Digging for melioidosis

New insights into the epidemiology and pathophysiology

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CHAPTER 13
GENERAL DISCUSSION
AND FUTURE DIRECTIONS
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The exact global burden of melioidosis has not yet been determined. A recent modelling study estimated the worldwide number of cases to be 165,000 per year with more than 50% mortality. Most of the cases remain unrecognized or maybe falsely allocated to other febrile illnesses. Furthermore, there is a discrepancy between melioidosis estimates and reported cases, explainable by the lack of awareness and under-developed or non-existent microbiological facilities. Melioidosis hotspots in Southeast Asia and Northern Australia are extensively described in the literature. However, there is a need to expand our knowledge on melioidosis beyond those geographic areas. More surveillance studies are necessary to enhance the understanding of the epidemiology of this highly neglected tropical infectious disease. Unfortunately, melioidosis fatality rates remain high, urgently mandating further research on pathophysiology to open up avenues to new treatment possibilities. In 1989, a landmark ceftazidime randomized control trial, showed that the usage of this drugs reduced mortality rates of melioidosis patients from 75% to 37%. Since then, various treatment options have stagnated in development pipelines and/or have been proven insufficiently effective to be adopted in everyday clinical practice. Overall, high incidence estimates, alarming mortality rates, and limited treatment options fully supports current efforts by public health officials and policy makers to make melioidosis a global healthcare priority.

This thesis adds to the growing understanding that melioidosis needs to be recognized as a deadly neglected tropical disease (NTD): by providing the first estimates of the burden of melioidosis worldwide, conducting surveillance studies in Africa, and fundamental research on bacterial pathogenesis and host-pathogen interactions. Here, a critical reflection will be given regarding the evidence provided with this thesis, with a focus on methodologic limitations and identification of important knowledge gaps. The most promising findings will also be highlighted in order to guide upcoming study designs and to suggest key areas for future research.

Section I - Burden of Melioidosis

The findings presented in Section I Chapter 2 of this thesis provide worldwide estimates of the global burden of melioidosis in terms of DALYs, including regions of South Asia, South America, and Africa. Putting this into perspective, the estimated global burden of melioidosis expressed in DALYs (4.64 million) is higher than leptospirosis (2.90 million), den
gue (2.86 million) and schistosomiasis (2.63 million). Whilst comparing melioidosis DALY estimates to DALYs of such officially recognized NTDs, we found no association between DALY burden of disease and the level of global investment in research and development in general (Figure 1).

**Figure 1** Global burden of a selection of neglected and emerging/re-emerging tropical diseases and corresponding investments in research and development

Leptospirosis is not officially recognized as a neglected tropical disease. Recognized neglected tropical diseases (by WHO) and leptospirosis are depicted in blue and melioidosis in red. **Source** Chapter 2 of this thesis.

Abbreviations: DALYs= disability-adjusted life years and WHO= World Health Organization
The findings in Chapter 2 are alarming, yet our study has several limitations. First, reliable global incidence and mortality data were scarce and therefore modelled on earlier estimates. Second, disability weights were unavailable for several disease states. Strikingly, there is no standardized disability weight for sepsis, a critical illness with a high disability, highlighting the importance of expanding and developing improved disability weights. Third, data on post-melioidosis sequelae is lacking, warranting the need to conduct studies on long-term disease outcomes. Consequently, we conducted an additional literature search and used surrogate post-sequelae data for disease states after sepsis/septic shock, musculoskeletal involvement, and brain abscess. An important study published by Prescott et al showed that at least one-in-six septic patients will develop post-sepsis sequelae. This highlights the need to focus more research on post-acute disease outcomes, not least in melioidosis. Lastly, reactivation of latent melioidosis does not seem to play a major role in the total burden; however, crucial data on this subject are missing and we were unable to determine exact figures. Despite these limitations, we argue that the systematic methodological approach we have undertaken has yielded more robust estimates than would otherwise have been obtained using limited source data of countrywide health statistics. The estimates described in Chapter 2 provide a clear motivation for considering melioidosis as an official NTD. In order to prioritize national epidemiological surveillance and strengthen development of laboratory capacity we need enhanced collaboration between international partners.

