The epidemiology and molecular characterization of
*Burkholderia pseudomallei*

in

Malaysian Borneo

By

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(B.Sc, M.Sc.)

A thesis submitted in fulfilment of the requirements for the degree of

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Institute of Advanced Studies

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DECLARATION

I hereby declare that the work herein, now submitted as a thesis for the degree of Doctor of Philosophy of the Charles Darwin University, is the result of my own investigations, and all references to ideas and work of other researchers have been specifically acknowledged. I hereby certify that the work embodied in this thesis has not already been accepted in substance for any degree, and is not being currently submitted in candidature for any other degree.

Yuwana Podin

December 2014
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**Dedication**
This thesis is dedicated to both my departed parents. I will not be what or where I am without your love and sacrifices. Thank you Papa and Mummy for being the wind beneath my wings.
DECLARATION OF AUTHOR’S CONTRIBUTION IN THE PUBLICATIONS ARISEN FROM THIS THESIS

This thesis is substantially my own work including developing the research questions, designing the methodology, applications of research permits, collecting and analysing data and interpreting the results, performed under the guidance of my supervisors Prof. Phil Giffard, Prof. Bart Currie and Dr. Mirjam Kaestli. I have written all the chapters in this thesis and the papers where I am the first author arisen from this thesis with contribution of wordings by my supervisors.

I hereby acknowledge the following contributions of co-authors in the publications arisen from this thesis:

Chapter 3: Automated biochemical characterization of *Burkholderia pseudomallei* from Malaysian Borneo

I applied for research permits and ethics approval from the various government agencies in Malaysia and Sarawak state for the collection of *B. pseudomallei* isolates used in this study, visited the hospitals that provided the clinical isolates, prepared and coordinated for the isolates to be transported from the various hospitals to the collaborative centre at Universiti Malaysia Sarawak and eventually to Menzies School of Health Research, conducted all the laboratory tests on the isolates, performed the data analysis and wrote the manuscript for publication and the chapter for this thesis. Dr. Mirjam Kaestli assisted in statistical analysis of the work in this chapter and provided critical input in the writing process of the manuscript. Ms. Nicole McMahon, Ms. Jann Hennessy and Mr. Robert Baird assisted in performing tests on the isolates using the Vitek 2 machine at different times. Dr. HieUng Ngian, Dr. JinShyan Wong, Dr. Anand Mohana, Dr. SeeChang Wong and Dr. Timothy William recruited the patients and provided clinical data of the patients whose isolates were used in this study. Mr. Mark Mayo assisted in re-culturing of the isolates at Menzies School of Health Research PC3 laboratory. Prof. Bart Currie conceived the idea and contributed in providing critical input as well as wording of some parts of the manuscript.
Chapter 4: Characterization of aminoglycoside and macrolide susceptible \textit{B. pseudomallei} from Malaysian Borneo

I applied for research permits and ethics approval from the various government agencies in Malaysia and Sarawak state for the collection of \textit{B. pseudomallei} isolates used in this study, visited the hospitals that provided the clinical isolates, prepared and coordinated for the isolates to be transported from the various hospitals to the collaborative centre at Universiti Malaysia Sarawak and eventually to Menzies School of Health Research, designed the reversion assay experiments, conducted all the laboratory tests and experiments on the isolates, performed the data analysis and wrote the manuscript for publication and the chapter for this thesis. Dr. Derek Sarovich assisted in the analysis of the whole genome sequence, provided intellectual input into the design of the experiments and provided critical input into the manuscript and chapter arisen from this work. Dr. Erin Price assisted in the analysis of the whole genome sequence of the isolates used in the study and provided critical input during the preparation of the manuscript and the chapter arisen from this work. Dr. Mirjam Kaestli assisted in the molecular testing of the isolates and provided critical input during the preparation of the manuscript and the chapter arisen from this work. Mr. Mark Mayo assisted in the culture of the isolates and provided critical input during the preparation of the manuscript and the chapter arisen from this work. Dr. KingChing Hii, Dr. HieUng Ngian, Dr. SeeChang Wong, Dr. IngTien Wong, Dr. JinShyan Wong, Dr. Anand Mohan, Dr. MongHow Ooi, Dr. TemLom Fam and Dr. Jack Wong recruited the patients and provided clinical data of the patients whose isolates were used in this study. Assist. Prof. Apichai Tuanyok and Prof. Paul Keim provided support in the whole genome sequencing of the isolates used in this study. Prof. Phil Giffard and Prof. Bart Currie conceived the idea and contributed in providing critical input and wordings into the manuscript preparation as well as the chapter arisen from this work.
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ABSTRACT

