ELUCIDATING THE GENETIC VARIANTS OF THE TYPE VI SECRETION SYSTEM 5 (T6SS-5) IN CLINICAL AND ENVIRONMENTAL STRAINS OF *Burkholderia pseudomallei* AND THEIR POTENTIAL CORRELATION WITH THE CLINICAL COURSES OF THE HUMAN DISEASES

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2024

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by

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Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

September 2024

ACKNOWLEDGEMENT

In the name of ALLAH, the most Merciful, the most Compassionate.

Alhamdulillah, all praise and thanks be to Him, the most gracious and the most merciful for his mercy that I can survive my PhD journey and complete this thesis successfully. First of all, I would like to express my deepest gratitude to my dearest supervisor, Professor Dr. Zakuan Zainy Deris, for his continuous guidance, support and patience towards me throughout this study. Special thanks also to my beloved co-supervisors, Assoc. Prof. Dr. Azian Harun and Assoc. Prof. Dr. Aziah Ismail. I am truly indebted and sincerely grateful to my lab mates and friends, especially Dr. Nik Zuraina, Yasmin, Iman, Syafiqah, Anis, and Dr. Engku Syafira, for providing me with valuable help in many aspects. My appreciation also goes out to my other lab mates, lecturers, and staff in the Departments of Medical Microbiology and Parasitology, USM. To my dear husband, Ahmad Nabil bin Zainodin, your continuous love and support are deeply appreciated. Thank you for being by my side throughout this journey. May Allah bless us with abundant happiness and beautiful moments together. To my beloved mother, Rohana binti Mat Noh, words cannot adequately express my appreciation for you ummi. And to my late father, Semail bin Bakar, whom I dearly miss, this is dedicated to you abi. I strive to make you proud, and how I wish you were here with me at the end of my PhD adventure. My deepest gratitude also goes to my siblings, in-laws, and dearest best friends. I am also gratefully acknowledged the Ministry of Higher Education Malaysia for Fundamental Research Grant Scheme (FRGS) with Project Code: FRGS/1/2019/SKK11/USM/02/5. Sincerely, I could not afford to pay for all the kindness from anyone who had involved directly or indirectly in my PhD journey. Again, “Jazakumullahukhairan kathira.”

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS AND SYMBOLS

-	negative or substraction
%	percentage
/	division or 'or'
~	approximately
+	positive or addition
=	equal to
±	plus-minus
≤	less than or equal to
≥	more than or equal to
°C	degree Celcius
µl	microliter
µm	micrometer
µM	micromolar
A	adenine
A_{260}	absorbance at 260 nm
A_{280}	absorbance at 280 nm
ATCC	American Type Culture Collection
BHI	brain-heart infusion
BimA	*Burkholderia* intracellular motility A
BLAST	Basic Local Alignment Search Tool
bp	base pair
B. pseudomallei	*Burkholderia pseudomallei*
C	cytosine
CDC	Centers for Disease Control
CFU	colony forming unit

CI	confidence interval
c-GAS	cyclic-GMP-AMP synthase
CPS	capsular polysaccharide
CTD	C-terminal domain
df	degree of freedom
dH_2O	distilled water
DM	diabetes mellitus
dNTPs	deoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
E. faecalis	*Enterococcus faecalis*
et. al.	*et alia* (and others)
F	forward or sense primers
g	gram
g	gravitational force
G	guanine
GC	guanine-cytosine
GSH	glutathione
HCl	hydrochloric acid
hcp	haemolysin co-regulated protein
H. pylori	*Helicobacter pylori*
IFN	Interferon
Indel	Insertion deletion
INFORMM	Institute for Research in Molecular Medicine
kDa	kilodaltons
K. pneumoniae	*Klebsiella pneumonia*

L	liter
LB	Luria-Bertani
LIS	laboratory information system
LOD	limit of detection
LPS	lipopolysaccharides
M	molar
Mb	million base pair
MDR	multiple drug resistant
MEGA	Molecular Evolutionary Genetics Analysis
mg	milligram
Mg^{2+}	magnesium ions
$MgCl_2$	magnesium chloride
MH	Mueller Hinton
ml	milliliter
MLST	multi-locus sequence typing
mm	millimeter
mM	millimolar
MNGC	multinucleated giant cell
Mg^{2+}	magnesium ions
$MgCl_2$	magnesium chloride
MH	Mueller Hinton
ml	milliliter
MLST	multi-locus sequence typing
mm	millimeter
mM	millimolar
MNGC	multinucleated giant cell
n	frequency or total

N	grand total
NaCl	sodium chloride
NaLC	N-acetyl-L-cysteine
NaOH	sodium hydroxide
NCBI	National Centre for Biotechnology Information
nm	nanometer
N. meningitidis	*Neisseria meningitidis*
OD_{600}	optical density at 600 nm wave length
OMV	outer membrane vesicles
P. aeruginosa	*Pseudomonas aeruginosa*
P. mirabilis	*Proteus mirabilis*
PCR	polymerase chain reaction
PubMLST	public database for multi-locus sequence typing
QS	quorum sensing
QUAST	quality assessment tool for genome assemblies
SD	standard deviation
SEM	scanning electron microscope
spp.	species
SNP	single nucleotide polymorphism
S. enterica serovar thypimurium	*Salmonella enterica serovar thypimurium*
S. epidermidis	*Staphylococcus epidermidis*
S. maltophilia	*Stenotrophomonas maltophilia*
S. viridans	*Streptococcus viridans*
ST	sequence type
STING	stimulator of interferon genes
T	thymine

T1SS	type I secretion system
T2SS	type II secretion system
T3SS	type III secretion system
T4SS	type IV secretion system
T5SS	type V-secretion system
T6SS	type VI secretion system
T_A	annealing temperature
Taq	*Thermus aquaticus*
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
USM	Universiti Sains Malaysia
UTIs	urinary tract infections
v	volts
vgrG	valine-glycine repeat protein G
WHO	World Health Organization
WGS	whole genome sequencing
V. parahaemolyticus	*Vibrio parahaemolyticus*
V. cholerae	*Vibrio cholerae*
×	times or multiply
X	hemin growth factor

LIST OF APPENDICES

MERUNGKAI VARIAN GENETIK SISTEM REMBESAN 5 JENIS VI (T6SS-5) DALAM STRAIN KLINIKAL DAN PERSEKITARAN *Burkholderia pseudomallei* SERTA KEMUNGKINAN HUBUNGKAIT DENGAN KEADAAN KLINIKAL PENYAKIT MANUSIA.

