CLINICAL CHARACTERISTICS, GENOTYPING AND MOLECULAR DETECTION OF

Burkholderia pseudomallei

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By

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LIST OF ABBREVIATIONS

Base pair	bp
Calcium chloride	Cl_2
Celsius	C^o
Centimeter	cm
Colony forming unit	CFU
Deoxyribonucleic acid	DNA
Enzyme-linked immunosorbent assay	ELISA
Ethylenediaminetetraacetic acid	EDTA
Femtogram	fg
Gram	G
Gravity	g
Hour	hr
Human immunodeficiency virus	HIV
Hydrochloric acid	HCl
Immunoglobulin	Ig
Indirect hemagglutination assay	IHA
Indirect immunofluorescent assay	IFA
Interleukin	IL
Liter	L
Magnesium chloride	$MgCl_2$
Microliter	µl
Micromolar	µM
Mililter	ml
Milligram	mg
Millimeter	mm
Millimolar	Mm
Minute	min
Multilocus sequence typing	MLST
Multiple linear regression	MLR
Nanogram	ng
Nanomolar	nM
Nontemplate control	NTC
Number	No
Optical density	OD
Phosphate buffered saline	BPS
Picogram	pg
Polymerase chain reaction	PCR
Pulsed-field gel electrophoresis	PFGE
Relative centrifugal force	RCF
Ribonucleic acid	RNA
Ribosomal RNA	rRNA
Round per minute	rpm
Second	sec
Sequence type	ST
Single linear regression	SLR
Sodium chloride	NaCl
Tryptone soya agar	TSA
Ultraviolet	UV
Unit	U
Weight per volume	w/v
World Health Organization	WHO

CIRI-CIRI KLINIKAL, PENJENISAN GEN DAN KAEDAH PENGESANAN MOLEKULAR BAGI *Burkholderia pseudomallei*

ABSTRAK

Burkholderia pseudomallei ialah bakteria saprofitik Gram negatif yang boleh menjangkiti manusia melalui saluran pernafasan, pengingesan atau melalui kulit, dan meyebabkan penyakit melioidosis. Patofisiologi dan gejala klinikal penyakit melioidosis bergantung kepada jumlah bakteria, cara jangkitan diperolehi serta faktor-faktor risiko yang ada pada manusia. Terdapat berbagai kaedah makmal yang melibatkan *B. pseudomallei* termasuklah ujian pengesanan, kajian epidemiologi molekular dan kajian patogenisiti yang menggunakan kaedah konvensional, immunologi dan molekular.

Kajian dalam tesis ini mendalami ciri-ciri klinikal dan faktor-faktor risiko penyakit melioidosis. Penentuan genotip dengan menggunakan kaedah multilocus sequence typing (MLST) telah dilakukan ke atas isolat klinikal *B. pseudomallei* untuk mengkaji kepelbagaian genetik serta perhubungan antara genotip dengan ciri-ciri klinikal, prognosis dan kesudahan penyakit melioidosis. Bagi mempertingkatkan kebolehkesanan *B. pseudomallei*, satu asai berasaskan tindak balas rantai polimerase (PCR) yang menyasarkan gen *sctq* dari kluster TTS1telah dibangunkan.

Dalam kajian yang melibatkan 158 kes, gejala klinikal utama yang diperolehi adalah infeksi paru-paru (n=65, 41.1%), infeksi kulit (n=44, 27.8%), artritis septik/osteomielitis (n=20, 12.7%) dan infeksi hati (n=19, 12%). Melioidosis bakteremik telah didapati dalam sebahagian besar pesakit (n=121, 76.6%). Seramai 124 pesakit (78.5%) mendapat melioidosis setempat (lokal), 45 pesakit (28.5%)

mendapat melioidosis multifokal, manakala seramai 34 pesakit (21.5%) menghidap melioidosis tanpa punca yang nyata. Abses organ dalaman serta fokus sekunder dalam paru-paru dan tisu badan juga banyak didapati dalam kajian ini. Enam puluh tujuh pesakit (41%) telah mendapat penyakit pada musim monsun lembap (tengkujuh). Kematian disebabkan melioidosis telah melibatkan seramai 52 pesakit (32.9%), manakala infeksi berulang melibatkan 4 pesakit (2.5%). Kajian ini juga menjumpai 12 kes kematian yang melibatkan pesakit yang tidak mendapat rawatan fasa akut. Faktor risiko bagi penyakit melioidosis dijumpai dalam sebahagian besar pesakit (n=133, 84.2%). Ini termasuk diabetes (74.7%), gangguan sistem imun badan (9.5%), kanser (4.4%) dan penyakit buah pinggang kronik (11.4%). Dalam analisis multivariat, penelah bebas bagi kes mortaliti melioidosis adalah kewujudan sekurang-kurangnya satu faktor morbiditi (OR 3.0; 95% CI 1.1–8.4), kejutan sepsis (OR 16.5; 95% CI 6.1–44.9) dan umur >40 tahun (OR 6.47; 95% CI 1.7–23.8).

Penjenisan gen menggunakan kaedah MLST, yang dilakukan ke atas 83 isolat *B. pseudomallei* telah menjumpai 32 genotip yang berbeza, termasuk 13 genotip yang baharu (*novel*), iaitu: ST1317, ST1318, ST1319, ST1320, ST1321, ST1322, ST1323, ST1324, ST1325, ST1326, ST1327, ST1358 dan ST1359. Kesemua genotip yang ditemui dalam kajian ini dihantar dan disimpan dalam pangkalan data MLST. Dalam kalangan 83 isolat yang diuji, kekerapan sesuatu genotip adalah dalam lingkungan 1-12 kali dan genotip yang paling lazim atau pradominan adalah ST54, ST371 dan ST289. Genotip-genotip sedia ada (*non-novel*) yang ditemui dalam kajian ini tidak didapati secara eksklusif di Malaysia sahaja, tetapi juga didapati di negara-negara sekitar yang lain dengan kekerapan yang berbeza. Walau bagaimanapun terdapat

genotip yang julung kali dijumpai di Malaysia iaitu ST371, ST164, ST47, ST306, ST55, ST376, ST402, ST507, ST368, ST369, ST10 dan ST168.

Menurut analisis data MLST, pokok filogenetik yang terhasil menunjukkan lebih daripada separuh genotip terkumpul dalam gugusan yang sama, termasuklah genotip-genotip dari Malaysia, Thailand, Singapore, Cambodia, Vietnam, Laos dan China. Genotip-genotip yang baharu juga terkumpul dalam gugusan yang sama dalam pokok filogenetik. Satu-satunya genotip unik yang bersendirian dalam pokok filogenetik ialah ST1326, yang juga genotip baharu. Kajian ini telah memperluaskan pangkalan data yang sedia ada bagi genetik populasi *B. pseudomallei* di Malaysia dengan menambahkan cabang-cabang genotip dan mewujudkan kluster atau gugusan baharu berikutan pengembangan klonal yang bercambah dari ST84.

Secara evolusi, tiada bukti perkaitan antara genotip *B. pseudomallei* dengan gejala klinikal melioidosis. Penumpuan mana-mana genotip terhadap sesuatu kesudahan klinikal juga tidak didapati dalam kajian ini. Kelainan dari segi kevirulenan atau tropisme juga tidak ditemui. Sepsis yang teruk (n=11) dilihat berhubung kait dengan tujuh genotip yang berbeza, manakala kejutan sepsis (n=29) dan abses (n=30) berpunca dari 17-18 genotip yang berlainan. Sepsis, pneumonia, ensefalopati serta infeksi organ lain dan kesudahan penyakit adalah berhubung kait dengan beberapa genotip yang berbeza-beza. Analisis bagi menentukan perkaitan antara kluster genotip dengan kesudahan klinikal juga tidak menunjukkan perkaitan yang signifikan secara statistik.

