MELIOIDOSIS IN KELANTAN: DISEASE OUTCOME, DISTRIBUTION AND GENOTYPING OF *Burkholderia pseudomallei* FROM PATIENTS AND ENVIRONMENTS

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by

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

℃	Degree Celsius
%	Percentage
<	Less than
>	Greater than
≥	Greater than or equal to
±	Plus-minus
=	Equal to
∞	Infinity
/	Division or ‘or’
e.g	*exempli gratia* (for example)
g	gram
L	Litre
cm	Centimetre
µm	Micrometre
µM	Micromolar
mM	Millimolar
cm	Centimetre
mL	millilitre
µL	Microlitre
mL/L	Milliliters per liter
mg/L	Milligrams per litre
mg/mL	Milligrams per millilitre
ng/µL	Nanogram per microlitre
U/µL	Unit per microlitre

rpm	Revolutions per minute
xg	Times gravity (Unit of relative centrifugal force)
min	Minute
V	Volt
Mb	Megabases
bp	Base pair
et al.	Et alia (and others)
pH	Exponential of the concentration of hydrogen ion
CDC	Centre for Disease Control and Prevention
PBE	Plant-associated beneficial and environmental
CDS	Coding sequences
GI	Genomic islands
rRNA	Ribosomal ribonucleic acid
BPSA	*B. pseudomallei* selective agar
BCSA	*B. cepacia* selective agar
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
MS	Mass spectrometry
API	Analytical profile index
RDT	Rapid diagnostic test
TAT	Turnaround time
PCR	Polymerase Chain Reaction
TTSS	Type III Secretion System
USM	Universiti Sains Malaysia
DM	Diabetes mellitus
DEBWorP	Detection of environmental *Burkholderia pseudomallei* Working Party
JEPeM	Human Research Ethics Committee

QC	Quality control
SPSS	Statistical Package for the Social Sciences
ArcGIS	Aeronautical Reconnaissance Coverage Geographic Information System
PPE	Personal protective equipment
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
Taq	*Thermos aquaticus*
TAE	Tris-acetate-EDTA
NCBI	National Centre for Biotechnology Information
SEA	Southeast Asia
ST	Sequence type
PubMLST	Public Database for Molecular Diversity and Microbial Genome Diversity
MLST	Multi-locus sequence typing
MLEE	Multi-locus enzyme electrophoresis
MEGA	Molecular Evolutionary Genetics Analysis
NNR	Nearest Neighbour Ratio
Bpc	*B. pseudomallei* complex
Bcc	*B. cepacia* complex

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MELIOIDOSIS DI KELANTAN: HASIL JANGKITAN, TABURAN DAN GENOTIP BAGI *Burkholderia pseudomallei* DARI PESAKIT DAN PERSEKITARAN

ABSTRAK

Melioidosis merupakan satu penyakit berjangkit disebabkan oleh bakteria tanah yang sangat patogenik dikenali sebagai *Burkholderia pseudomallei.* Penyakit berjangkit ini mempunyai spektrum klinikal tidak patognomonik yang meluas dengan kadar kematian yang tinggi di seluruh dunia. Melioidosis yang kurang didiagnos dan kurang dilaporkan telah menjadi beban global disebabkan kurangnya kesedaran dan pengetahuan dalam populasi. Melioidosis adalah endemik di Malaysia, bagaimanapun, terdapat kekurangan data berkenaan corak taburan penyakit dan agen penyebab. Oleh itu, kajian ini dilakukan untuk mengenal pasti hasil jangkitan penyakit, taburan dan genotip bagi pencilan *B. pseudomallei* di Kelantan, negeri di timur laut Malaysia. Data demografi dan klinikal pesakit telah dianalisis dengan perisian SPSS sementara hubungan genetik antara varian klinikal dan persekitaran dianalisis dengan kaedah MLST. Kajian terhadap 70 kes positif menunjukkan prevalens melioidosis yang tinggi dalam kalangan lelaki (32.9%, n = 23), golongan umur pertengahan (75.7%, n = 53) dan kumpulan etnik Melayu (98.6%, n = 69). Diabetes mellitus adalah komorbiditi utama di kalangan pesakit (65.7%, n = 46). Kadar kematian sebanyak 37.1% (n = 26) dilaporkan di mana melioidosis septisemia membawa kadar kematian yang tinggi. Melioidosis sistemik, pembentukan nanah dan sepsis/ kejutan septik dikaitkan dengan hasil pesakit secara signifikan. Daerah Kota Bharu dikenali sebagai kawasan panas melioidosis di Kelantan. Analisis MLST terhadap 33 pencilan klinikal dan dua pencilan persekitaran *B. pseudomallei* mendedahkan 15 jenis jujukan yang berbeza

termasuk tujuh jenis jujukan baru muncul. Kajian ini menunjukkan kepelbagaian genetik *B. pseudomallei* di Kelantan dengan kepelbagaian jenis jujukan yang tertinggi di Bachok (67%). Genotip yang paling utama dalam populasi kajian ini adalah ST371 (17.1%; n = 6). Walaupun jenis jujukan yang dilaporkan tidak berkaitan dengan sebarang daerah di Kelantan, semua strain didapati berkait rapat dengan pencilan yang telah dilaporkan di Asia Tenggara. Peta risiko yang telah dihasilkan dengan genotip sedia wujud dan genotip baru yang beredar dalam populasi adalah penting untuk rancangan pencegahan dan pengurusan melioidosis di Kelantan pada masa akan datang.

MELIOIDOSIS IN KELANTAN: DISEASE OUTCOME, DISTRIBUTION AND GENOTYPING OF *Burkholderia pseudomallei* FROM PATIENTS AND ENVIRONMENTS

ABSTRACT

Melioidosis is an infectious disease caused by highly pathogenic soil-dwelling bacteria known as *Burkholderia pseudomallei.* This infectious disease has a wide range of non-pathognomonic clinical spectrum with high mortality rates worldwide. Melioidosis is underdiagnosed and underreported which has become a global burden due to the lack of awareness and knowledge among the population. Melioidosis is endemic in Malaysia, however, there is paucity of data on the distribution pattern of the disease and the causative agent. Therefore, this study is conducted to identify the disease outcome, distribution and genotyping of *B. pseudomallei* isolates in Kelantan, the north-eastern state of Malaysia. The demographic and clinical data of patients were analysed by SPSS software while the genetic interrelationship between clinical and environmental variants was analysed by MLST method. Study of a total of 70 positive cases showed high prevalence of melioidosis among males (32.9%, n=23), middle-aged group (75.7%, n=53) and Malay ethnic group (98.6%, n=69). Diabetes mellitus was the major comorbidity among the patients (65.7%, n=46). Mortality rate of 37.1% (n=26) was reported, of which septicemic melioidosis carried high mortality rate. Systemic melioidosis, formation of abscess and sepsis/septic shock were significantly associated with the outcome of the patients. Kota Bharu district was identified as the hotspot area of melioidosis in Kelantan. MLST analysis of 33 available clinical and two environmental isolates of *B. pseudomallei* revealed 15 different sequence types (STs) including seven new emerging novel STs. This study indicates the genetic

diversity of *B. pseudomallei* in Kelantan with the highest ST diversity in Bachok (67%). The most predominant genotype in the research population is ST371 (17.1%; n=6). Even though the reported STs were not related to the districts in Kelantan, all strains were shown to be closely related to the previously reported Southeast Asia strains. The generated risk map with the variety of existing and novel genotypes circulating in the population is important for the future preventive and management plan of melioidosis in Kelantan.