Chapter 3, provides a comprehensive retrospective overview of culture confirmed imported melioidosis cases in a non-endemic country, The Netherlands, over a 25-year period. In total, 33 cases were identified, although this number is probably an underestimation. Clinicians may not be aware about melioidosis and disease manifestations are often unspecific especially in an early stage. In this study, 70% of the patients had one or multiple risk factors for melioidosis. This corresponds with changing traveler patterns wherein individuals with such risk factors are often able to travel (longer) and to more adventurous holiday destinations because of improved clinical therapies. Results, however, should be interpreted carefully as there is a chance of recall-bias and possible melioidosis cases might have been missed. In addition, due to a twenty-five-year study period, not all identification methods were standardized. Imported melioidosis is likely to increase in the light of increasing numbers of (immunocompromised) travelers, improvements in diagnostics and vigilance towards
the condition. This largest series of travelers with melioidosis to date, also shows that imported cases can serve as sentinels for non-endemic areas (e.g. Africa and South America) and help clinicians in their travel advice and clinical management.

Section II - In Search of Melioidosis in Africa

The studies described in Section II of this thesis add to our epidemiological knowledge of melioidosis in Africa. In Chapter 4, we start with a historical literature review of human, animal and soil studies on melioidosis in Africa. Reports on *B. pseudomallei* are limited highlighting the need for more studies on this continent. Therefore, we conducted a translational seroepidemiological, prospective fever and soil surveillance study, presented in Chapter 5. Here, we demonstrated the presence of *B. pseudomallei* in humans and soil in Gabon (Central Africa) for the first time. The low antibody rate combined with the low prevalence of *B. pseudomallei* cultured from blood, however, suggest that melioidosis is rare in this setting. During our serology study we made use of the commonly used indirect hemagglutination test (IHA), a simple serologic test to detect *B. pseudomallei* antibodies with low specificity and sensitivity, which could potentially have led to false-positive results. At the time of study initiation, the protein array test was not yet developed, but this promises to be a major improvement with high sensitivity and specificity. Nonetheless, this test is costly and difficult to perform in low-resource settings. There is an urgent need for an inexpensive, simple serologic assay with comparative sensitivity and specificity.

We hypothesized that potential differences in the virulence of *B. pseudomallei* strains could explain the variance in melioidosis incidence observed across the globe. Our data presented in Chapter 6 based upon ex vivo and in vivo studies, supports this notion. We found that the Gabonese *B. pseudomallei* isolate was significantly less virulent in vivo compared with the well-defined Thai isolate 1026b, yet our study was limited due to the small number of strains identified. Additionally, as we made use of C57BL6 mice, one should realize that the pathogenicity of *Burkholderia* isolates can vary among animals and that it does not always correlate with their virulence in humans. A larger analysis on the in vivo virulence and genetic expression between strains across the globe is required to gain insights into these variations. As well as incidence, the clinical presentation of melioidosis can also differ across countries. For example, parotitis is extremely
rare in Australia, in contrast to presentations seen in Southeast Asia. This is possibly related to ingestion being an uncommon mode of melioidosis transmission in Australia.\textsuperscript{2,13,20} Furthermore, data from a large Australian melioidosis cohort showed that the \textit{B. mallei}-like actin polymerization (bimA) gene is strongly correlated with neurological disease.\textsuperscript{21} Research highlights the importance of variably present virulence factors that can play a role in the pathogenesis of melioidosis,\textsuperscript{20} further underpinning the need for more data regarding melioidosis strains and presentations from South Asia, Africa, and South America. Our Gabonese \textit{B. pseudomallei} human strain was used in a larger research project in which WGS was performed to investigate the origin and virulence factors of \textit{B. pseudomallei} strains across the globe.\textsuperscript{20} This sequencing study revealed that \textit{B. pseudomallei} was previously spread via (slave) trade routes from Australia via Africa to (South) America centuries ago.\textsuperscript{20}