Asai PCR yang dibangunkan telah dioptimisasikan bagi mencapai tahap diskriminasi yang maksimum di antara *B. pseudomallei* dengan spesies-spesies bakteria lain dan juga mempertingkatkan aras kebolehkesanannya. Ujian spesifisiti secara *in-silico* menunjukkan padanan tepat dengan rujukan gen sasaran, manakala spesifisiti 100% didapati apabila diuji terhadap *B. pseudomallei* dan bakteria lain termasuk *B. thailandensis* dan *B. cepacia*.

Asai PCR ini juga menunjukkan sensitiviti 100% apabila diuji terhadap isolat-isolat dari genotip yang berlainan. Kepekatan DNA paling minimum yang dapat dikesan menggunakan asai *sct*-PCR ini ialah 100 fg/µl, manakala bilangan bakteria dalam darah yang paling sedikit yang berjaya dikesan ialah 18.2 X 10^5 CFU/ml. Oleh itu, asai PCR yang dibangunkan dalam kajian ini mampu untuk mengesan secara spesifik bakteria yang disyaki *B. pseudomallei* dari plat kultur dan juga secara langsung dari spesimen klinikal.

Kajian ini secara langsung telah memberikan satu ulasan meluas bagi penyakit melioidosis dan agen penyebabnya, *B. pseudomallei* dalam kalangan populasi yang menduduki bahagian timurlaut Semenanjung Malaysia. Ciri klinikal dan faktor risiko bagi penyakit ini tidak terhad kepada Malaysia sahaja dan genotip *B. pseudomallei* menunjukkan kepelbagaian yang berkait rapat dengan taburannya di persekitaran. Penemuan genotip baharu menggambarkan aktiviti serta ketakstabilan genetik bakteria ini yang boleh meramalkan kemunculan jenis-jenis bakteria baharu dengan kesan kevirulenan yang berbeza. Kajian ini juga menyokong kajian lepas yang mengandaikan bahawa faktor manusia dan persekitaran menjadi penyebab kepelbagaian gejala klinikal penyakit melioidosis ini. Bagi kaedah pengesanan secara

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CLINICAL CHARACTERISTICS, GENOTYPING AND MOLECULAR DETECTION OF

Burkholderia pseudomallei

ABSTRACT

Burkholderia pseudomallei is a saprophytic Gram-negative bacterium that infects human body through inhalation, ingestion or percutaneous inoculation and causes melioidosis. The pathophysiology and clinical presentations of melioidosis are influenced by *B. pseudomallei* load on exposure, route of infection and human risk factors. Laboratory methods for *B. pseudomallei* include various procedures applied for diagnosis, molecular epidemiology and pathogenicity studies using conventional, immunological and molecular techniques.

In this study, the clinical characteristics and risk factors of melioidosis were explored. Genotyping using multilocus sequence typing (MLST) was performed on clinical *B. pseudomallei* isolates to explore the degree of their genotypic diversity in the area of the study and to correlate the identified genotypes with clinical presentations and outcomes. In order to improve the laboratory detection of *B. pseudomallei*, a PCR-based assay targeting *sctq* gene of the TTS1 cluster has been developed.

In this study, which involved 158 cases, the principal clinical presentations reported were lung infection in 65 (41.1%), skin infection in 44 (27.8%), septic arthritis/osteomyelitis in 20 (12.7%) and liver infection in 19 (12.0%). Bacteremic melioidosis was seen in most cases (n=121, 76.6%). Focal melioidosis was seen in

124 (78.5%) of patients and multi-focal melioidosis was reported in 45 (28.5%) cases, while melioidosis with no evident focus was reported in 34 (21.5%) patients. Internal organ abscesses and secondary foci in lungs and/or soft tissue were common in this study. Sixty seven (41%) patients were admitted during the monsoonal wet season. Death due to melioidosis was reported in 52 (32.9%) of patients, while recurrent infections occurred in 4 (2.5%) patients. Twelve fatal melioidosis cases directly attributed to the absence of prompt acute-phase treatment were seen in this study. Predisposing risk factors were reported in 133(84.2%) patients, which included diabetes (74.7%), immune disturbances (9.5%), cancer (4.4%) and chronic kidney disease (11.4%). On multivariate analysis, the independent predictors of mortality were the presence of at least one co-morbid factor (OR 3.0; 95% CI 1.1–8.4), the occurrence of septic shock (OR 16.5; 95% CI 6.1–44.9) and age >40 years (OR 6.47; 95% CI 1.7–23.8).

Multilocus sequence typing of 83 *B. pseudomallei* isolates has revealed 32 different sequence types, of which 13 (40%) were novel, namely: ST1317, ST1318, ST1319, ST1320, ST1321, ST1322, ST1323, ST1324, ST1325, ST1326, ST1327, ST1358 and ST1359. All retrieved sequence types were deposited in MLST database. The frequencies of sequence types among the 83 isolates ranged from 1-12 observations with predomination of ST54, ST371 and ST289. All non-novel sequence types identified in this study were not exclusive for Malaysia; they were identified in other regional countries with different frequencies. However, some of these sequence types were firstly identified in Malaysia such as ST371, ST164, ST47, ST306, ST55, ST376, ST402, ST507, ST368, ST369, ST10 and ST168.

Based on MLST data analysis, the constructed phylogenetic tree revealed more than half of the sequence types clustering with each other, as well as with the sequence types from Malaysia, Thailand, Singapore, Cambodia, Vietnam, Laos and China. Majority of novel sequence types were clustered with each other along the phylogenetic tree. The only novel and singleton sequence type in this study was ST1326. The present study has expanded the former Malaysian database for *B. pseudomallei* population genetics by adding more branching sequence types and the creation of new cluster after predicted clonal expansion emerged from ST84.

There was no evidence of associations for *B. pseudomallei* sequence types with clinical melioidosis presentations on the evolution tree. No clustering of particular sequence types with a given clinical outcome was noted. In addition, there was no evidence of differential virulence or strain tropism. Severe sepsis (n=11) was caused by strains of 7 different sequence types, while both septic shock (n=29) and abscesses (n=30) were caused by strains of 17 and 18 different sequence types, respectively. Presentations with sepsis, pneumonia, different melioidosis types, septic encephalopathy and other organ infections and disease outcome were caused by strains of several different sequence types. Finally, two-way tables for all bacterial genotype clusters in relation with each clinical outcome were statistically insignificant and no risk estimate was reported for every genotype cluster for developing any of clinical outcomes.

PCR was optimized to achieve the maximal discrimination between *B. pseudomallei* and its genetic relatives and to enhance the best detection limit. The *in-silico* specificity test showed complete match with a reference sequence for the target gene.

sctQ-PCR showed 100% specificity once tested against *B. thailandensis* and *B. cepacia* and a panel of genetically related and non-related microorganisms DNA. The sensitivity was 100% after successful amplification of isolates of different MLST strains and genotypes. The lower concentration of purified *B. pseudomallei* DNA that was amplified by *sctQ*-PCR was 100 fg/µl, whereas the lowest number of bacterial cells detected in spiked blood specimens was 18.2 X 10^5 CFU/ml. The developed PCR assay was capable of specific identification of suspected *B. pseudomallei* from cultured plate or directly from clinical specimens.