ABSTRAK

Burkholderia pseudomallei, penyebab melioidosis, adalah endemik di Asia Tenggara dan tropika Australia. Walaupun pengetahuan dan pemahaman mengenai penyakit ini telah meningkat, kadar kematian masih tetap tinggi. Secara khususnya, faktor virulensi bakteria iaitu sistem rembesan jenis enamke-5 (T6SS-5) memainkan peranan penting dalam kemasukan bakteria ke dalam sel ini, namun varian genetiknya serta hubungannya dengan keadaan klinikal melioidosis masih kurang difahami. Dengan menggunakan PCR multipleks dan analisis penjujukan genom penuh (WGS), kajian ini bertujuan untuk menjelaskan variasi genetik T6SS-5 dan kemungkinan hubungkait dengan keparahan penyakit dan hasilnya. Tiga ujian PCR multipleks telah dibangunkan untuk pengesanan 18 gen dalam cluster T6SS-5 *B. pseudomallei* iaitu: Ujian1: *tss*C-5, *tag*D-5, *tss*A-5, *hcp*-5, *tss*B-5, *tss*F-5, dan *vgr*G-5; Ujian 2: *tss*L-5, *tss*J-5, *tss*G-5, *vir*A-5, *tag*B-5, dan *tag*AB-5; dan Ujian 3: *clp*V-5, *tss*E-5, *tss*M-5, *vir*G-5, dan *tss*K-5. Langkah pertama dalam membina ujian PCR ini adalah merancang primer khusus. Kekhususan dan kepekaan dinilai pada setiap set primer secara individu. Isolat klinikal *B. pseudomallei* digunakan untuk menilai ketepatan awal ujian PCR multipleks ini. Keputusan menunjukkan bahawa semua primer yang direka untuk kluster gen T6SS-5 adalah spesifik kepada bakteria yang berkenaan. Konsentrasi optimum primer untuk setiap ujian multipleks adalah berbeza untuk setiap pasangan primer, antara 2.5 – 20.0 µM. Suhu penyepulih-indapan optimum bagi ujian ini 61 °C. Had pengesanan untuk Ujian 1, Ujian 2, dan Ujian 3 telah ditentukan masing-masing

pada 10^3 CFU/ml, 10^8 CFU/ml, dan 10^7 CFU/ml untuk mengesan semua gen yang dijangkakan dalam setiap ujian multiplex. Saringan gen T6SS-5 telah dijalankan pada 88 isolat klinikal dan 2 isolat persekitaran. Hasil kajian menunjukkan bahawa majoriti isolat klinikal (83%; *n* = 73) dan semua isolat persekitaran (100%; *n* = 2) mempunyai semua 18 gen sasaran dalam genom mereka. Variasi kumpulan gen T6SS-5 dilihat dalam gen *tss*E-5, *tss*M-5, *vir*G-5, dan *tss*K-5. Walau bagaimanapun, tiada hubungan yang signifikan diperhatikan antara kehadiran gen-gen ini dan keadaan klinikal serta kesudahan penyakit melioidosis. Selain analisis PCR multipleks, WGS telah dilakukan pada 18 isolat, yang digolongkan kepada kumpulan pesakit yang sembuh (enam isolat), berulang (empat isolat), meninggal dunia (enam isolat), dan persekitaran (dua isolat). Sebanyak 1495 mutasi polimorfisme nukleotida tunggal (SNP) diedarkan di seluruh kelompok gen T6SS-5, dengan mutasi tertinggi ditemui dalam gen *clp*V-5. SNP tidak sinonim bagi *clp*V-5, *tss*M-5, *tss*A-5, dan *tag*AB-5 menunjukkan hubungan yang signifikan (nilai $p < 0.0001$, < 0.0001, 0.0067, dan 0.0207, masing-masing) dengan jangkitan bakteremia di kalangan pesakit. Dianggarkan 149 mutasi penambahan/pengurangan (indels) telah dikenal pasti, dengan kejadian yang ketara dalam gen *tss*A-5, *clp*V-5, *tag*AB-5, *tag*C-5, dan *tss*M-5. Walau bagaimanapun, tidak ada hubungan yang signifikan diperhatikan antara mutasi indels gen-gen ini dengan keadaan klinikal serta kesudahan penyakit melioidosis. Secara keseluruhannya, kajian ini menekankan kompleksiti patogenesis melioidosis dan kepentingan T6SS-5 sebagai penentu virulensi, serta keperluan untuk penyelidikan lanjut bagi mengungkap penentu virulensi ini. Memahami variasi genetik ini dapat membantu dalam membangunkan terapi bergerak sasaran atau diagnostik untuk melioidosis.

ELUCIDATING THE GENETIC VARIANTS OF THE TYPE VI SECRETION SYSTEM 5 (T6SS-5) IN CLINICAL AND ENVIRONMENTAL STRAINS OF *Burkholderia pseudomallei* AND THEIR POTENTIAL CORRELATION WITH THE CLINICAL COURSES OF THE HUMAN DISEASES.

ABSTRACT

Burkholderia pseudomallei, a pathogenic bacterium that causes melioidosis, is endemic in Southeast Asia and tropical Australia. Although knowledge and understanding of this neglected infectious disease have increased, the fatality rate remains high. Notably, the bacterial virulence factor type six secretion system-5 (T6SS-5) plays a significant role in this cellular internalization, yet its genetic variants and correlation with clinical outcomes of melioidosis remain unexplored. By employing multiplex PCR and whole genome sequencing (WGS) analysis, this study aimed to elucidate T6SS-5 genetic variations and their potential association with disease severity and outcome. Three multiplex PCR assays were developed for the detection of 18 genes within the T6SS-5 cluster of *B. pseudomallei* i.e. Assay 1: *tss*C-5, *tag*D-5, *tss*A-5, *hcp*-5, *tss*B-5, *tss*F-5, and *vgr*G-5; Assay 2: *tss*L-5, *tss*J-5, *tss*G-5, *vir*A-5, *tag*B-5, and *tag*AB-5; and Assay 3; *clp*V-5, *tss*E-5, *tss*M-5, *vir*G-5, and *tss*K-5. Designing specific primers was the first step in constructing these PCR assays. The specificity and sensitivity were assessed on each individual set of primers. Clinical *B. pseudomallei* isolates were used to evaluate the initial accuracy of these multiplex PCR assays. Results indicated that all the designed primers for T6SS-5 gene cluster were specific to the respective target bacteria. The optimized concentrations of primers for each multiplex assay were varied for each primer pair, between 2.5 – 20.0 µM. The optimum annealing temperature for these assay was 61 °C. The limit of detection for

Assay 1, Assay 2 and Assay 3 were determined to be at 10^3 CFU/ml, 10^8 CFU/ml, and 10^7 CFU/ml, respectively, to detect all the targeted genes in each multiplex assay. Initial evaluation of clinical isolates indicated that the assays were 100% accurate on both target and non-target bacteria (n = 28). Screening of T6SS-5 genes was conducted on 88 clinical and 2 environmental isolates. Results showed that majority of the clinical isolates (83%; n=73) and all environmental isolates (100%; n=2) were able to detect all the 18 target genes in their genomes. Variations in detection of the T6SS-5 gene cluster were seen in the *tss*E-5, *tss*M-5, *vir*G-5, and *tss*K-5 genes. However, there was no significance association observed between the presence of these genes and the types of clinical presentation and outcomes of the disease. In addition to multiplex PCR analysis, WGS was performed on 18 isolates, stratified into recovered (six isolates), relapsed (four isolates), deceased (six isolates) patient groups, and environment (two isolates). A total of 1495 single nucleotide polymorphism (SNP) mutations were detected across the T6SS-5 gene cluster, with the highest mutations found in the *clp*V-5 gene. The non-synonymous SNPs of *clp*V-5, *tss*M-5, *tss*A-5, and *tag*AB-5 show a significant association (p value < 0.0001, < 0.0001, 0.0067, and 0.0207, respectively) with bloodstream infection among the patients. Approximately 149 insertion/deletion (indel) mutations have been identified, with notable occurrences in *tss*A-5, *clp*V-5, *tag*AB-5, *tag*C-5, and *tss*M-5 with 36, 72, 16, 11 and 14 mutations respectively. However, there was no significance association observed between indels mutations of these genes with the clinical presentations and outcomes of melioidosis. In conclusion, this study highlights the complexity of melioidosis pathogenesis and the importance of T6SS-5 as a virulence determinant, as well as the need for further research to unravel this virulence determinant. Understanding these genetic variants could aid in developing targeted therapies or diagnostics for melioidosis.