Melioidosis is a potentially fatal disease caused by *Burkholderia pseudomallei* which is endemic in Malaysian Borneo. The general aim of this study is to elucidate the molecular epidemiology of *B. pseudomallei* in Malaysian Borneo. Consistent with the Wallace line theory of separation, genotyping showed Malaysian Borneo clinical *B. pseudomallei* isolates were more related to Southeast Asian strains than to Australian strains. Whole genome sequencing demonstrated that *B. pseudomallei* from Sarawak were very closely related to each other. Biochemical testing using VITEK 2 revealed that 25% of *B. pseudomallei* from Malaysian Borneo were misidentified as *B. cepacia*, suggesting that specificity of that identification system is regionally dependent. A major and unexpected finding was that 88% of Sarawak *B. pseudomallei* were gentamicin susceptible, with these *B. pseudomallei* being restricted to multilocus sequence type ST881 and its single locus variant ST997. A novel non-synonymous mutation was identified within *amrB*, an essential component of the AmrAB-OprA multi-drug efflux pump. Reversion of the mutation to the wild-type sequence confirmed the role of this mutation in conferring aminoglycoside and macrolide sensitivity. No environmental *B. pseudomallei* were isolated from Sarawak but other *Burkholderia* species were isolated, prompting the formulation of hypotheses to explain the lack of environmental *B. pseudomallei*. Although inconclusive, experiments showed antagonistic activities by other environmental *Burkholderia* spp. recovered from environmental sampling studies towards *B. pseudomallei* and also that gentamicin susceptible *B. pseudomallei* were slightly
less robust than gentamicin resistant strains in competing with other soil microorganisms. This thesis contributed to the understanding of the population structure of _B. pseudomallei_ in Malaysian Borneo, Southeast Asia and globally. The discovery of gentamicin susceptibility in Sarawak _B. pseudomallei_ has significant implications for laboratory diagnosis and environmental sampling of _B. pseudomallei_ in Malaysian Borneo and potentially in other melioidosis endemic regions. Although the exact distributions, quantification and potential environmental hazards and implications of _B. pseudomallei_ in Malaysian Borneo remain uncertain, these studies have led to important research questions now to be explored. Most immediate is further searching for the proposed existence of an as yet unidentified localized niche of _B. pseudomallei_ in Malaysian Borneo.
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>amrB-C1102G</td>
<td>Cytosine to guanine transition at 1102 nucleotide position of the amrB gene</td>
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<tr>
<td>AMX</td>
<td>Amoxicillin-clavulanic acid</td>
</tr>
<tr>
<td>ANOSIM</td>
<td>Analysis of similarities</td>
</tr>
<tr>
<td>AQIS</td>
<td>Australian Quarantine and Inspection Service</td>
</tr>
<tr>
<td>AS-PCR</td>
<td>Allelic-specific PCR</td>
</tr>
<tr>
<td>AZM</td>
<td>Azithromycin</td>
</tr>
<tr>
<td>Bcc</td>
<td><em>Burkholderia cepacia</em> complex</td>
</tr>
<tr>
<td>bimA&lt;sub&gt;Bm&lt;/sub&gt;</td>
<td>B. mallei-like BimA allele</td>
</tr>
<tr>
<td>bimA&lt;sub&gt;Bp&lt;/sub&gt;</td>
<td>B. pseudomallei BimA allele</td>
</tr>
<tr>
<td>BNAG</td>
<td>β-N-acetyl-glucosaminidase</td>
</tr>
<tr>
<td>bp</td>
<td>Base-pairs</td>
</tr>
<tr>
<td>BPSA</td>
<td>B. pseudomallei selective agar</td>
</tr>
<tr>
<td>BTFC</td>
<td><em>B. thailandensis</em>-like flagellum and chemotaxis biosynthesis gene cluster</td>
</tr>
<tr>
<td>C</td>
<td>Cytosine</td>
</tr>
<tr>
<td>C→G</td>
<td>Cytosine to guanine transition</td>
</tr>
<tr>
<td>CAZ</td>
<td>Ceftazidime</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical and Laboratory Standard Institute, USA.</td>
