# CLINICAL AND MOLECULAR CHARACTERIZATION OF *Burkholderia pseudomallei* ISOLATED FROM PATIENTS IN HOSPITAL SULTANAH NURZAHIRAH (HSNZ) AND TREATMENT OUTCOME

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## ABBREVIATION

| | |
|---|---|
| AMC | Amoxycillin-clavulanic acid |
| BHI | Brain heart infusion |
| DM | Diabetes Mellitus |
| DNA | Deoxyribonucleic Acid |
| HSNZ | Hospital Sultanah Nur Zahirah |
| HUSM | Hospital Universiti Sains Malaysia |
| IgA | Immunoglobulin A |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| IMR | Institute of Medical Research, Malaysia |
| IU/ml | International units per ml |
| MIC | Minimum inhibitory concentration |
| Min | Minute |
| MLST | Multilocus sequence typing |
| MLEE | Multilocus enzyme electrophoresis |
| ng | Nanogram |
| No | Number |

| | |
|---|---|
| OR | Odds ratio |
| PFGE | Pulsed-field gel electrophoresis |
| PCR | Polymerase chain reaction |
| pg | Picogram |
| RAPD | Random amplified polymorphic DNA |
| Rpm | Resolution per minute |
| TTS1 | Type III secretion system 1 |
| TMP-SMX | Trimethoprim-sulfamethoxazole |
| TBE | Tris borate EDTA |
| UPGMA | Unweighted Pair Group Method with Arithmetic Average |
| µM | Micromolar |
| µl | Microliter |

## ABSTRACT

## CLINICAL AND MOLECULAR CHARACTERIZATION OF *Burkholderia pseudomallei* ISOLATED FROM PATIENTS IN HOSPITAL SULTANAH NURZAHIRAH (HSNZ) AND TREATMENT OUTCOME

### Introduction

Melioidosis is a potentially fatal disease caused by environmental saprophyte, *Burkholderia pseudomallei*. It is commonly found in soil and surface water in endemic regions of Southeast Asia and a common cause of community acquired sepsis and pneumonia in the east coast of Peninsular Malaysia.

### Methodology

This is a prospective cohort study conducted to evaluate the demographic data, clinical features and treatment outcome of culture-proven melioidosis at Hospital Sultanah Nurzahirah, Terengganu from October 2016 till June 2017. Molecular confirmation of *B. pseudomallei* was performed using a PCR-based assay targeting *sctQ* gene of the TTS1 cluster. Twenty isolates were then subsequently genotyped using DNA sequencing of multiple gene loci to determine the genetic relatedness of the strains and to correlate the identified genotypes with clinical presentations and outcomes. The mortality rates of patients who receive early and appropriate empirical anti-melioidosis therapy was compared to those who did not receive appropriate empirical therapy. A total of 52 cases were included in the study. Patients' clinical data were obtained from electronic clinical notes.

**Results**

Ninety six percent of melioidosis patients in this study had at least one underlying illness with the most common being diabetes mellitus. Bacteremic melioidosis was seen in most patients (n=48, 92.3%). Majority of them had bacteremic localized infection (57.7%), followed by disseminated bacteremia (26.9 %) and bacteremia with no focal lesion (7.7%). The remaining four patients (7.7%) had non bacteremic localized infection. Community acquired pneumonia and fever of unknown origin were the common presentation on admission. Lung was the most common organ involved (61.5%), followed by liver (15.4%), spleen, central nervous system and soft tissue (9.6%), joint and urinary tract (3.8%). All the culture-proven isolates were successfully confirmed with PCR. Constructed phylogenetic tree from sequencing of multiple gene loci revealed genetic diversity among *B. pseudomallei* strains, and there is no clustering seen with the clinical presentation or outcome. The overall mortality rate was 53.9% (n=28/52) and all patients who died had bacteremic melioidosis. Only 15 out of 52 patients (28.9%) were prescribed appropriate empirical antibiotics, with corresponding mortality of 26.7%. The mortality was significantly higher (64.9%) in those who did not receive appropriate empirical antibiotic therapy.

**Conclusion**

The clinical manifestations of melioidosis in Terengganu state were diverse and comparable to other countries worldwide. *B. pseudomallei* genotypes showed genetic diversity indicating distribution of different strains in the environment. The overall mortality rate is high, particularly in those who did not receive early and appropriate empirical antibiotics. Thus, a high index of suspicion in endemic country and the early use of appropriate empirical antibiotic therapy is crucial to prevent death.

## ABSTRAK

## CIRI-CIRI KLINIKAL DAN MOLEKULAR *Burkholderia pseudomallei* YANG DIPENCILKAN DARIPADA PESAKIT DI HOSPITAL SULTANAH NURZAHIRAH (HSNZ) DAN HASIL RAWATAN

**Pengenalan**

Melioidosis adalah penyakit berpotensi maut yang disebabkan oleh saprofit alam sekitar, *Burkholderia pseudomallei*. Ia biasanya ditemui di dalam tanah dan permukaan air di kawasan endemik di Asia Tenggara dan merupakan punca utama yang menyebabkan penyakit sepsis dan radang paru-paru di Pantai Timur Semenanjung Malaysia.

**Tatacara**

Ini adalah kajian kohort prospektif yang dijalankan untuk mengkaji data demografi, ciri-ciri klinikal dan hasil rawatan penyakit melioidosis yang terbukti melalui kultur di Hospital Sultanah Nurzahirah, Terengganu dari Oktober 2016 sehingga Jun 2017. Pengesahan molekular *B. pseudomallei* dilakukan menggunakan ujian berasaskan PCR mensasarkan gen *sctQ* daripada kumpulan TTS1. Dua puluh isolat kemudiannya menjalani penentuan genotip menggunakan urutan DNA pelbagai gen *loci* untuk mengkaji kepelbagaian genetik dan untuk mengaitkan genotip yang dikenal pasti dengan ciri-ciri klinikal penyakit. Kadar kematian pesakit yang menerima terapi empirikal anti-melioidosis yang awal dan sesuai dibandingkan dengan pesakit yang tidak menerima terapi empirikal anti-melioidosis yang sepatutnya. Sejumlah 52 kes terlibat di dalam kajian ini. Data klinikal pesakit diperoleh daripada nota klinikal elektronik.