This study has provided major review for melioidosis and its causative agent, *Burkholderia pseudomallei* among selected population clusters residing in the Northeastern part of Peninsular Malaysia. Clinical presentations and risk factors of melioidosis were not unique for Malaysia and *B. pseudomallei* genotypes showed wide diversity that was correlated with the distribution of different strains in the environment. Novel sequence types indicated the genetic activity and instability of the bacteria which predicted the emergence of new strains that may harbor different virulence effect. This work kept on previous assumption that host and environmental factors were behind the diversity for clinical presentation of the disease. For molecular diagnosis, further upgrading and evaluation are needed to improve the performance of the assay.

CHAPTER ONE

INTRODUCTION & LITERATURE REVIEW

1.1. History of Nomenclature

The early history of *Burkholderia pseudomallei* discovery was started in 1911 when a pathologist Alfred Whitmore and his assistant Krishnaswami noticed unusual bacterial infection presentation in multiple organs during the postmortem examination of a Burmese man with pneumonia and described the case in the literature in 1912.

Subsequently, both authors described cases of fatal septicemic disease with multiple organs abscesses in 38 patients that were clinically similar to glanders disease in animal, which was identified in 1885, and the clinical condition was named Whitmore's disease.

In all cases, Gram negative bacilli were grown on peptone agar and potato slopes and were differentiated from glanders agent (*Pseudomonas mallei*) by rapid growth, motility, colony appearance on agar and lack of Strauss reaction in guinea pigs.

These characteristics were sufficiently distinctive to recognize the isolated bacilli as new species; *Bacillus pseudomallei* which was closely related to *P. mallei*. Since 1913, doctors Stanton and Fletcher had recognized the same disease in laboratory animals and humans and reported many cases as the first discovery of the disease in Malaysia.

Because the disease was clinically and pathophysiologically similar to glanders, the same doctors proposed the name melioidosis (*melis*: distemper of asses; *oid*: like; *osis*: a condition) which refers to glanders-like infection.

Melioidosis agent was known by several names over decades, including *Bacillus whitmori, Pfeifferella whitmori, Pfeifferella pseudomallei, Actinobacillus pseudomallei, Flavobacterium pseudomallei, Loefflerella whitmori, Loefflerella pseudomallei, Bacillus pseudomallei, Malleomyces pseudomallei* and *Pseudomonas pseudomallei* (Puthucheary, 2009; Currie, 2015a).

In 1992, with the molecular revolution of bacterial classification, Yabuuchi and co-workers proposed a new genus *Burkholderia* (named after Walter Burkholder who first described *Burkholderia cepacia*) based on the 16S rRNA sequences, DNA-DNA homology, composition of cellular lipid and fatty acid, and phenotypic characteristics.

The species *pseudomallei*, along with six species under this group, were transferred to the new genus and became *Burkholderia pseudomallei, Burkholderia mallei, Burkholderia solanacearum, Burkholderia gladioli, Burkholderia caryophylli, Burkholderia pickettii* and *Burkholderia cepacia* (Yabuuchi and Arakawa, 1993).

These findings, and in addition to the continuous speciation of other Pseudomonads under the genus *Burkholderia* were confirmed by recent molecular genotyping studies (Rainbow *et al.*, 2002; Godoy *et al.*, 2003).

Burkholderia genus belongs to the class Beta-Proteobacteria, order Burkholderiales and family Burkholderiaceae, and encloses more than 30 species that are mostly nonpathogenic soil-dwelling bacteria such as *B. thailandensis* which is genetically closed to few pathogenic species, such as *B. cepacia*, *B. mallei* and *B. pseudomallei* (Currie, 2015a).

Burkholderia pseudomallei is a small gram-negative, nonsporulating, straight or slightly curved bacillus, oxidase-positive, motile and aerobic. It exhibits bipolar staining (safety-pin appearance) due to accumulation of poly-β-hydroxybutyrate.

Bacterial cells are approximately 0.4-0.6 μm in width and 2-5 μm in length with two to four polar flagella. The bacterium is easily recovered on routine culture media and produces variable colony morphologies in appearance.

Previously, *B. pseudomallei* was classified by the Centers for Disease Control and Prevention (CDC) as category-B biothreat (Dance, 2008). However, *B. pseudomallei* was upgraded to Tier 1 Biological Select Agent status as a biothreat beside *Bacillus anthracis* and *Yersinia pestis.*

This ranking system is reserved for infectious agents that have the greatest risk to human and animal health and was declared by the U.S. Department of Health and Human Services (HHS) and known as Biological Select Agents or Toxins" (BSATs) (Butler, 2012).

B. pseudomallei K96243 genome contains two chromosomes: chromosome 1 (4.07 Mb) is composed of housekeeping genes involved with metabolism, motility and biosynthesis of cellular components. Chromosome 2 (3.17 Mb) genes are thought to be involved with adaptation within the environment and host.

The genome of *B. pseudomallei* is of great plasticity that leads to genetic divergence due to frequent mutations, gene transfer and recombination that influence strain to strain variation (Holden *et al.*, 2004).

Recently, many studies reported wide genetic diversity of *B. pseudomallei* (De Smet *et al.*, 2015; Nandi *et al.*, 2015), as well as cases of genetic divergence reported among isolates within the same melioidosis patient and indicated within-host adaptation (Price *et al.*, 2010; Limmathurotsakul *et al.*, 2014a).

In addition, morphological changes in *B. pseudomallei* colonies for a single strain was also observed and reported in strains isolated from various specimens types from melioidosis patients and has been associated with bacterial adaptation to adverse conditions (Chantratita *et al.*, 2007a; Tandhavanant *et al.*, 2010).

1.2. *B. pseudomallei* in the Environment and Associated Risk Factors

Burkholderia pseudomallei is a saprophytic organism which is able to survive for extended periods of time in variety of harsh environmental conditions like nutrient deficiency, different temperatures and pH and wide range of physical, chemical and biological factors (Cheng and Currie, 2005) and is capable of infecting the free-living protozoa *Acanthamoeba astronyxis* (Inglis *et al.*, 2000b). However, *B. pseudomallei* exclusively inhabits the environment in tropical regions of the world located between 20°N and 20°S lines of latitude and can be recovered from water and wet soils, particularly rice paddies (Cheng and Currie, 2005).

Many studies tested environmental samples to investigate for *B. pseudomallei* and understand its geographical distribution in Thailand (Wuthiekanun *et al.*, 2009), Northern Australia (Cheng *et al.*, 2008a), Taiwan (Chen *et al.*, 2010), Papua New Guinea (Warner *et al.*, 2008) PDR Lao (Wuthiekanun *et al.*, 2005c), Malaysia (Ellison *et al.*, 1969; Strauss *et al.*, 1969b) and Brazil (Rolim *et al.*, 2009) and found that *B. pseudomallei* was more common in rice paddies and farms (Wuthiekanun *et al.*, 1995; Kaestli *et al.*, 2009).