CHAPTER 1

INTRODUCTION

1.1 Study Background

Thousands of people in Southeast Asia and Northern Australia are at risk to be infected by deadly melioidosis. Melioidosis is a community-acquired infectious disease caused by *B. pseudomallei* which is common in tropical countries and has been described as a potential bioweapon and Tier 1 select agent by Centre for Disease Control and Prevention (CDC) (Wagar, 2016). In Malaysia, about 16.4% of seroprevalence melioidosis has been noted in a cross-sectional study within the year 2015 and 2019, while in Northeast Thailand, melioidosis has been described as the third most common cause of death due to infectious disease which surpassed only by HIV infection and Tuberculosis (TB) (Limmathurotsakul et al., 2010). During the past 10 years, 550 of melioidosis cases have been documented in Singapore, in which 20% of those cases has resulted in fatalities (Liu et al., 2015). While reports have only been gathered for entire nations, extensive statistics on the exact distribution and burden of melioidosis alongside its possible risk factors is not yet accessible in the Southeast Asia region (Selvam et al., 2022).

Melioidosis have frequently been correlated to exposure of contaminated water and soil which can be spread to human through direct contact especially through open wounds on the skin (Currie et al., 2010; Galyov et al., 2010; Limmathurotsakul & Peacock, 2011). Besides that, there are significant risks of *B. pseudomallei* infection for those who work in ecotourism, construction, and military bases (Pruekprasert & Jitsurong, 1991; Nathan et al., 2018). In Malaysia, people who work in the agricultural, forestry and fishing industries contribute for 2%–25% of melioidosis cases, while those

in the construction and trucking industries contribute for 3%–18% cases (Kingsley et al., 2016).

The impairment of host defence mechanisms due to drug therapies or other underlying diseases might be the predisposing factors for melioidosis (Currie et al., 2010; Limmathurotsakul et al., 2010). About 40% of melioidosis patients with pre-existing diabetes or newly diagnosed with it at the time of hospital presentation, making diabetes the primary risk factor for this deadly disease (Limmathurotsakul et al., 2010). Other chronic diseases such as chronic renal and lung diseases as well as tuberculosis are among common risk factors in melioidosis.

Melioidosis demonstrates a varied spectrum of non-specific clinical presentations ranging from mild, localized skin infections and soft tissue abscesses to more severe and often fatal symptoms including pneumonia, septic shock and occasionally, involvement of neurological problems (Sarovich et al., 2014). Common clinical manifestations of melioidosis associated with high mortality despite of antibiotic treatments received include fulminant septic shock, acute pulmonary infection known as pneumonia and unusual involvement of neurological complications (Hopf et al., 2014; Sarovich et al., 2014). Additionally, bacteraemia and community-acquired pneumonia are two most frequent diagnosis and clinical manifestations reported for melioidosis cases (Cheng & Currie, 2005; Chen et al., 2015; Stewart et al., 2017).

The ability of *B. pseudomallei* to penetrate, persist, attack, and adopt the host intracellular life cycle is the distinguishing features of its pathogenicity (Wiersinga et al., 2018). This pathogen is equipped with a broad spectrum of virulence factors that enable it to survive intracellularly and resist host defence systems and thus enable a

successful infection which then lead to clinical diseases. Flagella, fimbriae, lipopolysaccharide, capsular polysaccharide, quorum sensing, biofilm formation as well as secretion systems Type II, III, and VI are among well-known virulence factors in *B. pseudomallei* (Galyov et al., 2010). Each of them is vital and necessary for the pathogen to survive in both extracellular and intracellular of the host as well as the environmental persistence of the pathogen (Singh et al., 2013).

Type VI secretion system 5 (T6SS-5) is one of the virulence factors in *B. pseudomallei* and studies on this secretion system gene cluster has been a topic of growing interest. T6SS-5 particularly involves and act as the main virulence determinant in most of Gram-negative bacteria (Pukatzki et al., 2009). Fundamentally, T6SS-5 involves in host pathogen interactions in which it allows the intercellular spread of the pathogen while evading extracellular host immune response. It is an essential dependent bacterial killer which exports effector proteins and various toxins into the host cell which facilitates the pathogen to evade immune system, constrain the functions, facilitates multinucleated giant cell (MNGC) formation and lead to subsequent autophagic cell death of host cell (Ku et al., 2020). The effectors proteins and secreted proteins of T6SS-5 is vital to ensure successful colonization as well as infection of *B. pseudomallei* in the host cell (Hachani et al., 2016).

Research and studies focusing on the causative agent, *B. pseudomallei* regarding the virulence factors, host-pathogen interaction, identification of pathogenesis as well as bacteriology studies are crucial for discovery of new insights and correlation for better understanding of the disease. Therefore, this study focuses on T6SS-5 gene cluster, to reveal the genetic variation of this nanomachine and its correlation with the severity of clinical presentations and outcomes of the disease. Detection of this gene

cluster is important for the early screening of potential virulence strains of *B. pseudomallei* as well as to investigate the genetic variations and their correlations with clinical presentations and outcomes of melioidosis.

1.2 Rationale of Study

Melioidosis remains as a challenging infectious disease throughout the world, especially in its endemic hot spot country such as Malaysia. The detailed overview of the disease burden, clinical manifestations, and difficulties encountered in melioidosis diagnosis and treatment is not yet established. Based on the incidence and mortality rate in Malaysia, it has been reported that more than 2000 patients have died due to melioidosis each year, which is considerably higher than the death caused by TB or dengue fever (Kingsley et al., 2016; Nathan et al., 2018). Regardless advances of therapies received by the melioidosis patients in Malaysia, approximately 65% mortality rates have been reported by Puthucheary et al. and Zueter et al. Both studies concluded that bacteraemic which lead to subsequent septic shock as the robust expecting factor for mortality among melioidosis patients (Puthucheary et al., 1992; Zueter & Harun, 2018).

The main question in melioidosis therapy is why the patient presented with severe clinical presentations, relapse and having poor outcome? Critically, it remains ambiguous why certain individuals in endemic areas experience asymptomatic melioidosis, whereas others experience more severe clinical presentation? Very little is currently known about the virulence factors and pathogenesis of *B. pseudomallei*. One of the important virulent factors, T6SS-5 is associated with internalization and intracellular survival of the *B. pseudomallei*. However, is this gene cluster important to cause infection clinically? Many fundamental questions remain unanswered since the

discovery of the vital role of the T6SS-5 in *B. pseudomallei* host cell interaction over 10 years ago. Critically, deciphering the mode of action of the T6SS-5 poses a challenge for the field as it remains elusive.

The key aspect of *B. pseudomallei*'s virulence and pathogenesis that makes therapy more difficult is their capacity to penetrate, encounter, and multiply within host cells. Intracellular survival of the agent, *B. pseudomallei*, in both phagocytic and non-phagocytic cells is one of the reasons that allows the bacterium to evade host immune responses, as well as prevent the activity of antimicrobial chemotherapy. Bacterial T6SS-5 is an essential virulence factor for *B. pseudomallei* to enter and survive intracellularly, as well as facilitate the formation of multinucleated-giant-cell. Although there a lot of studies on molecular and virulent aspect of this secretion system *in vitro*, the relevant of this virulent factor and its genetic variants on clinical and environmental strains were not being investigated at the moment.

