Digging for melioidosis

New insights into the epidemiology and pathophysiology

Birnie, E.

Creative Commons License (see https://creativecommons.org/use-remix/cc-licenses):
Other

Citation for published version (APA):
CHAPTER 1

GENERAL INTRODUCTION
CHAPTER 1
GENERAL INTRODUCTION
The tropical infectious disease melioidosis is caused by the soil-dwelling Gram-negative bacillus *Burkholderia pseudomallei*.\(^1\)\(^,\)\(^2\) The clinical presentation varies from abscess formation to fulminant sepsis.\(^3\) Melioidosis is often misdiagnosed because of its diverse clinical presentation and sometimes mistaken for other infectious diseases, such as tuberculosis.\(^4\) Diagnosis requires awareness and specific microbiological facilities and expertise. Mortality of melioidosis can reach up to 50% in low resource settings, and many patients may even die before establishment of the diagnosis.\(^5\) B. *pseudomallei* has been identified as a category B bioterrorism agent by the Center for Disease Control and Prevention\(^6\) because of its high morbidity and mortality, poor antibiotic response, and easy aerosilization.\(^7\) Therefore, there is a need for better treatment options and vaccine candidates.

**History**

Melioidosis, was first discovered by Whitmore and his assistant Krishnaswamy in 1911 in Rangoon, Myanmar. They described a ‘glanders-like’ disease in morphine addicts and recognized a bacterium that complied with Koch’s postulates for the causative relationship between a pathogen and disease.\(^1\) In 1932, Stanton and Fletcher named the disease melioidosis which is Greek for “melis” (distemper of asses) and “eidos” (resemblance). In the last century various names have been given to this pathogen, ranging from: *Bacillus whitmori* (or *Bacilli de Whitmore*), *Malleomyces pseudomallei*, *Loefflerella pseudomallei*, *Pfeifferella whitmori*, *Pseudomonas pseudomallei*, and since 1992, *Burkholderia pseudomallei*.\(^4\)

**Epidemiology**

The known hotspots of melioidosis are located in Northern Australia and Northeast Thailand, with annual incidence rates up to 50 cases per 100,000 persons.\(^5\)\(^,\)\(^6\) Other potential endemic regions have been recognized – most importantly the well-documented emergence of melioidosis in the Northeast of Brazil.\(^6\) A recent modelling study estimated that worldwide 165,000 (95% credible interval 68,000-412,000) annual cases acquire melioidosis, from which 89,000 (95% CI 36,000-227,000) patients die, making it a more lethal disease than dengue (12,500) or leptospirosis (50,000).\(^7\) Moreover, it is predicted that melioidosis is significantly under-reported in 45 melioidosis-endemic countries and potentially prevalent in another 34 countries from where the disease is not yet reported.\(^7\) However, the true burden remains poorly understood. Melioidosis is not officially included into the list of Neglected Tropical Diseases generated by the World Health
Organization (WHO), although the disease has high fatality rates and is potentially preventable and curable. Hence, there is an urgent need to raise awareness about melioidosis. For example, the calculation of a representative disability-adjusted life year (DALY) would provide deeper understanding of the burden of melioidosis (Figure 1), and additionally enables a comparison with other diseases. Consequently, this would inform the public health agenda, leading to the formal recognition of melioidosis as a neglected tropical disease.

**Melioidosis in Africa**

Reports of patients with melioidosis in Africa are scarce, which most probably is the result of under recognition and under reporting. However, isolated reports on the presence of *B. pseudomallei* in East and West Africa (amongst others in Nigeria, The Gambia, Kenya, and Malawi) may very well represent “the Tip of the Iceberg” (Figure 2). Conventional diagnostic techniques easily miss *B. pseudomallei* often leading to the provision of empiric antibiotic treatment for sepsis that does not cover melioidosis. Unrecognized melioidosis could very well be an important cause of sepsis-associated mortality in tropical Africa, with fatalities within 48 hours post-infection. Multiple studies have highlighted our lack of understanding of the causative agents in patients admitted with fever apart from malaria, tuberculosis, and HIV; identifying emerging pathogens as a prominent cause of bloodstream infection in Africa. Overall, there is a clear need to better map the true occurrence of melioidosis in these regions.