A study concluded that the increased incidence of melioidosis in an area, might be partly correlated with the increased bacterial density in the environment of that area and also found that the increased exposure to bacteria was resulted from changes in behaviors of farmers and work style; such as farming techniques (Chantratita *et al.*, 2008b). In Australia, *B. pseudomallei* was found most commonly in wet soils to a depth of 25 to 45 cm with the proposal of moving to the surface with rising water

table during the monsoon events that increases the surface bacterial load and then increasing the infective dose that enhance the chance for severe acute infection (Grivas *et al.*, 2015). However, studies from Malaysia showed less correlation of melioidosis with rainfall and reported no significant association between month of patients' admission to hospital and mortality due to melioidosis (Sam and Puthucheary, 2007; Deris *et al.*, 2010).

1.3. Modes of Acquisition

Three common modes of *B. pseudomallei* entry were recognized in human and animals: inhalation of aerosolized bacteria, ingestion of contaminated food and water and percutaneous inoculation (Currie, 2015b). Inhalation of contaminated dust was initially thought to be the primary route of transmission of *B. pseudomallei* according to the earlier cases of respiratory melioidosis in soldiers exposed to dust in Vietnam (Howe *et al.*, 1971). Recent report was documented in Singapore for a case of inhalation-associated melioidosis in a returning tourist (Amadasi *et al.*, 2015). Animal studies also demonstrated the potential importance of aerosol inhalation model and reported high lethality observations (Nelson *et al.*, 2011; West *et al.*, 2012b). However, the overall incidences of inhalation melioidosis remain uncertain in comparing with other routes (Currie, 2015b). *B. pseudomallei* gain access through the direct contact of contaminated soil and water with percutaneous wounds or existing skin abrasions, ulcers, or burns (McLeod *et al.*, 2015). During massive weather events such as monsoonal storms, typhoons and tsunami, the route of bacterial entry might be shifted from inoculation to inhalation as the predominant route of transmission (Chen *et al.*, 2014).

In spite of conclusions made for the impossibility of the ingestion route for transmission (White, 2003; Dance, 2008), few reports described disease acquisition via ingestion of contaminated food or drink (Ketterer *et al.*, 1986; Currie *et al.*, 2001). However, rivers in tropical environments and unchlorinated domestic water supplies that may comprise high bacterial load expect to uncover for higher risk of ingestion melioidosis than previously thought. This was supported by previous literatures findings of melioidosis outbreaks reported in Thailand that were linked to contamination of potable unchlorinated water with *B. pseudomallei* (Limmathurotsakul *et al.*, 2014b; Thaipadungpanit *et al.*, 2014).

Less common melioidosis transmission routes have been reported including mother to child (Ralph *et al.*, 2004), sexual transmission (Thatrimontrichai and Maneenil, 2012), laboratory acquired (Schlech *et al.*, 1981), animals to humans (Choy *et al.*, 2000) and person-to-person transmission (Holland *et al.*, 2002).

1.4. Melioidosis: Pathology and Pathogenesis

Melioidosis has emerged over the past 20 years as an important cause of morbidity, mortality, and fatal community-acquired bacteremic pneumonia. Melioidosis pathophysiology and clinical presentations, degree of disease severity and outcomes are all influenced by *B. pseudomallei* load on exposure (infecting dose) that might be controlled by environmental risk factors; route of infection acquisition (inhalation, percutaneous inoculation or ingestion); virulence factors of the infecting *B. pseudomallei* strain and most importantly the host risk factors (Currie, 2015a).

1.4.1. Virulence Factors

Burkholderia pseudomallei is an intracellular pathogen which multiplies inside phagocytic and nonphagocytic cells and possesses many genes responsible for metabolism pathways and surface components synthesis such as lipopolysaccharide and capsular polysaccharide. The structural and functional aspects of *B. pseudomallei* components, their regulation, and their role in pathogenesis as virulence factors are still incompletely or poorly understood. Examples of *B. pseudomallei* virulence genes include those encoding for proteases, lipases, lecithinase, catalase, perioxidase, hemolysins, cytotoxins, adhesions, invasins, fimbriae and pili, and secretion systems types II, III and VI (Sarovich *et al.*, 2014a) (table 1.1).

Table 1.1: Examples for virulence factors of *B. pseudomallei* and their relative importance in melioidosis pathogenesis

Virulence factor	Description	Role during pathogenesis
Capsule	200-kD capsular polysaccharide composed of a homopolymer of-3)-2-*O*-acetyl- 6-deoxy-β- D-manno-heptopyranose	Required for epithelial attachment and survival and/or replication in macrophages and during bacteremia by reducing C3b-mediated opsonization and phagocytosis
Lipopolysaccharides	Unbranched heteropolymer made of D-glucose and L-talose units with the structure -3)-β-D-glucopyranose-(1–3)-6-deoxy-α- L-talopyranose.	Confers resistance against macrophage killing and promotes survival during bacteremia by preventing killing by the alternative pathway of complement
Flagellin	Surface-associated 43-kDa protein	Required for motility and optimal invasion of non-phagocytic cells
Fimbriae and pili-IV	Same structure of flagella	Bacterial attachment to epithelial surfaces and microcolony formation
Quorum sensing	Seven quorum-sensing molecules, including C8-HSL, 3-hydroxy-C8-HSL, 3-oxo-C8-HSL, C10-HSL, 3-hydroxy-C10-HSL, 3-oxo-C10-HSL, 3-hydroxy-C12-HSL, and 3-oxo-C14-HSL	Coordinate biofilm synthesis, virulence, and antibiotic resistance based on the local density of the bacterial population.
Secretion systems genes	Cluster of genes located in pathogenicity island gene cluster	Type II secretion system (T2SS): secretion of protease, lipase, and phospholipase C Type III secretion system (T3SS): inject host cells with bacterial toxin and so called toxin gun Type VI secretion system (T6SS): modulate intracellular persistence
Exoproteins	Phospholipase C (PLC): 77-kDa proteins putative nonhemolytic enzyme Metalloprotease: a 47-kDa protease	PLC: hydrolyse lipids, phosphatidylcholine and sphingomyelin Metalloprotease: have cytotoxic and proteolytic activities

(Cheng and Currie, 2005; Lazar Adler *et al.*, 2009; Galyov *et al.*, 2010)

In vivo infection models, immunological, cellular and molecular studies were done to understand the impact of each virulence element of the bacteria. The study of lethal effect for a given structural virulence factor is usually performed by preparing genetically-modified *B. pseudomallei* mutants for certain virulence factor and then used to infect a laboratory animal (Currie, 2015a). Another principle is to inject a laboratory animal with purified *B. pseudomallei*-antigen that represents certain structural virulence factor and demonstrate degree of induced passive protection (Currie, 2015a). Several studies were performed on passive immunity using capsule-mutants (Atkins *et al.*, 2002a), lipopolysaccharides-mutants (Jones *et al.*, 2002) and flagillin-mutants (Chua *et al.*, 2003) showed low virulence of these mutants and reported positive protection effect of the anti-sera of all antigens against lethal bacterial challenge. Genetically muted *B. pseudomallei* for enzymes, secretory and signaling systems were also studied, including quorum sensing (Ulrich *et al.*, 2004), secretion systems type II, III and VI (Warawa and Woods, 2005; Pilatz *et al.*, 2006) and proteases (Boddey *et al.*, 2007).

1.4.2. Bacterial Attachment and Cell Invasion

Burkholderia pseudomallei is transmitted from its environmental reservoir to human host and attached to epithelial cell layers of either the abraded skin, the mucosal surface of the lungs or gastrointestinal tracts where it initially attaches by the capsule and type 4 pili. Once the epithelial attachment is maximized, bacteria start forming pili-mediated microcolonies (Galyov *et al.*, 2010).