</tr>
<tr>
<td>Ct</td>
<td>Threshold cycle</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleoside triphosphates</td>
</tr>
<tr>
<td>DOX</td>
<td>Doxycycline</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assays</td>
</tr>
<tr>
<td>EPS</td>
<td>Exopolysaccharide</td>
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<tr>
<td>FAM</td>
<td>FAM blue reporter dye</td>
</tr>
<tr>
<td>G</td>
<td>Guanine</td>
</tr>
<tr>
<td>GEN</td>
<td>Gentamicin</td>
</tr>
<tr>
<td>GEN&lt;sup&gt;r&lt;/sup&gt;</td>
<td>Gentamicin resistance</td>
</tr>
<tr>
<td>GEN&lt;sup&gt;s&lt;/sup&gt;</td>
<td>Gentamicin sensitive</td>
</tr>
<tr>
<td>HBA</td>
<td>Horse blood agar</td>
</tr>
<tr>
<td>IFAT</td>
<td>Immunofluorescent assay test</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>IHA</td>
<td>Indirect hemagglutination assay</td>
</tr>
<tr>
<td>KAN</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>kb</td>
<td>Kilo base-pairs</td>
</tr>
<tr>
<td>LGT</td>
<td>Lateral gene transfer</td>
</tr>
<tr>
<td>MALDI-TOF MS</td>
<td>Matrix-assisted laser desorption ionization-time of flight mass spectrometry</td>
</tr>
<tr>
<td>Mbp</td>
<td>Mega base-pairs</td>
</tr>
<tr>
<td>MEM</td>
<td>Meropenem</td>
</tr>
<tr>
<td>MH</td>
<td>Mueller- Hinton’s agar</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimal inhibition concentration</td>
</tr>
<tr>
<td>MLST</td>
<td>Multilocus sequence typing</td>
</tr>
<tr>
<td>MLVA</td>
<td>Multilocus VNTR analysis</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>---------------------------------------------------------</td>
</tr>
<tr>
<td>MGBNFQ</td>
<td>Molecular-groove binding non-fluorescence quencher</td>
</tr>
<tr>
<td>NAGA</td>
<td>β-N-acetyl-galactosaminidase</td>
</tr>
<tr>
<td>NED</td>
<td>NED yellow reporter dye</td>
</tr>
<tr>
<td>NGS</td>
<td>Next-generation sequencing</td>
</tr>
<tr>
<td>nMDS</td>
<td>Nonmetric multidimensional scaling</td>
</tr>
<tr>
<td>NT</td>
<td>Northern Territory</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>orf2</td>
<td>Open reading frame 2</td>
</tr>
<tr>
<td>PC2</td>
<td>Physical Containment Level 2 (equivalent of BSL-2)</td>
</tr>
<tr>
<td>PET</td>
<td>PET red reporter dye</td>
</tr>
<tr>
<td>PFGE</td>
<td>Pulsed-field gel electrophoresis</td>
</tr>
<tr>
<td>PNAG</td>
<td>Poly-β-(1-6)-N-acetyl-glucosamine</td>
</tr>
<tr>
<td>R</td>
<td>Arginine</td>
</tr>
<tr>
<td>RAPD</td>
<td>Randomly amplified polymorphic DNA</td>
</tr>
<tr>
<td>RDH</td>
<td>Royal Darwin Hospital</td>
</tr>
<tr>
<td>rDNA</td>
<td>Ribosomal DNA</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RND</td>
<td>Resistance-nodulation-division</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SIMPER</td>
<td>Similarity percentages</td>
</tr>
<tr>
<td>SLV</td>
<td>Single locus variant</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>spp.</td>
<td>Species</td>
</tr>
<tr>
<td>ST</td>
<td>Sequence type</td>
</tr>
<tr>
<td>SXT</td>
<td>Trimethoprim-sulfamethoxazole</td>
</tr>
<tr>
<td>T</td>
<td>Threonine</td>
</tr>
<tr>
<td>T367R</td>
<td>Threonine to arginine substitution at amino acid position 367</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptic soy agar</td>
</tr>
<tr>
<td>TTS1</td>
<td>Type III secretion system</td>
</tr>
<tr>
<td>UMMC</td>
<td>University Malaya Medical Centre</td>
</tr>
<tr>
<td>UNIMAS</td>
<td>Universiti Malaysia Sarawak</td>
</tr>
<tr>
<td>US CDC</td>
<td>United States Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>USAMRU</td>
<td>United States of America Medical Research Unit</td>
</tr>
<tr>
<td>VNTR</td>
<td>Variable numbers tandem repeat</td>
</tr>
<tr>
<td>WGS</td>
<td>Whole genome sequencing</td>
</tr>
<tr>
<td>YLF</td>
<td>Yersinia-like fimbrial gene cluster</td>
</tr>
<tr>
<td>ΔCt</td>
<td>Threshold cycle difference</td>
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</table>
Chapter 1