*B. pseudomallei* is thought to be mainly present in areas where there is human activity, such as rice paddies and farm grounds containing crops or animals. The bacillus primarily occurs in soils over 10 cm deep; the rhizosphere (soil surrounding roots of plants), but during heavy rains (monsoon season) *B. pseudomallei* can come closer to the surface. The ability to detect *B. pseudomallei* in the soil greatly depends on appropriate soil sampling methods. For this reason, a consensus guideline for the sampling of soil to determine the distribution of the bacteria has recently been developed. See Box 1 for a detailed description of the bacterium *B. pseudomallei*. 

---

**Box 1**

See Box 1 for a detailed description of the bacterium *B. pseudomallei*. 

---

12
DALYs (disability-adjusted life years) are a measure of overall disease burden, expressed as the cumulative number of years lost due to ill-health, disability (YLD = Years Lost due to Disability) or early death (YLL = Years of Life Lost).

**Figure 1  Disability-adjusted life year**

\[
\text{DALY} = \text{YLD} + \text{YLL}
\]

**Figure 2  Melioidosis in sub-Saharan Africa**

(A) Melioidosis occurs between the 20°N and 20°S degrees of latitude with endemic hotspots in Asia, Australia and Brazil, but with uncharted territories in Africa. Isolated case reports are depicted as black dots. The colors of the countries were established on evidence-based consensus, ranging from green (complete absence) to red (complete presence). (B) Using environmental factors (temp, rainfall, soil, altitude, vegetation, population) to predict the distribution of *B. pseudomallei* in Africa. High suitability areas are depicted in red and low suitability areas in green. (C) In Africa, microbiological facilities should be enhanced and awareness should be increased. Melioidosis-endemic countries are shown in red (where disease is under-reported) or in pink (never reported the disease). Figure adapted with permission from Limmathurotsakul et al, Nat Microbiol, 2016 Jan1; 1(1)."

13
Digging for Melioidosis

Box 1 B. pseudomallei, the bacterium

*B. pseudomallei* is a facultative intracellular saprophyte with a broad arsenal of intrinsic virulence factors. *B. pseudomallei* belongs to the *Burkholderia* genus containing over 40 species and is part of the *B. pseudomallei* complex together with the genetically closely related *B. mallei* and *B. thailandensis*. *B. mallei* causes glanders in horses, but seldom disease in humans. *B. thailandensis* likewise rarely causes disease in humans and is generally considered less virulent (by a factor of > 100,000) than *B. pseudomallei* in murine studies.

*B. pseudomallei* consists of numerous genetically different isolates, which are found in specific endemic areas or countries. The genome of *B. pseudomallei* is one of the most complex sequenced bacterial genes up to date with two chromosomes of 4.07 and 3.17 megabase pairs. The genome comprises a core genome, which is shared between different *B. pseudomallei* strains, and various highly variable genomic islands (GIs). These GIs contain mobile genetic elements obtained by horizontal gene transfer, and are the source of the genetic diversity between strains. Genetically different *B. pseudomallei* strains are mapped with help of multilocus sequence typing (MLST) and whole genome sequencing (WGS). GIs are also associated with virulence of the bacterium, and could be the source of differences in virulence factors between strains found in different parts of the world. More importantly, *B. pseudomallei* can adapt to the genome of its host, in that way modifying its virulence and contributing to its survival.

Different potential virulence factors of *B. pseudomallei* are described. However, their role and importance in the pathogenesis of melioidosis remain largely unknown. Examples of potential virulence factors are lipopolysaccharide (LPS), flagelin, the quorum sensing (QS) system, the secretions systems, namely type III and VI, and the extracellular polysaccharide capsule. It has been hypothesized that virulence factors and their differential expression may change melioidosis disease presentation and outcome. For example, *B. pseudomallei* isolates carrying a *B. mallei* like bimA allele (bimAbm) are associated with increased persistence in phagocytic cells, increased virulence, and neurologic melioidosis, that are mainly found in Australia.

*B. pseudomallei* can evade the immune response due to its capacity to enter, survive and replicate in non-phagocytic and phagocytic cells, including neutrophils (Figure 3). After phagocytosis, the bacterium can escape from the endocytic vacuole and start replicating in the cytosol of the host cell. When free in the cytosol, *B. pseudomallei* is able to create a so called actin tail composed of actin filaments mediated by autotransporter bimABm. This allows the bacterium to move in the cytosol of the host cell and to form cell-membrane protrusions. A hallmark for melioidosis is the formation of multinuclear giant cells (MNGCs), which results directly in cell-to-cell spread enabling the bacterium to establish an infection while avoiding the host response.
Immune response