**Keputusan**

Sembilan puluh enam peratus daripada pesakit melioidosis dalam kajian ini mempunyai sekurang-kurangnya satu penyakit kronik, dan faktor risiko yang paling kerap ditemui

adalah diabetes mellitus. Melioidosis bakteremik dilihat pada kebanyakan pesakit (n = 48, 92.3%). Kebanyakan kes mempunyai jangkitan bakteremik setempat (lokal) (57.7%), diikuti dengan bakteremik multifokal (26.9%) dan bakteremia tanpa fokus yang nyata (7.7%). Empat pesakit (7.7%) mempunyai jangkitan lokal tanpa bakteremia. Radang paru-paru yang diperolehi daripada komuniti dan demam berpanjangan yang tidak diketahui punca adalah gejala klinikal yang selalu menyebabkan kemasukan ke hospital. Paru-paru adalah organ yang paling biasa terlibat (61.5%), diikuti oleh hati (15.4%), limpa, sistem saraf pusat dan tisu badan (9.6%), sendi dan saluran kencing (3.8%).

Semua isolat berjaya disahkan dengan PCR. Pokok filogenetik yang dibentuk daripada penjujukan pelbagai gen *loci* mendedahkan kepelbagaian genetik di kalangan isolat *B. pseudomallei*, dan tiada kluster yang dilihat dengan gejala klinikal atau hasilnya. Kadar kematian keseluruhan adalah 53.9% (n = 28/52) dan semua pesakit yang meninggal mempunyai melioidosis bakteremia. Hanya 15 dari 52 pesakit (28.9%) yang diberikan antibiotik empirikal yang sesuai, dengan kematian sebanyak 26.7%. Kematian pesakit yang tidak menerima terapi empirik antibiotik yang sesuai adalah jauh lebih tinggi (64.9%).

**Kesimpulan**

Kepelbagaian gejala klinikal melioidosis di negeri Terengganu adalah sama dengan negara-negara lain di seluruh dunia. Kajian genotip *B. pseudomallei* menunjukkan kepelbagaian genetik yang menunjukkan kewujudan strain yang berlainan dalam alam sekitar. Kadar kematian keseluruhan adalah tinggi, terutamanya bagi mereka yang tidak menerima antibiotik empirikal yang awal dan sesuai. Oleh itu, indeks kecurigaan yang tinggi di negara endemik dan penggunaan awal terapi empirikal antibiotik yang sesuai adalah penting untuk mencegah kematian.

# CHAPTER 1

# 1 INTRODUCTION

## 1.1 Background of the study

Melioidosis is caused by gram-negative saprophyte, *Burkholderia pseudomallei*, which is present abundantly in soil and surface water, especially in paddy fields, in endemic regions. *B. pseudomallei* is endemic in Southeast Asia and northern Australia, particularly Thailand, Malaysia and Singapore (Currie *et al.*, 2008; Puthucheary, 2009; Suputtamongkol *et al.*, 1999). A study in Pahang has shown the incidence of melioidosis is comparable with that in Northern Thailand which is 6.1 per 100 000 populations per year (How *et al.*, 2005).

*B. pseudomallei* is a small, gram-negative, oxidase-positive, motile, aerobic bacillus with occasional polar flagella. The mode of infection is by percutaneous inoculation, inhalation, aspiration or ingestion (Currie, 2015). Melioidosis mainly affects people who have direct contact with wet soils and have an underlying predisposition to infection. Susceptibility of the host is an important factor in acquiring the disease as it occurs predominantly in patients with underlying illness (Puthucheary, 2009). Diabetes mellitus was confirmed to be the most common underlying illness associated with melioidosis (60.9%); the other common predisposing conditions were chronic renal disease (20.8%), thalassemia (7.3%), history of previous trauma or surgery (6.9%), pulmonary tuberculosis (6.3%) and hematologic malignancy or solid tumor (4.2%) (Suputtamongkol *et al.*, 1999).

*B. pseudomallei* infection causes wide range of clinical presentations. Pneumonia was found to be the most common presentation of melioidosis in majority of the studies, but

there is a great clinical diversity, ranging from localized infection to fulminant sepsis with multi organ involvement. Most patients have bacteremia with severe disseminated disease, and high mortality rates despite recent advances in the antibiotic treatment regimens (Waiwarawooth *et al.*, 2008).

Treatment of melioidosis is often difficult and challenging, as response to treatment is often disappointingly low despite high dose parenteral antibiotics administration (White, 2003). *B. pseudomallei* is characteristically resistant to penicillin, ampicillin, first and second generation cephalosporin, gentamicin, tobramycin and streptomycin. An open-label randomized trial in Thailand comparing ceftazidime with conventional therapy (a combination of chloramphenicol, trimethoprim-sulfamethoxazole (TMP-SMX) and doxycycline) showed that ceftazidime caused reduction in 50% of overall mortality in severe melioidosis (White, 2003). Ceftazidime then become the drug of choice for initial intensive therapy for melioidosis. Imipenem has also been proved equivalent to ceftazidime in a large randomised trial (Simpson *et al.*, 1999). In another study Cheng *et al*. demonstrated that outcomes of patient with melioidosis treated with meropenem were similar to those treated with ceftazidime (Cheng *et al.*, 2004).