General Introduction

and

Literature Review
Chapter 1: General introduction and literature review

1.1 General introduction: Burkholderia pseudomallei and melioidosis

1.1.1 Burkholderia genus
The Burkholderia genus was formed in 1992 when seven members of the Pseudomonas species were transferred over to the newly formed Burkholderia genus (Yabuuchi et al., 1992). The name “Burkholderia” was coined to honour Walter Burkholder who discovered Pseudomonas cepacia which caused onion diseases in the 1940s and 1950s (Yabuuchi et al., 1992, Coenye and Vandamme, 2007). To date, the Burkholderia genus has over 60 species residing in diverse ecologies including environmental and biological niches (http://www.bacterio.net/burkholderia.html)(Coenye and Vandamme, 2003, Vandamme and Dawyndt, 2011). Most Burkholderia species (spp.) are considered plant pathogens, plant commensals and soil bacteria, but some have the ability to cause infections in humans and animals (Coenye et al., 2001, Coenye and Vandamme, 2003, Coenye and Vandamme, 2007, Mahenthiralingam et al., 2005). There are 17 closely related Burkholderia spp. that are categorised as Burkholderia cepacia complex (Bcc). While some Bcc members are considered plant pathogens, others are plant commensals and also bioremediation agents which degrade environmental pollutants (Mahenthiralingam et al., 2005). In addition to their roles in plants and environments, some Bcc have been reported to cause opportunistic infection in both immunocompromised and cystic fibrosis (CF) patients (Coenye and Vandamme, 2003, Coenye and Vandamme, 2007, Vandamme and Dawyndt, 2011).

1.1.2 Burkholderia pseudomallei
Burkholderia pseudomallei is the bacterium that causes melioidosis which was first described by Whitmore and Krishnaswami in Rangoon, Burma in 1911 (Whitmore and Krishnaswami, 1912). Due to the resemblance of the bacterium to Bacillus mallei which causes glanders disease, it was named Bacillus pseudomallei. Over the years, the bacterium was also known by different names such as Bacillus whitmorii (Bacille de Whitmore in French), Malleomyces
*Pseudomonas pseudomallei* and finally *B. pseudomallei* in 1992 (Yabuuchi et al., 1992). It is a non-spore forming motile gram-negative bacillus with bipolar staining, often described as resembling a safety pin in a Gram stain (Cheng and Currie, 2005). The genome of *B. pseudomallei* is comprised of two circular chromosomes with a combined length of 7.2 mega base-pairs (Mbp), encompassing approximately 5800 genes (Holden et al., 2004, Nandi et al., 2010). Chromosome 1 contains housekeeping genes for macromolecular biosynthesis, amino acid metabolism, cofactor and carrier synthesis, nucleotide and protein biosynthesis, chemotaxis, and mobility. Chromosome 2 contains genes for accessory functions such as adaptations to atypical conditions, iron homeostasis, secondary metabolism, regulation and horizontal gene transfer (Holden et al., 2004). Due in part to the high virulence of this organism and increased concerns for transmission by aerosolization, *B. pseudomallei* was classified as a Tier 1 select agent by the U.S. Centres for Disease Control and Prevention (US CDC) in 2012 (http://www.selectagents.gov/).

