Melioidosis
A Century of Observation and Research
Edited by Natkunam Keteesan
Melioidosis – A Century of Observation and Research
Dedicated to the

Pioneers and Teachers

who went before us

*Burkholderia pseudomallei* adhered to HeLa cell surface
(M. Rohde, Helmholtz Centre for Infection Research, Braunschweig, Germany)
Melioidosis
– A Century of Observation and Research –

Edited by
Natkunam Keteesan
James Cook University, Townsville, Australia
Preface

While organising the VIth World Melioidosis Congress in 2010 which coincided with the centenary of the description of melioidosis by Whitmore and Krishnaswami, a few colleagues suggested that we should consider compiling a monograph with information accumulated over a century. With the support of authors and section editors who devoted many hours of their time we have compiled and synthesised data on diverse aspects of melioidosis which we present in the ten different sections of this monograph.

The information presented in this monograph is not intended to be an up to date review on melioidosis; rather, we have endeavoured to provide the reader with a comprehensive insight into what has been achieved in the last hundred years. It is considered frivolous in today’s electronic age, where research excellence is judged by impact factors and citation indices, to request researchers to contribute to monographs that would be outdated even before being typeset. However, several investigators and clinicians, especially those working in melioidosis-endemic regions, recognise a need for such a publication.

Significant research funding has been made available in the last few years, since *Burkholderia pseudomallei* was classed as a potential bioterrorism agent. This has certainly provided a major impetus to better understand the pathogen and the host responses, providing an opportunity for development of novel diagnostic tools and treatment options. However, it is the delivery of affordable detection and treatment modalities that would have a positive impact on the significant majority of patients who contract the infection in endemic areas. Scientific innovations in this field should therefore be applicable in the ever expanding *B. pseudomallei*-endemic areas, which are mostly in low- to middle-income countries in the tropics.

Even as several important facets of the pathogen and the host response are being unravelled, there is still much that is unknown. Many controversial issues related to basic concepts on infection route, pathogenesis, detection methods and optimal treatment protocols have yet to be resolved. Our current knowledge of melioidosis has to be scrutinised and gaps in our understanding of the disease process that would significantly contribute to patient welfare have to be identified. This requires a multidisciplinary approach involving microbiologists, geneticists, pathologists, immunologists, pharmacologists, clinicians, intensivists, epidemiologists, and public-health experts. This compendium brings together a multidisciplinary panel of authors who have summarised the literature and suggest avenues for further research where appropriate.

It is hoped that to some readers the material in this monograph will provide adequate information and stimulate enthusiasm to carry out much needed research to answer many
of the unanswered questions that will ultimately aid in timely diagnosis and provide effective and affordable treatment options to patients.

I would like to take this opportunity to thank all the authors and section editors for their contribution and for the long hours devoted to this project. Thanks are also due to those who provided illustrations and figures for this monograph. Without the financial support provided for the VIth World Melioidosis Congress and the publication of this monograph by sponsors, this project would not have been possible. I would also like to acknowledge and thank those colleagues and students who proof read several sections of this monograph to reduce the number of errors. We would like to express our appreciation to the staff members of Elsevier who helped with the publication process.

Natkunam Ketheesan
Townsville, Australia
December 2011
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List of Contributors

Jonathon P. Audia, PhD
Assistant Professor
Department of Microbiology and Immunology
College of Medicine
University of South Alabama
Mobile, Alabama, USA
Other Burkholderia species

Anthony L. Baker, BSc
Graduate Researcher
Microbiology and Immunology
School of Veterinary and Biomedical Sciences
James Cook University
Townsville, Australia
Burkholderia pseudomallei in the environment

Greg J. Bancroft, BSc, PhD
Reader in Immunology
Department of Immunology and Infection
Faculty of Infectious and Tropical Diseases
London School of Hygiene and Tropical Medicine
London, UK
Pathogenesis and development of protection

Ifor R. Beacham, BSc, PhD, FASM
Professor
Institute for Glycomics
Gold Coast Campus
Griffith University
Gold Coast, Australia
Microbiology
List of Contributors

**John D. Boyce, BSc, PhD**
Senior Lecturer
Department of Microbiology
Faculty of Medicine, Nursing and Health Sciences
Monash University
Clayton, Australia
*Section Editor*
*Microbiology*

**Katrin Breitbach, PhD**
Clinical Microbiologist
Friedrich Loeffler Institute of Medical Microbiology
Ernst Moritz Arndt University Greifswald
Greifswald, Germany
*Pathogenesis and development of protection*

**Paul J. Brett, PhD**
Assistant Professor
Department of Microbiology and Immunology
College of Medicine
University of South Alabama
Mobile, Alabama, USA
*Other Burkholderia species*

**Mary N. Burtnick, PhD**
Assistant Professor
Department of Microbiology and Immunology
College of Medicine
University of South Alabama
Mobile, Alabama, USA
*Other Burkholderia species*

**Narisara Chantratita, BSc, PhD**
Lecturer
Mahidol-Oxford Tropical Medicine Research Unit
Faculty of Tropical Medicine
Mahidol University
Bangkok, Thailand
*Laboratory diagnosis and detection*
Wipada Chaowagul, MD
Professor
Department of Medicine
Sappasithiprasong Hospital
Ubon Ratchathani, Thailand

Clinical manifestations

Allen C. Cheng, FRACP, MPH, PhD
Associate Professor
Infectious Diseases Epidemiology
Monash University and Alfred Hospital
Melbourne, Australia;
Menzies School of Health Research
Darwin, Australia

Section Editor
Epidemiology; Clinical manifestations; Treatment

Ploenchan Chetchotisakd, MD
Professor
Division of Infectious Diseases and Tropical Medicine
Department of Medicine, Faculty of Medicine
Khon Kaen University
Khon Kaen, Thailand

Treatment

Wirongrong Chierakul, MD, PhD, Dip.
Deputy Head
Department of Tropical Medicine
Mahidol-Oxford Tropical Medicine Research Unit
Faculty of Tropical Medicine, Mahidol University
Bangkok, Thailand

Treatment

Bart J. Currie, FRACP, DTM&H
Professor in Medicine
Menzies School of Health Research
Darwin, Australia;
Infectious Diseases Department
Royal Darwin Hospital
Darwin, Australia

Section Editor
Epidemiology; Clinical manifestations; Treatment
List of Contributors

David A.B. Dance, MB, MSc, FRCPath
Clinical Research Microbiologist
Wellcome Trust-Mahosot Hospital-Oxford Tropical Medicine Research Collaboration
Vientiane, Lao People’s Democratic Republic;
Centre for Clinical Vaccinology and Tropical Medicine
Churchill Hospital, University of Oxford
Oxford, UK
Section Editor
Historical Overview; Epidemiology

David DeShazer, PhD
Senior Scientist
United States Army Medical Research Institute of Infectious Diseases
Bacteriology Division, Fort Detrick
Maryland, USA
Section Editor
Other Burkholderia species

Bob K. Ernst, BS, MA, PhD
Associate Professor
Department of Microbial Pathogenesis
Dental School
University of Maryland
Baltimore, USA
Microbiology

Yunn H. Gan, BSc, PhD
Associate Professor
Department of Biochemistry
National University of Singapore
Singapore
Pathogenesis and development of protection

Mindy B. Glass, BS
Microbiologist
Centers for Disease Control and Prevention
Atlanta, Georgia, USA
Laboratory diagnosis and detection
Melioidosis – A Century of Observation and Research

Brenda L. Govan, BSc, PhD
Head of Microbiology Department
Microbiology and Immunology
School of Veterinary and Biomedical Sciences
James Cook University
Townsville, Australia
Section Editor
Microbiology

Yuka Hara, PhD
Postdoctoral Fellow
Malaysia Genome Institute
UKM-MTDC Smart Technology Centre
Selangor, Malaysia
Microbiology

Kelly A. Hodgson, BSc
Graduate Researcher
Microbiology and Immunology
School of Veterinary and Biomedical Sciences
James Cook University
Townsville, Australia
Pathogenesis and development of protection

Tim J.J. Inglis, DM, PhD, FRCP A
Clinical Associate Professor and Medical Microbiologist
Division of Microbiology and Infectious Diseases
PathWest Laboratory Medicine
Perth, Australia;
School of Pathology and Laboratory Medicine
University of Western Australia
Perth, Australia
Burkholderia pseudomallei in the environment

Mirjam E. Kaestli, PhD
Senior Research Officer
Menzies School of Health Research
Tropical and Emerging Infectious Diseases Division
Darwin, Australia
Section Editor
Burkholderia pseudomallei in the environment
List of Contributors

**Paul S. Keim, PhD**
Professor and Director of Pathogen Genomics
MGGen, Northern Arizona University
Flagstaff, Arizona, USA;
TGen, Flagstaff, Arizona, USA
*Burkholderia pseudomallei in the environment*

**Natkunam Ketheesan, MD, PhD**
Professor of Infection and Immunity
Microbiology and Immunology
School of Veterinary and Biomedical Sciences
James Cook University
Townsville, Australia
*Chief Editor*
*Pathogenesis and development of protection*

**Prasong Khaenam, BSc, PhD**
Research Fellow
Cellular and Molecular Immunology Unit
Faculty of Associated Medical Sciences
Khon Kaen University
Khon Kaen, Thailand;
Systems Immunology Division
Benaroya Research Institute
Seattle, USA
*Laboratory diagnosis and detection*

**Gavin C.K.W. Koh, MD, MRCP, DTM&H**
Research Associate
Department of Medicine, University of Cambridge
Cambridge, UK;
Centre for Experimental and Molecular Medicine
Academic Medical Centre
Amsterdam, The Netherlands
*Clinical manifestations; Treatment*

**Ganjana Lertmemongkolchai, BSc, PhD**
Assistant Professor
Department of Clinical Immunology
Faculty of Associated Medical Sciences
Khon Kaen University
Khon Kaen, Thailand
*Laboratory diagnosis and detection; Pathogenesis and development of protection*
Avram Levy, BSc, PhD
Senior Scientist
PathWest Laboratory Medicine West
Perth, Australia
*Burkholderia pseudomallei in the environment*

Direk Limmathurotsakul, MD, MSc, PhD
Department of Tropical Hygiene
Faculty of Tropical Medicine, Mahidol University
Bangkok, Thailand;
Mahidol-Oxford Tropical Medicine Research Unit
Faculty of Tropical Medicine, Mahidol University
Bangkok, Thailand
Section Editor
*Clinical manifestations; Treatment; Burkholderia pseudomallei in the environment*

Pagakrong Lumbiganon, MD
Division of Infectious Diseases
Department of Pediatrics, Faculty of Medicine
Khon Kaen University
Khon Kaen, Thailand;
Srinagarind Hospital
Khon Kaen, Thailand
*Clinical manifestations*

Mark J. Mayo, BSc
Project Manager
Menzies School of Health Research
Darwin, Australia
*Burkholderia pseudomallei in the environment*

Jodie L. Morris, BSc, PhD
Senior Research Associate
Microbiology and Immunology
School of Veterinary and Biomedical Sciences
James Cook University
Townsville, Australia
Section Editor
*Pathogenesis and development of protection*
List of Contributors

Tannistha Nandi, BSc, PhD
Research Associate
Genome Institute of Singapore
Biopolis Street, Singapore
Microbiology

Sheila Nathan, BSc, DPhil
Professor
School of Biosciences and Biotechnology
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
Selangor D. E. Malaysia;
Malaysia Genome Institute
Selangor, Malaysia
Microbiology

Michelle Nelson, PhD
Principal Scientist
Defence Science and Technology Laboratory
Porton Down, Salisbury
Wiltshire, UK
Pathogenesis and development of protection

Robert E. Norton, MBBCh, FRCP A, MD
Director of Microbiology and Pathology
Townsville Hospital
Townsville, Australia;
School of Medicine and Dentistry
James Cook University
Townsville, Australia
Section Editor
Laboratory diagnosis and detection

Sharon J. Peacock, FRCP FRCPath PhD
Professor
Department of Medicine
Cambridge University, Addenbrooke’s Hospital
Cambridge, United Kingdom;
Department of Microbiology and Immunology
Faculty of Tropical Medicine
Mahidol University
Bangkok, Thailand
Section Editor
Clinical manifestations; Treatment; Burkholderia pseudomallei in the environment
Melioidosis – A Century of Observation and Research

Ian R. Peak, BA, PhD
Senior Lecturer
Institute for Glycomics
Gold Coast Campus
Griffith University
Gold Coast, Australia
Microbiology

Talima R. Pearson, PhD
Assistant Research Professor
Northern Arizona University
Flagstaff, USA
Burkholderia pseudomallei in the environment

Erin P. Price, BAppSci, PhD
Post-doctoral Research Fellow
Centre for Microbial Genetics and Genomics
Northern Arizona University
Flagstaff, USA
Burkholderia pseudomallei in the environment

Savithiri D. Puthucheary, MBBS, MHPed, FRCPath
Emeritus Professor
Faculty of Medicine
University of Malaya
Kuala Lumpur, Malaysia
Section Editor
Historical overview; Epidemiology

Deborah M. Ramsey, PhD
Research Technologist
Institute for Cellular Therapeutics
University of Louisville
Louisville, USA
Other Burkholderia species

Stuart C. Ramsay, MBBS, MD, FRACP
Queensland X-Ray PET/CT Centre
Mater Medical Centre
Brisbane, Australia
Laboratory diagnosis and detection
Catherine M. Rush, BSc, PhD  
Senior Lecturer  
Microbiology and Immunology  
School of Veterinary and Biomedical Sciences  
James Cook University  
Townsville, Australia  
Section Editor  
_Melioidosis in animals_

Mitali Sarkar-Tyson, PhD  
Principle Scientist  
Defence Science and Technology Laboratory  
Porton Down, Salisbury  
Wiltshire, UK  
_Pathogenesis and development of protection_

Herbert P. Schweizer, BS, MS, PhD  
Professor  
Department of Microbiology, Immunology and Pathology  
Colorado State University  
Fort Collins, USA  
_Treatment_

Stitaya Sirisinha, DMD, MS, PhD  
Emeritus Professor  
Department of Microbiology, Faculty of Science  
Mahidol University  
Bangkok, Thailand  
_Epidemiology; Pathogenesis and development of protection_

Suppiah P. Sivalingam, MSc, PhD  
Scientist  
Defence Medical and Environmental Institute  
DSO National Laboratories  
Singapore  
_Microbiology; Pathogenesis and development of protection_

Ivo Steinmetz, MD, DTM&H  
Professor  
Friedrich Loeffler Institute of Medical Microbiology  
Ernst Moritz Arndt University Greifswald  
Greifswald, Germany  
_Pathogenesis and development of protection_

Melioidosis - A Century of Observation and Research

Yupin Supputamongkol, MD
Professor
Siriraj Hospital, Mahidol University
Bangkok, Thailand
Clinical manifestations

Gladys Tan, PhD
Principal Research Scientist
Defence Medical and Environmental Research Institute
DSO National Laboratories
Singapore
Microbiology

Patrick Tan, MD, PhD
Group Leader
Genome Institute of Singapore
Singapore:
Associate Professor
Duke-NUS Graduate Medical School
Singapore
Microbiology

Annette Thomas, BSc, PhD
Principal Microbiologist
Biosecurity Queensland Tropical and Aquatic Animal Health Laboratory
Department of Primary Industries and Fisheries
Townsville, Australia
Melioidosis in animals

Richard W. Titball, BSc, PhD, DSc
Professor of Molecular Microbiology and Director of Research
University of Exeter
Exeter, UK
Pathogenesis and development of protection

Apichai Tuanyok, PhD
Assistant Research Professor
Department of Biological Sciences
Northern Arizona University
Flagstaff, USA
Microbiology, Burkholderia pseudomallei in the environment
Glen C. Ulett, BSc, PhD  
Senior Lecturer  
Centre for Medicine and Oral Health  
Griffith University, Gold Coast Campus  
Southport, Australia  
Pathogenesis and development of protection

David M. Wagner, BA, MS, PhD  
Associate Professor  
Department of Biological Sciences and Centre for Microbial Genetics and Genomics  
Northern Arizona University  
Flagstaff, USA  
Section Editor  
Burkholderia pseudomallei in the environment

Amanda L. Walsh, MSc, FIBMS  
Senior Scientist  
Health Protection Agency  
London, United Kingdom  
Laboratory diagnosis and detection

Jeffrey M. Warner, PhD  
Associate Professor  
Microbiology and Immunology  
School of Veterinary and Biomedical Sciences  
James Cook University  
Townsville, Australia  
Burkholderia pseudomallei in the environment

Tassili A.F. Weehuizen, MSc, MD  
Graduate Researcher  
Academic Medical Center  
Amsterdam, The Netherlands  
Pathogenesis and development of protection

T. Eoin West, MD, MPH  
Assistant Professor of Medicine  
Division of Pulmonary and Critical Care Medicine  
Department of Medicine, Harborview Medical Center  
University of Washington  
Seattle, USA  
Treatment; Pathogenesis and development of protection
W. Joost Wiersinga, MD, PhD
Physician
Academic Medical Centre, University of Amsterdam
Amsterdam, The Netherlands
Section Editor
Clinical manifestation; Pathogenesis and development of protection

Surasakdi Wongratanacheewin, PhD
Associate Professor
Department of Microbiology
Faculty of Medicine, Khon Kaen University
Khon Kaen, Thailand;
Melioidosis Research Centre
Khon Kaen, Thailand
Epidemiology

Donald E. Woods, BS, MS, PhD
Professor Emeritus
Department of Microbiology and Infectious Diseases
University of Calgary
Calgary, Canada
Pathogenesis and development of protection

Vanaporn Wuthiekanun, BS
Senior Microbiologist
Mahidol-Oxford Tropical Medicine Research Unit
Faculty of Tropical Medicine
Mahidol University
Bangkok, Thailand
Section Editor
Laboratory diagnosis and detection; Burkholderia pseudomallei in the environment
I
Historical Overview
Section I. Historical Overview

Editorial overview

David A.B. Dance

Regional Microbiologist (South West), Health Protection Agency, Devon, United Kingdom

It is fitting that a book like this, published to mark the centenary of the ‘discovery’ of melioidosis, starts with a historical overview. This enables the considerable advances in our knowledge of the disease and its causative organism, *Burkholderia pseudomallei*, to be seen in context, and also highlights some of the areas in which our knowledge is still sadly lacking.

The Section starts with a reproduction of the original description of the disease by Whitmore and Krishnaswami, published in the *Indian Medical Gazette* in July 1912. Anyone who is sufficiently interested in melioidosis to have picked up this book would be well advised to read this paper, and also to seek out the longer review published in the *Journal of Hygiene* the following year, if they have not done so before. Not only is the manuscript beautifully written and extremely readable, but it is also somewhat humbling to read the meticulous work undertaken by Whitmore and Krishnaswami in what must have been extremely difficult circumstances. Their names deserve to be better known in the annals of medical history.

The next Section details the milestones in the history of melioidosis. This has been divided into a series of distinct periods. Initially, we see the gradual recognition of the disease by British, French and Dutch doctors and scientists working in the various colonies of these countries around the globe. Then the disease is recognised in Australia, which, as it now appears, is where it probably originated. During the conflicts in Southeast Asia, it assumed prominence as a military medical problem, gaining the nickname of ‘Vietnam Time Bomb’, and for the first time attracting attention as a potential bioweapon. In the 1980s, the importance of melioidosis as a public health problem among the indigenous inhabitants of Southeast Asia and northern Australia began to emerge, especially in Thailand. In more recent years, the concurrent availability of modern molecular biological tools and an influx of funding associated with concerns about the potential use of *B. pseudomallei* by terrorists, has seen a dramatic surge of interest in, and understanding of, the basic biology of the organism. This is yet to be matched by our knowledge of the worldwide distribution, epidemiology and ecology of the disease.

Not surprisingly, these latter periods have spawned increasing numbers of research groups and publications on melioidosis, *B. pseudomallei* and *B. mallei*, which needed a focus for networking and discussion. The last Section (I.3) describes the evolution of
international meetings devoted exclusively to melioidosis and glanders. These started with an International Symposium in Kuala Lumpur in 1994, catalysed by Professor Savithiri Puthucheary, and every three years or so since then the growing scientific community working in the field has gathered to present and discuss their work and plan future collaborations. This is still a relatively small group of enthusiasts, but the quality and quantity of the science increases year on year, and the common bond shared by those who are working on these fascinating diseases is probably all the stronger as a result.
Section I.1

An account of the discovery of a hitherto undescribed infective disease occurring among the population of Rangoon

A. Whitmore, C.S. Krishnaswami
disease somewhat resembling but really easily distinguishable from glanders prevalent among the ill-nourished, neglected, wasters of the town. In April, 1912, we had occasion to perform a post mortem upon the body of a Burman aged 40 years; he had been admitted to hospital for fever of seven days' duration after a three-days' stay in hospital. During these three days his temperature had been high, ranging from 103° to 104° F. His thighs bore numerous marks of morphia injections and in connection with these injections were several subcutaneous abscesses. At the post mortem examination the principal lesion discovered was a peculiar cheesy consolidation of the lungs. The distribution and appearance of this consolidation were those of neither ordinary lobar pneumonia nor tubercular infection, and upon examining smears from the diseased patches a large number of non-Gram-staining bacilli of the size and shape of B. mallei, apparently unaccompanied by other microorganisms, were found to be present. As our minds were at that time intent upon the detection of glanders, it was not strange that we made a preliminary diagnosis of that infection in this case. We notified the M. O. H. of our suspicion, and he replied that a glanders infection would appear to be unlikely, as so far as he could trace the man, he had had no close contact with horses and had only quite recently been released from jail. In the meantime we had made cultures from the diseased lung. These cultures upon ordinary agar gave luxuriant growths; upon examining these growths after three days' incubation we found that they consisted of pure cultures of what appeared to be non-motile bacilli of the size and shape of those we had found in the lung smears. We were rather puzzled by the rapidity and luxuriance of the growth, otherwise we were quite satisfied that the bacilli would turn out to be B. mallei, and it was without my misgivings as to the results that we passed on to carry out the cultural and inoculation tests for the differentiation of this bacillus. An inoculation of a potato slope gave after 24 hours a light yellow growth, which although rather rapid and luxuriant for B. mallei was otherwise not unlike the growth to be expected from an inoculation with this bacillus. Twenty minutes of a 24 hours' broth culture were injected intraperitoneally into a male guinea-pig. The guinea-pig died within 36 hours, and to our disappointment no enlargement of the testicles was observed; the post mortem examination disclosed no signs of very acute peritonitis, although a small amount of free fluid was present, the omentum was rolled up and along the rolled up matted omentum were deposits of acute inflammatory lymph; there was also acute perihepatitis, but the spleen appeared normal. Smears from the general peritoneal cavity showed a few bacilli; in the matted omentum these were very numerous, in the liver smears there were a fair number, while in spleen smears only one or two could be seen.

From the omentum, liver and spleen pure growths of a very active motile bacillus were obtained. We were under the impression that the bacilli obtained from the diseased lung was non-motile, and therefore we were at first of opinion that the bacilli isolated from the guinea-pig was not that which had been inoculated but that during injection we had caused some injury to the intestinal tract and so set up a peritonitis with infection from the animal's own intestinal contents. We would not think that have abandoned this experiment as a failure had it not been that during the past few years we have seen much of the effects of an acutely fatal peritonitis the result of bowel injury. In considering the signs of peritonitis presented by the peritoneal cavity of this guinea-pig we felt satisfied with the simple view that they were due to a cell infection the result of bowel injury, and adopted an alternative view, namely, that the bacilli isolated from the human lung might lose its motility after a few days' cultivation upon artificial media and that we had been dealing from the first with a motile organism. This view proved to be correct, for upon examining the cultures from the guinea-pig a few days later it was found that their very active motility was now almost completely lost, and also that in young subcultures from our original lung cultures the bacilli were again actively motile. It now seemed possible that the bacillus which had caused the death of the guinea-pig and those which had been isolated from the diseased lung were of one and the same species, and if so it was plain that we were not dealing with B. mallei but with some other organism with which we were up to that time unacquainted, and that therefore if, as seemed very probable, the disease in the lung was the result of infection with this bacillus, it was a disease hitherto undescribed. The problem before us had changed entirely. Hitherto we had accepted the lung condition as that not, unusual in glanders infection and had been engaged in the simple task of substantiating such hypothesis by bacteriological investigation upon well-known lines. Now, however, we had to abandon all guides and set to work to elucidate not only the distinctive characters of an unknown bacillus, but also the symptoms and lesions set up by its infection of the human subject and the incidence and method of such infection.
Melioidosis − A Century of Observation and Research

actually returned the case as almost certainly glanders, but upon bacteriological investigation we had failed to confirm the provisional diagnosis: our bacteriological failure had been attributed to the fact that decomposition of the body was moderately far advanced, the motile bacilli isolated being classed as a post mortem contamination. In two cases, however, we had persevered with the bacteriological searches for some considerable time and had carefully noted our findings, so that by the help of this previous work we were in a position to approach any fresh case with a bacteriological knowledge fairly advanced. We had not long to wait before a second case presenting these curious lung lesions was met with in the mortuary. A Burman aged 30 years was admitted to hospital in a moribund condition with a history of fever of about a month's duration, and of dysentery during the last week: he died after less than 24 hours' stay in hospital.

P. M. notes:—An encysted body with numerous marks of morphia injections.

Lungs.—The left lung contained throughout both lobes numerous patches of the peculiar and characteristic consolidation, while in the right lung were a fair number of patches in the upper lobe with a few in the lower lobe.

The spleen was twice the normal size. The large bowel was extensively ulcerated, the ulcers being of the usual amebic dysentery type.

Other organs normal.

Cultures from both lungs and spleen gave luxuriant growths in pure culture of the bacilli under investigation. Animal inoculations with the bacilli isolated from these two cases were again undertaken. Male guinea-pigs were inoculated both intraperitoneally and subcutaneously with young broth cultures and similar results were obtained with both strains of the bacilli. The guinea-pigs inoculated subcutaneously died, or rather were moribund within 48 hours; in both there were enlargement and inflammation of the testicles due, as shown by P. M. examination, to an infection of the tunics vasculosa: the peritoneal cavities contained a very small amount of free fluid, in which a few bacilli were present, but there was no general purulent inflammation, though in each case the omentum was rolled up and matted by a white deposit of inflammatory lymph; acute perihepatitis was present, and the spleen was enlarged and presented small white points of inflammatory deposit; along the liver tract of inoculation was evidence of acute inflammation in the shape of a sort of caseous deposit with a hemorrhagic surrounding; the lungs were normal. In the case of subcutaneous inoculations the tissues around the site of inoculations became widely inflamed and distended within 18 hours, and the guinea-pigs were moribund upon the fifth day. Post mortem examinations showed that the most important observations were the extensive matted of the tissues around the site of inoculations, the matting being due to caseous inflammatory material, very little fluid pus being present. The spleens in both cases were enlarged and contained numerous very small white deposits. Pure cultures of the bacilli were obtained from all the diseased organs and tissues.

By the results of these animal inoculations and by cultural tests which will be shortly described, we were convinced that we had to deal with a species of bacillus which was not described in the ordinary books of reference, and with a disease of which no description had yet found its way into the text books of tropical or other medicine known to us. At first we thought that the disease was an infectious disease in which the lesions were limited to the lungs, but the occurrence of the following case enlarged our field of view:

A dead body of a Hindoo male, aged about 32 years, was picked up in the street and brought to hospital for post mortem examination. The body was poorly nourished, but there were no obvious signs of the foot, but there were no marks of morphia injection.

Lungs.—No obvious disease present in either lung.

Spleen.—Soft and friable and about 1 g normal in size.

Liver.—One or two very minute abscesses present, otherwise normal.

The case was signed up as death due to fever the result of some septic infection, and cultures from the spleen were made. These cultures gave a pure growth of the species of bacilli which we had isolated from the previous lung disease cases.

This case proved that the infection which we were investigating could occur apart from morphia injection, and that it was probably a septicemic disease in which microscopic lesions occurred in various organs, possibly more frequently in the lungs than elsewhere. Subsequent investigations have fully confirmed this view.

The characters of the bacillus, by which it may be certainly and rapidly distinguished from all other pathogenic bacilli known to us, are as follows:

The bacillus is about the size and shape of B. mallei. It stains readily with all the usual stains, but is not acid-fast, and does not retain the stain when stained by Gram's method.

Stained with Leishman's stain, it shows well-marked bipolar staining, the poles being stained purple and the body blue. This bipolar staining is clearly shown by the bacilli present in smears taken from inflammatory lesions, and is particularly useful as a rapid preliminary test for the presence of the infection.

Growth upon all the usual culture media is rapid and luxuriant, and the bacillus is both mobile and anaerobic, though more luxuriant under the former condition.
The cultural characteristics upon which we rely for a diagnosis are those upon ordinary nutrient agar, in broth, upon salted agar, upon gelatin and upon glycine agar. Upon ordinary agar, the growth appears in from 8 to 10 hours as moist, translucent, slightly raised colonies. In 24 hours these colonies become dry, with the middle portion wrinkled, and the colour is brown with a tinge of pink.

In broth the growth is not very luxuriant for the first 24 hours, although it can be seen as a diffuse faint haze as early as the end of the tenth hour; after 24 hours there is a general turbidity of the broth and a pellicle begins to be formed at the surface; the pellicle gradually thickens until at the end of the fourth or fifth day it is a tough, resistant, wrinkled skin.

Upon salted agar the growth appears within 24 hours as a thin layer, rather like a thin coating of white paint, but the characteristic point about the growth upon this medium is, that if a smear preparation be made the bacilli are found to be growing in dense feathery masses composed of very long filaments.

Upon glycine agar the growth in its early stages is similar to that upon ordinary agar, but at the end of the second day the lowest third of the culture begins to acquire a wrinkled appearance, and at the end of a week the whole growth has become humped up and rugose, rather as a growth of many bacilli.

Upon gelatin at a temperature of 18° to 22° C., the growth is rather slow, but in stab cultures the appearance is very characteristic; at the end of the third day there is visible growth along the whole track of the needle, while upon the surface it has spread out as a small white disc. By the fourth or fifth day liquefaction of the gelatin becomes apparent. This liquefaction occurs just beneath the white surface disc and forms here a small cup of liquefied gelatin, so that by the end of the first week or a little later the culture shows upon the top a cup of liquefied gelatin covered by a thick, wrinkled, pellicle, while along the rest of the stab is a white line of growth with extremely fine white dots distributed out into the surrounding clear gelatin.

Liquefaction has occurred in every case, but the actual rate of the liquefaction has varied considerably with the different strains of bacilli inoculated.

In young cultures upon all media the bacilli are actively motile, but this motility is almost entirely lost as the ages of the cultures advance. The motility is of a curious serpentine character. The results of animal inoculation have been briefly mentioned already. Tryptose-peptone has been the only medium used and inoculations have been made both intraperitoneally and subcutaneously. Both methods are useful for diagnostic purposes. In the former, we would call attention to the occurrence of a well-marked Strassmann’s reaction in the male pig, but to obtain a good reaction it is important to use only small doses of the bacilli, i.e., 2 minutes the ear-consensus broth culture is quite large enough a dose; if larger doses are used the animal is apt to die before the testicular reaction is marked.

A subcutaneous inoculation the rapid local induration is the most important feature; this induration is made by a marking of the tissues with a thick caesarean exudate, from which the bacilli can be readily isolated.

Feeding experiments have been carried out, and although for the purposes of diagnosis they are too protracted to be serviceable, yet they have proved of great value as final proof that the bacillus isolated from the lung and other lesions was the cause of the illness.

Infection with this bacillus has been demonstrated in 38 cases during the past ten months. A full description of each of these 38 cases appears to us to be beyond the scope of a short paper such as this; but we would note that 30 of these 38 subjects bore marks of morphia injections (though in two these marks were few and old); 24 of the cases were either dead or moribund when brought to hospital; and in 12 there were serious dysenteric lesions in addition to this bacillary infection. The bacillus has been isolated from the lungs, spleen, kidneys, heart’s blood, and urine after death, while in one case we isolated it from the peripheral blood during the patient’s lifetime.

In the majority of cases the microscopic lesions, if present, are characteristic and readily recognized after an experience of one or two cases. In the lungs the typical lesion is a patch of consolidation of about the size of a hazelnut, the central area of the consolidation is pale and generally soft and cheesy;—although not so soft as tubercular caseation,—while the outer zone hemorrhagic; such patches are distributed irregularly throughout the lungs and appear to have no special predilection for any particular lobe or part of a lobe, upon the cut surface of an infected lung they stand out above the level of the surrounding normal lung, and they are usually rather dry, though in a few cases there have been very small areas where the infiltrated tissues has broken down and minute abscesses have been formed; in two cases this separation had gone on still further to the formation of quite large cavities, but cavity formation is apparently rare. The acute patches of consolidation may coalesce so as to form very large areas of consolidation extending over many square inches. In such cases the individual, small, acute areas are frequently marked out by their hemorrhagic borders, but in other cases the whole of the extensive consolidation presents a uniform white cheesy appearance; it seems to us probable that these extensive areas of cheesy consolidation mean that the disease has run an acute or suddenly chronic course and we have provisionally classed them in our records as chronic cases;
and the other cases as acute. In favour of such a view is the fact that the bacilli are often scarce in the extensive cheesy areas, while in the small areas with well-marked homorrhagic zones the bacilli are extremely numerous. This lung consolidation in the "chronic" form could be easily mistaken for consolidation due to tubercular infection, but with slight experience it is not difficult to distinguish in the vast majority of cases between the two infections. The areas of acute consolidation seem to go so closely with the descriptions given of the lung lesions due to a glanders infection in man, and for such we at first mistook them.

Although the lungs are the most frequent sites of the lesions, yet microscopic lesions do occur in other organs. We have found lesions in the liver, spleen, and kidneys, and the bacilli was isolated once from a subcutaneous abscess.

The lesions in the liver and kidneys resemble in their broad features those occurring in the lung, that is to say, they consist of areas of a cheesy infiltration with a well-marked homorrhagic zone, and upon cutting the organ the diseased areas stand up a little above the surrounding normal surface. In one case both kidneys were practically destroyed by conflating areas of this inflammatory infiltration, but as a rule the areas of disease in these organs have been small and isolated. In the spleen the usual lesions were very minute abscesses; pus formation apparently taking place in this organ more readily than in the liver or kidney.

Up to this present knowledge of this disease has been drawn almost entirely from material obtained in the mortuary, and as the subject has in most instances been brought to hospital either dead or moribund, our opportunities for investigation upon the clinical side have necessarily been scanty. The only case in which the disease has been observed throughout its course is one reported to us by Captain Knapp, M.S., Superintendent of the Rangoon Central Jail. Captain Knapp has most kindly allowed us to record it in this paper:—

Patient, a Punjabi boy, aged 35 years, was admitted to jail as a prisoner upon a three years' sentence in September, 1910; he was in good general health, not a morphia injector nor an opium habitue, and he was passed fit for full work.

Admissions to hospital since coming to the jail:—

(1) February 10th, for a bad attack of acute colitis.
(2) February 11th, for acute conjunctivitis.
(3) February 18th, for a bad attack of acute dysentery in February.
(4) For slight fever on the 10th of June.
Nothing of note in the personal or family history.

Admitted to hospital over the first illness upon 28th of June, complaining of fever.

Condition upon admission.—Temperature 101° F., pulse 80, respiration 20, face flushed in the left cheek, and pleuritic pain on the left side complained of.

June 28th.—A single material parasite found in the blood.

July 3rd.—Spitum examined but tubercle bacilli not found.

July 3rd.—A swelling formed over the thyroid cartilage: on the 8th this swelling was incised and a little pus found. Reaction rules were now audible over the bases of both lungs.

On the 12th a swelling, which was incised on the 11th, formed over the right clavicle. On the 18th a superficial abscess was found over the right trochanter and incised. On the 20th the patient began to be markedly dejected, and dizziness was detected at the base of the right lung. On the 22nd there were signs of consolidation at the left base. On the 25th a swelling appeared over the left malleolus; incised on the 1st of August. The sputum was again examined, but neither pneumococci nor tubercle bacilli were found.

The patient died uneventfully on the 7th of August.

Post-mortem Examination.—All the abscesses had been superficial: there was a small acute abscess in the left side of the chest, no pus from this abscess and stained with methylene blue showed a few bacilli.

With the exception of the lungs, the other organs of the body were healthy. Both lungs were the seat of an extensive but patchy consolidation; but no pathological appearance so characteristic of this infection. The smaller patches were surrounded, as is usual, by hyperemic zones. From the lungs the bacilli were readily isolated in pure culture, and from the diseased patches showing bacilli present in large numbers.

The formation of the pulmonary abscesses made the resemblance to glanders exceedingly close, and as a matter of fact such diagnosis was suggested by Captain Knapp and negatived upon the grounds that there seemed to be no possible source of such infection in the jail.

The nature of the bacilli isolated from this case were of course determined by full bacteriological and animal inoculation tests.

This case alone is quite sufficient to show that this infection can attack an apparently healthy and vigorous man, but the majority of our mortuary cases have been ill-nourished emaciated men, among whom morphia injectors have so preponderated that we have unavoidably come to associate the infection with such a habit. It is probable that this assumption has blinded us to the fact of the clinical recognition of the infection in patients who are still inhabitants of the hospital wards; it must be remembered that we derive our mortuary experience chiefly from the ill-nourished, neglected, wretched who have neither relatives nor friends to bear the cost of their burial, and therefore such experience is but illadapted to afford reliable evidence as to the incidence of any disease.

In three cases suspicion of the infection has been entertained during the lives of the patients: in two of these cases the disease was so far advanced that they died within a very few hours of their admission to hospital, and there was no time for bacteriological examination; in the third case, a Mohammedan man, aged 52, was admitted to hospital for cellulitis of the scrotum. This was treated in the ordinary way, but the patient did not improve, and when seen by Captain Trumpp, three days after admission to hospital, the man's high fever and rapid breathing suggested to this officer a general septic infection of some sort, and, as the man had numerous marks of morphia injections, he reported the case to us as being possibly a case of the infection which we were investigating. A specimen of the man's sputum
was obtained, but upon examination yielded no evidence of the infection. A specimen of peripheral blood was withdrawn from an elbow vein; this small operation was carried out in the middle of the night, and as the patient was very delirious and no assistance could be readily obtained, it was not surprising that the tubes were contaminated and that we failed to isolate the bacillus from plate cultures. However, a few minutes of the citrated blood were inoculated beneath the skin of a guinea-pig; upon the third day after inoculation the pig was ill and there was considerable induration around the place of inoculation. In a smear from the造成的 material from this inoculated area the bacillus of typical appearance were numerous and pure cultures of the bacillus were readily obtained. The patient died, and though it was not possible to have a complete post mortem examination, yet we were able to examine the lungs, the liver, and the spleen. The lungs proved to be free from disease, but both liver and spleen contained typical lesions from which the bacillus was grown in pure culture. Unfortunately no culture was taken from the inflamed serosities, but there would seem to be no doubt, but that this case was a typical example of the infection, and it affords good proof that at any rate shortly before death, the infection is a systemic one, and can be diagnosed by a bacteriological examination of the blood during life.

The isolation upon 30 occasions of bacillus with constant and distinctive characters from pathological lesions, many of which were in themselves so peculiar in appearance as to suggest doubt as to their causation by any of the usual pathogenic bacteria, and the fact that in the majority of the lesions these particular organisms alone were present would be sufficient foundation for the hypothesis that we have been dealing with a particular and new infective disease. We think that it will be agreed that this disease and not hitherto been described, may rest solely upon our ignorance of the scientific literature, an ignorance which it is extremely difficult to avoid under the conditions of pathological work at present not uncommon in the East.

To sum up—
Our experience during the year seems to us to warrant the following conclusions—

That there is prevalent in Rangoon a peculiar septicemic or pyemic disease caused by an infection with a bacillus whose characters are so distinct from other known pathogenic bacteria that it can be readily isolated and certainly identified.

That the disease so far as present evidence goes is peculiarly prevalent among chronic morphia injectors.

That our clinical knowledge is at present so meagre that only a bare suspicion of such infection can be excuted by clinical signs, but that our bacteriological knowledge is sufficiently complete to allow such suspicion to be rapidly decided by the aid of the laboratory.

That in many cases the macroscopic appearance of the lesions caused by the infection are sufficiently characteristic to permit a confident diagnosis to be made immediately in the mortuary without the aid of bacteriology, though of course such aid should be sought wherever facilities for laboratory work exist.

That the disease has certain resemblances both clinically and bacteriologically to glands, but that the two infections can be readily differentiated if a proper bacteriological examination be carried out. Confusion would be due to a reliance upon the positive results of Straus's test, as a positive result with this test is common to the two infections.

There are, we fear, very many most important points which we have failed to touch upon in this paper; such omissions are in part due to the necessary limitations of space, and in part due to our still very imperfect knowledge of the disease, a knowledge particularly scanty upon the clinical aspects of the infection; but we hope that we have succeeded in bringing forward sufficient facts to stimulate the curiosity of pathologists working in other places, and that such work may very speedily be made plain, whether or not this infection is of merely local or of much wider interest.

Before concluding we would like to be allowed to record our thanks to Captain Kemp, M.B., to whose professional zeal and clinical acumen we owe the only complete account of a case of the infection.

To Mr. Blake for providing us with specimens of true glands.

To Dr. Marshall, Acting Health Officer, for his endeavours to trace the various cases reported to him.

And to Captain Crump, M.B., for the careful look-out which he has kept for us upon the admission of possible cases of the infection to the hospital wards.
Abstract

In the century since the first recognition of ‘Bacillus pseudomallei’ by Alfred Whitmore and Krishnaswami in Rangoon in 1911, many of their original observations have been confirmed. The disease was subsequently recognised by Stanton and Fletcher in the Federated Malay States, initially in laboratory animals and later in humans and wild animals, and they coined the term ‘melioidosis’. European researchers in Southeast Asia later identified the infection in the Dutch East Indies and French Indochina, where it was first shown to be an environmental saprophyte. The disease assumed military importance amongst Americans during the Vietnam War. Melioidosis was first reported in animals in Australia in 1949, but has subsequently been recognised increasingly in humans. Thailand was relatively late to identify indigenous cases, but is now the world leader for melioidosis, both in terms of incidence and research. The infection has largely remained a tropical problem with the exception of a bizarre episode in zoos and riding clubs in France in the 1970s. Research funding received a boost due to the potential for deliberate release of *Burkholderia pseudomallei*, and the publication of the organism’s genome in 2004 signalled a new and fertile era of research into the disease.

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1. The beginnings in Rangoon

This book celebrates the centenary of the discovery of melioidosis by Alfred Whitmore and C.S. Krishnaswami in Rangoon, described in the landmark paper reproduced in this monograph [1]. In this and the longer review published the following year in the *Journal of Hygiene* [2], which was selected as one of the seminal papers to mark the recent centenary of that journal, Whitmore elegantly described the pathological features of the disease and the characteristics of its causative organism. He astutely recognised the similarity of the disease to glanders, then a well recognised infection of horses, and of the new bacterium to its cause, “Bacillus mallei”. Recent taxonomic studies have confirmed this, demonstrating that *Burkholderia mallei*, as it is now known, is actually derived from a clone of Whitmore’s bacillus, *Burkholderia pseudomallei*, which has lost substantial amounts of its genetic material in the process of becoming adapted as an equine pathogen [3].
Born on 16th May 1876, Whitmore was educated at St. Bees’ school in Cumbria, Cambridge University and St. Mary’s Hospital, qualifying MB in 1902 and joining the Indian Medical Service the following year (Figure I.1). Shortly afterwards he was posted to Burma, where he established a pathology department in Rangoon General Hospital. Working with his assistant Krishnaswamy, in what must have been difficult conditions with rudimentary facilities (Figure I.2), the description of a completely new disease within such a short space of time was no mean achievement. It is difficult not to be impressed by the thoroughness of Whitmore’s work, or his beautifully descriptive prose. It has been suggested that Whitmore’s disease may have been the inspiration for Sir Arthur Conan Doyle’s description of ‘Tapanuli fever’ in ‘The Adventure of the Dying Detective’, first
published in 1913 [4,5]. The timing would certainly fit, particularly if Doyle had been present when Whitmore presented his findings at the 80th meeting of the British Medical Association in Liverpool in July 1912 [6], but unfortunately the attendance list has not survived.

This Section documents some of the major milestones in the history of melioidosis over the past century, but readers are referred to other publications for more detailed descriptions [7–10].

2. Colonial days

The first phase of the history of melioidosis was characterised by increasing recognition of the disease by doctors posted to Southeast Asia by the major European colonial powers. It was first recognised outside Burma in Malaysia, when William Fletcher (perhaps better known for the eponymous medium that is still used for the culture of leptospira) witnessed an epizootic amongst his laboratory animals in the Institute for Medical Research, Kuala Lumpur in 1913 (Figure I.3), although the relationship to Whitmore’s discovery was not appreciated until several years later. Working with a Canadian, A.T. Stanton (later Sir Thomas Stanton, Chief Medical Adviser to the Secretary of State for the Colonies), who was perhaps better known for his groundbreaking work on beri-beri, Fletcher went on to describe cases in humans and a wide range of animals in the Federated Malay States, which they described in a series of papers and a comprehensive monograph published in 1932 [11]. It was Stanton and Fletcher who coined the name ‘melioidosis’ to describe Whitmore’s disease in 1921, derived from the Greek work μηλίζ (meaning a distemper of asses) and ειδος (meaning shape or appearance) [12].

During the ensuing years, cases were described by the British in Ceylon (1927), the French in Indo-China (1925) and Madagascar (1936), and the Dutch in the Dutch East Indies (1929) and later the Caribbean (1957) [8].

Stanton and Fletcher considered that melioidosis was probably a zoonosis with a reservoir in rodents, and this view persisted for many years [7], but a number of observations by French workers in the Instituts Pasteur in Saigon and Hanoi, Vietnam, pointed to another possibility. They recognised that:
1. Cases of melioidosis often followed trauma with exposure to mud or aspiration of surface water.

2. The causative organism was almost never grown from rats during searches for plague bacilli. This led to a hunt for \textit{B. pseudomallei} in the environment, which was eventually successful when the organism was grown from guinea pigs whose scarified abdomens had been exposed to muddy pond water [13] and ultimately when it was isolated from environmental samples in artificial media by Chambon [14].

3. **Isolation of the bacterium in Australia**

   Initially it appeared that Whitmore’s disease was largely confined to Asia. Then, in 1949, it was discovered amongst sheep and goats in northern Australia by Cottew [15]. It was not long before human cases were described [16]. This led to debate as to how and when the organism had reached this new environment. Fournier maintained that it had probably been introduced by men and animals returning from war in the Pacific [17]. Recent phylogeographic molecular studies, however, have suggested that \textit{B. pseudomallei} is most likely to have evolved originally in Australia, transferring to Southeast Asia during a relatively recent glacial period [18,19]. The disease in Australia has remained an important veterinary problem, with human disease seen most frequently in the tropical regions of Australia including among the Aboriginal communities [10].

4. **The ‘Vietnam time bomb’**

   As conflict escalated in Indochina, melioidosis drew increasing attention as a military medical problem, first amongst French and subsequently American soldiers fighting in Vietnam (Figure I.4). This resulted from the exposure of large numbers of servicemen to the soil and surface water that harboured \textit{B. pseudomallei}, with battle wounds increasing the likelihood of infection. Helicopter crews also experienced a disproportionate share of infections, leading to the suggestion that this resulted from the inhalation of infectious dust and aerosols whipped up by the rotors [20].

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Fig. I.4. Left: Institut Pasteur Saigon. Right: Institut Pasteur, Hanoi.
The other fascinating aspect of melioidosis that became apparent during the Vietnam War was its potential for long periods of latency, the longest such period reported to date being 62 years [21]. American servicemen, exposed to the organism during a well defined period in an endemic area, would occasionally develop melioidosis long after returning to their native, non-endemic country. This gave rise to the nickname ‘Vietnam Time Bomb’, once even meriting mention in ‘Time’ magazine [22]. Even now this phenomenon, highly unusual amongst bacterial infections, is poorly understood.

5. Emergence of Thailand

Thailand had not featured amongst the early descriptions of melioidosis, perhaps partly because it was never a colony of any European power, although occasional cases which were probably acquired in Thailand were reported in adjacent countries [8,23]. The first case in a Thai was reported in 1955 by Chittvej et al. [24]. It was not until the 1980s, however, when interest and awareness in the disease was stimulated by Sompone Punyagupta and his colleagues in the Infectious Disease Association of Thailand, that the true picture began to emerge. As the number of indigenous cases recognised in Thailand grew, the Association organised a landmark conference in 1985 [25]. This meeting inspired collaboration between the Wellcome–Mahido–Oxford Tropical Medicine Research Programme and Dr Wipada Chaowagul and her colleagues in Sappasitprasong Hospital, Ubon Ratchathani. This highly productive programme, running continuously from 1986 to the present day, along with the work of many Thai clinicians and scientists from Khon Kaen and elsewhere, has helped to establish Thailand as a world leader in clinical and epidemiological research on melioidosis.

One of the first milestones resulting from these studies was the demonstration that the mortality of melioidosis could be dramatically reduced by some of the newer antibiotics, first demonstrated with ceftazidime in 1989 [26]. In fact, as a result of numerous prospective clinical trials conducted in Thailand, the evidence base for the treatment of melioidosis is now amongst the strongest for any bacterial infection.

Perhaps one of the less celebrated events in melioidosis history was the recognition in 1990, during studies of the isolation of B. pseudomallei from the environment in northeast Thailand, of strains of ‘arabinose-positive B. pseudomallei’ by Vanaporn Wuthiekanun. Eventually assigned to a new species [27], the avirulent B. thailandensis, as it is now known, has become a valuable laboratory tool, particularly in studies of B. pseudomallei pathogenesis.

6. L’affaire du Jardin des Plantes

The majority of reports of melioidosis have originated from tropical or sub-tropical countries. The most notable exception to this occurred in France during the 1970s and became known subsequently as ‘L’affaire du Jardin des Plantes’ [28]. Initially recognised as the cause of death in a Przewalski’s horse from the zoo at the Jardin des Plantes in Paris, B. pseudomallei was subsequently found in a number of other animals in zoos in the Paris area, and ultimately appears to have spread amongst horses in riding stables and
equestrian clubs across France, associated with extensive environmental contamination and even, anecdotally, a handful of human cases of melioidosis [28]. It has been suggested that the organism may have been introduced by a panda, presented by Mao Tse Tung to President Pompidou in 1973, although why it should have spread and persisted in an area so different from its normal environment is unclear. Eventually the outbreak burnt itself out, and we will probably never know the full truth about this bizarre and atypical episode in the history of melioidosis.

7. Biothreat

Both America and Russia had been interested in the potential for the development of \( B. \textit{pseudomallei} \) as a biological weapon [29]. Although it was never actually weaponised, its close relative, \( B. \textit{mallei} \), had been used by Germany against animals in neutral countries during the First World War and by the notorious Japanese Unit 731 in human experiments [29]. Ironically, probably one of the biggest ever boosts to global melioidosis research came when the US Centers for Disease Control classified \( B. \textit{pseudomallei} \) as a ‘category B’ select agent [30]. This has led to a huge growth of American interest in the organism and disease, due to the dramatic surge in the funding available for melioidosis research. This has also consequently led to an increase in the number of publications in the field.

8. From gharry ponies to genomes

A major milestone in the history of melioidosis was the publication of the genome of \( B. \textit{pseudomallei} \) in 2004, coinciding with the IV\textsuperscript{th} World Melioidosis Congress in Singapore [4]. This has given researchers almost endless opportunities for \textit{in silico} research and is helping to unravel aspects of evolution, pathogenesis, ecology and basic biology of this fascinating organism.

Over the past 20 years, new foci of melioidosis have continued to be identified around the world [10,31]. However, despite the extraordinary advances in our understanding of \( B. \textit{pseudomallei} \), in many places the disease it causes remains unrecognised, or has even been forgotten. Krishnaswamy reported in 1917 that about 1 in 20 post mortems in Rangoon General Hospital was a case of melioidosis [32]. Yet when Professor Nick White in 1998 arranged for a plaque to be erected in Rangoon General Hospital to celebrate the discovery of melioidosis, few if any Burmese doctors had heard of, let alone diagnosed, the disease. Only recently has it begun to be described again after an ‘absence’ of nearly 90 years [33].

9. Conclusion

Whitmore stayed in Rangoon until 1927, becoming one of the civil surgeons and attending the family of King Thibaw on more than one occasion, serving in the Army during the First World War, rising to the rank of Lieutenant-Colonel, and being instrumental in the establishment of the Burma Medical School. He was renowned as an original
and stimulating teacher with a keen sense of humour, described as “radiating something buoyant and joyous”, and he inspired great affection in students, patients and colleagues. When he retired from the Indian Medical Service he returned to the Pathology Department in Cambridge, where he continued to conduct research into cardiac pathology and teach right up to his death on 26th June 1946, despite undergoing major surgery in 1943. If he were able to read this book, he would be amazed at the extraordinary advances that have been made in our understanding of the genetics and pathogenesis of his bacillus. He would probably be less impressed by our failure over the past 100 years to reach a better understanding of the true burden of his disease amongst the rural poor throughout the tropics, or how to prevent it. Let us hope that the next 100 years will bring great advances in these areas.

References

Section I.3

World Melioidosis Congresses

Savithiri D. Puthucheary

Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Malaysia

Abstract

Since the original description of melioidosis in Rangoon, Burma, cases have been reported from many other countries and researchers began to look at the various aspects of the disease and the causative organism, *Burkholderia pseudomallei*. By the early 1990s, there was sufficient interest in melioidosis in the scientific and medical world and it was thought that the time was right to bring these researchers together at a forum. Thus the First International Symposium on Melioidosis, convened by the Malaysian Society of Infectious Diseases and Chemotherapy under the Chairmanship of this author, was held in Kuala Lumpur in April 1994. Eighty delegates attended the symposium and the papers presented were subsequently edited and published as a book. The success of this conference convinced others doing similar research to organise international conferences and an international movement was born. Subsequent congresses have been held in Thailand, Australia and Singapore.

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1. The beginnings

Since the original description of melioidosis in Rangoon, Burma, cases have been reported from many other countries. Both the Allied and Japanese soldiers suffered from melioidosis during World War II in Burma, Malaysia and Thailand. After World War II, sporadic reports of melioidosis appeared in the literature, with 10 cases from Malaysia being reported by Thin et al. [1].

During the 1980s, interest in the disease in Southeast Asia began to grow, stimulated particularly due to the work of the Infectious Disease Association of Thailand and the Wellcome-Mahidol University-Oxford Tropical Medicine Research Programme. It was at the First Western Pacific Congress of Infectious Diseases in Kuala Lumpur, Malaysia, in February 1989, that David Dance and the author first mooted the idea of having a special international forum or symposium devoted to melioidosis. It was felt that there was sufficient interest in melioidosis in the scientific and medical world and that the time was right to bring these researchers together at an international meeting. Five years later, in 1994 this dream finally became a reality. Since then, melioidosis researchers from
around the world have gathered every three years, and the growing size and nature of the meetings are a testament to the burgeoning interest in, and understanding of, this fascinating disease and its causative organism (Table I.1).

The first melioidosis conference was held in Malaysia. This historic event was convened by the Malaysian Society of Infectious Diseases and Chemotherapy (Figures I.5, I.6). At this meeting, the Chairperson formally paid tribute to Dr Les Ashdown and acknowledged the enormous contribution that he had made in the area of melioidosis. There were 80 participants who presented approximately 45 papers and selected papers from this symposium were subsequently edited and published as a monograph [2]. Due to the success of this conference an international movement was born!

2. The continuation of the international efforts

The II\textsuperscript{nd} International Congress was held in Bangkok in 1998 and “state of the art discoveries and trends towards the 21\textsuperscript{st} century” was the theme of this congress. There were more than 100 delegates at the congress held in Bangkok. The III\textsuperscript{rd} World Melioidosis Congress was held in late September 2001, in Perth, Australia. Due to the terrorist attacks on the Twin Towers of the World Trade Center in New York City earlier in the month there were travel restrictions and delays which impacted on the attendance at this meeting. The IV\textsuperscript{th} World Melioidosis Congress held in Singapore in 2004 coincided with the publication of the genome of \textit{B. pseudomallei}. At the V\textsuperscript{th} World Melioidosis Congress organised by the Melioidosis Research Center, in Khon Kaen University, Thailand pre-congress workshops were introduced. The two workshops held were highly successful. The VI\textsuperscript{th} World Melioidosis Congress held in Townsville, Australia commemorated the centenary since the description of melioidosis by Whitmore and Krishnaswami.

3. The future

Half a dozen world congresses! What of the future? Will they die a natural death like some other specialised meetings? There are many pros and cons. As the chairman of the first international gathering of melioidosis researchers, the author is of the view that the advantages outweigh the disadvantages for the following reasons:

- Specialised conferences bring together researchers interested in a speciality and not necessarily interested in a general conference
- There is no specific focus at a general conference with many different subjects and there is no common interest for in-depth discussion
- Networking is a very important part of research and the time factor in these special conferences allows for detailed and specific dialogue

\textit{Burkholderia pseudomallei} is a soil saprophyte in the tropical and sub-tropical regions but has spread to temperate countries by various means. Being an environmental saprophyte, it cannot be eradicated. Although we have studied this disease for more than a century now, we have no acceptable vaccine or an effective diagnostic tool. Neither has
Table I.1
Facts and figures of the melioidosis congresses 1996–2010

<table>
<thead>
<tr>
<th>Congress</th>
<th>Year</th>
<th>Location</th>
<th>Theme</th>
<th>Number of delegates</th>
<th>Presentations, oral (poster)</th>
<th>Chairman</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1994 (7–8 April)</td>
<td>Kuala Lumpur, Malaysia</td>
<td>Prevailing problems and future directions</td>
<td>80</td>
<td>45</td>
<td>Savithiri Puthucheary</td>
</tr>
<tr>
<td>III</td>
<td>2001 (26–29 Sep)</td>
<td>Perth, Australia</td>
<td></td>
<td>105</td>
<td>59 (37)</td>
<td>Tim Inglis</td>
</tr>
<tr>
<td>IV</td>
<td>2004 (16–18 Sep)</td>
<td>Singapore</td>
<td></td>
<td>180</td>
<td>56 (73)</td>
<td>May Ann Lee</td>
</tr>
<tr>
<td>V</td>
<td>2007 (21–23 Nov)</td>
<td>Khon Kaen, Thailand</td>
<td></td>
<td>350</td>
<td>58 (140)</td>
<td>Surasakdi Wongratanacheewin</td>
</tr>
<tr>
<td>VI</td>
<td>2010 (30 Nov–3 Dec)</td>
<td>Townsville, Australia</td>
<td>Celebrating a century of research</td>
<td>274</td>
<td>49 (133)</td>
<td>Natkunam Ketheesan</td>
</tr>
</tbody>
</table>
the pathogenesis of the disease been completely elucidated! Therefore it is the author’s view that the World Melioidosis Congresses should continue to be held in the future.

**References**

II
Epidemiology
Section II. Epidemiology

Editorial overview

David A.B. Dance\textsuperscript{a}, Savithiri D. Puthucheary\textsuperscript{b}

\textsuperscript{a} Health Protection Agency, Plymouth, United Kingdom
\textsuperscript{b} Department of Medical Microbiology, University of Malaya, Malaysia

This Section deals with the epidemiology of melioidosis. Although considerable advances in this field have been made over the past twenty five years, it has to be admitted that these are not as dramatic as those made in our understanding of the basic biology of \textit{Burkholderia pseudomallei}, thanks to the extraordinary tools of molecular biology.

The Section starts with a discussion of melioidosis as an emerging disease (Section II.1). Each year brings the recognition of cases of melioidosis in places where it has not previously been recognised, or the reporting of greater numbers of cases than have previously been apparent in a particular region. However, as melioidosis is primarily a disease of the rural poor, our understanding of the true global epidemiology is bound to be incomplete. To a considerable extent, this apparent emergence is likely to be due to better ascertainment as medical care and laboratory facilities are enhanced within endemic areas. Nonetheless, there are some places where surveillance for melioidosis is relatively complete, such as parts of northeast Thailand, northern Australia and Singapore. In some of these regions, the incidence of the disease definitely appears to be increasing, although there may still be an element of ascertainment bias. One of the most likely reasons for this increase is the growing numbers of those who have underlying diseases, such as diabetes, that make them susceptible to infection with \textit{B. pseudomallei}. Another is climate change, especially where this leads to a greater risk of severe weather events such as cyclones, typhoons and tropical storms, with flooding. Outside these well known endemic areas, melioidosis is known to occur throughout southeast Asia, the Indian sub-continent and parts of Central and South America and the Caribbean, but the true incidence is unknown, and nowhere is this more so than in Africa.