Additionally, T6SS-5 was activated throughout the course of the melioidosis, demonstrating the crucial role of this pathway in supporting *B. pseudomallei* survival and reproduction in the cell cytosol (Pilatz et al., 2006). To further understand how T6SS-5 gene cluster in *B. pseudomallei* infection correlates with clinical presentations and outcomes of melioidosis patients, the genetic variation of this virulence factor must be determined.

The previous publications only tested a few strains of *B. pseudomallei*. Therefore, the presence of T6SS-5 gene cluster in all clinical or environmental strains of *B. pseudomallei* and the environmental strains that probably less virulence to cause infections will also be tested in this study. Is the presence of any variations in gene characterizations may lead to different capability of the bacteria to cause different range

of clinical presentation and persistence of bacteria in the host? The relevance of the T6SS5 genetic variations to the severity of human diseases and outcome of the patients is also one of the interests of this present study.

The lack of recent information of T6SS-5 gene clusters particularly in B. pseudomallei is because it is understudied compared to other virulence genes. Better understanding of the molecular underpinnings of the T6SS-5, as a crucial virulence determinant of *B. pseudomallei* is significant to develop and facilitate approaches that can prevent the pathogen from surviving and multiplying in humans, and successively prevent the infection from occurred.

1.3 Research Questions

1. Is the intracellular virulence factor Type VI Secretion System 5 (T6SS-5) present in all clinical/environmental strains of *B. pseudomallei*? Is there any different in the genetic characteristic?
2. How genetic variations of T6SS-5 associate with the clinical presentations, and outcomes of melioidosis?

1.4 Hypothesis of Study

Type VI Secretion System 5 (T6SS-5) associated with internalization and intracellular survival of the *B. pseudomallei*. T6SS-5 gene cluster present in all clinical and environmental strains of *B. pseudomallei* and have correlation with severe clinical presentations, relapse and having poor outcome.

T6SS-5 is a critical virulence factor for *Burkholderia* pathogenesis in mammalian hosts and is implicated in generating cell-cell fusion that results in MNGC formation. T6SS-5 has also been revealed to be vital for intracellular proliferation in

Raw 264.7 murine macrophages. It has been demonstrated that a K96243 hcp5 mutant, which lacked the Hcp protein expression for the T6SS-5, has a considerably lower number of intracellular bacteria than the K96243 wild-type strain of *B. pseudomallei*.

The potential of the bacteria to induce a variety of clinical presentations and persistence of the germs in the host might alter depending on the existence of any differences in the characterizations of T6SS-5 gene cluster. According to previous studies, in animal models of acute infection, deletion of crucial T6SS-5 genes significantly reduced the virulence of *B. pseudomallei* and *B. thailandensis*.

1.5 Objectives of the Study

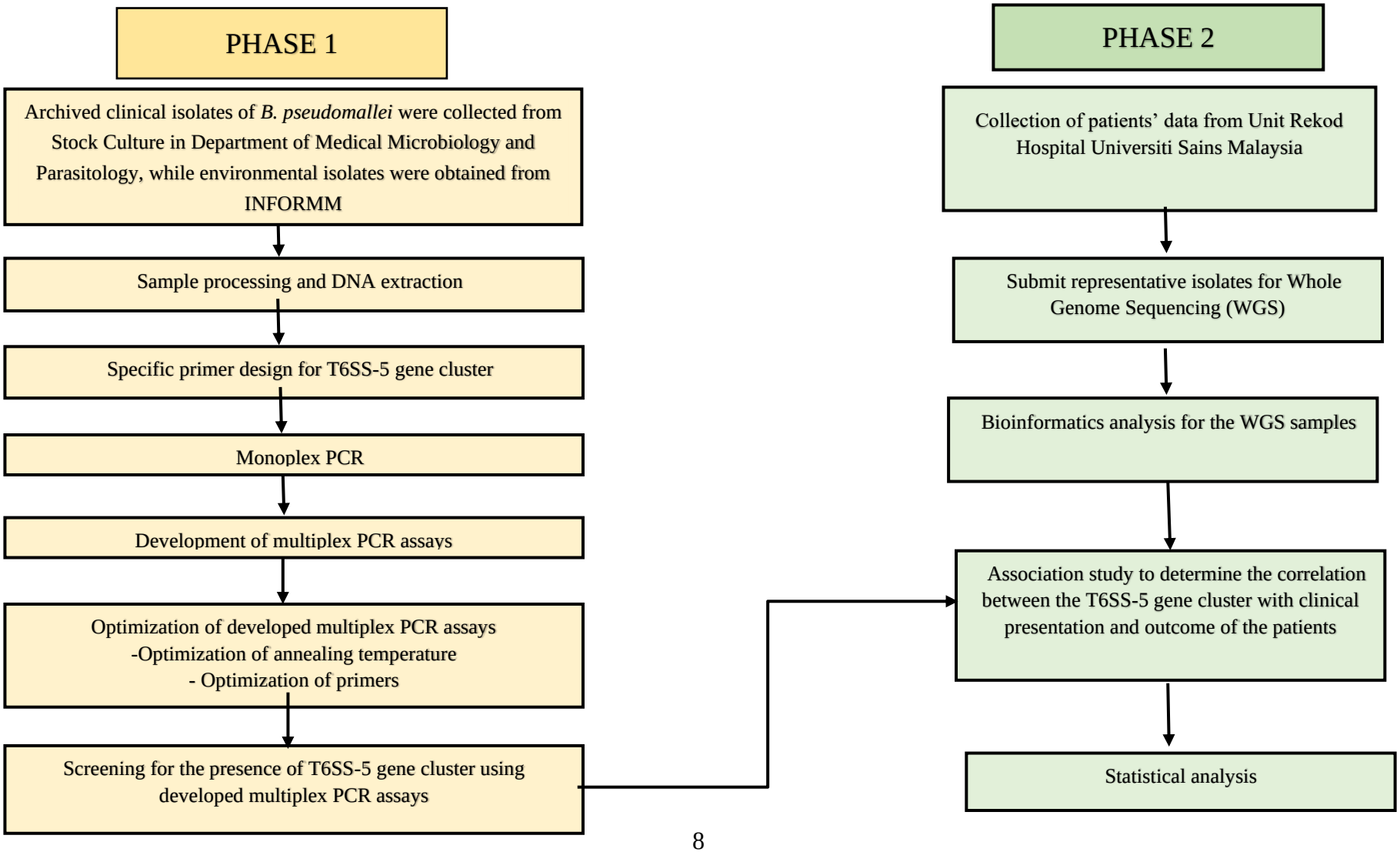
1.5.1 General Objectives

This present study is focused to investigate the association between the variation of intracellular virulence factor Type VI Secretion System 5 (T6SS-5) with the severity of clinical presentations and outcomes of *B. pseudomallei* infection.

1.5.2 Specific Objectives

1. To develop multiplex PCR for the detection of T6SS-5 gene cluster in *B. pseudomallei* isolates
2. To screen and to determine genetic characterizations of T6SS-5 gene cluster on clinical and environment isolates of *B. pseudomallei*
3. To determine the association between T6SS-5 gene cluster variations with severity of clinical presentation and outcome of melioidosis patients
4. To determine the genetic variation of T6SS-5 gene cluster using whole genome sequencing platform

1.6 Flow Chart of the Study



CHAPTER 2

LITERATURE REVIEW

2.1 Melioidosis

Melioidosis is a neglected tropical disease cause by *Burkholderia pseudomallei*. This disease responsible for considerable morbidity and mortality in human and animal populations as well as the ability to be easily spread (Wagar, 2016). Prior to the discovery of the *B. pseudomallei*, its aetiological agent was mistakenly attributed to a closely related species, *Burkholderia mallei* because the morphologies of the agent resembled *B. mallei* and the clinical manifestations closely similar to those seen in horses infected with glander disease (Stanton & Fletcher, 1925).