The recommended duration of intensive therapy is at least 10 to 14 days. Longer treatment course is required for critically ill patients, or for patients with extensive pulmonary disease, deep seated collections or organ abscess, osteomyelitis, septic arthritis and neurological involvement (Dance, 2014; White, 2003). Subsequent eradication therapy is necessary after the initial intensive phase to prevent recrudescence or relapse of melioidosis. *B. pseudomallei* is a facultative intracellular pathogen that can invade and replicate inside various cells, including polymorphonuclear leukocytes and macrophages.

The ability of *B. pseudomallei* to survive intracellularly explains the tendency for the patient to get relapse or recurrent disease (Dance, 2014; White, 2003).

## 1.2 Rationale of the study

Melioidosis is a disease of the rainy season in endemic areas like Malaysia. The association between surface water and melioidosis is supported by its association with monsoonal rains, and with occupational and recreational exposure to water and soil (Cheng and Currie, 2005). Terengganu state, which is located on the east coast of Peninsular Malaysia, has a heavy monsoon season from November to March every year. Thousands of people are at risk of contracting *B. pseudomallei* infection, which is a great public health concern and an important cause of community acquired sepsis in the east coast of Peninsular Malaysia. However, not many publications on melioidosis reported from Malaysia, in particular Terengganu state.

Melioidosis has a diverse spectrum of clinical presentations and can affect any organ. It is important to define the local demographic profiles, clinical characteristics and outcome of melioidosis because of regional differences that have been described in the prevalence of organ involvement. This study aimed to determine the demographic profiles, clinical and molecular characteristics, and treatment outcome of patients with melioidosis who attended Hospital Sultanah Nur Zahirah (HSNZ), Terengganu.

*B. pseudomallei* is easily recovered on standard culture medium but may be misidentified as *B. cepacia, B. thailandensis, Pseudomonas stutzeri* or other *Pseudomonas* species. Molecular methods are more accurate in species identification but are expensive for routine laboratory use. In this study, molecular method has been used to give accurate species

identification. The molecular identification is based on detection of *sctQ* gene which is located in Type III secretion system (TTS1) gene cluster, on chromosome no II. TTS 1 is a toxin delivery mechanism that allows pathogenic bacteria to inject toxic substances into cytoplasm of host's cells. A study in France showed amplification of *orf11*, a gene region located in the TTS1 is seen only in *B. pseudomallei* (Thibault *et al.*, 2004).

Molecular typing that shows clonality of isolates in animal and human clusters has revealed that the same outbreak strain can cause different clinical presentations, with host factors being most important in determining the severity of the disease (Bennett *et al.*, 2014). Two studies, however, have suggested that clinical presentation or outcome may depend on the strain type. A study by Pitt *et al.* found that certain ribotypes appeared to be associated with a higher mortality or risk of relapse (Pitt *et al.*, 2000). Another study by Norton *et al.* in 1998 that utilized multilocus enzyme electrophoresis (MLEE) and RAPD analysis suggested that soft tissue infections were restricted to one cluster and respiratory and neurological infections were seen in another cluster. In this study genotyping of *B. pseudomallei* by DNA sequencing of multiple gene loci has been performed to determine the genetic relatedness of the strains.

Early diagnosis of melioidosis and administration of appropriate antibiotic therapy is necessary as acute melioidosis is life threatening, especially in patient with bacteremia. Bacteremia and overall mortality rates have been high. More than half of the patients died within two days after admission, before bacterial cultures became positive (Dharakul and Songsivilai, 1999).

A retrospective study done in a tertiary hospital found that death due to melioidosis among bacteremic melioidosis was associated with shorter hospitalization, no identified underlying disease, and no anti-melioidosis therapy (Deris *et al.*, 2010). Eight out of 27 patients (29.6%) were not given anti-melioidosis therapy because identification of the causative agent was not made until after the patient died. Lack of clinical suspicion leading to delay in treatment was also described in a retrospective study done among paediatric melioidosis patients in Pahang, Malaysia (How *et al.*, 2005). Only one out of seven (14.3%) paediatric bacteremic melioidosis patient was treated empirically with active drugs for melioidosis. In this study, the mortality rates of patients who receive early and appropriate empirical anti-melioidosis therapy were compared to those with delayed empirical anti-melioidosis therapy and those who never received appropriate empirical antibiotic.

## 1.3 Literature review

### 1.3.1 History and taxonomy of *B. pseudomallei*

*Burkholderia pseudomallei* was discovered in 1911 when a pathologist; Alfred Whitmore and his assistant Krishnaswami noticed 'undescribed glanders-like illness' during routine post mortem examination of morphine addict corpse in Rangoon. Glanders is an abscess-forming infection in horses caused by *Burkholderia mallei*, which can occasionally affect human (White, 2003). In 1913 Fletcher discovered the disease in laboratory animals at the Institute for Medical Research (IMR) in Kuala Lumpur, Malaysia and later in 1917 Stanton first described similar infection in human patient from Kuala Lumpur. Both authors wrote a short monograph on the disease and its sporadic occurrence in Malaya up to 1932 (Puthucheary, 2009).

The term 'melioidosis' was created by Stanton and Fletcher in 1921, which derived from the Greek words 'melis' meaning 'a distemper of assess' and 'eidos', resemblance. This was because the disease resembles glanders, a chronic and debilitating disease of equines caused by *B. mallei*. The new organism could be differentiated from *B. mallei* by its relatively rapid growth, motility, and the lack of the Strauss reaction when it was injected into guinea pigs. They correctly predicted that this new bacterium was closely related to the organism that caused glanders, a finding that has only recently been confirmed by molecular studies.