Section II.2 provides greater detail about the epidemiology of melioidosis in Thailand, the country that describes the greatest number of cases worldwide (estimated at approximately 2000–3000 cases per year). The disease is especially common amongst rice farmers in the northeast of the country and is highly seasonal, with the majority of cases presenting during the rainy season, but events such as the tsunami of 2004 act as a reminder that \textit{B. pseudomallei} is present in soil throughout Thailand. The high
number of cases in Thailand has meant that the country has led the world in clinical and epidemiological research into the disease in recent years.

Section II.3 describes melioidosis in Malaysia and Singapore. After Whitmore’s original description of the disease in Burma, the greatest advances were made over the subsequent twenty years by Stanton and Fletcher in Kuala Lumpur, who coined the name ‘melioidosis’. Although melioidosis is probably still under-recognised in parts of Malaysia, in some places (such as Pahang), it is increasingly diagnosed and surveillance is more complete. It is more complete still in Singapore, which is a very different environment from rural northeast Thailand, because melioidosis is statutorily notifiable and the level of access to high quality healthcare and diagnostic facilities is universally high. Melioidosis in Singapore has particularly been associated with construction work, and a number of cases have also occurred in military recruits on training exercises.

This Section concludes with Section II.4 describing the epidemiology of melioidosis in Australia and the Pacific. Although melioidosis was not actually described in Australia until 1949, recent phylo-geographic studies have suggested that *B. pseudomallei* probably originated in Australia, spreading from there to southeast Asia and elsewhere. As in other countries, melioidosis in Australia is very much a rainy season disease, but case-clusters have been described in association with floods and cyclones and also with water supplies. Evidence from Australia has suggested that severe weather events may favour inhalation rather than inoculation as a route of infection with *B. pseudomallei*. The disease is most commonly found in tropical areas of Queensland and the Northern Territory, but local foci are found in temperate areas. The disease has also been reported in Papua New Guinea, Guam, New Caledonia and Fiji, and probably exists elsewhere in the Pacific.
Melioidosis represents an excellent example of an emerging disease in two respects: it is being reported increasingly in many countries; and it is being recognised for the first time in countries where it has not previously been described. However, the reported epidemiology reflects a complex interaction between genuine emergence and increasing recognition, as familiarity with the disease and its causative agent increases amongst clinicians and microbiologists respectively, and laboratory facilities and techniques improve. Genuine increases may relate to environmental, climatic or behavioural changes, or to increasing numbers of those predisposed to the infection as treatment of underlying conditions such as diabetes mellitus improves. The contrast between Thailand, where reported cases of melioidosis increase annually, and neighbouring Burma, where the disease was first reported 100 years ago but where it is now seldom if ever recognised, exemplifies the influence of factors other than true incidence on the perceptions of emergence. In recent years melioidosis has been increasingly recognised in the Indian sub-continent, China and Taiwan, several islands in the Pacific and Indian Oceans, and South America and the Caribbean. However, the great unknown remains the extent of melioidosis in Africa.

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1. Emerging diseases

The World Health Organisation defines an emerging disease as one that has appeared in a population for the first time, or that may have existed previously but is rapidly increasing in incidence or geographic range [1]. Melioidosis qualifies on all counts, however the extent to which this reflects genuine emergence or ‘pseudo-emergence’ is difficult to determine. This Section will consider the evidence for the emergence of melioidosis over the past 20 years since the global epidemiology of melioidosis was comprehensively reviewed [2]. Readers may also wish to refer to previous reviews of developments during this period [3–5].

2. Emergence vs. recognition

One of the main problems in defining whether a disease is truly increasing in incidence (emerging) or just being increasingly recognised (pseudo-emerging) is the accuracy, or
rather inaccuracy, of surveillance. In the case of melioidosis, accurate diagnosis relies on the awareness of clinicians of the disease, the availability of laboratory facilities that are sufficiently well developed to confirm the diagnosis (which usually requires isolation and identification of *Burkholderia pseudomallei*), and finally familiarity of laboratory staff with the disease and its causative organism. Thereafter, recognition of melioidosis in a particular country or region depends either on the reporting of cases in the published literature or through voluntary or mandatory surveillance systems. Each of these steps may fail, and in the case of melioidosis there is evidence of this happening at each step in the chain. This is what the author considers as the surveillance iceberg (Figure II.1). Even in countries with well developed surveillance systems, much of the iceberg (i.e. the true incidence of a disease) may be unrecognised because it is below the “water”.

Problems with recognising the true incidence of melioidosis are described in the subsections that follow.

![The Surveillance Iceberg](image)

Fig. II.1. The surveillance iceberg, showing that cases of an infection reported to national surveillance systems or in the literature may represent only a small proportion of the total number of cases occurring.

2.1. Awareness of clinicians

Melioidosis is still considered a rare and esoteric disease in many countries, and consequently it does not feature highly on the syllabus of medical schools or even postgraduate training for infection specialists, let alone General Practitioners. If clinicians are not aware of the disease, they will be unable to make the diagnosis. Even if they know of melioidosis, there are few clinical manifestations of the disease that are sufficiently specific for the diagnosis to be made on clinical grounds alone, with the possible exception of acute suppurative parotitis in children [6]. Perhaps the best example of this problem is Burma (Myanmar), where melioidosis was first discovered and where, in 1917, Krishnaswami reported that approximately 5% of the autopsies he performed in Rangoon were cases of the disease [7]. Yet in the late 1990s, few Burmese doctors had ever heard
of melioidosis (White, N.J., personal communication), and only now are cases beginning to be recognised once more [8].

2.2. Availability of laboratory facilities

*Burkholderia pseudomallei* is an environmental saprophyte found in the soil and surface water in endemic areas, which are mainly in the tropics. It is hardly surprising, therefore, that the people most at risk of acquiring melioidosis are the rural poor of these areas, especially those whose occupations bring them into contact with mud and water, such as rice farmers [2, 4]. Yet this is precisely the group that is least likely to have access to sophisticated medical care, including well developed diagnostic microbiology support. In the 1960s, melioidosis was seldom if ever diagnosed in northeast Thailand [4]. However, as medical facilities, including microbiology laboratories, improved in Thailand, by the 1980s it had become apparent that melioidosis was an important public health problem in the region [2]. Although it is possible that the incidence of the disease genuinely increased during those two decades, it is quite likely that it was there all the time but just not recognised because of the absence of diagnostic capacity.

2.3. Familiarity of laboratory staff with the disease and its causative organism

Even if clinicians have access to a laboratory and send the right samples, if laboratory staff are not familiar with *B. pseudomallei*, or prompted specifically to consider the possibility of melioidosis, the likelihood of the diagnosis being made is remote. Even in the best of laboratories, the organism can be dismissed as a contaminant by the unwary, or perhaps more accurately, those who have simply never seen *B. pseudomallei* or been taught about its characteristics. Wrinkled *B. pseudomallei* colonies can look like aerobic spore bearers (*Bacillus* spp.), which are relatively common contaminants of enrichment cultures of sterile fluids like blood cultures, and thus may be dismissed. If a Gram stain and oxidase test are done, the organism may be considered as a ‘pseudomonad’ or ‘*Pseudomonas* sp.’, but these are common environmental organisms and again frequently dismissed as contaminants without any further identification. The literature is full of cases where, with hindsight, *B. pseudomallei* was probably grown from clinical samples long before the diagnosis was realised. Even modern automated systems are far from foolproof when it comes to identifying things that are relatively rare in the western world, as organisms like *B. pseudomallei* may not have been used to construct their databases [9]. Once melioidosis has been recognised as endemic in a country, the chances of cases being diagnosed increases.

2.4. Surveillance systems

There are very few endemic countries in which melioidosis or *B. pseudomallei* are statutorily notifiable, Singapore (since 1989), endemic areas of Australia (since 1991), and Taiwan (since 2000) being notable exceptions. In these regions, melioidosis surveillance is likely to be relatively complete, because the majority of the population have access to modern diagnostic facilities and reporting of cases is mandatory. Some non-endemic
countries have made melioidosis notifiable, at least partly because of its potential use as a bioterrorist agent (e.g. United Kingdom in 2010) [10]. Even where comprehensive voluntary surveillance systems exist, many cases of melioidosis may go unreported [11]. Surveillance based on the published literature is likely to be even more incomplete, and so we have a very inadequate picture of the true incidence of melioidosis in most countries. Any discussion of melioidosis as an emerging disease must thus be considered in that light.

3. Reasons for emergence of diseases

Before considering the evidence for melioidosis emergence in particular, it is worth considering the various reasons which can lead to the genuine emergence of diseases in general, and how these might apply to melioidosis. These include:

- **Microbial adaption.** Although strains of *B. pseudomallei* undoubtedly can vary in virulence, since *B. pseudomallei* is an environmental saprophyte rather than an obligate animal pathogen, and is not spread from person to person, this seems relatively unlikely to account for the emergence of melioidosis in a given region. Clinical isolates are generally very heterogeneous and there are very few reported instances of clonal outbreaks of melioidosis [4] which might suggest microbial adaptation as a possible reason for emergence.

- **Changing human susceptibility.** As yet there is no evidence that HIV predisposes significantly to melioidosis [12]. Diabetes mellitus, however, is strongly associated with the disease, and as diabetes becomes more prevalent in endemic areas and diabetics survive longer due to improvements in treatment [13], the pool of highly susceptible people is continually increasing. This may be a particularly significant factor in India, estimated to have some 35 million diabetics [14].

- **Climate and weather.** There is little or no evidence to suggest that melioidosis is vector-borne, although environmental and climatic factors are clearly important in determining its distribution. The factors that make a particular environment suitable for *B. pseudomallei* are still poorly understood, although temperature is undoubtedly important given its predominantly tropical distribution. Rainfall, both total rainfall and, perhaps more importantly, severe weather events, is also increasingly apparent as a risk factor for melioidosis [15,16]. Global warming and climate change, particularly the increasing occurrence of typhoons and similar events, are likely to increase the range of melioidosis endemcity, the overall incidence, and the number of clusters.

- **Other natural disasters.** In the wake of the Asian tsunami of 2004, cases of melioidosis occurred amongst survivors in Thailand, Indonesia and Sri Lanka [17]. Whilst this in itself did not make a major and permanent impact on the numbers of cases worldwide, it acts as a reminder that *B. pseudomallei* is undoubtedly present but probably under-diagnosed in the affected regions, and that similar events may unmask cases again in future.

- **Change in human demographics and trade.** Although melioidosis very rarely spreads from one infected human or animal to another, increasing global travel must at least bring with it an increasing risk of exposure of those from non-endemic regions to
the disease and the potential for the contamination of new environments by infected people or animals excreting *B. pseudomallei*. Several cases and outbreaks amongst animals have occurred following their importation both to and from endemic areas, the stress of shipment being suggested as a potentially associated factor [18]. Cases have also been associated with changes in human behaviour such as eco-tourism and increasing recreational activities in endemic areas (ref. [19] and Puthucheary S.D., personal communication).

- **Economic development.** In the case of melioidosis, the factors that render an environment favourable for the survival and proliferation of *B. pseudomallei* are poorly understood [20]. In addition to the climatic factors mentioned above, pH of soil appears to be important [21], as do man-made environmental changes such as grazing [22]. It is possible that agricultural practices such as ploughing and the use of agro-chemicals may at least play a role [23]. Furthermore, a handful of outbreaks appear to have resulted from contaminated water supplies [23], although the overall impact of these on incidence is likely to be relatively small. Nonetheless, it is important to understand the factors that enable *B. pseudomallei* to contaminate a water supply so that relevant public health measures can be taken.

- **Breakdown of public health.** As there is no vaccine for melioidosis, and public health measures have never been shown to have any impact on the incidence of the infection, this is unlikely to play a major role in its emergence.

- **Poverty and social inequality.** Undoubtedly melioidosis is a disease of the rural poor. However, there is no evidence to suggest that increasing poverty directly increases the risk of the disease, and in any case, in many endemic areas such as Thailand, the socio-economic situation for rural populations has largely improved in recent times. In fact, it might be expected that the increasing automation of farming, which should reduce the amount of direct soil and water contact of farmers, would actually reduce the risk of melioidosis.

- **War and famine.** Melioidosis is undoubtedly a disease of military significance, as demonstrated during the Vietnam conflict [2], and it has also caused infections during training exercises involving contact with mud and water [24]. However, there is no evidence to associate any recent increases in the reported incidence of the disease with war.

- **Bioterrorism.** Although *B. pseudomallei* is considered a potential bioweapon, there is no evidence of it having been used as such, and little effort appears to have gone into its weaponisation [25].

- **Dam and irrigation system construction.** *B. pseudomallei* is undoubtedly an aquatic organism and irrigation systems may play a role in changing epidemiology. Recent cases in Brazil and Papua New Guinea have clearly been associated directly with water contact [23,26].

4. **Evidence of increasing incidence**

The best quality evidence for melioidosis as an emerging infection would be expected to come from countries where comprehensive surveillance is in place. For example, in
the wet season of 2009–10, the incidence of melioidosis in the Northern Territory of Australia was more than 3 times higher than expected [27]. Singapore experienced a significant increase in cases in 2004 following a period of heavy rainfall, strong winds and flash floods [28]. Australia, Malaysia and Singapore are covered in subsequent Sections. The strongest evidence for melioidosis as a genuinely emerging infection comes from Thailand. Although this is described in detail in Section II.2 a brief commentary is given below.

4.1. Thailand
Prospective clinical and epidemiological studies of melioidosis, based in Sappasithiprasong Hospital in Ubon Ratchathani, northeast Thailand, have been underway continuously since 1986. Between 1987 and 1991, the average annual incidence of melioidosis in Ubon province was estimated as 4.4 per 100,000 per year [29]. Between 1997 and 2006, this had nearly trebled to 12.7 per 100,000 per year, increasing incrementally from 8.0 to 21.3 per 100,000 between 2000 and 2006 [13], and continuing to increase annually to the present day (Peacock, S.J., personal communication), making it the second commonest infectious cause of death in this region after HIV/AIDS. The reasons for this are far from clear. There have been no major improvements in diagnostic techniques over this period, and case definitions have remained the same (based on culture positivity), so the increase is undoubtedly genuine. One possible artefactual factor might be increasing referral of patients suspected of having melioidosis to Sappasithiprasong Hospital, although there is no hard evidence to support this. There was actually a negative association between the total annual rainfall and the number of melioidosis cases observed in this study, although the frequency of severe weather events was not analysed. The authors speculate that the increase results primarily from increasing life expectancy, especially increasing numbers of people with conditions that predispose to melioidosis, such as diabetes. Clearly further studies are required.

4.2. Indian sub-continent
When the worldwide distribution of melioidosis was reviewed in 1991, only a handful of cases of the disease had been reported from the Indian sub-continent [1]. Nonetheless, the fact that they were reported at all implied that the disease was present, but a lack of awareness amongst clinicians and microbiologists meant that it was seldom recognised. This situation has been changing slowly, and there is increasing recognition that melioidosis is probably widely distributed throughout India and adjacent countries [14,30–33]. Surveillance is too inconsistent to determine whether this is true emergence, pseudo-emergence, or a combination of the two, but the increasing prevalence of diabetes in India might genuinely be increasing the numbers of people contracting the infection [14].

4.3. Taiwan and Southern China
The situation in southern mainland China, as far as melioidosis is concerned, is very unclear, although the disease is undoubtedly present there [34]. Much better studied,
however, is the position in Taiwan. Melioidosis was first recognised in Taiwan as recently as 1984, and then only in a patient who had probably acquired the disease in the Philippines [35]. Over the past two decades, however, there has been increasing recognition of indigenous melioidosis, especially in the south of Taiwan and in the Er-Ren River basin [36,37]. This was most marked in the period immediately after Typhoon Haitang, a category 5 super typhoon, that struck in 2005 [38], when the incidence rose to 70 per 100,000 [36]. The diversity of strains of *B. pseudomallei* present in soil in Taiwan [37] suggests that the disease has probably been endemic there for many years but unrecognised rather than being recently introduced.

5. New frontiers

In the last 20 years, melioidosis has been described for the first time in several places where it has never been reported before, or where it has not been reported for many years. Sometimes the evidence is dubious, particularly when this has been limited to the isolation of ‘*B. pseudomallei*’ from environmental samples without independent confirmation or detailed description of the method of identification [3]. A brief summary of these countries where melioidosis has definitely emerged or re-emerged over this period is given below.

5.1. Asia

In many countries that are contiguous with known endemic areas, such as Vietnam, Cambodia, Laos and Burma, it can be assumed that melioidosis has been continuously present but under-recognised for the various reasons given above, and so reports of increasing numbers of cases recognised in these countries almost certainly reflect increased ascertainment rather than true emergence [7,39–42]. Elsewhere in Asia, and throughout the Pacific region, the same is probably true. Exported cases of human and animal melioidosis are regularly reported from Indonesia [18,35] and the Philippines [18,43], and yet indigenous cases are rarely if ever reported there.

5.2. Pacific Islands

Over the past two decades, culture-positive melioidosis has been reported from New Caledonia and Papua New Guinea, and serological evidence of the disease has been found in Timor Leste [5,26].

5.3. The Americas

Perhaps the most interesting extensions of the known distribution of melioidosis since 1991 have occurred in Central and South America and the Caribbean. Cases up to 2006 were comprehensively reviewed by Inglis et al. [44]. There is no doubt that melioidosis is present on several Caribbean islands, including Aruba, Martinique, Guadeloupe, Puerto Rico, Costa Rica, and possibly the British Virgin Islands [44,45], and in addition to Panama and Ecuador, there have been unequivocal cases of melioidosis in Brazil [44],
Fig. II.2. The worldwide distribution of melioidosis. (Reproduced from Currie et al. [5], Trans R Soc Trop Med Hyg 2006;100(Suppl 1):S1–S4).
where *B. pseudomallei* is undoubtedly present in the environment [45], and cases probably exported from Honduras [5].

5.4. Africa

As far as the presence and incidence of melioidosis is concerned, Africa truly remains the ‘dark continent’. There are tantalising glimpses of the presence of *B. pseudomallei* in various parts of Africa, but confirmed cases are extremely rare. As diagnostic microbiology facilities are poorly developed throughout Africa, it is extremely difficult to have any real idea of the true incidence of the disease. Since 1991, only a single case of caprine melioidosis has been reported from South Africa [3], and a human case thought to be acquired in the Gambia has been reported from Spain [46]. Human cases have also been reported from Mauritius and Madagascar [5]. Very recently, a case likely to have been acquired in Ogun State, Nigeria, has been diagnosed in the United Kingdom and, interestingly, the isolate clustered with an environmental isolate from Niger on e-Burst analysis of multilocus sequence typing (MLST) type (Klein, J. and Kenna, D., personal communications). The presence of *B. pseudomallei* in environmental samples from Nigeria has also been reported, but not independently confirmed [47]. This begs the question as to how common melioidosis is in West Africa, although if it were common then it is surprising that exported cases are not recognised more frequently, especially given the regularity of migration between countries like Nigeria and the United Kingdom. Nonetheless, it is very likely that more of the iceberg remains to be revealed in Africa.

6. Conclusions

Despite the many advances made in understanding the basic biology and pathogenesis of *B. pseudomallei*, our understanding of the epidemiology and ecology of the organism remains poor. Most cases worldwide probably go unrecognised because they occur in people who have no access to diagnostic facilities capable of confirming the aetiology. The current state of our understanding of the global distribution of melioidosis is summarised in Figure II.2. In some areas there is unequivocal evidence that the disease is increasing in incidence, probably because of a combination of factors including an increase in the prevalence of conditions that enhance susceptibility to melioidosis, along with climate change resulting in more frequent severe weather events. In most places, however, any apparent increase in incidence is more likely to be due to increasing recognition rather than genuine emergence.

References

D.A.B. Dance / II.1. Melioidosis as an emerging disease


Epidemiology of melioidosis in Thailand

Surasakdi Wongratanaicheewin\textsuperscript{a}, Stitaya Sirisinha\textsuperscript{b}

\textsuperscript{a}Department of Microbiology and Melioidosis Research Centre, Khon Kaen University, Thailand
\textsuperscript{b}Department of Microbiology, Mahidol University, Bangkok, Thailand

Abstract

The first report of human melioidosis in Thailand appeared in 1955. However, it was not widely recognised until the Vietnam conflict, when a large number of American soldiers contracted melioidosis. The disease is acquired via skin abrasions or inhalation. It is endemic in northeast Thailand and a major cause of community acquired septicaemia with a high relapse rate. Patients with recurrent disease are more often associated with relapse than with re-infection. The disease affects all age groups, with higher prevalence in males and shows a strong correlation with levels of rainfall. Currently, the incidence is increasing, due to awareness and higher sensitivity of available diagnostic tests. Attempts have been made using molecular typing to correlate strain variation with clinical disease manifestations and/or severity with little success.

1. Introduction

The first case report on melioidosis in Thailand appeared in 1955 [1]. It was not widely recognised until the Vietnam war, with the death of American soldiers due to acute pneumonitis [2]. At present, some 3000 to 4000 cases of clinical melioidosis are estimated to occur each year throughout Thailand. The reported incidence has been increasing, probably due to increasing awareness, climate change and more sensitive and novel technologies used to detect the disease. Although a wide variety of animals have been shown to be susceptible to melioidosis, information on naturally occurring melioidosis in animals in Thailand is rather limited. The first case of bovine melioidosis in Thailand was reported in 1984 [3] where the bacteria were isolated from milk samples from dairy cattle with clinical mastitis.

2. Clinical epidemiology

Melioidosis is a disease of the rainy season in endemic areas. It mainly affects people who have direct contact with wet soils [4]. The disease is endemic in northeastern Thailand, where \textit{B. pseudomallei} is most readily recovered from the soil [5]. Between
Table II.1
Incidence of melioidosis in Thailand

<table>
<thead>
<tr>
<th>Yeara</th>
<th>Location of study</th>
<th>Incidence (per 100,000 population)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1997</td>
<td>Northeast Thailand</td>
<td>137.9</td>
<td>[5]</td>
</tr>
<tr>
<td>1997</td>
<td>Northern Thailand</td>
<td>18</td>
<td>[5]</td>
</tr>
<tr>
<td>1997</td>
<td>Central Thailand</td>
<td>13.4</td>
<td>[5]</td>
</tr>
<tr>
<td>1997</td>
<td>Southern Thailand</td>
<td>14.4</td>
<td>[5]</td>
</tr>
<tr>
<td>2007</td>
<td>Northeast Thailand</td>
<td>8.7 (2010)</td>
<td></td>
</tr>
</tbody>
</table>

\[\text{a} \quad \text{All age groups are represented, with the exception of Ubon Ratchathani [7] where the figures for 1986-2004 are for children under 15 years of age.}\]

\[\text{b} \quad \text{Wongratatnacheewin S, personal communication, 2010.}\]

1987 and 1991, the average incidence of human melioidosis in Ubon Ratchathani was estimated at 4.4 per 100,000 (Table II.1). The disease affected all ages, was 1.4 times more common in males than females, and showed a strong linear correlation with rainfall [6]. In the 1980s, melioidosis was estimated to account for 20% of cases and approximately 40% of deaths from community acquired septicaemia [9], with a relatively high rate of recurrence. Patients with severe disease had recurrences 4.7 times more frequently than patients with localised melioidosis [10], but it was later recognised that 25% of patients with recurrence had re-infection rather than a relapse [8]. A prospective cohort study of culture-confirmed melioidosis patients admitted to Sappasithiprasong Hospital in Ubon Ratchathani between 1997 and 2006 had an average annual incidence rate of 12.7 per 100,000 population [11]. A year-on-year increase in the annual incidence was observed, rising from 8.0 in 2000 to 21.3 in 2006. Several previous reports have shown other northeastern provinces including Khon Kaen, Nakhon Ratrasima, Buri Ram and Udon Thani to have large numbers of patients. Data from 19 provincial hospital laboratories in northeast Thailand in 2007 recorded 1,865 culture-confirmed cases of melioidosis (authors’ unpublished observations). This number is equivalent to an annual incidence rate of 8.7 per 100,000 population for northeast Thailand (Table II.1).

3. Age and gender distribution

The disease affects all ages (mean\(\pm SD\) of age: 52\(\pm\)14 years) and is 1.4 times more common in males than females [6]. Most patients are adults, although children and neonatal cases have also been reported [12], the mean age being 6.8 years with a range of 8 months to 15 years with acute suppurative parotitis being a unique characteristic reported in children in Thailand [13].
4. Mortality

The mortality of melioidosis in Thailand has varied considerably from 38% to 68% [9] depending upon the time of admission, underlying diseases and treatment protocols. In 2006, the mortality rate attributed to melioidosis in northeast Thailand was comparable to that attributed to tuberculosis [11].

5. Ecology and distribution

The bacterium is found in the soil and water mostly in northeast Thailand but rarely reported from the adjacent areas like central Thailand [14]. Within the culture-positive sites, the number of *B. pseudomallei* in soil and water was significantly higher in the northeast (median 230 CFU/mL) than in the central region (median 10 CFU/mL). In a survey in northeast Thailand in 1995, *B. pseudomallei* was recovered from 68% of the sites tested. In the wet season, the recovery rates increased steadily with increasing depth of soil down to 60 cm below the surface, whereas in the dry season, no bacteria were found on the surface but the numbers were uniformly high from soil taken between 30 cm and 90 cm [15]. The organism was also found in the soil and water (60.9–78.1%) in southern Thailand [16], and its presence in the south was confirmed by the occurrence of 6 cases of melioidosis in survivors of the 2004 tsunami [17]. However, it is possible that these were cases of relapse induced by stressful conditions.

A total of 3,585 soil samples were collected from 896 sites from four regions in Thailand in 1997, and *B. pseudomallei* was recovered from 4.4%, 6.1%, 20.4%, and 5.9% of the samples from the northern, central, northeastern, and southern regions, respectively [5]. There was a correlation between the presence of *B. pseudomallei* in the soil and the number of clinical cases. The infection rate in patients attending government hospitals in the northeastern region (137.9 per 100,000 inpatients) was significantly higher than those in the northern, central and southern regions [5]. The environmental isolates of *B. pseudomallei* were found to be genetically diverse, although there were some unique types [18]. The bacteria from agricultural land were also found to be genetically diverse despite the effects of flooding and processes associated with farming [19].

Although it is generally accepted that the occurrence of melioidosis in Thailand is associated with the presence of *B. pseudomallei* in the soil, the climatic, physical, chemical and biological factors which control the proliferation and survival of *B. pseudomallei* in the environment are controversial. It has been recently demonstrated that the organism was significantly associated with certain soil physicochemical parameters such as pH 5.0–6.0, moisture content >10, higher oxygen demand and total nitrogen content [20].

6. Modes of transmission and acquisition

The exact route of transmission of this disease is unknown but most cases of melioidosis in Thailand are probably acquired via skin abrasions or inhalation. There are also case reports of acute melioidosis presenting with pneumonia and septicaemia following
aspiration of contaminated water [21]. Suputtamongkol and associates [6] identified penetrating injury in 5.2% of cases and near-drowning in 0.5% of cases in northeast Thailand. Eighty one percent of cases with melioidosis occurred in rice farmers or their families, the risk being associated with occupational exposure to soil [6], nosocomial infections have been reported from some hospitals in certain endemic areas (Chetchotisakd P, personal communication). Vertical transmission [12] and human to human spread have also been reported, albeit rarely [22].

7. Predisposing factors

Melioidosis is significantly associated with underlying diseases such as diabetes, thalassaemia, and preexisting renal disease [6]. Kosuwon et al. [23] found that diabetes was the most common concurrent disease in melioidotic septic arthritis. The presence of specific risk factors such as diabetes, suggests that functional impairment in neutrophils may be important in the pathogenesis of melioidosis. Other risk factors reported in Thailand are excessive alcohol consumption, dengue hemorrhagic fever and systemic lupus erythematosus [4,6]. However, melioidosis does not seem to be associated with HIV infection [24].

8. Molecular epidemiology

Genotyping of 2,058 bacterial colonies isolated from 133 patients demonstrated that infection with more than one strain was uncommon [25]. A study of 577 isolates collected from all over Thailand, demonstrated 77 distinct ribotype patterns [26] and 12 major ribotypes were identified, of which types 3, 8 and 23 were the most common (278/577, 48.2%) in both clinical (217/371, 58.5%) and environmental isolates (61/206, 29.6%). Although three types were unique for environmental isolates, no unique clinical type was observed. On investigation of five genomic island (GI) regions from \textit{B. pseudomallei} strain K96243, of 186 isolates from northeast Thailand (83 environmental and 103 clinical isolates), the presence of each of the five GIs and cumulative GI number did not differ between environmental and disease-associated isolates [27]. Typing of the bacteria from various parts of the world by MLST revealed that sequence type (ST) 84 is the likely representative of \textit{B. pseudomallei} isolates originating from Southeast Asia, although a single clinical isolate having the same MLST type was isolated from a female patient from Australia whose travel history was unknown. However, ST60 was found in the environment both in Thailand and Australia [28]. There were 123 STs in 266 clinical and environmental isolates from Thailand and the 4 most commonly recovered were ST70 (n = 21), ST167 (n = 15), ST54 (n = 12), and ST58 (n = 11). The genotypes of invasive isolates were predominant (43.7%) within the 10 most commonly observed STs whereas only 25.3% of soil isolates belonged to these 10 STs [29] confirming the notion that genotypes are non-randomly distributed. Comparison of sequences of K96243 from Thailand with strain MSHR305 from Australia using E264 (\textit{B. thailandensis}) as reference [30], identified the variable homologous region between the two strains. K96243 was shown to possess a horizontally acquired \textit{Yersinia}-like fimbrial (YLF) gene
cluster from E264 but MSHR305 was similar to \textit{B. thailandensis}-like flagellum and chemotaxis (BTFC). In 517 isolates, BTFC group was found to be dominant in Australia and YLF group in Thailand. In addition, clinical isolates were more likely to belong to group YLF, whereas environmental isolates were more likely to belong to group BTFC.

9. Conclusion

Melioidosis is known to be highly prevalent in northeast Thailand, where \textit{B. pseudomallei} is also widely distributed in soil and surface water. Within Thailand, the incidence of the disease is increasing and it is estimated that at least 3,000 to 4,000 cases of clinical melioidosis occur each year. With new technologies and high throughput molecular typing methods, the epidemiology of this disease will become more apparent.

References


Section II.3
Epidemiology of melioidosis in Malaysia and Singapore

Savithiri D. Puthucheary
Department of Medical Microbiology, University of Malaya, Malaysia

Abstract

In 1913, Fletcher encountered Whitmore’s disease in laboratory animals at the Institute for Medical Research in Kuala Lumpur, Malaysia and in 1917 Stanton first described the infection in a human patient also from Kuala Lumpur. These authors wrote a monograph on the disease and its sporadic occurrence in Malaya up to 1932. The first reported case of acute melioidosis in Singapore was in 1931. Since then, cases have been reported in both humans and in a variety of animals in Singapore as well as in Malaysia. The epidemiology of melioidosis is similar in both countries and there has been a sharp increase in cases recently due to greater awareness and increased isolation rates by laboratories. Being developing countries, there is excessive soil disturbance, thereby creating conditions conducive for contact with *Burkholderia pseudomallei*.

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1. Introduction

Not long after Whitmore and Krishnaswami described melioidosis in Burma, in 1913, Fletcher encountered the disease in laboratory animals at the Institute for Medical Research in Kuala Lumpur, Malaysia. In 1917, Stanton first described the infection in a human patient from Kuala Lumpur and these authors wrote a short monograph on the disease and its sporadic occurrence in Malaya up to 1932 [1]. Since that time, cases have been reported in Malaysia in both humans and in a variety of animals such as sheep, buffalo, deer, monkey, gibbon, orang utan, kangaroo, camel, parrot, hamster, zebra and crocodile [2]. Singapore and Malaysia are geographically and demographically similar and linked, hence the epidemiology of melioidosis is similar in both countries. The first case of melioidosis in Singapore was reported in 1920 [1]. There has been a sharp increase in cases recently due to greater awareness and increased isolation rates by laboratories.

2. Ecology and distribution

In Malaysia, the organism has been isolated from soil and water from all states in West Malaysia [3]. Samples were taken from primary and secondary forests, wet rice fields and
recently cleared areas. The isolation rates were lower from the forested areas compared to the cleared areas of wet rice fields and newly planted oil palm plantations. Soil moisture was an important criterion in the isolation of the organism. The incidence of melioidosis peaks during the months of heavy rainfall in Thailand and Australia, but in Malaysia, although the percentage of water and soil specimens positive for *B. pseudomallei* was higher during increased rainfall [3], there appeared to be only a slight increase in the number of cases during the wet monsoon period. The correlation between rainfall and the occurrence of culture proven melioidosis was evident in a study carried out over a 30 year period [4], although this correlation appeared less strong than that reported in other countries. This may be due to various factors, including the different local patterns of rainfall intensity as well as increased exposure to the organism during ploughing and planting of the rice paddies in some endemic areas [5].

In Singapore, cases occur sporadically across the island throughout the year. There was no significant correlation between disease incidence and rainfall, and no significant difference in incidence between those living in flats or on properties with land [6].

In Malaysia, the organism has been isolated from soil at various depths below the surface, with soil moisture being an important criterion in the wide distribution of *B. pseudomallei* in surface and soil water of pH ranging from 2.8 to 7.4 [3]. Furthermore, soil temperature studies by the same authors showed temperature ranges of 26–31°C for cleared areas and 22–25°C for the forested areas. While forested areas may well have the moisture needed for growth, soil temperatures appear well below those needed for optimum growth, which might account for the greater yield of *B. pseudomallei* from cleared areas.

Soil and surface waters are highly complex ecosystems in which a vast range of physical, chemical and biological factors interact. The organism is, however, found to persist in some tropical regions better than others, leading to hyper endemic foci or “hot spots” such as northeastern Thailand and east Malaysia, where more cases are reported than from the rest of the country [5], although the exact reasons for this are far from clear. The tenacity of this soil saprophyte to survive in a hostile environment should not be underestimated. There is a homeostatic balance in nature between the host, pathogen and the environment, and any disturbance of this by logging or clearing of large tracts of vegetation will upset this equilibrium. In developing countries there is frequently excessive soil disturbance, thereby creating conditions conducive for contact with *B. pseudomallei*.

3. Clinical epidemiology

Malaysia is an endemic area for melioidosis but the true incidence of melioidosis in Malaysia is relatively unknown. Since melioidosis is not a notifiable disease in Malaysia, there are only a few reports available on the incidence in the country. However, it is known that cases are not evenly distributed, and anecdotally more cases are being reported from the so called “hyper-endemic” states such as Pahang, Kelantan, Sabah and Sarawak. In the state of Pahang, the annual incidence has been reported as 4.3 per 100,000 population per year (adults 6.0 and children 1.6 per 100,000 respectively [7]). In the southern state
of Johor, only 44 new cases were recorded over a period of five years [8] and there were 250 cases reported from the State of Sabah over seven years [9].

In the city state of Singapore, where melioidosis became a notifiable disease in 1989, the incidence varied between approximately 1 and 3 per 100,000 per year, but decreased during 1998–2007, with the exception of the first quarter of 2004, which was preceded by heavy rainfall, strong winds and flash floods [10].

In a comprehensive review of 98 septicaemic and 43 non-septicaemic cases studied over a period of 35 years in one institution, a bimodal distribution of age in both groups of patients was observed [5]. The age of patients ranged from 17 days to 79 years. The high incidence in the 10 to 30 year group possibly reflects greater environmental exposure during play or outdoor recreational activities. The peak age-specific incidence occurred from 41 to 59 years for both males and females in Malaysia. In a more recent study, the highest age-specific incidence rate of melioidosis in Singapore for adults was found to be greater than 45 years [10]. Patients ranged in age from one month to 97 years [10]. In every published case series on melioidosis, males have outnumbered females, but the proportions varied considerably (ratio of male:female from 5:1 to 1.4:1). This most likely reflects involvement in activities which lead to exposure to contaminated soil and water. In Malaysia, the male to female ratio was found to be 3.2:1 [11] and in Singapore the annual incidence rate for male patients was 2.8 to 7.2 times greater than that for females [10].

Ethnic differences in susceptibility to melioidosis were suggested in the Singapore Armed Forces, amongst whom the ethnic distribution was 69.6% Chinese, 17.4% Indians and 8.6% in Malays. The ethnic group-specific rates, however, were higher in the Indian and Malay groups than the Chinese by factors of 2.6 and 2.1 respectively [12]. In Malaysia, the morbidity rate was also highest amongst the Indians, lowest in Malays and intermediate in the Chinese [11]. In a more recent study, Malays formed the largest group (52.7%), followed by Chinese (13.7%) and Indians (12.3%) and the ethnicity was not available for 21.2% [13]. This ethnic distribution is similar to the present racial composition of Malaysia.

An overall mortality of 60% was recorded for 50 septicaemic cases from Malaysia [14]. In Singapore, during a 10 year period the overall case fatality rate was 16.2% (range 8.8–27.1%); patients with bacteraemic melioidosis had a significantly higher case-fatality rate of 25.8% than those without bacteraemia 5.5% [10].

4. Bacterial strains

Genotyping by pulsed field gel electrophoresis (PFGE) using SpeI restriction endonuclease of 146 sporadic clinical isolates of B. pseudomallei from the various states in Malaysia showed that strains were genetically diverse and heterogeneous [13]. It was observed that some pulsotypes were limited to the peninsular states of Malaysia whereas three types were isolated only from Sarawak, an east Malaysian state. Overall, there were genomic and molecular differences in the distribution of B. pseudomallei in the peninsular and East Malaysian states. At the same time, certain clusters of B. pseudomallei were present in some states and not in other states. Although the three pulsotypes appeared to be present only in the rural and padi-growing regions of Malaysia, it is difficult to say categorically
that they are from hyper-endemic regions as the number of isolates was not sufficient for the calculation of statistical significance [13].

5. Predisposing conditions

It has been recognised that *B. pseudomallei* behaves as an opportunistic pathogen. Exposure to the organism is widespread and yet disease is not that common, occurring predominantly in those with underlying predisposing conditions, suggesting that the susceptibility of the host is an important factor. In Malaysia, the majority of patients (76%) with clinically apparent melioidosis are recognised as having underlying diseases [14]. In Singapore, 75.5% had co-existing diseases, with diabetes (47.9%) being the most common [10]. Renal failure, renal calculi, retroviral infections, malignancy, steroid therapy, alcoholism, occupational exposure, trauma and parenteral drug abuse were also confirmed as important predisposing factors both in Malaysia and Thailand [14,15]. Underlying disease was seldom reported in cases from the Singapore Armed Forces [12] and in children and young adults from East Malaysia [16] suggesting that a substantial exposure to *B. pseudomallei* will cause infection even in healthy individuals.

6. Persons at risk

In endemic areas like Malaysia, acquisition is through outdoor activities, occupational exposure being high on the list; a variety of personnel such as farmers, forest rangers, construction site employees, drivers of heavy vehicles, carpenters and gardeners [14].

Travellers from non-endemic areas, such as New Zealand, to Malaysia are also at risk, especially with regard to eco-tourism (Puthucheary, S.D., unpublished observations). This is also true of other tourists and military personnel.

References

Section II.4

Epidemiology of melioidosis in Australia and the Pacific region

Allen C. Cheng\textsuperscript{a}, Bart J. Currie\textsuperscript{b}

\textsuperscript{a} Menzies School of Health Research, Darwin, Australia; Department of Epidemiology and Preventive Medicine, Monash University; Infectious Diseases Unit, Alfred Hospital, Melbourne Australia
\textsuperscript{b} Menzies School of Health Research; Northern Territory Clinical School and Infectious Diseases Department, Royal Darwin Hospital, Darwin, Australia

Abstract

Melioidosis was first described in Australia in sheep in 1949 and the following year in humans. Like most endemic countries, the incidence of melioidosis closely parallels the monsoonal wet season, with an annual incidence in the Top End of the Northern Territory of 16.5 cases per 100,000. However, case-clusters have been described, not only with extreme weather events such as floods and cyclones, but also with contaminated environmental foci. Localised temperate areas of endemcity are also described in Australia, with cases in southern Western Australia and in southeast Queensland. Sporadic cases of melioidosis have also been described in Papua New Guinea, Guam and Fiji. More recently, cases have also been reported in the Western Province in Papua New Guinea and in New Caledonia. Molecular studies suggest that isolates from Australia and the Pacific region are diverse, distinct from, and ancestral to, those in Thailand. Ongoing studies are stratifying risk based on soil sampling.

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1. Introduction

Melioidosis was recognised relatively recently in Australia, compared to its original description in southeast Asia in the early 20\textsuperscript{th} Century. Although there are low absolute case numbers, reflecting a low resident population in northern Australia, the per capita rate of disease is probably higher than in southeast Asia. Melioidosis is regarded as a disease of public health significance in the Top End of the Northern Territory, the Kimberley region of Western Australia and northern Queensland.

2. Historical aspects

Melioidosis was first recognised within Australia from an outbreak in sheep in 1949 in Winton, northern Queensland [1]. The following year, the first human case was
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49

described; a diabetic who died from septicaemic melioidosis in Townsville [2]. The first case in the Northern Territory was not noted until 1960 [3]. Subsequently, early case series were reported from Queensland [4] and the Northern Territory [5]. Studies by Annette Thomas, Les Ashdown and colleagues in the 1980s, and later clinicians in northern Australia, have increasingly recognised melioidosis as an important cause of community acquired sepsis in animals and humans. Melioidosis is implicated in a significant proportion of patients with pneumonia and/or bacteraemia in Darwin [6,7].

This apparent late emergence of such an important infectious disease in northern Australia led to suggestions that \textit{B. pseudomallei} may have colonised Australia from southeast Asia [8]. However, the high molecular diversity of isolates in the endemic area suggests that \textit{B. pseudomallei} may actually have originated in Australia and subsequently spread to southeast Asia [9].

3. Clinical epidemiology

The incidence of melioidosis is closely correlated with monsoonal rains; 88% of cases present during the wet season [10]. The severity of disease is also higher in the wet season, with rainfall greater than 125 mm in the 2 weeks prior to presentation a significant risk factor for pneumonia, bacteraemia, septic shock and death; this may suggest inhalation as a potential mode of acquisition [11]. However, percutaneous inoculation is still thought to be the primary mode of acquisition. Although outbreaks have been linked to potable water supplies in Australia, this does not necessarily implicate ingestion as a route of acquisition [12,13].

In the majority of cases it is difficult to establish an incubation period, as the time of acquisition is not defined. However, in a subset of 25 patients where a history of an inoculating event was obtained prior to presentation with acute melioidosis, the incubation period was defined as between 1 and 21 days (mean 9 days) [14]. The incubation period appears to be as short as a few hours in situations with a high inoculum, such as following near drowning [15]. An extended period between acquisition and presentation, either due to low grade chronic disease (defined as symptoms for more than 2 months) and extended latent infection has been reported for months or even many years [16–18]. However, the Darwin series suggests that around 88% of cases are acute, with chronic and latent disease only implicated in a small proportion of cases (16).

Clinical susceptibility factors are well described in melioidosis (Section IV). In contrast to patients in Thailand, only a minority of patients report occupational exposure. The mean age of cases is 49 years with 63–75% of cases in males [19,20]. Paediatric cases are uncommon in Australia, with only 4% of cases occurring in children under 15 years. With improved acute medical management of patients with severe sepsis due to melioidosis, and earlier recognition and administration of antibiotics, the case fatality ratio has been declining since the late 1980s. Overall mortality at the Royal Darwin Hospital has fallen from 30% (1989–94) to 9% (2005–9), but was reported as 25% in a recent series from Townsville (1996–2004) [20].
4. Distribution in Australia

Melioidosis is generally regarded as endemic in Australia in the region north of 20°S (including Far North Queensland, the Top End of the Northern Territory and the Kimberley region of Western Australia). Clinical cases are also reported in the Queensland city of Mackay (21°S), which is regarded as being within the endemic area [21]. Case ascertainment is likely to be good compared to some other endemic countries, as centralised microbiology facilities are widely available, there is a high awareness of melioidosis amongst medical staff and treatment is often provided in a small number of referral hospitals. Melioidosis has been a notifiable disease in the endemic areas of Australia since 2001 [21].

In the Top End of the Northern Territory, the incidence rate of culture-confirmed melioidosis was 16.5 per 100,000 between 1989 and 1999 [19]. In the 2001/2 season, incidence in the Top End (15.5 per 100,000) was higher than that in the Kimberley region (2.4 per 100,000) and Far North Queensland (3.9 per 100,000) [21]. Higher rates have been associated with high annual rainfall and severe weather events; in 1998, the incidence in the Top End was 41.7 per 100,000 [14,22]. In the Torres Strait islands in northern Queensland between 1995 and 2000, a rate of 42.7 cases per 100,000 was documented [23]. The incidence rate in Indigenous Australians appears to be higher than in non-Indigenous Australians (relative rate 3.0), and may relate to both increased exposure as well as a higher prevalence of comorbidities [22].

The distribution of clinical cases appears to be broadly correlated with the extent of environmental contamination of soil as well as the bacterial density [24,25]. Environmental sampling has revealed widespread presence of *B. pseudomallei* in samples of soil, mud and pooled surface water in northern Australia, including Queensland [26], around Darwin [27–30] and remote communities in the Northern Territory and Western Australia [31,32]. Ongoing studies are examining environmental factors associated with detection of *B. pseudomallei* nucleic acid in environmental samples, including soil disturbance, soil type, acidity, and the presence of livestock animals [29]. Additionally, strains of *B. pseudomallei* have also been isolated from bore water in the rural area near Darwin. The clinical significance of environmental bacterial contamination to individuals is not yet clear outside of an outbreak situation.

Although serological tests have been demonstrated to have poor sensitivity and specificity in clinical situations, the prevalence of positive serology is likely to reflect background exposure to *B. pseudomallei* on a population basis. Surveys of populations in northern Australia have demonstrated a relatively low prevalence of positive serology, compared to the prevalence seen in northeastern Thailand. For example, in Queensland, the proportion with positive serology in urban populations (up to 5%) was lower than in patients residing in rural locations or of Aboriginal or South Pacific origin (up to 10%) [33]. The prevalence of positive serology was similar to those found in the primarily Indigenous population of Arnhem Land in the Northern Territory (12.8%) [14]. These seroprevalences contrast with the much higher rates in immigrants to Queensland from southeast Asia (29%) [33] and the background seropositivity found in residents of the endemic area of Thailand [34,35].
5. Case clusters within the endemic area

A review of case clusters in the Northern Territory demonstrated that clonal outbreaks were associated with environmental point sources, whereas non-clonal case clusters were associated with extreme weather events [36]. Non-clonal case clusters have been described following extreme weather events, including tropical cyclone Thelma (1998/9) and flooding of the town of Katherine following tropical cyclone Les (1998) [36]. Similar observations have been made elsewhere in Australia and in Taiwan [21,37].

Two outbreaks have been linked to contamination of the drinking water supply, where disease control measures such as cleaning of the water supply, replacing damaged pipes and restoration/institution of chlorination led to a cessation of the outbreaks. In a remote community in Western Australia, 5 cases were detected over a 5 week period in a population of 300. Isolates from patients as well as a strain isolated from a tap were found to be clonal and contamination was attributed to repair of a poorly maintained reticulated water system in the community [12,38]. Another outbreak in a remote community in the Northern Territory was associated with 9 cases over a 28 month period. Typing of isolates found that strains from 6 patients were identical to a strain isolated from the unchlorinated community water supply, but different from those isolated from soil. The outbreak appeared to be terminated by remedial works to the water supply [13]. Neither chlorination or water pH appear to be associated with observed rates of melioidosis in other remote communities, although adequate chlorination is considered one important aspect of preventing future outbreaks from potable water [39].

A small outbreak, involving two mechanics working in the same small community, was attributed to contamination of a large container with detergent used for cleaning grease from hands [40].

6. Foci outside the endemic area

Sporadic cases and case clusters have occurred south of the endemic area in Australia, including the first cases in sheep in Winton (22ºS). Two foci have been reported in southern Queensland, in the Burnett River region (25.5ºS) and the Brisbane River Valley (27ºS). In the Burnett River region, an outbreak involving 159 cases of melioidosis in pigs over 3 years occurred following flooding and was attributed to a contaminated water supply drawn from the river [41]. Similarly, animal and human cases in the Brisbane River region occurred following flooding in 1974, possibly linked to cattle having previously been imported from northern Queensland [42]. Subsequent human cases linked to flooding in that same area have been described from the city of Ipswich (27.5ºS); the lack of clonality found on molecular typing of B. pseudomallei strains from the cases suggests that this temperate southeast Queensland focus may not be the result of a single recent introduction [43,44].

In contrast, a focus in temperate southwest Western Australia, in farms north west of Perth (31ºS), involved multiple animal cases since 1966 and a human case in 1991 [45]. Ribotyping of 10 of the isolates from animals and humans found that these strains were clonal, suggesting that this may have been the result of a single introduction into the
area [31]. In the Northern Territory, human cases are periodically reported in central Australia, including from the town of Tennant Creek and from some remote Indigenous communities.

7. Melioidosis in the Pacific region

The extent of melioidosis in Pacific island countries is not clear, as many countries have limited diagnostic capability. At least six cases of melioidosis have been reported from Port Moresby in Papua New Guinea [46–49] as well as one additional case in an ex-serviceman living in Brisbane [50] for whom the place of exposure was not clear. Small serological surveys in the Port Moresby region have not demonstrated antibodies to *B. pseudomallei* [46,51]. A focus of melioidosis has been studied in the Western Province of Papua New Guinea, with most cases being in children [52,53]. This suggests that melioidosis may well occur elsewhere in the country.

Cases of melioidosis have been described in several other Pacific countries, including Guam [54], Fiji [55] and more recently New Caledonia [56]. A serological study has also supported the likely occurrence of melioidosis in Timor Leste [57], although no culture-confirmed cases have been reported from that country. The only cases of melioidosis reported in New Zealand are in returned travelers or immigrants from other countries, including Fiji.

8. Control measures

In Australia, general advice regarding prevention is routinely provided in endemic areas. This includes the use of protective clothing and avoidance of contact with mud and pooled surface water, particularly for groups with significant comorbidities. Communication with the public and health care providers to raise awareness is performed during times of high risk. Outbreak investigations are uncommonly required but may be prompted by a cluster of cases in excess of historical seasonal incidence. Because clinical features may be non-specific, case definitions should be based on culture confirmation rather than solely on clinical syndromes or serological findings. Previous outbreaks have implicated environmental point sources, including soil and potable water supplies. Molecular typing techniques [pulsed-field gel electrophoresis, multiple loci variable-number tandem repeat (VNTR) analysis or multilocus sequence typing] may be helpful to link cases with each other or with environmental isolates.

References


III
Microbiology of *Burkholderia pseudomallei*
The following reviews describe some of the most important new molecular findings from the last decade relating to *Burkholderia pseudomallei* and the disease melioidosis. The rapid increase in new molecular technologies, coupled with the decreasing cost of specialised equipment and reagents, has allowed many research laboratories to access opportunities previously only available to large, well funded organisations. This recent explosion in molecular research has resulted in rapid advances in many fields of *B. pseudomallei* research, including genomics, characterisation of virulence determinants and identification of molecular biomarkers of disease.

Perhaps the most significant advance in the last decade is the determination of the *B. pseudomallei* genome. As stated by Nandi and Tan (in Section III.2), the 7.25 Mb *B. pseudomallei* genome is one of the most complex bacterial genomes ever sequenced. Since the completion of the K96243 genome in 2004, sequence data of multiple genomes has been made available to researchers, allowing information relating to strain differences and population diversity to be compiled. A particularly interesting feature discovered during the sequencing projects, was that the *B. pseudomallei* genome is distributed across two chromosomes (4.1 Mb and 3.2 Mb). Furthermore, there was a striking difference in the gene content between the two chromosomes, with Chr1 predominantly containing genes associated with core features of cellular growth and metabolism, and Chr2 predominantly containing accessory genes associated with adaptation and survival. These genome analyses have also shed significant light on the genetic mechanisms underlying the remarkable ecological diversity and environmental versatility of *B. pseudomallei* that has been so well described.

Tandem repeats and simple sequence repeats feature highly in the *B. pseudomallei* genome. A significant proportion of these repeats occur either within, or close to, genes encoding surface exposed or secreted proteins; these proteins may be critical for the interaction of the bacteria with either the host or the environment during pathogenesis or environmental survival. Tuanyok (in Section III.3) discusses the presence of a very large number of *B. pseudomallei* genomic islands and their association with tRNA gene loci. Their non-random occurrence throughout the genome strongly suggests that their insertion is mediated by tRNA genes, allowing site-specific recombination. Several studies...
have assigned virulence functions to genes present on genomic islands, as mutations have been shown to alter virulence-associated phenotypes, particularly adherence to epithelial cells. Other studies have suggested genomic islands may play a role in determining the clinical manifestation of melioidosis.

The relationship between clinical presentation and strain variation is a contentious one. As described by Govan (in Section III.1), a variety of molecular typing methods have been used in attempts to identify any correlation between clonality and disease outcome. Although strains have been found to group based on either clinical or environmental origin, or site of geographical isolation, no link between isolates and disease state has yet been established.

The search for critical *B. pseudomallei* virulence factors has been facilitated by the advent of new molecular technologies. Furthermore, the existence of numerous genome sequences has allowed comparative genomic predictions of factors involved in host-pathogen interactions. However, progress in this field continues to be incremental; virulence factor prediction has typically used information from other more well characterised pathogens as a starting platform. Progress has almost certainly been slowed by the fact that virulence is multifactorial and there is still some contention as to the best animal model for testing of virulence. Beacham et al. (in Section III.4) nicely summarise the current state of play in this field. This review also introduces the concept of *B. pseudomallei* as an accidental pathogen, where an environmental organism finds itself in an “accidental invasive state” within the human host. This is a fascinating concept, given credence by genome similarities of a range of environmental organisms and their pathogenic counterparts.

Specific virulence factors, such as the bacterial lipopolysaccharide (LPS) addressed by Ernst et al. (in Section III.5) have long been suggested to play a role in the pathogenesis of Gram-negative infections such as melioidosis. The LPS of *B. pseudomallei* has been reported to occur in two forms, resulting to some degree, in the antigenically heterogeneous nature of the organism. The intracellular signalling pathways triggered by *B. pseudomallei* LPS with respect to the specific roles of Toll-like receptors 2 and 4 are still under debate, and may vary due to the structural differences in the LPS produced by different isolates.

Bacterial pathogens have evolved a number of mechanisms to disrupt cellular interactions and host defences. The wide spectrum of clinical presentations attributed to *B. pseudomallei* is testament to the arsenal of pathogenic strategies employed by this organism. From an acute, fulminating septicaemia, to a dormant, latent state, *B. pseudomallei* is able to employ many different mechanisms to overcome or subdue the host immune system. Genomic and proteomic advances over the last decade have gone a long way to unravelling some of these systems, giving us hope that one day *B. pseudomallei* pathogenesis will be well understood and melioidosis will be a preventable disease of the past.
Molecular characterisation and classification of *Burkholderia pseudomallei*

Brenda L. Govan

School of Veterinary & Biomedical Sciences, James Cook University, Townsville, Australia

Abstract

Melioidosis presents with a broad spectrum of clinical presentations. Discussions on the correlations of disease presentation and severity with factors such as isolate virulence, host status and geographical location have been long and many. Additional issues such as latency and disease relapse serve to further complicate the picture. Molecular typing methods have been used to support a number of theories relating to such questions. They have also provided data regarding the evolutionary origins of *Burkholderia pseudomallei* isolates, in addition to defining the degree of clonality of multiple isolates from individual patients. More recently, molecular technology has been used in the search for biomarkers, molecular fingerprints used as diagnostic indicators of disease.

1. Taxonomy

Melioidosis is caused by the Gram-negative, motile, non-spore forming, and facultatively anaerobic bacillus known in current nomenclature as *Burkholderia pseudomallei*. The organism was first described by Whitmore and Krishnaswami in 1912 [1], following the investigation of 38 fatal cases of pneumonia in Rangoon and given the name *Bacillus pseudomallei*. This name was chosen due to the similar clinical and pathophysiological features of the disease to those of glanders, caused by *Bacillus* (now *Burkholderia*) *mallei* the causative agent of glanders. The term “melioidosis” is derived from the Greek root “melis” meaning “a distemper of asses” and “eidos”, meaning “resemblance”. Since this time, phenotypic studies have led to the organism being reclassified as *Bacillus whitmori*, *Malleomyces pseudomallei*, *Loefflerella whitmori*, *Pieżferella whitmori* and *Pseudomonas pseudomallei*. Most recently, Yabuuchi et al. [2] reclassified the organism into the new genus *Burkholderia* based on RNA and DNA sequencing data.

2. Microbiology and culture

*Burkholderia pseudomallei* exists in the environment as an opportunistic saprophyte. It is extremely nutritionally versatile, capable of utilising more than 85 different compounds.
as sole carbon sources and demonstrates a remarkable ability to persist in a nutrient free environment [3]. The most commonly used media for laboratory isolation is Ashdown agar [4], on which the organism typically appears as small, glistening purple colonies after 24 hours of culture, changing to rough, wrinkled colonies by 48 hours. However, there is a large degree of variability in the colonial morphologies between strains [5], potentially leading to misidentification of the organism. On blood agar, the colonies may appear creamy-white to grey or yellow-orange and they may be haemolytic. On both Ashdown agar and blood agar, colonies produce a characteristic sweet, musty odour, although this technique is not recommended for identification purposes.

*Burkholderia pseudomallei* is able to survive under low nutrient conditions, with organisms recovered from distilled water ten years after inoculation [6]. The ability of *B. pseudomallei* to survive in water likely contributes to its environmental persistence in endemic regions. However, the molecular mechanisms underpinning this are poorly understood. A comparative microarray analysis undertaken on cultures transferred from growth media to water, showed that the expression of a large number of genes was induced [7]. The genes were mostly involved in energy metabolism and membrane biosynthesis, although none were shown to be absolutely essential for survival in water.

*Burkholderia pseudomallei* is considered a possible biothreat agent and its ability to survive on artificial surfaces is an important component of the possible threat. To investigate this specifically, Shams et al. [8] analysed the viability of *B. pseudomallei* over time on a variety of materials. To enable inclusion of potentially viable-non-culturable cells, esterase activity was used as a measure of viability. The study demonstrated viability on surfaces such as glass, stainless steel and paper for up to 21 days, supporting previous studies reporting the low nutrient requirements of the organism.

The RpoS subunit of RNA polymerase is a bacterial alternative sigma factor and it functions to control the expression of genes encoding stress response proteins. A proteomics approach was used to identify the RpoS regulon in *B. pseudomallei*; 70 proteins were differentially expressed between the wild-type strain and an *rpoS* mutant [9]. The transcription of *rpoS* itself is regulated by growth phase, with peak levels occurring in stationary phase, or when the organisms undergo carbon starvation or oxidative stress. In addition to stress response genes, RpoS was found to be involved in regulating the transcription of virulence factor genes, such as the T3SS1 gene cluster and those encoding proteins involved in cell envelope biogenesis [9].

### 3. Evolution and genetic diversity

Early methods used to characterise and group *B. pseudomallei* strains included antimicrobial susceptibility, bacteriocin production or sensitivity and serotyping. More recently, molecular typing methods have proved useful in assigning *B. pseudomallei* strains into epidemiologically related groups. Even so, there is a high degree of variability in the sensitivity of these techniques and their ability to discriminate closely related strains, particularly when genome differences are minor. With the increasing availability of technologies such as DNA microarrays, high-throughput sequencing and proteomic...
tools, it is now possible to address questions related to microbial diversity in the natural environment.

The genome of \textit{B. pseudomallei} shares a marked degree of similarity with the genomes of \textit{B. mallei} and \textit{B. thailandensis}, despite the fact that \textit{B. mallei} is host restricted and not isolated from the environment, and \textit{B. thailandensis} is thought to be avirulent and isolated only from the environment [10]. A multilocus sequence typing (MLST) scheme developed to study evolutionary relationships between \textit{B. pseudomallei}, \textit{B. mallei} and \textit{B. thailandensis} found that the allelic profiles of \textit{B. pseudomallei} could be distinguished from avirulent \textit{B. thailandensis}, but clustered together with \textit{B. mallei} [11]. The authors suggested that \textit{B. mallei} may be a clone of \textit{B. pseudomallei}, as phylogenetically they would be placed within the same species. This correlates with the disease manifestation and degree of pathogenicity of the two species. Even among patients with melioidosis, there is considerable variation in disease presentation and severity, with a number of studies reporting regional variations in disease presentation [12,13]. A finding of less genetic diversity in clinical compared to environmental strains led to the suggestion that strains of \textit{B. pseudomallei} may differ in their ability to cause disease [14]. However, there is no convincing data demonstrating a correlation between disease presentations and bacterial strains [12]. Mechanisms causing variations in disease severity remain unclear, although differences between the genomes have been proposed [15].