CHAPTER 1

INTRODUCTION

1.1 Background of study

Melioidosis is a life-threatening infectious tropical disease that is endemic to Northern Australia and Southeast Asia including Malaysia (Cheng and Currie, 2005; Dance and Limmathurotsakul, 2018). Melioidosis is highly infectious but somehow remain neglected compared to the other highly pathogenic bacterial infection disease such as tuberculosis. The causative agent of infection for melioidosis infection is a Gram-negative bacterium called *Burkholderia pseudomallei.* A wide range and variety of clinical manifestations from the infection of this bacteria were reported. This leads to the lack of suspicion among medical practitioners resulting in late diagnosis and delayed treatment which eventually increase the rate of mortality among the patients.

Therefore, determining the common clinical manifestation and the disease outcome of the patients as well as the distribution pattern of the disease in Kelantan, Malaysia will help in further understanding the epidemiology of the disease in the population of this study. Thus, this will further help in proper treatments and improving the outcome of the patients.

B. pseudomallei is soil-dwelling bacteria that commonly found in rice paddy fields, ponds, stagnant streams and ground water (Baker *et al.*, 2011b). *B. pseudomallei* is an environmental saprophyte that has the ability to withstand extreme environmental conditions (Yabuuchi and Arakawa, 1993). Direct contact of the causative agent is believed to be the main transmission route of infection of melioidosis especially in the agricultural workers (Limmathurotsakul *et al.*, 2016).

In this study, it was hypothesized that the environments surrounding the residential area of the patients are high likely to be the source of infection. Molecular typing of the bacteria from previous studies worldwide also revealed that the same strains isolated from the clinical specimens were also found from the environments. However, to date, no studies has been conducted for the profiling of the bacterial strains and association of the genotypes for both clinical and environmental isolates from the same locality.

Determining the distribution of the *B. pseudomallei* isolates in environments is the key to develop the risk map of melioidosis among the districts in Kelantan. This information will help in the preventive plan as well as increasing the awareness of this highly pathogenic disease among general practitioners and healthcare workers in Kelantan. This will eventually lead to a faster diagnosis and better prognosis in future.

Different genetic diversity of strains are also postulated to be associated with the disease outcome and the geographical area. Therefore, identifying the predominant genotype(s) and the relatedness of the genotypes from humans and the environment will unravel aspects that remain unclear on this disease in the population.

1.2 Objectives of study

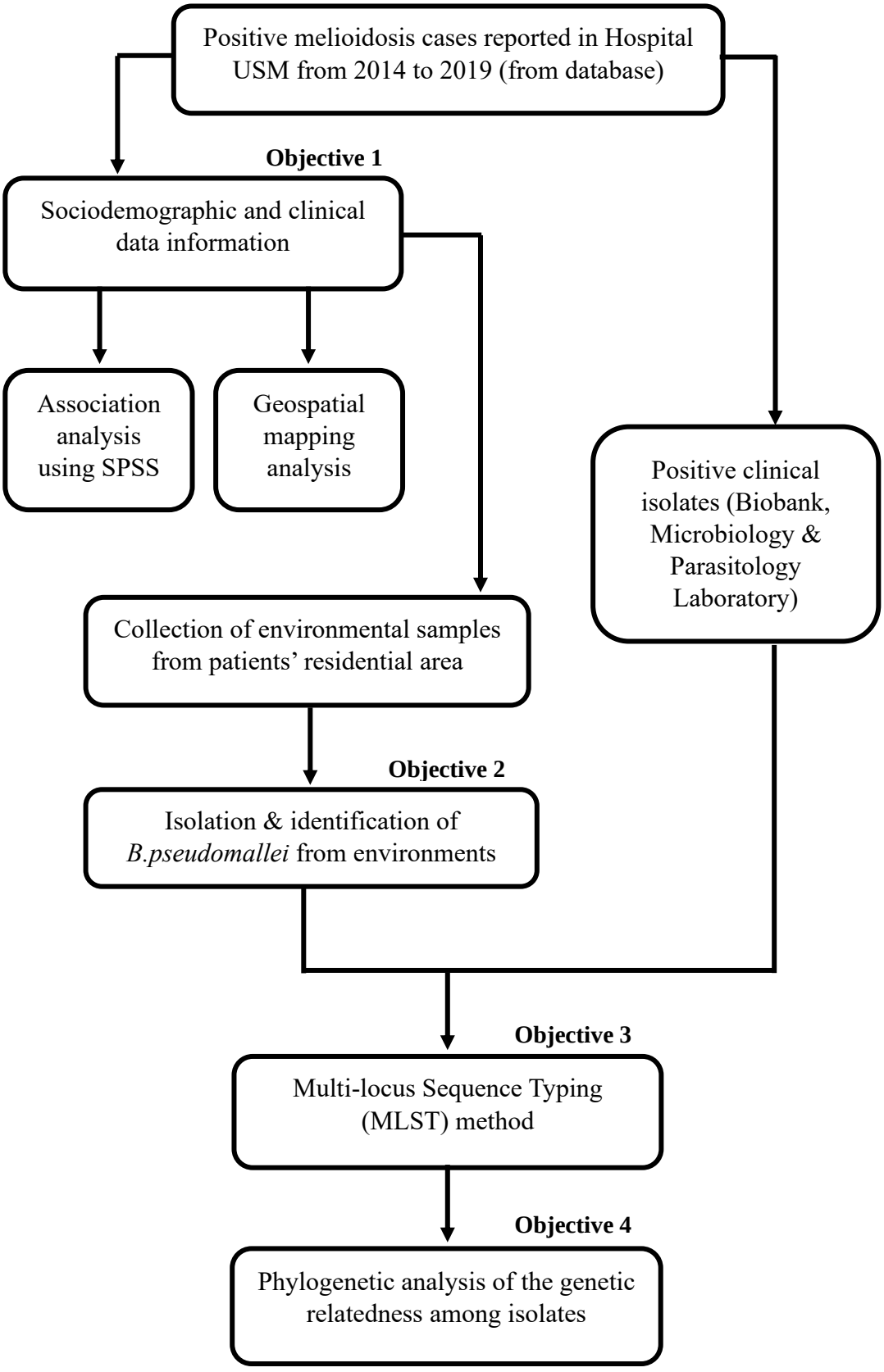
1.2.1 General objective

To study the disease outcome, distribution and genotyping of *Burkholderia pseudomallei* from humans and environments in Kelantan.