Conserved motifs on pathogens, including LPS, peptidoglycan, flagella, TTSS, and DNA, are recognized as pathogen-associated molecular patterns (PAMPs) by innate immune receptors, better known as pattern recognition receptors (PRRs) (Figure 4). The prime example of these PRRs, the toll-like receptors (TLRs), recognize PAMPs and trigger an inflammatory response in the central TLR adaptor protein myeloid differentiation primary response 88 (Myd88). Up-regulation of TLRs (TLR2, TLR4 and TLR5) leads to nuclear factor-κβ (NF-κβ) induced release of pro-inflammatory cytokines and subsequent activation of the immune response. Activation of the TLRs receptors expressed on myeloid cells 1 (TREM-1) triggers amplification of TLR-induced signaling, while activation of interleukin 1 receptor-associated kinase-like molecule (IRAK-M) dampens the immune response during melioidosis. Recognition of bacterial virulence factors and endogenous danger signals by the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) results in intracellular inflammasome activation of caspase-1 which causes pyroptosis (caspase-dependent cell death) and subsequently releases interleukin-1β (IL-1β) and interleukin-18 (IL-18). Both ILs are elevated in septic melioidosis.
In turn, the IL-18 produces interferon-γ (IFNγ), which generates a protective effect. Simultaneously, neutrophils, macrophages, and lymphocytes are recruited to the site of infection initiated by PRRs. Activated neutrophils play an essential role in early bacterial containment. In contrast, IL-1β has the potential to cause detrimental excessive neutrophil recruitment.

The production of IFNγ activates recruitment of T-cells leading to a cell mediated immune response. Knowledge about certain T cell subsets regulating the progression during melioidosis is limited. Human studies showed that decreased levels of CD4+ and CD8+ are correlated with mortality. Murine studies showed that particular CD4+ T cells are essential in both innate and adaptive response against B. pseudomallei infection. In contrast to other infections caused by similar pathogens (e.g. non-typhoidal Salmonella), there is no association between HIV and melioidosis. Additionally, B-cells start to produce antibodies, inducing the humoral response.

The coagulation system becomes activated by the release of pro-inflammatory cytokines tumor necrosis factorα (TNFα) and interleukin-6 (IL-6) and plays a role in melioidosis severity. Ample evidence shows there is a bidirectional interplay between inflammation and coagulation. Coagulation activation and fibrin deposition are of major importance in host defense against infection in an attempt to prevent further dissemination of infection. If inflammation induced blood coagulation is not sufficiently controlled it could become deleterious and consequently lead to thrombosis and hemorrhage, also known as the condition disseminated intravascular coagulation (DIC). In addition, the complement system becomes activated and facilitates host cellular homeostasis, opsonization, and elimination of bacteria.
Figure 4  Pathogenesis of B. pseudomallei

**Platelets**

Important cells missing from Figure 4 are platelets. Platelets (anuclear cells derived from megakaryocytes) are known for their role in hemostasis. Recently, it has become evident, that platelets can also play a significant role in the host defense. Platelets can directly kill bacteria by the release of antimicrobial peptides and recruit neutrophil and monocytes to the site of infection, as well as that they potentiate pathogen phagocytosis and killing by both cell types. Platelets can also stimulate neutrophil extracellular trap (NET) formation. NETs are DNA-networks released by neutrophils, which can capture bacteria. NET-related components are significant present in plasma of melioidosis patients and NETs induce antibacterial activity against *B. pseudomallei*. Besides interactions with immune cells, platelets act in close interplay with the coagulation system, potentiating coagulation and immunothrombosis (clot of platelets, fibrin and leucocytes capturing pathogens in blood vessels). Thrombocytopenia may dys-regulate immune response in septic patients, e.g. by influencing cytokine responses and by decreasing leucocyte adhesion signaling. However, the role of platelets during melioidosis remains to be elucidated (Figure 5).
**Clinical presentation**