*B. pseudomallei* is closely related to two other *Burkholderia* spp. namely *Burkholderia thailandensis* and *Burkholderia mallei* (Brett et al., 1998, Nierman et al., 2004). Despite its high genomic similarities to *B. pseudomallei*, *B. thailandensis* is an avirulent soil bacterium that is able to utilize arabinose as a sole energy source (Brett et al., 1998, Trakulsomboon et al., 1999). *B. mallei* is a zoonotic host-adapted pathogen affecting mainly equines and causes glanders disease (Nierman et al., 2004). Studies showed that *B. mallei* evolved from a single strain of *B. pseudomallei* ancestor through erosion of nonessential genomes responsible for environmental survival and became completely host-adapted. It was also shown that the genome of *B. mallei* is approximately 1.5 Mbp smaller than that of its ancestor (Godoy et al., 2003, Nierman et al., 2004, Losada et al., 2010).

*B. pseudomallei* is commonly found in the soil and water of melioidosis endemic regions, which include Southeast Asian countries, northern Australia and other tropical regions (White, 2003, Cheng and Currie, 2005). As more melioidosis areas are discovered, the knowledge on global distribution will keep expanding over time (Currie et al., 2008, Wiersinga et al., 2012).
1.1.3 Melioidosis

Coined by Stanton and Fletcher in 1921, melioidosis was derived from Greek which means ‘distemper of asses’ (Stanton and Fletcher, 1921). Patients with melioidosis can present with a spectrum of clinical presentations including localized skin abscess, acute or chronic pneumonia, genitourinary, bone, and joint infections and severe systemic sepsis (with or without foci of multiple abscesses in internal organs). Patients with septic shock may have a mortality of >90% (White, 2003, Cheng and Currie, 2005). Asymptomatic and chronic infections have been reported where the organism has been shown to evolve within the host (Price et al., 2010, Price et al., 2013a).

Mode of acquisition

The mode of acquisition of melioidosis is via percutaneous inoculation, inhalation or ingestion of *B. pseudomallei* contaminated wet soil or surface water or aerosols (Puthucheary and Vadivelu, 2002, Cheng and Currie, 2005, Wiersinga et al., 2012).

Inoculation has been suggested to be the main mode of acquisition. Individuals with occupational risks such as farmers and construction workers were reportedly exposed to *B. pseudomallei* through open wound or penetrating injuries (Chaowagul et al., 1989, Cheng and Currie, 2005, Kaestli et al., 2009, Wiersinga et al., 2012). In a study in the Top End, Australia, 25% of melioidosis patients were shown to have some history of inoculation via skin breakage prior to disease. Despite being acquired through skin inoculation, the range of disease in the patients in this study was not confined to merely subcutaneous symptoms as many patients presented with more severe illnesses (Currie et al., 2000b).

Inhalation was implicated as the primary mode of acquisition for melioidosis cases amongst helicopter crews of the United States armies in Vietnam in the 1960s (Howe et al., 1971). Since then, inhalation has been considered to be an important mode of acquisition especially during increased heavy rainfall and during extreme weather events such as strong winds, cyclones or typhoons (Puthucheary and Vadivelu, 2002, Currie and Jacups, 2003, Ko et al., 2007, Lo et al., 2009, Su et al., 2011).
The first implication of ingestion as a mode of acquisition was by Stanton and Fletcher in 1925 (Stanton and Fletcher, 1925). Although this route has not been described much in detail, reports of melioidosis outbreaks due to contaminated water supply have implicated ingestion as a mode of acquisition (Inglis et al., 1999, Currie et al., 2001). The high incidence of suppurative parotitis amongst paediatric patients mainly in South East Asia has been suggested to be associated with ingestion (Dance et al., 1989a) and a recent case control study from Thailand has supported this, together with the high rates of recovery of *B. pseudomallei* from domestic water supplies in that study (Limmmathurotsakul et al., 2013b).