1.2.2 Specific objectives

1) To identify the disease outcome of positive melioidosis patients and the distribution of the cases in districts in Kelantan by Geographical Information System (GIS) method
2) To isolate and identify *B. pseudomallei* in environmental samples
3) To determine the genotyping of *B. pseudomallei* isolates from patients and environment using Multi-locus Sequence Typing (MLST) method
4) To study the genetic relatedness between *B. pseudomallei* isolates from patients and environmental samples using phylogenetic tree analysis.

1.3 Research workflow



CHAPTER 2

LITERATURE REVIEW

2.1 Background and history of melioidosis

Melioidosis was first discovered by Alfred Whitmore and C. S. Krishnaswami in 1911 upon the post mortem of a morphia injector Burmese man with unusual presentation of bacterial infection in multiple organs in Rangoon, Burma. They observed a peculiar consolidation of the lungs which were neither lobar pneumonia or tubercular infection (Whitmore and Krishnaswami, 1912). The isolates were differentiated from *Bacillus mallei* by the motility, rapid luxuriant growth on peptone agar, wrinkled culture growth on glycerine agar, formation of pellicle and the long filamentous appearance (Whitmore, 1913). The infection by the bacillus was known as "Whitmore's disease".

After Burma (now known as Myanmar), the infection was then recognised in Kuala Lumpur, Malaysia by William Fletcher and Sir Thomas Stanton in 1913 (Stanton and Fletcher, 1921). Both of the researchers coined the melioidosis name in 1921 derived from the Greek word "*melis"* (distemper of asses) and "*eidos"* (resemblance). In 1932, melioidosis infections reported with up to 98% mortality were reported in South and Southeast Asia (Stanton and Fletcher, 1932).

At first, melioidosis was considered to be zoonotic with rodents as the reservoir. However in 1932, researchers in Saigon and Hanoi, Vietnam had found the source habitat of *B. pseudomallei* which was eventually in soil and water in the environment after the observation from cases that often occur due to exposure to mud or contaminated water.

An unexpected melioidosis outbreak infecting horses and other animals famously known as "*L'affaire du Jardin des Plantes*" had occurred in France during 1970s. The outbreak had caused the death of many horses in Jardin des Plantes zoo, Paris and spread amongst horses in equestrian clubs across France into other animals in the zoos of Paris (Ketheesan, 2012).

Melioidosis had been in attention when 343 American soldiers in Vietnam were infected during World War II. Latency period of infection was reported from the American soldier giving melioidosis the nickname of "Vietnam Time Bomb" (Ngauy *et al.*, 2005).

As *B. pseudomallei* had been recognised to be a potential bioterrorism agent, United States Centre for Disease Control and Prevention (US CDC) had classified the causative agent as a Category B select agent in 2002 together with *B. mallei*. It was believed to have potential to spread on large scale with multidrug resistance and had been one of the agents weaponized by Soviet Union before 1992 (Zilinskas, 2017). *B. pseudomallei* was then classified as Tier 1 select agent as this biological agent poses greater risk of deliberate misuse and severe threat to the public, animal and/or plant (Federal-Register, 2012; Zilinskas, 2017). The classification was done based on the concern of its difficulties to be diagnosed due to the ability to mimic other bacterial infections, high mortality, high potential to be aerosolized, wide range of antimicrobial resistance and no vaccine are available (Currie, 2015).

The announcement by CDC had become the catalyst and booster for research interest in the causative agent. Even though numerous discovery of useful information and knowledge was found, vaccine for melioidosis was remained unavailable due to reasons including the high complexity of the bacteria.

2.2 Nomenclature history of *Burkholderia pseudomallei*

The agent responsible for the melioidosis infection was first named *Bacillus pseudomallei* because of its morphology features that are most likely similar to *Bacillus mallei* (Whitmore, 1913). It was also known as *Bacillus whitmori* (or *Bacille de Whitmore*) naming after the researcher who discovered the bacteria, Alfred Whitmore. The bacteria was variously known with several names through times such as *Pfeifferella whitmori, Pfeifferella pseudomallei, Loefflerella whitmori, Loefflerella pseudomallei, Actinobacillus pseudomallei, Flavobacterium pseudomallei, Malleomyces pseudomallei* and *Pseudomonas pseudomallei* (Cheng and Currie, 2005).

Later in 1992, new genus named Burkholderia was proposed by Yabuuchi and his colleagues. The genus was named after Walter H. Burkholder. They proposed transferring seven species of the genus Pseudomonas homology group II to the new genus *(P. cepacia, P. mallei, P. picketti, P. solanacearum, P. gladioli and P. caryophylli* including *Pseudomonas pseudomallei*). *P. pseudomallei* was therefore now known as *Burkholderia pseudomallei* (Yabuuchi *et al.*, 1992).

2.3 Taxonomy of *Burkholderia pseudomallei*

Burkholderia pseudomallei is classified under the Beta-proteobacteria and order of Burkholderiales. It belongs to the family known as Burkholderiaceae. The genus Burkholderia consist of a large group of over 100 species and was proposed to be differentiated into two major groups which are Group A and Group B (Estrada-De Los Santos *et al.*, 2016; Suárez-Moreno *et al.*, 2012). Group A is known to comprise non-pathogenic plant-associated beneficial and environmental (PBE) with the potential benefit for agriculture or known as Paraburkholderia such as *B. xenovorans, B. terricola, B. fungorum* and *B. kururiensis* (Eberl and Vandamme, 2016).

Meanwhile, group B is consist of species that are predominantly pathogenic and opportunistic-pathogenic species to human, animal and plants including the species of *Burkholderia pseudomallei*, *B. mallei*, *B. thailandensis* and *Burkholderia cepacia* (Tuanyok *et al.*, 2017). The taxonomic classification of the *Burkholderia pseudomallei* is shown below (Whitmore, 1913).

Kingdom : Bacteria

Phylum : Proteobacteria

Class : Betaproteobacteria

Order : Burkholderiales

Family : Burkholderiaceae

Genus : Burkholderia

Species : *Burkholderia pseudomallei*

2.4 Bacteriology of *Burkholderia pseudomallei*

Burkholderia pseudomallei is a highly pathogenic saprophyte, aerobic and non-sporulating bacterium. It is flagellated small Gram-negative bacilli with the appearance of safety-pin like structure when viewed under microscope as shown in

Figure 2.1 (White, 2003). The bipolar staining structure can be observed due to the accumulation of poly-β-hydroxybutyrate. The bacterium size is approximate 0.8 μm in width and 1.5 μm in length (Yabuuchi and Arakawa, 1993).

B. pseudomallei is a motile and oxidase-positive bacteria. It is capable of withstanding extreme environmental conditions. It had been reported that extreme environmental condition results in phenotypic switching associated with complex shifts in colony morphology (Chantratita *et al.*, 2007; Paksanont *et al.*, 2018).

