant protein (standardized at 15 µg/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 \textit{B. pseudomallei}

4.1.1. Determination of the colony morphotypes of \textit{B. pseudomallei}

Seven different groups or colonial morphotypes of \textit{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 \textit{B. pseudomallei}

In order to compare the biofilm formation between \textit{B. pseudomallei} isolates, the relative fold differences of \textit{B. pseudomallei} when compared to \textit{B. thailandensis} ATCC 700388 was determined. \textit{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 \textit{B. pseudomallei} isolates based on the relative fold differences. \textit{P. aeruginosa} ATCC 27853 demonstrated 7.06 times higher OD$_{540}$ reading than \textit{B. thailandensis} ATCC 700388.
<table>
<thead>
<tr>
<th>Group</th>
<th>Image</th>
<th>Description; <em>No. of isolates</em> (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><img src="image1" alt="Image" /></td>
<td>Central rough surface with radiating wrinkling up to edge; <strong>31</strong> (40.8)</td>
</tr>
<tr>
<td>2</td>
<td><img src="image2" alt="Image" /></td>
<td>Mixture of rough, wrinkled and smooth surface with irregular edge; <strong>14</strong> (18.4)</td>
</tr>
<tr>
<td>3</td>
<td><img src="image3" alt="Image" /></td>
<td>Central rough surface with smooth circumference and edge; <strong>12</strong> (15.8)</td>
</tr>
<tr>
<td>4</td>
<td><img src="image4" alt="Image" /></td>
<td>Wrinkled central area with smooth circumference and edge; <strong>5</strong> (6.6)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>Image</th>
<th>Description; <em>No. of isolates</em> (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td><img src="image5" alt="Image" /></td>
<td>Convex, mucoid, with smooth colony surface; <strong>5</strong> (6.6)</td>
</tr>
<tr>
<td>6</td>
<td><img src="image6" alt="Image" /></td>
<td>Rough texture of entire colony with irregular edges; <strong>5</strong> (6.6)</td>
</tr>
<tr>
<td>7</td>
<td><img src="image7" alt="Image" /></td>
<td>Wrinkled surface of entire colony; <strong>4</strong> (5.2)</td>
</tr>
</tbody>
</table>

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

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
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

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<thead>
<tr>
<th>Variable</th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>Variance</th>
<th>Minimum</th>
<th>Q1</th>
<th>Median</th>
<th>Q3</th>
<th>Maximum</th>
<th>Range</th>
<th>IQR</th>
<th>Skewness</th>
<th>Kurtosis</th>
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<td>10.95</td>
<td>10.25</td>
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<td>8.4</td>
<td>11.08</td>
<td>43.21</td>
<td>41.25</td>
<td>5.7</td>
<td>2.24</td>
<td>4.67</td>
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<tr>
<td>Grp2</td>
<td>14</td>
<td>7.86</td>
<td>7.3</td>
<td>53.26</td>
<td>1.49</td>
<td>2.64</td>
<td>5.91</td>
<td>10.8</td>
<td>28.92</td>
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<td>8.16</td>
<td>2.05</td>
<td>5.01</td>
</tr>
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<td>5.36</td>
<td>21.87</td>
<td>23.09</td>
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<td>Grp5</td>
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<td>9.11</td>
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<td>4.4</td>
<td>4.81</td>
<td>8.89</td>
<td>22.51</td>
<td>22.57</td>
<td>18.17</td>
<td>17.71</td>
<td>0.46</td>
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<td>Grp6</td>
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<td>10.82</td>
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<td>15.37</td>
<td>17.91</td>
<td>12.46</td>
<td>9.11</td>
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<td>-0.43</td>
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<td>4.1</td>
<td>20.22</td>
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<td>23.57</td>
<td>18.07</td>
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<td>3.74</td>
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Table 4.2: ANOVA for biofilm formation of different groups

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<th>Average</th>
<th>Variance</th>
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<td>10.94548</td>
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<td>Column 3</td>
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<td>7.245</td>
<td>147.1487</td>
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<td>51.64</td>
<td>10.328</td>
<td>114.4505</td>
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<td>Column 5</td>
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<td>63.52</td>
<td>12.704</td>
<td>82.99638</td>
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<td>5</td>
<td>54.08</td>
<td>10.816</td>
<td>24.28318</td>
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<td>Column 7</td>
<td>4</td>
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<td>8.825</td>
<td>122.8218</td>
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</table>

### ANOVA

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<tr>
<th>Source of Variation</th>
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<th>df</th>
<th>MS</th>
<th>F</th>
<th>P-value</th>
<th>F crit</th>
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<td>37.33654</td>
<td>0.383398</td>
<td>0.887234</td>
<td>2.233171</td>
</tr>
<tr>
<td>Within Groups</td>
<td>6719.446</td>
<td>69</td>
<td>97.38328</td>
<td></td>
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<tr>
<td>Total</td>
<td>6943.465</td>
<td>75</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
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-II*, a fucose-binding lectin) and *C. violaceum* (*CV-II*, 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* (Wongtrakoon 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.