*B. pseudomallei* acquisition has also been associated with tsunami where survivors of the disaster in affected countries were presented with various symptoms including cutaneous infection, pulmonary involvement and septicaemia (Allworth, 2005, Athan et al., 2005, Chierakul et al., 2005, Nieminen and Vaara, 2005, Svensson et al., 2006, Othman et al., 2007, Currie et al., 2008, Arzola et al., 2007).

Other modes of acquisition are via laboratory-acquired infection (Green and Tuffnell, 1968, Schlech et al., 1981), contaminated detergent and other medical supplies (Punyagupta, 1989, Gal et al., 2004), breast milk (Ralph et al., 2004), person-to-person transmission (McCormick et al., 1975, Holland et al., 2002), possible sexual transmission (McCormick et al., 1975, Webling, 1980) and intra-uterine transmission (Abbink et al., 2001). Although rare, zoonotic human infections have also been reported (Low Choy et al., 2000).

**Treatment of melioidosis**

Due to both the intracellular nature of the organism after infection and its potential for latency, there are two phases in melioidosis treatment; a short-term intensive acute phase and a long-term oral eradication phase (Wiersinga et al., 2012). In general, the intensive acute phase treatment involves bactericidal drugs with or without post-antibiotic effect, while the eradication phase treatment involves bacteriostatic drugs (Estes et al., 2010, Wiersinga et al., 2012). The current recommended treatment of melioidosis consists of an intensive phase of at least 10 to 14 days of intravenous ceftazidime, meropenem, or imipenem,
followed by oral eradication therapy of trimethoprim–sulfamethoxazole for three to six months (Wiersinga et al., 2012).

**Drug susceptibility and resistance**

*B. pseudomallei* is intrinsically resistant to numerous antibiotics including first-generation and second-generation quinolones, all narrow-spectrum cephalosporins, all macrolides, most penicillins, all polymyxins, and aminoglycosides (Livermore et al., 1987, Dance et al., 1989c, Dance et al., 1989b, Jenney et al., 2001, Estes et al., 2010, Wuthiekanun et al., 2011), which limits treatment options for melioidosis. Although rare, aminoglycosides and macrolides sensitivity has been reported (Simpson et al., 1999, Trunck et al., 2009, Podin et al., 2014). *B. pseudomallei* is generally susceptible to carbapenems, some fluoroquinolones, and amoxicillin-β-lactamase inhibitor combinations, chloramphenicol, tetracyclines and trimethoprim-sulfonamides in vitro (Ashdown, 1988, Dance et al., 1989c, Wuthiekanun et al., 2011). However, clinical studies have shown that fluoroquinolones are associated with higher treatment failure rates (Chaowagul et al., 1997, Chetchotisakd et al., 2001, Steward et al., 2005, Wuthiekanun et al., 2011). Although uncommon, acquired resistance to ceftazidime during therapy has been documented and genetically characterised (Jenney et al., 2001, Chantratita et al., 2011, Sarovich et al., 2012a, Sarovich et al., 2012b).

**Relapse of melioidosis**

Relapse in melioidosis upon completion of treatment has been well documented (Puthucheary and Vadivelu, 2002, White, 2003, Cheng and Currie, 2005, Sarovich et al., 2014b). There have also been reports of reactivation of melioidosis for as long as 62 years after primary exposure to the causative agent (Ngauy et al., 2005). The propensity for relapse is caused by factors such as poor adherence to treatment, severe diseases, inadequate use of drug type during the intensive and eradication phase, and eradication phase of less than eight weeks (Chaowagul et al., 1993, Currie et al., 2000a). While most of the relapse cases were associated with reactivation of the original infecting strain, re-infection with a different strain has also been reported (Desmarchelier et al., 1993, Vadivelu et al., 1998, Currie et al., 2000a, Sarovich et al., 2014b).