The genomes of \textit{B. pseudomallei} C-141 and \textit{B. mallei} C-5 were compared by subtractive hybridisation [16] and the identified genome differences were compared to the genome sequence data available for \textit{B. pseudomallei} K96243. Some of the sequences absent from strain C-141 were also absent from the genome of strain K96243. However, a significant number of gene sequences absent from strain C-141, were found in both strain K96243 and \textit{B. mallei} C-5. Some of these sequences displayed similarity to mobile elements not previously described for \textit{B. mallei}. These results support the premise of genome-wide intraspecies variability of \textit{B. pseudomallei}.

A similar approach has also been used to identify differences between \textit{B. pseudomallei} and \textit{B. thailandensis} [17] and between \textit{B. mallei} and \textit{B. thailandensis} [18]. The various differences described in these studies included genes encoding proteins associated with biosynthesis, export and translocation of a capsular polysaccharide in \textit{B. mallei}, necessary for the development of glanders in animals. A comparison of two strains of \textit{B. pseudomallei} using subtractive hybridisation identified a number of differences relating to mobile genetic elements, including a temperate bacteriophage [19]. The authors suggested that bacteriophages may be major contributors to the genomic diversity of the species.

3.1. Environmental versus clinical strains

Genotyping of environmental and clinical strains has been performed in an attempt to provide an insight into the relationship between pathogenic human strains of \textit{B. pseudomallei} and the environmental populations from which they originate. Environmental sampling in Thailand was undertaken at disused sites [20], to eliminate anthropogenic events such as ploughing and addition of pesticides, as well as in
agricultural lands [21] used for rice cultivation. Each study reported genetic diversity within strains from the same sites, as MLST and pulsed field gel electrophoresis (PFGE) genotyping showed an uneven distribution of the genetic populations. However, the finding of dominant sequence types at each site suggests that microevolution is occurring through the movement of genomic islands and genetic rearrangements [20].

Apparent clustering of strain types, as determined by MLST, has been reported in northern Australia [15] suggesting regional geographical localisation. A study by Chen, reported that strains from Queensland and the Northern Territory did not share any common sequence types, although they could not be clearly delineated on a dendrogram [15]. Importantly, none of the isolates tested in that study were closely related to any from Southeast Asia, although predominant alleles were identified in strains from both regions. It has been suggested that _B. pseudomallei_ may have originated in Australia and moved to Southeast Asia via animal migration during the Miocene period about 15 million years ago when a land bridge joined the continents [12]. As the Australian and Southeast Asian strains are from different sequence types, but have similar major alleles, it has been hypothesised that as _B. pseudomallei_ is a relatively young species sufficient time has not yet elapsed to permit ancestral alleles to accumulate mutations that allow differentiation. Furthermore, this suggests that the different sequence types result primarily from recombination [15].

### 4. Molecular typing techniques

Ribotyping was one of the first molecular techniques used to differentiate _B. pseudomallei_ isolates. This method is based on the generation of specific patterns resulting from the hybridisation of ribosomal ribonucleic acid (rRNA) from _Escherichia coli_ to restriction endonuclease-digested DNA. Lew et al. [22] established a ribotyping technique investigating epidemiologically related isolates of _B. pseudomallei_ and demonstrated its suitability for epidemiological studies for this species. From a collection of 100 _B. pseudomallei_ strains, isolated from Australia and Thailand, 22 ribotypes were identified. Ribotype 1 included 29 isolates collected from the regions of Thailand and Malaysia. In contrast, the Australian isolates showed much greater diversity, constituting 19 different ribotypes. Some of these were restricted to a particular host species, geographic area or period of isolation.

An analysis of 74 clinical and 10 environmental strains from Thailand [23] demonstrated 10 distinct ribotypes and subtypes. Clinical strains were grouped primarily into ribotypes A to D, with the majority in A1 (64%). The lack of diversity among the clinical strains suggests that ribotype A1 had a regional prevalence in Thailand. Environmental isolates clustered within ribotypes E and F, again suggesting differences in the abilities of organisms to cause disease [23]. To further evaluate the ribotyping technique as an epidemiological tool, Currie et al. typed a select group of _B. pseudomallei_ isolates from Western Australia and the Northern Territory [24]. The group found that the isolates from Western Australia produced an indistinguishable ribotype pattern, however the isolates from the Northern Territory were different from the Western Australian isolates and from each other. These results suggested that there was a clonal introduction
of *B. pseudomallei* into a non-tropical region of Australia that persisted and disseminated over a 25 year period. This data also supports the possible environmental stability of certain ribotypes.

Random amplification of polymorphic DNA (RAPD) is a polymerase chain reaction (PCR)-based typing method which uses short, single, random primers. Using this method, Haase et al. [25] found *B. pseudomallei* isolates previously grouped into one ribotype were able to be further discriminated. However, both Currie et al. [24] and Haase et al. [25] found that isolates from Western Australia demonstrated strain stability using both ribotyping and RAPD techniques.

Pulsed field gel electrophoresis (PFGE) allows for the separation of large molecules of DNA between 10 and 2000 kb in length and is a more recent molecular technique for characterisation of *B. pseudomallei* strains. Vadivelu et al. [26] compared *B. pseudomallei* strains from melioidosis patients in Malaysia by both PFGE and ribotyping and found PFGE was able to differentiate between ribotypes, suggesting that ribotyping may be useful for providing broad epidemiologically-relevant groupings, while PFGE provides finer discrimination between strains [26]. These observations correlate with those of Currie *et al.*, who found that clinical strains collected in the endemic area of Northern Territory, only produced three distinct ribotypes [24].

Although PFGE has been shown to effectively discriminate closely related *B. pseudomallei* isolates, it is a technically difficult and time consuming technique. Furthermore, results from PFGE cannot be easily compared between laboratories. In contrast, MLST has been designed specifically to allow inter-laboratory comparisons. This technique uses PCR to amplify highly conserved housekeeping genes and the DNA sequences of these genes are determined and can then be entered into a WEB-based database to allow for the comparison of isolates for relatedness.

A study by Godoy et al. [11] compared the discriminatory power of PFGE and MLST using a collection of *B. pseudomallei* strains; both PFGE and MLST grouped the isolates into the same major clusters. A later study reported PFGE to be a more sensitive tool than MLST for detecting rapid genetic changes in *B. pseudomallei* [20], especially recombination-mediated changes. As MLST analysis only compares sequence diversity of a small number of generally highly conserved housekeeping genes, it will not identify genomic rearrangements occurring outside these genes. Such genomic rearrangements may be an important means of microevolution. In addition, recent data suggests eBURST, the conventional algorithm used to analyse such allelic profile data may be unreliable for organisms such as *B. pseudomallei* due to high recombination and mutation ratios [27].

### 5. Search for biomarkers

The use of diagnostic indicators of disease, such as blood or tissue-based biomarkers expressed differentially in infected and non-infected individuals, has existed for some time. The accuracy of such assays is dependent on the consistency of altered expression of such biomarkers, and our ability to measure these changes, in all cases of disease [28]. However, typical biological variation in infected hosts, resulting in heterogeneous
response to infection, complicates interpretation of these tests [29]. Therefore, selection of optimal biomarkers is critical for the success of such a diagnostic approach.

The relatively recent determination of the complete genome sequences of B. pseudomallei, B. thailandensis and B. mallei, provides a scaffold for us to understand the complete range of similarities and differences between strains and species. A detailed understanding of the overall genome divergence and intra-gene polymorphisms, both within and between species, should provide us with unique genetic signatures that will enable pathogens to be distinguished from their harmless relatives and also from one another.

Using two-dimensional gel electrophoresis, Wongtrakoongate and colleagues [30] identified 77 unique proteins expressed during the stationary phase of B. pseudomallei growth, permitting the construction of a proteomic reference map. The authors suggested that a number of the identified proteins might act as potential protein markers, capable of distinguishing B. pseudomallei from the other closely related species. A similar study using an evolutionary-based computational approach allowed the selection of a panel of B. pseudomallei antigens. Computational simulation of this data reported a prediction accuracy of at least 92% using 17 antigens [29].

Surface proteins play crucial roles in the host-pathogen interaction and have been exploited as vaccine candidates and diagnostic targets. A total of 35 immunogenic surface proteins were identified using a surface-biotinylation approach and 12 proteins were identified through screening with convalescent human sera. The majority of proteins were membrane or exported proteins. Many proteins were identified by both methods [31]. However, due to culturing in minimal media, this study may have produced biased results, resulting in identification of proteins predominantly associated with the stress response.

Pankla et al. [32] used DNA microarrays to generate genome-wide transcriptional profiles from whole blood of patients with septicaemic melioidosis and patients with sepsis caused by other pathogens. The study demonstrated the existence of a whole blood transcriptional signature capable of distinguishing patients with sepsis from healthy control subjects. Similar to the finding of Keasey et al. [28], this group found that the majority of changes were common to individuals with sepsis, regardless of the infectious agent. However, class prediction analysis was able to identify a candidate 37 transcript diagnostic signature that distinguished melioidosis-induced sepsis from sepsis caused by other organisms with 100% accuracy. This transcriptional signature was significantly enriched for genes encoding proteins involved in the MHC class II antigen processing and presentation pathway. The application of signatures such as those described above, may allow diagnosis of potentially fatal melioidosis prior to the onset of clinical symptoms.

The increasing level of technology and decreasing cost of molecular applications over the last decade has resulted in a rapid rise in our detailed understanding of B. pseudomallei and its close relatives at the molecular level. One important area of increased understanding has been in the evolutionary biology of the organism, with detailed MLST data coming from an ever increasing number of strains. However, as with many scientific advances, new questions continually arise just as others are answered. Important questions still remain regarding the biogeography of B. pseudomallei (in Section IX.1) and what genes are critical for its survival in the soil and during the
transition from environmental saprophyte to mammalian pathogen. Another decade of research as fruitful as the last, may lead to the resolution of these and many more currently unanswered questions.

References


Section III.2

The *Burkholderia pseudomallei* genome – an emerging model for microbial complexity and pathogen virulence

Tannistha Nandi\(^a\), Patrick Tan\(^a,b\)

\(^a\) Genome Institute of Singapore, Singapore, Republic of Singapore
\(^b\) Duke-NUS Graduate Medical School Singapore, Singapore, Republic of Singapore

Abstract

The 7.2 Mb *Burkholderia pseudomallei* genome represents one of the most complex bacterial genomes sequenced. Comprising two chromosomes, the genome contains large sets of genes and gene families encoding a vast array of biological functions, reflecting its clinical, ecological, and phenotypic diversity. Since 2004, the *B. pseudomallei* K96243 reference genome has been used by many researchers to identify new virulence genes and pathways, and comparative genomic comparisons between *B. pseudomallei* and related species such as *B. mallei* and *B. thailandensis* have shed light on the pivotal evolutionary events that have allowed *B. pseudomallei* to infect humans and other mammals. Over the past years, several other *B. pseudomallei* genomes have also been completed. Comparisons between these genomes have allowed delineation of the *B. pseudomallei* core and accessory genomes, and improved our understanding of how genome rearrangements, gene gain and loss, and single nucleotide polymorphisms can affect *B. pseudomallei* biology and virulence. In tandem with DNA-level genomic analysis, transcriptomic analysis of *B. pseudomallei* has also revealed that the *B. pseudomallei* genome is subject to high levels of regulation, with specific sets of genes only expressed under particular conditions. Future work will focus on the comparative analysis of large panels of *B. pseudomallei* strains, importantly drawn from non-clinical sources such as animals and the environment. Such information will further illuminate the *B. pseudomallei* genome and its contribution to bacterial diversity and flexibility.

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1. Introduction

Clinical data suggests that *B. pseudomallei* is primarily an opportunistic pathogen, with most melioidosis patients being elderly and diabetic [1]. Although Southeast Asia and Northern Australia have traditionally been considered the major endemic regions for *B. pseudomallei* [1,2], melioidosis outbreaks caused by the bacterium have also been reported in Brazil, Taiwan, and Hong Kong [2]. *B. pseudomallei* can be isolated from
many environmental sources such as soil, water, and air, and in endemic areas infections in humans are thought to be primarily acquired through direct contact (e.g. cuts through the skin) or inhalation of contaminated soil or water [1,3].

*Burkholderia pseudomallei* is well known for its striking ecologic and clinical versatility and can survive for prolonged periods in harsh environmental conditions such as desiccation, fluctuating temperatures, high salt, and lack of nutrients [1,4]. The ability of *B. pseudomallei* to persist in a myriad of diverse environments makes it a major source of community acquired infections in endemic areas [5]. Different strains of *B. pseudomallei* can display striking differences in virulence, biofilm formation, colony morphology, and growth rates [6]. Wide spectrums of symptoms are associated with human melioidosis, and *B. pseudomallei* infections can mimic many other disease conditions, often delaying accurate diagnosis [1]. The bacterium is resistant to broad spectrum antibiotics and is able to survive intracellularly in infected cells [1] and also in the extracellular milieu. Besides humans, *B. pseudomallei* is also capable of infecting many other species, including nematodes, amoebae, dolphins, birds, swine, sheep, and gorillas [7]. Taken collectively, the striking versatility of *B. pseudomallei* to grow in a variety of environments and hosts suggests that it would contain a highly complex genome.

2. The *Burkholderia pseudomallei* K96243 genome reveals high degrees of molecular complexity

The prediction of a complex genome was borne out by the completion of the first *B. pseudomallei* genome sequence in 2004. In a landmark study led by the Sanger Center [3], the genome sequence of a clinical isolate from Thailand (K96243) was determined and shown to be exceptionally large (7.25 Mb), placing it among the top 5% of sequenced microbial genomes to date. Computational analysis of protein coding genes in *B. pseudomallei* K96243 predicted an overall proteome size of 5728 proteins, similar to single celled eukaryotic species such as *Schizomyces pombe*. Strikingly, the *B. pseudomallei* genome was found to be distributed across two chromosomes, unlike most other prokaryotic species (other examples of microbes with two chromosomes include *Agrobacterium tumefaciens* and *Ochrobactrum anthropi*).

Intriguingly, the two chromosomes (4.1 Mb and 3.2 Mb) also appeared to differ substantially in gene content. Gene ontology analysis revealed that many core functions, such as those linked to central metabolism and cell growth were largely encoded on Chr 1, while accessory functions associated with adaptation and survival tended to be localised to Chr 2. To explain these functional differences, it was proposed that Chr 1 and 2 may have been acquired at different times by the *Burkholderia* ancestor, with Chr 1 representing the ancestral chromosome, and Chr 2 a later acquisition, being present initially as an accessory genetic element, but ultimately acquiring essential functions that rendered it an integral part of the *B. pseudomallei* genome. This two-step model of chromosomal acquisition is consistent with the observation that a greater number of orthologous genes are on Chr 1 compared to Chr 2 across several members of the *Burkholderia* family.
(e.g. *B. mallei*, *B. thailandensis* and *B. cepacia*), indicating that Chr 1 is likely the core chromosome, while Chr 2 is more variable [8].

Another important observation following analysis of the *B. pseudomallei* genome was the identification of several genomic regions exhibiting striking deviations in sequence composition (GC content, dinucleotide composition) from the general genome backbone. Such regions are referred to as “genomic islands”. Twelve genomic islands were identified on Chr 1, and four were identified on Chr 2, in total occupying 6% of the entire *B. pseudomallei* genome [3]. The atypical sequence features of genomic islands suggest that they have been acquired through horizontal gene transfer. A more detailed presentation of the *B. pseudomallei* genomic islands and their evolutionary and phenotypic implications is provided in Section III.3 of this monograph.

Bioinformatic analysis of the *B. pseudomallei* genome identified several genes likely to contribute to its various niche preferences and biological phenotypes. For example, *B. pseudomallei* exhibits a high degree of intrinsic resistance to commonly used antibiotics, including penicillins, macrolides and aminoglycosides. Resistance-nodulation-cell-division (RND) efflux pumps are recognised as major causes of multi-drug resistance in many Gram-negative bacteria, and *B. pseudomallei* has at least 10 operons that can code for efflux pump components; seven on Chr 1 and three on Chr 2. These include MexAB-OprM, MexXY-OprM, MexCD-OprJ, MexEF-OprN and AmrAB-OprM. The AmrAB-OprM efflux pump, was experimentally confirmed to confer resistance to *B. pseudomallei* against aminoglycosides and other macrolides. Furthermore, expression of several *B. pseudomallei* RND pumps can be induced by exposure to toxic compounds [9]. Resistance of *B. pseudomallei* to cationic peptides such as polymyxin B [3,10] may be due to another multidrug efflux pump, whose homologs have been shown to confer polymyxin resistance in *B. vietnammensis*. Besides efflux pumps, *B. pseudomallei* also has several clusters of secondary metabolism genes, mostly located on Chr 2, that may produce metabolites with antibiotic properties, as well as lipopeptides (such as surfactin), and siderophores to promote its survival in soil against competing microorganisms.

*Burkholderia pseudomallei* contains an impressive arsenal of genes likely to contribute to its broad host range and its ability to cause disease. The genome encodes several type IV pili (TFP) and fimbrial clusters which may be involved in adherence and recognition of host cells. Eight TFP-associated loci and six chaperone-usher type I fimbrial clusters have been identified in *B. pseudomallei* K96243 [3,11]. Experimental disruption of the *B. pseudomallei pilA* TFP gene, results in reduced bacterial adherence to human epithelial cells and decreased virulence in murine models of melioidosis [11]. The genome also contains several genes encoding proteins with identity to known haemagglutinins and surface proteins from other species. One such group is the Hep-Hag repeat family of proteins (Pfam domain PF05658) that are associated with haemagglutinins and invasins [12].

The *B. pseudomallei* genome contains three distinct type III secretion system (T3SS) loci, which can operate to facilitate pathogen invasion, intracellular survival, and intercellular spread. Two of these T3SS (T3SS1 and T3SS2) are homologous to the T3SS of the plant pathogen *Ralstonia solanacearum*, and one (T3SS3) is homologous to the *Salmonella* SPI-1 pathogenicity island. In a hamster model, the T3SS3 was
required for full virulence [13]. In contrast, a role for T3SS1 and T3SS2 in mammalian virulence remains to be convincingly demonstrated. Several gene clusters have been identified that encode proteins involved in the synthesis of surface carbohydrates (e.g. capsular polysaccharide, type II OPS) which function to protect the bacterium against host immune defenses. The *B. pseudomallei* capsule (type I O-PS) has been shown to be a critical virulence determinant in respiratory tract infections and an important antigen for generating Th1 immune responses commonly observed in systemic melioidosis [14]. Studies have also shown that lipopolysaccharide (LPS; type II O-PS) plays a critical role in virulence [15,16]. Interestingly, immunisation of mice with killed LPS type III O-PS or type IV O-PS and *B. pseudomallei* mutant strains unable to synthesise these molecules conferred different degrees of protection, demonstrating the immunological importance of the *B. pseudomallei* polysaccharide clusters, and their potential use as vaccine candidates [17]. Compared to other pathogenic bacteria with genomes of similar size (such as *B. pseudomallei* and *Y. pestis*), or comparable GC content, a fascinating feature of the *B. pseudomallei* genome is the presence of an unusually high number of tandem and simple sequence repeats (SSRs) [18,19]. In *B. pseudomallei*, tandem repeats appear to occur mostly in intergenic regions, while SSRs are primarily located within gene coding regions. Once again reflecting the differences between the *B. pseudomallei* chromosomes, *B. pseudomallei* Chr 2 has a high density of tandem repeats (~102.1 repeat arrays/Mbp) compared to Chr1 (~69.9 repeat arrays/Mbp). A large fraction of these repeats occur within or close to genes encoding surface or secreted proteins that may directly interact with host factors during pathogenesis, or with environmental factors during growth in the environment. Strain to strain variation in these repeats may represent an important mechanism of phase variation, facilitating the ability of *B. pseudomallei* to survive and adapt to host immune defences [20]. The availability of a complete *B. pseudomallei* genome has made it possible for research groups to rapidly identify gene homologs, and to test these homologs for possible functions in virulence. For example, type VI secretion systems, initially identified to be important virulence factors in *Pseudomonas aeruginosa*, were rapidly found to be present in *B. pseudomallei*, where they were also shown to play a role in virulence [21]. Such examples highlight the major impact the *B. pseudomallei* genome has played in the field in accelerating the discovery of putative genes.

3. Genomic comparisons between related *Burkholderia* species identifies genes related to pathogen evolution and niche specialisation

The genome sequences of two related *Burkholderia* species, *B. mallei* (strain ATCC 23344) and *B. thailandensis* (strain E264/ATCC700388), were completed either simultaneously with, or relatively soon after the *B. pseudomallei* K96243 genome. These three species represent distinct states of *Burkholderia* ecological niche adaptation; *B. pseudomallei* being an opportunistic pathogen, *B. thailandensis* an environmental saprophyte that is considered non-infectious to mammals, and *B. mallei* a host-restricted obligate pathogen (horses and other solipeds) [18,22,23]. Comparisons between the
genomes of these different species have allowed researchers to identify genes contributing to different steps in the evolution of pathogen virulence, and gain a better understanding of the cellular processes leading to increased niche specialisation.

A comparison of the B. pseudomallei and B. thailandensis genomes revealed only a few large scale genomic differences (after exclusion of genes in GIs), providing molecular explanations for previously known phenotypic differences between B. pseudomallei and B. thailandensis. For example, the ability of B. thailandensis, but not B. pseudomallei, to assimilate arabinose and xylose [23,24] was explained by the presence of distinct arabinose assimilation and xylose metabolism gene clusters in B. thailandensis, which were absent in B. pseudomallei. Genomic island 8 is found in place of the xylose metabolism cluster in B. pseudomallei, suggesting that a horizontal gene transfer has taken place in this region. This example thus provides an intriguing case where horizontal gene transfer has resulted in both gene gain (genes located on Genomic island 8) and gene loss (xylose metabolism genes). Another important large-scale gene replacement event involves a flagellar biosynthesis gene cluster present in B. thailandensis (BTFC – Burkholderia thailandensis like flagellar cluster) which has been replaced in B. pseudomallei by a group of fimbriae-related genes that have highest similarity to genes from Yersinia (YLF). Although an exact functional role for the YLF in B. pseudomallei virulence has yet to be experimentally determined, recent genomic comparisons between different B. pseudomallei strains have revealed the surprising finding that certain B. pseudomallei strains also carry a set of genes more similar to the BTFC than the YLF [25]. B. pseudomallei strains containing the YLF gene cluster are commonly recovered from clinical cases, whereas strains containing the BTFC gene clusters are more likely to be environmental isolates, supporting a potential role for the YLF in virulence.

Certain large scale gene differences have been identified between B. pseudomallei and B. thailandensis, and these are likely to have been pivotal to the pathogenic evolution of B. pseudomallei. Indeed, one example of a virulence related genetic change is the presence of the arabinose gene cluster. Genetically modified B. pseudomallei expressing the full arabinose operon exhibited a downregulation of gene expression in T3SS3 genes, and a reduction of virulence when these genetically modified strains were exposed to arabinose [26]. This finding suggests that the ability to assimilate and metabolise arabinose may have been important for the ability of B. pseudomallei to function as a mammalian pathogen. A further clear genetic difference between B. pseudomallei and B. thailandensis is the polysaccharide capsule gene cluster which has been shown to be an essential virulence determinant in B. pseudomallei [15,16]. Comparisons with the B. thailandensis genome revealed that the capsule gene cluster was likely non-randomly transferred into the B. pseudomallei genome, replacing a pre-existing gene cluster in B. thailandensis already dedicated to polysaccharide synthesis [22,23].

Beyond these large-scale differences however, both the B. pseudomallei and B. thailandensis genomes are in fact broadly similar and share large numbers of conserved metabolic genes and virulence components. For example, the B. thailandensis genome contains two type III secretion systems (T3SS2 and T3SS3), various antibiotic resistance genes, type IV pili genes, haemolysin-related genes, and genes encoding several adhesion factors
and proteases [23]. The presence of these virulence-associated genes in *B. thailandensis* suggests that *B. thailandensis* might also be a natural pathogen, perhaps of nematodes or amoebae, which the bacterium might encounter in soil [27,28]. However, a study comparing orthologous virulence genes between *B. pseudomallei* and *B. thailandensis* revealed that virulence genes were significantly more genetically divergent in nucleotide sequence between the two species compared to core metabolic genes or the rest of the genome [23]. This finding also raises the provocative possibility that a limited number of large scale alterations, coupled to fine scale alterations in shared orthologous virulence genes, may have been sufficient for *B. pseudomallei* to become a mammalian pathogen.

Compared to *B. thailandensis* and *B. pseudomallei*, the host-restricted pathogenic species *B. mallei*, has a significantly smaller genome size of 5.7Mb. Previous multi-locus sequence typing (MLST) analysis comparing *B. pseudomallei*, *B. thailandensis* and *B. mallei*, has suggested that while *B. pseudomallei* and *B. thailandensis* are distinct species, *B. mallei* is a clone of *B. pseudomallei* [29]. This evolutionary model is supported by the finding that almost all *B. mallei* genes have orthologs in *B. pseudomallei*, and that there are almost no *B. mallei*-specific genes (less than eight, mostly hypothetical proteins or phage integrases) [30]. Therefore *B. mallei* may have arisen as a founder population from a single *B. pseudomallei* strain, most likely after colonisation of an equine-like ancestral host. Several features in the *B. mallei* genome strongly suggest that the *B. mallei* genome is still undergoing active reductive evolution to support its existence in the host niche environment. Many genes required for environmental adaptation that were likely important for *B. pseudomallei* to survive in soil have been inactivated or deleted in *B. mallei*. For example, a 65-kb insert flanked by insertion sequence (IS) elements in *B. mallei* has disrupted the *fliP* gene, an essential gene for flagellum biogenesis, and a frameshift mutation in the flagellum motor gene *motB*, eliminates its functionality. These mutations are likely to account for *B. pseudomallei* being non motile and non flagellated [18]. These non-functional genes were likely required for *B. pseudomallei* to survive in the environment, but have become non-essential to the altered role of *B. mallei* as an obligate mammalian parasite, that now cannot survive outside its equine host. Genome-wide comparisons of chromosomal order between the three species also revealed that while *B. pseudomallei* and *B. thailandensis* genomes were highly syntenic with only a few large scale inversions, the *B. mallei* genome contains a significantly larger number of genome rearrangements compared to *B. pseudomallei* (Figure III.1). Most of these genome rearrangements in *B. mallei* are flanked by IS407A elements that can facilitate homologous recombination events, implicating IS element-mediated recombination as a major force for genetic change in *B. mallei*. Besides rearranging genomes, IS-mediated recombination could have also caused deletions of metabolic gene clusters via recombination between flanking IS elements, leading to an overall reduction in the size of the *B. mallei* genome [30]. Finally, the increased number of genome rearrangements in *B. mallei* may have also enhanced levels of genetic diversity in *B. mallei* protein coding genes, as genes localised near to the boundaries of genome rearrangement events have been shown to exhibit increased sequence diversity compared to genes located further away from breakpoint boundaries [8].
Fig. III.1. Genomic synteny of the (A) *B. pseudomallei* and *B. thailandensis* genomes and the (B) *B. pseudomallei* and *B. mallei* genomes. The *B. pseudomallei* and *B. thailandensis*/*B. mallei* genomes are depicted on the x and y axes respectively using Chromosome I & II forward strand. Inverted orientations of segments indicate regions of genomic inversion.

4. Beyond single strains – the *Burkholderia pseudomallei* accessory genome

Since 2004, advances in sequencing technologies have allowed the completion of more than 20 additional *B. pseudomallei* genome sequences. The availability of genome sequences for multiple strains of the one species has provided researchers with the means to probe the stability of the *B. pseudomallei* genome, and determine to what extent the *B. pseudomallei* genome varies between strains. The portion of the genome that is variably present between strains is often referred to as the “accessory genome”, while the “core genome” refers to the portion of the genome conserved across all strains. Defining the *B. pseudomallei* accessory genome is of important scientific and clinical value, as work in other pathogens has shown that genes on accessory elements contribute to important phenotypic differences between strains. For example, in other γ proteobacteria genera (*Escherichia*, *Pseudomonas*, *Vibrio*), the accessory genome can encompass up to 20% of all genomic content [31–33]. In *B. pseudomallei*, previous analyses have also suggested that the specific content of the *B. pseudomallei* accessory genome may play an important role in determining the clinical manifestation of disease in different patients with melioidosis [34].
Genomic studies probing the *B. pseudomallei* accessory genome have largely used two separate methods − array based comparative genomic hybridisation (aCGH) and whole-genome sequence (WGS) comparisons. In aCGH, genomic DNA from different *B. pseudomallei* strains have been compared to one another using microarrays containing probes representing some or all *B. pseudomallei* K96243 genes. One advantage of the aCGH approach is that it is rapid and cheap, allowing many strains to be compared simultaneously. However, a disadvantage of aCGH is that the information obtained is limited to the genomic content present on the microarray (typically the *B. pseudomallei* reference genome), such studies cannot interrogate regions of the genome that are not present in *B. pseudomallei* K96243. The first *B. pseudomallei* aCGH study was reported in 2004, where a *B. pseudomallei* K96243 microarray was used to compare 23 natural isolates of *B. pseudomallei*, *B. mallei*, and *B. thailandensis* to identify genes that were differentially present either between the different species, or between strains belonging to the same species [35]. This analysis identified several species-specific genes associated with diverse cellular functions, including nitrogen and iron metabolism, quorum sensing, and polysaccharide production.

Following this initial study, a more detailed aCGH analysis of a larger *B. pseudomallei* strain cohort (close to 100 clinical, animal and environmental *B. pseudomallei* isolates) was able to provide more accurate estimates of the *B. pseudomallei* accessory genome. Approximately 13% of the *B. pseudomallei* K96243 genome was found to be differentially present between the different *B. pseudomallei* strains. While some of these accessory elements were localised to the genomic islands, several other regions of genomic variation (referred to as genomic islets) were also identified. Because of the unidirectional nature of aCGH and the fact that the study did not include strains from geographically distinct areas (this study only focussed on Southeast Asian strains and did not include strains from Australia), this number (13%) almost certainly represents a lower limit with respect to the fraction of the *B. pseudomallei* genome that is variable. Interestingly, it was found that of the 16 genomic islands in the K96243 genome, only 14 were represented by accessory genes, while two genomic islands (7 and 14) were found in all strains, suggesting that these should be regarded as part of the *B. pseudomallei* core genome. At present, it is unclear why genomic islands 7 and 14 are stable, given that they also contain many hallmarks of recently acquired elements. Another intriguing finding from this study was that isolates associated with human melioidosis exhibited a tendency to harbour certain genomic islands, compared to isolates from either animal or environmental sources, suggesting that genes on these islands might facilitate colonisation of the human host. This hypothesis was further supported by experimental data where disruption of a fimbriae-like gene on genomic island 16, led to a significant decrease in the ability of *B. pseudomallei* to adhere to human cells. This supports the notion that the *B. pseudomallei* accessory genome may play a central role in adaptation and virulence [7].

Whole-genome sequence comparisons have also been used to define the *B. pseudomallei* core and accessory genomes. While this method has the advantage of being “open” in that sequences not found in the *B. pseudomallei* K96243 genome can be detected, its major limitation is that only sequenced genomes can be compared, which can limit the number of strains. Tuanyok et al. [36] studied five sequenced genomes of *B. pseudomallei*.
and identified 71 genomic islands differentially present across multiple *B. pseudomallei* strains, further emphasising the importance of these elements for shaping the genetic composition of individual strains and populations within this bacterial species. A more comprehensive whole-genome sequence analysis of 12 *B. pseudomallei* strains has also estimated that the *B. pseudomallei* accessory genome is likely to be at least 7500 genes, almost double the number of genes in the *B. pseudomallei* core genome [37]. This tremendous amount of genomic variation between different *B. pseudomallei* strains is consistent with the high level of phenotypic diversity and flexibility seen between different *B. pseudomallei* strains.

5. Genetic variation in the *Burkholderia pseudomallei* core genome

Another important area of investigation made possible by the availability of multiple *B. pseudomallei* genome sequences has been the role of fine scale molecular alterations, such as single nucleotide polymorphisms (SNPs) and small insertions or deletions (microindels) in contributing to the phenotypic versatility of *B. pseudomallei*. In contrast to the accessory genome studies, where large patterns of gene gain and loss can be inferred from using platforms such as variable amplicon typing and DNA microarrays, studying such fine-scale genetic variation events requires nucleotide-level sequence information of multiple strains. The fact that such data has only recently become available may explain why the impact of SNPs on virulence has been relatively understudied, despite SNPs being the most common cause of natural genetic variation among members of the same species. However, recent studies in pathogens such as group A Streptococcus have demonstrated an important role for such micro-level alterations in influencing pathogen virulence [38]. The first comprehensive nucleotide-scale comparative analysis of multiple *B. pseudomallei* genomes was reported by Nandi et al., 2010 [37], and described a *B. pseudomallei* core genome of 4909 genes, along with a *B. pseudomallei* “pan-genome” of 7500 genes. When the sequences of core genome genes were compared between the strains, it was revealed that each *B. pseudomallei* strain exhibited an average of approximately 8600 SNPs compared to the K96243 reference genome, demonstrating a significant amount of genetic variation in the *B. pseudomallei* core genome. Strains with the highest levels of genetic variation compared to K96243 were observed in isolates from Australia, the most geographically distant locale, while strains from Southeast Asia exhibited less genetic variation when compared to K96243, another Southeast Asian strain. The power of using genome-wide SNP distributions to infer phylogenetic relationships between *B. pseudomallei* strains and other related species was also shown by Pearson et al. [39], who used SNPs inferred from whole-genome sequencing of 43 *B. pseudomallei* genome sequences to establish robust strain to strain relationships, overcoming the confounding problems due to horizontal gene transfer and high levels of recombination. The SNP-based phylogeny also indicates an Australian origin for *B. pseudomallei*, with entry into Southeast Asia characterised by a single introduction event during a recent glacial period.

Beyond its use for phylogenetic studies, the patterns of genetic variation in the *B. pseudomallei* core genome could have functionally important implications. Evidence
for high levels of genetic dynamism in the *B. pseudomallei* core genome was established when it was found that a significant proportion of the *B. pseudomallei* core genome may be undergoing functional selection (otherwise known as positive selection). Importantly, the positively selected genes were not randomly distributed but were enriched in a few specific functional classes such as carbohydrate and secondary metabolism [37]. Genome comparisons between the *B. pseudomallei* strains also highlighted that many classical virulence genes such as adhesins, fimbrae, exopolysaccharides and type III secretion systems (T3SS) are part of the core genome [7], suggesting that small changes in these virulence genes and other core genome components might also influence *B. pseudomallei* mammalian pathogenicity. Studies of core genome genes undergoing evolutionary selection have identified new virulence genes and pathways including the Type IV pili (TFP), which are under positive selection [40]. It was shown that strains without a functional TFP4 locus exhibited significantly reduced virulence compared to parental *B. pseudomallei* K96243 wild-type controls, supporting a role for Type IV minor pilin activity in murine virulence. These results suggest that a subset of positively selected genes in *B. pseudomallei* may influence virulence in mammals [37]. Another virulence-related modification likely contributing to *B. pseudomallei* virulence was the finding that genes involved in host metabolite catabolism also showed signatures of positive selection. The taurine dioxygenase gene (*tauD*) involved in the catabolism of taurine, an animal-specific amino acid, was discovered to be both positively selected and exhibiting a species-specific expansion of several *tauD* gene members in *B. pseudomallei* [37]. These findings suggest that altered taurine metabolism, likely mediated by *tauD*, may represent a species-specific adaptation of *B. pseudomallei* that may have also facilitated its ability to survive in infected mammalian hosts [41]. Supporting this, taurine-exposed *B. pseudomallei* exhibited transcriptional up-regulation of ~280 genes, of which 40% (126 genes) have been previously associated with pathogenicity, host–cell interaction, or survival in diverse and challenging environments.

Whole-genome sequence comparisons between *B. pseudomallei* strains have also provided insights into clinical aspects of melioidosis. Genome comparisons have been performed on paired clinical isolates, where one sequence strain represented the primary infection, while the other represented the strain isolated during patient disease relapse, with an intervening period of typically three years. Surprisingly, these comparisons failed to reveal a significant number of newly acquired mutations in relapsed strains [37]. This finding suggests that the *B. pseudomallei* genome is likely to exhibit a high degree of stability during in vivo infection and persistence. However, it is worth noting that this finding which was based on genome analysis of *B. pseudomallei* coding genes, appears to contradict a related study examining the genetic evolution of *B. pseudomallei* during acute infection, where *B. pseudomallei* strains were compared using variable number tandem repeat typing (VNTR) from multiple sites of the same patients [42]. In this study, it was reported that *B. pseudomallei* mutates rapidly both in *vitro* and in *vivo* at tandemly repeated loci. These apparently conflicting findings may be due to differences in study design or the techniques used to study variation. Given the importance of understanding the mechanisms under which *B. pseudomallei* can persist in an infected host, this represents an area that needs to be further investigated.
6. Transcriptomics – Defining global regulation in *Burkholderia pseudomallei*

One final important area of biological research made possible by knowledge of the *B. pseudomallei* genome has been transcriptomics – the global study of gene regulation in *B. pseudomallei*. Information gleaned from transcriptomics has allowed researchers to identify the specific genes expressed by *B. pseudomallei* in response to different environmental signals and conditions. Such information is invaluable in enhancing our understanding of how *B. pseudomallei* is able to adapt and thrive in a multitude of environments. One of the first *B. pseudomallei* transcriptome studies involved comparing the transcriptomes of two different strains of *B. pseudomallei* [43]. Interestingly, this study revealed that genes differentially expressed between the strains were largely associated with the genomic islands, and in many cases could be interpreted as a consequence of differential gene gain and loss. This is significant in that the remainder of the *B. pseudomallei* genome appeared to be conserved in expression between the two strains, suggesting that the core transcriptional programme emanating from the *B. pseudomallei* core genome may be relatively stable.

Another study attempted to identify the global repertoire of genes in *B. pseudomallei* that are regulated during the process of growth in rich media. Rodrigues et al. [44] showed that approximately 17% of all *B. pseudomallei* genes display regulated expression during growth in rich medium, with broad waves of functionally coherent gene expression tightly associated with distinct growth phases and transition points. Virulence genes were found to be regulated across all growth phases and certain genes, including *serC*, were identified as potential novel virulence factors by virtue of their co-expression with other early-phase virulence genes. In addition, *B. pseudomallei* *serC* mutants are serine auxotrophs and display a dramatic attenuation of virulence in a mouse infection model compared to wild-type *B. pseudomallei*.

The *in vivo* gene expression profile of *B. pseudomallei* was analysed by comparing whole-genome transcriptomes of bacteria recovered from infected hamster organs, including liver, lung, and spleen following intraperitoneal and intranasal routes of infection with those from bacteria grown *in vitro* [45]. In this study, potential *B. pseudomallei* virulence genes such as a putative phospholipase C and a putative two-component regulatory system were differentially expressed, providing a better understanding of bacterial metabolism in response to the host environment during acute melioidosis.

7. Future directions and conclusions

The recent decade has seen a transformational shift in *B. pseudomallei* research due to the completion of the *B. pseudomallei* genome sequence. The availability of additional *B. pseudomallei* strain genomes has permitted analyses not previously possible, for example the identification of genes with signatures of positive selection. This genomic information will undoubtedly increase significantly in the near future, due to the increased throughput and reduced cost associated with new deep sequencing platforms that can permit sequencing of multiple strains simultaneously [46]. Future work will involve
analysis of larger *B. pseudomallei* strain panels, particularly those including more animal and environmental isolates, to gain a more complete understanding of the limits of *B. pseudomallei* variation in the wild. Another important goal will be the in-depth characterisation of the *B. pseudomallei* transcriptome, not just across protein coding genes, but for non-coding RNAs as well [46]. This latter work will also likely see rapid development, particularly with the availability of tiling arrays and RNA-seq platforms [46].

**References**


Section III.3

Genomic islands in *Burkholderia pseudomallei*

Apichai Tuanyok

Department of Biological Sciences and Center for Microbial Genetics and Genomics, Northern Arizona University, Flagstaff, Arizona, USA

Abstract

*Burkholderia pseudomallei* genomes are greatly diverse due to the high frequency of genetic recombination. Genomic differences are most likely associated with differential possession of genomic islands (GIs), the foreign, mobile and unstable genetic elements, which are mostly acquired through horizontal gene transfers. Studies have demonstrated that locations of GIs are not random, as many of them are associated with tRNA gene loci. The term “tRNA-mediated site specific recombination or tRNA-SSR” has been used to describe the mechanisms that facilitate this type of genetic recombination. A standardised nomenclature for GIs in *B. pseudomallei* has been proposed. The functional roles of most GIs are unknown. However, the differential presence of GIs across multiple *B. pseudomallei* genomes is predicted to be one of the major causes of the various clinical manifestations observed in melioidosis patients, as well as the widely diverse bacterial fitness and phenotypes observed between strains. This data indicates the importance of GIs in shaping the genetic composition of individual strains and populations within this bacterial species.

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1. Introduction

The availability of multiple *B. pseudomallei* genomes has enabled researchers to better understand the diversity of *B. pseudomallei* populations and provided insights into phenotypic differences between strains that can be linked to differential virulence. *B. pseudomallei* is known as one of the most recombinogenic bacterial species [1]. It is believed that the great genomic variation observed between *B. pseudomallei* strains is a result of its “open genome” [2], which recombines at a high frequency and readily incorporates genomic material from conspecific and other species. Genomic islands (GIs) are large DNA segments which have been observed to be differentially present in any of the *B. pseudomallei* genomes being compared. These observations were made from several studies even before the first complete genome of strain K96243 was published. Such initial studies used simple DNA-DNA hybridisation techniques (e.g. suppression subtractive hybridisation or SSH) to study the diversity of two different genomes. Several variable genomic loci e.g. a functional prophage in *B. pseudomallei* strain 1026b [3] a
A novel two-partner secretion (TPS) system in strain 08 [4], and 45 SSH-derived sequences used in a VAT (variable amplicon typing) scheme [5] were discovered. These discoveries showed the diversity of *B. pseudomallei* genomes, with the majority of these DNA segments located in the GIs. In 2004, with the completion of the *B. pseudomallei* K96243 genome, GIs were identified as a major feature of the *B. pseudomallei* genome for the first time. The high diversity of these GIs was further elucidated when many *B. pseudomallei* strains were tested by PCR [6]. The diversity of *B. pseudomallei* genomes became clearer when Sim et al., reported that 14% of the K96243 genes were part of the accessory genome because they were variably present or absent across 94 tested strains from Southeast Asia using CGH-microarrays; most of these accessory genome genes were GI genes [7]. However, even this 14% accessory genome identified from the comparison of Southeast Asian strains may not represent the maximal diversity of the *B. pseudomallei* population. Indeed, there are two distinct genomic groups in the *B. pseudomallei* population: Australian and Asian groups [8]. With this in mind, genomic analyses and GI identification in subsequent studies have used multiple genomes from both *B. pseudomallei* population groups [1,9].

### 2. Criteria for the identification and nomenclature of genomic islands

Genomic islands in *B. pseudomallei* can be observed as large variable chromosomal segments when at least two genomes are compared and displayed using bioinformatic tools such as Artemis Comparison Tool (ACT). Criteria for the identification of GIs have been proposed for most bacterial species [10] and can be successfully applied to the GIs in *B. pseudomallei* [6]. GIs are large inserts which have distinct percent G+C content compared to the rest of the genome, and usually contain mobility genes such as insertion sequence (IS) elements, and/or genes known to be involved in facilitating genetic recombination (e.g. integrase, transposase, and recombinase genes). Using these criteria, 16 GIs were identified in the K96243 genome [6]. In addition, when four new genomes (strains 1710b, 1106a, MSHR668 and MSHR305) were used in the analysis, as many as 71 different GIs were identified [9]. It is quite likely that the GIs identified in *B. pseudomallei* will continue to increase as additional genome sequences are used in the analyses. A standard nomenclature for GIs has been proposed based upon the differences in terms of locations and gene content between the newly identified GIs and the original 16 GIs in K96243 [9]. These criteria include (i) precedence: GIs found at the same relative GI locations and containing the same gene contents will be given the same name as the GIs previously identified in K96243; (ii) unique gene composition: GIs containing different gene content but located at the same reference genomic location will be given the same name as GIs in K96243 but with suffix differentiation (e.g. x.1, x.2, x.3) based upon the order of their discovery; and (iii) unique genomic location: novel GIs discovered at genomic locations located between two consecutive reference GI locations in K96243 will be named with a lower case letter (a to z) suffix indicating a new insertion position. Using these criteria, at least 128 different GIs have been identified so far in 11 *B. pseudomallei* genomes from strains K96243, 1710b, 1106a, MSHR668, MSHR305, MSHR346, Pasteur 52234, 406e, S13, Bp22, and MSHR1655 (Figure III.2).
Fig. III.2. Genomic locations (n=42) of 128 different GIs on chromosomes 1 and 2 in B. pseudomallei. Previously identified GIs from strains K96243, 1710b, 1106a, MSHR668, and MSHR305 are identified with black text [9]. Newly identified GIs from six additional genomes from strains MSHR346, Pasteur 52237, 406c, S13, Bp22, and MSHR1655 are identified with red text. Six new genomic hotspots for recombination (GI4a, GI5a, GI7a, GI8e, GI9d, and GI15f) were identified. The genomic location of two mutually exclusive genomic regions, BTFD (B. thailandensis-like flagella and chemotaxis gene cluster) and YLF (Yersinia-like fimbrial gene cluster; [8]) is also indicated.

3. Locations of genomic islands are not random

A number of studies have demonstrated that most GIs are located adjacent to tRNA genes. It is believed that GI insertions are mediated by tRNA genes. There are between 59 and 61 tRNA genes distributed throughout each B. pseudomallei genome. Recombination at a tRNA gene is usually initiated at its 3′ end sequence. It has been observed that most foreign genetic elements have a short DNA sequence (e.g. attP site of a bacteriophage genome) which is identical to the 3′ end sequence of a bacterial tRNA gene (attB site). This allows site-specific recombination at this particular genomic site and usually creates a short, direct repeat sequence of the tRNA gene downstream of the integration site. The term “tRNA-mediated site specific recombination or tRNA-SSR” has been used to describe this mechanism in B. pseudomallei [9]. These direct repeats are very common in B. pseudomallei prophages. Studies have demonstrated that a functional prophage in K96243 (or Myoviridae bacteriophage) has a direct repeat of 3′ end tRNA-Phe gene [6], while the 1026b prophage (or Siphoviridae bacteriophage) was flanked by a short direct repeat of tRNA-Pro [3]. The number of tRNA-SSR are varied among B. pseudomallei strains. At least eight, three, two, five and five tRNA-SSR events are present in the genomes of K96243, 1710b, 1106a, MSHR668, and MSHR305, respectively [9]. The same study suggested that tRNA-Met, Pro, Thr, Ala, and Arg serve as “genomic hotspots” for GI integration in B. pseudomallei.
4. Potential roles of genomic islands in virulence and fitness

The functional role of GI genes is not well characterised in *B. pseudomallei*. Only a few studies have demonstrated that GI genes may have defined functional roles in virulence. A study by Sim et al. [7] using CGH microarray analysis revealed that most strains isolated from patients with melioidosis were more likely to harbour certain GIs, compared to strains isolated from other sources (e.g. animal and environment). In addition, experimental mutation of a GI gene demonstrated that the mutant strain displayed an altered phenotype with reduced adherence to human epithelial cells. This gene, *fhaB3* (BPSS2053), encoded a filamentous haemagglutinin and is one of the most common GI genes in *B. pseudomallei*. Some *B. pseudomallei* strains may contain up to three copies of this gene, while most strains have one or two copies [9]. Furthermore, a study by Duangsonk et al. [5] demonstrated that one GI cluster was associated with strains causing severe disease, including a rare neurological melioidosis in Australia. This data suggests that the content of the GIs, or the accessory genome, may play an important role in determining the clinical manifestation of disease. In contrast, a study by a Tumpa et al. revealed that the presence of five selected K96243 GIs did not differ between environmental and disease-associated isolates [11].

In conclusion, the large and variable content of *B. pseudomallei* genomes strongly suggests that this bacterial pathogen has high recombination rates compared to other bacterial species. Major causes of this diversity include the variable presence of GIs islands which are abundant in *B. pseudomallei* genomes. Being both a soil saprophyte and a successful pathogen, it is believed that the ability of *B. pseudomallei* to grow and survive in various environmental conditions is associated with its large and flexible genome. Although the functional roles of most GI genes are not yet known, the acquisition and maintenance of certain GIs may provide a selective advantage under particular growth conditions, as these foreign DNA segments may contain genes that could benefit bacterial survival in the host and/or under extreme environmental conditions. Current evidence strongly suggests that the role of GIs in various studies, including population genetics, virulence, and environmental adaptation and fitness, warrants further investigation.

References


Section III.4

Virulence determinants in *Burkholderia pseudomallei*: Opportunist or accidental pathogen?

Ifor R. Beacham\textsuperscript{a}, Yuka Hara\textsuperscript{b}, Sheila Nathan\textsuperscript{b}, Ian R. Peak\textsuperscript{a}

\textsuperscript{a} Institute for Glycomics, Griffith University, Gold Coast campus, Queensland, Australia
\textsuperscript{b} Malaysia Genome Institute, UKM-MTDC Technology Centre, Selangor, Malaysia

Abstract

Although a number of predisposing host conditions have been identified, the progression from first encounter to disease state depends on the capacity of *Burkholderia pseudomallei* to survive and multiply within the host. Several approaches have been taken to identify ‘virulence factors’: techniques that either predictively test factors known to be important in other bacterial pathogens, or that seek to discover novel virulence factors. These have identified a range of bacterial factors involved in interactions with cultured cell lines, or which reduce virulence and/or systemic spread in animal models of infection. The increasing pace of comparative genomics will reveal significantly more insight into putative virulence factors. Major questions currently remain as to how bacterial factors contribute to evasion of the host immune system and what are the routes of dissemination from nasal or lung epithelia, to normally privileged sites, such as the brain. Questions also remain as to the selective pressures that lead to this environmental organism acquiring or increasing virulence in mammals.

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1. Introduction

*Burkholderia pseudomallei* is an environmental saprophyte, with a large genome that encodes genes enabling nutritional diversity and survival in a variety of niches, including soil and standing water. It is also a pathogen of many mammals, notably rodents, goats, sheep, alpacas, pigs, monkeys, dolphins and humans. Disease in humans has many presentations, collectively termed melioidosis. A feature of melioidosis in humans and mice is that virtually any organ may be infected, including lung, liver, spleen, brain, prostate, parotid, bone, and blood in the acute, septic, form of the disease. In humans and other animals, infection of the brain may result in neurological symptoms (‘neurological melioidosis’). Human neurological cases are apparently more common in northern Australia (approximately 5% of cases) than in Southeast Asia.

The ability of *B. pseudomallei* to survive in the environment, colonise a host and then cause disease raises many questions regarding the exact route of infection and dissemination within the host. For example, is adherence to epithelial cells involved as is
the case with many other bacterial pathogens? \textit{B. pseudomallei} is a facultative intracellular pathogen capable of replicating in professional phagocytes, including macrophage-like cell lines and also non-phagocytic cell lines; how does this property relate to virulence in animals?

What bacterial features or ‘virulence factors’ are associated with pathogenicity? The term “virulence factor” has a number of hues, but in this context we consider them to be those genes and proteins that directly enable initial colonisation, dissemination and/or immune evasion. Understanding all aspects of the life cycle of \textit{B. pseudomallei} will hopefully begin to shed light on the extraordinarily extensive organ tropism and presentations seen in human melioidosis, such as bone infection, neurological melioidosis and chronic infection. A comprehensive review is available on virulence determinants \cite{1} and here we highlight and overview the major virulence factors found to date and their significance. Finally, how does our current understanding of the determination of virulence inform us regarding the nature of \textit{B. pseudomallei} as a pathogen in mammalian hosts?

2. Searching for virulence factors

2.1. Searching the genome

\textit{Burkholderia thailandensis} is a relatively non-virulent species, but very closely related to \textit{B. pseudomallei}. Comparison of the genomes of the two species was therefore predicted to offer insight into virulence genes in the \textit{B. pseudomallei} genome not shared by \textit{B. thailandensis}. Subtractive hybridisation, prior to the genome sequences becoming available, revealed a number of candidate genes, foremost being genes clearly involved in capsule biosynthesis \cite{2,3}. This approach also indicated that there are a number of genomic islands (GIs) in \textit{B. pseudomallei} not present in \textit{B. thailandensis} \cite{2,4}.

Subsequent determination and analysis of the genome sequences indeed indicated that the capsule loci of the two strains are very different. Indeed, whilst the capsule of \textit{B. pseudomallei} has been characterised both genetically and biochemically, the presence of a capsule in \textit{B. thailandensis} has not been demonstrated. Direct comparison of the \textit{B. pseudomallei} and \textit{B. thailandensis} genomes \cite{5,6} also now provides a basis for predicting or confirming differences which relate to virulence although it is possible that the overwhelming factor is the presence/absence or structure of the capsule.

A major feature of the \textit{B. pseudomallei} genome is the presence of a large number of GIs (Section III.3). Furthermore, comparison of the sequences of many strains demonstrates that the array of GIs differs between strains. Are any factors necessary for virulence contained in these GIs? There are suggestions that the presence of some regions of the chromosome correlate with human, animal, or environmental isolation \cite{7}.

Some candidate virulence factors can be predicted by analogy to other pathogens. Many such genes have been identified based on similarity to genes from related bacteria, initially by DNA amplification using conserved primers and more recently by inspection of sequenced genomes. The genome sequence, not unexpectedly reveals a large number of candidate virulence factors, such as a large number of potential adhesins \cite{4}. With recent developments in the creation of unmarked deletion mutants, these can be tested in available models for disease and virulence.
2.2. Transposon mutagenesis

Signature-tagged mutagenesis is a method of searching the genome for genes that are required for virulence by identifying mutants which are not recovered from an infected animal. Indeed, the capsule locus was identified using this method [8]. Transposon mutagenesis followed by in vitro screening of the mutants for serum sensitivity, led to the identification of a locus involved in lipopolysaccharide (LPS) biosynthesis [9]. The screening of transposon mutants for their ability to form plaques on PtK2 cell monolayers has also been used to identify genes involved in intercellular spreading [10]. Nine such mutants were significantly attenuated for virulence following intranasal infection of mice. The identified genes included two that encoded hypothetical proteins. The first was encoded within the Salmonella-like type III secretion locus (bsa) and was involved in mediating bacterial escape from endocytic vacuoles while the second encoded a putative exported protein that was required for normal actin-mediated intracellular motility and intracellular growth in a macrophage-like cell line [10].

2.3. In vivo expression technology

In vivo expression technology is a method of identifying promoters active under specific conditions and it has been used to identify \textit{B. pseudomallei} genes induced following infection of the macrophage-like cell line RAW264.7 [11]. This strategy identified genes involved in manganese uptake and haem acquisition, and a type VI secretion (tss) locus, \textit{tss}-5. There is precedence for all of these functions being involved in virulence in other pathogens, although mutations introduced into \textit{tssH}, considered to play a role in protein export, and into the gene involved in haem utilisation, did not change the intracellular survival of bacteria as compared to the wild-type. However, intracellular replication was not observed in the wild-type strain (compare with [10]) so whether replication is compromised in these mutants remains to be determined. Interestingly, one of the mutants identified in the transposon screen for decreased plaque formation on PtK2 cells is defective in \textit{tssK} which is encoded within the macrophage inducible \textit{tss}-5 locus.

It has been recently proposed that infection of the upper respiratory tract is a significant route of infection in murine melioidosis [12]. Owen et al. [12] also provided evidence to suggest a direct route of infection to the brain from the olfactory epithelium, with implications for the pathogenesis of neurological melioidosis. This leads to the question of what determinants are responsible specifically for colonisation of, and replication within, the nasal cavity and respiratory and olfactory epithelia, and for brain infection. Unfortunately, identifying these determinants is a formidable task, since an in vitro screening method is not available.

3. Virulence factors of \textit{Burkholderia pseudomallei}

3.1. Surface glycans as major virulence determinants

Several loci encoding genes shown, or suggested to contribute to LPS biosynthesis have been described. Analysis of strains with mutations in some of these genes indicated that the mutants showed increased sensitivity to killing by rat, guinea pig (though not hamster)
and human sera [9,13] and had substantially reduced capacity to survive and replicate in human macrophages [13]. Such mutants are also attenuated in animal models of virulence via intraperitoneal infection [9,13]. Despite these convincing virulence deficits in animals, clinical isolates have been identified from China, Thailand and Australia which express no detectable O-antigen. The clinical profile of disease outcome or patient risk factors associated with these strains did not differ from that of O-antigen expressing strains. These isolates displayed no reduction in resistance to killing by human serum [14]. This suggests that for at least some strains, O-antigen is not required to infect and kill humans [14]. Interestingly, O-antigen is also an apparently dispensable “virulence factor” for B. mallei as some human and horse isolates also express a rough LPS lacking O-antigen [15].

Genes involved in capsule biosynthesis have been identified by subtractive hybridisation against DNA from B. thailandensis and by signature-tagged mutagenesis [2,3,8] and subsequently characterised [3,16]. Mutants deficient in capsule biosynthesis but competent for LPS biosynthesis (e.g. SR1015) are virtually avirulent in mice and Syrian hamsters [3,13,16] and are unable to survive in blood when inoculated either by the intraperitoneal or intranasal route [12,16]. However, they retain a serum resistant phenotype [3,13] presumably because they still express LPS which is primarily responsible for serum resistance in this genetic background. Consistent with this scenario, B. thailandensis is also serum resistant, but virtually avirulent, does not survive in blood [16] and lacks at least the same capsular polysaccharide as B. pseudomallei [9,17]. Why are capsule mutants unable to survive in blood? The available data suggests two reasons, both relating to interaction with phagocytes: interference with phagocytosis due to reduced complement activation [16] and increased survival in human macrophages [13]. However, in a double mutant, deficient in both capsule and LPS biosynthesis (SLR5, webB webE; [16]), addition of capsule can increase the serum resistance attributed to LPS deficiency; this is suggested to be due to in vitro inhibition of complement attack by decoy, which may or may not occur in vivo. In summary, both LPS and capsule are major virulence factors affecting LD50 and survival in the blood of animal models, and mediate serum resistance (LPS), reduced phagocytosis (capsule) and survival within phagocytes (LPS and capsule) in vitro.

3.2. Quorum sensing

Quorum sensing (QS) is a population density-mediated form of cell-cell communication via the production, release and detection of signaling molecules such as N-acyl-homoserine lactones (AHLs) and alkyl quinolones.

LuxI proteins are responsible for AHL biosynthesis and LuxR transcriptional regulators, following association with their cognate AHL(s), mediate gene repression or expression. The B. pseudomallei genome has three luxI homologues, which encode the AHL synthases (BpsI1−3, but variously designated), and five luxR homologues that encode transcriptional regulators [18]. These regulators become activated upon binding their cognate AHL and subsequently mediate transcription of QS-regulated genes. The synthase genes mediate the synthesis of multiple AHLs including C8, 3-oxo-C8,
3-hydroxy-C10 and 3-hydroxy-C12 AHLs, with bpsI1 mutants unable to produce C8 and 3-oxo-C8 AHLs [19].

The phenotype of *B. pseudomallei* mutants defective in AHL-mediated QS is most simply interpreted through analysis of synthase mutants, as regulatory mutants may have pleiotropic effects including regulation of synthase genes. Several reports variously indicate that bpsI1 and bpsI3 influence virulence either in terms of time to death or LD50 in mice or hamsters [18,20], or in terms of lung and liver colonisation following aerosol delivery in mice [18]. It should be pointed out, that aside from cell density-regulated gene expression (as related to virulence genes for example), QS might also be used to sense the flow dynamics of the environment (“environmental sensing”) in order to avoid wasteful synthesis of extracellular products; if secreted proteins, for example diffuse quickly away from the cell in a high flow rate environment they would not provide the breakdown products in the vicinity of the cell to allow for efficient uptake [21]. The population-wide response would be a consequence of environmental sensing. Interestingly, QS does commonly regulate extracellular products, including at least one secreted enzyme in *B. pseudomallei* [20]. Further studies on the role of AHL-mediated QS in the context of the infected host or within phagocytic cells are required to confirm and define a role in virulence.