Type III secretion system effectors assist bacterial invasion by saving the engulfed bacteria from autophagy killing via promoting the disruption of phagosomal membranes and facilitate the escape of bacteria into the host cytosol. Now the free bacterial cells in the cytosol start replication by the promotion from Type VI secretion system effectors. Once bacterial quorum sensing system alert for critical density threshold level for *B. pseudomallei* build up within host cell, *B. pseudomallei* induce apoptosis to lyse the host cell in order to escape and invade another cell (Lazar Adler *et al.*, 2009) (figure 1.1). As the invasion progresses, *B. pseudomallei* spread to body organs via the lymphatic vessels, either carried within macrophages or as free bacteria in the circulation with protection against circulating antibodies mediated by capsule and lipopolysaccharides (Lazar Adler *et al.*, 2009).

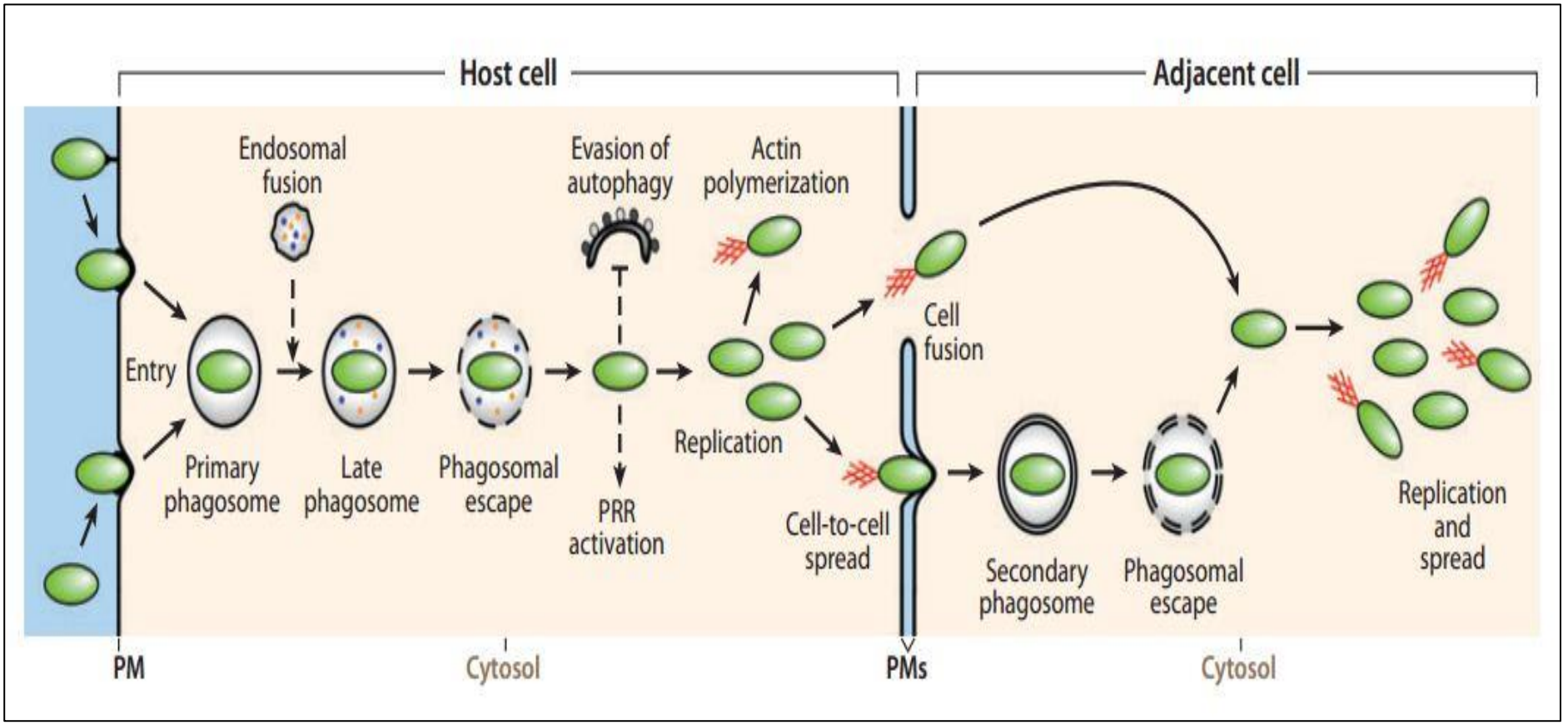

Figure 1.1: Demonstration of infection events beginning with cellular attachment and evading auto-phagosomes then invading adjacent cell. PM: plasma membrane, PRR: pathogen recognition receptor. Adapted from Galyov *et al.*, (2010).

1.4.3. Host Immune Response

Burkholderia pseudomallei induces humoral and cell-mediated responses during disease stages. Individuals who were exposed to *B. pseudomallei* develop IgA, IgM, and IgG antibodies that are increased during acute infection and their levels correlate with the severity of the disease. During severe melioidosis, the levels of pro-inflammatory cytokines and immunoregulatory cytokines indicate an extensive activation of the cellular immune response. In addition, the cellular immunity plays a key role in controlling the infection in asymptomatic seropositive individuals (Sarkar-Tyson *et al.*, 2009) (figure 1.2).

After melioidosis recovery, high levels of antibodies remain elevated for years in patient's serum, suggesting continuous exposure to *B. pseudomallei* or sequestration of bacteria in intracellular sites of latency (Gan, 2005). In spite of the role of cell-mediated immune response in the neutralizing and protection against disease progression, there is no definitive evidence for the protective immunity against melioidosis which allow reinfection occurrence with a different *B. pseudomallei* strain after successful treatment (Currie, 2015b).

Several studies showed the association of elevated levels of various inflammatory mediators with the severity of melioidosis. Nuntayanuwat *et al.*, (1999) found an association between severe melioidosis and TNF-α gene allele 2 which was represented by higher production of TNF-α.

In a study, mouse model was used for melioidosis and showed that TNF-α or IL-12 neutralization has increased mice susceptibility to infection; in that study treated mice with monoclonal anti–IFN-γ antibodies died more quickly which indicated the importance of IFN- γ for protection (Santanirand *et al.*, 1999). Toll-like receptors (TLR) role has been proposed and confirmed in the innate immune response in melioidosis (West *et al.*, 2012a).

Evasion mechanisms were recognized to enable *B. pseudomallei* to escape from host immune response by decreasing immune response stimulation through expression of unusual lipopolysaccharide structures (Utaisincharoen *et al.*, 2000), intracellular spreading by actin polymerization (Breitbach *et al.*, 2003), complement inhibition by bacterial capsule (Reckseidler-Zenteno *et al.*, 2005), escaping from endosome to cytosol (Stevens *et al.*, 2002), induction of macrophage apoptosis (Sun *et al.*, 2005) and inhibition of cytokines release (Utaisincharoen *et al.*, 2001).