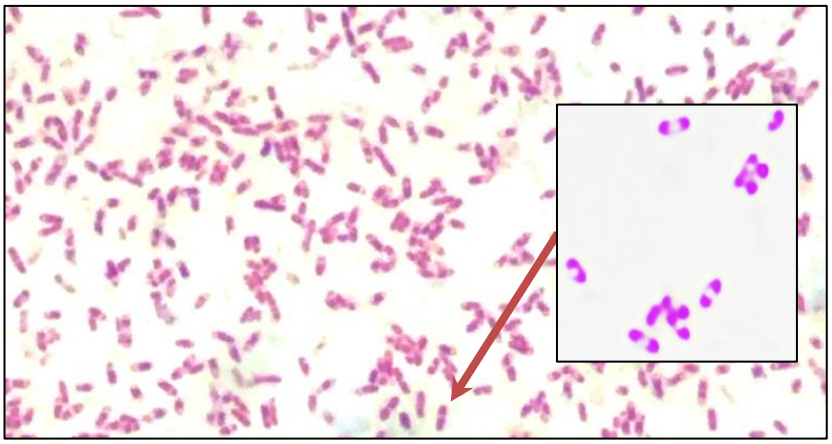


Figure 2.1 Safety pin characteristic with bipolar staining of *Burkholderia pseudomallei* under microscopy image adopted from Kandhasamy et al. (2020) and Gassiep et al. (2020) under 100X magnification

2.4.1 Morphology characteristic of *Burkholderia pseudomallei*

Burkholderia pseudomallei was reported with variety of colony morphology that occur due to alteration of surface determinant expression for the adaptation process. Wide genetic diversity and genetic divergence of *B. pseudomallei* were reported among isolates of similar patients indicating the within-host adaptation (Limmathurotsakul *et al.*, 2014a; Price *et al.*, 2010). On the selective media, Ashdown's agar, the morphology types were classified from wrinkled or smooth, dry or mucoid, pale or dark purple, round to irregular edge shape and the size of the colony (Chantratita *et al.*, 2007; Chen *et al.*, 2009).

Seven major colony morphotypes were observed in which type I was the morphotype that gives rise to other morphotypes. The 7 major morphotypes were shown in Figure 2.2. However, *B. pseudomallei* was reported to form highly wrinkled circular purple colonies on Ashdown's media that had been used for general identification of clinical laboratories in endemic countries (Chen *et al.*, 2009). Wrinkled colony morphotypes had shown to possess higher virulence compared to non-wrinkled morphotypes when tested in liver tissues (Chen *et al.*, 2009).

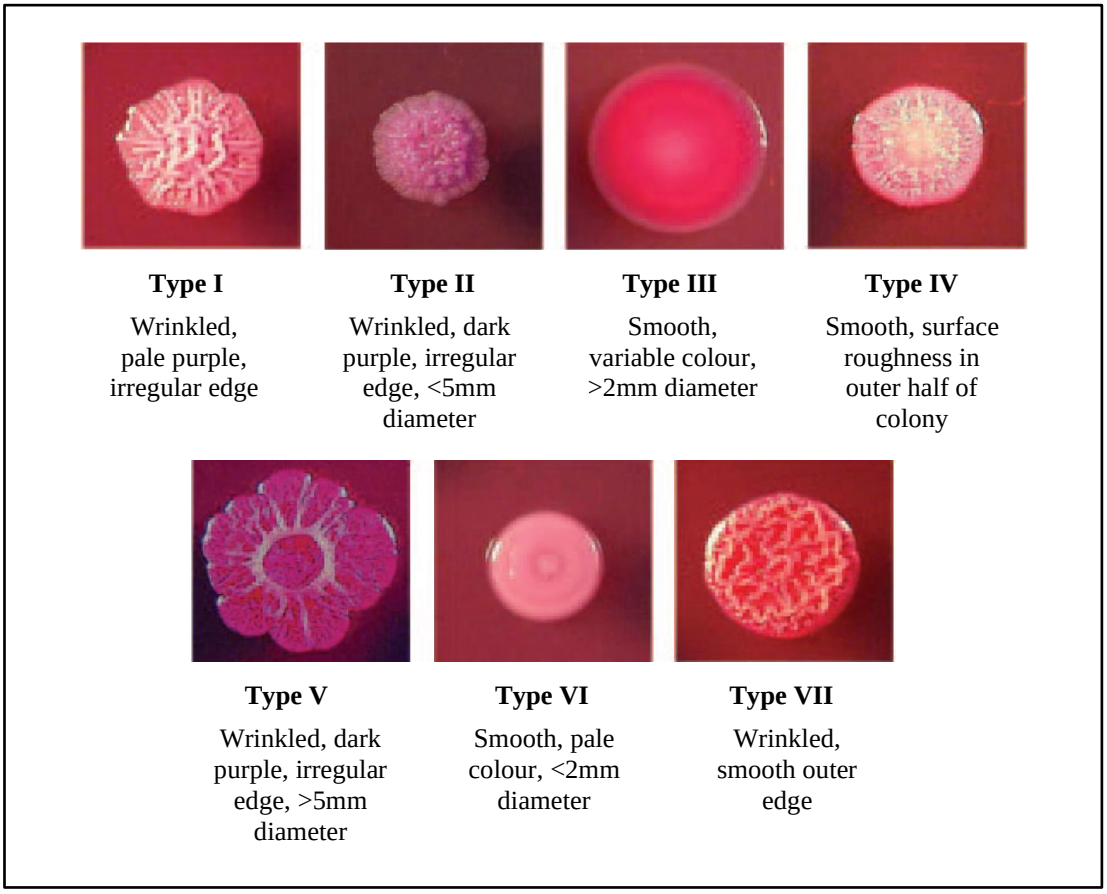


Figure 2.2 Major colony morphotypes of *Burkholderia pseudomallei* on selective Ashdown's agar after 4 days at 37°C in air. Adapted from Chantratita *et al.* (2007).

2.4.2 Genome of *Burkholderia pseudomallei*

The genome of *B. pseudomallei* is composed of two circular replicons (Chromosome 1 and chromosome 2) with a total size of 7.3 Mb which is relatively large and complex from the other prokaryotic genome (Figure 2.3). Holden *et al.* (2004) found that chromosome 1 (4.07 Mb) and chromosome 2 (3.17 Mb) consist of 3,460 and 2,395 coding sequences (CDSs) respectively. Chromosomes 1 involves in core functions including metabolic activity, mobility, cell growth, chemotaxis biosynthesis and motility. Meanwhile, chromosome 2 carries genetic elements encodes in accessory functions for adaptation and survival in extreme conditions of different niches (Galyov *et al.*, 2010).