Another significant challenge with melioidosis is that it also exhibits symptoms and presentations that mimic to those of other common illnesses such as tuberculosis, leptospirosis and enteric fever (Valsalan et al., 2008; Lipsitz et al., 2012; Yazid et al., 2017; Nakkawita et al., 2022). This might result in a misdiagnosis and subsequent ineffective treatment for the melioidosis patients. Besides that, *B. pseudomallei* also poses a high level of antibiotic resistance due to several antibiotic efflux pumps. If proper diagnosis and treatments cannot be made promptly, this frequently leads to death (Moore et al., 1999; White, 2003; Chan & Chua, 2005).

Melioidosis also has been seen in many animals especially in the tropical region. Currently, melioidosis has been recognised as a sporadic disease since many cases associated with this infection has been reported throughout the world. Host susceptibility and clinical manifestations between animals are varies thus increase the risk of misidentification with other infections. The source of melioidosis among animals

usually due to contaminated soil or water while the transmission between animals are very rare (Sprague & Neubauer, 2004).

Several prominent virulence factors in *B. pseudomallei* have already been discovered, including the type VI secretion system 5, cytotoxin *Burkholderia* fatal factor 1, and the Bsa type III secretion system cluster 3, and capsular polysaccharide I (Reckseidler et al., 2001; Wiersinga et al., 2006; Cruz-Migoni et al., 2011; Burtnick et al., 2011). However, the broad range of melioidosis manifestations has led to hypotheses that variable virulence factors may impact the severity and outcome of the disease (Sarovich et al., 2014).

Currently, the diagnosis depends mostly on culture method, which takes some time to accomplish and to get the results. Given the aggressive progression of melioidosis, this duration might be inadequate to diagnose as well as to treat the patients. Other than that, there is no vaccination currently available to prevent against *B. pseudomallei* infection.

2.1.1 Brief History of Melioidosis in Malaysia

Melioidosis was first discovered and reported over 100 years ago in 1911 in Burma. It was initially identified as an infectious pseudoglanders disease by the British pathologist, Alfred Whitmore and his assistant Krishnaswami (Whitmore & Krishnaswami, 1912). They discovered a novel pathogen that met Koch's postulates, according to which the pathogen is established to cause uncommon bacterial infection as well as autopsy investigation of the infected Burmese patients at the Rangoon General Hospital.

After the pathogen was isolated and cultured on peptone agar and potato slants, it was revealed to be similar to the bacterium causing glanders disease, *Burkholderia*

mallei. However, in terms of quick growth and motility, the bacillus that was isolated from autopsy specimens of the infected patients showed distinctive criteria from *B. mallei*. The pure culture of this pathogen caused deadly lung lesions, which were the same disease presentation found in patients when inoculated into guinea pigs (Stanton & Fletcher, 1925).

Subsequently, the disease was known as Whitmore's disease as well as "glanders-like" disease. In 1921, Stanton and Fletcher suggested the name melioidosis, which derived from the combinations of the Greek terms "melis","oeid" and "osis", which mean distemper of asses, resemblance, and abnormal disease, respectively. This refers to the pathogen causing the disease, which bears a resemblance to the clinical characteristics and disease presentation of glanders (Stanton & Fletcher, 1925).

In Malaysia, the Institute for Medical Research (IMR) in Kuala Lumpur has recorded the first melioidosis case in 1913, which involved an acute animal outbreak involving laboratory rabbits and guinea pigs (Stanton & Fletcher, 1925). Human melioidosis was also documented in Kuala Lumpur involving the two earliest cases that presented with melioidosis symptoms; their post-mortem swabs revealed the presence of *Bacillus whitmori*, and subsequently in 1932, a total of 39 cases of melioidosis have been described also in Kuala Lumpur (Stanton & Fletcher, 1925; Thin et al., 1970). Since these discoveries, melioidosis has been documented as endemic in this country.

In the late 1980s and early 1990s, melioidosis researches and publications started to accelerate with a plethora of manuscripts, particularly reviews and clinical reports (Puthucheary et al., 1981; Puthucheary et al., 1992). Molecular microbiology, genetics, and pathogenesis were later added to the list of study areas of interest after improved resources, funding, and qualified local specialists became available (Francis

et al., 2006; Su et al., 2008; Chua et al., 2010; Podin et al., 2014; Mariappan et al., 2017).

2.1.2 Epidemiology of Melioidosis

Melioidosis caused by environmental saprophytes was reported to be highly endemic in northern Australia and Southeast Asia regions, including Malaysia and its neighbouring countries (Dance, 2000; Arushothy et al., 2024). However, melioidosis has become increasingly well documented and worldwide endemicity is also recognised in many tropical areas, including the Cambodia, Hong Kong, Indian Sub-continent, Laos, southern China, and Taiwan, with sporadic cases reported in Brazil, the Middle East, and islands in the Caribbean (Dance, 2000; French et al., 2020).

Despite improved detection as well as surveillance of infection, the exact scope of *B. pseudomallei*'s global distribution is still elusive, particularly the extent to which melioidosis has become widespread beyond historical regions, and the exact moments and routes of global expansion (Currie, 2015). Reports of environmental *B. pseudomallei* isolates or melioidosis cases from Central and South America, Africa, and South Asia indicate that the bacteria are found in the tropics worldwide (Lennings et al., 2018; Meumann et al., 2024). It is frequently isolated from moist environments, including from moist surface water, especially from monsoon drains, or rice fields. According to studies, whole types of soils, including grave soil and high salinity soil, as well as geographical factors like climate change that include heavy rainfall, tropical cyclones, and astropical storms, strongly contributed to the elevated isolation of *B. pseudomallei* (Palasatien et al., 2008; Limmathurotsakul et al., 2013; Limmathurotsakul et al., 2016; Kaestli et al., 2016; Meumann et al., 2024).

Limmathurotsakul et al., have described approximately 165,000 incidences of human melioidosis globally each year, according to a predictive modelling study, with 89,000 people dying from the condition, representing a 54% fatality rate. The study also demonstrated melioidosis cases in animal and human together with the involvement of *B. pseudomallei* environmental isolates to predict the global distribution as illustrated in Figure 2.1 (Limmathurotsakul et al., 2016; Meumann et al., 2024). In a 2019 study, melioidosis was predicted to have a worldwide burden of 4.64 million disability-adjusted life-years, surpassing several other neglected tropical diseases acknowledged by the World Health Organisation (WHO) (Birnie et al., 2019).

The estimated fatality of melioidosis is comparable to measles (95,600 people per year), but greater than that of leptospirosis (50,000 people per year) and dengue (12,500 people per year), for which numerous international health organisations have designated these illnesses as high priority diseases. The study also anticipated that melioidosis is critically underreported in 45 endemic countries, and that it is likely prevalent in other 34 nations where the disease has certainly not been reported. Only three countries have equivalent national surveillance data for melioidosis to the estimations, which comprise of Singapore, Australia, and Brunei Darussalam (Limmathurotsakul et al., 2016).

Despite hundreds of cases of melioidosis have been documented in Malaysia, the actual prevalence of the disease in this endemic country remains unknown (Arushothy et al., 2024). The distribution amongst the states in Malaysia, and even within the same state, was predicted to vary (Mohan et al., 2017). Current estimates vary from 6.1 to 16.4 cases per 100,000 populations yearly (How et al., 2005; Zueter et al., 2016). Paediatric melioidosis among Malaysian have been recorded at a range of

0.6 to 4.1 cases per 100,000 children per year, with the highest prevalence in central Sarawak (Mohan et al., 2017).