In Chapter 7, we performed an extensive environmental surveillance study and detected no \textit{B. pseudomallei}, but multiple new \textit{B. thailandensis} sequence types in the soil of the West African country, Sierra Leone. \textit{B. thailandensis} infection in humans have been occasionally reported in the US and Asia;\textsuperscript{22,23} however, the true clinical importance remains to elucidated. If disease can be attributed to \textit{B. thailandensis}, this would be of great importance to the melioidosis research community and additionally would call for stricter biocontainment conditions.\textsuperscript{24} Our study was hampered by the lack of standard blood culture services for febrile patients across Sierra Leone. This prevented targeted soil sampling studies centered around possible melioidosis cases, which is the current practice to gain better yields. In addition, the possibility of sampling error cannot be ultimately dismissed both for our environmental surveillance in Sierra Leone (Chapter 7) and our study in Gabon (Chapter 5), although consensus guidelines were followed.\textsuperscript{25} Moreover, \textit{B. pseudomallei} bacteria present in the soil could have been viable but not in a culturable state, making it difficult to identify using culture methods. Nonetheless, \textit{B. pseudomallei} was also not detected by multi-target qPCRs.\textsuperscript{17} Finally, some \textit{B. thailandensis} isolates showed cross-reactivity with the \textit{B. pseudomallei} latex-agglutination test, similarly described in Chapter 5. Interestingly, several sequence types (ST) contained a \textit{B. pseudomallei}-like capsular polysaccharide (BTCV).\textsuperscript{26} This is of major importance, as the latex-agglutination test is recommended as standard screening methodology within the environmental consensus guideline and possibly detecting false positive \textit{B. pseudomallei} colonies.\textsuperscript{25}
very recent study also highlighted that this guideline does not appear to be equally sensitive in every location.\textsuperscript{27} This study advises to first use enrichment culture and then PCR as a screening methodology, followed by culture methods.\textsuperscript{27} Nevertheless, the current consensus guideline developed to standardize the work and to determine the distribution of \textit{B. pseudomallei} in the environment is still considered the gold standard.\textsuperscript{25}

\textit{Section III - Pathogenesis of Melioidosis and Development of a Novel Vaccine}

In Section III, we focused on host-pathogen interactions during melioidosis. In Chapter 8, we showed that thrombocytopenia is an independent predictor of mortality in a large cohort of human melioidosis patients, even after correction for multiple confounders. This corresponds with previous Gram-negative sepsis studies\textsuperscript{28-30} and is in line with our findings in a smaller patient cohort with melioidosis, described in Chapter 9. The association between thrombocytopenia and mortality may be a reflection of disease severity. However, disease severity scores were not collected, negating our ability to correct for this important confounder. Nonetheless, previous research in sepsis patients has illustrated an independent association between thrombocytopenia and enhanced mortality\textsuperscript{31-33} and moreover linked changes in immune response in septic patients with low platelet counts.\textsuperscript{28}

Most human studies are limited in inferring causality. Therefore, we made use of our experimental mouse model. In this pneumonia derived sepsis model, we infected mice intranasally with a low dose of \textit{B. pseudomallei}, which gradually multiplies in the lung and slowly disseminates throughout the body (Chapters 6, 8, 10, and 11). Similar to humans, mice infected with \textit{B. pseudomallei} developed marked thrombocytopenia and platelet depletion was associated with increased mortality and impaired host response (Chapter 8). These results are in line with other murine studies of Gram-negative infection using \textit{Escherichia coli},\textsuperscript{34,35} \textit{Klebsiella pneumoniae}\textsuperscript{29} as well as patients with sepsis.\textsuperscript{28} In our study, we made use of a platelet depleting antibody in order to deplete platelets to less than 5\% of normal. This model is often used to examine the effects of platelets on the host defense.\textsuperscript{29} However, it is important to note that the thrombocytopenia in these mice is more severe than the thrombocytopenia seen in patients with melioidosis.

As defects in neutrophils can play a crucial role in the host defense against \textit{B. pseudomallei} infection,\textsuperscript{36} we determined whether platelets medi-
ate their protective effect via the recruitment of neutrophils. During melioidosis neutrophil recruitment can influence outcome, mostly at later stages of infection.\textsuperscript{37} Interestingly, we observed an early effect of neutrophil recruitment during platelet depletion. The effect size, however, was modest (Chapter 8). Nonetheless, this is in line with a previous study during infection with \textit{Pseudomonas}.\textsuperscript{38} In contrast, platelets did not influence neutrophil recruitment during a \textit{Klebsiella} sepsis model.\textsuperscript{39} These findings are of interest as individuals with diabetes are at twelve times higher risk for contracting melioidosis, possibly explained by the impaired function of neutrophils.\textsuperscript{2,39} Diabetics show defects in neutrophil adhesion, chemotaxis and intracellular killing.\textsuperscript{40} Platelets can induce neutrophil extracellular trap (NET) formation, influencing bacterial growth.\textsuperscript{41,42} Chapter 8, however, indicated that platelet depletion did not impair NET formation, similar to a previous study on NETs in melioidosis.\textsuperscript{43}

