GENTAMICIN-SENSITIVITY TESTING OF *BURKHOLDERIA PSEUDOMALLEI* CLINICAL ISOLATES FROM SARAWAK

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(44353)

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Fakulti Sains dan Teknologi Sumber
Universiti Malaysia Sarawak

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Gentamicin-sensitivity Testing of *Burkholderia Pseudomallei* Clinical Isolates from Sarawak

Tan Pei Chin (44353)

A thesis submitted in fulfillment for the award of the Degree of Bachelor of Science with Honours in Resource Biotechnology

Faculty of Resource Science and Technology

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Last but not least, thanks to my beloved family and friends for being there to support and understand me throughout the research project.
DECLARATION

I hereby declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. It is original and is the result of my work, unless otherwise indicated or acknowledged as referenced work. This thesis has not been submitted at this or any other university or academic institution for any other degree or qualification.

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Program of Resource Biotechnology
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<td>B. pseudomallei</td>
<td>Burkholderia pseudomallei</td>
</tr>
<tr>
<td>B. thailandensis</td>
<td>Burkholderia thailandensis</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standards Institute</td>
</tr>
<tr>
<td>Etest</td>
<td>Epsilometer test</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive Care Unit</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>MLST</td>
<td>Multilocus sequence typing</td>
</tr>
<tr>
<td>PBPs</td>
<td>Penicillin-binding proteins</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PenA</td>
<td>Class A β-lactamase</td>
</tr>
<tr>
<td>PPE</td>
<td>Personal protection equipment</td>
</tr>
<tr>
<td>RND</td>
<td>Resistance nodulation and cell division</td>
</tr>
<tr>
<td>rRNA</td>
<td>Ribosomal RNA</td>
</tr>
<tr>
<td>SNP</td>
<td>Single-nucleotide polymorphism</td>
</tr>
<tr>
<td>ST</td>
<td>Sequence type</td>
</tr>
<tr>
<td>SYBR</td>
<td>Synergy Brands, Inc.</td>
</tr>
<tr>
<td>TMP /SMX</td>
<td>Trimethoprim-sulphamethoxazole</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptone Soy Agar</td>
</tr>
<tr>
<td>UHQ</td>
<td>Ultra-High Quality</td>
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Gentamicin-sensitivity Testing of Burkholderia pseudomallei Clinical Isolates from Sarawak
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ABSTRACT

Burkholderia pseudomallei is the causative agent for melioidosis disease. Melioidosis is a potentially fatal disease which is difficult to treat due to its inherent resistance to many antibiotics such as aminoglycosides, β-lactams, macrolides, and polymyxins. Laboratory diagnosis of this disease includes antibody detection, antigen detection, molecular techniques, and also bacterial culture using selective medium. Gentamicin is one of the selective agents utilized in the selective medium as the bacterium is resistant to it. Recently, there was a study which discovered that there were gentamicin-sensitive mutant clinical strains from central Sarawak. Hence, this study was conducted in order to do preliminary test for gentamicin-susceptible clinical B. pseudomallei strains from Sarawak. Antibiotic susceptibility test was carried out using disk diffusion tests to identify the gentamicin sensitivity properties of 112 B. pseudomallei clinical isolates collected from selected Sarawak district hospitals. The finding of this study indicates that the prevalence of gentamicin sensitivity of the isolates tested is 76%. This preliminary study result plays a crucial role in the first phase as to support continuous surveillance of gentamicin-susceptible B. pseudomallei strains in Sarawak. This will prevent misdiagnosis of melioidosis cases which then reduce potential errors in antibiotics therapy and possible deaths.

Keywords: Burkholderia pseudomallei, melioidosis, gentamicin, Sarawak, disk diffusion

ABSTRAK


Kata kunci: Burkholderia pseudomallei, melioidosis, gentamicin, Sarawak, disk diffusi
1.0 INTRODUCTION

Melioidosis is an endemic disease in tropical and sub-tropical areas, caused by a gram-negative saprophytic bacterium called *Burkholderia pseudomallei* (Cheng and Currie, 2005; Podin et al., 2014). It was reported as a potential bioterrorism weapon by Centers for Disease Control and Prevention (2012). Generally, transmission of melioidosis is through inoculation, inhalation and ingestion of contaminated source. A research conducted by Currie et al. (2010) indicated that melioidosis has a wide range of clinical manifestation ranging from acute, subacute to chronic forms. Melioidosis can be potentially fatal to both animals and humans. Lipsitz et al. (2012) mentioned that intensive-phase treatment and eradication-phase treatment are included in the treatment of melioidosis. Intensive-phase treatment also known as intravenous therapy where its main purpose is to avoid mortality of melioidosis patient particularly in severe case while eradication-phase is targeted on preventing relapse of melioidosis (Pitman et al., 2015). Unfortunately, there is no vaccine for melioidosis to date (Cheng and Currie, 2005).