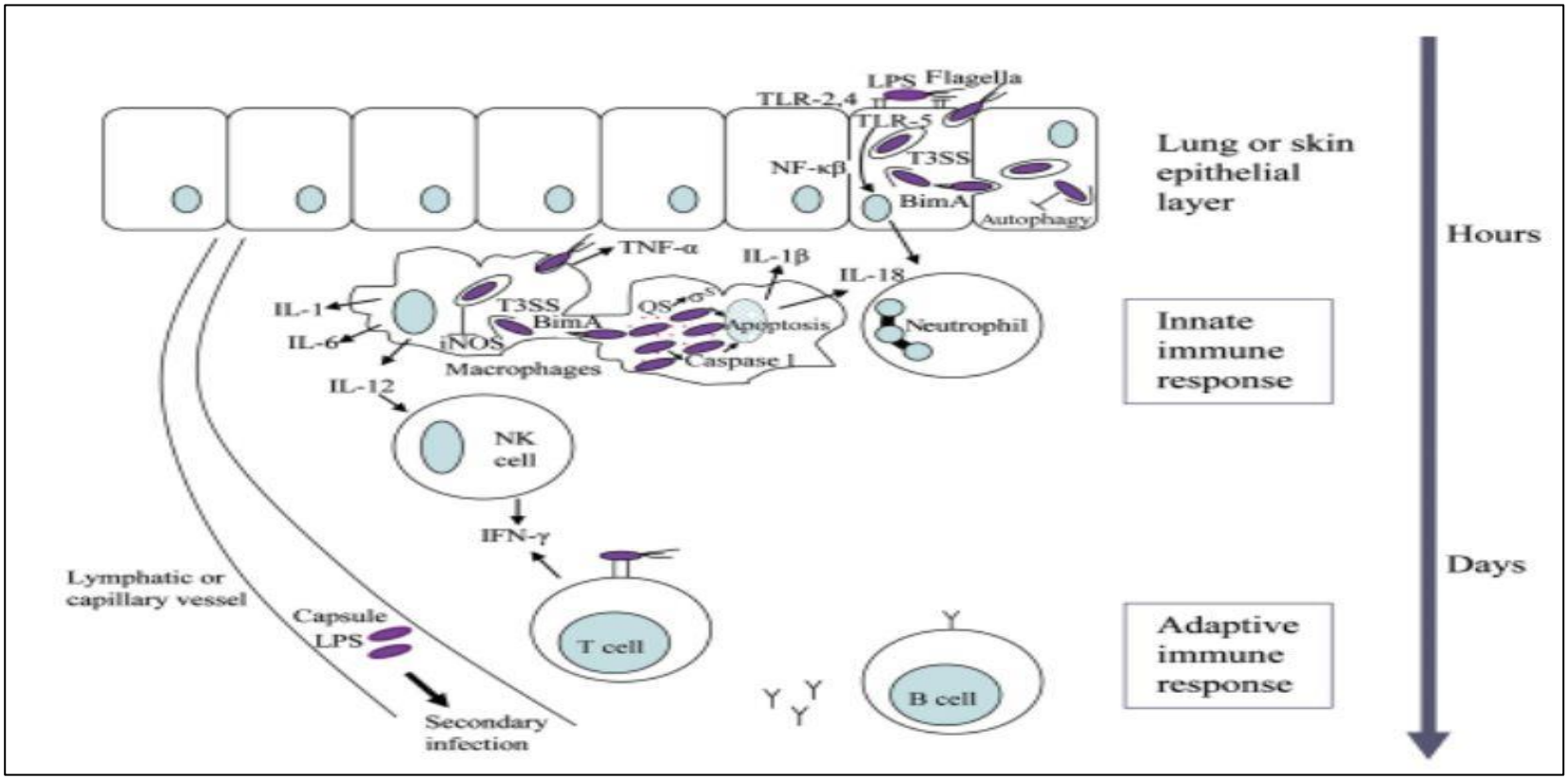

Figure 1.2: Cellular infection, spread and host immune response. Adapted from Lazar Adler *et al.*, (2009)

1.4.4. Clinical Manifestations

Melioidosis is a multi-system infection with non-specific signs and symptoms. The clinical spectrum of melioidosis is broad, therefore, several clinical classifications were proposed and identified the infection as bacteremic versus nonbacteremic, multifocal versus localized, and acute versus chronic melioidosis (Currie, 2015a).

Melioidosis cases were categorized according to The Infectious Disease Association of Thailand as: bacteremic multifocal infection with septicemia; bacteremic localized infection with septicemia; non-bacteremic localized infection and melioidosis with transient septicemia (Leelarasamee and Bovornkitti, 1989).

B. pseudomallei strains differ in their ability to cause disease and the outcome depends on the immune status and response of the infected host. Melioidosis incubation period is influenced by the mode of infection, inoculation dose, host risk factors, and strain virulence. The incubation period of melioidosis starts from the event of infection and extends until the onset of symptoms appearance but still has not been accurately estimated. An incubation period of 1-21 days (mean 9 days) was suggested to precede acute melioidosis, which is consistent with a series of nosocomial cases from Thailand (Puthucheary, 2009). Chronic melioidosis is defined as illness with symptoms duration of more than 2 months (Currie *et al.*, 2000).

In inhalation melioidosis, the infection is primarily localized and started from the lungs and could be asymptomatic or subclinical and the bacteria may colonize the lungs for latent stay. On the other hand, the infection could be aggressive and is

characterized by severe pneumonia and bronchitis leading to chest pain and shortness of breath and may spread to pleural fluid (Currie, 2015a). An evidence-based definition for inhalation melioidosis was recently described (Cheng *et al.,* 2013). In case of infection dissemination from the lungs, *B. pseudomallei* can enter blood stream and disseminates to other organs where the pathogen may colonize and cause deep seated abscesses (Cheng *et al.*, 2013) (figure 1.3).

Upon percutaneous inoculation, localized inflammatory process started with the production of large amounts of pus exudate consisting of neutrophils and edema fluid that leads to local suppuration accompanied by fever, rigors, and malaise. Suppurating abscess in the skin layers may involve the deeper tissues of muscles and bone which is common in untreated cases. In general, for inoculation melioidosis, the infection started with skin abscess followed by bacteremia and secondary organ abscesses (Currie, 2015b).

Acute melioidosis is more associated with the environmental and individual risk factors, whereas reactivated latent infections are associated with individual risk factors (Pitt *et al.*, 2007; Lazar Adler *et al.,* 2009).

The mortality of acute melioidosis is 30-47% (Peacock *et al.*, 2011); untreated bacteremic melioidosis increases the mortality rates to 80-90% within two days of hospital admission (Lowe *et al.,* 2013). In endemic areas, melioidosis had mortality rate at 9%, in Australia (Currie *et al.,* 2010) and 34% in Malaysia (Hassan *et al.,* 2010). Pneumonia and sepsis are the most severe clinical manifestations of melioidosis (White, 2003). In addition, pneumonia is the most common presentation

of melioidosis (Cheng and Currie, 2005). In Thailand, melioidosis cause 20% of community-acquired septicemia which accounts for 39% of fatal septicemia cases (Chaowagul *et al.*, 1989) and 36% of fatal community-acquired pneumonia (Boonma *et al.*, 1990). In addition, melioidosis was responsible for most cases of fatal community-acquired septicemic pneumonia in tropical Australia (Currie *et al.*, 2010).

The recurrent infection is defined as a new presentation of acute culture-confirmed melioidosis after recovery from previous infection upon completion of antibiotic therapy regime. Recurrent infection occurs in 6–13% of melioidosis cases due to relapse of the infection by the same bacterial strain rather than reinfection with a new bacterial strain (Currie *et al.*, 2000; Maharjan *et al.*, 2005). In Thailand, the overall relapse rate was up to 30% per year of survivors who had severe melioidosis (Chaowagul *et al.*, 1993). While in Malaysia, the rate of relapse or recurrence was approximately 13% over a period of 5 years (Puthucheary, 2009). In Australia, Sarovich *et al.*,(2014b) had reported improved recurrent melioidosis rate in Darwin hospital over 23 years and documented recurrent infection in 39/679 (5.7%); in which 29 patients suffered relapsed infection, while the rest got re-infected with new *B. pseudomallei*.