The genome of *B. pseudomallei* had been revealed to consist of 12 putative genomic islands (GIs) on chromosome 1 and 4 putative GIs on chromosome 2 (Holden *et al.*, 2004). There are very high differences found between the two chromosomes except for the rRNA clusters regions. High genomic plasticity of the *B. pseudomallei* had been revealed to be due to extensive recombination, frequent mutation and horizontal gene transfer resulting in high genetic heterogeneity between strains (Holden *et al.*, 2004; Price *et al.*, 2010; Tumapa *et al.*, 2008; Wiersinga *et al.*, 2018).

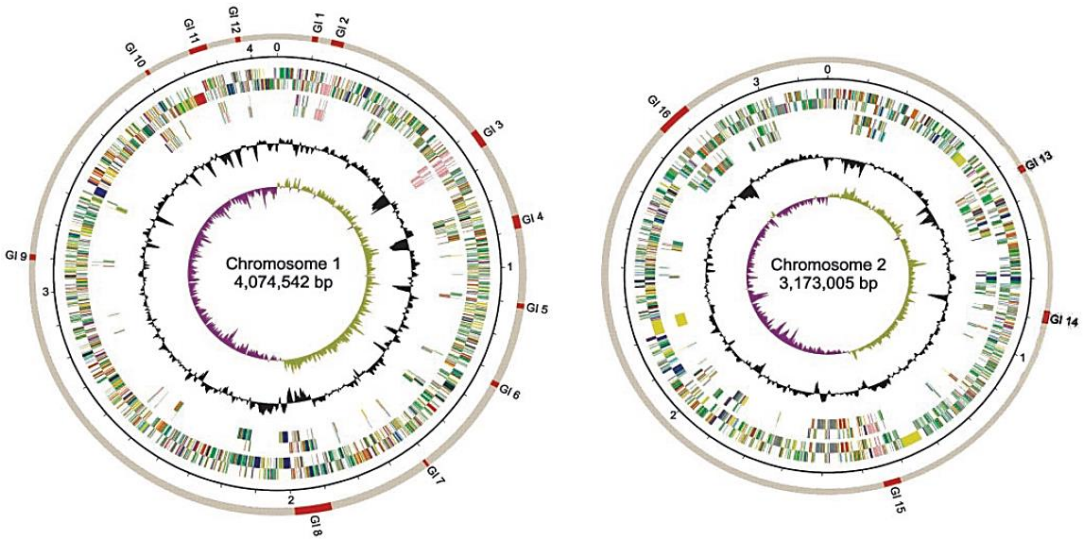


Figure 2.3 Large and small circular chromosomes with the genome size of *Burkholderia pseudomallei.* Adopted from Holden *et al.* (2004)

2.4.3 Ecology and survivability of *Burkholderia pseudomallei* in environment

The optimum temperature of *B. pseudomallei* is 37-42℃ which corresponds to the predominant growth in countries with tropical and sub-topical climates (Dance, 2000). *B. pseudomallei* has the unusual ability to withstand extreme environmental conditions for months to years and remain viable (Paksanont *et al.*, 2018). This ability is in parallel to the survivability of the bacteria throughout the dry season in endemic countries. Besides, certain strains were reported to be able to survive as low as 5ºC under laboratory conditions for a long period (Yabuuchi and Arakawa, 1993).

B. pseudomallei was reported to grow in waterlogged, heavy clay soils. This clay-type soil supports bacterial persistence by retaining the water content and nutrients compared to the well-drained, light sandy soils (Inglis and Sagripanti, 2006). Clay loam soil-type is found in the rice-paddy field thus it appears ideal for the *B. pseudomallei* growth and was corroborated by the findings in previous studies (Chuah *et al.*, 2017; Musa *et al.*, 2016; Wang-Ngarm *et al.*, 2014).

The optimum pH level for growth of *B. pseudomallei* is between 6.5 to 7.5 which is slightly acidic. Nevertheless, this bacteria are able to grow in a wide range of extreme pH conditions between pH 2 to 9 (Dance, 2000). Even though a significant reduction of growth was observed in the pH 4 and pH 8, *B. pseudomallei* was found to persist in the environment (Wang-Ngarm *et al.*, 2014). In Northeast Thailand, high bacterial burden has been reported in unusual acidic soil (Inglis and Sagripanti, 2006).

B. pseudomallei has the ability to survive in saline environmental conditions. In Thailand, *B. pseudomallei* has been isolated in the northeast where the region is rich in saline soil and water (Duangurai *et al.*, 2018). An increase in salt stress was found

to be associated with an increase in heat resistance, plaque formation and also oxidative resistance as a result of changes in gene expression due to the stress response (Pumirat *et al.*, 2017)

A study on the survival of *B. pseudomallei* in distilled water after being maintained at room temperature for 16 years was done where the bacteria was found viable however in a non-culturable state (Pumpuang *et al.*, 2011). The bacteria were remain alive in a very low metabolic activity and did not replicate, however, have the ability to become culturable after resuscitation. On the environmental surfaces; polyethylene, stainless steel and paper are similarly higher survivability compared to paper with up to 7 days (Shams *et al.*, 2007). *B. pseudomallei* was able to survive in some contaminated beverages such as sports and energy drinks for up to 4 weeks (Wuthiekanun *et al.*, 2020).

The ability to survive in a wide range of conditions helps in the transmission of the melioidosis infection due to the persistence of *B. pseudomallei* in the environments since environments are known as the main reservoir for the infection.

2.5 Modes of transmission

B. pseudomallei is inhabited in the environment where the bacteria are mostly found in soil and water but rarely found in the air. Common transmission routes of melioidosis are identified as subcutaneous inoculation, ingestion and inhalation of contaminated droplets (Currie, 2015; Limmathurotsakul *et al.*, 2016).

Direct inoculation of contaminated soil or water via penetrating wounds and skin breakage is known as the main transmission route especially among farmers and gardeners in developing countries (Currie *et al.*, 2000b; Limmathurotsakul *et al.*, 2016;

Liu *et al.*, 2015). A previous study in Australia had shown that 25% of the patients had acquired the infection through skin abrasion (Currie *et al.*, 2000b). Skin inoculation-acquired infection was also observed in splenic abscess patients in India (Gupta *et al.*, 2021).

Airborne transmission had been reported among American helicopter crew soldiers presented with pulmonary melioidosis after the Vietnam War due to the inhalation of dust in the environment which had been initially thought of as the primary mode of acquisition of melioidosis (Howe *et al.*, 1971; Koponen *et al.*, 1991). Acquisition of the bacteria in laboratory by inhalation with a variety of incubation periods, doses and acuteness of the disease during experimentation had been recorded (Green and Tuffnell, 1968). In Zoynan region of Taiwan, melioidosis clustered cases were highly increased after typhoon whereby the contaminated cropped fields were believed to generate the aerosols with contaminated *B. pseudomallei* and carried by wind to the dense populated area (Chen *et al.*, 2015; Hsueh *et al.*, 2018). A case on the inhalation-associated melioidosis cases acquired during helicopter flight had been reported in a tourist returned from Singapore (Amadasi *et al.*, 2015).