A second intercellular communication system, discovered and characterised in the opportunistic pathogen *Pseudomonas aeruginosa*, involves 4-hydroxy-2-alkylquinolones (HAQs) that coordinate group activities, and hence are quorum sensing molecules. Several of these are involved in the regulation of virulence genes in this bacterium [22,23]. An analogous biosynthetic system has been described in *Burkholderia* species, including *B. pseudomallei* [24] where the HAQs are methylated at the 3 position (HMAQs; [24,25]. As well as direct regulation of gene activity, HMAQs and HAQs may regulate the production of AHLs and hence potentially, indirectly, regulate QS-regulated activities [25]. The possible role of this QS system in the virulence of *B. pseudomallei* remains to be fully explored.

### 3.3. Determinants involved in survival and replication in cell lines

An intriguing feature of the infection of cell lines by *B. pseudomallei* is the formation of actin tails which propel the bacteria both intracellularly and from one cell to another, effecting intercellular spreading and the formation of multinucleated giant cells [26]. The gene responsible for actin polymerisation, *bimA*, has been identified [27]. Further, a transposon mutant in a gene encoding a hypothetical protein which reduced actin tail formation and intercellular spread was attenuated for virulence, indicating that actin-mediated intracellular motility is a significant virulence determinant [10].

The *bsa* locus encodes a type III secretion system that strongly resembles secretion systems found in *Salmonella* (encoded within pathogenicity island I) and *Shigella*, where they are involved in cell invasion and escape from endocytic vesicles. Mutants with disruptions in genes encoding components of the secretion and translocation apparatus (*bsaZ* and *bipD*) showed virtually no replication and actin tail formation in a macrophage-like cell line and a *bsaZ* (or, using proposed standardised nomenclature, *sctU*) mutant
was highly attenuated in the hamster model following intraperitoneal infection [28,29].
A mutant deficient in the putative type III translocator protein, BipB, also showed reduced
multinucleated giant cell formation in a macrophage-like cell line and reduced plaque
formation (intercellular spreading) in HeLa cells, and was highly attenuated in mice
following intranasal infection. However, it should be noted that the likely polar nature of
this bipB mutant could also give rise to reduced expression of bipCD [30]. This secretion
system is also present in B. thailandensis where it appears to play a similar role, as
tested in HeLa cells [31]. B. thailandensis, although of highly reduced virulence relative
to B. pseudomallei, is lethal to C57BL/6 mice over four to five days following aerosol
infection with a suitable concentration of bacteria. Using this model, it was demonstrated
that a bsaZ mutant was avirulent relative to the wild-type strain, strongly implicating the
bsa-encoded type III secretion system in virulence. Therefore, the cumulative evidence
is strong that the bsa-encoded type III secretion system is involved in both intracellular
events and virulence.

A model has been proposed for B. mallei infection in which type III secretion is
required for escape to the cytoplasm and type VI secretion and BimA are required
for subsequent actin polymerisation and intercellular spread [32]. From the data
available, such a model could also apply to B. pseudomallei infection, particularly as
B. pseudomallei type III secretion mutants fail to rapidly escape the phagosome. In
summary, there is mounting evidence that the capacity to replicate in macrophage-
like cells, and possibly epithelial cell lines in vitro, is related to virulence. However,
more detailed analyses of infection in animal models, with an array of mutants are
required to further substantiate and define the role of the bsa locus in dissemination and
pathogenesis.

3.4. Is Burkholderia pseudomallei an adherent organism?
A number of studies have shown that B. pseudomallei can adhere to various cell lines. This
includes an involvement of certain pili, encoded by pilA, in mediating bacterial cell-cell
interaction which numerically increases bacterial adherence to a human cell line due to adher-
ence of microcolonies [33]. The generality of this mode of adherence amongst strains
is not clear and PilA might be termed an accessory virulence factor. However, in general
the extent of bacterial adherence to cultured cell lines, relative to what is commonly
regarded as significant adherence for the case of a host-adapted pathogen, is unclear.
Recent experiments that attempted to quantitatively compare the extent of B. pseudomallei
adherence to that of E. coli K-12 (commonly regarded as insignificantly adherent) indi-
cated only a low level of adherence (unpublished data). Furthermore, no B. pseudomallei
adhesins have yet been unambiguously identified. It is possible that adherence is not
required for invasion of epithelial barriers; instead, immunological damage or dissemina-
tion via macrophages may be responsible for tissue and host invasion. Alternatively, the
discovery of adherence awaits studies with appropriate primary cells or in vivo studies.

3.5. The possible role of flagella
The role of flagella in virulence has been tested using flagellin-deficient (fliC) mutants in
animal infection experiments in several laboratories. A consensus emerges, that following
challenge via the intraperitoneal route, flagella are not required for virulence [13]. However, via the intranasal route in BALB/c mice, there is evidence that flagella are required for virulence as indicated by increased LD$_{50}$ and reduced dissemination to spleen and lung [34].

4. *Burkholderia pseudomallei*: primary pathogen, opportunist or ‘accidental pathogen’

What does our current knowledge of virulence determinants in *B. pseudomallei* tell us about the nature and evolution of *B. pseudomallei* as a pathogen? Pathogens are generally defined as organisms capable of causing disease in infected hosts [35,36]. Defining features of an opportunistic pathogen are 1) that disease is only caused in a host which is compromised in its immune defences; and 2) that it does not depend on the host for long term survival [35]. Further, similar to commensal organisms, opportunists do not have the capacity to breach intact host cell barriers, which distinguishes them from primary, or obligate, pathogens. Of course, even commensals may cause infections if introduced into an inappropriate, normally sterile site.

There are well defined risk factors which suggest that for many human melioidosis cases, the pathogen is acting opportunistically and host immune dysfunction or deficit contributes to disease. However, healthy individuals with no apparent risk factors are also susceptible to systemic melioidosis, indicating that *B. pseudomallei* has the capacity to breach host cell barriers and disseminate, thus acting as a primary pathogen. Conversely, *B. pseudomallei* is primarily an environmental organism, and clearly does not depend on the host for survival.

In view of the seemingly ambiguous nature of *B. pseudomallei* as a pathogen, part primary pathogen and part opportunist, a third category should be considered: Is *B. pseudomallei* an accidental pathogen? This term has been suggested for pathogens which “do not gain an evolutionary advantage as a result of the infection” [36]. It has also been applied to commensals, such as *Haemophilus influenzae* and *Neisseria meningitidis*, when disease results from ‘accidental’ or ‘incidental’ invasion of inappropriate niches such as blood, the middle ear or meninges [37,38]. This term signifies that evolutionary pressures are overwhelmingly those that favour survival as a commensal and that spread to other sites does not normally lead to replication.

The concept of accidental virulence has also been cogently argued by Casadevall and Pirofski [39], mainly in the context of eukaryotic pathogens acquired from the environment. In their terminology, virulence determinants such as surface glycans are ‘dual use’ virulence determinants, required for survival both in the environment and the host [7,40], including the commensal state (the host ‘environment’) and the ‘accidental’ invasive state. Such a view is emphasised by the recent genome sequencing of deep vent ε-proteobacteria, which share many genes that are characterised as virulence determinants in their pathogenic counterparts [41]. Applying the logic of Casadevall and Pirofski [39] the notion of dual use virulence determinants is also applicable to *B. pseudomallei*’s environmental niche and its ‘accidental’ invasive state.
So for *B. pseudomallei*, it may be suggested that the overwhelming evolutionary pressures for survival are environmental and the capacity for human and animal infection is accidental [6]. When infection of a mammalian host occurs, infection of blood and distant sites occurs. Survival in the blood depends on capsule and LPS, components which are clearly a feature of environmental organisms and potentially required to resist amoebal predation and dessication [39,40,42]. Experimental studies on *Legionella pneumophila* and *Mycobacterium avium* have established a link between virulence and growth in amoebae [42]. Experimentally, *B. pseudomallei* can also survive in amoebae, although the virulence factors contributing to survival and replication in these cells have not been well studied. Such a requirement may include type III and type VI secretion. These secretion systems (the *bsa* locus and five out of six type VI loci) as well as other candidate virulence determinants, such as pili and QS genes, are also present in the relatively non-virulent *B. thailandensis*, consistent with their (at most) ‘dual use’ status. As well as interaction with amoebae in the environment, interaction with fungi may provide, or have provided, selective pressure for survival since extant *Burkholderia* species are known endosymbionts of some fungal species [43]. Selection for the capacity to survive in protozoa, fungal cells and plant cells [44] may have provided the ‘dual use’ virulence factors that contribute to *B. pseudomallei*’s ability to breach animal mucosal and immune defences.

Simplistically, there are three potential outcomes of a host-microbe encounter: (1) rapid clearance of the bacteria without dissemination to a new host or environment; (2) death of the host; or (3) a balance, with bacteria establishing and being shed to the environment. Accidental virulence implies no selective advantage to colonising and causing damage to the host animal. Given that transmission of *B. pseudomallei* from one host to another is considered extremely rare, the success of a clone can be measured by its capacity to return to its environmental niche. Can it be imagined that any of these scenarios would lead to selection of traits involved in infection of mammalian hosts?

1. If, following infection of mammals, the bacteria are completely cleared before excretion, secretion, or other dissemination to the environment, then no evolutionary advantage accrues and infection is effectively a dead end. In this case, clearly the infecting lineage will not be successfully propagated back to the environment, and whatever ‘accidental’ or ‘dual use’ virulence factors which occur in that strain that allow it to establish and replicate in the host are offering no clear advantage to subsequent environmental or within-host survival.

2. Host death is frequently a result of overwhelming bacterial growth. When a wild or feral animal is infected (e.g. goats and pigs) and dies from the infection, then the dead host animal represents a localised high concentration of bacteria derived from a single strain. The capacity of *B. pseudomallei* to survive within a corpse (animal or human) has not been investigated, but its survival for long periods in many different media is well documented. The high, localised, bacterial concentration thus deposited to the environment suggests that death of the host confers an “accidental” survival and propagation outcome for that clone once it is returned to the environment. Of course, the infection of a mammalian host also confers on a bacterial clone the capacity to be transported; thus, mammalian infection is a dissemination strategy allowing
the bacteria to be deposited to distal environmental niches. This could be especially important if the capacity of *B. pseudomallei* to remain latent or contained within granulomatous lesions for very long periods is considered: a host may have travelled far from initial geographical site of infection before disease occurs. Long periods of latency are a well known human manifestation of infection. In pigs, often the only sign of infection is culture positive granulomatous or nodular lesions apparent at slaughter [45]. Pigs are widespread feral pests in northern Australia and other tropical regions, and are well known for their capacity to travel widely and disturb the ground (presumably contributing to acquisition by inhalation or ingestion when rooting in *B. pseudomallei*-endemic soils). Thus, infection of mammalian hosts could be seen to confer advantages of providing localised, high concentrations of bacteria and the capacity to populate distant niches. Therefore, phenotypes that are permissive for mammalian infection could be selected.

3. The selective advantage of bacteria “hitching a ride” (transportability) is even more apparent if it is accepted that *B. pseudomallei* can cycle through live mammalian hosts and be shed either during acute infection, or over longer periods of chronic infection [6]. In this scenario, the bacteria colonise a host, replicate and are continuously shed into the environment until disease resolution or host death. The potential for bacterial shedding has been known for at least two decades for humans. Wuthiekanun and colleagues [46] showed that infected human patients may be culture positive from both urine and anal swabs, even when the patients presented with apparently localised lung or parotid infections. In animals, goats frequently exhibit gut lesions, suggesting faecal excretion of bacteria is likely, and could potentially result in cycling of bacteria between the environment and animals. In addition to urine or faecal excretion, skin or foot lesions have been cited as a possible cause of bacterial cycling between animals and soil [47]. Thus, if indeed cycling between host and environment does occur, it is likely that genes which increase the organisms potential to colonise and cause shedding infections in animal hosts would be positively selected for and distinct lineages or clades should be apparent that are more frequently isolated from distinct niches: mammalian versus environmental. Some evidence of this is the identification of genetically distinct environmental and clinical clades, based on whole genome microarray analysis of many strains [7]. The clades are distinguished by presence or absence of genes in the accessory genome. Furthermore, genes differentially present in the clinical clade are mainly in GIs, whose presence or absence varies between strains but could play a qualitative role in virulence. This study provides insight into a dynamic genome with selection occurring for strains associated with human infection. This selection may have occurred in the challenging external environment (leading incidentally or accidentally to an increased capacity for human infection), or in mammalian hosts via cycling through animals including humans [7]. The latter prospect has been considered less likely for a number of reasons, including evidence for the genetic stability of *B. pseudomallei* in the human host and evidence for positive selection, predominantly in genes overtly involved in environmental survival [6]. However, the impact of environment-host cycling on selection for enhanced host infection has not been experimentally examined.
In summary, *B. pseudomallei* may be regarded as an accidental pathogen, an opportunist, or a pathogen in which selection in humans and animals favours virulence determinants for replication and spread via cycling between niches. Perhaps, at this moment in its evolutionary journey, it has features of both an accidental pathogen and an opportunistic pathogen.

5. Conclusions

Disease in humans is a multifactorial process. Several classical approaches have been used that have identified a number of genes that affect aspects of virulence in *in vitro* or *in vivo* models. The relative contribution of each potential virulence factor to disease outcome is difficult to quantify in a clinical setting, and the dispensability of each to human infection will only become clear, if or when, clinical strains lacking each or all of the virulence factors are isolated (as has already occurred for strains lacking O-antigen). Alternatively, further studies on *B. thailandensis* involving gene addition would be valuable. Additional extensive sequencing will identify if there is a subset of the pan-genome more frequently associated with virulence in humans. In the meantime, the further development of animal models, and the characterisation of factors that contribute to bacterial evasion of protozoal killing, or capacity to survive predation by helminths, may be at least as successful as searching for classical virulence determinants derived from the opportunist paradigm.

Acknowledgements

IRB and IRP acknowledge the support of the NHMRC; and the Griffith Medical Research College, a joint programme of Griffith University and the Queensland Institute of Medical Research, Herston, Qld, Australia. We also acknowledge the support of all our collaborators.

References


Lipopolysaccharide as a virulence factor of *Burkholderia pseudomallei*

Bob Ernst\textsuperscript{a}, Suppiah P. Sivalingam\textsuperscript{b}, Gladys Tan\textsuperscript{b}

\textsuperscript{a} Department of Microbial Pathogenesis, University of Maryland-Baltimore, Baltimore, Maryland, USA
\textsuperscript{b} Defense Medical and Environmental Research Institute, DSO National Laboratories, Singapore

**Abstract**

Lipopolysaccharide (LPS), an outer membrane molecule of Gram-negative bacteria, is the most common bacterial component implicated in initiating sepsis. *Burkholderia pseudomallei* LPS has been suggested to play a role in the pathogenesis of melioidosis because patients with septicemic melioidosis show significantly elevated levels of proinflammatory cytokines, the release of which is known to be activated by LPS in other bacterial infections. Recognition of LPS by Toll-like receptors depends on the structure of lipid A. Modification of this moiety under hostile environments allows Gram-negative bacteria to escape immune recognition. Recent research indicates that the LPS of *B. pseudomallei* may have unique structural characteristics, resulting in it being weakly immunogenic, hence allowing the bacteria to evade host recognition and facilitate prolonged intracellular survival in host cells. This review will examine the role of *B. pseudomallei* LPS in the virulence of *B. pseudomallei* and the pathogenesis of melioidosis.

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1. Discovery of bacterial lipopolysaccharide

The discovery of lipopolysaccharide began in the eighteenth century with the search for fever- and disease-producing substances associated with unhealthy situations [1]. In the early 1890s, Pfeiffer [2,3], a collaborator of Robert Koch at the Institute for Infectious Diseases in Berlin, discovered that lysates of heat-killed bacteria of the cholera-inducing infectious agent *Vibrio cholerae* caused toxic shock reactions in guinea pigs. Unlike an exotoxin, this product was not secreted by the bacteria and thus it was named endotoxin, a term still used today.

In time, endotoxin became the focus of a vast inquiry into molecular mechanisms of microbial pathogenesis [4]. It was determined that all Gram-negative bacteria carry endotoxins on their surface, and they are essential for bacterial growth and survival. With the development of techniques for the extraction and preparation of endotoxins in the 1930s to 1940s, relatively pure samples were obtained. These consisted of polysaccharide and lipid components with small amounts of protein and were thus
termed lipopolysaccharide (LPS) [1,5]. Today the terms endotoxin and LPS are used synonymously. We now know that lipopolysaccharide is a critical structural component of the outer leaflet of the outer membrane of most Gram-negative bacteria and it contains the O-antigenic polysaccharide determinants critical for serological strain differentiation.

With the purification techniques for LPS at hand, the structures of both the lipid A and the polysaccharide core components of LPS were established in the 1980s. Lipid A, the hydrophobic anchor of LPS, is a glucosamine-based phospholipid that makes up the outer monolayer of the outer membranes of most Gram-negative bacteria [6]. Further studies on lipid A have established it as being responsible for the endotoxic activity associated with LPS [7]. When released from the bacterial cell, endotoxins induce a great variety of pathophysiological effects in higher organisms. However, not all endotoxins are toxic, just as not all bacteria are pathogenic.

2. Clinical importance of bacterial lipopolysaccharide

The endotoxins of Gram-negative bacteria have been studied for many years as they are the typical trigger of the inflammatory response following infection. The most severe clinical manifestation of melioidosis is septic shock, which is often associated with pneumonia and bacterial dissemination to distant sites [8]. Lipopolysaccharide is the most common bacterial component implicated in initiating septic syndrome [9]. *B. pseudomallei* LPS has been suggested to play a role in the pathogenesis of melioidosis as patients with septicaemic melioidosis show significantly elevated levels of pro-inflammatory cytokines, the release of which is known to be activated by LPS in other bacterial infections [10,11], and display high levels of anti-LPS antibodies [12]. These antibodies may give a level of protection against melioidosis as the level of anti-LPS antibody was found to be significantly higher in melioidosis patients who survived than those who died and was also higher in patients with non-septicaemic melioidosis than those with septicaemic melioidosis [13]. In an *in vitro* model, using antibodies to the *B. pseudomallei* LPS O-antigen promoted phagocytic uptake and bacterial killing [14]. These antibodies were also shown to develop during human infections and may facilitate clearance of the organisms *in vivo*.

3. Structure of lipopolysaccharide

3.1. Common lipopolysaccharide structural patterns

Lipopolysaccharide, the major outer membrane component of Gram-negative bacteria plays an important role in bacterial pathogenesis. LPS has three structural regions: O-antigen, core, and lipid A (Figure III.3). O-antigen and core consist of polysaccharide chains, whereas lipid A (the bioactive component of LPS or endotoxin) is primarily composed of fatty acids and phosphate substituents bonded to a central glucosamine dimer [15,16]. The structure of LPS isolated from numerous Gram-negative bacterial species has been elucidated using both mass spectrometry and nuclear magnetic resonance (NMR) techniques. This analysis has shown that the O-antigen portion of LPS
Lipid A is the most variable structurally, followed by the core and lipid A. Phenotypically, bacteria are characterised as rough or smooth depending on the absence or presence of the O-antigen portion of LPS, respectively. The core domain of LPS is normally attached to lipid A with the first residue being a unique eight-carbon carbohydrate, keto-deoxyoctulosonate (KDO). Attachment of the O-antigen is bacterial species specific with the sugar heptose commonly present. Finally, the common base lipid A structure consists of a β-(1,6)-linked diglucosamine backbone with phosphates attached at the 1 and 4' positions, amide-linked fatty acids attached at the 2 and 2' positions, and ester-linked fatty acids attached at the 3 and 3' positions. As an example,
the fatty acids attached directly to the diglucosamine backbone for lipid A isolated from *E. coli* is 3-OH C14:0. However, wide variability in the individual fatty acids attached at these positions is observed depending on the individual bacterial species. In addition, the base structure of lipid A can be modified by the addition or removal of carbohydrate(s) and fatty acids moieties, removal of the terminal phosphate residues, and/or variation in the length and/or order of fatty acid chains. It has been previously shown that many of these additional modifications to lipid A alter pathogen interactions with the host innate immune system [17].

3.2. Structure of Burkholderia pseudomallei lipopolysaccharide

To date, the complete LPS structure from a single *B. pseudomallei* isolate has not been determined. However, individual portions of the LPS molecule have been elucidated. Perry et al. [18] determined the structure of the O-antigen polysaccharide portion from LPS isolated from two clinical isolates (304b and 824a) and Novem et al. [19] the structure of the lipid A component from a single clinical isolate (KHW). No structural analysis of the core component of LPS has been carried out as of yet.

The structure of the O-antigen (also known as type II O-PS) has been determined by NMR spectroscopy for two strains of *B. pseudomallei* [18]. Interestingly, strain 304b produced two unique O-antigens, whereas strain 824a only synthesised a single structure. The first O-antigen from strain 304b is an unbranched high-molecular-weight polymer of 1,3-linked 2-O-acetyl-6-deoxy-β-D-manno-heptopyranose residues. The second O-antigen is an unbranched polymer of repeating disaccharide units having the structure -3)-β-D-glucopyranose-(1−3)-6-deoxy-α-L-talopyranose-(1-n). In addition, approximately 33% of the L-6dTalp residues bear 2-O-methyl and 4-O-acetyl substituents while the other L-6dTalp residues carry only 2-O-acetyl substituents. Analysis of the O-antigen from strain 824a identified only the first O-antigen structure. These results were similar to the structural analysis of the O-antigen obtained from *B. pseudomallei* strain 57576 by Knirel et al. [20].

Using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to determine the laddering pattern for LPS from 1327 clinical and environmental isolates of *B. pseudomallei*, it was determined that 97% of all isolates tested displayed a single LPS laddering profile (Type A) [21]. Two percent of the isolates had a second laddering profile (Type B) and 1% were shown to be rough (i.e. lack of O-antigen). No structural analysis was determined for any of the strains to confirm if the Type A and B laddering profiles were similar to those determined previously.

Using higher order mass spectrometry, Novem et al. [19] structurally characterised the lipid A component from the *B. pseudomallei* isolate, KHW. Lipid A from this isolate consisted of a β-(1′,6)-linked diglucosamine backbone with phosphates at the 1 and 4′ positions, amide-linked fatty acids at the 2 and 2′ positions, and ester-linked fatty acids at the 3 and 3′ positions. The amine-linked fatty acids were shown to be 3-OH C16, whereas the ester-linked fatty acids were 3-OH C14. A single acly-oxyl-acyl fatty acid (either a C14 or a 2-OH C14) was present at the 2′ position fatty acid residue. The phosphate moieties could be further modified by the addition of a positively charged
sugar, aminoarabinose. The chain lengths of the amine-linked fatty acids attached to the
*B. pseudomallei* lipid A (3-OH C16) is two carbons longer than those of *S. typhimurium*
and *E. coli* and may play a role in altered recognition by the host innate immune system.
Interestingly, this is nearly the same structure identified for the lipid A component of
LPS from other *Burkholderia* subspecies including *B. caryophylli* [22], *B. cepacia* [23],
and *B. multivorans* [24] potentially suggesting a common lipid A structure for most
*Burkholderia* species.

4. Antigenic heterogeneity of *Burkholderia pseudomallei* lipopolysaccharide

*Burkholderia pseudomallei* is considered to be a homogenous species, but there are
studies documenting variations in colony morphology, antimicrobial susceptibility and
biochemical profiles. *B. pseudomallei* LPS was recently reported to be physicochem-
ically and antigenically heterogeneous [25]; a factor which could explain the false
negative serological results which occur in some individuals with *B. pseudomallei*
infections [25,26].

*Burkholderia pseudomallei* LPS has been reported to exist in two different forms;
S-form and R-form, differing in the chemical structure of their O-polysaccharide (O-PS)
component [26,27]. Much structural diversity exists in O-chains, particularly the types
and number of sugars within a unit, the nature of the linkages of the sugars, and the
number of repetitive units. In addition to polysaccharide heterogeneity, lipid A structures
may show structural variability in the degree of acylation, the pattern of substitution
of the two lipid A phosphates, and the nature of the fatty acids. An artifactual source
of heterogeneity can be introduced by excessive hydrolytic conditions used for lipid A
preparation resulting in the loss of some fatty acids or of the glycosidic phosphate. The
result of such diversity provides the serotype specificity of each bacterial strain.

Two distinct biotypes of *B. pseudomallei* have been distinguished by their ability to
utilize L-arabinose. Some environmental isolates are able to utilize L-arabinose (Ara+) while some are not. However, all clinical isolates identified to date are unable to utilize
L-arabinose (Ara-) [25,26]. Lipopolysaccharides isolated from Ara^- and Ara^+ biotypes
were indistinguishable as measured by SDS-PAGE profiles and immunoreactivity with
immune sera or monoclonal antibodies [26]. However, a subsequent study by the same
group using a monoclonal antibody against *B. pseudomallei* LPS showed several strains
produced variations in the LPS profiles by Western blot. Regardless of the varying profiles,
all LPS preparations exhibited similar endotoxic activity [25] The availability of specific
monoclonal antibodies, capable of distinguishing the more virulent Ara^- *B. pseudomallei*
from the Ara^+ counterpart, has provided an additional tool that may be used to further
study the virulence of these organisms.

5. Biological activities of *Burkholderia pseudomallei* lipopolysaccharide

5.1. Molecular recognition of *Burkholderia pseudomallei* lipopolysaccharide

The TLR2 is typically associated with the recognition of lipoproteins, lipotechoic acid
and glycolipids, while TLR4 is widely regarded as the receptor for Gram-negative LPS.
Using HEK293 cells stably transfected with TLR2/CD14 and TLR4/CD14, Wiersinga and colleagues [28] reported that although both TLR2 and TLR4 contributed to the cellular responsiveness to \textit{B. pseudomallei} strain 1026b in vitro, the LPS of \textit{B. pseudomallei} 1026b was detected only by TLR2. However, two subsequent studies have indicated that \textit{B. pseudomallei} LPS is indeed recognised by TLR4 [19,29]. In the study by West et al. [29], HEK293 cells transiently transfected with murine or human TLR2, TLR2/1, TLR2/6 or TLR4 and co-receptors CD14 and MD-2 were stimulated with LPS purified from two clinical isolates, BP-1 and K96243, as well as with lipid A from BP-1. In both murine and human transfected cells, robust TLR4-dependent NF-κB activation was identified in response to LPS from the \textit{B. pseudomallei} strains and purified BP-1 lipid A. No evidence of TLR2 stimulation by the same ligands was observed. In addition, it was shown that MD-2 is required for this TLR4-mediated signaling, as identical stimulation of cells transfected with human TLR4 and CD14, with \textit{B. pseudomallei} LPS and BP-1 lipid A in the absence of MD2, did not yield any activation [28]. Similarly, Novem and coworkers [19] demonstrated that LPS purified from \textit{B. pseudomallei} strain KHW, elicited NF-κB activation in a TLR4-dependent manner, when human TLR4-MD4-CD14-transfected HEK293 cells were stimulated with the LPS. Toll-like receptor 2-dependent activation of NF-κB was not observed when the human-TLR2-transfected cells were stimulated with the same LPS, indicating that the LPS of \textit{B. pseudomallei} activates the TLR4 complex, but not TLR2.

The discrepant findings reported here could potentially be explained in three ways. Firstly, as highlighted by West et al. [29], the co-transfection of MD-2 into HEK293 cells is important since this molecule is not endogenously expressed by HEK293 cells and is essential for the TLR4-dependent signalling. The absence of MD-2 in the assays performed by Wiersinga et al. [28] might contribute to the lack of recognition in CD14-TLR4-transfected cells. Secondly, the ability of some LPS preparations to activate TLR2, might be attributed to the presence of contaminating lipoproteins [30]. \textit{B. pseudomallei} LPS is typically extracted using the modified hot aqueous-phenol extraction method [31]. However, using this method the LPS of \textit{B. pseudomallei} partitions into the phenol phase together with the proteins. This method of isolation may result in an LPS preparation which could be contaminated with proteins [30]. Novem and coworkers (unpublished data) have shown that \textit{B. pseudomallei} LPS extracted using this traditional method potently activates both TLR2 and TLR4. Extensive purification of the LPS fraction is therefore required to remove the contaminating proteins and to eliminate recognition by TLR2, especially for biological assays in which the presence of minute quantities of contaminants may confound results [32]. Lastly, since the studies described above used different \textit{B. pseudomallei} strains, it is possible that there are strain-specific LPS structural differences which influence recognition by TLR2 and TLR4.

### 5.2. Induction of cytokine production by Burkholderia pseudomallei lipopolysaccharide

\textit{Burkholderia pseudomallei} LPS has been reported to exhibit weaker macrophage activation by at least one order of magnitude, as compared to enterobacterial LPS [33].
However, *B. pseudomallei* LPS was shown to elicit stronger mitogenic activity in murine splenocytes than the enterobacterial LPS. Using the mouse macrophage cell line RAW 264.7, Utaisincharoen and colleagues [34] demonstrated that *B. pseudomallei* LPS induced significantly less nitric oxide (NO) and TNF-α production than RAW macrophages stimulated with LPS from *E. coli* or *Salmonella typhi*. Furthermore, the time required for production of significant amounts of NO or TNF-α in response to *B. pseudomallei* LPS, was at least 30 minutes longer than that observed following activation with *E. coli* LPS (<5 minutes) or *S. typhi* LPS. The delay in NO release correlated with a reduced rate of iNOS protein expression and also a reduction in the level of iNOS and TNF-α mRNA. These results suggest that compared to *E. coli* LPS, signal transduction initiated by the interaction of *B. pseudomallei* LPS with the macrophage cell surface is delayed, resulting in an additional delayed release of the mediators NO and TNF-α, potentially allowing the bacterium to evade the innate host immune defence. In accordance with these results, Novem et al. [19] also reported that both murine (RAW 264.7) and human macrophage (THP-1) cell lines produce lower levels of TNF-α, IL-10 and IL-6 in response to *B. pseudomallei* LPS, than in response to *B. thailandensis* LPS; this reduced immunological activity was attributed to the *B. pseudomallei* lipid A structure.

West and coworkers [29] demonstrated that there was no TNF-α production by TLR4−/− bone marrow-derived macrophages in response to *B. pseudomallei* LPS. However, stimulation of TLR2−/− cells with the same ligand induced the production of more TNF-α than when wild-type cells were similarly stimulated. A similar profile was observed for MIP-2 production. The observation that proinflammatory cytokine production in response to *B. pseudomallei* LPS or lipid A was augmented in the absence of TLR2, suggested that this might be an IL-10-mediated phenomenon. However, no impairment in IL-10 production by TLR2−/− cells in response to *B. pseudomallei* LPS or lipid A was identified, rather the overall pattern of IL-10 production was similar to that observed for TNF-α production. Taken together these results indicate that cytokine production by macrophages stimulated with *B. pseudomallei* LPS or lipid A is primarily dependent on TLR4, but augmented in the absence of TLR2.

The O-antigen of *B. pseudomallei* LPS also plays an important role in modulating the host response as a mutant lacking the O-antigenic polysaccharide moiety, was more susceptible to macrophage killing during the early phase of infection than the parental wild-type strain [35]. This increased susceptibility to killing was associated with increased stimulation of the transcription factors Y701-STAT-1 and IRF1, and the concomitant production of increased levels of inducible nitric oxide synthase (iNOS).

6. Conclusions

The finding that a high level of anti-LPS antibodies were key to conferring protection against fatal melioidosis in patients, suggests that *B. pseudomallei* LPS may play a significant role in the pathogenesis of the disease. In addition, increased susceptibility of an LPS mutant of *B. pseudomallei* to macrophage killing, as compared to the wild-type parental strain, provides compelling evidence that LPS has a crucial role in modulating
the host response. Recognition of the LPS moiety by TLRs and the subsequent induction of weak immune responses in various assays have been well documented. However, there is a paucity of knowledge on the structural characterisation of the *B. pseudomallei* LPS molecule and to date, the complete LPS structure of a single strain of *B. pseudomallei* has not been determined. Future research efforts in this area will shed more light on the importance of LPS in regulating the virulence of *B. pseudomallei*.

References


IV
Clinical manifestations of melioidosis
Melioidosis is essentially a different disease in different patients. Although during his initial postmortem examinations, Whitmore found melioidosis to be “essentially a lung disease,” on further examination he concluded that “just as in typhoid fever though the most obvious lesions are confined to the intestine, the infection is general; so also in this disease, with its gross and obvious lesions restricted to the lungs, the local nature of the lesions gave no clue to the possible extent of the infection throughout the body” [1]. Although the organism may be distributed widely in the body, clinically recognised foci of infection are usually more limited in number. The severity of infection can range from asymptomatic disease to fulminant septic shock. Although presentations can lie anywhere on this continuous spectrum, clinicians have traditionally categorised melioidosis into different presenting syndromes.

Most presentations are with acute melioidosis, with a relatively short duration of symptoms of days to weeks that may be difficult to distinguish from other causes of acute sepsis, where a clinical focus of infection may or may not be evident. Acute melioidosis is described in further detail in Section IV.1. Chronic melioidosis, where the duration of symptoms is more than two months, usually presents either as an undiagnosed non-healing skin sore or with pulmonary infection clinically similar to tuberculosis. Recurrent disease following apparently successful treatment was thought mostly due to relapse involving the original infecting strain, but more recent studies suggest that this may also involve re-infection following repeat exposure. Latent disease, where disease only occurs many years following exposure, is analogous to tuberculosis but is poorly understood. These issues are discussed further in Section IV.2.

Melioidosis is known to have a predilection for particular groups of people. Whitmore and Krishnaswami recognised that there was a possibility of selection bias in their initial descriptions of melioidosis affecting patients who injected morphia intravenously, as they noted that this group of patients “were over-represented amongst the subjects on whom (we were) permitted to conduct autopsies” [2]. More carefully controlled studies since have defined that patients with diabetes, hazardous alcohol intake, renal disease, chronic
lung disease and immunosuppression have an increased risk of disease, although the precise mechanism of susceptibility is not known. The exception is disease in children where comorbidities are uncommon; although presentations may be similar to those found in adults, specific paediatric syndromes are recognised, particularly parotid disease in Thailand, lymphadenitis, and encephalomyelitis in Australia.

Infection also requires exposure to the causative organism, which was the clue that alerted Whitmore to reconsider the diagnosis of *Burkholderia mallei* infection in his first case; “glanders infection appeared improbable; as, so far as he could discover, the man had been released from the jail only a short time previously and had had no close contact with horses” [1]. It was not appreciated until much later that *B. pseudomallei* is mostly found in pooled surface water and mud in the rainy monsoonal season in endemic areas [3]. However, a lack of an exposure history does not preclude the possibility of melioidosis; Singapore, a highly urbanised city where 88% of the population live in high-rise flats, still reports moderately large numbers of cases each year [4]. The risk factors in adults are described in further detail in Section IV.3, and paediatric disease in Section IV.4.

The reasons for the heterogeneity in severity and pattern of organ involvement between patients and between regions are not known. It is assumed that it could be due to differences in the magnitude of the infecting bacterial load, strain differences in the organism, host differences and differences in the mode of acquisition. Whatever the cause, the clinical diversity of disease has several important implications for researchers and clinicians.

The protean manifestations can make it difficult for clinicians to distinguish melioidosis from other bacterial infections on the basis of clinical findings. This is a particular problem as the currently available methods for rapid diagnosis while awaiting culture results are either not applicable, such as in deep tissue infection where samples for diagnosis are not easily accessible (direct immunofluorescence can be performed on pus, sputum or urine) or not specific (serology with indirect haemagglutination). This is important, as standard empiric antibiotic regimens are not generally active against *B. pseudomallei*. However, because mortality is largely due to the complications of sepsis and is similar to sepsis due to other causes, the principles of early management should apply to all critically ill patients in this setting. The potential involvement of many different organs makes it imperative to screen for sites of covert foci, particularly involving the abdominal and pelvic viscera.

Researchers need to be mindful of which group of patients is being studied. Studies of pathogenesis need to account for the diversity of organ involvement and severity, as well as the disparate risk factors known to be associated with melioidosis. The wide spectrum in disease severity has particular relevance for design of clinical trials. This is well-illustrated in the control arms of various studies, all conducted in Thailand and where participants received a ceftriaxone-based treatment; in these studies, mortality varied from 14% to 87% [5,6]. Because early treatment is probably one of the strongest determinants of outcome, delays in treatment may arise if a confirmed diagnosis is required for enrolment. One solution is to base enrolment on clinical definitions, such as criteria for severe sepsis, with an *a priori* analysis of the subgroup with confirmed melioidosis.
The diversity of clinical presentations associated with melioidosis has been well-recognised since its initial descriptions. Although the label “the great mimicker” [7] is probably an overused moniker in medicine, having also been applied to syphilis, systemic lupus erythematosus (SLE), and human immunodeficiency virus, amongst other diseases, this heterogeneity in clinical presentation has significant implications for both clinicians and researchers. However, with early diagnosis, appropriate antibiotics and availability of state-of-the-art intensive care therapy, patients with melioidosis who do not have an identified risk factor are very unlikely to die from their infection [8].

References

Section IV.1

Clinical features of acute melioidosis

Bart J. Currie\textsuperscript{a,b}, Wipada Chaowagul\textsuperscript{c}, Allen C. Cheng\textsuperscript{a,d,e}

\textsuperscript{a} Menzies School of Health Research, Darwin, Australia
\textsuperscript{b} Northern Territory Clinical School and Infectious Diseases Department, Royal Darwin Hospital, Darwin, Australia
\textsuperscript{c} Sappasithiprasong Hospital, Ubon Ratchathani, Thailand
\textsuperscript{d} Department of Epidemiology and Preventive Medicine, Monash University, Melbourne, Australia
\textsuperscript{e} Infectious Diseases Unit, Alfred Hospital, Melbourne, Australia

Abstract

The majority of infections with \textit{Burkholderia pseudomallei} are asymptomatic. In disease, the incubation period, clinical presentations of melioidosis, and outcomes are thought to be determined by a combination of bacterial load infecting the individual, putative \textit{B. pseudomallei} strain differences in virulence, mode of infection and, most importantly, host risk factors for disease. Over 85\% of cases of melioidosis are acute presentations, with an incubation period of 1 to 21 days (mean, nine days). Mortality varies depending upon disease severity and access to diagnostic and therapeutic facilities, ranging from over 90\% in patients presenting with disseminated disease and septic shock in under-resourced rural locations, to potentially zero in patients without any risk factors who present with localised disease (for example, cutaneous melioidosis). Bacteraemia occurs in 40 to 60\% of cases, and around half of patients present with pneumonia. Other presentations include: cutaneous melioidosis; septic arthritis and/or osteomyelitis; genitourinary presentations, most notably prostatic abscesses; suppurative parotitis, seen commonly in children in Thailand; bacteraemia without an evident clinical focus; and melioidosis meningo-encephalitis, as seen more commonly in Australia than elsewhere. Internal organ abscesses are common, as is secondary bacteraemic spread to other foci irrespective of the primary presentation.

1. Introduction

The earliest descriptions of melioidosis documented the fulminant end of the clinical spectrum, with fatal sepsis with abscesses throughout both lungs and in many organs \cite{1}. At the other end of the spectrum are asymptomatic infections and localised skin ulcers or abscesses, without systemic illness. Howe and colleagues classified melioidosis as “acute”, “subacute”, and “chronic” \cite{2}. The Infectious Disease Association of Thailand summarised 345 cases into these four categories \cite{3,4}:  

1. Disseminated septicaemia (45\% of cases, 87\% mortality)  
2. Nondisseminated septicaemia (12\% of cases, 17\% mortality)
3. Localised infection (42% of cases, 9% mortality)
4. Transient bacteraemia (0.3% of cases)

From the Darwin prospective melioidosis study it was estimated that 88% of cases of melioidosis were acute presentations and 12% had chronic illness on presentation, defined as symptoms being present for over two months [5]. Overall 97% of cases were considered to result from recent infection, and only 3% were likely activation of disease from a latent focus years after prior infection. From analysing specific infecting events, the incubation period for acute melioidosis was established as being 1 to 21 days (mean, nine days) [5].

The incubation period, clinical presentations of melioidosis, and outcomes are thought to be determined by a combination of bacterial load infecting the individual, putative \textit{B. pseudomallei} strain differences in virulence, mode of infection and, most importantly, host risk factors for disease [6]. Inhalation of \textit{B. pseudomallei} is associated with more severe disease, higher mortality, and a greater proportion of presentations with pneumonia than is melioidosis following percutaneous inoculation. In northeast Thailand, over half of teenagers are seropositive for \textit{B. pseudomallei} [7]. Furthermore, in that region it was estimated that, for children, only about one in 4600 antibody-producing exposures results in clinical infection [8]. Therefore, the vast majority of infections with \textit{B. pseudomallei} appear to be subclinical. The high rates of background seropositivity in populations resident in endemic areas limit the use of serological tests in the diagnosis of melioidosis; although some clinical findings may be strongly suggestive (such as, hepatosplenic abscesses in Thailand) of the disease. In general treatment should be based upon definitive isolation of the organism on culture.

The proportion of patients with bacteraemia is relatively similar across different series. Overall bacteraemia rates in melioidosis have been documented at 41% in Thailand [9], 50% in Singapore [10], 60% in north Queensland, Australia [11] and 46% in the Northern Territory, Australia [12].

Mortality rates have been 49% in the large Thai study carried out between 1986 to 2004 [9], 65% in bacteraemic patients in Malaysia between 1976 to 1991 [13], 16% in Singapore between 1998 to 2007 [10], 22% in Taiwan between 2000 to 2005 [14], 25% in north Queensland between 1996 to 2004 [11], and 19% in the Northern Territory between 1989 to 1999 [12]. The most important determinants of survival from melioidosis are the absence of defined risk factors [15] (Section IV.3), and early diagnosis and access to appropriate antibiotics such as ceftazidime [16] and meropenem or imipenem [17].

In patients presenting with melioidosis and septic shock, the mortality varies from around 50% in regions where early, goal-directed resuscitation and state-of-the-art intensive care therapy are available to over 90% in some rural locations. In many melioidosis-endemic regions, renal replacement therapy and other resources for managing the metabolic abnormalities and organ dysfunction seen in severe sepsis are limited and without these, mortality in septiccaemic melioidosis will remain high [9,18,19]. In locations with access to state-of-the-art intensive care therapy, death from melioidosis in a patient without risk factors should be extremely uncommon provided there is timely diagnosis and institution of appropriate antibiotic therapy [12].
The clinical presentations of patients with melioidosis presenting to hospitals in northern Australia is summarised in Table IV.1. Whatever the initial presentation, secondary bacteraemic spread to distant sites is common.

### Table IV.1
Clinical presentations and outcomes of melioidosis in northern Australia

<table>
<thead>
<tr>
<th>Clinical Presentation</th>
<th>Total Number</th>
<th>Total Deaths (Mortality)</th>
<th>Bacteraemic Number</th>
<th>Bacteraemic Deaths (Mortality)</th>
<th>Non-Bacteraemic Number</th>
<th>Non-Bacteraemic Deaths (Mortality)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Septic shock</td>
<td>111</td>
<td>59 (53%)</td>
<td>99</td>
<td>49 (49%)</td>
<td>12</td>
<td>10 (83%)</td>
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<tr>
<td>Pneumonia</td>
<td>85</td>
<td>44 (52%)</td>
<td>75</td>
<td>36 (48%)</td>
<td>10</td>
<td>8 (80%)</td>
</tr>
<tr>
<td>Genitourinary</td>
<td>9</td>
<td>5 (56%)</td>
<td>8</td>
<td>4 (50%)</td>
<td>1</td>
<td>1 (100%)</td>
</tr>
<tr>
<td>Osteomyelitis/septic arthritis</td>
<td>4</td>
<td>2 (50%)</td>
<td>4</td>
<td>2 (50%)</td>
<td>0</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>No focus</td>
<td>13</td>
<td>8 (62%)</td>
<td>12</td>
<td>7 (58%)</td>
<td>1</td>
<td>1 (100%)</td>
</tr>
<tr>
<td>Non-septic shock</td>
<td>403</td>
<td>17 (4%)</td>
<td>181</td>
<td>13 (7%)</td>
<td>222</td>
<td>4 (2%)</td>
</tr>
<tr>
<td>Pneumonia</td>
<td>177</td>
<td>9 (5%)</td>
<td>78</td>
<td>8 (10%)</td>
<td>99</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>Genitourinary</td>
<td>64</td>
<td>2 (3%)</td>
<td>39</td>
<td>2 (5%)</td>
<td>25</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Skin abscess(es)</td>
<td>64</td>
<td>0 (0%)</td>
<td>1</td>
<td>0 (0%)</td>
<td>63</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Soft tissue abscess(es)</td>
<td>14</td>
<td>0 (0%)</td>
<td>1</td>
<td>0 (0%)</td>
<td>13</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Neurological</td>
<td>14</td>
<td>3 (21%)</td>
<td>3</td>
<td>0 (0%)</td>
<td>11</td>
<td>3 (47%)</td>
</tr>
<tr>
<td>Osteomyelitis/septic arthritis</td>
<td>12</td>
<td>0 (0%)</td>
<td>6</td>
<td>0 (0%)</td>
<td>6</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Other</td>
<td>58</td>
<td>3 (5%)</td>
<td>53</td>
<td>3 (6%)</td>
<td>5</td>
<td>0 (0%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>514</strong></td>
<td><strong>76 (15%)</strong></td>
<td><strong>280</strong></td>
<td><strong>62 (22%)</strong></td>
<td><strong>234</strong></td>
<td><strong>14 (6%)</strong></td>
</tr>
</tbody>
</table>

*Updated from Currie et al. [12].

b 7 blood cultures not done, 3 blood cultures negative.

c Blood culture not done.

2. Pneumonia and respiratory infection

Pneumonia is the commonest clinical presentation of patients with melioidosis in all studies, accounting for around half of cases. Acute melioidosis pneumonia has a spectrum from severe septic shock (mortality up to 90% or higher) to mild undifferentiated pneumonia with low mortality. Septicaemic patients present acutely unwell with high fevers and prostration and often little initial cough or pleuritic pain.

As observed on chest X-rays in these patients, diffuse nodular infiltrates often develop throughout both lungs. The infiltrates coalesce, cavitate, and progress rapidly, consistent with the caseous necrosis and multiple metastatic abscess formation seen at autopsy. Non-septicaemic patients with pneumonia have a more predominant cough, usually with dyspnoea and productive sputum. Their chest X-rays show discrete, and usually progressive, consolidation in one or more lobes. In melioidosis-endemic regions, pneumonia with upper lobe consolidation warrants consideration of melioidosis, although lower lobe infiltrates are also common.
It was thought until recently that a colonisation state did not exist for *B. pseudomallei*, with culture of *B. pseudomallei* from sputum or throat always reflecting disease. It has become evident, however, that *B. pseudomallei* can both colonise airways and cause disease in patients with cystic fibrosis (CF) and bronchiectasis [20]. The similarity to infection with *B. cepacia* in patients with CF is of concern, given the association of *B. cepacia* with more rapid deterioration in lung function. Patients with CF travelling to melioidosis-endemic locations should be warned of the risk of melioidosis, and this should be considered if they become sick after returning.

3. Sepsis without a clinical focus

Bacteraemia without an evident focus occurs in around 10% of presentations, with severity of illness ranging from rapidly-fatal septic shock to a clinically very mild febrile illness with low mortality. Severity appears to be more closely related to the development of sepsis-related organ dysfunction rather than the presence of bacteraemia *per se*. Secondary pneumonia, septic arthritis and osteomyelitis can develop in these patients, sometimes becoming clinically evident a week or more after commencement of appropriate antibiotic therapy.

4. Skin and soft tissue infection

A common presentation of melioidosis is with skin ulcer(s) or abscess(es). Bacteraemia and death are uncommon in these patients [21]. Indeed there are documented cases of cutaneous melioidosis who have cleared their infection before commencement of antibiotics [21]. Skin lesions can be single or multiple and are non-specific in appearance, ranging from dry, crusted papules or larger skin sores to frank purulent abscesses which may subsequently discharge and ulcerate. Classical cellulitis can occur in melioidosis but is very uncommon as a presenting illness. Multiple small pustules are sometimes seen in severe melioidosis sepsis as a result of bacteraemic spread to the skin.

5. Bone and joint infection

Presentation with septic arthritis or osteomyelitis also occurs, or one of these can develop after the patient has presented with another primary diagnosis, usually pneumonia. Any joint or bone can be involved and sequential septic arthritis in multiple joints can occur, necessitating repeated joint exploration for therapeutic aspirations and washouts. Secondary septic arthritis and osteomyelitis usually result from bacteraemic spread.

6. Visceral abscesses

Whatever the primary presentation, abscesses in internal organs are not uncommon in melioidosis. Spleen, kidney, prostate, and liver abscesses are particularly common. Where available, a computed tomograph (CT; Section V.4) of the abdomen and pelvis is useful in all melioidosis patients to detect these internal abscesses. While prostatic abscesses
and larger liver abscesses usually require drainage, most others respond to the prolonged antibiotic regimen. Abscesses in muscles, such as the psoas, also are seen and usually do not require drainage provided the primary infection is adequately treated. Secondary brain abscesses following another primary presentation can occur.

7. Parotid infection

Parotid involvement has been described in approximately 6% of all cases, but appears to be a characteristic presentation in Thai children in whom suppurative parotiditis is described in up to 40% of cases [22,23] (Section IV.4). Apart from involvement of distant organs, local complications include facial nerve palsy, rupture into the external auditory canal and involvement of bone. Parotitis is extremely rare in Australia.

8. Urinary tract infection

Isolation of *B. pseudomallei* in urine appears to be common in patients with melioidosis, with one Thai study demonstrating isolation of the organism in 28% of cases. It is routine to culture urine in suspected melioidosis. Therefore, it is possible that a testing bias may be present by which patients with suspected urinary tract involvement were more likely to have cultures performed. There is a correlation between the quantitative bacterial load in urine and mortality, suggesting that urinary *B. pseudomallei* may be a “spill over” phenomenon resulting from occult bacteraemia. In northeastern Thailand, renal tubular acidosis is endemic and is strongly associated with nephrolithiasis. Non-diabetic renal disease appears to be associated with urinary tract involvement, probably reflecting calculi acting as a nidus of infection.

Prostatic melioidosis appears far more common in Australia than elsewhere, with prostatic abscesses present in 20% of all male patients with melioidosis [24]. Some abscesses are asymptomatic and found on CT scan in patients who present with pneumonia or septicaemia without an evident focus. Most patients have a primary genitourinary presentation with fevers, abdominal pain, dysuria, and, sometimes, diarrhoea. Acute urinary retention often develops and requires catheterisation until the prostatic abscess has been drained.

9. Neurological melioidosis

Neurological melioidosis is an uncommon but dramatic presentation described in Australia but, apparently rarely, if ever, seen in Thailand. This accounts for around 4% of cases in Australia, with the distinctive clinical features being brainstem meningo-encephalitis, often with cranial nerve palsies (especially the VIth and VIIth nerves) and together with peripheral motor weakness of varying severity up to quadraparesis. The CT scan is often normal, but dramatic changes are seen on magnetic resonance imaging (MRI), most notably diffusely increased T₂ signal in midbrain, brainstem and spinal cord. Occasionally, presentation can be with acute flaccid paraparesis from myelitis without clinically-evident brain involvement [25,26]. Recent animal studies suggest that
infection may occur through colonisation of nasal olfactory epithelium and direct spread into the brain [27]. Macroscopic brain abscesses can also occur, probably resulting from bacteraemic spread, and these are described from outside Australia [28]. Frank meningitis is rarely seen in melioidosis.

10. Other presentations

The ability of *B. pseudomallei* to cause infections in almost any organ and the wide diversity of clinical presentations reflect the propensity of *B. pseudomallei* for bacteraemia and for intracellular survival and cell-cell propogation. It has been noted however, that endocarditis has yet to be reported with melioidosis [29]. Unusual foci of melioidosis infection described in case reports or case series include mycotic aneurysms, lymphadenitis mimicking tuberculosis, mediastinal masses, pericardial collections, mastitis, epididymoorchitis, adrenal abscesses and intestinal-associated masses with lymphadenopathy (which may reflect ingestion of *B. pseudomallei*).

11. Regional differences in presentation

Although there may be biases in presentation and ascertainment between Australia and Thailand, several obvious differences are evident between these two regions. In Thailand, visceral abscesses appear to be more common than in Australia. Parotid disease, common in Thailand, has only been described once in Australia. Conversely, prostatic abscesses, found in a fifth of Australian male cases, appear to be uncommon in Thailand. Similarly, the dramatic presentation with neurological melioidosis, which is described in 4% of Australian cases, appears to be rare in Thailand. The reasons for these differences, whether due to strain tropism or as yet unidentified behavioural differences that may result in a differing mode of acquisition, are not yet known.

References


Chronic melioidosis, relapse and latency

Direk Limmathurotsakul\textsuperscript{a}, Gavin C.K.W. Koh\textsuperscript{b,c}, Sharon J. Peacock\textsuperscript{a,c}, Bart J. Currie\textsuperscript{d}

\textsuperscript{a}Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand
\textsuperscript{b}Center for Experimental and Molecular Medicine, Academic Medical Center, University of Amsterdam, The Netherlands
\textsuperscript{c}Department of Medicine, University of Cambridge, Addenbrooke’s Hospital, University of Cambridge, UK
\textsuperscript{d}Menzies School of Health Research, Charles Darwin University, Australia

Abstract

Chronic melioidosis is the presence of symptoms for two or more months prior to presentation, and may mimic many diseases, including tuberculosis and cancer. Latent melioidosis is a retrospective term used to describe individuals who develop clinical features of infection many months or years after exposure to \textit{Burkholderia pseudomallei} and presumed infection. There is usually no recollection of a febrile illness around the time of exposure in these cases, and the onset of clinical melioidosis is often associated with a deteriorating host immune response. Recurrent melioidosis is the development of a further episode of \textit{B. pseudomallei} infection and is due to relapse following failure to eradicate the organism, or re-infection with a new \textit{B. pseudomallei} strain. Risk factors for relapse include short duration and choice of antimicrobial therapy for the primary infection; no risk factors have been identified for re-infection other than re-exposure to \textit{B. pseudomallei}. Understanding specific risk factors for relapse and re-infection could result in better preventive strategies.

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1. Introduction

Melioidosis was first described in Burma in 1911 in what was essentially a postmortem series of Burmese morphine addicts, and the syndrome described was that of a fulminant disease with a dire prognosis. While the most common presentation is acute septicemic infection, the manifestations of this disease are extremely broad (Tables IV.2, IV.3) and include a chronic form. Chronic melioidosis is often localised (for example, a lesion in the lung or an abscess), but may be impossible to differentiate on clinical grounds alone from other chronic bacterial infections, such as tuberculosis, or from cancer. Another rare presentation is activation of latent melioidosis with the onset of clinical manifestations months or years after exposure to the bacterium, without recollection of any illness at the
Table IV.2
Definition of common terminology used to describe clinical melioidosis

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute melioidosis</td>
<td>Melioidosis with duration of symptoms less than 2 weeks prior to presentation</td>
</tr>
<tr>
<td>Subacute melioidosis</td>
<td>Melioidosis duration of symptoms between 2 weeks and 2 months prior to presentation</td>
</tr>
<tr>
<td>Chronic melioidosis</td>
<td>Melioidosis with duration of symptoms longer than 2 months prior to presentation</td>
</tr>
<tr>
<td>Latent melioidosis</td>
<td>Asymptomatic B. pseudomallei infection that the organism remains dormant in the body for weeks or months, but which may become active at some indefinite point in the future</td>
</tr>
<tr>
<td>Activation of latent melioidosis</td>
<td>Melioidosis that present itself months or years after the last known exposure to B. pseudomallei</td>
</tr>
<tr>
<td>Recurrent melioidosis</td>
<td>The development of new symptoms and signs of melioidosis after an initial response to parenteral therapy. Recurrence may be due to relapse or re-infection.</td>
</tr>
<tr>
<td>Relapse</td>
<td>Recurrent melioidosis arising from failure to eradicate the original infecting strain</td>
</tr>
<tr>
<td>Re-infection</td>
<td>Recurrent melioidosis due to infection by a new strain after re-exposure to environmental B. pseudomallei</td>
</tr>
</tbody>
</table>

Table IV.3
Examples of deprecated terminology previously used to describe clinical melioidosis

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subclinical infection</td>
<td>Subclinical infection has been used to mean “latent infection”, as well as “minimally symptomatic/asymptomatic B. pseudomallei infection and none of the organisms are left in the body”</td>
</tr>
<tr>
<td>Recrudescence</td>
<td>Recrudescence has been used to mean “early relapse”, “relapse during treatment” and all “relapse”</td>
</tr>
</tbody>
</table>

Relapse, the development of new symptoms and signs of infection after a satisfactory response to therapy for melioidosis, is the single most important complication in patients who survive the initial episode. This may occur despite prolonged oral eradicative antimicrobial treatment. Re-infection is also well-described, and represents infection with a new strain of B. pseudomallei in an individual with a past history of melioidosis. Distinguishing between relapse and re-infection is important, since they have different implications for disease control and clinical management, but requires bacterial genotyping (to compare isolates from the two episodes), which is not readily available.
in many areas where melioidosis is endemic. The term “recurrence” (Table IV.2) has been used to describe a new episode of infection in an individual with a past history of melioidosis, and does not distinguish between relapse and re-infection. The mortality from recurrence is slightly lower than that for primary infection, at 24% in Thailand [2] or 15% in Australia [3].

2. Chronic melioidosis

Chronic melioidosis has been defined as an illness with symptoms for longer than two months duration at the time of presentation [3]. The time element in this definition may be associated with access to healthcare, and may vary greatly even within the same country. From a practical point of view, it is useful to recognise that chronic melioidosis is indolent, non-fulminant and most often localised without evidence of systemic disease. Fever, sweats and weight loss may be prominent, raising the suspicion of tuberculosis or lymphoma. The indolence of chronic melioidosis may be a function of the individual host immune response, differences in the virulence of the infecting organism, or mode of acquisition and magnitude of the bacterial inoculum [4].

Chronic melioidosis accounts for 12% (30/252) of all cases of melioidosis in northern Australia [3], and 8% (194/2,471) in northeast Thailand (unpublished data), but the majority of cases reported in non-endemic areas, including Europe and North America, are subacute or chronic [5,6]. One explanation for this could be that in these group of patients (such as the US war veterans) most who developed acute fulminant melioidosis during active service in Vietnam were either treated and recovered or died due to the disease overseas, thereby selecting for more chronic forms of disease in those returning home.

Chronic melioidosis may progress over many months or years, with periods of alternating remission and relapse. Any solid organ may be involved, and acute deterioration with bacteraemia may occur. The majority of patients present with fever and weight loss, together with symptoms specific to the organ involved: for example, chronic cough, chronic skin lesions, or cold abscesses. Patients with chronic pulmonary melioidosis may present with haemoptysis and pleuritic chest pain, and chest radiography usually shows apical cavitary disease with or without infiltration (mimicking tuberculosis). Other presentations include pleural effusions/empyemas or masses, which may mimic lung cancer [5]. Chronic skin lesions usually present as abscesses, which may be hot or cold, or chronic wounds that fail to heal. They may be associated with a history of penetrating injuries and may mimic Staphylococcus aureus infection. Cold abscesses are more commonly observed in diabetic patients, and the infection may extend locally to adjacent joint, bone or muscle [7].

The main problem associated with chronic melioidosis is delay in diagnosis. Chronic melioidosis may mimic many infectious diseases or cancers, and empirical treatment for these conditions is unlikely to include agents active against B. pseudomallei. As with all types of melioidosis, the diagnosis of chronic melioidosis is made by isolating and identifying the causative organism. There are three clues which, taken together, may lead the clinician to suspect this diagnosis: (1) a travel history to an area where
B. pseudomallei is present in the environment, (2) underlying risk factors for melioidosis, such as diabetes or chronic kidney disease, and (3) an infection refractory to normally-effective antimicrobial treatment; for example, B. pseudomallei normally would not be expected to respond to treatment with first-line anti-tuberculosis drugs. These clues may be difficult to elucidate, because the travel history may be some distance in the past and around a quarter of melioidosis patients have no identified underlying disease. The attending physician needs to have a high index of suspicion, and careful liaison with the hospital microbiologist is necessary. Similar to acute melioidosis, samples taken for culture should include blood, urine, sputum, throat swabs, wound or surface swabs and pus from any collection, regardless of clinical presentation. This is an important exception to usual culture practices, where normally sterile site cultures are considered of greater diagnostic value than those from colonised sites. This is because B. pseudomallei is not a member of the normal flora, and the presence of even a single colony from any sample in an individual with an infective process is diagnostic for melioidosis. An abdominal ultrasonogram may reveal multiple hepatosplenic abscesses, which is highly suggestive of melioidosis.