During the last century this gram negative saprophytes has been variously known as *Bacillus pseudomallei, Bacillus whitmorii, Malleomyces pseudomallei* and *Pseudomonas pseudomallei*. With the molecular evolution of bacterial classification, Yabuchi and co-workers proposed a new genus *Burkholderia* in 1992 (named after Walter Burkholder who

first described *Burkholderia cepacia*). The species *pseudomallei*, along with six other species were transferred to the new genus and became *Burkholderia pseudomallei* (Cheng and Currie, 2005). In the latter half of 20$^{th}$ century, *B. pseudomallei* emerged as an infectious disease of major public health importance in Southeast Asia, (including Thailand, Malaysia and Singapore) and Northern Australia. *B. pseudomallei* has been designated a tier 1 overlap select agent by the U.S. Centre for Disease Control and Prevention (CDC) and U.S Department of Agriculture and Animal and Plant Healh Inspection Service (USDA/APHIS) (Hemarajata *et al.*, 2016).

### 1.3.2 Epidemiology of Melioidosis

Melioidosis is regarded endemic to Southeast Asia and Northern Australia, especially in the tropical region which correspond approximately to the latitude of between 20˚N and 20˚S (Cheng and Currie, 2005). Dance *et al.* noted that published case report and series are likely to represent only the 'tip of iceberg' as culture facilities are not available in most of rural tropics where in the infection is likely to be prevalent (Dance, 2000).

Melioidosis is predominantly occurring during the rainy season in endemic areas. It mainly affects people who have direct contact with wet soils and have underlying predisposition to infection (White, 2003). *B. pseudomallei* has been isolated from soil and water from all states in West Malaysia by Strauss and co-workers in 1969. Soil moisture was found to be an important factor in the isolation of the organism; this is because the water table rises to the surface carrying with it the bacteria that normally reside deep inside the soil (Puthucheary, 2009). The risk of disease is roughly proportional to the concentrations of organisms in the soil.

Melioidosis may present at any age; however peak incidence was found to be in 4$^{th}$ and 5$^{th}$ decade of life, coinciding with development of underlying predisposing illness. In the northeast Thailand, the average annual incidence was estimated to be 4.4 per 100 000, but this number is increasingly steadily with the demographic transition, since improved health services and economic condition allow people to live longer (White, 2003).

Most of the population in endemic areas of Southeast Asia have antibodies to *B. pseudomallei*, but these antibodies have not been shown to be protective (Patel *et al.*, 2011). Melioidosis may result either from host defence failure due to underlying disease or drugs, or from an encounter of especially large inoculum, for instance during major trauma or near-drowning (White, 2003).

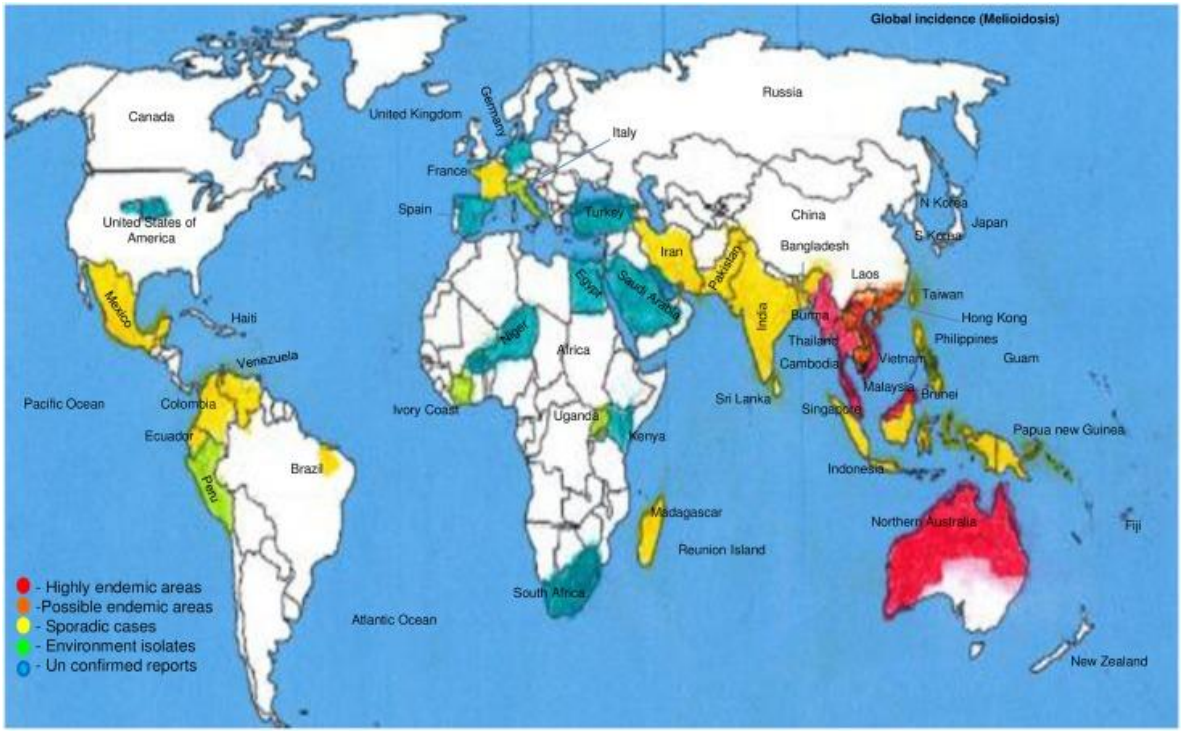



Figure 1.1: Worldwide distribution of melioidosis. Adapted from (Currie, 2015).

### 1.3.3 Risk factors for melioidosis

*Burkholderia pseudomallei* behaves as an opportunistic pathogen; as exposure to the organism is widespread in endemic area and yet the disease is not that common. It occurs predominantly in immunocompromised individuals with underlying disease for instance diabetes mellitus and renal disease. This suggests that susceptibility of the host is an important factor in acquiring the disease. A prospective melioidosis study over 25 years in Australia reported that 113 of the 115 deaths cases were directly attributed to one or more risk factors (Currie *et al.*, 2010).