In Australia, melioidosis was originally described in 1949 in sheep before was found in human in the subsequent year (Cottew, 1950; Rimington, 1962). In the Northern Australia, epidemiological studies have found an average yearly incidence of 19.6 cases per 100,000 people, with incidences varying from 5.4 to 41.7 in extreme weather conditions (Cheng & Currie, 2005). An exceptionally heavy rainfall year in that particular area within the year 2009 and 2010 led to the world's highest annual incidences reported, 50.2 cases per 100,000 populations (Parameswaran et al., 2012). Even though the mortality rate for melioidosis in Australia appears to be declining with an average rate of 14%, its incidence is still increasing (Currie et al., 2010). A recent study reported a fatality rate of 6% in the setting of prolonged intravenous treatment (Sullivan et al., 2020; Currie et al., 2021).

A report from northeast Thailand between 1987 and 1991 found an annual prevalence of 4.4 incidents per 100,000 people (Suputtamongkol et al., 1994). A peak incidence of 21.3 per 100,000 populations in 2006, with an annual average of 12.7 (Limmathurotsakul et al., 2010). A hospital here recorded high mortality rates among admitted melioidosis patients, with the mean annual mortality rate of 42.6% (Limmathurotsakul et al., 2010). Another study in 2018 indicated the fatality rate of admitted patients for the overall melioidosis cases in public hospital in Thailand was still high, between 30 to 35% (Hinjoy et al., 2018).

A study conducted in Singapore from 2003 to 2014 reported an overall melioidosis incidence of 1.1 cases per 100,000 populations, with incidence declining by 10% annually (Pang et al., 2018). In addition, investigations regarding severe

community-acquired pneumonia have shown a declining proportion of the overall prevalence of microbiologically confirmed infections due to bacteria from 24% to 13% between 1989 and 1993 and between 2003 and 2005, respectively (Tan et al., 1998; Poulose, 2008). The increased infrastructure for flood mitigation, rainwater drainage, and water sanitation have been highlighted as the factors contributing to the current decrease (Pang et al., 2018).

The exact burden of melioidosis in Malaysia has not been thoroughly identified or has been neglected in most states due to inadequate facilities in terms of diagnostic testing, bacteriological culture prior to antibiotic therapy, and insufficient awareness among microbiologists and physicians. Improved diagnostic expertise, skilled laboratory personnel, and nationwide surveillance should be helpful to detect melioidosis as well as to treat melioidosis patients properly.

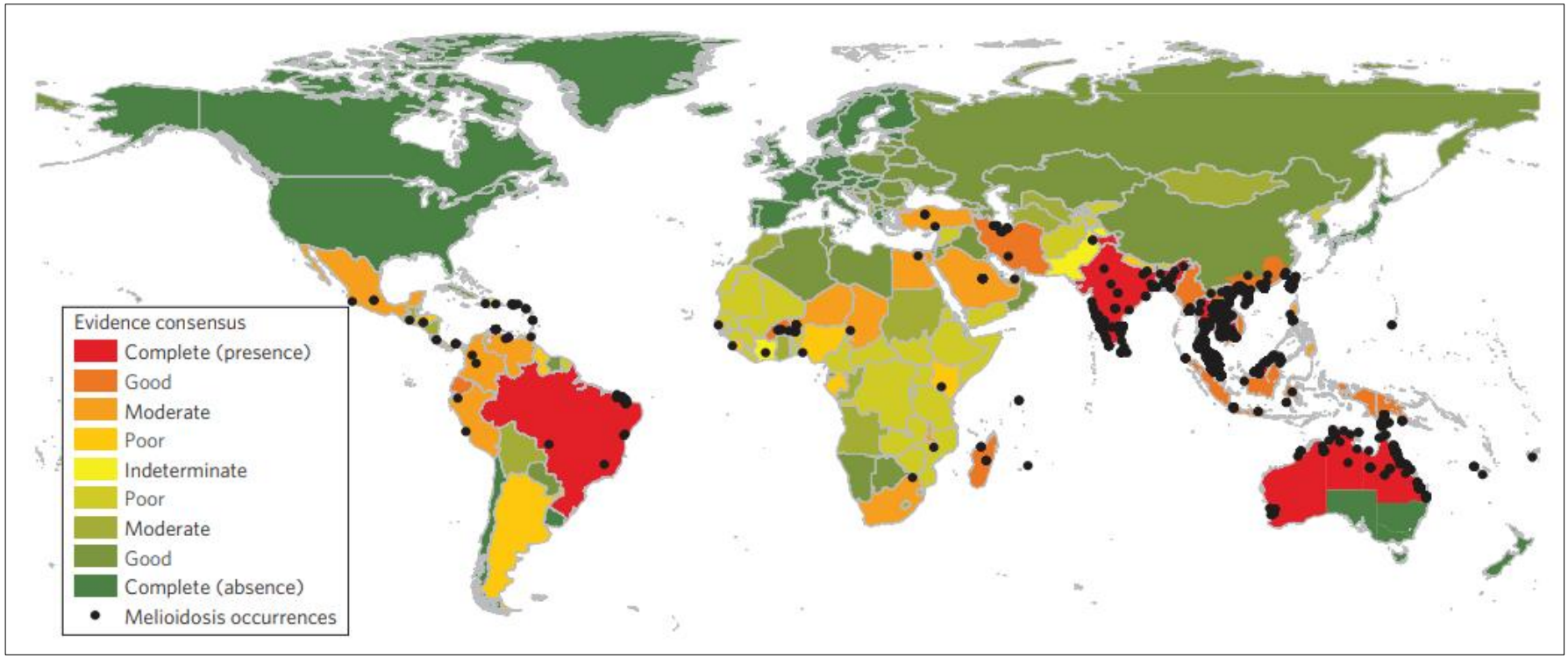


Figure 2.1. Global distribution of melioidosis based on evidence-based consensus (Adopted from Limmathurotsakul *et al.*, 2016

2.1.3 Human Exposure to the Disease

Skin inoculation, ingestion, and inhalation are the main routes of human infections. Therefore, melioidosis cases are commonly associated with those who had come into contact with contaminated soil and water, especially during occupational or recreational disclosure (Barnes & Ketheesan, 2005; Currie *et al.,* 2010; Galyov *et al.,* 2010; Limmathurotsakul & Peacock, 2011). People who work in eco-tourism, agriculture and forestry, construction and military base have high risks of *B. pseudomallei* infection due to the exposure to contaminated soil and water (Pruekprasert & Jitsurong, 1991; Nathan *et al.*, 2018; Meumann *et al.,* 2024).

In the *B. pseudomallei* endemic area, even simple activities of everyday life might be risk factors for melioidosis. A randomised case-control study in Thailand indicated farming in rice fields, going barefoot more than a few times a week, swimming in lake, and having wounds open on the skins, are risk factors of the infection as concerns to skin inoculation (Suputtamongkol *et al.,* 1999; Limmathurotsakul *et al.,* 2013). Ingestion of infection was most usually connected with eating contaminated food and drinking untreated water. Inhalation incidents were linked to outside exposure to rain or dust (Limmathurotsakul *et al.,* 2013).