Particularly in areas where melioidosis is not endemic, failure to give the microbiologist advanced warning may lead to B. pseudomallei being disregarded by the laboratory as an environmental contaminant, or overgrown by other bacteria. If the clinical specimens are obtained from a sterile site, for example, blood, cerebrospinal fluid, or pus from deep abscess, the large wrinkled colonies seen on commonly used agars (similar in appearance to what is seen on a “dropped plate”) may be disregarded as environmental contamination. If samples are collected from non-sterile sites, for example, sputum or superficial wound swabs, the organism is unlikely to be recognised or may be overgrown by other bacteria unless selective media are used (for example Ashdown or B. cepacia-selective media) [8].

There are no published trials on the antimicrobial treatment of chronic melioidosis as a specific entity, and recommendations are essentially the same as for acute melioidosis. The exception to this is that, in rare cases, intravenous therapy may be unnecessary if the symptoms are mild and the infection is localised (cross-sectional imaging by computed tomography or magnetic resonance imaging may be necessary to confirm this) and the patient can be started directly on oral eradication therapy (Section VI). All abscesses or collections should be drained, and any infected prosthetic or foreign material must be removed. Patients with chronic melioidosis usually respond well to the treatment, and the prognosis is better than for acute melioidosis [3]. Fatality was observed in none of 30 patients (0%) with chronic melioidosis in Australia [3], and is around 25% in Thailand (unpublished data).

3. Latent infection

The term “latent melioidosis” is used here to define individuals with asymptomatic B. pseudomallei infection. This term can only be used retrospectively when infection becomes clinically apparent, sometimes many years after exposure. The longest period reported is 62 years between the time of last known exposure to B. pseudomallei and
the development of active disease [1]. It is not clear whether cases suffer a mild, non-specific, self-limiting primary episode around the time of exposure that is long forgotten by the patient, or whether there is no primary episode and it is merely an exceedingly long incubation period. The strongest evidence for latent melioidosis comes from reports originating in non-endemic areas [5]. In endemic areas, activation of latent melioidosis may rarely be diagnosed in patients who were previously asymptomatic but who had a history of an abnormality on chest radiography (that is often assumed to be due to *Mycobacterium tuberculosis* infection), but that is clearly involved in the infective process when presenting with melioidosis. Latency is also a possible explanation for patients who are seropositive for *B. pseudomallei* prior to admission to hospital for an unrelated illness, and who then develop melioidosis following admission [3]. There is no test for latency, and it is unclear whether positive serology could be used to identify latent melioidosis prior to activation; large longitudinal studies of healthy people who are seropositive for *B. pseudomallei* are needed to answer this question.

Activation of latent melioidosis may form only a small proportion of patients with melioidosis, although the total number of people with latent melioidosis in any population is unknown. In endemic areas, such as northern Australia, only 3% (8/252) of melioidosis patients were considered to be due to activation of latent infection [3]. Although there are a number of case reports of activation of *B. pseudomallei* in Vietnam veterans and in travellers from endemic areas [5], they are small in comparison to the estimated 225,000 seropositive soldiers who returned from Vietnam [9] or the total number of people who visit endemic regions of the world annually.

We know very little about the mechanisms by which *B. pseudomallei* establishes a quiescent nidus in the body. It has been hypothesised that *B. pseudomallei* may lie dormant within macrophages, giant cells, biofilm, or granulomatous tissue. As activation from a persistently abnormal chest radiograph has been observed [3], it has been suggested that the quiescent nidus must lie in the organ involved, in a state similar to that seen in tuberculosis [4].

When latent melioidosis is activated, it presents most frequently as pulmonary melioidosis, and the clinical picture may be as a fulminant form as observed in acute melioidosis [3,5]. However, the preponderance of pulmonary presentations may be a result of focussing on patients who had abnormal chest X-rays prior to presentation. The range of clinical manifestations seen is similar to acute melioidosis, and it is difficult to find any features that discriminate between acute melioidosis and activation of latent melioidosis [6]. Activation of latent infection may follow another unrelated infection, other illnesses, major surgery, or deterioration of host immunity. Activation has been described following influenza A, staphylococcal endocarditis, trauma, burn, steroid administration, cancer, diabetes, naproxen-induced acute renal failure, radiotherapy, and lead encephalopathy from petrol sniffing [1,3,4]. Patients who present with activation of latent melioidosis should be treated as for acute melioidosis.

The diagnosis of latent infection is not currently possible. For asymptomatic persons, positive serology may be due to previous exposure to either *B. thailandensis* or *B. pseudomallei*, and does not necessarily imply either latent infection or active disease. Studies looking for cross-reactivity between *B. thailandensis* and *B. pseudomallei* have
not produced consistent results [10]. The proportion of healthy individuals with positive serology is very high in endemic areas [4], which suggests that most of the exposure to *B. pseudomallei* may be asymptomatic, or induces only a mild infection and the individual does not present to healthcare. Compared to the known incidence of culture-confirmed melioidosis, it is estimated that only one in 4,600 antibody-producing exposures result in clinical infection [11]. Nonetheless, it may be prudent to educate asymptomatic patients who have persistently high antibody levels to *B. pseudomallei* about the nature of melioidosis. Currently there are no recommendations available for treatment of suspected latency.

4. Recurrent melioidosis: relapse or re-infection

Recurrent melioidosis is defined as the re-appearance of active disease after completion of acute phase treatment. A definitive diagnosis of recurrent infection is based on the isolation of *B. pseudomallei* from a clinical specimen taken from a patient who has completed a course of parenteral antimicrobial treatment and has documented evidence of recovery from a primary episode of melioidosis. The diagnosis of recurrence sometimes needs to be made on the basis of strong clinical evidence, but in the absence of any microbiological evidence. However, some patients may remain culture-positive for a prolonged period of time after completion of parenteral antimicrobials, despite the administration of appropriate oral eradicative treatment. This does not represent recurrence. Likewise, patients with melioidosis may temporarily deteriorate within the first month after the start of oral eradicative treatment, and this reflects only fluctuations in the course of recovery from the primary episode after switching from parenteral treatment to oral eradicative treatment. There is, therefore, a role for clinical judgment in defining when a recurrence has occurred. Studies in Australia and Thailand observe the culture-confirmed recurrence rate at between 5% and 25% [2,3].

Recurrence may be caused by relapse secondary to failure to completely eradicate the bacterium responsible for the primary infection, or it may be due to re-infection following exposure to a new bacterial strain. Many studies have treated recurrence as a homogenous condition, with cases being assumed to have resulted from activation or relapse of a persistent focus of infection [12]. Distinguishing between the two is important, since the relevant outcome in clinical trials of oral therapy for melioidosis is relapse and not re-infection. If all recurrent infections are assumed to be relapses, the attending physician may be tempted to switch to less-effective second-line treatment, for example, amoxicillin–clavulanic acid, on the assumption that the recurrence is a failure of first-line oral eradicative treatment. Inappropriate use of less-effective second-line drugs exposes patients with re-infection to an unnecessary risk of relapse. Moreover, the physician may fail to educate the patient about measures for preventing re-infection.

Genotyping studies suggest that re-infection is a relatively common cause of recurrent melioidosis. By comparing bacterial isolates from the first and second episode of melioidosis, several small studies of recurrent melioidosis (5 to 35 patients) found that 6% to 20% of recurrent infections were caused by isolates that were genetically different from the primary isolate [13,14]. However, two possible sources of error are: (1) if
simultaneous infection with more than one strain of *B. pseudomallei* were common, and different strains from the primary and recurrent episode were picked by chance for genotyping, and (2) if the population genetic structure of *B. pseudomallei* in the local environment was highly clonal, then a patient could be repeatedly-infected by the same bacterial clone. The former would lead to misclassification of relapse as re-infection and *vice-versa* for the latter. A prospective study of 133 patients with culture-proven melioidosis found that co-infection with multiple *B. pseudomallei* genotypes is rare (less than 2%), and does not represent a significant source of error [15]. The genetic diversity of *B. pseudomallei* in the environment is very high, even in small areas [16,17]. This suggests that re-infection by the same strain is unlikely.

In Ubon Ratchathani, northeast Thailand, a cohort of 116 first recurrences was studied using a combination of pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). 74% of recurrent infections (86 episodes) were identified as relapses, and 26% as re-infections (30 episodes) [18]. The high proportion of re-infections may be a function of the long patient follow-up, with the longest interval between primary infection and recurrence being more than 10 years. The median time from initiation of oral eradicative treatment to relapse was 26 weeks (interquartile range [IQR] 10 to 72 weeks), while median time to re-infection was 111 weeks (IQR 59 to 164 weeks). Overall, 90% (63 of 70) of first recurrences occurring within 12 months of the primary infection were relapses, and this number decreased to 65% (13 of 20) in the second year and 50% (16 of 32) more than two years after the primary episode.

Similar to acute melioidosis, any solid organ may be involved, and the presentation may be acute and fulminant [19]. However, the same body site or organ is usually involved in both relapse and re-infection. A recent study found that, in each patient with recurrent infection, the organs involved during the first and second episode of melioidosis are more similar than would be expected by chance [20].

A simple scoring system has been described that enables clinicians to determine the most probable cause for recurrence (Figure IV.1) [19]. This may help determine the cause of recurrent infection in melioidosis-endemic regions where genotyping facilities are seldom available. Briefly, relapse is associated with previous inadequate treatment and time from start of eradicative treatment to time of recurrence of less than one year; re-infection is associated with renal insufficiency and presentation during the rainy season, factors which are scored using a predefined number based on their presence or absence. A total score of greater than five predicts re-infection, while a score of less than five predicts relapse. This score may help identify the cause of recurrent melioidosis and therefore help to individualise oral eradicative treatment and management (Section VI).

5. Risk factors for relapse and re-infection

The risk factors for recurrent melioidosis have been defined previously and include severity of the primary episode, the antimicrobial drugs used to treat the primary episode, and antimicrobial treatment duration [3,12]. However, these studies treated recurrence as a homogenous condition, with cases assumed to have resulted from activation of a persistent focus (i.e. relapse). Most of the risk factors listed in this section are
Fig. IV.1. Four predictors of re-infection and relapse for patients with recurrent melioidosis. Points can be determined for each of the predictors using the figure. Factors associated with re-infection give a positive score, while factors associated with relapse give a negative score. The total score is reached by adding the points together for these four variables. A total score of more than or equal to 5 indicates re-infection as a probable cause of recurrent melioidosis, while a total score of <5 is predictive for relapse.

Therefore based on the results of a single study that distinguished between relapse and re-infection [2].

The choice and duration of oral antimicrobial therapy are the most important determinants of relapse, followed by blood culture positivity and multifocal distribution [2]. Compliance with oral medication is likely to be crucial. The lowest risk of relapse was associated with 12 to 16 weeks of oral treatment, which supports the recommended minimum duration of oral treatment of 12 to 20 weeks. Amoxicillin–clavulanate is associated with an increased risk of relapse compared to the first-line
regimen (trimethoprim–sulfamethoxazole [TMP-SMX]-based therapies), but remains an alternative for patients with intolerance to TMP-SMX based regimens, and continues to be used first-line to treat children and pregnant women in Thailand. Fluoroquinolone-based regimens and doxycycline monotherapy are associated with a high risk of recurrence [21,22]. The risk factors associated with relapse [2], and previously reported risk factors of recurrent infection as a whole [3,12], are similar.

No risk factors have been identified for re-infection [2]. The lack of identified risk factors may relate to a lack of statistical power of the study, or the fact that relevant variables were not included; however, the incidence of re-infection in melioidosis survivors was 1,280 per 100,000 person-years, which is two orders of magnitude higher than 12.7 per 100,000 person-years in the general population (unpublished data). This indicates that risk factors for re-infection do exist. The most obvious risk factor is exposure to \textit{B. pseudomallei} in the soil, which was not specifically examined by this study, but which is a \textit{sine qua non} for melioidosis. Minimising exposure to the organism is a realistic option for reducing risk in this group.

General guidelines to reduce exposure have been recommended to farmers, who are the highest at-risk group. This should be applied to all patients who survive from primary melioidosis. However, rice farming involves wading in mud, together with exposure of the arms. Wearing full-length boots and gloves is not always practical. One alternative strategy includes mechanising the process of rice farming, to make it less dependent on human labour and, therefore, reducing direct contact with the soil. Another attractive strategy is to eradicate \textit{B. pseudomallei} from the soil. The former requires proof of economic benefit and, probably, government support for the initial capital outlay. There is no immediately obvious vaccine candidate and the ultimate goal in prevention would be eradication of \textit{B. pseudomallei} from the environment.

6. Conclusion

Chronic melioidosis is an indolent disease capable of mimicking a wide variety of other diseases, particularly tuberculosis. Mortality from chronic melioidosis is low, provided appropriate therapy is initiated promptly. Latent melioidosis is an entity that is postulated to exist, as active disease may appear many years after exposure. Currently, there is neither means to diagnose latent melioidosis, nor a recommended antimicrobial treatment for individuals who are suspected to have it.

Recurrent melioidosis may be due to relapse or re-infection. Severity of the primary infection and duration and type of oral antimicrobial treatment are the most important risk factors for relapse. The management of relapse and re-infection are broadly similar, but distinguishing between the two has widely differing implications for disease control and prevention.

References


Section IV.3

Clinical risk factors for melioidosis

Allen C. Cheng\textsuperscript{a,b,c}, Direk Limmathurotsakul\textsuperscript{d}, W. Joost Wiersinga\textsuperscript{e}, Yupin Supputamongkol\textsuperscript{f}, Bart J. Currie\textsuperscript{a,g}

\textsuperscript{a}Menzies School of Health Research, Darwin, Australia
\textsuperscript{b}Department of Epidemiology and Preventive Medicine, Monash University, Melbourne, Australia
\textsuperscript{c}Infectious Diseases Unit, Alfred Hospital, Melbourne, Australia
\textsuperscript{d}Department of Tropical Hygiene and Mahidol–Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand
\textsuperscript{e}Academic Medical Centre, University of Amsterdam, Amsterdam, The Netherlands
\textsuperscript{f}Siriraj Hospital; Mahidol University, Bangkok, Thailand
\textsuperscript{g}Northern Territory Clinical School and Infectious Diseases Department, Royal Darwin Hospital, Darwin, Australia

Abstract

Although melioidosis can affect previously healthy patients, several comorbidities appear to be strongly associated with disease. Case control and population-based studies have defined an increased risk of melioidosis in patients with diabetes, hazardous alcohol intake, renal disease, immunosuppression and thalassaemia. These specific comorbidities provide clues to the pathogenesis of melioidosis, suggesting that immune dysfunction specifically affecting innate immunity, such as neutrophil function, is an important mechanism rather than conditions affecting adaptive immune function, such as infection with human immunodeficiency virus (HIV). Despite the demonstration of a cell-mediated response to \textit{Burkholderia pseudomallei}, HIV infection does not appear to be a significant risk factor for disease, further supporting that adaptive immunity may have a limited role in protecting against infection. Exposure to mud and pooled surface water in endemic areas is an important and potentially preventable risk in susceptible patients.

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1. Introduction

Although melioidosis can affect otherwise healthy patients, a number of clinical risk factors appear to be important and associated with an increased risk of developing melioidosis (Tables IV.4, IV.5). These may provide clues to the pathogenesis of this disease, which remains poorly understood. Like many infectious diseases, risk factors can be divided into three components (Figure IV.2):
### Table IV.4

Established risk factors for melioidosis

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Australian studies</th>
<th>Thai studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes mellitus</td>
<td>37% of patients are diabetic, mainly type 2. Associated with a RR of 13 [1−3]</td>
<td>23–60% of patients have diabetes, with an OR of 4.8–5.9 when compared to hospital-based controls [4,5]</td>
</tr>
<tr>
<td>Thalassaemia</td>
<td>Not reported</td>
<td>α-thalassaemia trait common in Thailand (44%) but disease less common (7–8%); case–control studies in Thailand estimate RR 10.2 [4–6]</td>
</tr>
<tr>
<td>Aboriginality</td>
<td>Population-based study in Australia estimate RR 2.7–8.1; assumed to relate to exposure to soil/water [1−3]</td>
<td>Not relevant</td>
</tr>
<tr>
<td>Male gender</td>
<td>All series in Australia, Thailand, Malaysia and Singapore demonstrate male preponderance [2−4,7]</td>
<td>All series in Australia, Thailand, Malaysia and Singapore demonstrate male preponderance [2−4,7]</td>
</tr>
<tr>
<td>Soil/water exposure</td>
<td>25% of patients report a inoculating injury [8]. Incidence closely linked to rainfall and flooding [9,10]</td>
<td>Rice farmers constitute 81% of patients in Thailand, RR risk in case–control study estimated at 3.3 [4,5]</td>
</tr>
<tr>
<td>Renal disease</td>
<td>Patients with renal impairment or failure comprise 10% of Australian series [1], with RR of 3.2 [3]</td>
<td>Renal disease (renal failure and calculi) described in 20–27% and associated with increased risk of melioidosis (OR 2.9) [4,5]</td>
</tr>
<tr>
<td>Alcohol</td>
<td>Excessive alcohol use documented in 39% of Australian patients with RR 2.1–6.7 in case–control and population-based study [1−3]</td>
<td>Less prevalent in Thai patients (12%) [4]</td>
</tr>
<tr>
<td>Kava use</td>
<td>Use of <em>Piper methysticum</em> root documented in 8% of Australian series [1] but not associated with pneumonia in case–control study [11]. Strongly associated with prostatic disease, but possibly confounded by geographically restricted pattern of use [12]</td>
<td></td>
</tr>
<tr>
<td>Chronic lung disease</td>
<td>Present in 27% of Australian patients [1] with RR of 4.3 [3]</td>
<td>Not described</td>
</tr>
</tbody>
</table>

RR Relative Risk; OR Odds Ratio.
### Table IV.5
Other reported risk factors associated with melioidosis

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Level of evidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Splenectomy</td>
<td>Often related to thalassaemia [6,13,14]</td>
</tr>
<tr>
<td>Aplastic anaemia, febrile neutropenia</td>
<td>Case reports [4,15,16]</td>
</tr>
<tr>
<td>Chronic granulomatous disease</td>
<td>Case reports [17,18]</td>
</tr>
<tr>
<td>Mycobacterial disease</td>
<td>Case reports of patients with atypical mycobacteria, ( M. \text{tuberculosis} ) or ( M. \text{leprae} ) infection may suggest common host susceptibility [4,19–21]</td>
</tr>
<tr>
<td>Renal transplantation</td>
<td>Case report; patient also diabetic [22]</td>
</tr>
<tr>
<td>Systemic lupus erythematosus or steroid use</td>
<td>Case reports; also associated with immunosuppressives [4,23–25]. Steroid-containing herbal remedies documented in up to 10% of Thai patients [5].</td>
</tr>
<tr>
<td>Glucose-6-phosphatase deficiency</td>
<td>Case reports [4]</td>
</tr>
<tr>
<td>Haemosiderosis</td>
<td>Case reports [26,27]. One unreported case of pulmonary haemosiderosis secondary to mitral valve disease (Currie B, personal communication)</td>
</tr>
<tr>
<td>Cystic fibrosis</td>
<td>Multiple reports from travelers to endemic areas [28–31]</td>
</tr>
</tbody>
</table>

---

**Fig. IV.2.** Interaction between host, bacterial and environmental factors in melioidosis.
1. Host susceptibility factors; patients with melioidosis have a higher prevalence of diabetes, heavy alcohol use, renal disease and possibly specific immunological defects.

2. Bacterial factors; although a number of virulence determinants have been defined in vitro and in animal studies, bacterial factors strongly associated with severity or particular sites have not been defined. However, there are strong regional differences both in bacterial phylogeny and disease manifestations (such as, parotid abscesses in Thailand), suggesting that strain variation in tropism or virulence may be present.

3. Environmental factors; exposure to *B. pseudomallei* in endemic areas is presumably a *sine qua non* of melioidosis, however unusual cases in patients with specific immunological defects have occurred in areas not previously known to have autochthonous cases suggesting that the organism may be more widespread than previously appreciated, perhaps at very low concentrations.

Factors associated with melioidosis (that is, the comorbidities that are more commonly found in patients presenting with melioidosis than in the general population who are at risk), should be distinguished from factors associated with mortality (that is, the comorbidities more commonly found in fatal cases of patients with melioidosis). Although there are few fatalities in Australian patients without comorbidities [1], no specific comorbidity has been associated with mortality, and markers of the severity of illness are much stronger predictors of outcome [32]. This is likely to reflect poor outcomes from severe sepsis in patients with a decreased physiological reserve with chronic underlying diseases.

### 2. Diabetes

Diabetes is the most commonly identified risk factor in patients with melioidosis: up to 60% of patients with melioidosis have pre-existing or newly diagnosed type 2 diabetes mellitus [1,2,4,5,33]. Case control studies suggest that diabetes is associated with an odds ratio of 4.6 to 5.9 compared to hospital-based controls, and, in a population based study, was associated with a relative risk of 12.9 to 13.1 [2,3,5]. For a significant number of patients, melioidosis may be the first presentation of previously unrecognised diabetes. However, a distinction should be made between diabetes and acute hyperglycaemia due to a stress response.

The mechanism of the association between diabetes and risk of developing melioidosis is unclear. Hyperglycaemia has been associated with defects in neutrophil function [1,4,34] and is known to play a key role in the pathogenesis of melioidosis [35,36]. It has recently been shown that polymorphonuclear neutrophils (PMN) from otherwise healthy diabetic Thai subjects display impaired phagocytosis of *B. pseudomallei*, reduced migration in response to interleukin-8, and an inability to delay apoptosis when compared to PMN of healthy non-diabetics [37]. Earlier studies have argued against a role of endogenous or exogenous insulin in the pathophysiology of melioidosis [38,39]. Interestingly, despite being a strong risk factor for the acquisition of melioidosis, diabetes was found to be independently associated with survival outcome in one small retrospective study [40]. This may suggest that the infective threshold is decreased in diabetic patients.
but disease may not necessarily be severe once established. However, further confirmation of this phenomenon is required.

3. **Hazardous alcohol intake**

In two Australian series, 39% to 42% of patients with melioidosis were reported to have excessive alcohol consumption [1,33], and a relative risk of 2.1 to 6.7 in controlled studies [2,3]. In Thailand, a retrospective case control study did not find an association with alcohol use [4]. The mechanism by which alcohol intake may be related to the acquisition of melioidosis is not known, in particular, whether susceptibility is due to chronic alcohol use, acute intoxication or factors associated with heavy alcohol use, such as aspiration, chronic liver disease or malnutrition. Studies have established that acute alcohol intoxication and malnutrition are associated with neutrophil dysfunction in animal studies [41,42]. Acute intoxication is obviously correlated with long term use of alcohol, and the prevalence of chronic liver disease is not known in this group due to the insensitivity of clinical markers and underdiagnosis of chronic liver disease.

4. **Renal disease**

In northern Australia, 10% of patients with melioidosis have renal failure, and renal failure is associated with a relative risk of melioidosis of 3.2 [3]. Chronic renal failure is also associated with a variety of immunological impairments, including impairment of neutrophil function [43]. In northeastern Thailand, distal renal tubular acidosis is endemic and often complicated by renal calculi [44]; 8% of patients with melioidosis had renal calculi and 5% of patients had chronic renal failure. Renal disease (renal failure or renal calculi) was associated with an odds ratio of 2.6 to 2.9 when compared to hospital-based controls [4,5]. Renal disease was only associated with a mildly increased risk of urinary tract involvement [40].

5. **Indigenous status**

The finding that Aboriginal Australians have a relative rate of melioidosis three times higher than non-Indigenous Australians [3] does not necessarily invoke genetic susceptibility as an explanation, particularly as melioidosis has been shown to affect a diverse group of residents and travellers to endemic areas. Indigenous Australians are at an increased risk of a number of infectious diseases that are thought to relate to a lack of access to health care, poor educational status and socio-economic deprivation; a greater proportion of the population is likely to be exposed to *B. pseudomallei* through activities such as hunting, and the prevalence of risk factors for melioidosis, particularly diabetes, is much higher in this group.

6. **Immunosuppression and immunological defects**

Immune dysfunction specifically affecting neutrophils, such as neutropenia or chronic granulomatous disease appear to be more strongly associated with susceptibility to
melioidosis. Case studies have noted an intriguing association with chronic granulomatous disease that provide support for the key role of neutrophils in protection against melioidosis [17,18]. On the basis of this hypothesis, granulocyte colony-stimulating factor, a cytokine shown to increase neutrophil number and function, was studied as an adjuvant to antibiotics. Although observational data with a historical control appeared to be associated with a substantial fall in mortality in northern Australia [45], no significant differences were seen in a clinical trial in Thailand [46].

There have been several reports of melioidosis as a cause of febrile neutropenia [15,16], as a complication of aplastic anaemia [4] or following chemotherapy [47]. Other isolated reports of melioidosis have been noted in patients with cancers, including hepatocellular carcinoma and lung cancer [48–50]. It is questionable however if these observations are specific for melioidosis. In the near future new data will probably become available on the genetic susceptibility of the host to infection with *B. pseudomallei*. New polymorphisms in innate immunity genes that are associated with the risk of acquiring infection have already been described for a large number of other bacterial infections [51]. It is already known that a polymorphism in the tumour necrosis factor-alpha (TNF-α) gene (the −308 TNF-α promoter polymorphism), which is related to severity of disease for several other infectious diseases, is associated with both the occurrence and severity of melioidosis [52].

Despite cell-mediated immunity being implicated as a mechanism of resistance to melioidosis [53,54], infection with HIV does not appear to be a major risk factor [55]. The authors are not aware of any reports of melioidosis associated with defects of the humoral immune system, and antibodies specific to *B. pseudomallei* have not been shown to be protective. The use of steroids is associated with an increased risk of melioidosis; this includes steroid-containing herbal remedies (“yaa chud”) in Thailand, documented in up to 10% of Thai patients [5]. Corticosteroids have wide ranging effects on the immune system, including reducing migration of neutrophils to sites of inflammation by down-regulation of endothelial and neutrophil receptors [56]. Remarkably, melioidosis is also uncommonly reported following organ transplantation and in association with connective tissue disease [22–24]. Case reports of melioidosis and previous or subsequent mycobacterial infection (*Mycobacterium tuberculosis*, *M. terrae*, *M. leprae*) may reflect a common host susceptibility to these intracellular pathogens [4,19–21].

Clinical thalassaemia (β-thalassaemia/haemoglobin E or haemoglobin H disease) seems to be strongly associated with melioidosis in Thailand [4,5]. Thalassaemia trait is found in approximately 40% of Thai patients and does not appear to be associated with melioidosis [5]. Reports of patients with haemosiderosis may suggest a common mechanism, as iron is known to impair phagocytic cell function [26,27].

7. Respiratory disease

Chronic lung disease was reported in 27% of Australian patients with melioidosis [1] and associated with an increased risk of melioidosis in a population-based study [3], but smoking was not noted to be a risk factor in Thailand [5]. Patients with chronic lung disease are more likely to have pneumonia (unpublished data). Patients with cystic fibrosis
appear to be at risk of chronic airways colonisation analogous to disease associated with *B. cepacia* [28]. Anecdotal cases have been noted of similar chronic disease in patients with bronchiectasis not related to cystic fibrosis (unpublished data).

8. Kava and prostatic disease

In the Australian series, the consumption of kava, an extract of the root of the plant *Piper methysticum* introduced as an alternative to alcohol, was reported in 8% of patients [1]. Kava use, particularly in the East Arnhem region of northern Australia, appears to be specifically associated with prostatic disease [28]. It is not clear whether this is due to kava directly or a particular behaviour placing patients at risk of melioidosis in this region; however, it does not appear that this prostatic tropism is due to a geographically restricted strain [57].

9. Exposure

Melioidosis occurs when susceptible individuals are exposed to *B. pseudomallei*, and the risk is probably dependent on the inoculating concentration and the extent of exposure. Rice farming in northeast Thailand is a well described occupational risk factor for exposure; farmers spend many months of the year planting and re-planting seedlings in flooded rice paddies. Additionally, the concentration of *B. pseudomallei* appears to be highest in this region of Thailand [58]. Melioidosis can also be associated with recreational exposure, such as sporting injuries in contaminated grounds (unpublished data).

Specific incidents where an otherwise healthy individual is exposed to high infecting inoculum could also cause infection. This includes laboratory workers exposed to pure culture of *B. pseudomallei* in accidents, near drowning incidents, extreme weather events (such as flooding associated with tropical cyclones) and the Indian Ocean tsunami of 2006 where several cases were reported in returning travellers [9,59–61].

10. Management of patients with risk factors for melioidosis

In general, patients are advised to be aware of the risk of melioidosis, particularly during extreme weather events or monsoonal rains. Where possible, diabetic patients should wear protective footwear to reduce exposure to mud and pooled surface water. Protective clothing has been shown to reduce the risk of a number of infectious and non-infectious diseases in rice farmers but are not widely used in Thailand [62]. Travellers (and their clinicians) should be aware of the possibility of melioidosis presenting with febrile illnesses after return from endemic areas, even after many years [63]. Asymptomatic patients with a history of exposure in endemic areas do not generally require screening or post-exposure prophylaxis. Screening may be indicated in some specific situations, such as, planned immunosuppression or following laboratory accidents, depending on local clinical guidelines. Patients with positive serology detected incidentally or localised
nodules should have cultures of throat and rectal swabs, urine and sputum to exclude active chronic melioidosis.

A common clinical problem is the management of the patient who is found to have positive serology for melioidosis incidentally or on pre-immunosuppression screening prior to cancer chemotherapy or organ transplantation. Patients with or about to undergo immunosuppression and their treating clinicians should be informed of the risk of reactivation of melioidosis in those with positive serology. Measures should also be taken to avoid exposure to mud and pooled surface water. In melioidosis-endemic settings, empiric antibiotic protocols for febrile neutropenia should include antibiotics active against \textit{B. pseudomallei}, such as ceftazidime or meropenem.

In Australia, several other measures are commonly instituted to reduce the risk of melioidosis in immunocompromised patients:

1. Where possible, administration of adjuvant chemotherapy is delayed until after the monsoonal season (or the patient’s return to the endemic region is delayed, if treatment is administered elsewhere).

2. For patients with serological evidence of prior exposure (indirect haemagglutination titre $\geq 1:40$), common clinical practice is to obtain a baseline chest X-ray and cultures for \textit{B. pseudomallei} to exclude active infection and then, if negative, to prescribe long-term cotrimoxazole (for example, 800/160 mg bd).

Although low-dose cotrimoxazole is common practice to prevent \textit{Pneumocystis jiroveci} pneumonia, high-dose cotrimoxazole carries the risk of bone marrow suppression and delayed haematological recovery; second-line agents with activity against \textit{B. pseudomallei} include doxycycline or coamoxiclav. In Thailand where the prevalence of positive serology is much higher, the appropriate serological threshold is undefined and secondary prophylaxis is not routinely provided.

References


Section IV.4

Paediatric melioidosis

Pagakrong Lumbiganon\textsuperscript{a}, Allen C. Cheng\textsuperscript{b,c,d}, Bart J. Currie\textsuperscript{b,e}

\textsuperscript{a} Division of Infectious Diseases, Department of Paediatrics, Faculty of Medicine, Khon Kaen University, Thailand
\textsuperscript{b} Menzies School of Health Research, Darwin, Australia
\textsuperscript{c} Department of Epidemiology and Preventive Medicine, Monash University, Melbourne, Australia
\textsuperscript{d} Infectious Diseases Unit, Alfred Hospital, Melbourne, Australia
\textsuperscript{e} Northern Territory Clinical School and Infectious Diseases Department, Royal Darwin Hospital, Darwin, Australia

Abstract

Melioidosis has similar clinical manifestations in children but comorbidities are less common than in adults. A significant risk factor for disease is near drowning, which may be associated with a very short incubation period and development of septicemia. In Thailand, parotid disease is a common presentation, and often requires surgical drainage. Lymphadenitis and skin infection are also common presentations which may be unrecognised. An encephalomyelitis syndrome is a rare but distinct presentation described in Australia. Relapse appears to be less common than in adults.

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1. Introduction

Melioidosis in children is less common than in adults. Children younger than 15 years of age account for 5.3% to 17% of the total cases in most series [1,2]. Most of the clinical presentations are similar but the associated comorbidities are less common in children than in adults. Two distinct paediatric syndromes are described. Septicaemic illness is associated with a fulminant course and may be associated with comorbidities or near drowning. Localised disease (Figure IV.3), primarily involvement of parotid, skin, subcutaneous tissue or lymph nodes, is associated with a better prognosis. Neurological disease is uncommon but a recognised syndrome in Australia.

The largest paediatric series consists of 55 children with culture-confirmed melioidosis from Thailand. Of these, 20 patients had septicemia, while 35 patients had localised infection, with the median age of patients being 11 and 6 years, respectively [3]. Eleven patients (55%) in the septicemic group had underlying diseases, which included diabetes, haematologic malignancy, chronic renal failure, and prolonged corticosteroid administration (for the treatment of aplastic anaemia and nephrotic syndrome). In patients
with localised infection, none of the patients had comorbidities. Two patients in the septicaemic group developed rapidly progressive pneumonia and septic shock three days after near drowning in natural water sources. The most common organ involvement was the lung (75%). In localised melioidosis, suppurative parotitis was the most common manifestation (40%). Other common infections included skin and subcutaneous abscesses, and lymphadenitis, with no fatalities.

2. Risk factors in children

Where present, the most common underlying diseases associated with melioidosis in children include diabetes, chronic renal failure, and being treated with immunosuppressive agents [3−5]. Another significant risk factor for disease is near drowning, which may be associated with a very short incubation period and a rapidly progressive pneumonia with septicaemia. It is associated with a high mortality [3,6]. Other uncommon chronic diseases associated with melioidosis, include cystic fibrosis [7] and chronic granulomatous disease [8]. Patients with these conditions travelling to endemic areas may also be at risk.

3. Septicaemia

The most common organ involvement in septicaemic melioidosis is the lung. The patient usually presents with acute onset of fever and tachypnoea that progresses rapidly to severe sepsis. Even in previously healthy children septicaemic melioidosis is associated with high mortality rates [9]. Chest X-rays most commonly show bilateral alveolar infiltration. Other findings include lung abscess or unilateral alveolar infiltration. There may be abdominal pain with liver and/or splenic abscesses. Other common secondary sites of
Table IV.6
Organ involvement in paediatric patients with septicaemic and localised melioidosis

<table>
<thead>
<tr>
<th>Organ involvement</th>
<th>Septicaemia (n=20), % (n)</th>
<th>Localised infection (n=35), % (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>75 (15)</td>
<td>8.6 (3)</td>
</tr>
<tr>
<td>Skin</td>
<td>25 (5)</td>
<td>31.4 (11)</td>
</tr>
<tr>
<td>Spleen</td>
<td>20 (4)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Liver</td>
<td>15 (3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Bone and joint</td>
<td>15 (3)</td>
<td>5.7 (2)</td>
</tr>
<tr>
<td>Muscle</td>
<td>5 (1)</td>
<td>0 (2)</td>
</tr>
<tr>
<td>Genitourinary tract</td>
<td>5 (1)</td>
<td>0 (2)</td>
</tr>
<tr>
<td>Lymph node</td>
<td>5 (1)</td>
<td>14.3 (5)</td>
</tr>
<tr>
<td>Parotid gland</td>
<td>0 (0)</td>
<td>40 (14)</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>10 (2)</td>
<td>5.7 (2)</td>
</tr>
</tbody>
</table>

infection include the genitourinary tract, bone and joint although almost any organ in the body could be affected (Table IV.6). In some cases, diffuse pustular skin lesions observed may be associated with systemic sepsis; biopsies of these skin lesions, as well as blood cultures are commonly culture positive. In severe infection multiple sites are involved [1]. In some children septicaemia without an obvious focus of infection has also been observed.

4. Skin infection

Skin infection is the second most common presentation of melioidosis in children. Primary skin infection can occur. It usually presents insidiously as a skin nodule after blunt trauma, which might be tender but with no sign of inflammation. Therefore, it could be misdiagnosed as a non-infectious lesion. There may be no fever or other systemic symptoms and in some cases, the infection can be treated successfully by incision and drainage of abscess without appropriate antibiotic therapy. This type of skin lesion is chronic and is usually mild, even being diagnosed after a year of illness. There is also usually no bacteraemia or septicaemia. Cellulitis is uncommon.

In endemic areas, traumatic injury leading to open wounds may become contaminated with soil or water and lead to skin and soft tissue infection that may progress to involve the underlying tissue and bone. Multiple pustular lesions can occur in different sites due to haematogenous spread, but this is an uncommon complication [10,11].

5. Parotitis

Suppurative parotitis presents with fever and painful, unilateral swelling of the cheek without preceding respiratory symptoms. The illness may last for a few days to a week before the patient presents for medical attention. During the first few days of illness, it can be misdiagnosed as mumps, but the involvement is usually unilateral and the
swelling and tenderness of the parotid gland lasts for more than a week. Fluctuance is demonstrated late in the course of illness. There may be purulent discharge at the opening of Stensen’s duct when applying pressure around the opening. Involvement of the facial nerve (either directly, or at the time of surgery) may result in paralysis. In some cases, extensive spread of the abscess may occur, involving the parapharyngeal and deep temporal fossa, periorbital cellulitis and conjunctivitis [3,12,13]. Bacteraemia or septicaemia due to \textit{B. pseudomallei} infection of the parotid is rare [14].

6. Lymphadenitis

Melioidosis of the lymph nodes usually presents as gradual, painful or painless, unilateral lymph node enlargement, and most commonly involves the head and neck region, in particular, the cervical lymph node [3]. Systemic signs and symptoms other than fever are usually absent. It could be misdiagnosed as tuberculosis, infection with nontuberculous mycobacteria or as a malignancy [15]. Lymph node involvement is not usually associated with bacteraemia or severe sepsis, but infections at other sites of the body can occur following lymphadenitis.

An uncommon syndrome of fever, sore throat and exudative tonsillitis followed by cervical lymphadenitis can also occur in paediatric patients. The patient may be misdiagnosed and treated for streptococcal pharyngitis. Diagnosis can be made by culture of \textit{B. pseudomallei} from the tonsillar exudates and/or pus aspirated from the cervical lymph node [16].

7. Involvement of the central nervous system

Brain abscesses occasionally occur in paediatric melioidosis [17]. It could be the initial presentation, but more commonly, it is a secondary manifestation following pneumonia. Surgical drainage may be required. Florid meningitis is rare, but in Australia an encephalomyelitis syndrome has been described as a distinct, although rare, presentation of melioidosis [18]. Patients present with fevers, headache and sometimes with mild neck stiffness. Initially the patient could be conscious. The most dramatic changes include focal cranial nerve signs, such as a VIth or VIIth nerve palsy, ophthalmoplegia, cerebellar abnormalities and peripheral limb weakness. Magnetic resonance imaging could be used to confirm extensive infection involving the brainstem and/or cerebellum, often extending into the upper spinal cord. Clinical progression to quadriplegia and severe bulbar paralysis requiring ventilation can occur. Even those patients requiring prolonged ventilation can improve and survive, with varying levels of disability. Occasionally, presentation can involve isolated myelitis, with acute flaccid paraparesis and urinary retention without other clinical evidence of brainstem involvement.

8. Neonatal melioidosis

Melioidosis has been described in neonates, although this is not common [19–22]. Presentations include non-specific sepsis, pneumonia, and meningitis. All patients described in a report of five cases of neonatal melioidosis from Thailand, were premature
infants presenting between birth to 14 days after birth. The organism was cultured from blood in four patients and from cerebrospinal fluid culture in the other. In two of the patients the infection was fatal [20]. Mother-to-child transmission of *B. pseudomallei*, probably as a result of trans placental infection, has been reported [23]. Transmission via breast milk also has been observed [24]. Nosocomial acquisition of the organism via contaminated antiseptic solutions is another possible mode of transmission [20].

9. Treatment of paediatric patients

Similar to treatment in adults with severe melioidosis, in children an initial intensive treatment phase is commonly followed by a prolonged eradication phase to reduce the risk of relapse. Preferred treatment regimens vary between Australia and Thailand. In Thailand, intravenous ceftazidime, with or without trimethoprim–sulfamethoxazole (TMP-SMX), is used in the intensive phase, although in mild disease, oral treatment alone may be sufficient. TMP-SMX (4/20 mg/kg twice daily) is used as the preferred oral agent. In Australia, either ceftazidime or meropenem is used in the intensive phase, with meropenem preferred for severe disease or central nervous system (CNS) involvement as penetration across the blood-brain barrier is higher than with ceftazidime. A higher dose of TMP/SMX (8/40 mg/kg twice daily, with folic acid) is used in Australia.

For children allergic or intolerant to first-line therapy, an alternative is used which requires more frequent dosing of co-amoxiclav with supplemental amoxicillin (a total dose of 20/5 mg/kg three times a day). For localised infection without severe systemic symptoms or visceral organ involvement, such as skin abscess or lymphadenitis, a shorter course of eradication treatment of six to eight weeks is usually successful. In mild melioidosis, oral treatment alone may be sufficient in children [10,11]. Parotid abscesses often require incision and drainage. However, a recognised complication of parotid surgery is trauma to the facial nerve, which may result in temporary or permanent paralysis. Multiple, small, visceral abscesses, particularly involving the liver or spleen, can be generally managed without surgery.

Relapse in children appears to be less common than in adults. A report of long-term follow-up of 889 patients with melioidosis in adults found a relapse rate of 9.7% and re-infection in 3.4% [25]. Little data exists on relapse rates in children; a report of treatment and long term follow-up of 25 children with cultured proven melioidosis found only one case with possible relapse [10].

Physicians treating children who live in endemic areas, travelled to such areas, or who have a history of exposure to soil and water in the endemic area, should be familiar with the common clinical syndromes associated with paediatric melioidosis. The differential diagnosis of melioidosis should be strongly suspected in children who present with suppurative parotitis, lymph node abscess or chronic draining abscess from the skin even in previously-healthy children.

References

V
Laboratory diagnosis and detection
Section V. Laboratory diagnosis and detection

Editorial overview

Robert Norton\(^a\), Vanaporn Wuthiekanun\(^b\)

\(^a\) Pathology Queensland, Townsville Hospital, Townsville, Australia
\(^b\) Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

The identification of *Burkholderia pseudomallei* is not usually a significant problem for laboratories in endemic regions. However, the increase in travel to and from endemic areas, will make it inevitable that laboratories, otherwise unfamiliar with the organism, would be confronted with diagnostic dilemmas relating to its identification. Early diagnosis is critical to ensure optimal therapy. Mortality rates of up to 70% are reported where treatment is either delayed or inappropriate. Even when the diagnosis and treatment is timely, mortality rates of up to 20% are reported.

The culture of relevant clinical material remains the mainstay of diagnosis and good communication between the clinician and the laboratory is essential to achieve this. Blood, urine, sputum and pus collected for culture before the administration of antibiotics will provide the highest yield. Throat swabs can modestly improve the rate of culture positivity particularly in an endemic region. Cerebrospinal fluid conversely is usually culture negative even in proven neurological disease. It should be noted that *B. pseudomallei* isolated from any site is always significant. The Gram stain appearance of *B. pseudomallei* may show bipolar Gram-negative staining. This is however neither universal nor unique and cannot be relied on to make a diagnosis. Direct immunofluorescence of sputum and pus is used in some centres but is not available commercially and is observer dependent as in most immunofluorescent techniques.

The use of specific selective culture media in the isolation of *B. pseudomallei* from non-sterile sites is discussed in this review. A variety of media have been trialled but Ashdown’s medium, originally described by Leslie Ashdown, remains the most reliable. However, it may not universally be commercially available. The organism is an aerobic Gram-negative bacillus which will grow easily on most media including Columbia horse blood agar and MacConkey agar. Presumptive identification can be made in most instances with simple bench tests. An oxidase positive, Gram-negative bacillus, resistant to gentamicin and colistin, sensitive to amoxicillin-clavulanic acid with a characteristic colonial morphology should be presumptively identified as *B. pseudomallei*. The use of a recently described latex assay using a monoclonal antibody is discussed in this review and shows considerable promise. Confirmation of identity is usually done using automated
or semiautomated methods, such as the bioMérieux API 20 NE and Vitek systems. The relative merits of these are discussed.

The use of molecular methods to identify isolates has been proposed. We feel that it is generally unnecessary but it can be a useful adjunct particularly in laboratories unfamiliar with the organism.

Serology has an important but limited role in the diagnosis of melioidosis. The drawbacks of the indirect haemagglutination assay (IHA) are discussed along with other serological assays currently used. Delay in the appearance of IHA antibodies in up to 50% of culture positive patients remains a major drawback in its reliability as a diagnostic tool. The reliable direct detection of *B. pseudomallei* using molecular methods from clinical material, such as blood, remains elusive and requires more research.

Determination of sites of infection has therapeutic implications. Since it is not infrequent to find infection sites and occult abscesses a variety of tissue and organs in patients with melioidosis, appropriate imaging techniques should be used in assessment of patients.

The microbiology laboratory plays a critical role in the accurate and timely diagnosis of melioidosis. The availability of simple bench tests and culture will greatly improve the diagnostic yield and management in endemic regions which invariably may have limited facilities. Laboratories in non-endemic regions need to be aware of this important pathogen and its characteristics.
Isolation and identification of *Burkholderia pseudomallei* in clinical samples

Mindy B. Glass\(^a\), Amanda L. Walsh\(^b\)

\(^a\) Centers for Disease Control and Prevention, Atlanta, USA
\(^b\) Health Protection Agency Centre for Infections, London, UK

Abstract

*Burkholderia pseudomallei* can be recovered from a variety of clinical samples, both sterile sites and those with a normal flora. Microscopy may show bipolar or unevenly stained Gram-negative bacilli. Isolation rates from sites with a normal flora (throat swabs, sputum, superficial wound swabs, etc.) may be increased by the use of selective media such as Ashdown medium; selective media for the isolation of *B. cepacia* can also be used. Simple screening tests can be applied to colonies with an appearance consistent with *B. pseudomallei* and identification confirmed using biochemical tests such as those in the API 20NE (bioMérieux, France) strip. Rapid identification techniques, such as direct immunofluorescent microscopy and latex agglutination, can significantly reduce the time to diagnosis but are unavailable commercially. Susceptibility testing should include the antibiotics co-trimoxazole, doxycycline, co-amoxiclav and ceftazidime, together with carbapenems. Future research into rapid diagnosis will be discussed.

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1. Introduction

Whether in endemic areas, where doctors are familiar with the clinical presentation of melioidosis, or in parts of the world where it is rarely seen, the laboratory detection and identification of *B. pseudomallei* is crucial to the correct diagnosis of this infection and administration of appropriate antimicrobial treatment to ensure patient survival. Proper diagnosis of melioidosis relies upon good laboratory practices to ensure that appropriate collection and processing of specimens and isolation and identification procedures promote the confirmation of *B. pseudomallei*.

Biosafety Level (BSL) 2 or equivalent Containment Level (CL) 2 practices, equipment and containment may be used when working with clinical specimens suspected or known to contain *B. pseudomallei*. All work should take place inside a biosafety cabinet and gloves should be worn. When working with cultures, especially those using high-aerosol risk procedures or procedures where large quantities of the organism are required, work should take place according to BSL-3/CL-3 containment procedures and should include...
There have been two laboratory-acquired cases of melioidosis where infection occurred by aerosol and skin exposure [1]. Persons with diabetes should use extreme caution when working with B. pseudomallei as this has proven to be the most commonly associated risk factor for naturally acquired disease [2]. All specimens from patients with suspected melioidosis should be handled with the appropriate safety considerations.

2. Clinical samples and processing

The culture of B. pseudomallei from any specimen in a patient with suspected melioidosis remains the diagnostic “gold standard”. Because of the range of clinical presentations in melioidosis, a variety of patient specimens may be submitted; key specimens to collect include blood, throat swabs, respiratory secretions, urine, and when available, pus and swabs from wounds or lesions. B. pseudomallei grows well on most routine laboratory media and can be isolated from sterile sites using standard techniques. Specimens from non-sterile sites can benefit from the use of selective media which help to promote the growth of B. pseudomallei while reducing the growth of other organisms. A number of selective media have been developed to facilitate growth of B. pseudomallei from sites with mixed flora. Examples include Ashdown agar [3], B. pseudomallei selective agar (BPSA) [4], and a medium designed to be both selective and differential to distinguish between B. pseudomallei and B. cepacia [5]. Of these, Ashdown agar, containing glycerol, crystal violet and gentamicin, is probably the most widely used, although it has limited commercial availability. Recognising the need for commercially available selective media which would be useful for isolation of B. pseudomallei, some studies looked at the utility of B. cepacia selective media [6,7] for this purpose as the two organisms are so closely related. Different formulations of B. cepacia agars have been shown to be effective and these media can be reliably employed for respiratory samples (Mast Diagnostics, UK; Oxoid, UK).

Blood for culture is the most common specimen used in the diagnosis of melioidosis. The collection of blood cultures should occur prior to commencement of antimicrobial treatment. Samples are inoculated into blood culture broths either for manual processing or a continuously monitored automated blood culture system. Positive detection of growth in blood culture occurring in the first 24 hours indicates high bacterial concentrations in the blood and in turn higher risk of mortality. If a blood culture becomes positive within 24 hours, the mortality rate has been found to be as high as 74% compared with a 41% mortality rate after 24 hours of culture. Quantitatively, if there are more than 100 colony forming units (CFU)/ml of B. pseudomallei in blood, mortality rates reach 96% where 1 CFU/ml of B. pseudomallei has a relative mortality rate of 42% [8].

Isolation of B. pseudomallei from urine cultures is considered significant. Highlighting the utility of this specimen, a study by Limmathurotsakul et al. [9] found urine to be the only positive, direct specimen in 70 out of 171 cases. Higher B. pseudomallei counts in urine also correlate with higher mortality rates. When urine cultures were found with >10^5 CFU/ml, mortality rates were 71%. Urine can be plated directly but should also be centrifuged and the pellet cultured. Urine cultures may become positive sooner than
blood culture in bacteraemic patients with low numbers of organisms. Routine media for urine culture, such as MacConkey and Cystine Lysine Electrolyte Deficient (CLED), will support the growth of *B. pseudomallei*.

*Burkholderia pseudomallei* can colonise the respiratory tract and since throat carriage does not occur outside of clinical disease, throat swabs have proven diagnostically useful. Throat swabs should be collected by careful swabbing of the pharynx and fauces with a cotton-tipped swab. Swabs should be inoculated directly on a selective media such as Ashdown agar in addition to inoculation of a selective broth [10], which can increase sensitivity. They are particularly important specimens in paediatric patients or in cases where a sputum culture is not available. Throat swabs were found to be 36% sensitive and 100% specific and may detect an additional 6% of cases where sputum culture is negative [11].

Respiratory secretions, including sputum specimens and tracheal aspirates, are collected into sterile containers and then inoculated into selective broth and onto non-selective and selective agars. These may include Ashdown agar if available, or selective media for isolation of *B. cepacia*. Positive sputum cultures are associated with an increased mortality in patients with melioidosis and may continue to be positive in follow-up cultures even with appropriate antimicrobial treatment [12]. Collection of pus, or swabs from wounds, ulcers, skin lesions or other infected sites should be taken when relevant. Inoculation into enrichment broths prior to plating on routine and selective media enhances detection.

All clinical samples should be directly plated onto sheep or horse blood agar and Ashdown’s agar and incubated in air or CO\(_2\) for 37ºC for 2 to 4 days. If inoculated into selective enrichment broth this should be incubated at 37ºC for 48 hours before subsequent subculture onto blood agar and Ashdown’s agar. Incubation at 42ºC can increase the selectivity of the enrichment [13]. Though culture may take days to a complete identification, it has an advantage over rapid techniques in that it allows for antimicrobial susceptibility testing.

### 3. Microscopy

Gram staining (with neutral red as counterstain) directly on clinical specimens or with isolates is valuable to presumptively identify *B. pseudomallei* (Figure V.1). It is a non-spore forming Gram-negative bacillus with uneven or bipolar staining, commonly referred to as a safety pin appearance. Note, however, that this is not pathognomonic. The bacilli are 0.8 to 1.5 \(\mu\)m in size, slender and vacuolated with rounded ends. A smear from clinical specimens may show only a few bacilli. In cases where antimicrobial treatment has been administered, *B. pseudomallei* may appear as long Gram-negative filaments in stains directly on clinical specimens [9]. The utility of the Wayson stain for the detection of bipolar staining *B. pseudomallei* has been assessed but was not found to be useful [14].

A direct immunofluorescent antibody test has been developed that can directly detect *B. pseudomallei* in clinical specimens, including sputum, wounds, pus and urine. Using a purified polyclonal anti-*B. pseudomallei* IgG conjugated to fluorescein isothiocyanate (FITC), the specimen is examined by incident light fluorescence. A positive specimen shows bacilli with a strong and bright green appearance. This technique can generate
a specific result in under two hours, but requires specialised and expensive microscopy equipment. Used directly on isolates, this method is 100% sensitive and specific. When tested on sputum, wound swabs, urine, pus or fluid, this method was 66–73% sensitive and 99% specific when compared to culture [15,16]. This technique has been further refined into a rapid test that detects \textit{B. pseudomallei} within 10 minutes and showed no significant change in performance from the standard test. Both the standard and rapid immunofluorescent methods were found to be 100% sensitive and specific when tested directly on blood culture [16].

4. Isolation

Blood cultures may become positive after any duration of incubation; when evidence of growth is noted, and Gram stain shows Gram-negative slender bacilli. Following subculture onto solid media, plates should be examined daily for up to 4 days. In a pure growth, ‘typical’ colonies usually develop by 48 hours, although colony development may be slower, in mixed cultures (Table V.1). The most characteristic feature of \textit{B. pseudomallei} is its metallic sheen, and the usual progression to dry and wrinkled colonies (Figure V.2). Colony variation in terms of colour and degree of wrinkling is common. Some isolates may be smooth and shiny, and mucoid variants may also be seen. A characteristic sweetish earthy odour may be noted.

5. Identification

Problems with identification of \textit{B. pseudomallei} are common where the disease is rarely seen, and this is often due to misidentification or dismissal as a contaminant. Colonial appearance as described above may be suggestive, and if antibiotic susceptibilities have already been performed, the unusual pattern may also give a clue as to the organism’s
Table V.1

Typical appearance of *Burkholderia pseudomallei* on different culture media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Colony size*</th>
<th>Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood agar</td>
<td>&lt;1 mm to 3 mm</td>
<td>Creamy colonies, resembling ‘coliform’, may be mucoid. Non-haemolytic. Slight metallic sheen. Becoming dry and wrinkled (Figure V.2a).</td>
</tr>
<tr>
<td>MacConkey</td>
<td>0.5–2 mm</td>
<td>Resemble a weak lactose fermenter, usually flat. May develop sheen.</td>
</tr>
<tr>
<td>CLED</td>
<td>Pinpoint to 3 mm</td>
<td>Clear blue colonies if single, pale yellow if confluent. Flat with matt surface, metallic sheen, becoming dry and wrinkled.</td>
</tr>
<tr>
<td>Ashdown’s</td>
<td>Pinpoint to 3 mm</td>
<td>First visible growth is clear to pale pink with slight sheen. Becoming darker pink to purple, with increasing dryness and wrinkling. Colony variants are common (Figure V.2b).</td>
</tr>
<tr>
<td>BPSA</td>
<td>1–3 mm</td>
<td>Agar colour and colonies are reddish pink, metallic, becoming dry and wrinkled.</td>
</tr>
<tr>
<td><em>B. cepacia agar</em> (PC)</td>
<td>1–3 mm</td>
<td>Agar colour turns pink in areas of heavy growth, colonies white to yellow, metallic, becoming dry and wrinkled (Figure V.2c).</td>
</tr>
<tr>
<td><em>B. cepacia</em> selective agar (BCSA)</td>
<td>1–3 mm</td>
<td>Agar colour turns yellow in areas of heavy growth, colonies white to creamy, metallic, becoming wrinkled only in the centre.</td>
</tr>
</tbody>
</table>

* 18 to 72 hours following culture.

identity. It is susceptible to co-amoxiclav, chloramphenicol, tetracyclines, co-trimoxazol, and ceftazidime, but resistant to gentamicin, colistin, and ampicillin. Results from simple screening tests are useful for presumptive identification. *B. pseudomallei* is oxidase positive, has a metallic sheen on blood agar, is resistant to gentamicin and colistin, but susceptible to co-amoxiclav (this latter aids differentiation from *B. cepacia*) [17,18].

Further identification carried out in routine laboratories is based on biochemical testing, while specialised tests, such as molecular detection or fatty acid profiling, are offered only in reference facilities. Substrate utilisation panels, be they manual, automated or semi-automated, are commonly used in diagnostic laboratories for the identification of non-fermenting Gram-negative bacilli, and there are many reports of these leading to misidentification of *B. pseudomallei*. Examples of such systems include API 20E® and 20NE® (bioMérieux, France), VITEK® 1 and 2 (bioMérieux), Rapid NF Plus® (Remel, USA), Phoenix® (Becton Dickinson, USA), Microscan® Walkaway 96 (Dade Behring, USA) and Microbact® 24E (MedVet, Australia). With all these commercial systems, *B. pseudomallei* is most often misidentified as *B. cepacia*, *Pseudomonas fluorescens*, *P. aeruginosa*, or *Chromobacterium violaceum*.

Ashdown [19] initially used the API 20E and found it useful, however, subsequent reports [17,20] did not concur. The API 20NE has probably had the widest use, and in some endemic settings, performs very reliably, correctly identifying 99% of isolates [21].
Fig. V.2. Colony morphology of *Burkholderia pseudomallei* on (a) blood agar, (b) Ashdown’s agar and (c) *B. cepacia* agar.
Other studies have not found it so consistent, identifying as few as 37% of isolates \[22\]. In comparison with the Microbact 24E, Inglis \[23\] showed that the API 20NE misidentified up to 20% of \textit{B. pseudomallei} isolates, most commonly as \textit{C. violaceum}. This finding might account for reported cases of apparent \textit{C. violaceum} sepsis in melioidosis endemic areas. The inability of the Vitek 2 to discriminate between \textit{B. pseudomallei} and other non-fermentative Gram-negative bacilli or \textit{B. cepacia} has also been described \[24\]. However, this has since been reportedly corrected by software adjustments. The use of an older (>36 hour) culture in both the API 20NE and Vitek systems can improve the rate of identification.

It is essential for isolates of \textit{B. pseudomallei} to be correctly identified to ensure proper management and appropriate treatment of patients with melioidosis; prolonged courses of antibiotics are required to minimise the likelihood of relapse. Stringent use of the screening tests for presumptive diagnosis, followed by referral of all such isolates to an expert facility, irrespective of the result of the identification panel, will ensure that a correct diagnosis is reached.

6. Agglutination tests and rapid antigen detection

While culture remains the gold standard of identification for \textit{B. pseudomallei}, attempts have been made to develop rapid methods that will reduce the time to diagnosis of melioidosis. Latex agglutination assays, developed using various polyclonal and monoclonal antibodies to \textit{B. pseudomallei}, can be tested directly on clinical specimens reducing the time burden required by culture-based methods. Four main latex agglutination assays (Table V.2) have been described in the literature \[25–29\]. The most commonly used latex agglutination assay was developed using both polyclonal and monoclonal antibodies specific for the 200 kDa exopolysaccharide of \textit{B. pseudomallei}. This assay was found to 95.1% sensitive and 99% specific on blood culture specimens from patients with community acquired pneumonia gathered from 12 hospitals in northeast Thailand \[27\]. A study performed by Wuthiekanun et al. \[28\] recommended

<p>| Table V.2 |
| Comparison of latex agglutination assays |</p>
<table>
<thead>
<tr>
<th>Latex Agglutination Antibody</th>
<th>Tested On</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal and Monoclonal, Anti-200 kDa exopolysaccharide</td>
<td>Blood culture and isolates</td>
<td>95–100%</td>
<td>97–100%</td>
<td>[21,27,28]</td>
</tr>
<tr>
<td>Monoclonal, Anti-200 kDa exopolysaccharide</td>
<td>Isolates</td>
<td>100%</td>
<td>100%</td>
<td>[25]</td>
</tr>
<tr>
<td>Monoclonal, 3015, Anti-30 kDa protein</td>
<td>Blood culture</td>
<td>95–100%</td>
<td>86–100%</td>
<td>[26,29]</td>
</tr>
<tr>
<td>Monoclonal, Bps-L1, Anti-lipopolysaccharide</td>
<td>Blood culture and isolates</td>
<td>100%</td>
<td>100%</td>
<td>[28,30]</td>
</tr>
</tbody>
</table>
the use of this assay over the anti-lipopolysaccharide assay in a clinical setting, since it is able to differentiate \textit{B. pseudomallei} from \textit{B. thailandensis}. Although this assay was later shown by Amorncahi et al. [21] not to be able to differentiate \textit{B. pseudomallei} from \textit{B. mallei}, this is not of great concern since infection with \textit{B. mallei} is extremely rare in humans [21]. None of these assays are available commercially and therefore, are unlikely to be used in laboratories that rarely encounter the organism. In endemic areas however, they would have a role in assisting with confirmation of the diagnosis.