<table>
<thead>
<tr>
<th>UniProt Accession</th>
<th>Protein names (in UniProt)</th>
<th>Gene names</th>
<th>Organism</th>
<th>Length of amino acid</th>
<th>Protein existence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q63TB1</td>
<td>Putative oxidase</td>
<td>BPSL2056</td>
<td><em>Burkholderia pseudomallei</em></td>
<td>855</td>
<td>Predicted</td>
</tr>
<tr>
<td>Q63ME4</td>
<td>Putative uncharacterized protein</td>
<td>BPSS0713</td>
<td><em>Burkholderia pseudomallei</em></td>
<td>411</td>
<td>Predicted</td>
</tr>
<tr>
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<td>Q63L84</td>
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<td>BPSS1488</td>
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</tr>
<tr>
<td>Q63JR7</td>
<td>Putative sugar-binding protein</td>
<td>BPSS1649</td>
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<td>806</td>
<td>Predicted</td>
</tr>
<tr>
<td>Q63IP7</td>
<td>Putative outer membrane protein</td>
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<td><em>Burkholderia pseudomallei</em></td>
<td>301</td>
<td>Predicted</td>
</tr>
<tr>
<td>Q7NX84</td>
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<td>CV_1744</td>
<td><em>Chromobacterium violaceum</em></td>
<td>114</td>
<td>Proven (Zinger-Yosovich <em>et al.</em>, 2006)</td>
</tr>
<tr>
<td>Q9HYN5</td>
<td>Fucose-binding lectin PA-IIIL</td>
<td><em>lecB</em> PA3361</td>
<td><em>Pseudomonas aeruginosa</em></td>
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<td>Proven (Gilboa-Garber, Katcoff, &amp; Garber, 2000)</td>
</tr>
<tr>
<td>Q05097</td>
<td>PA-I galactophilic lectin</td>
<td><em>leca paI</em>L</td>
<td><em>Pseudomonas aeruginosa</em></td>
<td>122</td>
<td>Proven (Gilboa-Garber &amp; Sudakevitz, 1982)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Hypothetical Protein</th>
<th><em>B. mallei</em></th>
<th><em>B. thailandensis</em></th>
<th><em>B. oklahomensis</em></th>
<th><em>B. cepacia complex</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>BPSSL1958</td>
<td>√</td>
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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

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

* 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.](image)

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

<table>
<thead>
<tr>
<th>Hypothetical protein</th>
<th>Organism</th>
<th>Predicted Molecular Weight (dalton)</th>
<th>Amino Acid Length</th>
<th>Protein Successfully Expressed (Yes / No)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>UniProt Accession</td>
<td>Gene names</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Q63TB1</td>
<td>BPSSL2056</td>
<td>B. pseudomallei</td>
<td>88,867</td>
</tr>
<tr>
<td>2</td>
<td>Q63ME4</td>
<td>BPSS0713</td>
<td>B. pseudomallei</td>
<td>46,443</td>
</tr>
<tr>
<td>3</td>
<td>Q63M93</td>
<td>BPSS0767</td>
<td>B. pseudomallei</td>
<td>11,689</td>
</tr>
<tr>
<td>4</td>
<td>Q63L84</td>
<td>BPSS1124</td>
<td>B. pseudomallei</td>
<td>36,249</td>
</tr>
<tr>
<td>5</td>
<td>Q63K77</td>
<td>BPSS1488</td>
<td>B. pseudomallei</td>
<td>30,182</td>
</tr>
<tr>
<td>6</td>
<td>Q63JR7</td>
<td>BPSS1649</td>
<td>B. pseudomallei</td>
<td>83,187</td>
</tr>
<tr>
<td>7</td>
<td>Q63IP7</td>
<td>BPSS2022</td>
<td>B. pseudomallei</td>
<td>32,343</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control protein</th>
<th>Organism</th>
<th>Predicted Molecular Weight (dalton)</th>
<th>Amino Acid Length</th>
<th>Protein Successfully Expressed (Yes / No)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>UniProt Accession</td>
<td>Gene names</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Q7NX84</td>
<td>CV-IIL (CV_1744)</td>
<td>Chromobacterium violaceum</td>
<td>11,972</td>
</tr>
<tr>
<td>2</td>
<td>Q9HYN5</td>
<td>lecB</td>
<td>Pseudomonas aeruginosa</td>
<td>11,863</td>
</tr>
<tr>
<td>3</td>
<td>Q05097</td>
<td>lecA</td>
<td>Pseudomonas aeruginosa</td>
<td>12,893</td>
</tr>
</tbody>
</table>

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-IIL) 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.
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 of 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*. 
REFERENCES


Burkholderia pseudomallei and diagnosis of melioidosis. *Journal of Clinical Microbiology, 46*(2), 568-573.


LIST OF PUBLICATIONS AND PAPERS PRESENTED

PUBLICATIONS


PATENT

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

POSTER PRESENTATIONS


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