Two outbreaks with fatality due to contamination of unchlorinated bore water supply had occurred in Australia (McRobb *et al.*, 2013). Infection caused by contaminated drinking water was reported in Thailand (Limmathurotsakul *et al.*, 2014b). Aspiration-related melioidosis cases were also reported among tsunami survivors in Thailand and patients with history of near-drowning episodes with various clinical manifestations (Arzola *et al.*, 2007; Chierakul *et al.*, 2005; Lim *et al.*, 2016).

Zoonotic melioidosis which is the infection transmitted from animals such as primates, iguana, and canine to humans were reported even though it was rare (Damrongsukij *et al.*, 2021; Elschner *et al.*, 2014; Kasantikul *et al.*, 2016; Ryan *et al.*, 2018). Other rare transmission routes between human to human had been reported from a mastitis mother to child through breastfeeding, sexual transmission, healthcare-associated infection and lab-acquired transmission (Aziz *et al.*, 2020; Green and Tuffnell, 1968; Thatrimontrichai and Maneenil, 2012).

2.6 Period of acquisition

Incubation period of melioidosis is 1-21 days however clinical manifestation may develop within hours (Maluda *et al.*, 2020). The average reported incubation period was 9 days (Currie *et al.*, 2000c). Nevertheless, a prolonged period before the clinical manifestations from the exposure was reported with the latency of 18 and 28 years in Vietnam Air Force retiree and World War II veteran respectively (Koponen *et al.*, 1991; Mays and Ricketts, 1975). The longest reported incubation period was 62 years from an ex-prisoner of Japanese army during World War II with the history as railroads labour in Singapore, Malaysia, Burma and Thailand (Ngauy *et al.*, 2005).

The incubation period is depending on the amount of bacterial inoculum, acquisition route and host risk factors (Wiersinga *et al.*, 2018). High doses of inoculum were associated with shorter incubation period for infection (Chierakul *et al.*, 2005). Recurrence of the infection was high likely to occur among patients regardless of adequate antimicrobial treatment (Cheng and Currie, 2005; Wiersinga *et al.*, 2006).

2.7 Melioidosis and its association to risk factors

2.7.1 Host factors

The highest reported risk factor for melioidosis infection is the presence of comorbidities. Up to 80% of the infected patients were having one or more underlying diseases in Australian adults howbeit low in children (Currie, 2015). In Malaysia, 77-90% of the patients were at risk (Deris *et al.*, 2010; Hassan *et al.*, 2010). Presence of at least one comorbidity was identified in 5% of paediatric patients (McLeod *et al.*, 2015).

Diabetes mellitus (DM) is the most important host factor reported with more than 60% in Thailand, up to 89% in Malaysia and 37% in Australia and was highly associated to melioidosis (Churuangsuk *et al.*, 2016; Currie *et al.*, 2010; Limmathurotsakul *et al.*, 2010b; Zueter *et al.*, 2016). Diabetic patients have significantly 12-fold greater risk of infection compared to non-diabetics (Carey *et al.*, 2018; Shah and Hux, 2003). Thalassemia was also one of the significant factors with up to 11-fold higher risk for melioidosis in Thailand and 140 cases per 100,000 annually among paediatric patients in Malaysia (Fong *et al.*, 2015; Suputtamongkol *et al.*, 1999).

Prolonged steroid therapy, immunosuppressive and immunocompromised patients were at risk for infection (Wiersinga *et al.*, 2018). Heavy alcohol consumption was significantly associated with melioidosis in Australia but low in other countries (Sathkumara *et al.*, 2018). Other identified comorbidities among melioidosis patients were malignancy, systemic lupus erythematosus, chronic kidney disease, liver and lung failure and heart failure (Gassiep *et al.*, 2020; Rahim *et al.*, 2019).

Human melioidosis occurs in people of all ages ranging from new born to 92 years (Stewart *et al.*, 2017). General peak specific incidence occurs in adults aged between 40 to 69 years for both males and females (Nathan *et al.*, 2018; Suputtamongkol *et al.*, 1994). Median age of incidence was 50 years in the Southeast Asia region, 48 years in the Western Pacific region, 37 years in the American region, 49 years in the African region and 60 years in the Mediterranian region (Birnie *et al.*, 2019b).

Male was reported to predominate in melioidosis worldwide with 58.5% in Thailand to 84% in Singapore compared to female (Limmathurotsakul *et al.*, 2010b; Pang *et al.*, 2018). Male is at higher risk compared to female probably due to the higher exposure to the environment (Wiersinga *et al.*, 2018).

2.7.2 Environmental factors

Melioidosis is associated to the high environmental exposure among the people (Kingsley *et al.*, 2016). *B. pseudomallei* are hardy environmental saprophytes that resist temperature extremes, acidic and alkaline. It can be highly found in natural reservoirs with water and moist soil such as rice paddy fields, rivers, ponds, waterholes and farms (Baker *et al.*, 2011b; Currie and Kaestli, 2016). Therefore, people with high occupational exposure to the reservoirs such as farmers, gardeners, forestry workers, construction workers and military personnel are at greater risk for melioidosis (Inglis *et al.*, 2006; Kingsley *et al.*, 2016; Shrestha *et al.*, 2019). Besides, extreme climatic changes such as typhoons, heavy rainfall and flood are believed to increase the risk of melioidosis infection among people (Parameswaran *et al.*, 2012; Zueter *et al.*, 2016).

Outdoor activities such as eco-travelling and sports also increase the risk for acquisition of melioidosis Approximately 22% of the patients were infected due to recreational activities (Currie *et al.*, 2010). However, the association of the activities was not well-established (Hill *et al.*, 2013).

2.8 Epidemiology

2.8.1 Melioidosis in worldwide

Melioidosis is an endemic disease in approximately 46 countries and began to be reported to more than 33 countries as a potential endemic (Almog *et al.*, 2016; Gassiep *et al.*, 2020; Limmathurotsakul *et al.*, 2016). It was reported to be originated from Australia and is highly spread into Southeast Asia (Wiersinga *et al.*, 2018). It is highly prevalent in Northern Australia, Thailand, Malaysia, Singapore, Vietnam and is increasingly recognized in other parts of the world. However, the true incidence of melioidosis worldwide remains unknown (Gassiep *et al.*, 2020).

Melioidosis, an under-diagnosis and under-reporting disease, had been a major issue and global burden worldwide. A modelling study done had estimated the worldwide annual incidence of melioidosis with approximately 165,000 cases per year and 89,000 (54%) fatality cases. The predicted fatality is comparable to measles with 95,600 individuals and is higher than fatality due to dengue fever and leptospirosis with 12,500 and 50,000 individuals per year, respectively (Wiersinga *et al.*, 2018). The actual annual reported cases were revealed as approximately 1,300 which was only <1% of the estimation cases (Limmathurotsakul *et al.*, 2016).