Diabetes mellitus is the most frequent predisposing condition for melioidosis. Up to 50% of patients with melioidosis have diabetes mellitus, usually Type II diabetics with uncontrolled blood glucose level before infection (White, 2003). In Malaysia, Northeast Thailand and Singapore, diabetes mellitus was the most frequently reported predisposing condition with up to 60% of patients having pre-existing or newly diagnosed type 2 diabetes (Puthucheary, 2009). In Malaysia about 70-89% of melioidosis patients had diabetes mellitus (Deris *et al.*, 2010; Hassan *et al.*, 2010; How *et al.*, 2005).

Other recognized risk factors in acquiring melioidosis include chronic renal failure, malignancy, immunosuppressive treatment, in particular steroids, alcoholism, occupational exposure, thalassemia, chronic liver and lung disease and neutropenia (Dance, 2000; Puthucheary, 2009). The underlying immune dysfunction in the aforementioned risk factors lead to wide range of immune deficits including phagocytic defects, diminished humoral and cellular immune responses, and diminished cytokine production.

### 1.3.4 Modes of acquisition

Melioidosis is a disease involving all age group but commonly occurs in people between the ages of 40 to 60 years. There are three recognized modes of acquisition for *B. pseudomallei*; inhalation of contaminated dust, ingestion and direct inoculation (Puthucheary, 2009). The modes of acquisition as well as size of inoculum are likely to be responsible for the pattern and severity of disease. Conditions that can cause exposure to high inoculum of the bacteria, such as near drowning, are associated with shorter incubation period. Inoculation is now believed to be the major mode of acquisition. Minor wounds to the feet of rice farmers are common during planting and harvesting season. Occasional unusual modes of transmission have been reported in the literature; including lab acquired infection, sexual transmission, vertical and perinatal transmission (Cheng and Currie, 2005).

### 1.3.5 Pathogenicity

#### 1.3.5.1 Virulence factors

*B. pseudomallei* is a resilient bacterium that can survive in a very harsh environment. In human, it behaves as an opportunistic intracellular pathogen which multiplies inside phagocytic and non-phagocytic cells. It may remain latent for many years before reactivation into active infection (Puthucheary, 2009). The structural and functional aspect of *B. pseudomallei* components, their regulation and role in pathogenesis are still incompletely or poorly understood. Examples of *B. pseudomallei* virulence gene include those encoding for proteases, lipases, lecithinases, catalase, peroxidase, hemolysis, cytotoxins, adhesions, inasins, fimbriae and pili and secretion systems type II, III and VI (Sarovich *et al.*, 2014).

*B. pseudomallei* is able to polymerize actin, and to spread from cell to cell, causing cell fusion and the formation of multinucleated giant cells. It also produces a highly hydrated glycocalyx polysaccharide capsule, which helps to form slime. This capsule facilitates formation of micro colonies and creates protection from penetration of antibiotics (Yabuuchi *et al.*, 1992). In addition, *B. pseudomallei* also possesses a unique fusogenic type-VI secretion system that is required for cell to cell spread and virulence in mammalian hosts. It is one of the first Proteobacteria identified to have an active type-VI secretion system and the only organism identified that contains up to six different type-VI secretion systems. The bacterium also expresses a toxin called lethal factor 1 (Kespichayawattana *et al.*, 2000; Toesca *et al.*, 2014; Wiersinga *et al.*, 2006).

*B. pseudomallei* is transmitted from environment to human host and attached to the epithelial cell layers of either the abraded skin, the respiratory tract mucosa or gastrointestinal tract mucosal layers. The attachment is aided by the bacterial capsule and type 4 pili, followed by formation of pili-mediated micro colonies once the optimal epithelial attachment is obtained (Galyov *et al.*, 2010). Type III secretion system effectors assist bacterial invasion by disrupting the phagosomal membrane and facilitate the bacterial escape into the host cytosol (Lazar Adler *et al.*, 2009).

#### 1.3.5.2 Role of host immune response

*B. pseudomallei* appears to be resistant to serum bactericidal components. Even though the alternative complement pathway is activated, resulting in phagocytosis, it is to be able to evade phagosome-lysosome fusion and destroy the phagosome membrane as soon as 15 minutes after ingestion. It is also resistant to the effects of the terminal complement

membrane attack complex. It is established that *B. pseudomallei* can survive and multiply within professional phagocytes, including macrophage or monocyte and neutrophil cell lines. The comorbidities recognized as risk factors for melioidosis such as diabetes mellitus has been demonstrated to cause impaired chemotaxis, phagocytosis, oxidative burst and killing activity. Similar defects have been observed in association with chronic renal failure, thalassemia and high alcohol intake (Cheng *et al.*, 2007).

*B. pseudomallei* induces humoral and cell-mediated responses during disease stage. Individual who were exposed to *B. pseudomallei* develop IgA, IgM and IgG antibodies that are increased during acute infection and their levels correlate with the severity of disease. High levels of antibodies remain elevated for years in patient's serum after meliodosis recovery, suggesting continuous exposure to *B. pseudomallei* or sequestration of bacteria in intracellular sites of latency (Gan, 2005). It is assumed that *B. pseudomallei* survival in professional phagocytes play a role in the site of latent infection, little is known regarding the precise localization of latent intracellular *B. pseudomallei*.

During severe melioidosis, the levels of pro-inflammatory cytokines and immunoregulatory cytokines indicate an extensive activation of cellular immune response (Cheng *et al.*, 2007). Despite cell-mediated immune response in the neutralizing and protection against disease progression, there is no definitive evidence for the protective immunity against melioidosis which allow reinfection occurrence with a different *B. pseudomallei* strain after successful treatment (Currie, 2015).