The direct detection of antigen in clinical specimens would be of significant clinical benefit. Enzyme-linked immunosorbent assays (ELISAs) have been developed to detect a \textit{B. pseudomallei} exotoxin at the range of 16 ng/ml, and a 40 kDa protein in the range of 4 ng/ml in culture supernatants but neither has been evaluated in a clinical setting [31]. An assay developed to detect \textit{B. pseudomallei} antigen in urine, performed with 81% sensitivity and 96% specificity and was able to detect antigen in the range of 12.2 ng/ml [32]. A sandwich ELISA using the monoclonal antibody, 5F8, reactive to a 200 kDa surface antigen of \textit{B. pseudomallei} was developed and performed with 75% sensitivity and 98% specificity when tested on clinical specimens. It could detect antigen at 8 ng/ml or $1.8 \times 10^4$ CFU/ml. None of these antigen detection systems are available commercially and essentially have not been able to supersede the effectiveness of culture-based methods [31].

7. Susceptibility testing and reporting

In clinical microbiology laboratories, susceptibility testing should be performed using the most commonly administered antimicrobial agents, with appropriate Clinical and Laboratory Standards Institute (CLSI) guidelines [33]. \textit{B. pseudomallei} is resistant to many antibiotics, including first-, second-, and third-generation cephalosporins, aminoglycosides, pencillins, and polymyxin [8]. Susceptibility testing should include a carbapenem (meropenem or imipenem), ceftazidime, co-trimoxazole (TMP-SMX), doxycycline, and co-amoxiclav; meropenem, imipenem, or ceftazidime are the preferred drugs for the initial parenteral phase of treatment, while co-trimoxazole (TMP-SMX), doxycycline, and co-amoxiclav are routinely used for long term oral eradication therapy and post-exposure prophylaxis [1]. No interpretive guidelines exist for disk diffusion testing of \textit{B. pseudomallei}. Zone diameters are usually adapted from data for related organisms, such as non-Enterobacteriaceae or \textit{Pseudomonas} species. While disk diffusion is cheap and convenient, it should only be used in cases where laboratories are unequipped to perform CLSI recommended methods. Caution needs to be exercised in interpreting zone diameters for cotrimoxazole as overgrowth can lead to the incorrect reporting of resistance. E-test gradient diffusion testing, though not specifically addressed by the CLSI, uses the same breakpoints as broth microdilution minimal inhibitory concentrations (MIC). A validation of the E-test for \textit{B. pseudomallei} is needed. For broth microdilution MIC testing, CLSI does have breakpoint guidelines for \textit{B. pseudomallei}. Susceptibility tests should be repeated with isolates obtained from multiple sites and during antimicrobial therapy to monitor for development of resistance [13]. Resistance
is rare but has been observed for ceftazidime, chloramphenicol, tetracycline and cotrimoxazole.

8. Pitfalls and future directions in diagnosis

Melioidosis is a clinically diverse disease with many presentations. Pitfalls in identification can include technical unfamiliarity with the disease and organism, overgrowth of competing organisms from non-sterile sites, and misidentification by manual and automated commercial identification systems. Misidentification may in turn lead to occupational exposure and potential infection of laboratory personnel. Few of the rapid techniques developed to aid in the diagnosis of melioidosis have been tested extensively in endemic settings. For rapid tests to succeed, they must be widely available, fast, inexpensive and highly sensitive and specific. Increasing the global awareness of melioidosis will contribute towards a greater understanding of the true burden of the disease. This awareness can be achieved by education and support of microbiologists and clinicians from national laboratories down to those in rural and under-resourced areas. Encouraging research on improving techniques for isolation, identification and rapid diagnostic methods can help to reduce the time to identification of *B. pseudomallei* and in turn ensure appropriate treatment and survival of patients with melioidosis.

References

Section V.2

The serological diagnosis of melioidosis

Narisara Chantratita\textsuperscript{a}, Ganjana Lertmemongkolchai\textsuperscript{b}, Vanaporn Wuthiekanun\textsuperscript{a}, Robert Norton\textsuperscript{c}

\textsuperscript{a} Mahidol–Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand
\textsuperscript{b} Department of Clinical Immunology, Khon Kaen University, Khon Kaen, Thailand
\textsuperscript{c} Pathology Queensland, Townsville Hospital, Townsville, Australia

Abstract

Serology has a limited role in the diagnosis of melioidosis. Methods for serodiagnosis include the indirect haemagglutination assay (IHA), enzyme linked-immunosorbent assay (ELISA), immunofluorescent assay (IFA) and the immunochromatographic cassette test (ICT). The IHA is currently the most common test used but interpretation in endemic areas is difficult because of the presence of antibodies in apparently healthy individuals. The IHA assay is poorly standardised, with variation in \textit{Burkholderia pseudomallei} strains, detection threshold and protocols for the polysaccharide antigen preparation. The ELISA and IFA \textit{IgG} assays have similar sensitivities. The ELISA uses crude or purified aqueous protein extracts while the IFA uses bacterial cell wall components. The ELISA and IFA \textit{IgM} assays are used as markers of disease activity. Cassette based ICT assays have been evaluated but lack sensitivity. More specific antigens are currently being investigated as candidates to facilitate early serological diagnosis.

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1. Introduction

The diagnosis of melioidosis is currently best confirmed by the isolation of \textit{B. pseudomallei} from blood, urine, sputum, pus or other clinical specimens. Serology has a role in supporting a clinical diagnosis or in identifying disease relapse. The most commonly used serological methods are the indirect haemagglutination assay (IHA), the immunofluorescent antibody test (IFAT) and the enzyme linked-immunosorbent assay (ELISA). The IHA uses a polysaccharide antigen derived from heat-killed whole bacteria, the IFAT uses whole-killed organism dotted on a slide and the ELISA uses a crude sonicated, aqueous protein extract [1,2].

The IHA is currently the most common test used to quantify the human antibody response after exposure to \textit{B. pseudomallei}. The IHA assay is poorly standardised, with variation in \textit{B. pseudomallei} strains and protocols for antigen preparation in different
laboratories. Detection thresholds or cut-off values can differ between laboratories. Several studies have evaluated the utility of the IHA for the serodiagnosis of melioidosis. These indicate that test accuracy is too low for both diagnosis and monitoring of response to antibiotic treatment. The interpretation of the IHA for melioidosis in endemic areas is difficult because of the presence of antibodies in apparently healthy individuals.

Background IHA antibody levels have been observed in healthy individuals living in endemic regions [3−5] and in patients after recovery from melioidosis [2]. For example, Ashdown and Guard [3] showed that an average of 5.7% of 9,047 individuals from North Queensland had a titre of 1:40 or greater for the IHA. Antibody levels can also persist indefinitely after primary infection. Using the IHA alone therefore makes it difficult to distinguish between past and present infection. The main value of the IHA is as a screening test for non-acute melioidosis. The reported sensitivity of the IHA in diagnosing acute melioidosis has varied between 50% and 85%. The specificity of the IHA remains high at between 92% and 100%, depending upon the cutoff titre [2].

The ELISA IgG has a reported sensitivity of 95% and a specificity of 81%, with no difference noted between acute and convalescent sera [2]. The ELISA IgM has been reported to be a good marker for melioidosis disease activity, rather than as a diagnostic test for acute melioidosis [2]. Conversely, the IFAT−IgM has been shown to be of relevance in the diagnosis of acute disease as well as being a marker of disease activity [6]. The IFAT has a similar sensitivity and specificity to the ELISA but requires the use of a fluorescent microscope and can be subjective, particularly when used by inexperienced observers.

The distribution of immunoglobulin classes in melioidosis has been described using a culture filtrate antigen. Essentially IgG was predominant in septicaemia with IgG1 and IgG2 being the major subclasses. In localised infection, IgA and IgM predominated [7]. It has been suggested that the detection of IgG1 and IgG2 isotype antibodies might be useful in monitoring the status of infection.

The need for early, rapid and simple diagnostics for melioidosis, has led to the investigation of novel antigens and systems for serodiagnosis. None of these however, are currently commercially available.

2. Indirect haemagglutination assay

The IHA is currently the most common test used to quantify the human antibody response after exposure to B. pseudomallei. The test was first described for diagnosis of melioidosis in 1965 [8]. Preparation of materials for the IHA test is simple [1]. However, work with B. pseudomallei requires a Biosafety Level 3 (BSL-3) facilities, as it is a National Institute of Allergy and Infectious Diseases (NIAID) category B pathogen that causes serious disease which can be acquired by the aerosol route.

The IHA assay is poorly standardised, with variation in B. pseudomallei strains and protocols for antigen preparation in different laboratories. The protocol at the Mahidol-Oxford Tropical Medicine Research Unit (MORU) in Thailand uses pooled heat-killed antigens of two clinical B. pseudomallei isolates, strains 199a and 207a [9], while other laboratories use a combination of three or five local isolates [10,11]. B. pseudomallei
antigen is used to sensitise sheep red blood cells, after which sensitised and non-sensitised cells control are incubated with human test serum. Prior to use, serum is inactivated at 56°C, preabsorbed for non-specific sheep cell agglutinins, then serially diluted two-fold from 1:10 to 1:10,240 on a 96 well U-bottom microtitre plate. The plate is mixed gently and agglutination results read after leaving at room temperature for two hours. The antibody titre is recorded as the highest dilution to show agglutination. Cut-off values differ between laboratories and depending on the purpose of the test. The diagnostic cut-off for Thailand has been set at $\geq 1:160$ [9,12], while the threshold cut-off in Australia is $\geq 1:40$ to $\geq 1:160$ [10,11]. Several studies have evaluated the utility of the IHA for the serodiagnosis of melioidosis. These indicate that test accuracy is too low for both diagnosis and monitoring of response to antibiotic treatment. A recent study evaluating the IHA as a diagnostic test in 322 suspected melioidosis cases in northeast Thailand reported a sensitivity of 73% and specificity of 64% [13,14]. The poor sensitivity of the IHA for the diagnosis of melioidosis has also been reported in two studies in Australia. The use of the IHA on admission serum samples from 275 patients with culture-confirmed melioidosis presenting to the Royal Darwin Hospital, Northern Territory of Australia had a sensitivity of 56% using a threshold titre of 1:40 and 41% at a threshold titre of 1:160 [10]. Among those patients who had an initially negative IHA titre, 68% had a subsequent seroconversion. The IHA was persistently positive after recovery from illness in the majority of patients [10]. A second study in Townsville Hospital, in Queensland, Australia, reported that 51% of 140 patients with culture-confirmed melioidosis were seropositive at presentation [11]. Repeated IHA testing demonstrated four possible outcomes: seroconversion following an initial negative IHA (15%), persistently positive IHA (42%), persistently negative IHA (26%), or an initial positive IHA reverting to a negative result upon convalescent testing (17%). IHA positivity has been shown to be associated with diabetes, while a negative IHA test was often found in patients with melioidosis bacteraemia [11].

Due to the heat-stable nature of the antigen preparation used in the IHA it has been suggested that one of the major antigens responsible for the immunoreactivity is lipopolysaccharide (LPS). Supportive evidence for this comes from a study demonstrating that antibody titres could be absorbed by phenol-water extracted LPS from a single strain, and that this blocked the reactivity of serum using red cells sensitised with crude extracts of other strains [15]. However, the IHA has also been shown to have poor test correlation with other assays that used a range of defined or crude antigens in ELISA format. Correlations with IHA of 0.5 to 0.6 were obtained for ELISA using affinity-purified antigen, exopolysaccharide, LPS, crude \textit{B. pseudomallei} and crude \textit{B. thailandensis} antigens [13].

Interpretation of IHA for melioidosis in endemic areas is difficult because of the presence of antibodies in apparently healthy individuals. Several studies in Thailand have demonstrated that over 10% of healthy individuals have an IHA antibody titre that falls above the cut-off level [4,9,12,13]. The increasing prevalence of IHA antibodies with age has been used as a marker of the intensity of exposure. An extensive study of 2,214 children in northeast Thailand reported a sharp rise in antibody from birth to 4 years followed by 60% to 70% having an IHA antibody titre thereafter [9]. The intensity of
exposure was reported to be higher in Udon Thani than in Ubon Ratchathani, Thailand with 24% as opposed to 11% of patients becoming seropositive each year [16]. In north Queensland, rates of background IHA seropositivity range from 2.5% to 5.7% [3,11,17]. A seroprevalence rate of 17.0% using a cut-off titre $\geq 1:40$ was reported for 407 East Timorese evacuated to Darwin, Australia, in September 1999 [18]. Fifty-three out of 200 healthy blood donors in Malaysia showed positive antibody titres ($\geq 1:40$) against $B. \textit{pseudomallei}$. Seven percent had an IHA titre of 1:40, while 8.5% had a titre $\geq 1:160$ [5]. IHA was used to determine exposure of children to $B. \textit{pseudomallei}$ in Siem Reap, Cambodia and it was found that 16% had a positive titre [19]. A serologic survey of healthy adult immigrant workers from Myanmar in Thailand, indicated that exposure to $B. \textit{pseudomallei}$ is common in this region. Antibodies were detectable by IHA in 757 (78%) of 968 adults, of whom 69 (7%) had an IHA titre $\geq 1:160$ [20]. High background seroprevalence of antibodies to $B. \textit{pseudomallei}$ may be the result of repeated exposure to the bacterium in the environment. An alternative explanation for background seropositivity is cross-reactivity to other closely-related species such as $B. \textit{thailandensis}$ or other Gram-negative bacteria [21–23]. A recent study using $B. \textit{thailandensis}$ as the antigen in an IHA, demonstrated that there was some cross-reactivity in serum samples from patients with culture confirmed melioidosis [24].

3. Enzyme linked immunoassay and the immunofluorescent antibody test

The ELISA for the detection of both IgG and IgM antibodies to $B. \textit{pseudomallei}$ was developed and assessed by Ashdown [2]. This was an indirect ELISA which used a sonicated, heat inactivated antigen, derived from a pool of eight strains of clinical isolates of $B. \textit{pseudomallei}$. This ELISA IgG has a reported sensitivity of 95% and a specificity of 81%, with no difference noted between acute and convalescent sera. The ELISA IgM assay using this antigen, has been reported to be a good marker for melioidosis disease activity, rather than as a diagnostic test for acute melioidosis [2]. Since then, a variety of antigens have been trialled in the ELISA IgG and IgM detection systems. These have included an affinity purified antigen [13,25], exopolysaccharide and LPS [13] and a culture filtrate antigen [7]. A comparison of sensitivities and specificities is provided in Table V.3.

The methodology for the indirect ELISA (IgG and M) has been as described in detail by Ashdown [3]. The antigen preparation used in the ELISA is derived from a cell suspension of $B. \textit{pseudomallei}$ isolates inactivated by heating at 80°C for one hour and then sonicated. Following centrifugation and filtration, the filter supernatant (antigen) is obtained and used to coat the ELISA plates. The immunofluorescent antibody test (IFAT) for the detection of IgG and IgM in melioidosis was also described by Ashdown [6]. Essentially, whole $B. \textit{pseudomallei}$ cells were used as antigen with serial sera dilutions in PBS. Specific fluorescence at a serum dilution of $\geq 1:10$ for IgG was considered positive, while IgM titres of $\geq 1:40$ together with relevant clinical findings were considered diagnostic of active infection. Sensitivities of between 91% and 95% and specificities of between 95% and 100% have been reported using IFAT [2,26]. While useful in a
Table V.3
A comparison of sensitivities and specificities of different antigens preparations used in ELISA assays for the detection of antibodies in melioidosis

<table>
<thead>
<tr>
<th>Antigen used in ELISA (antibody isotype)</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude sonicated filtrate (IgG)</td>
<td>90%</td>
<td>99%</td>
<td>[2]</td>
</tr>
<tr>
<td>Affinity purified antigen (IgG)</td>
<td>85.7%</td>
<td>82.5%</td>
<td>[25]</td>
</tr>
<tr>
<td>Affinity purified antigen (IgM)</td>
<td>63.5%</td>
<td>81.8%</td>
<td>[25]</td>
</tr>
<tr>
<td>Affinity purified antigen (IgG)</td>
<td>63.5%</td>
<td>81.8%</td>
<td>[25]</td>
</tr>
<tr>
<td>Culture filtrate antigen (IgG)</td>
<td>96%</td>
<td>94%</td>
<td>[7]</td>
</tr>
<tr>
<td>Culture filtrate antigen (IgM)</td>
<td>74%</td>
<td>99%</td>
<td>[7]</td>
</tr>
<tr>
<td>Lipopolysaccharide (IgG)</td>
<td>66%</td>
<td>81%</td>
<td>[13]</td>
</tr>
<tr>
<td>Exopolysaccharide (IgG)</td>
<td>64%</td>
<td>74%</td>
<td>[13]</td>
</tr>
</tbody>
</table>

reference laboratory, the IFAT would be impractical to introduce in resource poor settings or in regions where the condition is rarely encountered.

4. Immunochromatographic card test

In an attempt to obviate the need for dedicated serological expertise and equipment in the serodiagnosis of melioidosis, an immunochromatographic card test (ICT) was developed. This detected both IgG and IgM in a simple to use flow assay card format. It required minimal equipment, little training and could be used at the bedside.

The use of ICT was assessed by Cuzzubbo [27] utilising a culture filtrate antigen of *B. pseudomallei* and was shown to have a sensitivity of 100% for IgG and 93% for IgM, with both assays having a specificity of 95%. The test kit used in that study, differed from the cassette prototype kit subsequently used, in that it was a strip test. The antigen however was similar. Another study [28] using the prototype melioidosis ICT kit, showed a sensitivity of 77% for IgG and 88% for IgM with specificity of 90% and 69% respectively. Because of the discrepancy, a further study was carried out which confirmed a low sensitivity of 50.6% for IgG and 72% for IgM. The specificities were 97.4% and 72% for IgG and IgM respectively [29]. A subsequent prospective study further confirmed the low sensitivity of the assay [14]. Given the relatively poor sensitivity of the prototype melioidosis ICT, it was not further developed and consequently the kit is not currently commercially available.

5. Novel antigen candidates for future serological assays

The efficiency of currently available serological assays to detect antibodies to *B. pseudomallei* is still limited since there is cross-reactivity with related *Burkholderia* species, especially *B. mallei*. In addition, the sensitivity and specificity need to be improved [13,30]. The main reason for this problem is the use of crude *B. pseudomallei* antigens in these assays. In endemic areas, seropositivity in the population can be either due to
active disease or previous exposure to *B. pseudomallei* [14,31]. There have been various attempts to define purified antigens for the rapid diagnosis of *B. pseudomallei* infection. Antigen detection for exotoxin and cell components yielded favourable results, but required further validation.

Cell wall components including LPS, 30kDa protein, and exopolysaccharides (EPS) have been tested. LPS is highly conserved among *B. pseudomallei* species [32,33]. However, the use of LPS and/or EPS for ELISA does not provide higher sensitivity or specificity in comparison to the use of crude antigens in IHA [13]. Moreover, the pattern of LPS varies according to geographical area and clinical origins. Bip, a virulence factor in type III secretion system (T3SS) of *B. pseudomallei*, is highly conserved among these species [34,35]. By genomic sequence analysis, BipD is similar to IpaD of *Shigella* (26% identity; 36% similarity) and SipD from *Salmonella* (27% identity; 39% similarity) [35,36]. T3SS protein assemblies span the inner membrane, the periplasmic space, the peptidoglycan layer, the outer bacterial membrane, the extra-cellular space and the target cell membrane [34,37,38]. The biological function of T3SS protein is to deliver effector proteins into the target host cell in an ATP-dependent manner [37,38]. The diagnostic potential of Bip has been determined in patients with melioidosis. The indirect ELISA using BipD as an antigen demonstrated poor sensitivity (42%) but high specificity (100%) [39].

The outer membrane protein OmpA was shown to be a good candidate in an endemic area demonstrating both high sensitivity (95%) and specificity (98%) over BipD or BPSL0972, a hypothetical protein of *B. pseudomallei* [39]. A recombinant truncated flagellin as the antigen for indirect ELISA offered 93.8% sensitivity and 96.3% specificity [40]. However, due to the cross-reactivity with other organisms, this antigen needs to be further evaluated. The potential of these proteins in serodiagnosis is still under review as these assays have been conducted on a very small sample size and due to the possible cross-reactivity with other related organisms. A study using a polysaccharide microarray platform, a high throughput technology, identified O-saccharide antigens from different bacterial strains of *B. pseudomallei*. This result indicated the low efficiency of these antigens for specific antibody detection and the methodology needs to be improved to circumvent the false-positive results observed [41]. The immunoscreening of a *B. pseudomallei* genomic expression library identified 109 proteins that were reactive against a pooled serum of five infected patients [41].

The use of bioinformatics is an alternative approach to predict the probability of candidate antigens in the serodiagnosis of melioidosis. An effort to use *in silico* analysis identified 12 immunoreactive proteins in human convalescent sera [42]. Experimental evidence demonstrating the precise nature of these markers is required to explore the potential of candidate antigens in the diagnosis of melioidosis. Newer technologies have brought the potential of identifying more sensitive and specific antigens that might be used in the serodiagnosis of melioidosis. Large scale field studies are needed to assess the utility of these agents which have the potential to improve diagnosis and impact on the management of patients with melioidosis.
References


Abstract

The diagnosis of melioidosis relies upon culture. This can take up to four days and errors in identification by standard identification systems can occur. The importance of Burkholderia pseudomallei as both a potentially fatal pathogen and as a bioterrorism agent, has led to the development of polymerase chain reaction (PCR) based detection methods. These have targeted a number of genes and include type III secretion system genes (orf2, orf11, orf13 and SCU2), 16S and 23S rRNA genes, flagellin C gene (fliC), ribosomal protein subunit S21 gene (rpmU) and a single nucleotide polymorphism in a putative antibiotic resistance gene (P27). When used to confirm the identity of B. pseudomallei, PCR has been shown to be very specific and sensitive. When used for direct detection from clinical specimens however, the sensitivities and specificities of a number of assays have been less promising.

1. Introduction

Culture and phenotypic identification are currently the gold standard for the diagnosis of melioidosis. This is both time consuming and reliant upon the presence of viable organisms in a clinical sample. In addition, misidentifications occur particularly if early cultures are used. Serological tests can be falsely negative for acute sera in up to 50% of patients with bacteremia. They can also be falsely positive in endemic regions. The necessity for rapid and accurate diagnosis of melioidosis is highlighted by the high mortality associated with this condition. A variety of PCR detection methods have been developed using both conventional and real time formats. The advent of real time systems has demonstrated increased sensitivity, rapidity and ease of use over conventional PCR methods. Compared to conventional PCR, real-time PCR has the added advantage of automation with a marked reduction in the risk of carryover contamination. Post-PCR manipulation is also avoided. Real-time PCR is however relatively expensive and requires
experienced staff. Both of these are generally not easily available in regions where melioidosis is endemic.

Unlike some other pathogens which can be colonisers (e.g. *Streptococcus pneumoniae, Staphylococcus aureus*) in certain clinical specimens, the detection of *B. pseudomallei* in any clinical specimen would imply a high likelihood of clinical disease. There are essentially two potential roles for molecular detection of *B. pseudomallei* in the clinical diagnostic laboratory. The first is in the confirmation of the identity of an isolate which has some characteristics of *B. pseudomallei* but cannot be confirmed as such by phenotypic methods. The second is the direct detection of *B. pseudomallei* from clinical specimens such as blood, pus, urine or sputum. This has proven to be problematic, with some studies showing limited sensitivity.

The key features of a molecular detection system for *B. pseudomallei* would require the choice of a target which is sufficiently specific so as to distinguish it from other *Burkholderia* species (*B. cepacia, B. mallei*), while at the same time not being affected by the evolving heterogeneity of *B. pseudomallei* strains.

## 2. The use of molecular methods to confirm organism identity

Molecular confirmation is a more attractive approach due to rapidity, high sensitivity and specificity, less subjective decision making, and high resolution of identification. The technique is not only used for identification, but can also be applied to investigate the relationship of possibly related organisms. In addition, some molecular markers can be used to determine the genetic modification that accounts for the genetic diversity and evolution of the organism, such as resistance to antibiotics and pathogenicity [1−5]. The use of molecular markers has a higher specificity and sensitivity than protein antigens, as the nucleotide sequence is exact and unique and is not affected by the post-translational modification found at the protein level.

The high sensitivity of PCR-based assays is due to the ability to amplify a low copy number of bacterial DNA within a specimen, and high specificity is based on the unique DNA sequence of the marker used. The capability of identification ranges from family, genus, species, subspecies, and strains of the organism and is dependent upon the type of PCR used [6]. These can be performed using a conventional, real-time, high throughput, or *in silico* approach.

The most powerful and definitive method is DNA sequencing but it is expensive, laborious, and time consuming. Molecular targets for identification of *B. pseudomallei* have been revealed by different methods. Generally, the procedure starts with the selection of a conserved and specific marker by genetic typing and sequence alignment tools, such as basic local alignment search tool (BLAST, www.ncbi.nlm.nih.gov), and primers or probes are designed for the selected targets [7−9]. Recently, a Tool for Oligonucleotide Fingerprint Identification (TOFI) programme was developed as an easier and faster approach to design probes, especially for DNA microarray to discriminate *B. mallei* and *B. pseudomallei* [10,11]. This pipeline combines a genome comparison tool, probe design software, and sequence alignment programme together. *In silico* hybridisation can be performed with this system.
### Table V.4
Molecular markers for PCR-based identification of *Burkholderia pseudomallei*

<table>
<thead>
<tr>
<th>Target genes</th>
<th>Methodology</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Limit of detection</th>
<th>Specimen types</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>16s rDNA</td>
<td>C</td>
<td>100%</td>
<td>100%</td>
<td>–</td>
<td>Spiked blood</td>
<td>[12]</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>41.37%</td>
<td>47.37%</td>
<td>–</td>
<td>Blood</td>
<td>[13]</td>
</tr>
<tr>
<td></td>
<td>RT</td>
<td>–</td>
<td>100%</td>
<td>40 ge</td>
<td>Spiked blood</td>
<td>[14]</td>
</tr>
<tr>
<td>23s rDNA</td>
<td>C</td>
<td>20%</td>
<td>–</td>
<td>–</td>
<td>Purified DNA</td>
<td>[12]</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>34.48%</td>
<td>58.82%</td>
<td>–</td>
<td>Blood</td>
<td>[13]</td>
</tr>
<tr>
<td></td>
<td>SN</td>
<td>100%</td>
<td>100%</td>
<td>–</td>
<td>Purified DNA</td>
<td>[15]</td>
</tr>
<tr>
<td></td>
<td>SN</td>
<td>100%</td>
<td>99%</td>
<td>–</td>
<td>Purified DNA</td>
<td>[16]</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>BAL</td>
<td>[17]</td>
</tr>
<tr>
<td>LPS</td>
<td>C</td>
<td>–</td>
<td>100%</td>
<td>0.5 fg</td>
<td>Blood</td>
<td>[18]</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>31.03%</td>
<td>100%</td>
<td>–</td>
<td>Blood</td>
<td>[13]</td>
</tr>
<tr>
<td>T3SS1 cluster</td>
<td>RT</td>
<td>91%</td>
<td>95%</td>
<td>–</td>
<td>CS</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>65%</td>
<td>100%</td>
<td>–</td>
<td>CS</td>
<td>[20]</td>
</tr>
<tr>
<td></td>
<td>LAMP</td>
<td>44%</td>
<td>98.4%</td>
<td>38 copies</td>
<td>CS</td>
<td>[21]</td>
</tr>
<tr>
<td>fliC</td>
<td>RT</td>
<td>–</td>
<td>–</td>
<td>40 ge</td>
<td>Spiked blood</td>
<td>[14]</td>
</tr>
<tr>
<td>orf11 of T3SS1</td>
<td>RT</td>
<td>86.7%</td>
<td>88.2%</td>
<td>–</td>
<td>CS</td>
<td>[22]</td>
</tr>
<tr>
<td>BPSS1187</td>
<td>RT</td>
<td>100%</td>
<td>100%</td>
<td>5 fg</td>
<td>Purified DNA</td>
<td>[23]</td>
</tr>
<tr>
<td></td>
<td>RT</td>
<td>100%</td>
<td>100%</td>
<td>50 fg</td>
<td>CS</td>
<td>[22]</td>
</tr>
<tr>
<td>BPSS2089</td>
<td>RT</td>
<td>100%</td>
<td>100%</td>
<td>50 fg</td>
<td>CS</td>
<td>[22]</td>
</tr>
<tr>
<td>mrpA</td>
<td>C</td>
<td>100%</td>
<td>100%</td>
<td>10 fg</td>
<td>Purified DNA</td>
<td>[25]</td>
</tr>
<tr>
<td>rpsU</td>
<td>RT</td>
<td>–</td>
<td>–</td>
<td>400 ge</td>
<td>SB</td>
<td>[14]</td>
</tr>
<tr>
<td>lpxO</td>
<td>C</td>
<td>89%</td>
<td>100%</td>
<td>–</td>
<td>Purified DNA</td>
<td>[16]</td>
</tr>
<tr>
<td>phaC</td>
<td>C</td>
<td>100%</td>
<td>–</td>
<td>–</td>
<td>Positive with</td>
<td>[16]</td>
</tr>
</tbody>
</table>

**Gene-specific PCRs have been developed (Table V.4). Unfortunately, they are not able to replace bacterial culture and susceptibility testing. Small subunits of ribosomal RNA gene (rDNA), especially 16s rDNA, is useful for discriminating *B. pseudomallei* from related species [12–17]. Real-time PCR is faster, and offers higher sensitivity and specificity in comparison to conventional PCR. It is therefore, easier for implementation in a routine laboratory [16]. The loop-mediated isothermal amplification (LAMP) method**
is an alternative assay which is simpler, inexpensive and has a high specificity for *B. pseudomallei* identification but more improvement in sensitivity is required [21].

3. The use of molecular methods for direct detection from clinical specimens

The direct detection of bacterial molecules from clinical specimens such as blood, pus, sputum, urine, and body fluids, remains a challenge for the diagnosis of melioidosis. Most attempts focus on using molecular methods, especially PCR assay for detecting bacterial DNA. The viability of bacteria in the samples does not affect PCR results. It is therefore, more useful than culture and biochemical testing, particularly for patients who have previously received antibiotics [20]. There are many commercial systems, including reagent kits and DNA extraction machines, that could be utilised to obtain DNA from samples. The overall running time for PCR is quicker than that of bacterial culture and biochemical testing. Thus, detection of *B. pseudomallei* DNA from clinical specimens facilitates the diagnosis and treatment of melioidosis. Currently, clinical specimens offer a lower efficiency of molecular detection when compared to purified or crude DNA [22]. A validation of the PCR assay with bacteria spiked blood, demonstrated higher limits of detection, indicating the assay required higher amounts of bacterial DNA in the samples, compared to isolated DNA from a pre-enriched culture [14]. False positive or false negative results may be due to impurities or dilution of host DNA in clinical specimens. However, commercial DNA purification systems could be used to eliminate PCR inhibitors in the samples. The inadequacy of bacterial DNA in the sample needs to be considered as a factor in improving the sensitivity of assays [19,20].

There are several factors which need to be addressed in improving the quality of *B. pseudomallei* identification from clinical specimens by PCR. Firstly, candidate markers must provide high sensitivity and specificity. Most markers offer high specificity but the sensitivity is variable [13,19–22]. T3SS1 gene cluster is a possible marker providing a practical assay which is more than 90% sensitive and specific. It was found that more than one type of sample from the same patient could improve detection [19]. Secondly, real-time PCR is a more effective variant of PCR, providing higher sensitivity and specificity. Additionally it is faster and a technically easier process than a conventional PCR [19]. Lastly, the type of specimens could also influence the outcome of the assay due to different characteristics of specimen. For instance, PCR for sputum is the most robust and is achievable in the presence of high bacterial numbers in the sputum [19,20]. Samples such as bronchoalveolar lavage require larger sample volume [17]. While direct detection of *B. pseudomallei* may not have enough sensitivity, pre-enrichment of culture could overcome this problem. Crude DNA or cell lysate could be extracted by heating bacterial colonies at 95 to 100°C for 10 to 15 minutes. The overall process is quicker than the conventional procedure for detection of the bacteria. In addition to the conventional PCR assays, high throughput technologies such as microarray has been developed for the identification of bacteria including *B. pseudomallei* [26–28]. DNA, RNA, and protein expression can be determined based upon the type of microarray used [26]. Apart from determining bacterial antigens, human transcriptomes and proteomes expressed in response to *B. pseudomallei* infection may alternatively provide a molecular target and
candidate antigens for the diagnosis of melioidosis [29,30]. The molecular signature of \( B. \) pseudomallei infection is a dynamic status reflecting the pathophysiology of the infection in a patient. This may not only be useful in predicting the severity of illness but also in selecting appropriate adjunct therapies [30]. Further evaluation is still required with larger sample pools and more robust techniques. Currently, none of these molecular techniques may be effective enough to replace culture-based approaches.

References


Medical imaging in melioidosis

Stuart Ramsay
Queensland X-Ray, Townsville, Australia

Abstract

Melioidosis can involve multiple organ systems. Abscesses in internal organs may present with poorly localising symptoms or signs. Patients with sepsis, respiratory failure or an altered state of consciousness due to central nervous system involvement may not give a coherent history or accurately localise sites of pain. Musculoskeletal and soft tissue disease can be overlooked in the race to save the patient’s life. Hence, assessment of the distribution of infection in melioidosis can be difficult. Appropriately directed medical imaging has an important role in: identifying early the sites of infection, allowing appropriate management decisions to be made; assessing patients whose conditions deteriorate; assessing those who do not respond to treatment. X-ray, computerised axial tomography (CT), ultrasound and magnetic resonance imaging (MRI) are very helpful in confirming sites of infection. Nuclear medicine bone scanning and white cell scanning have an important role to play in multifocal infections because of their functional nature and capacity to examine the whole body. Positron emission tomography (PET) scanning is useful in infectious disease, but remains untested in patients with melioidosis.

1. Introduction

The treatment of melioidosis requires accurate assessment of the presence, extent and severity of infection. However, clinically, this may not be simple. Patients with sepsis, respiratory failure or an altered state of consciousness due to central nervous system (CNS) involvement frequently cannot give a coherent history or accurately localise sites of infection and pain. As a consequence of an initial focus on life-saving treatment, musculoskeletal and soft tissue involvement can be overlooked. In subacute or chronic disease, visceral abscesses in the liver, spleen, kidney and prostate, may be present, with poorly localising symptoms or signs. In addition, disease may progress during treatment, with evolution of new sites of infection.

Medical imaging plays a number of important roles in the diagnosis of melioidosis. Firstly, it assists in the initial identification of the presence, location and extent of infection. Secondly, it is important in the assessment of patients whose clinical condition deteriorates or patients who do not respond to treatment. Finally, interventional radiology is an important adjunct to antibiotic and supportive therapy, as it can assist aspiration of material for diagnostic culture and therapeutic drainage of sites of infection.
2. Medical imaging

2.1. Pulmonary and thoracic disease

In melioidosis the lungs are most frequently affected, being involved in about half of patients [1,2]. In acute infection, the chest X-ray (CXR) and computerised axial tomography (CT) of the chest, show pulmonary consolidation indistinguishable from other pneumonias (Figure V.3a). Hence in endemic regions *Burkholderia pseudomallei* infection should be considered in any case of acute pneumonia. In Darwin, Australia, melioidosis is the second most common cause of community acquired pneumonia [3]. There is a predilection for the upper lobes [4], but other lobes are not infrequently affected. Pulmonary nodules of varying sizes can be seen (Figure V.3b), and these may enlarge or coalesce. Cavitation may also occur (Figure V.3c). The combination of pulmonary nodules
and cystic cavitation may mimic staphylococcal infection [5]. In chronic disease, the upper lobe preponderance, associated with cavitation and or pleural effusions, can make the radiographic appearance indistinguishable from tuberculosis [4], though melioidosis often spares the lung apices, and is less frequently associated with pleural effusion or lymphadenopathy [6]. Pneumothorax can be seen if a cavitating lung lesion ruptures into the pleural space [6]. Pericarditis is relatively rare [1], and its functional significance is best assessed with echocardiography.

2.2. Intraabdominal disease

Hepatic and splenic abscesses occur, are often multiple [1], and are best confirmed with ultrasound or CT (Figure V.3d). These abscesses may be septated [6]. In endemic regions, the finding of multiple small, discrete, target-like lesions and/or large multiloculated lesions in the spleen and liver is highly suspicious for melioidosis [7,8], particularly when found in association with pneumonia [9].

In Queensland, Australia and in some Asian centres genitourinary infection occurs in about 10% of patients [1,2]. Renal abscesses from melioidosis can be imaged with ultrasound or CT, but their imaging features are identical to those caused by other pathogens [7]. Prostatitis and prostatic abscesses also occur, being relatively frequent in the Northern Territory where CT has proven useful in their diagnosis [1]. However, particularly in older men, the CT and transrectal ultrasound changes found in B. pseudomallei prostatic infection overlap those of age related changes, making definitive diagnosis difficult. White cell scanning has been used to confirm prostatic inflammation [10], but ultimately diagnostic aspiration under transrectal ultrasound guidance, or confirmation based on cultures taken during therapeutic drainage or direct surgical intervention, may be required.

Patients undergoing peritoneal dialysis can develop intraperitoneal B. pseudomallei infection [10]. CT and ultrasound are helpful in these patients to assess for loculated intraperitoneal collections. CT and ultrasound also have an important role in directing diagnostic and therapeutic drainage procedures in intra-abdominal abscesses.

2.3. Central nervous system disease

Brain abscesses are well described in melioidosis, secondary either to haematogenous spread or direct invasion from adjacent infectious sites (including sinuses and calvarial osteomyelitis) [1]. Encephalitis usually involves the cerebrum, but in the Northern Territory, Australia brainstem encephalitis is well recognised [6,11]. Contrast CT demonstrates intracerebral abscesses as rim enhancing lesions, and can identify associated sinusitis or orbital infection. Cerebritis, encephalitis and meningitis are better identified on contrast enhanced magnetic resonance imaging (MRI) due to superior soft tissue contrast resolution. MRI diffusion weighted imaging can help differentiate cerebral abscesses from other space occupying lesions [12]. Currie [11] reported that CT scans may appear normal in melioidosis encephalitis, but MRI shows extensive increased signal intensity on T2-weighted scans, suggestive of oedema, and variable gadolinium enhancement. MRI is also superior to CT for evaluating the brainstem [6,11].
Spinal disease usually manifests as vertebral osteomyelitis, but *B. pseudomallei* infection can occur within the spinal canal. This can be extramedullary [10], but occasionally intramedullary lesions are identified (Figure V.4). MRI is superior to CT for evaluating disease within the spinal canal.

### 2.4. Musculoskeletal disease

In tropical Queensland, Australia only 4% of patients with melioidosis present with bone and joint infection, but this presentation occurs in up to 12% in some other regions [1]. X-ray, Nuclear Medicine bone scanning, CT, MRI and ultrasound all have a role to play in assessing musculoskeletal infection in melioidosis, but the imaging findings are not specific to *B. pseudomallei* infection. Nuclear medicine bone scanning and white cell scanning are particularly useful in multifocal infections, such as melioidosis, because of their capacity to examine the whole body.

A syndrome of combined osteomyelitis and septic arthritis can occur (Figure V.5a) and can prove very resistant to treatment, with some cases experiencing recurrent relapsing...
infection for years [10,13]. Involvement of the leg, particularly the metaphyseal region of the long bones, is most common, followed by infection of the vertebrae [6,10,13]. Some muscle involvement, such as psoas abscess, can result from direct spread from adjacent bone [6]. However, unlike many other bacteria, \textit{B. pseudomallei} can cause localised intramuscular infection without associated bone or joint disease (Figure V.5b).

2.5. Other soft tissue disease

Abscesses may also occur in the skin, subcutaneous tissues and lymph nodes (Figure V.6). The skin abscesses may ulcerate. Patients with multiple skin and subcutaneous abscesses can be surprisingly well and are frequently not bacteraemic [2]. Parotitis (bilateral in 10%) has been reported frequently in children in some regions [14], and may progress to abscess formation, best imaged with ultrasound. Parotid disease is less frequent in adults. Innumerable case reports have described melioidosis abscesses in almost every conceivable body tissue. The imaging features of such lesions are typically nonspecific, representing the expected findings of an abscess within the organ of interest. However these reports must be kept in mind when an abscess is found in an unusual soft tissue location, or in conjunction with pneumonia.

3. Whole body functional imaging techniques for assessing disease extent

Melioidosis is not infrequently a multiorgan disease. In north Queensland 28% of patients present with involvement of more than one organ system [2]. Some medical imaging
techniques allow the specific identification of inflammatory foci throughout the entire body in a single scanning episode. This capability has the potential to be useful in melioidosis.

3.1. Radiolabelled white cell scans

The patient's own white blood cells are labelled with a gamma emitting radioisotope and reinjected into the blood stream, distributing to sites of active inflammation. The resultant focal accumulation of radioisotope at inflammatory sites can be imaged with a gamma camera (Figure V.6). In melioidosis, white cell scanning accurately identifies and localises musculoskeletal, soft tissue, and other visceral involvement, and can show unexpected foci of infection [10]. In 20% of cases, white cell scanning provides information that is not obtained clinically. Unfortunately, pulmonary involvement and hepatic and splenic disease, are not well demonstrated with this technique, so it cannot be used as the only imaging modality in suspected or proven melioidosis.

Because of the accuracy of white cell scanning in identifying foci of infection throughout the body, this imaging technique is useful in the initial workup of patients with severe melioidosis. In patients with cutaneous ulceration or superficial skin abscesses, but no clinical indication of systemic infection, white cell scans are probably not initially indicated. Whilst white cell scans do accurately identify sites of infection in this subgroup, this has not proven to be of additional clinical benefit. However, if these patients fail to respond to first-line treatment, or if their condition deteriorates, white cell scanning should be considered.

3.2. Positron emission tomography and computerised axial tomography scanning

18F-Fluorodeoxyglucose (FDG) PET scanning is used to assess disease extent and activity in many forms of malignancy. FDG is also taken up by inflammatory cells; uptake is particularly avid in primed or activated monocytes/macrophages [15], but also occurs in neutrophils [16]. PET can be used to image infection [17] and to localise infective and inflammatory foci in individuals with fever of unknown origin [18]. PET/CT scanning allows accurate co-localisation of hypermetabolic FDG avid foci identified with PET with the precise anatomical detail of CT. This imaging modality has the capacity to be very useful in melioidosis as it can accurately localise sites of infection/inflammation and measure their inflammatory activity. One possible important advantage of PET over white cell scanning is that PET is useful in lung infection [17]. The potential clinical usefulness of FDG PET in melioidosis is supported by a report where it was used in a case of proven B. pseudomallei infection to confirm isolated involvement in the prostate. However, the precise clinical role of PET/CT in melioidosis and its cost effectiveness have not yet been evaluated.

4. Conclusion

Many of the imaging findings in melioidosis are characteristic of infection within the involved organs, rather than being specific for B. pseudomallei infection. However, the
presence of suggestive imaging findings, in combination with an appropriate clinical syndrome, is highly suggestive of melioidosis. Hence, when working in an endemic area, clinicians and imaging specialists must have a high index of suspicion for this disease. In addition, they must be aware of the different clinical and imaging patterns of melioidosis so that when B. pseudomallei is suspected or proven to be the causative organism, the most appropriate imaging investigations are utilised. Imaging specialists outside of endemic areas also need to be aware of these patterns so that melioidosis is recognised when it does occur in unusual geographic locations.

References

VI
Treatment of melioidosis
Section VI. Treatment of melioidosis

Editorial overview

Direk Limmathurotsakul\textsuperscript{a,b}, Sharon J. Peacock\textsuperscript{c,d}

\textsuperscript{a} Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand
\textsuperscript{b} Mahidol–Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand
\textsuperscript{c} Department of Medicine, University of Cambridge, Addenbrooke’s Hospital, University of Cambridge, United Kingdom
\textsuperscript{d} Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand

\textit{Burkholderia pseudomallei} is notoriously difficult to eradicate from the human host, as reflected by the duration of antimicrobial treatment given to patients with melioidosis; parenteral therapy is given for 10 to 14 days (or longer if clinically indicated), followed by oral therapy for 12 to 20 weeks. In Section VI.1 the evidence for first and second-line antimicrobial therapy during the initial parenteral phase of treatment is reviewed. The in-hospital mortality rate is the outcome against which different parenteral antimicrobial regimens have been compared. Recommended first-line parenteral therapy is ceftazidime, with the use of a carbapenem drug as first-line therapy in Australia for those patients presenting with severe melioidosis. There is \textit{in vitro} evidence that meropenem is better than ceftazidime, and an important uncertainty is whether treatment with a carbapenem drug is associated with a survival benefit compared with ceftazidime. An on-going randomised controlled trial in northeast Thailand is addressing this question but the results will not be known for several years. Some patients require prolonged parenteral antimicrobial therapy, and clinical pointers suggesting the need for prolonged therapy are discussed, together with indicators that are commonly used to determine when treatment can be switched from parenteral to oral therapy.

In Section VI.2 the evidence for oral antimicrobial therapy for melioidosis is reviewed. The aim of this stage of treatment is to eradicate viable \textit{B. pseudomallei} and reduce the risk of relapse of infection from a persistent nidus. The rate of relapse is the outcome against which different oral antimicrobial regimens have been compared. The recommended treatment regimen is trimethoprim–sulfamethoxazole (TMP-SMX) with or without doxycycline. Doxycycline is added in some centres but not others, and the need for this is being addressed by a randomised controlled trial that is currently nearing completion in Thailand. The recommended regimen for pregnant women and adults who are intolerant of TMP-SMX is amoxicillin–clavulanate (AMC), although this has been associated with a higher rate of relapse. Children may receive either AMC (Thailand) or...
TMP-SMX (Australia). The need for prolonged oral therapy for mild localised disease is an unresolved question, as is the optimal duration of treatment for paediatric melioidosis, which is often localised and relatively mild. No research has been conducted on patient compliance and strategies to improve this, an important omission since this is likely to be crucial to the likelihood of cure.

In Section VI.3 the management of patients with severe melioidosis in an intensive care setting is addressed. Management of sepsis is pivotal to patient outcome from severe melioidosis, with a difference in mortality rate between high-income and low- and middle-income countries that is likely to be due in large part to differences in the availability of intensive care facilities. Although many regions of the world where melioidosis is endemic currently do not have adequate intensive care facilities, this situation is changing in many developing countries. This review provides timely information to clinicians working in settings where investment in healthcare is high or increasing. Much of the evidence presented is obtained from studies in which the study population had sepsis from a range of different pathogens, since little evidence exists for the management of severe melioidosis per se. Granulocyte colony stimulating factor (G-CSF) has been part of the treatment protocol for patients with severe melioidosis in one of the centres in Australia, but the addition of G-CSF to antimicrobial therapy has not been shown to be of benefit in a study carried out in Thailand. The benefit that might be derived from this adjunctive therapy in other healthcare settings is not known. As other adjunctive agents that reduce death from bacterial sepsis are licensed for use in the developed world, the challenge will be to determine what if any role these have in lower income settings in treating melioidosis.

In Section VI.4, the management of accidental laboratory exposure to B. pseudomallei is addressed. Diagnostic laboratories worldwide are increasingly likely to isolate B. pseudomallei from clinical specimens, and laboratory research involving the use of this species is increasing. Guidelines are required to provide laboratory and medical professionals with background information on the organism, actions required prior to working with it, assessment of risk factors and actions required in the event of accidental exposure to B. pseudomallei, and the management of seroconversion. The evidence on which to formulate these guidelines is largely absent and current recommendations are largely based on expert opinion.

In the final Section (VI.5) the mechanisms involved in B. pseudomallei antimicrobial resistance are reviewed. This bacterial species is intrinsically resistant to a range of antimicrobial agents, and acquired resistance may also arise during therapy. Genome sequence analyses have provided an indication of possible mechanisms of intrinsic and acquired resistance to antimicrobial compounds, but to date only a few have been experimentally confirmed. Of several putative β-lactamases encoded by B. pseudomallei, a Class A β-lactamase confers resistance to amoxicillin and other β-lactams, and mutant variants confer resistance to ceftazidime. Efflux pumps belonging to the resistance nodulation cell division superfamily are widespread and, when expressed, bestow resistance to aminoglycosides, chloramphenicol, fluoroquinolones, macrolides, tetracyclines and trimethoprim. Penicillin-binding protein mutations cause ceftazidime resistance, while intrinsic polymyxin B resistance is due to outer membrane exclusion.
properties. Other factors, such as biofilm formation and intracellular latent lifestyle, are likely to contribute to persistence of *B. pseudomallei* in the presence of antimicrobial drugs to which the organism is susceptible *in vitro*. 
Outcome following melioidosis remains poor despite more than two decades of clinical research. The overall mortality rate is 40% in northeast Thailand (half of which occur within 48 hours of presentation) and 19% in Australia. If melioidosis is suspected, antimicrobial agents that are effective against *Burkholderia pseudomallei* should be included in the initial empirical regimen. After confirmation of the diagnosis, parenteral antimicrobial therapy should be given for a minimum of 10 days. Clinical evidence supports the use of ceftazidime or a carbapenem drug as first-line treatment. Carbapenem antibiotics have theoretical advantages over ceftazidime, but there is no clinical evidence to date of the superiority of carbapenem.

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1. Introduction

Parenteral antimicrobial therapy is required for a minimum of 10 to 14 days at the start of the prolonged course of antibiotics required to cure melioidosis. *B. pseudomallei* is intrinsically resistant to many antimicrobial drugs used for the empirical treatment of suspected Gram-negative sepsis. The agent used most widely for the treatment of melioidosis is parenteral ceftazidime, a choice supported by clinical trials carried out in Thailand [1,2]. However, the mortality rate for severe septicemic melioidosis remains high (up to 90%) in many countries where this infection is endemic. The search continues for a more effective antibiotic and/or adjunctive therapy that would improve mortality, but much of the excess mortality in some areas of Asia may be related to a combination of late patient presentation, delay in time between admission and providing the first dose of effective antibiotic, and restricted availability of intensive care facilities for patients who develop septic shock and its sequelae.

2. In vitro antimicrobial susceptibility

*Burkholderia pseudomallei* is intrinsically resistant to many antimicrobial drugs, including first, second and third-generation cephalosporins, aminoglycosides, penicillins and
polymyxin. The organism is usually susceptible to ceftazidime, the carbapenem antibiotics, amoxicillin–clavulanate (AMC), doxycycline and trimethoprim–sulfamethoxazole (TMP-SMX). In vitro susceptibility of *B. pseudomallei* to a range of antimicrobial drugs is summarised in Table VI.1.

The carbapenems are the most active antibiotics against *B. pseudomallei in vitro*. Of these, biapenem has been shown to have the lowest minimum inhibitory concentration (MIC) (MIC	extsubscript{90} 0.25 mg/L) [11], followed by doripenem, imipenem, panipenem, and meropenem (MIC	extsubscript{90} of 0.75 mg/L, 0.5–1.0 mg/L, 1.0–1.6 mg/L, and 0.78–2.0 mg/L, respectively) [7,15]. Carbapenem drugs exert bactericidal activity against *B. pseudomallei*, including isolates with reduced susceptibility to ceftazidime and/or AMC [11]. Carbapenem-resistant *B. pseudomallei* have not been reported to date.

*Burkholderia pseudomallei* is highly resistant to almost all penicillins, including agents with anti-pseudomonas activity, such as temocillin, carbenicillin and ticarcillin [3,8,10]. Resistance is due to the production of chromosomal β-lactamases [18], which can also hydrolyse almost all cephalosporins, but not ceftazidime. The addition of clavulanic acid or sulbactam improves the activity of penicillins and cephalosporins against *B. pseudomallei*. Cefoperazone has the poorest activity among the third generation cephalosporins against *B. pseudomallei* (mean MIC 16 mg/dl) [4,10,18], but the MIC is reduced by four- to eight-fold to clinically-achievable concentrations by the addition of clavulanate or sulbactam [14,18]. Laboratory evaluation of cefoperazone/sulbactam demonstrated an MIC	extsubscript{90} of 4 mg/L for 50 *B. pseudomallei* clinical isolates from Malaysia [14], and 3 mg/L for 100 clinical isolates from Thailand [19]. Ticarcillin/clavulanic acid, ampicillin/sulbactam, piperacillin/tazobactam, cefoperazone/sulbactam and AMC are also highly active against *B. pseudomallei* strains *in vitro* [4,8,11]. Ureidopenicillins are semi-synthetic penicillins with activity against *Pseudomonas aeruginosa*. Azlocillin and piperacillin, but not mezlocillin, have been shown to have activity against *B. pseudomallei* [3,5,9].

*Burkholderia pseudomallei* is susceptible to TMP-SMX, with rates of resistance among clinical isolates of 10% to 13% in Thailand and 0% to 3% in Australia [9,20]. The bactericidal activity of ceftazidime has been reported to be antagonised *in vitro* by the addition of other bacteriostatic agents, including TMP-SMX [21]. However, this was not confirmed by a subsequent study [22]. Fluoroquinolones have poor activity *in vitro*, and should not be used for the treatment of melioidosis (Table VI.1).

### 3. Pharmacokinetics and pharmacodynamics

β-Lactam antibiotics do not exhibit a post-antibiotic effect against *B. pseudomallei*, and the serum drug level should be maintained above the MIC	extsubscript{90} throughout the dosing interval. In a study of the pharmacokinetic and pharmacodynamic effects of continuous infusion of ceftazidime versus intermittent bolus dosing, 34 patients suspected to have septic caemic melioidosis (of whom 20 were culture positive for *B. pseudomallei*) were randomised to receive ceftazidime by either bolus injection or by constant infusion following a loading dose. Simulations based on this data and the ceftazidime susceptibility of the related *B. pseudomallei* isolates indicated that administration by constant infusion
Table VI.1
*In vitro* susceptibility pattern of *Burkholderia pseudomallei* against a range of antibiotics

<table>
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<th>Antibiotics</th>
<th>No. of isolates</th>
<th>MIC Range</th>
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<td>64</td>
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<td>0.25–3.13</td>
<td>0.78–1</td>
<td>0.78–2</td>
<td>100</td>
<td>[7,9,11,15]</td>
</tr>
<tr>
<td>Panipenem</td>
<td>197</td>
<td>0.06–3.13</td>
<td>0.5–1.56</td>
<td>1–1.56</td>
<td>100</td>
<td>[7,11]</td>
</tr>
<tr>
<td><strong>Aminoglycosides</strong></td>
<td></td>
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</tr>
<tr>
<td>Amikacin</td>
<td>211</td>
<td>16–256</td>
<td>64</td>
<td>128</td>
<td>5.9</td>
<td>[3]</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>268</td>
<td>8–&gt;256</td>
<td>128</td>
<td>64–128</td>
<td>0–0.5</td>
<td>[4,10]</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>295</td>
<td>8–256</td>
<td>16–32</td>
<td>16–64</td>
<td>16–55.7</td>
<td>[3,4,10]</td>
</tr>
<tr>
<td><strong>Fluoroquinolones</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>790</td>
<td>0.064–32</td>
<td>2–4</td>
<td>2–8</td>
<td>9.5–95</td>
<td>[3–5,7,8,13,16]</td>
</tr>
<tr>
<td>Clinafloxacin</td>
<td>71</td>
<td>0.25–2</td>
<td>0.5</td>
<td>1</td>
<td>–</td>
<td>[12]</td>
</tr>
<tr>
<td>Enoxacin</td>
<td>131</td>
<td>3.13–64</td>
<td>6.25–8</td>
<td>6.25–32</td>
<td>0</td>
<td>[7,16]</td>
</tr>
<tr>
<td>Fleroxacin</td>
<td>97</td>
<td>1.56–12.5</td>
<td>3.13</td>
<td>6.25</td>
<td>–</td>
<td>[7]</td>
</tr>
<tr>
<td>Gatifloxacin</td>
<td>71</td>
<td>1–32</td>
<td>4</td>
<td>16</td>
<td>21.1</td>
<td>[12]</td>
</tr>
<tr>
<td>Grepafloxacin</td>
<td>71</td>
<td>0.5–32</td>
<td>4</td>
<td>8</td>
<td>15.5</td>
<td>[12]</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>71</td>
<td>0.5–32</td>
<td>2</td>
<td>4</td>
<td>–</td>
<td>[12]</td>
</tr>
<tr>
<td>Plefloxacin</td>
<td>57</td>
<td>4–64</td>
<td>–</td>
<td>16</td>
<td>0</td>
<td>[10]</td>
</tr>
<tr>
<td>Temafloxacin</td>
<td>297</td>
<td>0.25–32</td>
<td>3.13–8</td>
<td>6.25–16</td>
<td>9.5–60.3</td>
<td>[7,8]</td>
</tr>
<tr>
<td><strong>Macrolides</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Azithromycin</td>
<td>100</td>
<td>&lt;0.5–&gt;256</td>
<td>256</td>
<td>256</td>
<td>3</td>
<td>[17]</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>100</td>
<td>4–&gt;256</td>
<td>128</td>
<td>256</td>
<td>3</td>
<td>[17]</td>
</tr>
<tr>
<td><strong>Doxycycline</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>431</td>
<td>0.19–16</td>
<td>1</td>
<td>1.5–4</td>
<td>97.3–100</td>
<td>[3,9,13]</td>
</tr>
<tr>
<td><strong>Chloramphenicol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>562</td>
<td>4–&gt;256</td>
<td>8–12.5</td>
<td>8–32</td>
<td>84–100</td>
<td>[3,4,7,9,10]</td>
</tr>
<tr>
<td><strong>Trimethoprim–</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>sulfamethoxazole</strong></td>
<td>374</td>
<td>0.25–32</td>
<td>12.5–16</td>
<td>4–&gt;32</td>
<td>78–98.2</td>
<td>[4,7,9,10,13]</td>
</tr>
</tbody>
</table>
could provide adequate dosing with a significant dose reduction and cost saving. Ceftazidime infusions are now being used in Darwin, Australia, to facilitate home intravenous (IV) therapy after discharge from hospital. The cost of peripherally-inserted central catheter lines and infusors, together with the cost and infrastructure required to support IV therapy, prohibits their use in most melioidosis endemic regions.

A pharmacokinetic study of AMC indicated that serum concentrations of clavulanic acid sufficient to inhibit *B. pseudomallei* in the presence of amoxicillin persist for only around three hours following a 1.2g IV bolus dose of AMC [23], suggesting that the dosing interval for IV AMC should not exceed four hours. A subsequent PK/PD study of patients with melioidosis supported by a Monte-Carlo Simulation suggested that the usual dosing interval used in other infections, six- or eight-hourly, is not appropriate for the treatment of melioidosis [24].

4. Clinical trials of parenteral therapy for melioidosis

Six randomised controlled trials have been published to date on initial parenteral antimicrobial treatment of patients with melioidosis (Table VI.2). The first treatment regimens to be used were based on *in vitro* susceptibility patterns and experience accumulated during the Vietnam War. One of the first combinations of chloramphenicol, kanamycin and novobiocin was superseded in the 1970s by a combination of chloramphenicol, tetracycline or doxycycline and TMP-SMX. Therapy was given for a period ranging between six weeks and six months and was associated with a mortality rate of over 70%. Practice changed following the publication of two open randomised controlled trials of severe melioidosis conducted in Thailand between 1986 and 1989 [1,28]. The first study compared ceftazidime with a combination of chloramphenicol, doxycycline and TMP-SMX. The ceftazidime-treated group had a 50% reduction in mortality (37% versus 74%). The second trial compared ceftazidime plus TMP-SMX with a combination of chloramphenicol, doxycycline and TMP-SMX, and reported a mortality rate of 19% versus 47%, respectively [28]. Ceftazidime, with or without TMP-SMX, became the standard of care thereafter for the initial therapy of patients in Thailand. Uncertainty over whether TMP-SMX was necessary during initial treatment has since been addressed by a randomised trial of ceftazidime alone versus ceftazidime plus TMP-SMX. The addition of TMP-SMX to ceftazidime therapy during the initial treatment of severe melioidosis did not reduce the acute mortality rate, although there were more switches in therapy in the ceftazidime monotherapy group [29], and rates of relapse did not differ between the two groups during long term follow-up [30]. A retrospective review of 1,353 Thai patients with melioidosis demonstrated that the mortality rate among patients who were initially treated with either cefotaxime or ceftriaxone was significantly higher (71%) than those who were treated with ceftazidime (42%) or AMC (54%) [25], suggesting that cefotaxime and ceftriaxone should not be used to treat melioidosis.