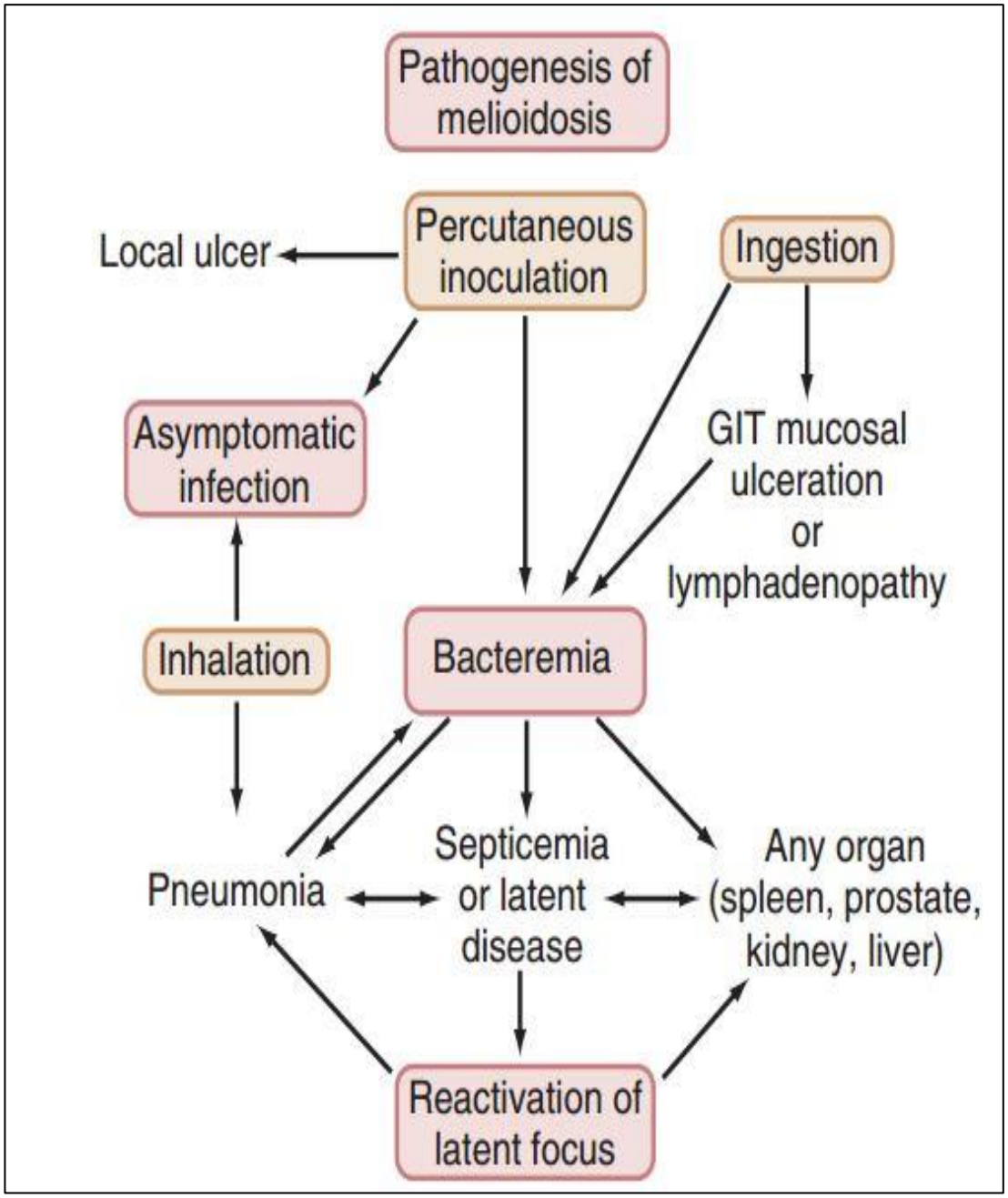

Figure 1.3: Clinical involvement of melioidosis from the starting point of entry. Adapted from Currie (2015a).

1.4.5. Human Risk Factors

Many of melioidosis cases occur in individuals with pre-existing illnesses; up to 80% of melioidosis patients have one or more risk factors, whereas risk factors are less common in children (Currie, 2015b). In Australia, a prospective melioidosis study over 25 years reported that 113 of the 115 death cases were directly attributed to one or more known risk factors (Currie *et al.*, 2010). In the same country, a 24-year prospective study was done recently to review the clinical presentations and outcomes of pediatric melioidosis. Out of 820 patients, 45 pediatric cases were identified, representing 5% of melioidosis. About 84% of children had no recognized risk factors for melioidosis (McLeod *et al.*, 2015). In Malaysia, the percentage range of patients at risk was 77-90% (How *et al.*, 2005; Deris *et al.*, 2010; Hassan *et al.*, 2010).

The recognised risk factors in melioidosis patients were type II diabetes mellitus, chronic renal failure, dengue hemorrhagic fever, immunosuppressive treatments, in particular steroids, α-thalassemia, chronic liver and lung disease and neutropenia (Dance, 2008). There is no evidence that human immunodeficiency virus infection predisposes to melioidosis (Cheng and Currie, 2005). However, the association of melioidosis with diabetes is strong and may increase the risk of the infection by up to 100-fold (Suputtamongkol *et al.*, 1994). Pre-existing or newly diagnosed type-2 diabetes mellitus is the most frequently reported predisposing factor for melioidosis (Puthucheary, 2009). In Malaysia about 70-89% of melioidosis patients had diabetes (How *et al.*, 2005; Deris *et al.*, 2010; Hassan *et al.*, 2010), 37% in Australia (Currie *et al.*, 2010) and 53-60% in Thailand (Suputtamongkol *et al.*, 1999).

Melioidosis affects all age groups. In Malaysia, it was found that the peak age-specific incidence of melioidosis occur from 41–59 years for both males and females (Puthucheary, 2009), but in Thailand it was 60-69 years for men and 50-59 years for women (Suputtamongkol *et al.*, 1994). Males are at higher risk of gaining the infection than females; probably because of differences in exposure to bacterial reservoir such as soil during rice farming. The male-female ratio for melioidosis was higher in all studies in Malaysia (Hassan *et al.*, 2010), Australia (Currie *et al.*, 2010), Thailand (Limmathurotsakul *et al.*, 2010b),and Singapore (Lo *et al.*, 2009). However, rapid diagnosis, appropriate antibiotics, and proper sepsis management will reduce the rate of death in melioidosis patients with predisposed risks and death is extremely uncommon in those without identified risk factors for melioidosis (Deris *et al.*, 2010).

1.5. Epidemiology of Melioidosis

Melioidosis occurs predominantly in Southeast Asia, northern Australia, the Indian subcontinent and China (figure 1.4). Cases were first described in Burma 1912, Malaysia 1913, Singapore 1913, Vietnam 1925 and Indonesia 1929 (Leelarasamee, 2000). Although Thailand and Australia have the highest endemicity of melioidosis, it was not documented in Thailand until 1955 and in Australia until 1949. In Thailand, the annual melioidosis incidence rate was 4.4 per 100,000 (Suputtamongkol *et al.*, 1994) with frequently documented cases from the northeastern provinces (Limmathurotsakul *et al.*, 2010b).