Figure 2.4 shows the estimated fatality per year and reported cases of melioidosis worldwide. Australia, Brunei and Singapore had shown the most comparable data to the estimates. Countries such as Indonesia, Bangladesh and China showed large differences between the estimated and actual reported cases (Limmathurotsakul *et al.*, 2016; Wiersinga *et al.*, 2018).

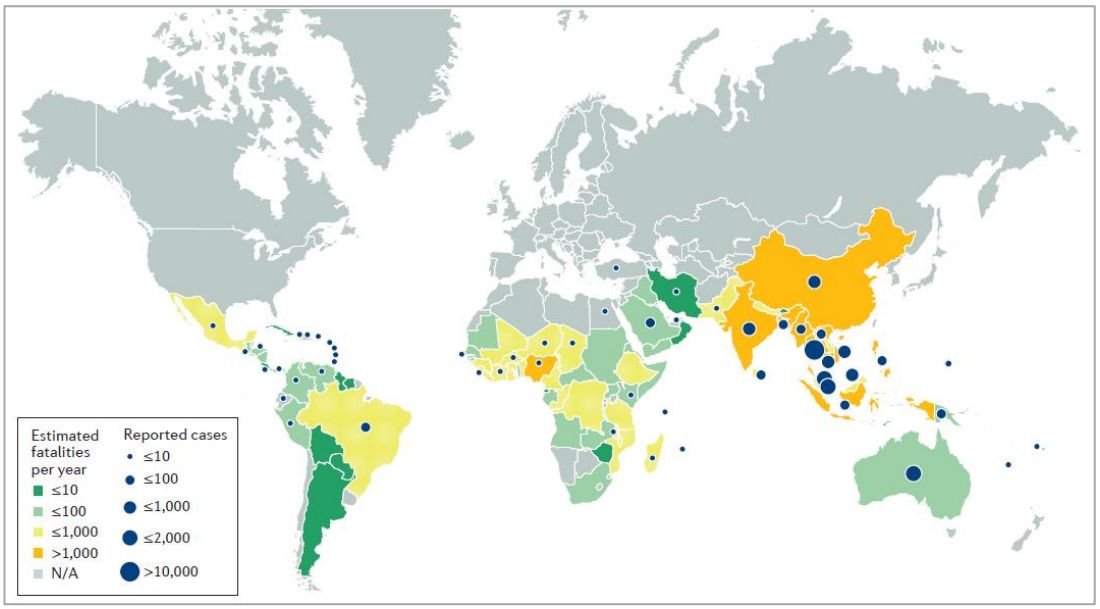


Figure 2.4 The estimated fatality per year and reported cases of melioidosis worldwide. Adopted from Wiersinga *et al.* (2018).

In Australia, the average incidence of melioidosis per year was reported 19.6 cases per 100,000 population and increases between 5.4 to 41.7 during severe climatic changes in Northern Territory (Cheng and Currie, 2005). The highest documented incidence was 50.2 cases per 100,000 population and 102.4 among the indigenous population after heavy rainfall occurred in Northern Territory from 2009 to 2010 (Gassiep *et al.*, 2020; Parameswaran *et al.*, 2012).

Thailand is the highest reported country with melioidosis in the Southeast Asia region. Melioidosis is a notifiable disease in Thailand since 2002 (Kongkaew *et al.*, 2017). The average rate of incidence reported from 2012 to 2015 was 3.95 per 100,000

population per year where Northeast Thailand was the highest with 8.73 per 100,000 per year (Hantrakun *et al.*, 2019). In Southern Thailand, the incidence rate was 36.5 per 100,000 inpatients (Churuangsuk *et al.*, 2016). In general, 35% to 42% of mortality was reported in general hospitals in Thailand (Chaowagul *et al.*, 1993; Hinjoy *et al.*, 2018).

In Singapore, melioidosis also had been registered as a notifiable disease thus increasing the awareness and improving the diagnosis and prognosis of the disease (Sim *et al.*, 2018). The average annual prevalence rate was 1.1 per 100,000 and was higher in Malay (2.4 per 100,000) and Indian (2.1 per 100, 000) (Pang *et al.*, 2018). In Brunei, the overall annual incidence rate was 16.3 per 100,000 population with Temburong district as the highest reported rate (37.93 per 100,000 population). Mortality in Brunei was reported up to 27% (Pande *et al.*, 2018).

In Vietnam, at least 18 out of 25 northern provinces were widely distributed with melioidosis but no clear prevalence rate was reported (Trinh *et al.*, 2018). In Indonesia, the overall fatality rate was 43% where Java was reported with the highest culture-confirmed (Tauran *et al.*, 2018). Meanwhile, in Philippines, only sporadic cases among travellers returning from Philippines were documented with mortality rate of 14.6% and 4.9% recurrence cases (San Martin *et al.*, 2018).

In South Asia, no clear distribution was documented however, the mortality reported from few centres was 20%. Bangladesh and Sri Lanka are recognized as endemic to melioidosis. In contrast, melioidosis was not conclusive in Nepal, Bhutan, Afghanistan and Pakistan (Mukhopadhyay *et al.*, 2018). Mortality in Bangladesh and Sri Lanka were 27% and 21% respectively (Chowdhury *et al.*, 2018; Corea *et al.*, 2018;

Sathkumara *et al.*, 2018). Higher mortality of 25.8% was reported in southern India (Basheer *et al.*, 2021).

Sporadic cases of melioidosis had been reported in Central America and Caribbean Island mostly among travellers returning to their home countries (Araúz *et al.*, 2020; Sanchez-Villamil and Torres, 2018). In South America, sporadic cases had been documented in Ecuador, Venezuela, Colombia and Peru except for northeastern Brazil which is endemic (Benoit *et al.*, 2015; Rolim *et al.*, 2018). Increasing in the melioidosis distribution in future is predicted and be influenced by the increase in the prevalence of DM worldwide (Figure 2.5). High population movements resulting in the establishment of new areas and the formation of anthrosol are also believed to be contributed to the melioidosis distribution (Gassiep *et al.*, 2020; Gopalakrishnan, 2021).