The carbapenem antibiotics have advantages over ceftazidime based on its *in vitro* evidence of bactericidal activity, rather than bacteriostatic activity, and a lower MIC against *B. pseudomallei*. A clinical trial comparing imipenem and ceftazidime reported no
Table VI.2
Summary of randomised controlled trials for the parenteral treatment of patients with severe melioidosis\(^a\)

<table>
<thead>
<tr>
<th>Place and time</th>
<th>Treatment arms</th>
<th>Sample size (^b)</th>
<th>Culture confirmed (^b)</th>
<th>Mortality</th>
<th>Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubon Ratchathani, Thailand, Sep 1986–Jul 1988</td>
<td>Both arms given iv for at least 7 days (chloramphenicol 6 hourly, doxycycline 12 hourly, all others 8 hourly)</td>
<td>161 (15)</td>
<td>65</td>
<td>ITT 77% vs 49%</td>
<td>50% reduction in mortality ((P=0.009, 95% CI 19–81%))</td>
<td>[1]</td>
</tr>
<tr>
<td></td>
<td>Conventional (chloramphenicol 100 mg/kg/day, doxycycline 4 mg/kg/day, and TMP/SMX 10/50 mg/kg/day)</td>
<td>69</td>
<td>31</td>
<td>74%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ceftazidime 120 mg/kg/day</td>
<td>77</td>
<td>34</td>
<td>34%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Khon Kaen, Thailand, Sep 1986–Jan 1989</td>
<td>Both arms given iv for at least 10–14 days (chloramphenicol 6 hourly, doxycycline 12 hourly, all others 8 hourly)</td>
<td>136</td>
<td>64 (3)</td>
<td>ITT NP</td>
<td>61% reduction in mortality ((P=0.039))</td>
<td>[2]</td>
</tr>
<tr>
<td></td>
<td>Conventional (chloramphenicol 100 mg/kg/day, doxycycline 4 mg/kg/day, and TMP/SMX 8/40 mg/kg/day)</td>
<td>NA</td>
<td>34</td>
<td>47%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ceftazidime 100 mg/kg/day and TMP/SMX 8/40 mg/kg/day</td>
<td>NA</td>
<td>27</td>
<td>18.5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ubon Ratchathani, Thailand, Jan 1989–Oct 1992</td>
<td>Both arms given iv for at least 7 days</td>
<td>379</td>
<td>212</td>
<td>44% (ITT 46% vs 43%)</td>
<td>No difference in mortality ((P=0.4))</td>
<td>[25]</td>
</tr>
<tr>
<td></td>
<td>Ceftazidime 120 mg/kg/day, 8 hourly</td>
<td>186</td>
<td>106</td>
<td>47%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amoxicillin/clavulanate 120/40 mg/kg/day, 4 hourly</td>
<td>193</td>
<td>106</td>
<td>46%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ubon Ratchathani, Thailand, Jul 1994–Nov 1997</td>
<td>Both arms given iv every 8 hours until clinically improved (usually a minimum of 10 days)</td>
<td>296(^c)</td>
<td>214</td>
<td>34.8% (ITT NP)</td>
<td>No difference in mortality ((P=0.96))</td>
<td>[26]</td>
</tr>
<tr>
<td></td>
<td>Ceftazidime 120 mg/kg/day</td>
<td>148</td>
<td>106</td>
<td>37.7%</td>
<td>Less treatment failure ((41.3% vs 20.3%, P=0.01))</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Imipenem/cilastatin 50 mg/kg/day</td>
<td>148</td>
<td>108</td>
<td>36.1%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Information on mortality is based on ITT analysis unless otherwise stated.
\(^b\) Data for culture confirmed are based on the number of patients with positive cultures.
\(^c\) Includes 27 patients who died before the primary endpoint was reached.
Table VI.2, continued

<table>
<thead>
<tr>
<th>Place and time</th>
<th>Treatment arms</th>
<th>Sample size b</th>
<th>Culture confirmed b</th>
<th>Mortality</th>
<th>Results</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Khon Kaen, Thailand, Jul 1995–Apr 1999</td>
<td>Both arms given iv every 8 hours, duration not clearly stated</td>
<td>219</td>
<td>102 (2)</td>
<td>ITT NP</td>
<td>No difference in mortality (adjusted difference 0.9%, ( P = 0.696 ))</td>
<td>[19,27]</td>
</tr>
<tr>
<td></td>
<td>Cefazidime 100 mg/kg/day and TMP/SMX 8/40 mg/kg/day</td>
<td>NA</td>
<td>50</td>
<td>14%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cefoperazone/sulbactam 25/25 mg/kg/day and TMP/SMX 8/40 mg/kg/day</td>
<td>NA</td>
<td>50</td>
<td>18%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ubon Ratchathani and Khon Kaen, Thailand, 1999–Oct 2003</td>
<td>Both arms given iv every 8 hours until clinically improved (usually minimum of 10 days)</td>
<td>449</td>
<td>241</td>
<td>25.8% (ITT 25.1% vs 26.6%)</td>
<td>No difference in mortality (stratified ( P = 0.59 ), Wilcoxon test)</td>
<td>[28]</td>
</tr>
<tr>
<td></td>
<td>Cefazidime 120 mg/kg/day (+ placebo in Ubon Ratchathani)</td>
<td>223</td>
<td>118</td>
<td>22%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cefazidime 120 mg/kg/day and TMP/SMX 10/50 mg/kg/day</td>
<td>226</td>
<td>123</td>
<td>20%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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a Abbreviations: iv, intravenously; ITT, intention-to-treat; NA, not available; NP, analysis not presented.
b Number of patients excluded is listed in parentheses.
c 8 patients enrolled twice due to relapse.
difference in survival overall, or after 48 hours. There was a higher but non-significant difference in treatment failure which required switching therapy after 48 hours in the ceftazidime group. This trial was stopped prematurely due to interruption of imipenem supply, and as a result was underpowered. A retrospective review of the use of meropenem concluded that it may be associated with improved outcomes in patients with severe sepsis associated with melioidosis, although this study was confounded by several concomitant factors relating to changes in intensive care management. A prospective multicentre evaluation of meropenem versus ceftazidime is underway in northeast Thailand, and is expected to conclude in 2014.

Parenteral AMC is available in Thailand (although not in Australia), and represents a possible empirical therapy for suspected bacterial sepsis when the possible cause could include *B. pseudomallei* but when other pathogens including Gram-positive pathogens and anaerobes have not been ruled out. AMC has been evaluated in an open, randomised, controlled study comparing parenteral ceftazidime (using standard dosing every eight hours) and AMC (amoxicillin 120 mg/kg/day and clavulanate 40 mg/kg/day, divided into a four-hourly dosing schedule) [27]. The mortality rate was comparable between the two groups, but AMC was associated with a higher rate of treatment failure requiring a switch to an alternative antimicrobial drug, and an increase in morbidity and length of hospital stay.

A clinical trial comparing cefoperazone/sulbactam plus TMP-SMX versus ceftazidime plus TMP-SMX demonstrated comparable mortality rates, bacterial clearance rates, mean duration of defervescence and median duration of parenteral treatment [2,31]. Cefoperazone/sulbactam has the potential advantage that it can be given every eight hours for the treatment of melioidosis (compared with four-hourly for AMC), and has a broad-spectrum of activity including Gram-positive bacteria such as *Staphylococcus aureus*, which is a common cause of community-acquired septicaemia in northeast Thailand. However, cefoperazone/sulbactam has only undergone clinical evaluation in combination with TMP-SMX, and the use of cefoperazone/sulbactam alone is not supported by clinical evidence.

In all clinical trials of acute melioidosis, the mortality rate during the first 48 hours of treatment has not differed between different treatment schedules. This suggests that reduction in the mortality rate requires consideration of other issues, such as the time taken to commence effective antimicrobial therapy, and the availability and use of intensive care facilities.

5. Management guidelines for the initial parenteral therapy of melioidosis

In areas where melioidosis is known or suspected to occur, empirical treatment for suspected Gram-negative sepsis should include an antimicrobial drug that is effective against *B. pseudomallei*. This is particularly important in high risk groups, such as agricultural workers and individuals with predisposing conditions such as diabetes mellitus and chronic kidney disease. A summary of antimicrobial treatment recommendations for patients with culture proven melioidosis is provided in Table VI.3. Recommended regimens differ between centres in Thailand and Australia. In Thailand,
<table>
<thead>
<tr>
<th>Drugs</th>
<th>Dose</th>
<th>Mode of administration</th>
<th>Interval</th>
<th>Usual adult dose</th>
<th>Adjusted dose</th>
<th>Note</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceftazidime</td>
<td>120 mg/kg/day</td>
<td>iv bolus</td>
<td>8 hourly</td>
<td>2 g every 8 hours</td>
<td>Renal impairment</td>
<td>1st line regimen in Thailand</td>
</tr>
<tr>
<td></td>
<td>4 mg/kg/h (12 mg/kg loading)</td>
<td>continuous infusion</td>
<td></td>
<td>5.4 g on the 1st day then 4.8 g/day</td>
<td>Renal impairment</td>
<td></td>
</tr>
<tr>
<td>Amoxicillin/clavulanate</td>
<td>120/40 mg/kg/day</td>
<td>iv bolus</td>
<td>4 hourly</td>
<td>2.4 g loading followed by 1.2 g every 4 hours</td>
<td></td>
<td>Alternative empirical treatment in suspected cases</td>
</tr>
<tr>
<td>Cefoperazone/sulbactam plus TMP/SMX</td>
<td>25/25 mg/kg/day 8/40 mg/kg/day</td>
<td>iv bolus</td>
<td>8 hourly</td>
<td></td>
<td>Renal impairment</td>
<td>Alternative 1st line regimen in Khon Kaen, Thailand</td>
</tr>
<tr>
<td>Imipenem/cilastatin</td>
<td>60 mg/kg/day</td>
<td>iv infusion</td>
<td>8 hourly</td>
<td>1 g every 8 hours</td>
<td>Renal impairment</td>
<td>2nd line regimen for treatment failure in Thailand</td>
</tr>
<tr>
<td>Meropenem</td>
<td>75 mg/kg/day</td>
<td>iv infusion</td>
<td>8 hourly</td>
<td>1 g every 8 hours</td>
<td>Renal impairment</td>
<td>1st line regimen in Royal Darwin Hospital, Australia</td>
</tr>
</tbody>
</table>
first-line therapy is ceftazidime at a dose of 120 mg/kg/day, which in an adult equates to 2 g ceftazidime every eight hours. Continuous infusion is an alternative. A carbapenem drug can be used in patients with a history of hypersensitivity to penicillin or a cephalosporin. The usual dose for adults is 1 g every eight hours. This regimen is also an appropriate choice for patients who fail to respond to ceftazidime treatment. In Australia, patients may be treated with ceftazidime, but patients with severe sepsis are given meropenem (25 mg/kg/day, or 500 mg infused every eight hours for adults) in combination with 300 µg of granulocyte colony-stimulating factor (G-CSF) IV for 10 days. An alternative choice for empirical treatment when Gram-positive bacterial infection cannot be ruled out is high dose AMC. Cefoperazone/sulbactam plus TMP-SMX could also be used. In the event that melioidosis is confirmed, patients on AMC should be switched to ceftazidime or a carbapenem drug.

Intravenous antimicrobial therapy is given for at least 10 days before switching to oral antimicrobial drugs. There are no hard and fast rules about when to switch to oral agents, although the patient must have made a clear clinical improvement, with no evidence for persistent sepsis, and absence of fever for 48 hours. Prolonged intravenous treatment is frequently required for patients with disseminated infection, involvement of the central nervous system (CNS) or bone and joint, and for patients with deep-seated abscesses that cannot be drained. Cefoperazone/sulbactam should not be used in patients with CNS infection due to the relative poor penetration across blood-brain barrier [32]. The median fever clearance-time in survivors ranges from 7 to 12 days, and a persistently high fever in a patient who is otherwise improving does not merit a switch in parenteral antimicrobial therapy. A patient who has clinical deterioration or persistently positive blood cultures should be viewed as failing treatment, at which stage further imaging should be undertaken to investigate the possibility of the presence of abscesses and a change in antimicrobial therapy made. The development of antimicrobial resistance during treatment with ceftazidime or AMC is uncommon. Over a three-year period, acquired resistance to ceftazidime or AMC was identified in 1.3% of cases for each drug [33].

Adverse side effects of ceftazidime and cefoperazone are similar to other β-lactam antibiotics and these drugs are generally well tolerated, although the development of a coagulopathy, including hypoprothrombinaemia and bleeding, has been reported in up to 20% of the cases. Ceftazidime is excreted via the kidney, and the dose should be adjusted according to creatinine clearance for patients with renal insufficiency [34]. Imipenem is generally well tolerated. Nausea and vomiting can occur and may be more common when used at high dose. Seizures represent a more serious, dose-related side effect of imipenem, a problem reported less frequently with the use of meropenem [26]. However, seizures have never been reported as an adverse event in patients treated with imipenem for melioidosis.

6. Conclusions

Melioidosis is difficult to treat and is associated with a high mortality rate. The choice of effective antimicrobial drugs is limited and consists of ceftazidime or a carbapenem drug. Alternatives are AMC or a combination of cefoperazone/sulbactam plus TMP-SMX,
although AMC is associated with a higher failure rate. The results of a clinical trial comparing ceftazidime and meropenem will resolve an ongoing question about whether or not these drugs show clinical equivalence. Neglected but important areas of study should include determination of the reasons for the higher death rate observed in Asia compared with Australia and the evaluation of affordable strategies for rapid commencement of antimicrobial therapy.

References


1. Introduction

Recurrent infection is the most serious complication of melioidosis after fatality. Recurrence is defined as “new presentation of acute melioidosis after resolution of symptoms and completion of intensive intravenous antimicrobial therapy for melioidosis”. Recurrence occurs in 5% to 25% of patients with melioidosis who survive the acute phase [1,2]. Ineffective drug regimens, poor drug adherence and short duration of treatment are important risk factors for recurrent infection [1,2].
Various drug combinations have been used for oral eradicative treatment with the aim of reducing the rate of recurrence. In 1981, Rode and Webling suggested a four-drug combination of tetracycline or doxycycline, chloramphenicol and trimethoprim–sulfamethoxazole (TMP-SMX) [3], and Puthecheary et al. recommended a combination of tetracycline and chloramphenicol for at least six months to prevent recurrent infection [4]. The four-drug regimen was adopted widely and became the standard against which later regimens have been compared. Chloramphenicol has been found to be unnecessary [5] and is not used. Today, TMP-SMX-based regimens of 12 to 20 weeks’ duration are considered first-line, with amoxicillin–clavulanate (AMC) held in reserve for those in whom TMP-SMX is contraindicated or not well tolerated.

2. Pharmacodynamics and pharmacokinetics of antimicrobials for oral treatment

The therapeutic options for eradication therapy in melioidosis are limited by the innate resistance of *Burkholderia pseudomallei* to many oral antimicrobials. In addition, *B. pseudomallei* clinically may fail to respond to those drugs to which it is sensitive in vitro [5,6]. The agents currently used for oral eradicative treatments are TMP-SMX, doxycycline and AMC. The minimum inhibitory concentrations (MICs) of clinical isolates and serum drug concentrations according to the dosages of these four drugs are summarised in Table VI.4.

Trimethoprim–sulfamethoxazole is currently the first-line choice for eradicative treatment. TMP and SMX work synergistically to inhibit thymidine synthesis and therefore DNA replication. TMP-SMX could enter bronchial secretions, the prostate, has good penetration into walled abscesses [15] and will also pass the blood brain barrier, all features which make TMP-SMX an attractive drug for the treatment of melioidosis. Although TMP-SMX may interfere with folic acid metabolism and is theoretically harmful to the foetus, a retrospective study of 186 pregnancies found no excess congenital abnormalities [16]. Given the proven efficacy of TMP-SMX-based regimens and the high mortality associated with recurrent melioidosis, it may be argued that the current practice of avoiding TMP-SMX in pregnancy is over-cautious. TMP-SMX is primarily excreted by the kidneys and is contraindicated in patients with renal failure. High doses of TMP-SMX are needed to maintain serum levels above the MIC for *B. pseudomallei* (Table VI.4). Pharmacodynamic simulations have also highlighted the possibility that the usual dosage of TMP-SMX may not be adequate for isolates with intermediate resistance to TMP-SMX (MIC 2/38 mg/L) [11].

Doxycycline is a semi-synthetic tetracycline with a long half-life (18 to 22 hours). In common with other tetracyclines, it inhibits protein synthesis by binding to the 30S subunit of the bacterial ribosome. The tetracyclines are contraindicated in pregnancy and childhood (due to staining of the teeth and bones of the unborn foetus or child) and are absolutely contraindicated in renal failure. Although doxycycline has long been used with TMP-SMX in oral eradicative treatment regimens, in vitro studies show that TMP-SMX and doxycycline are antagonistic [17,18]. The possibility of omitting doxycycline from the treatment regimen is currently being evaluated in a randomised controlled clinical trial (ISRCTN86140460; http://www.controlled-trials.com/isrctn/pf/86140460).
### Table VI.4
Population MIC of clinical isolates, dosage and serum drug concentrations of four currently used active antimicrobial agents

<table>
<thead>
<tr>
<th>Antimicrobial</th>
<th>Population MIC [7–10]</th>
<th>Dosage and serum drug concentration</th>
</tr>
</thead>
</table>
| Trimethoprim–sulfamethoxazole  
(susceptible $\leq 1/19$ mg/L;  
intermediate = $2/38$ mg/L;  
resistant $\geq 4/76$ mg/L) | **Resistant** (MIC $\geq 4/76$ mg/L)  
13% (258/1976) to 14% (23/144) of Thai isolates  
6% (3/50) of Singapore isolates  
0% (0/170) to 2.5% (2/80) of Australian isolates  
**Intermediate** (MIC = $2/38$ mg/L)  
10% (202/1976) of Thai isolates  
16% (8/50) of Singapore isolates  
3% (4/170) of Australian isolates | Thai weight-based regimen (<40 kg [body weight], 160/800 mg every 12h; 40–60 kg, 240/1200 mg every 12h; >60 kg, 320/1600 mg every 12h) and Australian regimen (320/1600 mg every 12h) achieve adequate drug concentrations for strains with an MIC $\leq 1/19$ mg/L, but may not achieve adequate concentrations for strains with an MIC of $2/38$ mg/L [11] |
| Doxycycline  
(susceptible $\leq 4$ mg/L;  
intermediate = $8$ mg/L;  
resistant $\geq 16$ mg/L) | **Resistant** (MIC $\geq 16$ mg/L)  
3% (6/211) of Thai isolates  
0% (0/50) of Singapore isolates  
1% (2/170) of Australian isolates | 100 mg every 12 hours, achieves a maximum serum concentration of doxycycline ($c_{\text{max}}$) of 2.6 mg/L (n = 20) [12] |
| Amoxicillin–clavulanate  
(susceptible $\leq 8/4$ mg/L;  
intermediate = $16/8$ mg/L;  
resistant $\geq 32/16$ mg/L) | **Resistant** (MIC $\geq 32/16$ mg/L)  
0% (0/211) of Thai isolates  
0% (0/50) of Singapore isolates  
0.5% (1/170) of Australian isolates | The usual dose of 1000/250 mg every 8 hours is suboptimal. By pharmacodynamic modelling, only 42% of patients will achieve 90% T $>$ MIC, and 12% of patients will have 50% T $>$ MIC or worse [13] |

**MIC** = minimum inhibitory concentration (lowest drug concentration needed to inhibit bacterial growth of a specific isolate).  
% T $>$ MIC = proportion of the dose interval above MIC.  
Breakpoints for resistance are those recommended by CLSI [14].
Amoxicillin–clavulanate is used as second-line therapy to eradicate *B. pseudomallei*. Amoxicillin lyses bacteria by inhibiting cell-wall synthesis, but it is normally inactivated by β-lactamases produced by *B. pseudomallei*. Clavulanate inhibits class A β-lactamases, and thus enables amoxicillin to act. *B. pseudomallei* possesses class C and D β-lactamases which are not inhibited by clavulanate, although their clinical relevance remains unclear. Unless high doses of AMC are given, the serum drug concentration is inadequate [13]. The effectiveness of AMC is lower compared to that of a four-drug regimen (TMP-SMX, doxycycline and chloramphenicol), possibly because of poor intracellular penetration, particularly into alveolar macrophages. These macrophages are considered to be an important nidus for persistent infection. In addition, although the dose of AMC is very high for the treatment of melioidosis, it may only reach the required serum concentration in less than half of patients (Table VI.4) [11,13]. Another important limitation is that AMC does not cross the blood brain barrier in clinically useful concentrations, preventing its use in neurological infection.

Of these commonly used drugs, only AMC is bacteriocidal, while TMP-SMX and doxycycline are bacteriostatic. Although many *B. pseudomallei* isolates may be susceptible to ciprofloxacin, ofloxacin and azithromycin *in vitro* [7,8,19], clinical trials have found these drugs to be ineffective *in vivo* [6]. There is also some *in vitro* evidence to justify using other tetracyclines (for example, minocycline) and some new cephalosporins (for example, cefixime and cefetamet) [7,19]. These drugs however, have not yet been evaluated in clinical trials.

### 3. Clinical trial evidence for eradication phase antimicrobial therapy

From the mid-1980s, a four-drug combination of chloramphenicol, doxycycline and TMP-SMX was the recommended oral treatment for eradication of *B. pseudomallei* infection in Thailand. The recommended duration of treatment ranged from three to six months [20]. Chloramphenicol was normally prescribed only for the first month; however, the rate of adverse drug reactions from this combination was high. The pill burden associated with this regimen was large, which may have affected compliance. A number of alternatives have been evaluated in clinical trials and are summarised in Table VI.5.

A pilot study of AMC monotherapy was conducted from 1986 to 1989 at Sappasithiprasong Hospital, northeast Thailand [21]. The intended duration of therapy was a minimum of eight weeks, but AMC was given to 46 patients for a median duration of only 7.5 weeks. Culture proven recurrence occurred in 13 of 46 (28%) cases after a median follow-up of six months. The drug was well tolerated despite the high doses used and was thereafter considered an alternative for children, pregnant women, patients who were allergic to any drug within the four-drug regimen and for TMP-SMX resistant *B. pseudomallei*. An open label randomised controlled trial comparing AMC with the four-drug regimen was conducted from 1989 to 1992 [22], and a total of 101 patients were enrolled. Because of the high recurrence rates seen in the pilot study [21], the treatment duration was extended to 20 weeks. Recurrent melioidosis occurred in 2 of 52 (4%) patients in the oral four-drug regimen group and 8 of 49 (16%) patients in the
Table VI.5
Clinical trials of eradication phase oral antibiotics for the treatment of melioidosis

<table>
<thead>
<tr>
<th>Year of study</th>
<th>Study type</th>
<th>Regimen (dose)</th>
<th>Minimum duration of treatment (weeks)</th>
<th>Number of patients</th>
<th>Proportion of recurrent melioidosis (%)</th>
<th>Estimated recurrent rate (per 100 person-year)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1986–1989</td>
<td>Pilot</td>
<td>AMC (27/13.5 mg/kg/day)</td>
<td>8</td>
<td>46</td>
<td>28%</td>
<td>72</td>
<td>[21]</td>
</tr>
<tr>
<td>1989–1992</td>
<td>RCT</td>
<td>AMC (60/15 mg/kg/day) versus Chloramphenicol (500 mg every 6 h for the first 8 wks), doxycycline (100 mg every 12 h) and TMP-SMX (160/800 mg every 12 h)</td>
<td>20</td>
<td>49</td>
<td>16%</td>
<td>13</td>
<td>[22]</td>
</tr>
<tr>
<td>1991–1995</td>
<td>Pilot</td>
<td>Ciprofloxacin (500 mg every 12 h) or ofloxacin (300 mg every 12 h)</td>
<td>12</td>
<td>57</td>
<td>14%</td>
<td>49</td>
<td>[3]</td>
</tr>
<tr>
<td>1994–1997</td>
<td>RCT</td>
<td>Doxycycline (100 mg every 12 h) versus Chloramphenicol (500 mg every 6 h for the first 4 weeks), doxycycline (100 mg every 12 h) and TMP-SMX (160/800 mg every 12 h)</td>
<td>20</td>
<td>43</td>
<td>26%</td>
<td>20</td>
<td>[23]</td>
</tr>
<tr>
<td>1997–1998</td>
<td>RCT</td>
<td>Ciprofloxacin (500 mg every 12 h) and azithromycin (500 mg once daily) versus Doxycycline (100 mg every 12 h) and TMP-SMX (160/800 mg every 12 h)</td>
<td>12</td>
<td>32</td>
<td>22%</td>
<td>36</td>
<td>[6]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>20</td>
<td>3%</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Year of study</td>
<td>Study type</td>
<td>Regimen (dose)</td>
<td>Minimum duration of treatment (weeks)</td>
<td>Number of patients</td>
<td>Proportion of recurrent melioidosis (%)</td>
<td>Estimated recurrent rate (per 100 person-year)</td>
<td>Reference</td>
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<td>--------------</td>
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<td>---------------------------------------</td>
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<td>-----------</td>
</tr>
<tr>
<td>1998–2002</td>
<td>RCT</td>
<td>Doxycycline (100 mg every 12 h) and TMP-SMX (160/800 mg every 12 h)</td>
<td>12</td>
<td>89</td>
<td>8%</td>
<td>7</td>
<td>[24]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chloramphenicol (500 mg every 6 h for the first 4 wks), doxycycline (100 mg every 12 h) and TMP-SMX (160/800 mg every 12 h)</td>
<td>12</td>
<td>91</td>
<td>10%</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>2005–present</td>
<td>RCT</td>
<td>TMP-SMX (10/50 mg/kg/day: for patients of &lt;40 kg [body weight], 160/800 mg every 12 h; 40–60 kg, 240/1200 mg every 12 h; and &gt;60 kg, 320/1,600 mg every 12 h) and doxycycline (100 mg every 12 h)</td>
<td>20</td>
<td>~312</td>
<td>NA</td>
<td>NA</td>
<td>ISRCTN</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TMP-SMX (similar dose)</td>
<td>20</td>
<td>~312</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

AMC = amoxicillin–clavulanate. NA = not available yet. RCT = randomised controlled trial. TMP = trimethoprim. SMX = sulphamethoxazole.

<sup>a</sup> Only culture-confirmed recurrent melioidosis was considered.

<sup>b</sup> Estimated from mean, median or total duration of follow-up reported in the publications.

<sup>c</sup> ISRCTN86140460; http://www.controlled-trials.com/isrctn/pf/86140460.
oral AMC group (RR 0.4, 95% confidence interval 0.2–1.2) after a median follow-up of 18 months. The investigators undertaking the study concluded that AMC was safer and better tolerated, but was less effective than the four-drug regimen. The minimum duration of oral treatment was thereafter recommended for 12 to 20 weeks.

Oral ciprofloxacin and ofloxacin monotherapy were evaluated in a study conducted from 1991 to 1995 at Sappasithiprasong Hospital [5]. A total of 57 patients were enrolled into the study and 13 treatment failures (29%) were observed. Of these, five were treatment failures that occurred during the primary episode, eight had recurrence after an initial response, and, in one patient, fluoroquinolone resistance developed within a week of starting treatment. The combination of ciprofloxacin and azithromycin was compared to TMP-SMX plus doxycycline in an open-label randomised controlled trial (RCT) conducted from 1997 to 1998 [6]. Of 65 patients enrolled, 7 of 32 (22%) patients in the ciprofloxacin group and in 1 of 33 (3%) patients in the three-drug group recurrence of the infection was observed. Taken together, these two studies provide strong evidence that fluoroquinolones are ineffective in the oral eradication treatment of melioidosis.

Doxycycline monotherapy was used successfully in Australia during an outbreak of melioidosis in 1990 to 1991 [25]. An RCT was conducted from 1994 to 1997 to compare doxycycline alone against the four-drug regimen (TMP-SMX, doxycycline and chloramphenicol) [23]. Of the 87 patients enrolled, 1 of 44 (2%) patients randomised to the four-drug regimen and 11 of 43 (26%) patients randomised to doxycycline monotherapy had recurrent infection (HR 12.0, 95% confidence interval 1.4–100.8). Doxycycline resistance developed in one patient who was treated with doxycycline monotherapy. Based on the evidence from this study, doxycycline monotherapy cannot be recommended.

Chloramphenicol is the most toxic of the oral agents used in melioidosis, and this was the primary motivation behind an RCT comparing the four-drug regimen against three-drugs (minus chloramphenicol) that was conducted from 1998 to 2002 [24]. There was no difference in recurrence, but the three-drug regimen was associated with much lower rates of side effects. The duration of oral therapy was significantly associated with recurrent infection. Patients receiving treatment for less than 12 weeks had a 5.7-fold increase in recurrence or death. The three-drug regimen is now the standard treatment for oral eradication treatment for *B. pseudomallei* infection in Thailand.

More recent evidence suggests that recurrent melioidosis may not be the best primary outcome in clinical trials that compare oral treatment regimens because recurrent infection has two distinct causes: (i) relapse due to failure to eradicate the original infecting organism, or (ii) re-infection due to new acquisition of *B. pseudomallei* from the environment. The primary goal of oral eradication therapy is to prevent relapse and not re-infection. It is very likely that re-infection could be an important cause of recurrent infection, particularly after the oral therapy is completed. Reanalysis of data from previous clinical trials conducted in Thailand, which looked at relapse and re-infection as distinct phenomena, also found that treatment durations under 12 weeks were associated with relapse, that AMC was associated with an increased risk of relapse, and that fluoroquinolone-based regimens and doxycycline monotherapy were associated with a
higher risk of relapse [2]. Specific risk factors for re-infection were not found, and re-infection also occurs during oral eradicative treatment [2].

In Australia, concerns about side effects and potential antagonism between antimicrobial agents motivated clinicians there to give doxycycline monotherapy or TMP-SMX monotherapy [8]. After doxycycline monotherapy was found ineffective [1,23], TMP-SMX has been used as the standard treatment in Australia despite the lack of randomised-control trial evidence [1]. A double-blind randomised controlled trial comparing the three-drug regimen with TMP-SMX alone is currently underway in Thailand (ISRCTN86140460; http://www.controlled-trials.com/isrctn/pf/86140460). Based on the findings of previous trials [2,11], the intended duration of therapy was increased to 20 weeks and a weight-based regimen for TMP-SMX was instituted (Table VI.5). The study aimed to enroll 635 patients who will complete a minimum of one year follow-up.

4. Management guidelines for the eradication phase

The recommended regimen for the eradication phase of melioidosis therapy is TMP-SMX, with or without doxycycline. Parenteral treatment should only be stopped after a minimum of 10 to 14 days and when there is clear evidence of clinical improvement (some patients may even require more than 90 days of continuous parenteral treatment). Current treatment recommendations differ between Australia and Thailand; however, in both countries, the TMP-SMX component is expected to achieve adequate serum concentrations for isolates sensitive to TMP-SMX (MIC $\leq 1/19$ mg/L) [11]. The recommended regimen in Australia is a TMP-SMX dose of 320/1600mg every 12 hours, while in Thailand, a weight-based regimen of TMP-SMX plus doxycycline is recommended (Table VI.6).

Amoxicillin–clavulanate is used as a second-line agent, particularly for pregnant women (where TMP-SMX is relatively contraindicated), for those with hypersensitivity reactions to TMP-SMX or doxycycline, and for those who are intolerant of TMP-SMX (particularly with neutropenia or gastrointestinal intolerance). In Thailand, AMC is also used as first-line for children under eight, because doxycycline is relatively contraindicated. Based on pharmacodynamic and pharmacokinetic modeling, the recommended AMC dose should be 20/5mg/kg every eight hours (Table VI.6) [13,26]. Twice daily doses or formulations containing amoxicillin/clavulanate ratios greater than 4 to 1 (such as “Duo Forte” or “XR” formulations) are not recommended [13,26]. AMC does not pass the blood brain barrier and should therefore not be used in patients with neurological melioidosis [27]. Treatment options are limited for neurological melioidosis patients with TMP-SMX-intolerance or who are infected with a TMP-SMX resistant strain. Prolonged parenteral therapy (perhaps via a peripherally inserted central catheter) should be the first choice, but when this is impossible, the next best option is probably combination therapy with chloramphenicol and doxycycline.

The recommended duration of oral eradicative treatment (for both TMP-SMX based regimens and for AMC) is for at least three to six months in Australia and 20 weeks in Thailand [28]. It remains unclear whether a shorter duration of therapy may be adequate
Table VI.6
Recommended antimicrobial therapy in Thailand for eradication phase

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First-line treatment</strong></td>
<td></td>
</tr>
<tr>
<td>Weight 40–60 kg and creatinine clearance &gt;30 ml/min</td>
<td>TMP-SMX (80/400 mg) 3 tabs every 12 h plus doxycycline (100 mg) 1 tab every 12 h</td>
</tr>
<tr>
<td>Weight &lt;40 kg or creatinine clearance 15–30 ml/min</td>
<td>TMP-SMX (80/400 mg) 3 tabs every 12 h plus doxycycline (100 mg) 1 tab every 12 h</td>
</tr>
<tr>
<td>Weight &gt;60 kg and creatinine clearance &gt;30 ml/min</td>
<td>TMP-SMX (80/400 mg) 4 tabs every 12 h plus doxycycline (100 mg) 1 tab every 12 h</td>
</tr>
<tr>
<td>If creatinine clearance &lt;15 ml/min or</td>
<td>Use AMC</td>
</tr>
<tr>
<td>AST/ALT &gt;5 times upper limit of normal</td>
<td></td>
</tr>
<tr>
<td><strong>Second-line treatment</strong></td>
<td></td>
</tr>
<tr>
<td>Weight &lt;60 kg and creatinine clearance &gt;30 ml/min</td>
<td>Amoxicillin–clavulanate (1000/250 mg) every 8 h</td>
</tr>
<tr>
<td>Weight &gt;60 kg and creatinine clearance &gt;30 ml/min</td>
<td>Amoxicillin–clavulanate (1500/375 mg) every 8 h</td>
</tr>
<tr>
<td>Creatinine clearance 10–30 ml/min</td>
<td>Amoxicillin–clavulanate (1000/250 mg) every 12 h</td>
</tr>
<tr>
<td>Creatinine clearance &lt;10 ml/min</td>
<td>Amoxicillin–clavulanic acid (500/125 mg) every 12 h</td>
</tr>
</tbody>
</table>

for patients with mild, localised disease. Close follow-up and monitoring of adherence to drug therapy are crucial, as these appear to be the most important factors in determining recurrence [1,2]. Patient education about the need for compliance is critical. For patients with hepatosplenic abscesses, abdominal imaging (Section V.4) should be repeated at 12 and at 20 weeks after the start of oral therapy. Therapy should be extended indefinitely until the lesions have resolved. In some instances treatment duration could exceed for over one year. Surgical drainage or excision should be considered for lesions that are slow to resolve. Patients with melioidosis should be educated about the risk of relapse after apparent cure, the risk of re-infection, and measures to prevent these complications.

5. Problems arising during oral eradicative treatment

During the first two weeks of oral eradicative treatment, improvement may fluctuate and therefore regular evaluation is required. Enlargement of an abscess or the appearance of new collections (especially in skeletal muscle or joints) is possible and does not necessarily represent treatment failure or recurrence. Parenteral treatment need not be re-instituted, particularly if the general condition of the patient is improving. Sputum, throat swabs or collections may remain culture-positive for many months after clinical response to treatment and are less useful for guiding management. However, positive repeat blood culture should be interpreted as failure of oral eradicative treatment, and parenteral treatment should be re-started promptly.

Because of the high dose and long duration of the TMP-SMX based regimen, patients often experience adverse effects. Trimethoprim is generally well tolerated, but
sulphonamide rashes are common and, at the extreme end of the spectrum, may cause toxic epidermal necrolysis (TEN) or Stevens-Johnson syndrome (SJS). All oral drugs should be stopped upon the first appearance of skin rash or hypersensitivity, and the patient’s condition should be reevaluated. Rashes frequently reappear when patients are rechallenged with TMP-SMX and it is usually best to move directly to second-line therapy. Gastrointestinal irritation is commonly observed, and a variety of strategies may allow TMP-SMX to be reintroduced. Antiemetic drugs could be used to control the vomiting, the doses can be split (for example, from four tablets every 12 hours to two tablets every 6 hours), or by moving TMP-SMX dosing from pre- to post-prandial. TMP-SMX may also cause hyperkalaemia, hyponatraemia, hepatitis and elevations of serum urea or creatinine. Therefore, regular investigations to monitor for these complications are essential. Fatigue is also a common side effect. Bone marrow suppression is another common side effect, as are other blood dyscrasias, which respond rapidly to withdrawing TMP-SMX. Folate supplement is always prescribed during TMP-SMX treatment in Australia to reduce the risk of bone marrow suppression and to treat folate deficiency which is often found in the Indigenous population.

The most common adverse effect of doxycycline is gastrointestinal irritation, which may manifest as diarrhoea or oesophageal ulceration. Both doxycycline and TMP-SMX may cause photosensitivity and make patients more prone to sunburn. Recommendations to avoid exposure to sunlight and use of sunscreen should be provided to every patient who is prescribed TMP-SMX with or without doxycycline. Hypersensitivity reactions may necessitate the withdrawal of doxycycline, whereupon the clinician has the option of continuing with TMP-SMX monotherapy or switching to AMC. Doxycycline may induce severe onycholysis, but resolves after stopping doxycycline.

Amoxicillin–clavulanate is generally well tolerated. The most common side effect is diarrhoea. However, in some cases nausea, skin rashes and urticaria have also been observed. Hypersensitivity reactions to AMC may present with skin rash, urticaria, exfoliative dermatitis and, in rare cases, SJS. Whenever such reactions occur, the drug should be stopped and patients should be reevaluated. Mild reactions may be controlled with antihistamines.

Any severe febrile illness in a patient who has previously had melioidosis or with recurrence of symptoms similar to those of the primary episode should be treated for recurrence until proven otherwise. Patients with recurrent infection require a detailed review of history, including duration of each drug used, clinical progression and results of any bacterial cultures, and compliance and any lifestyle modification made by the patient to reduce exposure to environmental *B. pseudomallei*. A comprehensive panel of specimens should be collected for *B. pseudomallei* isolation, including blood, urine, throat swab, respiratory secretions, pus and wound or surface swabs, and an abdominal ultrasound should be performed (Section IV). If no definite alternative diagnosis can be made, then a full course of empirical therapy for *B. pseudomallei* (parenteral treatment and 20 weeks of oral therapy) should be provided.

Development of resistance to *B. pseudomallei* has been observed in vivo. Although infrequent, relapse may be associated with new resistance to TMP-SMX, doxycycline or AMC that develops during the course of treatment [1,29]. Secondary drug resistance
has been observed more frequently in patients treated with doxycycline monotherapy and fluoroquinolones [1,29].

6. Eradicative therapy in recurrent melioidosis

In recurrent melioidosis, the standard first-line treatment (TMP-SMX-based regimens) should still be used if the organism isolated during the recurrence is susceptible, but differentiation between recurrence and re-infection may allow that patient’s management to be individualised [29] (Section IV.2). For patients who have relapsed, efforts should be focussed on drug adherence and completion of a course of therapy of adequate duration. The second-line therapy, AMC, is less effective and should therefore be used only where TMP-SMX treatment failure is believed to have occurred. For patients believed to have re-infection as the cause of their recurrence, first-line eradicative treatment should be used and efforts should be focussed on prevention of further re-infection.

7. Conclusion

A prolonged period of oral therapy (minimum 12 to 20 weeks) is required after completion of parenteral treatment to prevent relapse. TMP-SMX-based regimens are the treatment of choice. TMP-SMX monotherapy is used in Australia, while TMP-SMX plus doxycycline is used in Thailand. Chloramphenicol is no longer routinely used. AMC is an alternative for pregnant women and for those with hypersensitivity reactions to TMP-SMX or doxycycline. In children, TMP-SMX is used as first-line in Australia, but in Thailand, AMC is used. AMC is associated with a higher risk of recurrence compared to TMP-SMX based regimens. The high doses used and the long duration of treatment worsen the side effects and reduce tolerability, and the management and prevention of adverse events are important in improving patient compliance. Recurrence remains a risk despite optimal therapy, and patients should be educated about the importance of drug adherence.

References


Section VI.3
Management of patients with severe melioidosis in intensive care

T. Eoin West\textsuperscript{a}, Allen C. Cheng\textsuperscript{b,c,d}

\textsuperscript{a} Division of Pulmonary and Critical Care Medicine, Department of Medicine, Harborview Medical Center, University of Washington, Seattle, WA, USA
\textsuperscript{b} Menzies School of Health Research, Darwin, Australia
\textsuperscript{c} Department of Epidemiology and Preventive Medicine, Monash University, Melbourne, Australia
\textsuperscript{d} Infectious Diseases Unit, Alfred Hospital, Melbourne, Australia

Abstract

Melioidosis frequently presents as severe sepsis or septic shock. Appropriate management therefore dictates familiarity with standard sepsis therapies and approaches to intensive care. These treatments include early and aggressive haemodynamic management using intravenous fluids, vasopressors, and inotropes in a goal-directed fashion to maximise oxygen delivery to peripheral tissues. In the critically ill patient, oxygen consumption can be reduced with antipyretics, sedation, and mechanical ventilation. The role of glycaemic control and adjunctive treatments such as corticosteroids, activated protein C and granulocyte colony stimulating factor (G-CSF) are controversial. G-CSF has been formally studied in melioidosis patients, but the level of evidence for adjunctive treatments is insufficient. Mechanical ventilation for respiratory failure or acute lung injury may be required, necessitating an understanding of basic ventilator therapies. Treatment of acute renal injury and use of appropriate prophylactic therapies is also essential. Most, if not all, may be key factors in the reduction of the high mortality rate from melioidosis in developing countries.

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1. Introduction

Melioidosis may present with a wide spectrum of severity, but patients with severe sepsis have a high mortality. The term “sepsis” implies a systemic inflammatory response to presumed infection, and severity is determined by the presence of acute organ dysfunction. The highest mortality is found in patients with shock. Early management of severe sepsis is focussed on optimising oxygen delivery to ischaemic tissues, while definitive treatment can be delivered in the form of eradication of the infectious source.

The recently updated “Surviving Sepsis Campaign” guidelines are one attempt at setting a standard of care for sepsis management [1]. Although there has been some
controversy regarding the recommendations, the development of the guidelines and the level of evidence supporting some recommendations, most interventions targeting early management of septic patients are less contentious. However, the majority of studies on which these recommendations are based were undertaken in high resource countries and may not be entirely applicable to health care in resource-limited settings. Nonetheless, the general principles apply to management of the severely ill patients with melioidosis, regardless of setting.

2. Acute resuscitation
The interventions delivered within the first hours of presentation are critical in determining outcome. Multiple studies have demonstrated improvements in outcome associated with the implementation of a “sepsis bundle” designed to improve timeliness and quality of care [2,3]. Initial care should expeditiously target optimisation of oxygen availability to tissues and control of the source of infection. Oxygen delivery to tissues is dependent upon cardiac output and arterial oxygen content. In turn, oxygen content is largely a function of blood haemoglobin concentration and arterial oxygen saturation. Interventions that bolster these physiologic measures include: the provision of supplemental oxygen, fluid resuscitation, vasopressors and inotropes, and transfusion of red blood cells. Interventions that reduce overall oxygen consumption include: administration of antipyretics, sedation and initiation of mechanical ventilation. Most importantly, the severely ill patient with suspected melioidosis should be viewed as an emergency, with every effort made to normalise the physiological anomalies as rapidly as possible to maximise chances for survival.

This concept of “correcting the physiology” is paramount to the successful management of severe melioidosis. Clinical measurements, such as blood pressure, oxygen saturation, and urine output, should be performed repeatedly during resuscitation to assess the adequacy of each intervention. Even when each of these measures has been normalised, tissue delivery may be inadequate, requiring further resuscitation. An additional marker of inadequate oxygen delivery is low mixed, or central, venous oxygen saturation (<65% or <70% respectively), indicating very high oxygen extraction from arterial blood. A single centre clinical trial found a large mortality benefit associated with a specific resuscitation strategy using invasive measures of central oxygen delivery to titrate therapies [4]. However, central venous oxygen saturations are measured using indwelling central venous catheters, which may not be available in many settings where melioidosis is endemic. Another important marker is serum lactate, which is elevated during tissue hypoxia and can be readily measured in peripheral blood in a clinical lab or using a point-of-care device. Recent evidence suggests that targeting a reduction in this marker by 10% may be as good an endpoint for resuscitation as central venous oxygen saturations in resuscitation from sepsis [5]. Accordingly, serial measures of serum lactate may help to guide resuscitation of severely ill patients with melioidosis.

2.1. Oxygen
Hypoxaemia is frequently a presenting sign of severe infection. In most settings, hypoxaemia is diagnosed by pulse oximetry (noninvasive measurement of the arterial
oxygen saturation). Decreased arterial oxygen saturation indicates an impaired arterial oxygen content and therefore, reduced oxygen delivery. All patients with severe melioidosis should be administered supplemental oxygen titrated to maximise the arterial oxygen saturation. Oxygen delivery by nasal prongs, simple face mask, non-rebreathing mask, or even by positive pressure ventilation may be necessary. Repeated measurement of oxygen saturation with a pulse oximeter is essential in these patients because the respiratory status and oxygen requirement can change quickly over the course of resuscitation.

2.2. Intravenous fluids

The high proportion of patients who have severe acidosis on presentation indicates that tissue hypoxia is common in severe melioidosis. This can occur in the setting of preserved arterial oxygen saturations, suggesting that other measures to improve oxygen delivery, such as the administration of intravenous fluids, are necessary. Even in the absence of hypotension, intravenous fluids will correct the decreased intravascular volume in severe sepsis, improve preload, and augment cardiac output. Thus, severely ill patients with melioidosis should all receive a volume challenge, even if their blood pressure is preserved. The rapid infusion of intravenous crystalloids (typically one to two litres in the first hour) may be sufficient to restore circulatory volume. Many patients require up to five litres in the first six hours [4]. The preferred fluid is isotonic crystalloid: Hartmann’s (or Ringer’s lactate) may be most suitable as the infusion of large volumes of normal saline may exacerbate metabolic acidosis. Colloid infusions are an acceptable alternative. Dextrose containing solutions are not indicated for resuscitation. The optimal endpoints of fluid resuscitation are debated; however, decreased urine output, flat jugular veins, cold peripheries and delayed capillary refill are clues that indicate to low intravascular volume. In higher resource settings, invasive measures of cardiac filling using direct measurement of central venous pressure (CVP) are frequently used. In this case, targeting a CVP of 8 to 12 mm Hg is reasonable. Frank hypotension (mean arterial pressure less than 60 mm Hg) mandates aggressive intravenous volume challenge and frequent reassessment as additional intravenous fluids and vasopressors may be required.

2.3. Vasopressors

Hypotension in melioidosis that persists despite intravenous fluid challenge is an indication that vasomotor tone is inadequate and defines septic shock. This is associated with extremely high mortality. To maintain tissue perfusion, additional interventions are necessary. Thus, after restoration of intravascular volume with fluids, intravenous vasopressors such as norepinephrine or dopamine should be administered to maintain a mean arterial pressure of at least 65 mm Hg, or higher in a patient with a history of hypertension. Measurement of blood pressure by an indwelling arterial catheter provides constant monitoring but is not essential as long as frequent assessment is performed to prevent catastrophic undertreated hypotension or iatrogenic hypertension. Vasoactive agents are preferably administered by a central venous catheter, although in urgent situations peripheral venous access may be used if central venous access is not possible.
Sites of peripheral catheters should be monitored carefully for any evidence of drug extravasation, which can be highly toxic to tissues.

2.4. Inotropes

Following efforts to optimise cardiac filling with volume repletion and vasopressor agents, oxygen delivery may still be inadequate. Often in severe sepsis, myocardial function is dramatically depressed, inhibiting cardiac output. Echocardiography provides direct evidence of myocardial function but persistently elevated lactate levels or low central venous oxygen saturations (indicating high oxygen extraction) after correction of the other key components of oxygen delivery suggest that augmentation of cardiac output may be desirable. These patients may benefit from inotropic agents, such as dobutamine titrated to improve cardiac output, although sometimes dobutamine causes a decrease in blood pressure that requires concurrent vasopressor infusion.

2.5. Red blood cells

Because oxygen content and oxygen delivery depend on haemoglobin, severe anaemia contributes to tissue hypoxia in sepsis. Thus, in critically septic patients with melioidosis, consideration of red blood cell transfusion is reasonable to optimise oxygen delivery, particularly if there is evidence of persistent tissue hypoxia, such as low central venous oxygen saturations or elevated lactate. Repletion of red blood cells in this setting must be balanced with data showing that aggressive transfusion strategies in the critically ill are deleterious [6]. Generally, a haemoglobin level of 100 g/L is accepted as a reasonable target in the acute management of severe sepsis.

2.6. Reduction of oxygen consumption

Efforts to reduce oxygen consumption in the setting of tissue hypoxia require targeting the hypermetabolic state associated with severe sepsis. Patients with melioidosis who are febrile should be administered antipyretics. Despite a growing literature indicating detrimental effects of excessive sedation in critically ill patients, judicious sedation to lower metabolic demands in the acute phase of severe sepsis resuscitation is appropriate when tissue oxygen delivery remains inadequate. For patients with frank or impending respiratory failure, mechanical ventilation to reduce the work of breathing may be indicated.

2.7. Early antibiotics

It is well established that prompt antibiotic administration and rapid drainage of collections of pus are required in managing the severely septic patient [7]. Mortality in septic shock increases for each hour antibiotic therapy is delayed. Outcomes are linked not only to rapid antibiotic initiation but also to suitability for the underlying infection. Where melioidosis is suspected, it is imperative to maintain a high index of suspicion and provide appropriate antibiotics to cover the full range of potential pathogens, as culture
results typically take two to three days to become available. General guidelines are to administer antimicrobials within one hour of presentation [8]. Where possible, control of the source of sepsis by identification and removal of any collections of infection should be considered. In melioidosis, this is particularly pertinent given the protean manifestations of disease and predilection for abscess formation in a variety of organs. In particular, drainage is commonly required for prostatic abscesses and empyema. For abscesses in other sites, including liver, spleen and lung, drainage may not be required depending on an assessment of response to antibiotic treatment.

3. Antibiotics and pharmacological adjuvants

3.1. Granulocyte colony-stimulating factor

Granulocyte colony-stimulating factor is a growth factor that stimulates the production of neutrophil precursors and activates mature neutrophils. Because several of the well established melioidosis risk factors, such as, diabetes, chronic alcohol use, and renal failure, may be associated with functional neutrophil defects, there is a strong rationale for the use of G-CSF [9]. While G-CSF therapy does not reduce mortality in pneumonia, multilobar pneumonia and severe sepsis of other causes [10–12], mortality from severe melioidosis was reduced compared to historical controls in an Australian study [13]. In a placebo-controlled trial conducted in patients with severe sepsis due to melioidosis in northeastern Thailand, overall mortality was high in both G-CSF-treated and placebo-treated groups (70% vs 87%, respectively) and was not significantly different [14]. However, G-CSF was associated with longer survival. Therefore, in severe melioidosis, G-CSF may “buy time,” although the high overall mortality suggests that it does not change the ultimate outcome, particularly where resource constraints limit supportive care.

3.2. Activated protein C

The role of recombinant activated protein C (APC) in the treatment of severe sepsis of any cause remains unclear. Limited data suggests that severe melioidosis is characterised by depletion of protein C [15] and recombinant APC has been used successfully in isolated patients [16]. Despite showing initial promise in reducing mortality in patients with sepsis, the role of recombinant APC in sepsis has been controversial and the clinical benefit now seems to be limited to only certain groups of patients [17–19]. Although treatment with APC could be considered where available, for severely septic patients with melioidosis with multiple organ failure, patients should be carefully assessed for risk factors that may predispose them to bleeding [20].

3.3. Steroids

As profound modulators of the host immune system, steroids have long been used in management of sepsis and are the subject of intensive and ongoing debate. Currently, the benefit of steroids (hydrocortisone ~300 mg/day in divided doses) appears to be
restricted to the subset of patients with shock, refractory to fluid resuscitation and vasopressors [21]. In these individuals, steroid therapy accelerates time to resolution of shock. The role of steroids in acute lung injury, an inflammatory pulmonary process that commonly results from sepsis, is also highly contentious. Complications from steroid therapy to consider include: myopathy, increased risk of infection and hyperglycaemia. Therefore, the present indications for steroid therapy in the critically ill patient with melioidosis are probably relatively narrow. These may be associated with hypoadrenal crisis and hypotension, mimicking or complicating septic shock. Any concern for hypoadrenal crisis should precipitate prompt therapy with stress dose corticosteroids, ideally after performing a corticotrophin stimulation test to confirm the diagnosis.

4. Organ support

Following the acute resuscitation phase of sepsis management, many critically ill patients require continued haemodynamic support in the form of intravenous fluids and vasopressor agents. There is a complex interplay between optimisation of cardiovascular, pulmonary and renal parameters. Shock unequivocally requires treatment with fluids (until adequate intravascular filling is achieved), vasopressors (for persistent hypotension) or inotropes (in the setting of myocardial depression). Improvement in cardiovascular parameters generally results in improved renal blood flow. However, total body volume overload is common after aggressive resuscitative efforts, which can impede organ perfusion. Ill patients become progressively hypoalbuminaemic, exacerbating the lack of intravascular oncotic pressure and worsening total body fluid overload. Sometimes renal replacement therapy is necessary to correct this imbalance. The lungs are particularly sensitive to extravascular fluid loads. In acute lung injury complicating sepsis, a fluid restrictive strategy maintaining an even net fluid balance is preferred, after initial resuscitation and treatment of shock [22]. For the critically ill patients with melioidosis with acute lung injury who have recovered from shock and are off vasopressor medications, it is advisable to limit the volume of fluids administered to the essential minimum. For patients who have received large volumes of fluid during initial resuscitation, it is also prudent to consider diuresis.

4.1. Mechanical ventilation

Respiratory failure is a common complication of severe sepsis, typically due to systemic illness, pneumonia, or acute lung injury [23,24]. In severe melioidosis, 70% of patients require mechanical ventilation for respiratory failure [14] but in resource constrained settings, the ability to mechanically ventilate patients safely and monitor treatment is often limited. Where ventilators are available, many do not have minute ventilation alarms or permit provision of positive end-expiratory pressure to improve gas exchange. Ideally, arterial blood gas measurements and pulse oximeters should be used to monitor the adequacy of ventilation and oxygenation. However, these are often unavailable in low resource environments. Furthermore, sub-optimal ventilator care may be associated with ventilator associated pneumonia [25,26]. If initiation of mechanical ventilation for
respiratory failure associated with melioidosis is feasible, then achieving a pH of 7.25 to 7.40 and PaO$_2$ of at least 60 mm Hg (oxygen saturation of at least 90%) is appropriate. In acute sepsis resuscitation where oxygen delivery is a concern, increasing oxygen saturations further may be appropriate. Simple strategies such as raising the head of the bed at least 45 degrees, decrease the incidence of nosocomial pneumonia. If acute lung injury is suspected (based on bilateral pulmonary infiltrates not attributable to cardiogenic failure and a PaO$_2$/fraction of inspired oxygen concentration ratio of <300), targeting a tidal volume of 6 ml/kg ideal body weight (or even lower to keep plateau pressures less than 30 cm H$_2$O) substantially reduces mortality [27]. Careful monitoring is required as the use of this technique typically induces hypercapnia and sometimes intolerable hypoxaemia. Noninvasive ventilation may be an alternative strategy in such settings to avoid the need for invasive mechanical ventilation but requires further study in patients with sepsis [28,29].

4.2. Renal replacement therapy

Acute kidney injury frequently complicates severe sepsis. Renal replacement therapy (RRT) is indicated for uraemia, volume overload, severe electrolyte abnormalities and severe metabolic acidosis, common manifestations of severe melioidosis [14]. There is no consensus on the optimal timing or mode of RRT but considering this therapy earlier in the hospital course of the severely ill patient may be prudent. Continuous versus intermittent therapies seem to be equivalent in acute kidney injury, but continuous modalities remove fluid more slowly and may be better tolerated in unstable patients. However, in low resource countries, RRT is prohibitively expensive and often unavailable. While peritoneal dialysis is appealing because of its relative simplicity, a small Vietnamese study of RRT in patients with malaria and sepsis demonstrated that peritoneal dialysis was inferior to haemofiltration and in fact less cost effective [30].

5. Other treatments

5.1. Prophylaxis against venous thromboembolism

Critically ill patients, including those with sepsis, are at high risk for venous thromboembolism (VTE) [31]. Although some have argued that the incidence of VTE is lower in non-Caucasian populations [32], more recent evidence suggests that this may not be the case in post-operative and medical patients [33,34]. Pharmacological agents such as unfractionated or low molecular weight heparin reduce but do not eliminate the risk of VTE. Both types of heparin seem equally efficacious, although there may be less major bleeding attributable to low molecular weight heparin [35]. However, this is offset by the increased cost. In severe melioidosis, consideration of VTE prophylaxis is therefore warranted.

5.2. Stress ulcer prophylaxis

Bleeding from peptic “stress” ulcers occur at a rate of 1% to 4% in patients with severe sepsis but mechanical ventilation and coagulopathy increase the risk dramatically.
Intravenous histamine-2 receptor (H2) blockers and oral proton pump inhibitors are both effective in reducing clinically significant bleeding rates, and the higher gastric pH may decrease rates of nosocomial pneumonia [36,37]. Enteral nutrition may also reduce the incidence of stress ulceration in critically ill patients. Patients with severe melioidosis, especially if not receiving enteral feeding, and mechanically ventilated or coagulopathic, should be prescribed a H2 blocker (intravenously or orally) or an oral proton pump inhibitor.

5.3. Sedation and analgesia

Sedation and analgesia are required not only to reduce oxygen demands but also to reduce the discomfort of illness and intervention. The use of standardised protocols to maintain adequate sedation reduces the duration of ventilation and stay in the intensive care unit [38]. Intermittent dosing of medications and daily interruptions in sedation may reduce the length of hospital stay of critically ill patients without adverse effects [39,40]. Importantly, severe melioidosis with multiple organ failure may be rapidly fatal. Therefore, consideration should be given to setting limits to intervention and instituting palliative measures where appropriate.

5.4. Nutrition

Feeding of critically ill patients provides substrate during a catabolic state and, when administered enterally, may reduce the incidence of infection by promoting gut immune function. There is an increasing trend toward early feeding in critically ill patients in high-resource settings. Although enteral feeding carries a risk of aspiration and usually requires an enteral feeding tube, this route is preferred over parenteral feeding. Provision of nutrition parenterally carries additional risks, such as intravascular infection, and requires close monitoring of electrolytes and should be reserved for malnourished patients who cannot tolerate enteral nutrition. In severe melioidosis, aspiration risk should be carefully assessed, and if indicated, enteral feeding begun at low rates.

5.5. Glycaemic control

There is an abundance of evidence suggesting that hyperglycaemia is associated with poor outcomes in severely ill patients. Hyperglycaemia may occur as a manifestation of the acute stress response in severe illness in non-diabetic patients, although there is a high prevalence of underlying diabetes mellitus in patients with melioidosis. Although maintaining blood glucose control seems appropriate in this patient group, the glycaemic targets in severely septic patients are controversial. Very intensive control may be associated with hypoglycaemia, and the use of insulin infusions requires a high level of monitoring. Despite reducing mortality in surgical patients in intensive care [41], studies have not confirmed the benefits of intensive glycaemic control (4.4 to 6.1 mmol/L) in medical patients [42,43]. Whether more modest blood glucose targets are beneficial remains to be determined. In the absence of additional data to guide the clinician caring for the critically ill patients with melioidosis, efforts should be made to avoid florid hyperglycaemia or hypoglycaemia using the most appropriate insulin administration system.
6. Conclusions

Treatment of severe melioidosis requires an aggressive approach beyond simply the administration of antibiotics. Because organ dysfunction associated with a systemic inflammatory response, severe sepsis, is a syndrome common to many infections, the principles of severe sepsis management should be invoked even before the diagnosis of melioidosis is made. Even in low resource settings, many of the fundamental elements of sepsis management can be implemented. Prompt interventions to optimise oxygen delivery, to maintain homeostasis and support for failing organs, combined with frequent reassessment and adjustment of therapies, are critical in determining outcome. Good supportive care, such as VTE prophylaxis, stress ulcer prophylaxis, and nutrition, are key to reducing complications associated with serious illness. The adjuvant use of G-CSF does not appear to be associated with significant benefits, and other adjuvant treatments, including activated protein C and intensive glycaemic control, remain controversial and the subject of ongoing investigations.