### 1.3.6 Clinical manifestations

*B. pseudomallei* causes wide range of clinical signs and symptoms, thus has been dubbed 'the great mimicker'. The symptoms and signs of melioidosis can range from asymptomatic, benign localized infection to a rapidly fulminant and fatal septicemia. Severe melioidosis may present as disseminated septicemia, non-disseminated septicemia or localized infection. Clinical classification of melioidosis has been controversial since melioidosis is a multi-system disease (Puthucheary, 2009). The type of presentation may be influenced by the magnitude of exposure, mode of acquisition, host factors and risk factors. Recognition of melioidosis based on clinical presentation can be challenging, and a delay in diagnosis can result in fatality.

Melioidosis is divided into acute and chronic cases based on the onset of the illness. Acute cases are those patients with symptoms of less than two months. In most patient, mean incubation was nine days (range 1 to 21 days). Chronic melioidosis is defined as an illness where symptoms have lasted for longer than two months at presentation (Foong *et al.*, 2014).

Pneumonia is the most common presentation of melioidosis accounting up to half of all cases, but there is a great clinical diversity, ranging from localized skin ulcers or abscess to fulminant sepsis with multiple skin and soft tissue, deep organ or musculoskeletal abscesses (Waiwarawooth *et al.*, 2008). Almost any organ system may be affected including lungs, kidneys, spleen, liver, prostate, parotids and brain (Foong *et al.*, 2014).

A study in HUSM in 2009 revealed that the main clinical presentation was fever (83.2%), with 66.7% of patients had lung involvement. Other presentations include cough, scrotal

swelling, Fournier's gangrene, urinary retention, neck cellulitis, abdominal distention, jaundice, vomiting and seizure (Deris *et al.*, 2010). Chou *et al.* conducted a retrospective study at Tainan Municipal Hospital, and found that the lungs were the most common site of infection in patients with bacteremic melioidosis and rapidly progressive community acquired pneumonia was major cause leading to mortality (Chou *et al.*, 2007). The most common site of infection was the lung (70%), followed by the genitourinary tract (13.3%), peritoneum (6.7%), meninges (3.3%), skin (3.3%) and aorta (3.3%). There were also 20% of cases without a primary site being identified.

### 1.3.7 Laboratory Diagnosis

Laboratory diagnosis of *B. pseudomallei* includes conventional, molecular and immunological methods.

#### 1.3.7.1 Conventional Identification

Isolation of *B. pseudomallei* from patients remains the 'gold standard' in the diagnosis of melioidosis. The organism is non-fastidious and will grow on almost all routine media, but with non-sterile specimens, *B. pseudomallei* can be overgrown by contaminating flora due to paucity of the organisms especially from deep-seated abscess. Cultures typically become positive in 24 to 48 hours (this rapid growth rate differentiates the organism from *B. mallei,* which typically takes a minimum of 72 hours to grow).

The rate of successful isolation of *B. pseudomallei* from non-sterile sites like sputum or pus aspirate is increased if they are placed into colistin-containing transport media called Ashdown's broth or directly plated onto gentamicin-containing Ashdown agar. Both types of media facilitate the selective growth of *B. pseudomallei* and inhibit the growth of contaminants (Currie, 2015; Puthucheary, 2009). The use of selective media, such as Mac-

Conkey agar, Ashdown agar, *B. pseudomallei* selective agar (BPSA), or *B. cepacia* selective agar (BCSA), is recommended, particularly for tissues or any specimen expected to be contaminated with normal flora, such as respiratory secretions.

*B. pseudomallei* colonies are initially smooth, however become wrinkled with prolonged incubation after three to five days. It has a metallic appearance, and possesses an earthy odour. It appears as colorless lactose non-fermenter colonies on MacConkey agar. On Gram staining, the organism is a Gram-negative rod with a characteristic "safety pin" appearance (bipolar staining). It exhibits bipolar staining due to accumulation of poly-β-hydroxybutyrate. It is oxidase positive, motile, grow on 42˚C and produce neutral-alkaline reaction on triple sugar iron biochemical test. On sensitivity testing, the organism appears highly resistant (it is innately resistant to a large number of antibiotics including colistin and gentamicin) and that again differentiates it from *B. mallei*, which is in contrast, exquisitely sensitive to a large number of antibiotics.

Conventional screening panel can be combined with substrate utilization kits, such as Vitek-1 and Vitek-2 systems (bioMérieux, France) and the analytical profile index system for non-enterobacteriaceae (API 20 NE system). API 20 NE (bioMérieux, France) is a standardized system for non-enteric gram-negative bacteria. It comprises of eight conventional tests (nitrate, tryptophan, glucose acidification, arginine, urea, esculin, gelatin and PNPG) and 12 assimilation test (glucose, arabinose, mannose, mannitol, N-acetylglucoseamine, maltose, gluconate, caprate, adipate, malate, citrate and pheny lactate) supplied with background database. Every test is assigned a digit so that the complete profile of 20 digits is given in the identification database which will give the most probable

identification. The API 20 NE has high accuracy rate and easy to handle (Weissert *et al.*, 2009).

Vitek 2 system showed sensitivity of 69% with common low discrimination identification finding, whilst API 20 NE correctly identified 87% of 56 and 99% of 800 isolates respectively in two studies (Foong *et al.*, 2014)x. In general, API 20NE detection rate of *B. pseudomallei* isolates in Thailand was up to 99% and in Australia was 37-98% (Hoffmaster *et al.*, 2015). In another study, Vitek 2 GN card detected 47 (78.3%) out of 60 confirmed clinical isolates which was lower than that of API 20 NE that was 52 (86.7%) (Deepak *et al.*, 2008).

Figure 1.2: Colony of *B. pseudomallei* on MacConkey agar

### 1.3.7.2 Molecular diagnosis

*B. pseudomallei* may be misidentified as *B. cepacia, B. thailandensis, P. stutzeri* or other *Pseudomonas* species. Molecular methods are more accurate in species identification but are expensive for routine laboratory use. Several molecular methods were developed for the identification of *B. pseudomallei*, including gene sequencing, DNA microarray, isothermal DNA amplification and polymerase chain reaction (PCR).