The characteristics of the saprophytic bacterium, *B. pseudomallei* include gram-negative, intracellular, bipolar staining, vacuolated, slender, has rounded ends, and oxidase positive (Cheng and Currie, 2005). Also, it has motile rod-shaped (Yabuuchi et al., 1992), nitrate reduction (Brook et al., 1997), and aerobic (Wiersinga et al., 2006). *B. pseudomallei* is normally present under the surface of soil and it can turn into airborne when found in surface water and mud after rain. The optimum pH for its growth is neutral to mild acidic condition. *B. pseudomallei* is well adapted and can survive in a wide range of hostile conditions, for instance, it can survive for 30 days in waterless soil (Tong et al., 1996), acidic condition up to pH4.5 (Dejsirilert et al., 1991) and detergent solution (Gal et al., 2004). There are several diagnostic methods that have been developed in order to identify *B.*
**pseudomallei** and diagnose melioidosis, namely, culture-based method, antibody detection, antigen detection, and molecular techniques (Cheng and Currie, 2005).

A recent study done by Podin *et al.* (2014) has discovered that 86 % of *B. pseudomallei* clinical isolates in Sarawak are gentamicin-sensitive. It shown higher prevalence of gentamicin-sensitive *B. pseudomallei* in Sarawak as compared to Thailand where it was only around 1 in 1000 clinical isolates reported to be gentamicin sensitive (Trunck *et al.*, 2009). According to Lipsitz *et al.* (2012), clinical diagnosis or manifestation solely could not surely confirm that a patient is affected with melioidosis. This is mainly due to the broad range of non-distinct clinical presentations of melioidosis. Hence, laboratory diagnosis plays a crucial role. As *B. pseudomallei* is fundamentally resistant to gentamicin, it is one of the most important selective agents used to detect *B. pseudomallei* in the laboratory diagnosis of melioidosis. The first problem statement here is that the presence of gentamicin-sensitivity of *B. pseudomallei* could affect the laboratory diagnosis of melioidosis either in Sarawak or potentially other endemic areas. Subsequently, this could affect the diagnosis of melioidosis with clinicians and laboratory personnel who are unaware of the possibility of gentamicin-sensitive *B. pseudomallei* will lead to misdiagnose of melioidosis case. Misdiagnosis of cases could lead to ineffective empiric treatment (Lipsitz *et al.*, 2012) which can be fatal to the patients. Secondly, there is also lack of research data done on the prevalence of gentamicin-sensitivity *B. pseudomallei* clinically from selected hospitals in Sarawak. The data is important to increase awareness of the clinicians about the existence of gentamicin-susceptible *B. pseudomallei* in Sarawak.
Therefore, the objectives of this study are:

1. To test *B. pseudomallei* clinical strains from Sarawak for strains that possess gentamicin-sensitive characteristics through antimicrobial susceptibility method using disk diffusion which is a biochemical and morphological related test.

2. To investigate the prevalence of gentamicin-sensitivity in *B. pseudomallei* clinical strains from selected hospitals in Sarawak.
2.0 LITERATURE REVIEW

2.1 Melioidosis

2.1.1 Melioidosis Background

Alfred Whitmore first described his eponymous disease—Whitmore’s disease together with his assistant C. S. Krishnaswami in Rangoon in 1911 (Whitmore and Krishnaswami, 1912; Whitmore, 1913). It is now known as melioidosis. The term melioidosis is derived from Greek “melis” and “eidos” (Stanton and Fletcher, 1921) which describe a disease similar with glander-like disease. Glander is a disease caused by 
Burkholderia mallei and predominantly affects equines (Khan et al., 2013). Whereas, as stated by Podin et al. (2014), melioidosis is a multiorgan infectious disease originated from tropical climates, particularly in south east Asia and northern Australia and mostly cause disease in human and animals. In Sarawak, Malaysia, it was stated by Sarawak Health Department as cited in Sibon (2011), a total of 343 reported melioidosis cases in 2010.