Melioidosis is mainly acquired by percutaneous inoculation, inhalation, and ingestion.[1] People who are routinely in contact with water and soil, such as rice farmers, are primarily at risk for developing melioidosis. The average age of melioidosis patients is between 40 and 60 years, but the disease can affect all age groups. The disease is mainly observed in Southeast Asia and Northern Australia[5] and peaks during the rainy sea-
Melioidosis is considered to be an opportunistic infection; 80% of patients have one or more known risk factors, mainly diabetes mellitus. Other risk factors include excessive alcohol intake, chronic lung, kidney and renal disease, use of immunosuppressive drugs, malignancy, and thalassemia. The mean incubation period ranges from 1-21 days (average 9 days). Infection with *B. pseudomallei* can result in acute (>85%), chronic (10%), or latent infection. Most *B. pseudomallei* infections will be cleared by immunocompetent individuals and only those cases who present with clinical symptoms (acute or chronic) will be considered as having melioidosis. The clinical presentation of melioidosis varies from localized skin abscesses to fulminant septicemia mimicking clinical features of tuberculosis and cancer. This can hinder the diagnosis; therefore, melioidosis is consequently known as “the Great Mimicker.” During acute infection half of the melioidosis cases present with pneumonia and 20% with sepsis, a life threatening organ dysfunction caused by a dysregulated immune response to infection. In total, 40-60% of melioidosis patients have bacteremia on admission. Abscess development in internal organs are common, especially in the spleen, liver, prostate, and kidneys. Geographical differences in disease manifestations are described, but remain poorly understood. For example, parotitis is mainly evident in Thai children, which is possible related to ingestion. In Australia, neurologic melioidosis is often observed, linked to differences in virulence factors of *B. pseudomallei*. The infection route, strain, virulence factors, and risk factors will all effect the clinical presentation and consequently melioidosis morbidity and mortality. Early detection and adequate treatment of melioidosis can have significant impact on the outcome.

**Diagnosis and treatment**

Culture from any site (e.g. blood, sputum, urine) remains the gold standard in *B. pseudomallei* diagnosis, despite its low sensitivity (60%). The majority of routinely used culture media in microbiological settings can grow *B. pseudomallei*, however, the bacterium may easily be ignored as a contaminant, or be misdiagnosed as a *Pseudomonas* spp, particularly in non-endemic areas. The use of multiple standard biochemical tests is recommended as misidentification may occur frequently otherwise. The latex-agglutination test is very useful in screening suspected colonies. Microbiological laboratories are increasingly using matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) mass spectrometry for identification. Molecular methods have been defined, including 16srDNA
Figure 6  Clinical presentation of melioidosis

The most common clinical presentations of melioidosis.
Abbreviations: CNS infection = central nervous system; MSK = Musculoskeletal; SSTI = skin-and soft tissue infection; Abscess formation = intra-abdominal abscess (mostly liver, spleen).
sequencing and specific PCR’s; however, these techniques are often not available in low-resource settings. The most common serology test used to detect antibodies against *B. pseudomallei* is the indirect-hemagglutination test (IHA). Nevertheless, sensitivity and specificity is limited as healthy individuals can have positive titers due to cross reactivity with other *Burkholderia* spp.\(^{53}\)

The therapy of melioidosis consists of an initial acute phase treatment of intravenous ceftazidime or meropenem (10-14 days), followed by an eradication phase with oral trimethoprim-sulfamethoxazole, amoxicillin-clavulanic acid, or doxycycline to prevent relapse or recrudescence of melioidosis (3-6 months).\(^2\) Such long and intensive therapy is often not affordable or available in melioidosis-endemic areas. Nonetheless, guidelines recommend a therapy of at least 10 days intravenously, with the exception for those patients who have localized skin infections.\(^2\)

Currently, no licensed melioidosis vaccine for humans is available, despite efforts on vaccine development made by the research community.\(^{64}\) In murine studies, live attenuated mutants of *B. pseudomallei* are one of the most effective vaccine candidates, inducing broad, long-lasting humoral, and cell-mediated immunity.\(^{65}\) However, one of the main concerns is that such mutants may be able to establish latent infection and revert to virulence.\(^{65,66}\) Killed or subunit vaccines (purified antigens) induce a strong antibody specific response, though they do not elicit strong cell-mediated immunity and may therefore be unable to clear intracellular bacteria.\(^{65}\) To overcome this, DNA vaccines have been developed with the ability to generate both antigen-specific antibody and cell-mediated response, but those have not been proven sufficiently effective yet.\(^{66,67}\)
Figure 7  Typical appearance of *B. pseudomallei* colonies on two types of agar