Exposure to the bacterium does not necessarily result in an infection, as only 1 in 4600 seroconversion-associated exposures produced clinical melioidosis as reported by Currie 2015 (Currie, 2015). The general period of incubation for *B. pseudomallei* is approximately 1 to 21 days, with a mean incubation period of just 9 days (Currie *et al.,* 2010). However, the bacterium can remain dormant within host cells for a long period of time causing persistent infections to the host. Recurrence of the melioidosis frequently occurs in those who have low immunity such as elders and

immunocompromised patients (See *et al.*, 2017). Figure 2.2 illustrates the human exposure to *B. pseudomallei* infection.

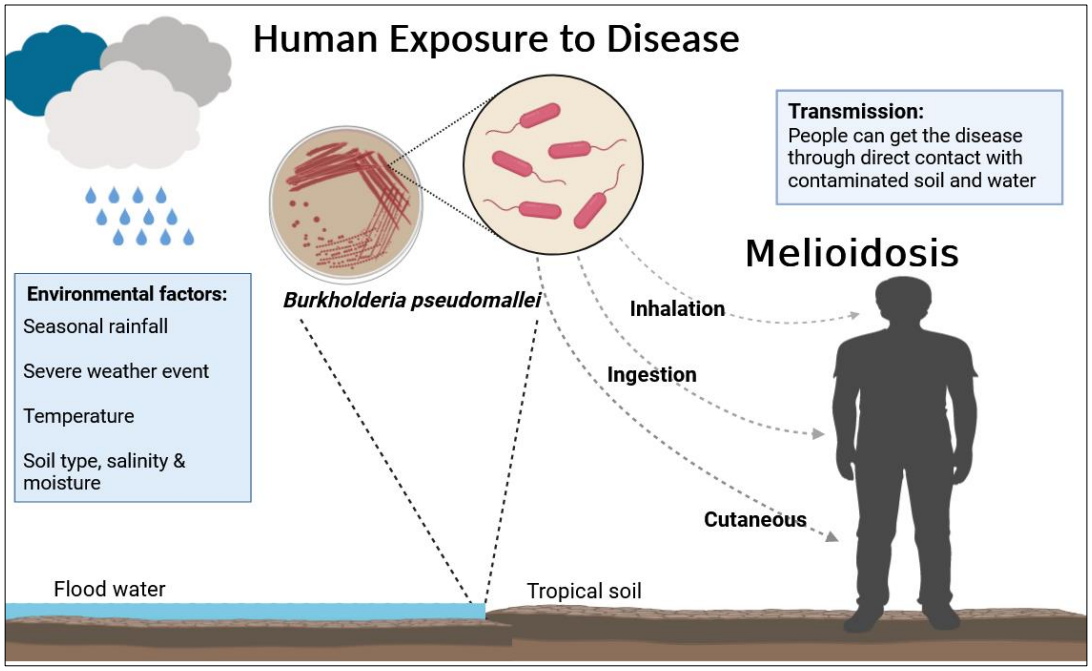


Figure 2.2 The illustration of the human exposure to *B. pseudomallei* infection. Humans acquire melioidosis by inhalation, ingestion or/and cutaneous inoculation. Rainfall, flood and contact with soil are among the environmental risk factors.

2.1.4 Risk Factors of Melioidosis

Melioidosis is frequently linked to several underlying risk factors, including health status, stress factors, and other variables such as gender, weather, and profession. The health status of an individual is often associated with severity and occurrence of melioidosis. Diabetes mellitus is the most prominent risk factor, and other diseases such as chronic renal disease, chronic lung disease, liver disease, malaria and thalassemia might increase their susceptibility to *B. pseudomallei* infection (Chaowagul *et al.*, 1993; Currie *et al.*, 2010; Jabbar & Currie, 2013; Selvam *et al.*, 2022).

Diabetes mellitus patients are at a higher risk of infection compared to non-diabetic patients (Toh & Chan, 2019; Currie *et al.*, 2021; Chantratita *et al.*, 2023). The proportional risks of sepsis and cellulitis caused by infection are significantly increased in diabetic patients (Shah & Hux, 2003; Carey *et al.*, 2018). The prevalence rates of diabetes ranging up to 75% in Malaysian cases, 17 to 47% in Thai cases, and 37 to 56% in Australian cases (Suputtamongkol *et al.*, 1994; Currie *et al.*, 2004; Churuangsuk *et al.*, 2016; Zueter *et al.*, 2016; Stewart *et al.*, 2017). Diabetes can particularly increase the risk of infection by impairing and reducing immunological functions such as phagocytosis, chemotaxis, cytokine response, as well as bacterial killing (Geerlings & Hoepelman, 1999; Graves & Kayal, 2008). Chanchamroen et al in 2009 have demonstrated that individuals with multiple risk factors, for instance, diabetes patients who are highly exposed to contaminated soil and water, are more prone to *B. pseudomallei* infection and have a higher prevalence of melioidosis compared to those with a single risk factor (Chanchamroen *et al.*, 2009).

Thalassemia and chronic renal disease are associated with elevated risk of *B. pseudomallei* infection due to defective innate immune system, particularly in the activity of neutrophils and macrophages (Jabbar & Currie, 2013; Teawtrakul *et al.*, 2015). According to Malaysian paediatric cases, up to 40.0% of patients had thalassemia major. However, infection rates among them were reduced by chelation therapy (Fong *et al.*, 2015; Selvam *et al.*, 2022).

Lifestyle with excessive use of alcohol and drug addiction often lead to immune-suppress host, are also highly susceptible to *B. pseudomallei* infection (Sridharan *et al.*, 2021; Mohapatra & Mishra, 2022). The risk of melioidosis in people who consumed alcohol is often associated with binge drinking instead of chronic liver disease, with high blood alcohol levels at the time of *B. pseudomallei* results in hindering defenses against bacterial replication and transmission. This is in accordant with previous findings involving alcohol intoxication and associated with reduce neutrophil function in melioidosis (Gluckman & MacGregor, 1978; Zhang *et al.*, 1999; Currie et al., 2010; Moreno *et al.*, 2019).

People who are involved in activities with relatively limited outdoors engagement seems to be resilient to the disease, whereas those in occupations that involved greater outdoor exposure were prone to get infected with melioidosis. For instance, the findings show that rice farmers are the most vulnerable to infection due to potentially prolonged exposure to soil sources and thus a higher risk of inoculation, inhalation, or ingestion with *B. pseudomallei* (Wuthiekanun *et al.*, 1995; Kaestli *et al.*, 2009; Abu Hassan *et al.*, 2019; Selvam *et al.*, 2022). Figure 2.3 illustrates the risk factors of melioidosis.