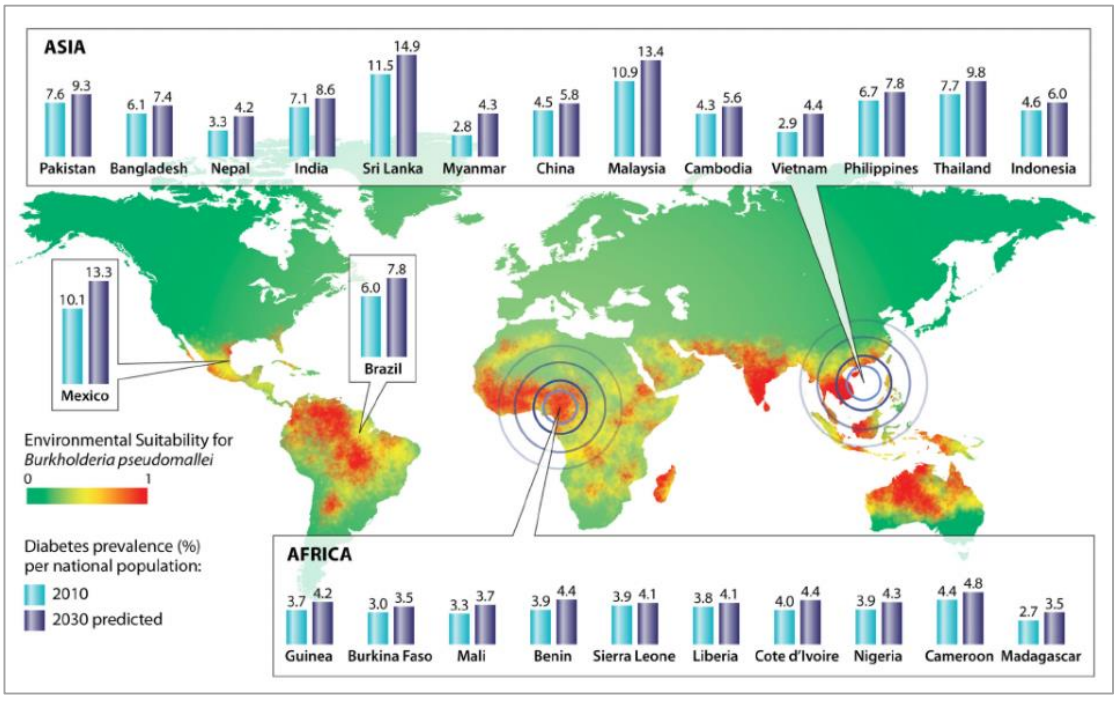


Figure 2.5 The predicted prevalence of melioidosis and diabetes mellitus as the major risk of the infection by 2030. Adopted from Gassiep *et al.* (2020).

2.8.2 Melioidosis in Malaysia

Melioidosis is endemic in Malaysia. Albeit thousands of cases of melioidosis had been reported, the actual incidence of the disease remains unclear. Distribution between states of Malaysia and even between the same state was expected to differ from each other (Mohan *et al.*, 2017).

Melioidosis had been reported in most of the states including Pahang, Johor, Kuala Lumpur, Kelantan, Kedah, and East Malaysia (Ali *et al.*, 2020; Fong *et al.*, 2017; Roslani *et al.*, 2014; Tan and Lee, 2021; Thabit *et al.*, 2020; Thong and Arul, 2016; Yazid *et al.*, 2017). Apparently, states with active and high agricultural activity were increasingly reported with melioidosis as shown in Figure 2.6. Melioidosis is actively reported from Pahang and Sabah due to good health and registry management of the states regarding this infection (Nathan *et al.*, 2018).

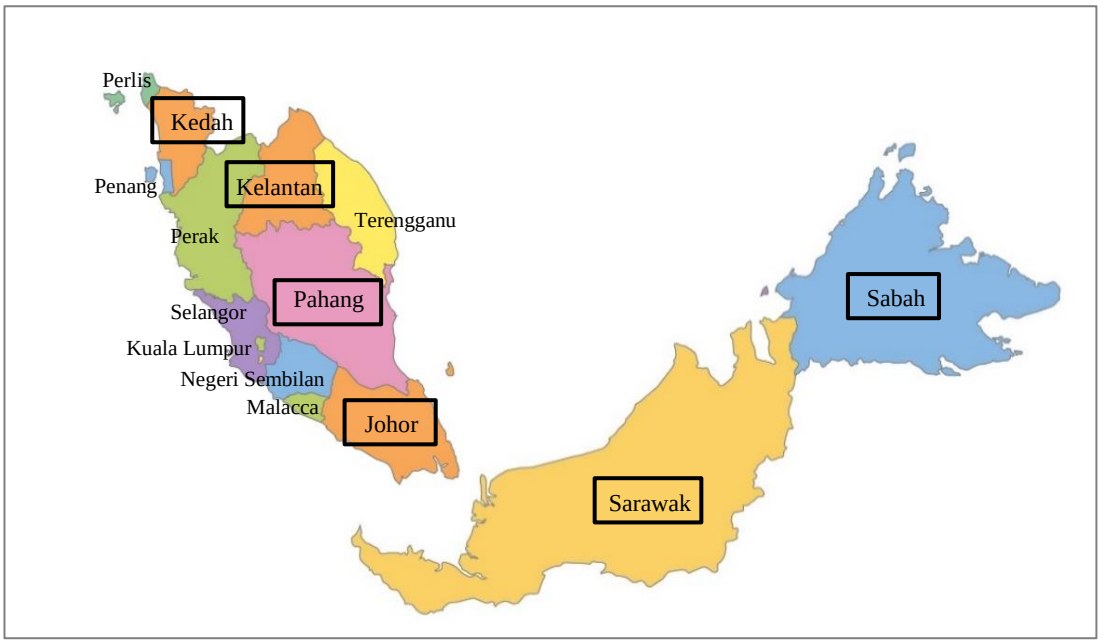


Figure 2.6 States with highly reported melioidosis infection in Malaysia. Adapted from (Nathan *et al.*, 2018)

Kedah; located at the border and near to Thailand which is the highest endemic melioidosis country in Southeast Asia, is well-known as the largest rice producer in Malaysia. (Hassan *et al.*, 2010). The incidence rates per year from 2014 to 2019 was 2.56 to 4.84 per 100,000 with mortality rates of 0.99 to 2.25 per 100,000 (Ali *et al.*, 2020). Meanwhile, Pahang is the largest state of peninsular Malaysia and agriculture as the main economic activity was recorded with incidence rates of 6.1 to 100,000 population per year (Nathan *et al.*, 2018). An increase in mortality was associated with the increase in mean monthly rainfall in Kedah (Hassan *et al.*, 2010).

In Sabah, the incidence rate reported was 2.57 per 100,000 population (Suleiman *et al.*, 2014; Tan and Lee, 2021). In Sarawak, the average annual rate of prevalence among pediatric melioidosis was 4.1 per 100,000 children of age less than 15 years old (Nathan *et al.*, 2018). Overall incidence rate of melioidosis 8.0 per 100,000 population was reported in Bintulu, Sarawak (Fong *et al.*, 2017).

Kelantan, located in north-eastern Malaysia, is also one of the rice-producing states with more than 60,000 hectares of paddy fields (Yazid, 2015). Kelantan is experiencing heavy monsoon annually from November to March where high admission of melioidosis patients was observed (Zueter *et al.*, 2016).

The peak incidence of age in Malaysia was 40 to 60 years with the median age of 44 to 51 years (How *et al.*, 2005; Kingsley *et al.*, 2016; Nathan *et al.*, 2018; Pagalavan, 2005; Zueter *et al.*, 2016). Malay was reported as the highest ethnicity with melioidosis infection compared to others (Zueter *et al.*, 2016).