We investigated mice lacking platelet GPI\textsubscript{b}\textalpha to study which platelet receptors were involved. We found an impaired host defense at the primary site of infection in GPI\textsubscript{b}\textalpha knock out mice, but not to the same extent as platelet depleted mice. In line with this, a recent study found a protective role for platelet GPI\textsubscript{b}\textalpha during Gram-negative pneumosepsis, using a GPI\textsubscript{b}\textalpha blocking antibody.\textsuperscript{44} Interestingly, we found that platelet counts in IL\textsubscript{4R}/GPI\textsubscript{b}\textalpha mice\textsuperscript{45} were reduced, limiting the interpretation of these results. Platelets can play a pivotal role in the prevention of bleeding, especially at the site of infection and inflammation.\textsuperscript{29,46} Platelet depletion resulted in bleeding in the lung during \textit{B. pseudomallei} infection. In contrast to \textit{Klebsiella} infection,\textsuperscript{39} melioidosis also induced severe bleeding when platelet counts were \textless5\% and lung bleeding was already seen at an early time point (24 hours after infection). Perhaps, infection with \textit{B. pseudomallei} causes more severe damage to tissue as well as vascular integrity, which requires more platelets to prevent bleeding. In melioidosis patients, however, bleeding complications are rarely observed.\textsuperscript{2,3} The importance of the risk of bleeding in human melioidosis remains to be elucidated.

In Chapter 9, we examined potential drivers of thrombocytopenia in melioidosis patients. We showed that VWF antigen levels were higher in melioidosis patients compared to controls. However, this was not associated with thrombocytopenia, indicating that excessive VWF is not the primary driver of thrombocytopenia in melioidosis. Another potential driver of thrombocytopenia is diffuse intravascular coagulation (DIC).\textsuperscript{47} Activation of the coagulation pathway may have been deleterious when the triggered blood coagulation is insufficiently controlled leading to DIC.
and microvascular thrombosis. Nonetheless, the median DIC score in this melioidosis cohort was 3 (2-4) indicating that DIC is not overt. More studies focusing on DIC in melioidosis patients are needed. In addition, hemophagocytosis lymphohisticytosis (HLH) or macrophage activation syndrome (MAS), a life-threatening condition of excessive immune activation, has been postulated to drive sepsis-related thrombocytopenia. Unfortunately most of the markers to score MAS were not available for our patients, making this an interesting area for further exploration in a prospective study design. Patients with sepsis and associated MAS, both presenting as cytokine storms, might benefit from interleukin-1 receptor blockade (anti-IL-1). Interestingly, anti-IL-1ra as well as anti-IL-1b enhances protection against experimental melioidosis. Our study was limited due to multiple factors. First, we only included diabetic patients in our study. This decision was based on the fact that diabetes is associated with abnormalities of coagulation, anticoagulation and fibrinolysis. Additionally, most melioidosis patients have diabetes and earlier research already extensively investigated the effect of diabetes on coagulation during melioidosis. Second, the number of patients in this analysis was restricted due to financial constraints. We call for more animal and human studies to understand the causes of thrombocytopenia in septic melioidosis patients, which can help inform our clinical practice and treatment.