As for the transmission of melioidosis, Cheng and Currie (2005) reported that inoculation is the main mode of acquisition. This can happen during direct contact with contaminated water and soils where the bacteria are found. There is a higher risk for infection with melioidosis if there is skin abrasion at the time of contact. According to Cheng and Currie (2005), it is also possible to acquire B. pseudomallei by breathing in contaminated dusts or water droplets as well as ingestion of water contaminated with the bacteria. However, ingestion is only considered as a source of inoculation or inhalation as it is not an important mode of transmission.
2.1.2 Clinical Presentations of Melioidosis

In a study by Currie et al. (2010), there were 540 melioidosis cases being analyzed over 20 year-period, predominant presentation was pneumonia (51%), then genitourinary infection (14%), skin infection (13%), bacteremia without evident clinical focus (11%), septic arthritis/osteomyelitis (4%) and the least was neurological melioidosis (3%). Additionally, the other 4% was melioidosis with no evidence and clinical focus (Currie et al., 2010). The clinical presentations of melioidosis vary between different individuals. As stated by Cheng and Currie (2005), its clinical manifestations and severity may depend on the bacterial strains, the host immune response and the acquisition.

Relapsing course of melioidosis also been described, it was mentioned that the appropriate choice and duration of antibiotic therapy are the keys to prevent recurrence in melioidosis (Chaowagul et al., 1993). Melioidosis has high mortality rate which can be up to 40% (Wiersinga et al., 2012) where a mortality rate of 44% for melioidosis in Malaysia (Pahang) was reported by How et al. (2005). Likewise, Cheng and Currie (2005) stated that the mortality of recurrent melioidosis case is almost the same as the initial episode.

The incubation period upon exposure to B. pseudomallei has not yet been clearly defined. However, it is reported that the time range is in between a couple of days (Chierakul et al., 2005) to the longest reported 62 years (Ngauy et al., 2005). The incubation period mostly depends on the type of infection, inoculating dose, risk factors of patient and also strain virulence (Wiersinga et al., 2012).

Melioidosis infects both animals and human. Animal species which are most commonly infected include sheep, cattle, pigs (Ouadah et al., 2006), while there were also some reported in horses, dogs, monkeys, deer, cows, rats, orangutans, kangaroos, buffaloes, camels, zebras, gibbons, wallabies, llamas, koalas, cats, mules, parrots, hamsters, rabbits,
crocodiles, guinea pigs, ground squirrels, seals, and dolphins (Sprague and Neubauer, 2004). Likewise, it is possible for zoonotic transmission to occur (Choy et al., 2000; Elschner et al., 2014). For human, susceptibility to melioidosis is in particular high risk for certain groups of people with unique conditions for example, diabetes mellitus, thalassaemia, renal disease, chronic lung disease, liver disease, and aboriginality as well represent one of the high incidence factors (Cheng and Currie, 2005). The mean age group for development of melioidosis is presumable between 40 and 60 years (Limmathurotsakul and Peacock, 2011). Unlike melioidosis in animal, it is very rare for this disease to transmit between human and human. Although weaken of the cell-mediated immunity can result in susceptibility to melioidosis, HIV is not categorized as the major risk factor (Chierakul et al., 2004).

2.1.3 Clinical Management of Melioidosis

The initial treatment of melioidosis usually consists of intravenous intensive phase of therapy which commonly utilize ceftazidime or carbapenem (either meropenem or imipenem). This treatment typically lasts for 10-14 days in Australia and Thailand, however, it could extend to 4 weeks or more in severe cases. It is accompanied with frequent checking of liver function tests, urea and electrolytes, full blood examination such as number of eosinophil as well as cardiopulmonary resuscitation (Currie, 2014). Patients with negative blood cultures and termination of fever would indicate the improvement of progress in therapy which they are allowed to initiate the eradication-phase treatment. At least 12 weeks of oral antimicrobial therapy should be taken by the melioidosis patients in eradication-phase. The therapy would include trimethoprim-sulphamethoxazole (TMP/SMX) given orally. Amoxicillin/clavulanic acid or doxycycline could replace TMP/SMX in the cases that patients cannot tolerate with it (Lipsitz et al., 2012). Nevertheless, their rate of melioidosis relapsing is higher as compared with TMP/SMX treatment (Limmathurotsakul et al., 2006).
Although vaccine has not yet been developed for melioidosis, related intensive researches are carried out in animal models (Wiersinga et al., 2012). Hence, preventive measures are crucial instead to avoid this disease especially for occupational and recreational targets. Likewise, earlier clinical identification and better management of severe sepsis as well could lead to the recovery of melioidosis (Cheng and Currie, 2005).