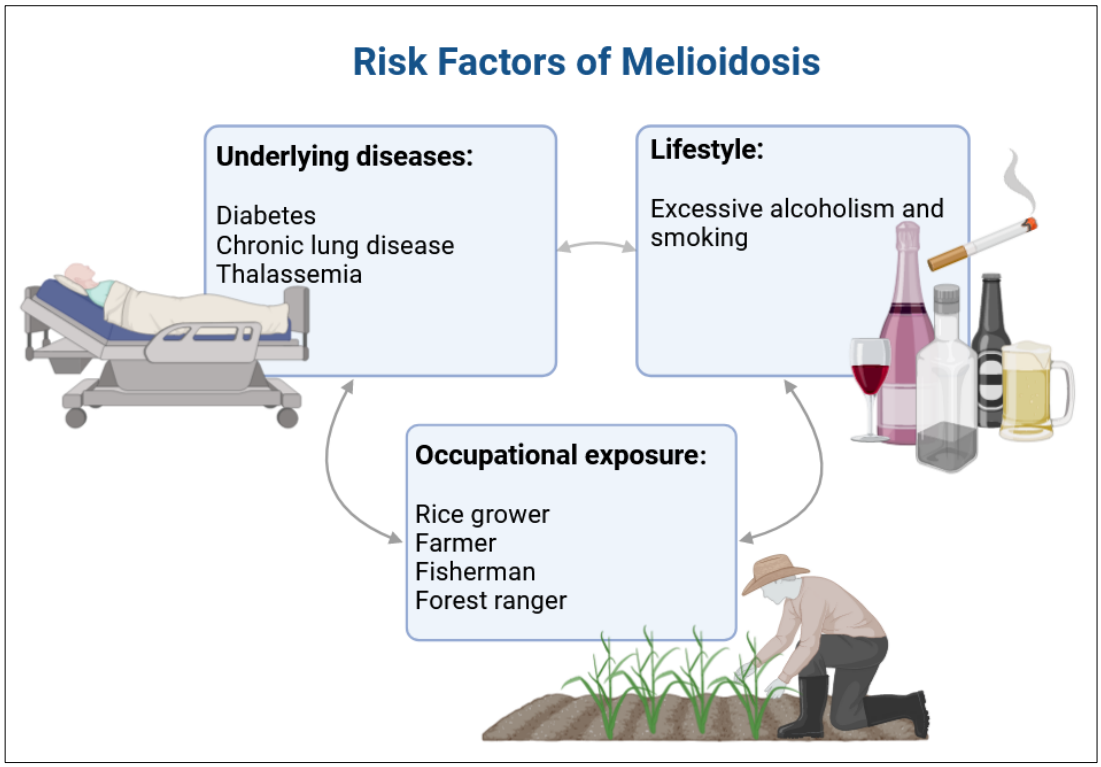


Figure 2.3 The illustration for the risk factors of melioidosis.

2.1.5 Clinical Manifestations of Melioidosis

The route of infections, bacterial loads, virulence of *B. pseudomallei* strain and host risk factors may influence the clinical presentations and outcome of melioidosis (Currie, 2015; Gassiep *et al.,* 2020). Melioidosis presents with wide spectrum of non-specific clinical manifestations. The severity varies from mild, localized skin infections and soft tissue abscesses to a more severe and chronic infection with fatal symptoms. Community-acquired pneumonia and bacteraemia are the most common clinical presentation and the most frequent microbiological diagnosis of melioidosis respectively *(Cheng & Currie, 2005; Currie et al., 2015; Chen et al., 2015;* Stewart *et al.,* 2017). Pneumonia, septic shock and involvement of neurological problems are common clinical manifestations of melioidosis associated with high mortality rates even with antibiotic treatments (Hopf *et al.,* 2014; Sarovich *et al.,* 2014).

A broad range of clinical presentations and severity of melioidosis may be attributed to one or a combination of three reasons: variety in bacterial strains (which includes the presence or absent of virulence factors), variation of acquisition, and variance in the immune response of the host. A vast amount of research demonstrates that host variables, notably age and comorbidities, are critical in defining disease pattern. Despite the occurrence of regional diversity in disease pattern indicates that bacterial variation may be essential, molecular investigations have not yet proven this. As outlined above, variations in the mechanism and degree of acquisition, is believed to have an underestimated role in determining illness clinical presentation (Cheng & Currie, 2005).

Fever was the most consistent symptom and was present in 96.8% patients, with pulmonary melioidosis as the most prevalent manifestation, accounting for

approximately 34.7% of all cases (Vidyalakshmi *et al.*, 2012). Chronic pulmonary melioidosis has been hypothesised to be obtained through cutaneous exposure, which leads to hematogenous spread to the lung as well as poses high risk of morbidity (Ali *et al.*, 2020).

Melioidosis is distinguished by the difficulty of eradicating the pathogen. Fever recovery duration is frequently prolonged, with a median of 8 days, antibiotic medication should be taken for about 12 to 20 weeks, and recurrence is observed in approximately 10% of melioidosis patients despite appropriate antimicrobial intervention (Currie *et al.*, 2000; Cheng & Currie, 2005; Tandhavanant *et al.*, 2010). Localized melioidosis is considered when the infection occurred and confined to one area of the body while disseminated infection is considered when the bacteria spread to many parts of the organs or body. Bacteremia is referred to a positive blood culture with or without one localised infection, whereas disseminated melioidosis was described as a positive blood culture with two or more localised infections (Churuangsuk *et al.*, 2016). Figure 2.4 illustrates the wide ranges of clinical presentation of melioidosis.

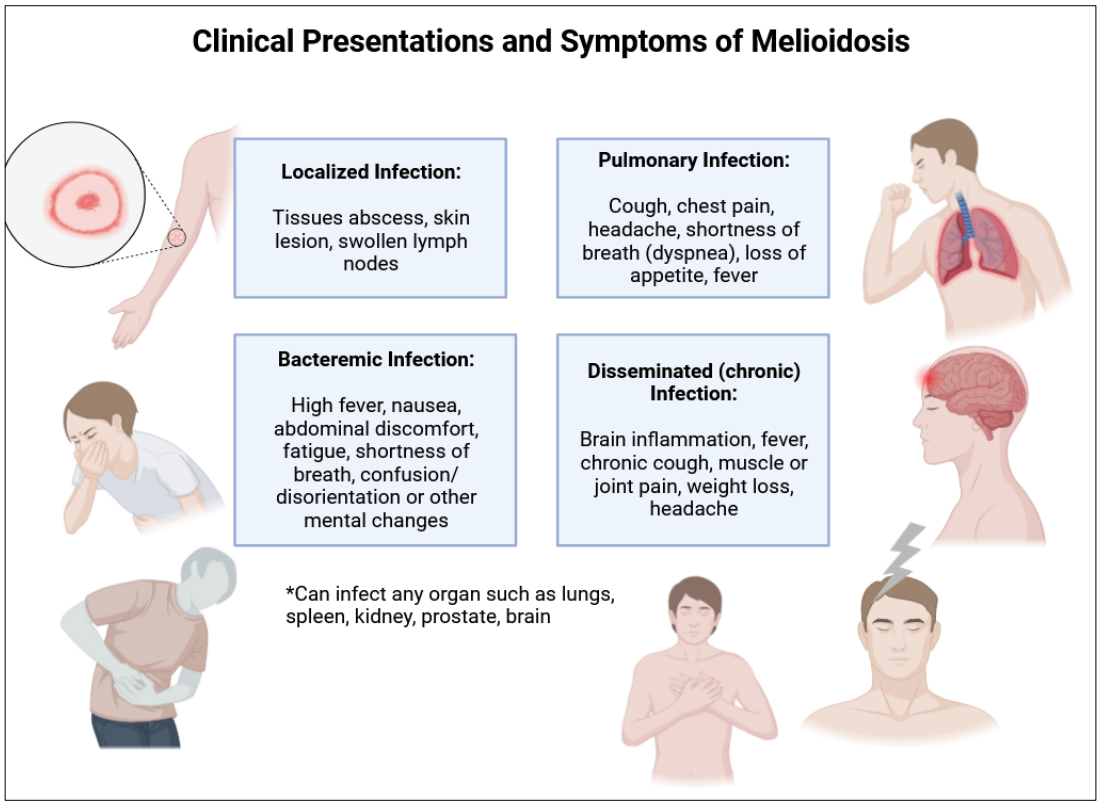


Figure 2.4 Wide ranges of clinical presentation of melioidosis. These include localised, pulmonary, bacteremia and disseminated infections. Some of these clinical presentations might overlap.