The antibiotic therapy for melioidosis patients - an intensive intravenous phase (minimum two weeks) followed by a long oral eradication phase (three months) – has a potentially profound effect on the microbiota. Furthermore, increasing research on this subject demonstrates a beneficial effect of the intestinal microbiota on the host defense against infection. In Chapter 10, we showed that the gut microbiota is a potential modulator of the host response during experimental B. pseudomallei infection. Of interest, we described that the systemic inflammatory response in itself can lead to marked alterations in the gut microbiota. Additionally, we found a potential protective effect of a healthy gut microbiota on the host defense against B. pseudomallei, where alveolar macrophages derived from antibiotic pre-treated mice showed a diminished capacity to phagocytose B. pseudomallei. Alveolar macrophages constantly adapt to their environment and are potentially affected by circulating compounds derived from the intestinal microbiota. This is supported by earlier reports. Disturbances in the microbiome may induce an altered phenotype of alveolar macrophages, leading to the observed decreased
phagocytosis capacity. This is in line with previous findings from a pneumococcal mouse model.\textsuperscript{56} Potentially, this can be linked to the induction of trained immunity in alveolar macrophages, as shown by a recent study in a viral infection model.\textsuperscript{59,60}

Study limitations need to be considered. First, the choice of our antibiotic regime may have influenced the phenotype, although the antibiotics chosen have a proven track record based on earlier experiments.\textsuperscript{8-50} Second, a malaria mouse model recently showed that supplier differences could additionally effect the intestinal microbiota and induce different host response.\textsuperscript{61} Further studies using mice from different suppliers are necessary to expose the influence of such confounding. Third, respiratory tract microbiota differences may play a part in our observed phenotype.\textsuperscript{62} We did not, however, find any differences in the lung microbiota between control and antibiotic treated mice. Last, the true clinical picture during human melioidosis is very different from our controlled murine experimental setting; comorbidities, medications, and inter-individual differences all influence the interplay between the microbiota and the innate immune system. It would be of interest to study the microbiota during melioidosis in a clinical setting.

Currently, no effective vaccine for the prevention of melioidosis have been implemented.\textsuperscript{63} Live attenuated mutants of \textit{B. pseudomallei} have proven to be the most effective candidates and are currently considered as the gold standard.\textsuperscript{64} However, significant safety concerns need to be acknowledged\textsuperscript{64} as there is a potential risk of reversion into a virulent phenotype.\textsuperscript{65} In Chapter 11, we explore an alternative approach assessing the development and testing of DNA vaccines during experimental \textit{B. pseudomallei} infection. DNA vaccines are able to produce antigen-specific antibodies, a cell-mediated response, without the potential for reversion to virulence.\textsuperscript{66,67} We found that a single dose of a fliC DNA vaccine intranasally yielded >1,000-fold lower bacterial loads and increased rate of survival. Unfortunately, the intranasally administered DNA vaccine led to undesired weight loss in some of the mice during this experiment, due to which four mice died and one was euthanized because of reaching a humane end point. We experienced this in one of the four experiments presented in Chapter 11. Additionally, we only investigated the vaccine in one mice species (C57BL6 mice) using one \textit{B. pseudomallei} (1026b) strain. Melioidosis vaccine consensus guidelines, however, require the use of different challenges and mice strains, as virulence can vary greatly and consequently disease severity and manifestation. Moreover, such guidelines require the use of
different inoculation routes and diabetic mice models, since diabetes is the most important risk factor and most patients acquire the infection by percutaneous inoculation, inhalation, or ingestion. For these reasons, we have an ongoing collaboration with the National Institutes of Health (NIH), who currently assists in follow-up testing of our vaccine including the comparisons with other vaccine candidates. The NIH will make use of different \textit{B. pseudomallei} and mice strains, whilst performing subcutaneous as well as intranasal vaccination and challenges. These experiments are currently ongoing. Importantly, our vaccine can be of great significance in the setting of bioterror threat reduction because it has the potential to rapidly protect humans and administration is user-friendly.
FUTURE DIRECTIONS