PCR-based techniques have been very useful in diagnosis of melioidosis, however it requires some level of instrumentation and expertise. Culture-based methods are time-consuming and may lead to misidentification while serology-based tests which are both faster and less sophisticated may be rendered less reliable in view of presence of high background titre levels in endemic country and cross-reactivity with other organisms.

Various PCR assays have been developed for detection of *B. pseudomallei*. The gene targets include the *orf2* gene of TTS1gene cluster (Winstanley and Hart, 2000), 16S ribosomal RNA (rRNA) (Brook *et al.*, 1997; Dharakul *et al.*, 1999), 16S-23S rRNA (Kunakorn *et al.*, 2000), 23S rRNA (Tkachenko *et al.*, 2003), serine metalloprotease (*mprA*) (Neubauer *et al.*, 2007), lipopolysaccharide (LPS) gene (Rattanathongkom *et al.*, 1997), flagellin C (*fliC*) and ribosomal protein subunit S21 (*rpsU*) (Tomaso *et al.*, 2005).

The sensitivity and specificity of PCR is generally excellent when tested against purified bacterial DNA, but the sensitivity decline when testing clinical specimen. This discrepancy might be attributed to the presence of DNA inhibitors in the clinical specimen (Kaestli *et al.*, 2012).

#### 1.3.7.3 Molecular typing

Various molecular typing tools have been performed to investigate the epidemiology of melioidosis and to explore genetic relatedness among *B. pseudomallei* isolates, including pulsed-field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) analysis, and ribotyping. However, these methods are not well suited for inter-laboratory comparisons, and molecular typing procedures that use nucleotide sequence data rather than DNA fragment patterns are increasingly being used (Godoy *et al.*, 2003).

MLST is based on allelic differences present at seven housekeeping genes, and is ideal for genetic analysis due to its reproducibility, low rate of genetic change in the allelic sites and allows for comparison between strains typed at different laboratories through an internet-based database (http://www.pubmlst.net/) (Cheng and Currie, 2005). The different sequences at each of the seven loci are assigned different allele numbers, and the series of integers that corresponds to the allele numbers at the seven loci define the allelic profile of a strain. MLST has become an established technique for the unambiguous and precise characterization of isolates and for epidemiological purposes (Godoy *et al.*, 2003).

MLST appears to have a discriminatory ability similar to that of PFGE, but provides data that are much more easily compared than PFGE data and is ideal for comparison of isolates characterized in different laboratories and for the detection of strains with international distribution (Godoy *et al.*, 2003).

### 1.3.8 Treatment of melioidosis

*B. pseudomallei* is intrinsically resistant to wide range of antibiotics which include some β lactam, aminoglycosides and macrolides. Therapeutic approach of melioidosis comprise of two phases; acute phase treatment to resolve severe acute infection to avoid death due to overwhelming sepsis. The second maintenance phase treatment is administered for eradication of residual intracellular infection to avoid relapse (Currie, 2015). Current international guidelines suggest intensive phase treatment with a minimum of 10 to 14 days of intravenous antibiotics followed by three to six months of oral antibiotics for eradication phase (Pitman 2015). Options for the intensive phase include ceftazidime or carbapenem; whilst options for the eradication phase include trimethoprim-sulfamethoxazole (TMP-SMX), doxycycline or amoxicillin-clavulanate.

For acute melioidosis, treatment with intravenous ceftazidime 50 mg/kg (up to 2 g every 8 hours) has been recommended in uncomplicated infection. In cases of severe acute infections with central nervous system involvement, treatment failure or persistent bacteremia, imipenem and meropenem 25 mg/kg (up to 1 g every 8 hours) are given intravenously. The duration of intensive phase may be extended to more than 4 weeks in severe case of melioidosis (Dance, 2014). Longer treatment is required for critically ill patients, or for extensive pulmonary disease, deep seated collections or organ abscess, osteomyelitis, septic arthritis and neurologic melioidosis. In the past 20 years, several studies have shown that the mortality rate has been reduced to 19-37% with high dose ceftazidime or imipenem therapy for at least 2 weeks. White *et al.* demonstrated that ceftazidime could reduce overall mortality by 50% in a randomized control trial while Cheng *et al*. demonstrated that outcomes of patient with melioidosis treated with meropenem were similar to those of ceftazidime-treated patient (Cheng *et al.*, 2004; White, 2003).

Carbapenems kill *B. pseudomallei* more rapidly than cephalosporins. High dose imipenem has been shown in a comparative trial in Thailand to be at least as effective as ceftazidime for severe melioidosis, with no differences in mortality rate and fewer treatment failures in those given imipenem (Simpson *et al.*, 1999). Observational data from Australia have suggested that meropenem produces better outcomes in severe melioidosis than ceftazidime which has led to the recommendation that meropenem is the drug of choice for melioidosis in septic shock (Cheng *et al.*, 2004).