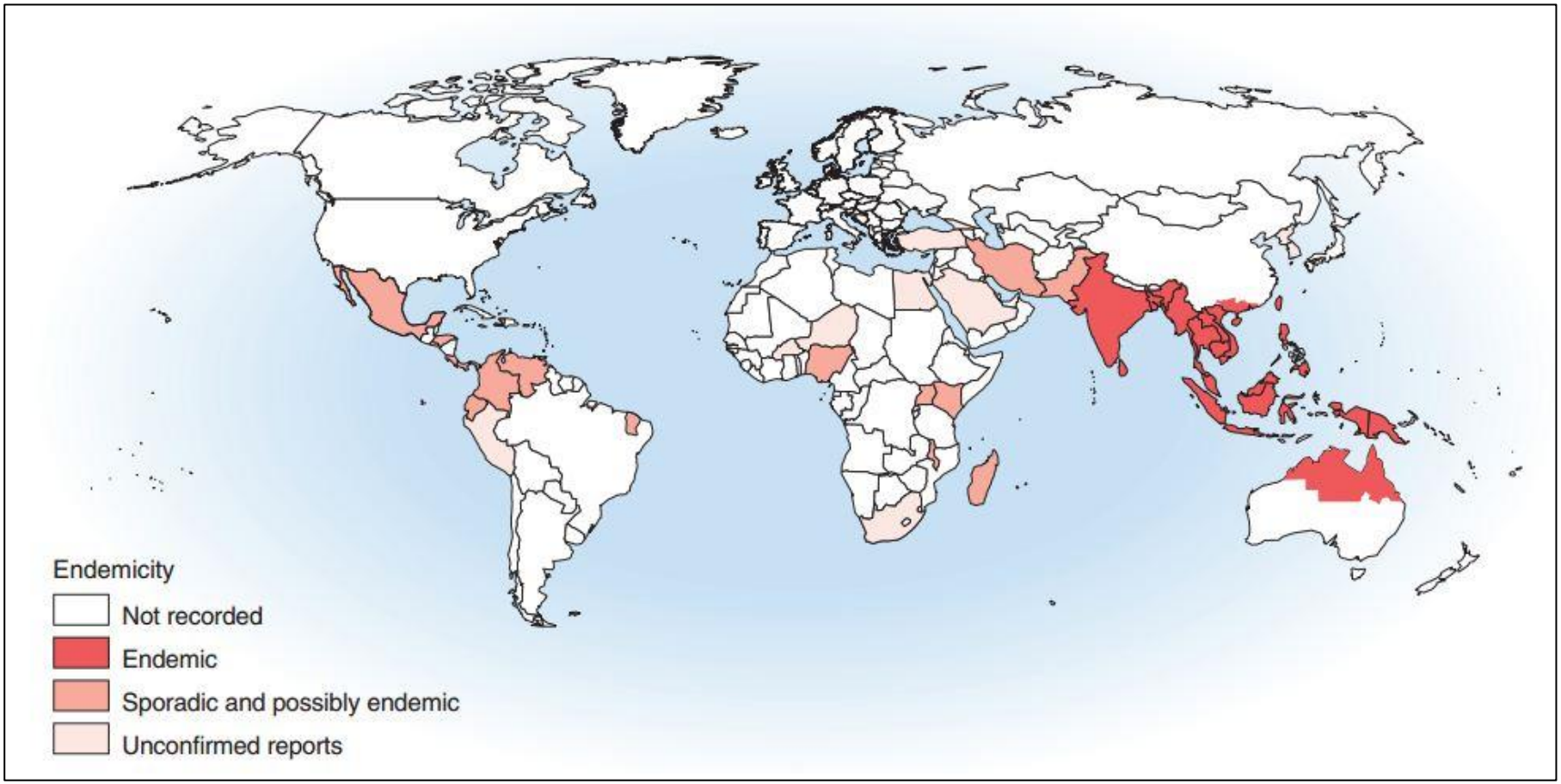

Figure 1.4: Worldwide distribution of melioidosis. Adapted from Currie, 2015a.

The epidemiological studies have defined an annual incidence rate of 16.5 per 100,000 and reported 540 melioidosis cases over 20 years in tropical Australia (Currie *et al.*, 2010). In Northeastern Malaysia, 35 melioidosis cases were reported over 5 years (Deris *et al.*, 2010) and the annual incidence rate was 6.1 per 100,000 population in central state of Malaysia (How *et al.*, 2005).

The history of melioidosis in Malaysia began with description of infection in animal by Stanton and Fletcher as far back as 1913, and then human cases were subsequently published in 1932. Strauss *et al.*, (1969a) has conducted a sero-survey using indirect haemagglutination assay in 1964 to 1966, and reported 7.3% seropositivity. In Malaysia the sero-prevalence of melioidosis using different serological procedures and was 7% (Vadivelu *et al.*, 1995). More recent Deris *et al.*, (2010) described the clinical characteristics and outcomes of 35 melioidosis identified in Kelantan state over 5 years. In Pahang state, 63 confirmed cases of melioidosis were identified over a year and the annual incidence rate of melioidosis was calculated as 6.1 per 100,000 of population (How *et al.*, 2005). In Kedah state, the incidence of melioidosis was 16.35 per 100,000 populations per year, which was remarkably much higher than the rates recorded in other Southeast Asian countries (Hassan *et al.*, 2010).

Melioidosis is now well recognized in epidemiology studies and case series documented from Singapore (Lo *et al.*, 2009), Brunei (Pande and Kadir, 2011), Laos (Phetsouvanh *et al.*, 2001), Vietnam (Parry *et al.*, 1999), China (Yang, 2000), Taiwan (Chen *et al.*, 2004), Myanmar (Wuthiekanun *et al.*, 2006c), Cambodia (Wuthiekanun *et al.*, 2008) India (Dance *et al.*, 1995), and Sri Lanka (Corea *et al.*,

2012). Although melioidosis is highly endemic in parts of Southeast Asia, cases were rarely reported in Indonesia; Tauran *et al.*, (2015) reported three cases of fatal melioidosis occurred between 2013 and 2014 beside seven cases reviewed from old literature. It was assumed that melioidosis could be endemic but still under-recognized throughout Indonesia and recommended that considerations should be given to make melioidosis a notifiable disease in Indonesia.

Cases of melioidosis from outside the classic endemic regions were increasingly being documented. This includes sporadic cases for human or animal infections or environmental isolates from the Middle East, Europe, Africa, the Caribbean, Brazil and in the Americas (Inglis *et al.*,2006; Salam *et al.*, 2011; Borgherini *et al.*, 2015; Wiersinga *et al.*, 2015).

1.6. *B. pseudomallei* Laboratory Methods

Laboratory methods for *B. pseudomallei* include conventional, molecular and immunological procedures that are applied for infection diagnosis, molecular epidemiology and pathogenicity studies (Hoffmaster *et al.*, 2015).

1.6.1. Laboratory Diagnosis

The highly variable presentations of melioidosis make the clinical diagnosis impossible without the aid of confirmatory laboratory testing. Melioidosis should be suspected in cases of febrile illnesses presenting with respiratory failure, multiple subcutaneous lesions, or tuberculosis-like radiological pattern (Puthucheary, 2009).