In Search of Melioidosis in Nigeria

We hypothesize that the largest burden of melioidosis in Africa is present in Nigeria, given its favorable climate and population density.\(^1\) Another important driver is the high and increasing prevalence of diabetes, the main risk factor for melioidosis.\(^2\) Moreover, melioidosis is also known as rice farmer fever. In Nigeria, rice is increasingly cultivated in order to meet increasing local demands. A modelling study estimated that each year around 13,000 Nigerians suffer from melioidosis of which the majority will not survive.\(^1\) In close collaboration with the WHO, we organize the First African Melioidosis Workshop in collaboration with the University of Lagos, in Lagos, Nigeria in the Spring of 2019. During this workshop, we will bring together regional and international experts to raise awareness on melioidosis and \textit{B. pseudomallei}, to create, enhance, and develop capacity across Nigeria and Africa. Visit www.melioidosisafrica.com for more information. This workshop will in addition serve as a kick-off event for the Nigeria melioidosis study funded by a personal research grant from the \textit{European Society of Clinical Microbiology and Infectious Diseases} (ESC-MID). This study will be translational, combining environmental, clinical and seroepidemiological surveillance studies of \textit{B. pseudomallei} in Nigeria. This study is part of the African Melioidosis Network; a larger study focusing on understanding the ecology and epidemiology of \textit{B. pseudomallei} in different African countries. The pilot project will provide some first insights into the prevalence of \textit{B. pseudomallei} infections in potentially affected areas in Nigeria.

The Road Ahead

To prevent and decrease mortality and morbidity from melioidosis there is a need for better granularity in the worldwide distribution of this disease. Alongside increasing awareness, it is important to further improve microbiological facilities, to conduct more research on underlying pathophysiology and host-pathogen interactions, and to identify potential new treatment targets or vaccines. Due to the saprophytic nature of melioidosis and the fact that it can also affect a wide range of animal species, a One Health approach would be ideal. Table 1 summarizes the most important themes that need to be addressed in the future. We advocate that studies should employ standardized methodologies and be freely accessible across
Group photo of the First African Melioidosis Workshop (funded by the WHO), Sheraton, Lagos, Nigeria.

Practical laboratory session.

Kick-off: *In search of Melioidosis in Nigeria* - a pilot project.
research groups to maximize success of scientific, clinical, and public health goals. An important recommendation of this thesis is that melioidosis should be recognized as an official NTD. This would be a useful starting point for future trajectories aiming at reducing incidence, mortality or potentially eliminating melioidosis.

Table 1  Questions for further research

**Epidemiology**

What is the geographical distribution of *B. pseudomallei* and *B. thailandensis* across the globe (with a special focus on high risk areas, such as Nigeria, Indonesia, and India)?

What are the true incidence and mortality rates of melioidosis in endemic areas?

What is the role of rhizosphere microbiota in the abundance of *B. pseudomallei* and could associated insights contribute to reducing exposure of this bacterium in the soil?

To what extent does/will global warming, severe weather events, soil changes, and urbanization influence the burden of melioidosis?

**Virulence and Pathogenesis**

How can differences in disease presentation combined with whole genome sequencing enhance our understanding of the phylogeny and virulence of *B. pseudomallei* and *B. thailandensis*?

Can differences in virulence across *B. pseudomallei* species explain observed variance in the origin and clinical presentations of melioidosis across the globe?

What is the true clinical importance of *B. thailandensis* infection?

Is macrophage activation syndrome of clinical importance in melioidosis?

What is the composition and the role of the gut microbiota in patients with melioidosis?

Why are people with diabetes twelve times more at risk for contracting melioidosis?

Why does HIV not seem to be a risk factor for melioidosis, in contrast to diseases like tuberculosis?

Why does the global antibiotic resistance pandemic has not crossed over to *B. pseudomallei*?
Diagnosis and Therapy

What diagnostic test is the quickest, most sensitive, specific, and accessible way to detect melioidosis in patients?

How can diagnostic microbiological facilities be improved, especially in low-recourse settings?

Which biomarkers can be of diagnostic, prognostic, and therapeutic value in melioidosis?

Can therapeutic modulation of the microbiota accelerate the recovery of melioidosis patients?

How can we develop more affordable effective treatment strategies for melioidosis?

How can we shorten the duration of melioidosis treatment and prevent relapse or will this lead to similar disappointing results as tuberculosis trials?

What is the true clinical relevance of latent melioidosis?

What are the most important post-melioidosis sequelae and how should these be managed?

Prevention

Which vaccine is the most effective to prevent the development of melioidosis?

What is the best route of vaccination, via the skin or via the airways?

How can prevention strategies and awareness programs targeted towards at risk groups be developed and implemented?

Which integrated multi-disciplinary "One Health" approach will be most effective in the prevention and control of melioidosis, and eventually eliminate melioidosis?
REFERENCES