Characteristic appearance of *B. pseudomallei* mixed with *Escherichia coli*. The bacteria were isolated from non-sterile clinical samples. (A) Blood agar: *B. pseudomallei* appeared to be creamy, non-hemolytic and resembling a coliform (day two). The colonies become dry and wrinkled on day four and show a metallic sheen. *E. coli* has similar morphology and overgrowth to *B. pseudomallei*. (B) Ashdown agar: the first visible growth is pinpoint and the color is clear to pale pink (day two). The colonies become darker pink to purple, flat, dry and wrinkled with a definite sheen (metallic) on day four. *E. coli* was inhibited by gentamicin in the agar and did not appear here. (C) The difference between *B. pseudomallei* and *B. thailandensis* colonies are depicted on Ashdown agar. On the left, a typical *B. pseudomallei* morphology is shown. The middle panel shows *B. thailandensis* colonies and the right panel a *B. thailandensis* with Bps like CPS. Parts A–B Photograph of *B. pseudomallei* on two different agars are a courtesy of Premjit Amornchai, Mahidol-Oxford Tropical Medicine Research Unit, Thailand. Part C Photographs of *B. pseudomallei* on Ashdown agar are a courtesy of Patpong Rongkard, Mahidol-Oxford Tropical Medicine Research Unit, Thailand.
AIM AND OUTLINE OF THIS THESIS

Our ultimate aim is to decrease incidence and mortality rates from melioidosis by increasing awareness of the disease, improving diagnostic facilities, and paving the way for novel effective treatment and prevention strategies using a systematic and translational approach.

Our key objectives are:

(I) To quantify the burden of melioidosis, both globally and nationally in the Netherlands;

(II) To study the occurrence of B. pseudomallei in sub-Saharan Africa and to provide new insights into the phylogeny and virulence;

(III) To obtain novel insights into the pathogenesis of melioidosis and possibilities for vaccine development.

Section I describes the global burden of melioidosis. In Chapter 2, we quantify the global burden of melioidosis in terms of DALYs, which was unprecedented to date. By combining the modelled estimates of the global incidence and mortality of melioidosis with a systematic review of the published literature on its clinical impact, we calculated the DALYs for melioidosis for the year 2015 by age, sex and country. Chapter 3 provides a retrospective overview of all imported human melioidosis cases in a non-endemic country, the Netherlands. We systematically reviewed all B. pseudomallei positive culture results in Dutch medical microbiology laboratories from 1985-2018.

Section II focuses on the burden of melioidosis in sub-Saharan Africa by using a systematic and translational approach. Chapter 4 provides an overview of the known literature on human, animals, and soil of B. pseudomallei in Africa. In Chapter 5, we hypothesized that B. pseudomallei and B. thailandensis are present in the Central African country of Gabon, potentially causing unrecognized disease, due to a lack of microbiologic facilities and awareness. We conducted a seroprevalence study, an environmental survey, and set up microbiology facilities for B. pseudomallei detection at a referral hospital in Gabon. Chapter 6 determines the difference in patterns of virulence of our Gabonese isolated B. pseudomallei both ex vivo and in vivo using a mouse model of experimental melioidosis. Chapter 7 deter-
mines whether *B. pseudomallei* and *B. thailandensis* are present in the soil of central Sierra Leone with the use of an environmental surveillance study.

In **Section III**, we shift our focus to the pathogenesis of melioidosis and the development of a potential novel vaccine. In **Chapter 8**, we assess the role of platelets in the host response against *B. pseudomallei* infection in a large cohort of culture-proven melioidosis patients and in platelet depleted mice. **Chapter 9** studies the importance of platelets in a small cohort of melioidosis patients and examines the relevance of Von Willebrand factor and ADAMTS13 in melioidosis patients. **Chapter 10** determines the composition and function of the intestinal microbiota during experimental melioidosis. **Chapter 11** examines the use of a new rapid plasmid DNA vaccine against *B. pseudomallei* flagellin as a potential candidate for the prevention against aerosolized melioidosis. The results of our investigations are summarized in **Chapter 12**. In **Chapter 13**, we discuss our findings and provide suggestions for the future.
SECTION I
BURDEN OF MELIOIDOSIS

CHAPTER 02
global burden

CHAPTER 03
the Netherlands

SECTION II
IN SEARCH OF MELIOIDOSIS IN AFRICA

CHAPTER 04
Africa

CHAPTER 05
Gabon: soil, fever & serosurveillance

CHAPTER 06
virulence of Gabonese strain

CHAPTER 07
Sierra Leone: soil surveillance

SECTION III
PATHOGENESIS OF MELIOIDOSIS & DEVELOPMENT OF NOVEL VACCINE

CHAPTER 08
the role of platelets in host defense

CHAPTER 09
dause of thrombocytopenia

CHAPTER 10
intestinal microbiota

CHAPTER 11
DNA vaccination
REFERENCES


33. Harley VS, Dance DA, Drasar BS, Tovey G. Effects of Burkholderia pseudomallei and other Burkholderia species on eukaryotic cells in tissue culture. Microbiol 1998; 96(384): 71-93.