2.2 Burkholderia pseudomallei

2.2.1 B. pseudomallei Background

The aetiology agent of melioidosis is B. pseudomallei. The bacterium is called as B. pseudomallei since 1992 (Yabuuchi et al., 1992). It was previously known as Bacillus pseudomallei, Bacillus whitmorri or Bacille de Whitmore, Malleomyces pseudomallei, and Pseudomonas pseudomallei (Cheng and Currie, 2005). B. pseudomallei is categorized in Burkholderia genus in which Burkholderia mallei, Burkholderia cenocepacia, Burkholderia thailandensis (B. thailandensis), and Burkholderia oklahomensis are all the pathogenic members of B. pseudomallei (Wiersinga et al., 2012). B. thailandensis is generally similar to B. pseudomallei. Differences between them include less pathogenicity of B. thailandensis and only B. pseudomallei can assimilate arabinose (Smith et al., 1997; Lertpatanasuwan et al., 1999).

Research by Pearson et al. (2009) indicates that the population of B. pseudomallei is subdivided by Wallace’s Line which mainly distributed into Asia and Australia regions. They stated that most probably B. pseudomallei originated from an ancient supercontinent, Gondwanaland which was then distributed to other regions through the breakup of Gondwanaland as in accordance with Gondwana hypothesis or the other possibility that B.
pseudomallei might be originated from Australia to southeast Asia during the time when both regions were connected through a land bridge (Cheng et al., 2004) which after that dispersed as result of animal migration approximately 15 million years ago (Miocene period). Dispersion of B. pseudomallei can occur through animals or humans and the sequence types (ST) from both northern Australia and Southeast Asia were very diverse (Cheng et al., 2004).

Furthermore, one of the distinctions of this bacterium is that it possesses two large chromosomes where normal bacterium only has one. This enhances its adaptability extensively yet it was described by Holden et al. (2004) as a formidable pathogen due to its high survival rate over varies harsh conditions.

2.2.2 Virulence Factors of B. pseudomallei

B. pseudomallei is well adapted and can survive in a wide range of hostile conditions, for instance, acidic condition up to pH4.5 (Dejsirilert et al., 1991) and detergent solution (Gal et al., 2004). Capsule (Reckseidler et al., 2001), Type II secretion system (DeShazer et al., 1999) and Type III secretion system (Pilatz et al., 2006), as well as Type IV pilin (Essex-Lopresti et al., 2005) are all considered as virulence factors of this bacteria.

Production of glycocalyx polysaccharide capsule is one of the virulent determinants for this bacterium (Steinmetz et al., 1995). The complement factor Cb3 is unlikely to opsonize the surface of bacterium with the presence of capsule which then allows the bacterium to form microcolonies and alter the organism’s phenotype, later, contributing to antibiotic resistance properties (Vorachit et al., 1993).

B. pseudomallei also produces proteases, catalase, lipases, lecithinase, peroxidase, hemolysins, superoxide dismutase, a cytotoxic exolipid, and a sidophore which lead to its
resistance to various cell defenses like complement lysosomal defensins and cationic peptidases (Cheng and Currie, 2005).

2.2.3 Laboratory Diagnosis of *B. pseudomallei*

Culture-based method, antibody detection, antigen detection, and molecular techniques are the diagnostic methods for identification of *B. pseudomallei* (Cheng and Currie, 2005). As melioidosis consists of a broad range of non-distinct clinical presentations, hence laboratory diagnosis is extremely important for clinicians to detect melioidosis. Appropriate diagnosis which result in accurate detection of *B. pseudomallei* is essential as delay in diagnosis could cause fatal to the patient (Wiersinga et al., 2012).