Waiwarawooth *et al.* has conducted a retrospective study in 2008 to evaluate the epidemiological data and clinical outcome of culture-proven melioidosis at Chonburi

Hospital, Thailand, form January 2001 to December 2006. The case fatality rate was found to be high, particularly in patient who had acute severe lung infection with bacteremia, and treated with inappropriate antibiotic therapy. The overall mortality rate was 47%. Male sex, bacteremia, lung infection, acute onset of disease and treatment with inappropriate antibiotics were the factors found to significantly correlate with higher mortality rate. The mortality was 63.41% in those who received inappropriate therapy; in which the mortality was 31.8% in those receiving delayed appropriate therapy, and 100% mortality in those who never received appropriate therapy. The mortality was lower in patients who received appropriate antibiotic therapy. Clinical awareness, high index of suspicion and prompt effective treatment of high-risk patients was reported reduce the mortality (Waiwarawooth *et al.*, 2008)

## 1.4 Objective(s) of the research

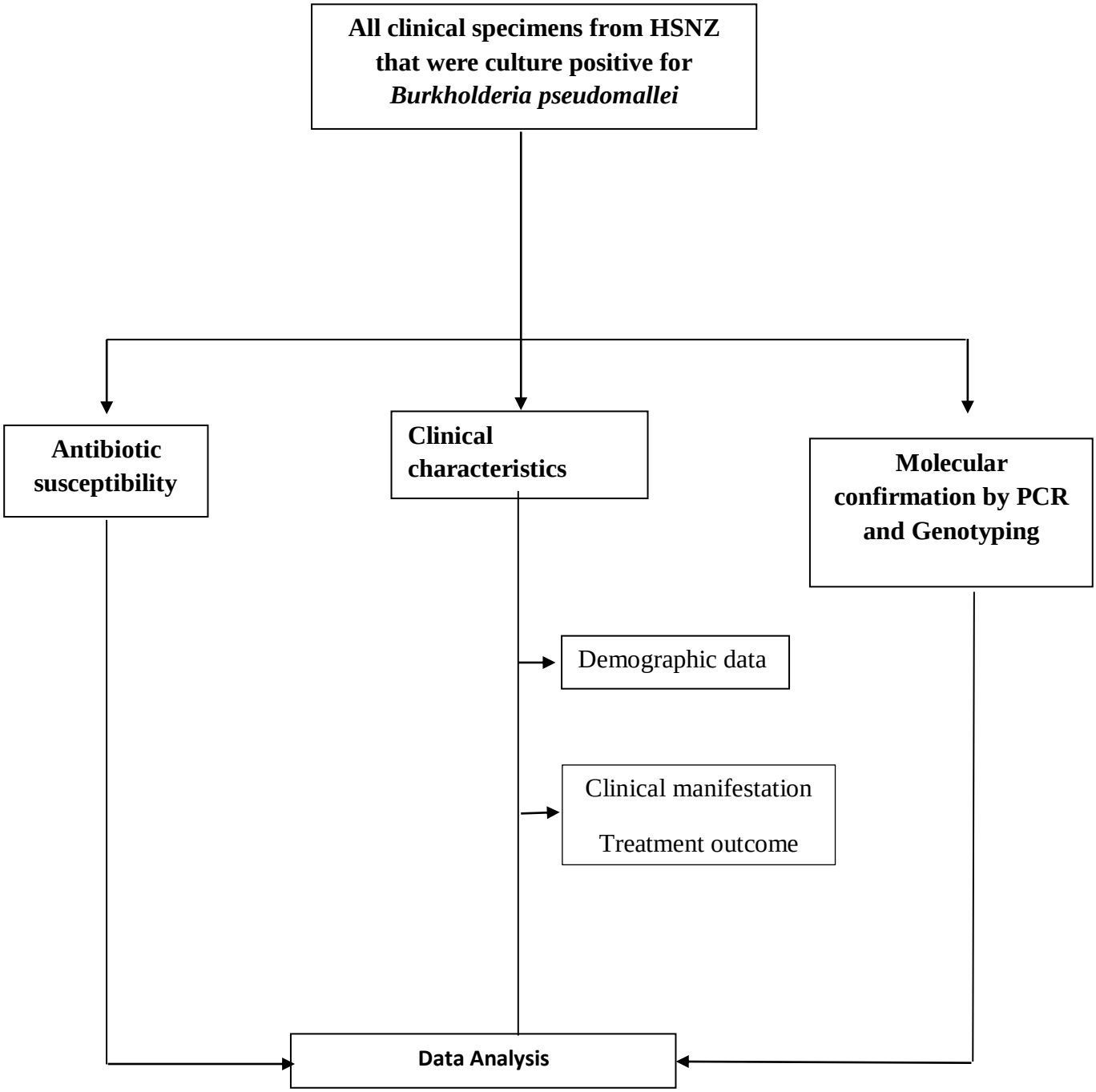
**General objective:**

To study clinical and molecular characteristics, antibiotics susceptibility patterns and treatment outcomes of *Burkholderia pseudomallei* infection in hospitalized patients in Hospital Sultanah Nur Zahirah (HSNZ).

**Specific objectives:**

1. To determine the demographic data and clinical characteristics of patients with melioidosis in HSNZ.
2. To identify *Burkholderia pseudomallei* isolates using molecular technique (PCR).
3. To perform genotyping of *Burkholderia pseudomallei* isolates and to correlate with clinical presentation.
4. To determine the antibiotic susceptibility pattern of *Burkholderia pseudomallei* isolates in HSNZ.
5. To determine the association between treatments outcome of patient with melioidosis in HSNZ with empirical antibiotic therapy received.

## 1.5 Flow chart of the study



# CHAPTER 2

# 2 METHODOLOGY

## 2.1 Study design

A descriptive cross-sectional study was conducted at Hospital Sultanah Nur Zahirah (HSNZ) from October 2016 until June 2017.

## 2.2 Reference population

All *Burkholderia pseudomallei* isolated in HSNZ.

## 2.3 Source population

All *B. pseudomallei* isolates isolated in HSNZ from October 2016 until June 2017.

## 2.4 Sampling frame

Patients who received treatment in HSNZ; whom clinical specimen grew *B. pseudomallei* collected from October 2016 until June 2017 that fulfil the inclusion and exclusion criteria.

## 2.5 Inclusion criteria

All confirmed *B. pseudomallei* isolated from any clinical specimens including blood, sputum, pus, swab, tissue, urine and CSF.

## 2.6 Exclusion criteria

Repeated isolate of *B. pseudomallei* discovered from similar or different sites during the same admission or within four weeks period are excluded.