Commonly, melioidosis is diagnosed through isolation of the bacterium from patient’s samples such as urine, blood, pus, and body fluid (Haque, 2010). It is then cultured on the selective medium to select for the presence of *B. pseudomallei*. Apart from the aforementioned medium which used for the growth of *B. pseudomallei* in laboratory, Ashdown agar also represents one of the media used to conduct culture-based method for melioidosis diagnostic and it is used extensively particularly in melioidosis endemic areas. Its ingredients include tryptase soy agar with glycerol, crystal violet, neutral red, and gentamicin (4 mg/liter). Later, colistin was added into Ashdown agar to make a modification. Besides, selective broth also available to be utilized for diagnosis of melioidosis from the site of throat, rectal, and wound swabs (Wuthiekanun et al., 1990). *B. pseudomallei* first presents as smooth appearance on agar plate while aging colonies turns dry, wrinkled, and metallic on further incubation. The identity of *B. pseudomallei* can also be recognized through its characteristics which have been mentioned earlier on.

Antibody response can be detected for *B. pseudomallei* through indirect hemagglutination assay (IHA) or ELISA which they have been applied clinically as a
practical way to diagnose melioidosis (Cheng and Currie, 2005). The complement fixation test is also specific and sensitive method for melioidosis diagnostic as mentioned by Nigg and Johnston (1961), only it is rather an older method.

Cheng and Currie (2005) described that antigen detection includes the use of latex agglutination or immunofluorescence. It can be carried out either on specimens or on culture supernatant (Cheng and Currie, 2005). For rapid immunofluorescent test, this method has the ability to identify B. pseudomallei before culture (Gilad et al., 2007).

Molecular method aims to diagnose the presence of B. pseudomallei in a clinical specimen or for confirmation of the purified isolates. Some of the molecular methods are 16S ribosomal RNA real-time polymerase chain reaction (PCR) (Chantratita et al., 2007), 23S ribosomal DNA (Lew and Desmarchelier, 1994), real-time PCR of type III secretion system genes (Thibault et al., 2004b), TaqMan allelic-discrimination assay (U’Ren et al., 2005), and sequencing of the groEL gene (Woo et al., 2001). As mentioned by Richardson et al. (2012), PCR is more rapid in the detection of melioidosis, nevertheless, it is less efficient when tested on blood samples.

Moreover, other methods such as using bacterial fatty acid methyl ester (FAME) profile analysis by gas-liquid chromatography (GLC), substrate utilization panel (API 20NE) also have been reportedly being used for laboratory diagnosis of B. pseudomallei (Inglis et al., 2005).

Furthermore, antibiotic susceptibility test is also a crucial method used in clinical experiment especially for preliminary test, for instance, qualitative disk diffusion and quantitative gradient diffusion (Epsilonmeter test (Etest)) (Reller et al., 2009).
2.3 Antibiotics and *B. pseudomallei*

2.3.1 Antibiotic Resistance of *B. pseudomallei*

*B. pseudomallei* is intrinsically resistant to β-lactams antibiotics, for example, as stated by Wiersinga *et al.* (2012), first-generation and second-generation cephalosporins; also aminoglycosides, such as, gentamicin, tobramycin and streptomycin; polymyxins, for instance, colistin; penicillin; ampicillin (Wiersinga *et al.*, 2012) as well as macrolides like erythromycin (Sarovich *et al.*, 2012a). As stated by Wiersinga *et al.* (2012), the latest antibiotics such as ertapenem, tigecycline, and moxifloxacin shown restricted *in vitro* activity against clinical strains of *B. pseudomallei*. There are different types of resistance mechanisms conferring to its resistance properties such as inactivation of enzyme, target deletion or alteration of target sites, efflux from the cell, cell exclusion, and drug sequestration. As stated by Schweizer (2012), chromosomally encoded genes are the major driving factor of all resistance in *B. pseudomallei* recorded up to year 2012. However, most of the resistance mechanisms are only putative. Also, resistance somehow evolves from the frequent and common use of the antibiotic, particularly in endemic areas, for instance, ceftazidime resistance has been reported in Australia (Sarovich *et al.*, 2012a), Malaysia (Sam *et al.*, 2009), and Thailand (Chantratita *et al.*, 2011; Sarovich *et al.*, 2012b).

Enzyme inactivation is one form of antimicrobial resistance mechanism of *B. pseudomallei*. Modifications such as acyltransfer, glycosylation or phosphorylation as well as cleavage are including in the mechanism of this enzymatic inactivation (Wright, 2005). Following the report by Schweizer (2012), cleavage of β-lactam antibiotics is the only recorded clinically important enzyme inactivation mechanism in *B. pseudomallei* which it is done by chromosomally encoded class A β-lactamase, PenA. PenA in *B. pseudomallei* confers resistance to some of the β-lactam antibiotics such as amoxicillin and carbenicillin.