References


Section VI.4
Management of accidental exposure to *Burkholderia pseudomallei*

Sharon J. Peacock\textsuperscript{a,b}, Bart J. Currie\textsuperscript{c,d}

\textsuperscript{a} Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand
\textsuperscript{b} Department of Medicine, Addenbrooke’s Hospital, University of Cambridge, United Kingdom
\textsuperscript{c} Menzies School of Health Research, Charles Darwin University, Darwin, Australia
\textsuperscript{d} Northern Territory Clinical School, Royal Darwin Hospital, Darwin, Australia

Abstract

Diagnostic laboratories worldwide are increasingly likely to isolate *Burkholderia pseudomallei* from clinical specimens, and laboratory research involving the use of this species is also increasing. Guidelines are required to provide laboratory and medical professionals with background information on the organism, actions required prior to working with it, assessment of risk factors and actions required in the event of accidental exposure to *B. pseudomallei*, and the management of seroconversion and culture-confirmed melioidosis. The evidence on which to formulate these guidelines is largely absent and current recommendations are based on expert opinion. These are presented here, together with current supporting evidence from animal models.

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1. Introduction

The number of investigators acquiring and working on strains of *B. pseudomallei* is growing, and research laboratories require guidelines in readiness for an accident in which one or more individuals may require post-exposure prophylaxis (PEP). Workers in clinical diagnostic laboratories may also unwittingly be exposed to *B. pseudomallei* before its identity is recognised, as exemplified by recent reports [1,2]. Guidelines on the actions required in the event of accidental exposure have been developed by a group of clinicians and laboratory workers who have many years of experience working with naturally acquired *B. pseudomallei* infection [3]. These guidelines are applicable to any setting where an exposure event has occurred, either in a research or diagnostic laboratory. This review does not deal with actions required in the event of a bioterrorist event.

2. Published cases of culture-proven laboratory acquired melioidosis

Two cases of culture-confirmed laboratory acquired melioidosis reported in the literature illustrate the practices that led to exposure and the time from exposure to onset.
of symptoms [4,5]. The first case resulted from cleaning up a centrifuge spill of
*B. pseudomallei* culture with bare hands. Clinical features of melioidosis (chills, fever,
malaise, tenderness in the right axilla, and right-sided pleuritic chest) occurred three
days later [4]. The second case was associated with performing antimicrobial susceptibility
testing on two apparent *B. cepacia* isolates (one of which was *B. pseudomallei*) [5].
Fever, pleuritic chest pain, a productive cough, and right calf swelling developed four
days later. Inhalation of an infectious aerosol was considered the likely route of infection
in both cases, although the first case could have inoculated an ulcerative lesion present on
her right index finger. Of note, the risk of laboratory acquired infection with accidental
exposure to the related glanders bacterium, *B. mallei*, is considered to be possibly greater
than that for *B. pseudomallei*, with a recent case reported from the United States [6].

3. Reported cases of accidental laboratory exposure to *Burkholderia pseudomallei*

A further two published reports document accidental laboratory exposure to *B. pseudo-
mallei* in the setting of a diagnostic laboratory in the United States (U.S.), and provide
a description of how these were investigated and managed [1,2]. In the first exposure
event [1], a total of 17 laboratory workers manipulated *B. pseudomallei* cultures
and all took PEP, starting a median of two days after exposure. Sixteen workers
completed a three-week regimen of trimethoprim–sulfamethoxazole (TMP-SMX), and
one completed a three-week regimen of doxycycline. Serum specimens were obtained
for anti-*B. pseudomallei* antibody testing from all exposed workers at one, two, four,
and six weeks after reported exposure. Serologic testing was performed by using an
indirect haemagglutination test (IHA), with a positive result defined as a titre $\geq 1:40$. Two
laboratory workers had borderline results with IHA titres of $\leq 1:20$ for *B. pseudomallei* on
their first serum samples, both were born in the U.S. and neither demonstrated an increase
in titre six weeks after exposure. None of the exposed laboratory workers developed
symptoms consistent with melioidosis during the next five months. The second report
detailed two separate exposure events in which nine workers were offered prophylaxis
and three accepted [2]. Serological testing was performed and was negative, and none of
the exposed developed symptoms consistent with melioidosis.

4. Actions required prior to working with *Burkholderia pseudomallei*

Good laboratory practice will prevent the majority of laboratory accidents involving
exposure to *B. pseudomallei*. The organism should be handled by trained personnel within
a Biosafety Level 3 (BSL-3) facility (or national equivalent), using laboratory practices
specified by the respective national legislative and institutional biosafety committees.
Work should be conducted in a Biological Safety Cabinet (BSC). Gloves should always
be worn when manipulating these microorganisms. Respiratory protection must be used
during centrifugation or when handling infected animals. Sealed cups should be used in
all centrifuges and these should be opened only in a BSC. More complete descriptions of
safe work practices, personal protective equipment and engineering controls associated
with such laboratories in the United States can be found in ‘Biosafety in Microbiological
and Biomedical Research Laboratories (BMBL), which can be accessed from the Centers
for Disease Control and Prevention (CDC) website [7]. Elsewhere, practice should follow
the relevant national guidelines. General international guidelines are provided by the
functioning at BSL2 may isolate B. pseudomallei from a variety of sample types. In
this event, all work should be transferred to appropriate containment facilities as soon
as B. pseudomallei is suspected, and if the bacterial identity is confirmed, a report made
to the relevant authority and a risk assessment of potential exposure of laboratory staff
carried out.

All B. pseudomallei isolates in current use in the research laboratory setting should
be tested to establish susceptibility to meropenem, ceftazidime, TMP-SMX, doxycycline
and amoxicillin–clavulanate (AMC). This choice is based on the fact that the last three
drugs listed may be used for PEP, and ceftazidime or meropenem are the drugs of choice
for the initial treatment of melioidosis. This information should be immediately available
to safety and medical staff following an exposure event. Work with known antimicrobial-
resistant B. pseudomallei strains should be avoided, unless resistance issues are the focus
of research.

Before starting planned work with B. pseudomallei, baseline serum samples should
be obtained from all workers, and logged and stored at −80°C in a secure location.
Testing of these samples is only necessary in the event of a subsequent exposure incident.
Serum banking must be done in a manner that ensures the privacy of the employees and
security of the specimens. Assessment of host risk factors for melioidosis in laboratory
personnel is based on studies of naturally acquired melioidosis in Thailand and northern
Australia. These include: diabetes mellitus, excessive alcohol consumption, chronic
renal failure, and chronic lung disease [9]. Individuals who are immunocompromised
through disease or prescribed drugs (including steroids) are also at increased risk.
Human immunodeficiency virus (HIV) infection does not appear to constitute a risk
factor [10]. Staff with risk factors for melioidosis should be informed of this fact, the
risks formally explained and documented, and alternative work options discussed and
provided where requested. Melioidosis should be considered in the differential diagnosis
in any laboratory staff with an identified risk factor who develops a febrile illness with
features suggestive of bacterial sepsis, irrespective of history of an exposure event in the
laboratory.

All laboratories where B. pseudomallei is known, or likely to be handled should have
agreed arrangements for the provision of occupational health support and should prepare,
in conjunction with their occupational health colleagues, a written protocol as to how
staff will be handled both prior to and following exposure incidents. This must include
the arrangements for immediate medical assessment following an incident. The specific
physician with experience in treating patients with melioidosis who is willing to be
consulted about individual incidents should be identified in advance. The laboratory safety
officer should determine regional or national laboratories that offer a serological service
based on the IHA or other validated assays to determine exposure to B. pseudomallei.
In the U.S. serum specimens should be shipped to the respective state health department
for testing at CDC, following consultation with the Bacterial Zoonoses Branch at CDC.
This information should be in place as a matter of routine and available in the event of an incident.

5. Actions following accidental exposure to *Burkholderia pseudomallei*

If the accident involves dispersal of bacteria into the air (such as spill in a centrifuge), everyone in the laboratory should be alerted and immediately evacuated. Directly after exposure, the site of contamination or inoculation should be washed with copious amounts of water followed by an appropriate cutaneous disinfectant, according to local policy. The designated safety officer for the laboratory should be informed immediately after washing the site. The details of the organism (the bacterial strain, its antimicrobial susceptibility and likely concentration of organisms in the culture) should be noted. High risk exposure events are inhalation, inoculation (puncture), or aerosols into the eye, but all exposure events should be taken seriously and treated as potentially ‘significant’. Following decontamination, exposed individuals must immediately report to the prearranged local hospital or clinic. The exposed individuals or their supervisor should describe the species and strain of organism (including its susceptibility pattern, if available), the type of exposure, and other pertinent information, to the attending medical staff. The individual should be interviewed regarding drug allergies and current health status including risk factors for melioidosis. A risk assessment (Table VI.7) should be carried out to determine whether the laboratory incident poses a low or a high risk. The list of incidents provided in Table VI.7 gives a framework for this distinction, but it may also be necessary to obtain expert advice. All individuals involved in a high risk incident should immediately commence post-exposure prophylaxis. For individuals involved in a low risk incident, the decision to give PEP should be based on the presence of known risk factors for melioidosis. Individuals with known risk factors should commence PEP, while individuals with no known risk factors should be managed with post-exposure monitoring alone.

6. Post-exposure prophylaxis

Post-exposure prophylaxis has been recommended previously following laboratory exposure to *B. pseudomallei* [1,2], although evidence for its efficacy in humans is lacking. Animal experimental data for the efficacy of prophylaxis or treatment given within 48 hours of exposure provides a weak body of evidence on which to base recommendations [11–15]. Comparison of these studies demonstrates that there is a lack of standardisation in nearly all aspects of experimental design, including the strain of *B. pseudomallei* used, the inoculating bacterial dose, the route of bacterial challenge, the route of antimicrobial treatment, and the time that antimicrobial therapy was started when a treatment regimen (rather than a prophylaxis regimen) was the stated aim. Based on a study that compared the antimicrobial drugs in current use for the oral eradication phase of human melioidosis (TMP-SMX, doxycycline and AMC) given either 48 hours before challenge, or PEP given either immediately or 10, 24 or 48 hours post-challenge, TMP-SMX performed best, followed by doxycycline [15]. AMC performed very poorly in this study but the dosing schedule was twice daily. It is highly likely based on the
Table VI.7
Risk assessment of laboratory incidents involving *Burkholderia pseudomallei*

<table>
<thead>
<tr>
<th>Low risk</th>
<th>High risk</th>
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<tbody>
<tr>
<td>Inadvertent opening of the lid of an agar plate growing <em>B. pseudomallei</em> outside a biological safety cabinet</td>
<td>The presence of any predisposing condition without proper personal protective equipment (PPE): diabetes mellitus; chronic liver or kidney disease; alcohol abuse; long term steroid use; haematological malignancy; neutropenia or neutrophil dysfunction; chronic lung disease (including cystic fibrosis); thalassaemia; any other form of immunosuppression</td>
</tr>
</tbody>
</table>

Inadvertent sniffing of agar plate growing *B. pseudomallei* in the absence of contact between worker and bacterium

Needlestick or other penetrating injury with implement contaminated with *B. pseudomallei*

Splash event leading to visible contact of *B. pseudomallei* with gloved hand or protected body, in the absence of any evidence of aerosol

Bite or scratch by experimental animal infected with *B. pseudomallei*

Spillage of small volume of liquid culture (<1 ml) within a functioning biological safety cabinet

Splash event leading to contamination of mouth or eyes

Contamination of intact skin with culture

Generation of aerosol outside biological safety cabinet (e.g. sonication, centrifuge incident)

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*a* The assignment of sniffing of agar plates to low or high risk has been the subject of debate. Authors of the published guideline maintain that sniffing colonies growing on solid agar in the absence of physical contact with the organism represented a low risk [3], but CDC have categorised this as a high risk activity during previous investigations of accidental laboratory exposure [1,2].

pharmacological properties of this drug that this schedule would have led to marked under-dosing and prolonged periods during which the drug concentration was below the bacterial minimal inhibitory concentrations. While the published literature supports the use of TMP-SMX for both treatment and prophylaxis, the current evidence base is grossly inadequate, and further studies are required to support the development of evidence-based guidelines for prophylaxis.

All those involved in high risk incidents and low risk incidents (Table VI.7) in those with risk factors for melioidosis should be offered PEP, following an explanation of risks and benefits. PEP should be prescribed by a physician, who should monitor the individual for evidence of adverse drug effects. The risks of PEP are not insignificant and should be balanced against the likely risks of infection in any given incident. The evidence on which guidelines for PEP is offered is based on expert opinion. The following recommendations are based on the available evidence from animal models and from efficacy of treatment for naturally acquired melioidosis. If the organism is susceptible, and the exposed individual does not have a documented allergy to the specific antimicrobial agent, oral TMP-SMX
Table VI.8
Recommended *Burkholderia pseudomallei* post-exposure antibiotic prophylaxis

<table>
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<tr>
<th>Antibiotic(s)</th>
<th>Dosage</th>
<th>Frequency</th>
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<tbody>
<tr>
<td>Trimethoprim–sulfamethoxazole</td>
<td>TMP-SMX orally 8–40 mg/kg/dose</td>
<td>Every 12 hours</td>
</tr>
<tr>
<td>(TMP-SMX)</td>
<td>2 × 160–800 mg (960 mg) tablets if more than 60 kg,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 × 80–400 (480 mg) tablets if 40 kg–60 kg, and</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1 × 160–800 mg (960 mg) OR 2 × 80–400 (480 mg) tablets if adult</td>
<td></td>
</tr>
<tr>
<td></td>
<td>less than 40 kg</td>
<td></td>
</tr>
<tr>
<td>Doxycycline</td>
<td>2.5 mg/kg/dose up to 100 mg orally</td>
<td>Every 12 hours</td>
</tr>
<tr>
<td>Amoxicillin–clavulanate</td>
<td>Amoxicillin–clavulanate 20/5 mg/kg/dose. Equates to 3 × 500/125 tabs if</td>
<td>Every 8 hours</td>
</tr>
<tr>
<td></td>
<td>&gt;60 kg, and 2 × 500/125 tabs if ≤60 kg</td>
<td></td>
</tr>
</tbody>
</table>

is the agent of first choice and should be given twice daily at the doses presented in Table VI.8. If the organism is resistant to TMP-SMX or the patient is intolerant, the choice is between oral doxycycline or AMC. Although doxycycline has been used previously in a case of laboratory exposure [1], some physicians favour AMC. Doxycycline monotherapy is associated with a significantly higher rate of relapse and treatment failure compared with a conventional oral combination of chloramphenicol, TMP-SMX and doxycycline for the treatment of melioidosis [16]. AMC treatment has a lower relapse rate than doxycycline monotherapy for the treatment of melioidosis, but a lower cure rate than conventional oral therapy [17,18], the mainstay of which is TMP-SMX.

The mean incubation period in one study of naturally acquired melioidosis in Australia was nine days (range 1 to 21 days), but only 25% of patients remembered a specific inoculation event [19]. The incubation period can be as long as 62 years [20]. A period of three weeks of PEP is suggested, the basis for which is a consensus between clinicians who are experienced in treating melioidosis in Thailand and Australia. Fluoroquinolones should not be used for PEP since clinical trial evidence indicates that their use in the treatment of melioidosis is associated with an unacceptably high relapse rate [21,22].

### 7. Post-exposure monitoring

The exposed individual should be instructed to present for medical attention if he/she becomes ill, and to mention the possible exposure event when he/she is seen again by the physician. Most important is early recognition of a febrile illness with or without a cough. Self-recording of temperature should be performed twice daily for 21 days by all individuals following accidental exposure. In the event of a febrile illness (>38°C) and/or development of a cough or progressive inflammation at the site of a known inoculation event, blood cultures (initially two sets from different venipuncture sites), sputum, throat swab and urine culture (using selective bacterial culture medium such as Ashdown medium or *B. cepacia* agar) [23], and chest X-ray should be performed immediately. A serum sample should be taken on the day of the exposure event and stored
and saved for testing in parallel with the baseline (pre-employment) and convalescent
sample, as the identification of seroconversion requires comparison of paired (acute and
convalescent) sera. Serum samples following the first collection on the day of exposure
should be carried out at one, two, four and six week intervals after exposure. The
baseline (pre-employment) and first two serum samples (day 1 and week 1) should be
tested in parallel for the presence of antibodies after one week, and subsequent samples
tested (in parallel with the earlier samples) as they are collected. Seroconversion, with
the development of an antibody response, indicates exposure. Although traditionally a
four-fold rise in titre is used to diagnose an infectious disease, any reproducible rise
between two samples should be used as an indicator of seroconversion resulting from
*B. pseudomallei* exposure, since there is unlikely to be an alternative explanation. Positive
results should be verified by repeating the test. Some individuals with culture-proven
melioidosis do not have detectable antibodies, so an exposed or even a sick individual
with melioidosis may have a negative serological test. In a prospective study of patients
with melioidosis in Darwin, Australia, only 56% of patients had an IHA titre on admission
that was $\geq 1:40$ [24]. Of those initially seronegative, 68% subsequently seroconverted
after admission [24], emphasising the importance of serial serology testing.

Individuals who have resided in an area endemic for melioidosis, or who have
travelled to such areas, may have pre-existing antibodies to *B. pseudomallei*. This will
be demonstrated by the presence of antibodies in the pre-employment sample. There is
no evidence to guide the interpretation of a series of titres following exposure in those
individuals who are already positive. A rise in titre is likely to indicate a new exposure,
although this may not necessarily be related to the laboratory event. An absence of change
in IHA titre in exposed individuals should not be assumed to indicate no exposure to
*B. pseudomallei*. Given the complex nature of interpretation in such a situation, experts
in the field should be consulted.

The antibody test with which people have most experience in endemic regions is the
IHA [24–26]. The results are provided as either being negative or as a specific titre. Titres
are determined in two-fold rising dilutions, usually to a maximal dilution of 1:10,240. The
accuracy of low titres in the assessment of exposure to *B. pseudomallei* is not known.
A titre of 1:20 may indicate exposure but may also be a false-positive result, and should
be repeated on further samples in conjunction with careful clinical assessment. A titre
of 1:40 has been used previously during the investigation of an accidental laboratory
exposure [1], and is likely to provide a more robust measure of true exposure. The
IHA tests for *B. pseudomallei* exposure should be undertaken in laboratories which have
experience in performing such tests. No other available diagnostic tests described in the
literature for the diagnosis of melioidosis perform consistently better than the IHA.

8. Management of seroconversion

In the event that an individual seroconverts following laboratory exposure to *B. pseudomallei*, further clinical evaluation and an extended course of antimicrobial treatment is
recommended. Given the lack of evidence to guide management of individuals under
such circumstances, this is based on expert consensus. A physician should assess the
patient and investigate (samples for culture, blood tests, imaging) if clinically indicated. In individuals who seroconvert but remain asymptomatic and culture-negative, the PEP agent should be continued for a total of 12 weeks, during which the individual should be monitored for adverse drug reactions and clinical manifestations of melioidosis. Follow-up should be continued after cessation of PEP with the duration of follow-up based on clinical judgment.

References


Abstract

Despite \textit{Burkholderia pseudomallei}'s recognised intrinsic antimicrobial resistance which complicates therapy, remarkably little is known about the underlying mechanisms. While rates of resistance in response to antibiotic therapy are relatively low, acquired resistance has been observed and can cause treatment failure. Genome sequence analyses have provided an indication of possible mechanisms of intrinsic and acquired resistance to antimicrobial compounds, but only a few have been experimentally confirmed to date. Of several putative $\beta$-lactamases encoded by \textit{B. pseudomallei}, a Class A $\beta$-lactamase confers resistance to amoxicillin and other $\beta$-lactams, and mutant variants confer resistance to ceftazidime. Efflux pumps belonging to the resistance nodulation cell division superfamily are widespread and, when expressed, bestow resistance to aminoglycosides, chloramphenicol, fluoroquinolones, macrolides, tetracyclines and trimethoprim. Penicillin-binding protein mutations cause ceftazidime resistance. Intrinsic polymyxin B resistance is due to outer membrane exclusion properties. Other factors, such as biofilm and intracellular latent lifestyle, contribute to \textit{B. pseudomallei}'s antimicrobial resistance.

1. Introduction

Because of \textit{B. pseudomallei}'s intrinsic resistance to many antibiotics, melioidosis therapy is difficult and must be continued for extended periods of time \cite{1}. The limited spectrum of antibiotics available for the treatment of melioidosis and the emergence of resistant strains during antibiotic therapy \cite{2,3} call for a better understanding of underlying resistance mechanisms to enable proper therapeutic interventions. Furthermore, successful and suitable treatment of infections caused by bioterrorism events involving strains having acquired resistance determinants, whether by natural means or by malicious genetic engineering, may be impossible if the underlying resistance mechanism(s) cannot be readily identified. The paucity of research and knowledge in this area also makes it impossible to rapidly detect resistance mechanisms in clinical isolates or in maliciously engineered strains. In 2001, Bart Currie and co-workers stated that the results of one of their studies on antibiotic susceptibility of \textit{B. pseudomallei} from tropical northern
Australia “... emphasise the need for microbiological monitoring so that the emergence of resistance can be detected early and appropriate treatment modifications made.” [3]. 

*B. pseudomallei* genome sequence analysis has provided an indication of possible mechanisms of resistance to antimicrobial compounds, but less than a handful have been experimentally confirmed to date [4]. Although relatively rare, resistant mutants have been found in all comprehensive *B. pseudomallei* strain collections that contain isolates from diverse geographic regions (e.g. Australia, Southeast Asia) and sources (for example, clinical versus environmental) [5]. This brief review will highlight the current state of knowledge of *B. pseudomallei* antimicrobial resistance mechanisms. Much of the work described in this Section is in progress.

2. Resistance mechanisms in Gram-negative bacteria

Bacteria possess diverse mechanisms that contribute to intrinsic and/or acquired resistance, either alone or in concert. First, the bacterial cell wall, notably the outer membrane of Gram-negative bacteria, forms a formidable barrier for antimicrobials, which are thus excluded from (efficient) entry to the cellular compartments containing the respective targets. Second, bacteria express enzymes which either covalently modify (for example, by acetylation, adenylation or phosphorylation) or degrade the antimicrobial. Third, target modification by mutation or enzymatic modification either obliterates binding or reduces the affinity of the antimicrobial for the target. Fourth, overproduction of the target raises the threshold required for its inhibition. Fifth, acquisition of targets that are resistant to an antimicrobial allows bypass of the native, susceptible target. Sixth, active efflux of the antimicrobial from the cell depletes its intracellular concentrations to subtherapeutic levels. High-level bacterial antimicrobial resistance often involves synergy between diverse mechanisms. For example, active efflux from the cell is most effective in bacteria with good exclusionary properties. Intrinsic resistance is caused by determinants that are always present and expressed in the cell. Acquired resistance either involves expression or emergence of existing determinants after mutational events, or acquisition of new determinants by horizontal gene transfer from another organism. Although for the most part not well-characterised, *B. pseudomallei* possess all of these antimicrobial resistance mechanisms.

3. Exclusion from the cell

*Burkholderia pseudomallei* is intrinsically resistant to cationic antimicrobial peptides, including polymyxin B, but the mechanisms of resistance are not fully understood. For polymyxin B, experimental evidence points to the fact that lipopolysaccharide (LPS) O-antigen and LPS outer core components play important roles in this resistance [6]. These possibly act as a protective barrier preventing polymyxin B from interacting with potential binding sites residing in the LPS inner core and lipid A regions. The LPS core was also implicated in resistance to other cationic peptides. Mutants defective in the *lytB* gene were polymyxin B resistant, possibly due to membrane permeability alterations caused in part by changes in phospholipid and/or peptidoglycan synthesis. Both groups of
mutants, that is, those affecting LPS synthesis and the lytB mutants, showed altered outer membrane protein profiles, further suggesting that the integrity of this permeability barrier plays a crucial role in antimicrobial peptide resistance. While studies on the roles of porin proteins in *B. pseudomallei*’s antimicrobial resistance are sparse, at least one purified porin, Omp38, facilitated the permeation of charged antibiotics in liposome reconstitution assays [7]. It can thus be postulated that, analogous to what has been observed in other Gram-negative bacteria, porins may play a yet to be recognised role in resistance to some antimicrobials.

4. Enzymatic resistance mechanisms

The only enzymatic resistance mechanisms noted in *B. pseudomallei* to date include a number of Ambler Class A, B and D β-lactamases that are encoded by the K96243 and other *B. pseudomallei* genomes [4]. However, the function of only two enzymes (Class A PenA and Class D Oxa-57) has been confirmed experimentally [8–10].

PenA is present and expressed in prototype *B. pseudomallei* strains. The enzyme confers resistance to numerous β-lactam antibiotics, including ampicillin, amoxicillin, carbenicillin, piperacillin and others, but little to no resistance against ceftazidime, imipenem and meropenem. Several reports have described a role of PenA in acquired ceftazidime resistance in patients treated with this antibiotic [8,11]. This increased resistance is accompanied by reduced activity against other β-lactams. One of the mutations previously identified in clinical isolates with high level ceftazidime resistance was a cysteine to tyrosine substitution at position 69 of PenA [11] which was subsequently verified in other strains (unpublished data from the author’s laboratory). While this mutation generated ceftazidime resistance, it increased susceptibility to other β-lactams (for example, amoxicillin and imipenem), which enables alternate therapeutic options. A laboratory-generated ceftazidime resistant mutant contained a proline to serine change at position 167 of PenA, but this mutation has not yet been reported in clinical isolates [12].

Although susceptible to hydrolysis by PenA, the activities of β-lactams such as amoxicillin can be significantly potentiated by the β-lactamase inhibitor, clavulanic acid. Mutants that are insensitive to inhibition by clavulanic acid have been described, and in one study, the causative mutations were mapped to *penA* [8,9]. Recent experimental evidence demonstrates that PenA is secreted via the twin arginine transport (TAT) system. PenA TAT signal sequence and *tatABC* deletion mutants were equally susceptible to PenA substrates than a Δ*penA* mutant.

It has been speculated that *penA* transcription may be regulated by the product(s) of one of the two genes, *BPSS0944* and *BPSS0948*, located upstream and downstream of *penA* and encoding putative LysR regulators (Figure VI.1) [9]. However, unpublished preliminary data from the author’s laboratory shows that deletions of these regulatory genes do not affect *penA* transcription. Therefore, the regulation of PenA expression remains unknown.

A few reports describe an initial characterisation of the class D β-lactamase of *B. pseudomallei*. While the cloned enzyme has activity against a number of β-lactam
5. Resistance due to target mutation

Incidences ascribing antibiotic resistance to target mutations in \textit{B. pseudomallei}, including target overproduction, are rare. While clinical ceftazidime resistance is rare (<0.2%) [1], ceftazidime therapy of human melioidosis for originally ceftazidime-susceptible strains of \textit{B. pseudomallei} can lead to emergence of resistant variants whose resistance is not always caused by PenA mutations. It was recently found that some ceftazidime resistant strains isolated from patients with melioidosis in Thailand in whom ceftazidime therapy failed exhibit a severe \textit{in vitro} growth defect (unpublished data). When compared with the original ceftazidime susceptible isolates from the same patients, this resistance and growth defect was shown to apparently be associated with a large (>70 kb) recurrent deletion of a region of chromosome 2. Within this region lie two genes encoding putative penicillin binding proteins (PBPs), BPSS1219 and BPSS1240. These two PBPs are paralogs of PBP3 (BPSSL3031), located on the large chromosome. PBP3 proteins have a high affinity for ceftazidime in comparison to other PBPs, and the growth defect and accompanying ceftazidime resistance most likely stems from missing one or two of these PBPs. With respect to strain 1026b, it subsequently was shown that deletion of PBP3-BPSS1219, but not BPSS1240, caused a slow-growing, filamentous phenotype. It is therefore likely that the PBP3-BPSS1219 is a major ceftazidime target and that its mutation, either by deletion or possibly by alteration or overproduction, can cause ceftazidime resistance.

6. Resistance due to target overproduction

An example for target overproduction as a resistance mechanism is the over-expression of PenA which decreases susceptibility to most \beta-lactams. This is consistent with the finding that derepression of PenA resulted in a general increase in resistance to \beta-lactam antibiotics. However, the clinical significance of these findings, if any, remains unknown.

7. Efflux-mediated resistance

K96243 and other \textit{B. pseudomallei} genomes encode an arsenal of efflux pumps, including 10 pumps belonging to the resistance nodulation cell division (RND) family [4], which play major roles in clinically significant antibiotic resistance in non-enteric bacteria [13].

antibiotics and its transcription was elevated in ceftazidime resistant mutants, its role in clinically relevant \beta-lactam resistance has yet to be established [10].
The operons encoding these pumps are present and expressed to some degree in many strains, but only three pumps have been characterised in detail: AmrAB-OprA [14], BpeAB-OprB [15], and BpeEF-OprC [16] (Figure VI.2). The genetic make-up of these three operons is typical of those encoding RND efflux pumps found in Gram-negative bacteria. They all encode a gene for the cytoplasmic membrane RND transporter (AmrB, BpeB and BpeF), an outer membrane channel protein (OprA, OprB and OprC) that physically interacts with the RND transporter, and a periplasmic membrane fusion protein (AmrA, BpeA and BpeE) that stabilises the efflux complex. Located upstream of the efflux operon are regulatory genes that either encode repressors (AmrR and BpeR) or an activator (BpeT). There are variations on this theme. For example, not all efflux operons encode cognate outer membrane channel proteins and the corresponding pump proteins interact with other outer membrane channel proteins encoded elsewhere on the genome. Similarly, some efflux operons are regulated by proteins encoded by distant rather than local regulatory genes. In fact, AmrAB-OprA and BpeEF-OprF expression are regulated by local and other regulatory mechanisms.

AmrAB-OprA was the first efflux pump characterised in \textit{B. pseudomallei} and it is the main player in this bacterium’s high intrinsic aminoglycoside and macrolide resistance [14]. A careful analysis using isogenic mutants revealed that this pump also extrudes other antibiotics, including fluoroquinolones and tetracyclines (Table VI.9) [17]. The intrinsic tetracycline resistance bestowed by AmrAB-OprA in prototype strains probably is clinically insignificant, since most \textit{B. pseudomallei} strains contain and express this pump yet are susceptible and respond to doxycycline treatment. While AmrAB-OprA is expressed at detectable levels in prototype strains, exposure to certain substrates can select for mutants over-expressing this efflux system. For example, \textit{B. pseudomallei} strain 1026b is susceptible to the experimental ketolide cethromycin but exposure to the antibiotic selects for resistant mutant derivatives due to AmrAB-
Table VI.9
Antibiotic resistance mechanisms demonstrated in *Burkholderia pseudomallei*

<table>
<thead>
<tr>
<th>Antibiotic* or inhibitor class</th>
<th>Exclusion from the cell</th>
<th>Enzymatic modification/degradation</th>
<th>Target mutation</th>
<th>Target overproduction</th>
<th>Efflux</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoglycosides</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>β-lactams</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>+</td>
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<tr>
<td>Chloramphenicol</td>
<td></td>
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<td></td>
<td>+</td>
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<tr>
<td>Clavulanic acid</td>
<td></td>
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<td></td>
<td></td>
<td>+</td>
</tr>
<tr>
<td>Fluoroquinolones</td>
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<td></td>
<td>+</td>
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<tr>
<td>Macrolides</td>
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<td></td>
<td>+</td>
</tr>
<tr>
<td>Tetracyclines</td>
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<td>+</td>
</tr>
</tbody>
</table>

*a Antibiotics tested were: Aminoglycosides, gentamicin and kanamycin; β-lactams, ampicillin, amoxicillin, carbenicillin, ceftazidime, imipenem, meropenem and piperacillin; Chloramphenicol; Fluoroquinolones, ciprofloxacin and norfloxacin; Macrolides: erythromycin, clarithromycin and clindamycin; Tetracyclines, tetracycline and doxycycline.

OprA over-expression [18]. These AmrAB-OprC over-expressing mutants do not contain mutations in *amrR* or the *amrA-amrA* intergenic region, indicating involvement of another, yet unknown regulatory mechanism. While the majority of *B. pseudomallei* strains are aminoglycoside resistant, susceptible variants do exist. Analysis of three aminoglycoside susceptible strains from northeast Thailand revealed that two of them did not express AmrAB-OprA and one variant contained a large (>131 kb when compared to the sequenced strain K96243) chromosomal deletion of the *amrAB-oprA* containing region [19]. Such mutants may not be as rare as originally thought, since clinical diagnosis of *B. pseudomallei* still relies on Ashdown agar which contains gentamicin as its main selective ingredient, and susceptible variants will obviously be missed using this medium. AmrAB-OprA is not required for virulence, since strains lacking this efflux pump cause human disease and are virulent in a murine melioidosis model [19].

BpeAB-OprB was originally described in Singapore strain KHW and reported to play major roles in high-level resistance to aminoglycosides and macrolides [15]. However, recent results indicate that in 1026b, and some other strains, BpeAB-OprB does not efflux aminoglycosides [17]. There is considerable substrate overlap between AmrAB-OprA and BpeAB-OprB. Both pumps extrude macrolides, fluoroquinolones and tetracyclines, and contribute to intrinsic resistance to these antibiotics. To a lesser extent, BpeAB-OprB also effluxes chloramphenicol. Again, the intrinsic tetracycline and chloramphenicol resistance conferred by BpeAB-OprB probably is clinically insignificant, since most *B. pseudomallei* strains contain and express this pump yet are susceptible to doxycycline and chloramphenicol, and respond to treatment by these antibiotics. In strain KHW, this
pump is required for optimal production of a number of virulence factors as well as
excretion of acyl-homoserine lactone quorum sensing molecules, but again these findings
could not be verified using strain 1026b and its mutant derivatives [17]. The reason(s) for
these disparate findings remain unknown. BpeAB-OprB expression is negatively regulated
by the BpeR repressor. Although bpeAB-oprB transcription can be detected in prototype
strains, bpeR mutants contain significantly elevated transcript levels, which are accompa-
nied by elevated antibiotic resistance. Resistance levels to clinically significant antibiotics
like doxycycline are still below breakpoints that would compromise treatment [15].

Using a surrogate, drug-sensitised Pseudomonas aeruginosa strain, it was shown
that BpeEF-OprC effluxes chloramphenicol and trimethoprim [16]. When expressed in
B. pseudomallei bpeT and other regulatory mutants, BpeEF-OprC confers resistance
to fluoroquinolones, chloramphenicol and tetracyclines (unpublished data from the author's laboratory). Transcription of bpeEF-oprC is inducible by chlor-
amphenicol, doxycycline and trimethoprim, but not fluoroquinolones. Moreover, cross-
resistance to inhibitory concentrations of chloramphenicol and doxycycline is inducible
by sub-inhibitory concentrations of trimethoprim. These findings may have implications
regarding the design, implementation and outcome of melioidosis combination therapies.
Of the three B. pseudomallei efflux pumps characterised to date, BpeEF-OprC potentially
has the greatest clinical impact as it confers resistance to therapeutically significant
antibiotics, including trimethoprim and doxycycline. This notion is supported by the
finding that BpeEF-OprC expression is responsible for the trimethoprim resistance
observed in many Thai strains (unpublished data). However, BpeEF-OprC expressing
strains remain susceptible to trimethoprim–sulfamethoxazole.

The B. pseudomallei K96243 genome also encodes a homologue (BPSL2468) of the
NorM efflux pump that confers polymyxin B resistance in B. vietnensis [20]. However,
since high-level polymyxin B resistance is attributed to cell permeability barriers, it is
unlikely that efflux is a major player in polymyxin B resistance. This notion is supported
by the finding that NorM may only contribute to polymyxin B resistance under certain
growth conditions [20].

8. Other factors affecting drug resistance and tolerance

Aside from the traditional resistance mechanisms discussed thus far, there are other factors
affecting antimicrobial susceptibility. Many of these are linked to lifestyle, altered modes
of growth and changes in the physical environment, but studies in this area are sparse
with B. pseudomallei.

A well-known factor affecting antibiotic susceptibility is the biofilm mode of growth. Not
surprisingly, B. pseudomallei cells grown in a biofilm or under biofilm stimulating
conditions exhibited significantly increased antibiotic resistance [18,21]. It is known that,
akin to Mycobacterium tuberculosis, B. pseudomallei can survive for extended periods
of time in a dormant state, when the bacteria presumably reside intracellularly [22].
In this state, bacteria are non-replicating and thus, for the most part, not susceptible
to conventional antibiotic treatment. While the factors and mechanisms governing the
transition from the replicating to the non-replicating state and maintenance of the
persistent lifestyle are largely unknown, the parallels to \textit{M. tuberculosis} suggest altered metabolic patterns, anaerobiosis and other factors may play a role [23]. A recent study showed that, again akin to \textit{M. tuberculosis}, isocitrate lyase is a persistence factor required for maintenance of latent infections [23]. This work also suggested that isocitrate lyase inhibitors can force a transition from the latent into an acute state of infection, which can then be treated with conventional antibiotics.

A consequence of antibiotic treatment \textit{in vitro} and \textit{in vivo} can be the emergence of colony variants that are highly antibiotic resistant. The emergence of such variants is not limited to treatment with a specific antibiotic, since drug resistant small colony variants (SCVs) were isolated after \textit{in vitro} exposure to ceftazidime, ciprofloxacin and gentamicin, as well as from ceftazidime-treated mice [24]. Once isolated, SCVs were relatively stable in the absence of continued antibiotic selection and were cross-resistant to chemically-unrelated antibacterial agents. Revertants to the parental colony morphology exhibited parental susceptibility patterns. The molecular basis for the SCV phenotype, and the clinical significance of SCVs, is unknown.

Antimicrobial susceptibilities may also change in response to stress conditions. During an analysis of \textit{B. pseudomallei} secreted proteins, it was shown that salt stress (exposure of \textit{B. pseudomallei} to 150 mM NaCl), induced 19-fold expression of a \(\beta\)-lactamase-like protein [25]. Salt stressed bacteria showed a significantly greater survival rate after ceftazidime exposure.

9. Conclusions

Since it was listed as a select agent in the United States less than a decade ago, recognition of the potential use of \textit{B. pseudomallei} as a bioterrorism and emerging infectious disease agent led to an infusion of funds into research into this important pathogen, and a proliferation of research groups, especially in the United States. While the main thrust of the newly funded research is to improve diagnostics and therapeutics, as well as perhaps develop vaccine strategies, concerted efforts are being made to understand mechanisms of drug resistance and/or tolerance at the molecular level.

\textit{Burkholderia pseudomallei} is intrinsically resistant to numerous antimicrobials, and \textit{in vivo} acquired resistance can develop via many different mechanisms. Research into mechanisms of resistance development not only supports the discovery of new anti-\textit{B. pseudomallei} therapeutics but also provides the basis for diagnosis of resistance emergence in clinical settings and detection of maliciously engineered strains.

References


VII
Pathogenesis and development of protection
Section VII. Pathogenesis and development of protection

Editorial overview

W. Joost Wiersinga\textsuperscript{a,b}, Jodie L. Morris\textsuperscript{c}

\textsuperscript{a}Center for Infection and Immunity Amsterdam (CINIMA), Center for Experimental and Molecular Medicine, \textsuperscript{b}Department of Medicine, Academic Medical Center, Amsterdam, the Netherlands \textsuperscript{c}Microbiology and Immunology, School of Veterinary and Biomedical Sciences, James Cook University, Townsville, Australia

Determination of the pathogenesis of an infectious disease is very important for the development of diagnostics, therapeutics and for investigating possible candidates for vaccines. The five reviews included in this Section provide an insight into our current understanding of the immunopathogenesis of melioidosis.

Section VII.1 provides an overview of experimental models of melioidosis. Characterisation of microbial pathogenesis relies heavily on the use of animal models. Animal infection models have played an important role in investigations of the pathogenesis of melioidosis. A wide spectrum of animal models for \textit{Burkholderia pseudomallei} infection has been used for investigations ranging from non-mammalian \textit{Caenorhabditis elegans} to non-human primates. Although larger animal models may mimic the human condition more closely, research data on such models are limited. Most of our understanding on different aspects of melioidosis, including host immunity, disease pathogenesis and drug and vaccine design, has come from rodent models.

Initial host–pathogen interactions set the scene for subsequent disease progression. \textit{B. pseudomallei} has to overcome mucosal and epidermal defences to initiate infection following inhalation, percutaneous inoculation, or ingestion. \textit{B. pseudomallei} is largely an opportunist, causing infection in situations where the hosts’ immune system is impaired, the infecting strain is highly virulent, the inoculating dose is sufficiently high, or any combination of these factors. In Section VII.2 a range of bacterial metabolic pathways that are upregulated to facilitate survival of \textit{B. pseudomallei} within the host are discussed. \textit{B. pseudomallei}, like other facultative intracellular bacteria, possesses various mechanisms which enable it to evade or resist the host defence mechanisms, allowing multiplication to occur even within host phagocytes. \textit{B. pseudomallei} is able to flourish within the cytoplasm of infected cells by suppressing key innate host defences. The pathogen is also able to rapidly escape from the phagosome through the formation of actin-based membrane protrusions which extend to neighbouring cells.

A range of putative virulence factors of \textit{B. pseudomallei}, including structural surface components such as lipopolysaccharide, capsular polysaccharides, outer membrane
proteins and flagella, have been described and are discussed in Section VII.3. B. pseudomallei also produces a range of secreted antigens that facilitate host cell invasion and intracellular survival. While phenotypic variants are common for B. pseudomallei, the significance of variant morphology in the immunopathogenesis of disease remains uncertain. However, an extensive repertoire of virulence factors enables the pathogen to survive under a range of hostile conditions, and contributes to pathogen recognition receptor-mediated dysregulation of the immune response.

The role of the immune system is pivotal in orchestrating the host response that ultimately defines the outcome of infection; and is discussed in more detail in Section VII.4. Immunity to an infectious agent requires coordination between the innate and adaptive immune responses. Interest in understanding how B. pseudomallei attempts to evade the immune responses of the host to cause infection has lead to a number of studies investigating the ability of specific components of the innate and adaptive immune system to recognise and respond to B. pseudomallei. Our current understanding of why certain individuals are more susceptible to this disease and the possible impact of selected risk factors on the host immune response to the pathogen is limited. B. pseudomallei has been proven to be a highly evolved bacterium adapted for intracellular survival. Investigations of host and B. pseudomallei interactions have found that coordinated and robust innate immune responses, namely the efficient function of macrophages and neutrophils and appropriate activation of adaptive immune responses involving CD4+ and CD8+ T cells during the later stages of infection, contribute to the development of immunity against B. pseudomallei.

Strategies for the development of vaccines are discussed in Section VII.5. There is currently no licensed vaccine against melioidosis, although B. pseudomallei is considered to be a potential biowarfare agent. Some progress has been made in characterising the immune responses in individuals exposed to B. pseudomallei and mechanisms of resistance to infection are being studied in animal models. Both antibody and CD4+ T cells appear to play an important role in protection from disease. A number of approaches to vaccine development have been proposed, including the use of live attenuated mutants, killed cell and subunit vaccines.
Section VII.1
Models of infection

Suppiah P. Sivalingam\textsuperscript{a}, Glen C. Ulett\textsuperscript{b}, Michelle Nelson\textsuperscript{c}
\textsuperscript{a}Defence Medical and Environmental Research Institute, DSO, National Laboratories, Singapore
\textsuperscript{b}School of Medical Science, Centre for Medicine and Oral Health, Griffith University, Australia
\textsuperscript{c}Defence Science and Technology Laboratory, Porton Down, Salisbury, United Kingdom

Abstract
Experimental infection models are intended to mimic the biological phenomenon of a disease that occurs in humans. A wide spectrum of animal models for \textit{Burkholderia pseudomallei} infection have been reported, ranging from non-mammalian \textit{Caenorhabditis elegans} to large mammalian models, including non-human primates (NHP). Host susceptibility, virulence mechanisms, host–pathogen interactions and disease patterns differ widely among the different model systems. Although large animal models including goat, pig, and NHP may mimic the human counterpart more closely, research data on such models are limited. In contrast, small animal models of melioidosis have contributed significantly to understanding different aspects of host immunity, disease pathogenesis and drug/vaccine development. It remains unclear which animal models of melioidosis best reflect the disease pattern in humans. This review provides an overview of the various models used in \textit{B. pseudomallei} infection, highlighting their advantages and drawbacks.

1. Use of model systems in disease management
Animal models have traditionally played critical roles in the safety and efficacy testing of prophylactic and therapeutic products. Prophylactic antibiotic regimens have prompted the need for animal models for novel therapeutic regimens for accidental laboratory exposure or malicious releases during bioterrorism events \cite{1}. While animal models can indeed be used to predict clinical outcomes of therapeutic interventions, they still have significant shortcomings.

The treatment study conducted by Ulett et al. \cite{2} used a murine model to compare the efficacy of ceftazidime and cefepime, alone or with cotrimoxazole, in the therapy of melioidosis. This study showed that ceftazidime in combination with cotrimoxazole was the most effective treatment, and demonstrated the maintenance of plasma antibiotic levels at relevant concentration using dosing modification. In another study \cite{3}, ciprofloxacin, gatifloxacin and moxifloxacin given for 14 days did not provide good post-exposure protection to BALB/c mice following subcutaneous inoculation of \textit{B. pseudomallei}, even when treatment was initiated six hours post-challenge. Ciprofloxacin and doxycycline
given 48 hours before or immediately after the intraperitoneal bacterial challenge in a mouse model raised the median lethal dose. However, *B. pseudomallei* was recovered from surviving animals and relapse of infection was observed in some treated animals up to five weeks later [4].

Melioidosis prophylaxis has only been addressed in animal experiments. While post-exposure prophylaxis following laboratory exposure to *B. pseudomallei* has been recommended previously, there is little evidence for its efficacy in humans [5]. A recent study evaluating the efficacy of oral administration of commonly used melioidosis drugs demonstrated amoxicillin-clavulanic acid, doxycycline and cotrimoxazole provided the most effective pre- and post-exposure prophylaxis, rescuing 100% of the animals when administered 48 hours pre-exposure, and within the first 24 hours post-infection, as assessed by survival rates and elimination of bacteria from the lung. In the same study, doxycycline had significant efficacy but amoxicillin-clavulanic acid was the least effective. Data from this study suggested that the recommended cotrimoxazole could indeed be used as first-line agent for prevention and treatment of acute and chronic disease [6].

Various factors, such as bacterial strain type, routes of inoculation and dose, play an important role in the outcome of prophylaxis and treatment. Host strain type is also another important factor to consider. Thus, although no animal study can completely replace controlled human clinical trials, data derived from such studies provide useful information that could be extrapolated to human subjects. Investigational prophylactics and therapeutics for many biological threat-agents, such as *B. pseudomallei*, fall into this category.

2. Nematodes

A wide variety of animal models have been used over the years to investigate *B. pseudomallei* infection, ranging from *Caenorhabditis elegans* to large mammalian models, including non-human primates (NHP). *C. elegans* was originally pioneered as a model for the study of neurobiology by Sydney Brenner nearly 40 years ago and it has since been used extensively as a model organism against many different pathogens [7]. *C. elegans* has also been used as a host model for *Burkholderia* species, complementary to those already in existence. Studies of the nematodes response to infections have revealed the existence of host susceptibility, virulence mechanisms and host–pathogen interactions, all of which employ evolutionarily conserved signalling pathways and produce an array of effector molecules, some of which are similarly conserved.

Unlike higher mammalian models, *C. elegans* can be easily maintained and manipulated in the laboratory, and is also cost-effective. Furthermore, the completion of whole genome sequencing of *C. elegans* [8] offers additional advantage to study an array of molecular genetics and signalling pathways. Tan et al. [9,10] were the first to report the use of *C. elegans* as a model for *Pseudomonas aeruginosa* pathogenesis, and subsequently many other pathogens were demonstrated to infect and/or kill *C. elegans*. O’Quinn and colleagues identified a novel approach to study the pathogenesis of *Burkholderia* species using this non-vertebrate model [11]. Their study demonstrated that *B. thailandensis*, which is avirulent for humans, kills *C. elegans* readily while *B. mallei*, which is
virulent for humans, is non-pathogenic in *C. elegans*. Therefore, the findings of O’Quinn et al. [11] seem to suggest little correlation with *Burkholderia* species virulence in vertebrates and *C. elegans*.

In 2002, Gan and colleagues reported on the use of *C. elegans* to study the pathogenesis of *B. pseudomallei* and *B. mallei* [12]. The authors found that *C. elegans* was susceptible to infection by a broad range of *Burkholderia* isolates. The effect of virulence of different clinical isolates of *B. pseudomallei* was similar in both *C. elegans* and mammals. Both *B. mallei* and *B. pseudomallei* strain, KHW, exhibited a nematocidal and virulence effect, whereas *B. thailandensis* exhibited decreased virulence in nematodes and avirulence in mammals.

Studies by both O’Quinn et al. [11] and Gan et al. [12] have contrary results for susceptibility within the *C. elegans* model. However, both studies have consistently demonstrated that *C. elegans* can be killed by various *Burkholderia* species, and that living bacteria are required for a maximal killing effect. Nevertheless, it is now known that both *B. pseudomallei* and *B. mallei* are virulent in vertebrates, whereas *B. thailandensis* is identified to be clinically avirulent [13].

The genetic tractability of the nematode *C. elegans* model offers many advantages to study host–pathogen interactions. However, the disadvantages limiting the use of this model are: (i) the lack of adaptive immune system, (ii) the presence of only a few, or no, specialised cells equivalent to macrophages and neutrophils, and (iii) an inability of *C. elegans* to survive at 37°C. Although *C. elegans* may not be a suitable candidate as a complete experimental system, it is still a very useful model for the identification of virulence factors in bacteria, and the identification of bacterial mutants with increased and/or decreased virulence.

3. Small animal models for melioidosis

3.1. The initial studies

Some of the first small animal models of human melioidosis were those described by Stanton and Fletcher [14,15]. They used rats, goats and several other species to describe disease presentations of melioidosis in animals [16]. Their early insights into differential host susceptibility were subsequently investigated further by numerous groups, in studies that have provided the foundations for the various small animal models of human melioidosis used in current research. Over the past 15 years, in particular, small animal models have been described in some detail and have provided insight into the pathogenesis of melioidosis, virulence factors of *B. pseudomallei*, potential vaccines and efficacy of antibiotics for treatment and prevention of disease. The most widely used small animal models have been those utilising rats, mice, and hamsters [13,17–21]. Rats are relatively resistant to *B. pseudomallei* and most recently were used to draw comparisons between melioidosis and pulmonary tuberculosis [22]. Hamsters, in contrast, are highly susceptible to *B. pseudomallei* and have been used in a series of studies on host susceptibility, virulence factors and immunoprotection, largely over the past decade [23–26]. Mice provide the most intriguing of small animal models for melioidosis since they display varying levels of innate susceptibility and resistance,
depending upon the strain of mouse. The most widely studied are the BALB/c and C57BL/6 inbred strains, which were first described as suitable small animal models by the group led by Professor Robert Hirst [19], followed by many others [20,27–30]. In addition to mouse strain, the severity of disease in small animal models depends on the route of infection and, most importantly, the virulence of the individual *B. pseudomallei* strain [19,31,32]. Beginning in 1997, application of small animal models of melioidosis has been particularly useful in comparing the pathogenicity of *B. pseudomallei* to that of the closely related *B. thailandensis*, which rarely causes disease in humans and is essentially avirulent in small animal models [33,34]. Many key insights into the unique traits of *B. pseudomallei* have been obtained from studies on small animal models, which have provided not only much needed evidence of immunological aspects of disease and potential vaccine targets, but also a rudimentary understanding of host susceptibility and pathogenesis of *B. pseudomallei* infection as it occurs in humans.

### 3.2. Small animal models for studying host susceptibility

Susceptibility to *B. pseudomallei* infection is a complex balance between innate and adaptive immune responses, bacterial virulence, and the state of health of the host. Susceptibility studies in small animal models, particularly in mice, have shown a spectrum of host susceptibility phenotypes, ranging from exquisite susceptibility to relative resistance in different host strains. In the BALB/c and C57BL/6 mouse model of acute and chronic human melioidosis the difference in susceptibility of these strains is marked [19,27]. Acute susceptibility in BALB/c mice stems from uncontrolled growth of *B. pseudomallei* in blood, spleen and liver [19], and is associated with poor antimicrobial function of macrophages [35,36], and an overwhelming bacteraemia that leads to severe inflammation and tissue necrosis [28,37]. Cellular infiltrate in infected organs in acutely infected BALB/c mice comprises accumulation of neutrophils, in contrast to macrophages that are more pronounced in C57BL/6 mice [38]. *B. pseudomallei* induces caspase-1-dependent cell death in macrophages [39], and C57BL/6 mice made caspase-1-deficient are rendered highly susceptible to infection [36], illustrating a key role for caspase-1 in resistance in mice. Survival of *B. pseudomallei* is also affected by the autophagy pathway of cell death in epithelial cells and macrophages, which may influence disease [40]. However, the roles of autophagy, caspases, and other mediators of programmed cell death, which influence bacterial disease pathogenesis in many other infections [41], remain uncertain with regards to susceptibility of BALB/c mice to *B. pseudomallei*. A unique aspect of susceptibility to *B. pseudomallei* is that the susceptibility of mice is not related to genes, such as Nramp1 [42] that control innate resistance to other bacterial infections [27]; rather, fulminating disease in mice appears to be due to an excess rather than a lack of inflammation [43]. In recent years the impact of route of infection in small animal models has garnered attention and several groups have shown the dissimilar impact of intravenous (i.v.), intranasal (i.n.), intraperitoneal (i.p.), and inhalation challenge on disease outcome in mice. With the exception of one model based on nebulised *B. pseudomallei* [29], the differential susceptibility of BALB/c and C57BL/6 mice to systemic (i.v.) *B. pseudomallei* infection is mirrored in models of mucosal and
An important element of resistance in C57BL/6 mice is that the degree of resistance, and thus nature of disease (acute, subacute, chronic, convalescence, latency can be established in C57BL/6 mice), varies considerably depending on the virulence of the challenge \( B. pseudomallei \) strain. C57BL/6 mice succumb to infection with virulent \( B. pseudomallei \) within weeks of infection [19], but other less virulent strains are cleared after several weeks and infection is either completely cleared or contained as a latent asymptomatic state with seroconversion, which can last for many months [27,28,44]. Future studies will hopefully determine whether infection is completely cleared in such mice; particularly given that specific antibodies are produced and may aid protection [45−47]. A more recent model of differential host susceptibility in rats that mimics acute and chronic human melioidosis has proven useful to quantitate differences in virulence between \( B. pseudomallei \) strains during acute and chronic disease [22].

One of the risk factors commonly associated with susceptibility to melioidosis is diabetes mellitus, and this aspect of host susceptibility has been investigated in small animal models by the group led by Professor Donald Woods, as reviewed elsewhere [48]. Their studies used a unique rat model of diabetes to show that insulin impairs the growth rate of \( B. pseudomallei \) and this directly influences disease severity. In a model of streptozotocin-induced diabetes in infant rats [49], they demonstrated that faster growth of \( B. pseudomallei \) observed in sera from infant diabetic rats compared to controls was associated with increased susceptibility to sepsis [50]. Experiments using insulin-depleted human sera confirmed that the infant diabetic rat model is an excellent one with which to study facets of human susceptibility to \( B. pseudomallei \) related to diabetes [50].

### 3.3. Small animal models for studying virulence factors and pathogenesis

The gamut of virulence of individual \( B. pseudomallei \) isolates is broad; when assessed in BALB/c mice isolates exhibit a spectrum of LD\(_{50}\) values ranging million-fold [31]. Furthermore, virulence of \( B. pseudomallei \) in mice does not easily correlate with disease presentation or outcome in patients, nor the source of bacterial isolates [31]. One of the common disease presentations in humans infected with \( B. pseudomallei \) is pneumonia, which stems from the natural route of infection of environmental exposure via inhalation of contaminated dust [51]. Several small animal models have been useful to provide insight into respiratory melioidosis [21,52,53], and knowledge that the bacteria adhere to buccal epithelial cells [54] and replicate in nasal-associated lymphoid tissue [55] is relevant in such models. In a model of respiratory infection in BALB/c mice using aerosolised bacteria, as developed by Ulrich et al. [25], quorum sensing was shown to contribute to virulence of \( B. pseudomallei \). A separate study using a \textit{C. elegans} host system [11,12] confirmed that \( B. pseudomallei \) mutants lacking quorum sensing ability are attenuated when co-cultured with \textit{C. elegans} [56], although the relevance of this model to human melioidosis has been debated [13]. Nevertheless, quorum sensing also contributes to virulence in Syrian hamsters after i.p. infection [25]. At present it is unknown how quorum sensing counteracts host immune defence mechanisms in these models; however, it may be associated with secretion of exoproducts [56].
During systemic *B. pseudomallei* infection in mice, neutrophils are recruited to sites of local inflammation [38,57] and are critical for resistance to disease [58]. However, *B. pseudomallei* can replicate inside many cell types [59], including neutrophils [60,61]. The contribution of such intracellular lifecycles to virulence has been explored using various *in vitro* models by several groups, as reviewed elsewhere [62]. In a series of experiments in mice it was collectively demonstrated that *B. pseudomallei* defective in various pathways associated with intracellular survival (including Bsa T3SS, actin-tail formation) are attenuated [63–65]. In BALB/c mice, mutants in type III secretion system (T3SS) BipD were also found to be attenuated after respiratory and i.p. infection [64], and mutants for BipB, which drives multinucleated giant cell formation, are attenuated after i.n. challenge [66]. More recently, studies in BALB/c mice have shown that *B. pseudomallei* may gain access to the central nervous system through the olfactory epithelium as a means of initiating invasive disease [55].

The capsular polysaccharide produced by *B. pseudomallei* contributes to virulence in small animal models by increasing serum survival and resistance to phagocytosis [26]. A genomic approach combined with a hamster model, as outlined in [13], was the first to identify capsule as a critical virulence factor of *B. pseudomallei* [26,49,67]. The capsule contributes to virulence during respiratory infection in BALB/c mice [53,55], and mutants defective in capsule production are also attenuated in mice after i.v. or i.p. infection [68,69]. *B. pseudomallei* also produce flagella, which imparts motility to the bacteria [70]. In one study, there was no difference in virulence between an isogenic flagella-deficient mutant and wild-type *B. pseudomallei* in diabetic rat and hamster infection models [70]. However, a more recent study of respiratory infection in BALB/c mice showed a flagella-deficient mutant was attenuated, compared to wild-type following i.n. infection. The mutant was also less virulent in an i.p. infection model [71], showing a role for flagella in virulence in mice. Importantly, these studies illustrate that distinct small animal models may impact pathogenesis studies. Thus, interpretations should consider the role and limitations of the chosen model in relation to human disease. Phenotypic plasticity is another characteristic of *B. pseudomallei* that has been associated with virulence, and is observed as colony phenotype switching between isogenic morphotypes [31,72]. Different morphotypes exhibit different virulence *in vivo* [72], and prolonged exposure to iron was identified in one study as a possible trigger for colony phenotype switching that may lead to attenuation of some *B. pseudomallei* isolates in mice [31].

### 3.4. Small animal models for studying immune responses and immunity

Since the early studies of mice and hamsters by Dannenberg and Scott in the 1950s, several groups have utilised mouse and hamster models to analyse elements of host immunity and protection in melioidosis [18,47,73,74]. Analysis of gene expression of cytokines has been described in several mouse models of human melioidosis [37,75,76]. While there is a protective role for some elements of a Th1 response including interferon gamma (IFN-γ) in mice, comparing Th1-response-prone C57BL/6 mice with Th2-response-prone BALB/c mice does not link Th1–Th2 responses with resistance and susceptibility to *B. pseudomallei* [19,27,37]. Tumour necrosis factor (TNF)-α.
comprises part of the cytokine expression profile to \textit{B. pseudomallei} in mice \cite{37} and humans \cite{77,78}, and it plays a role in resistance to infection \cite{79,80}. However, TNF-\(\alpha\) is produced in lesser amounts in response to \textit{B. pseudomallei} lipopolysaccharide (LPS) as compared to other bacterial LPS \cite{81}. In mice, the LPS receptor Toll-like receptor (TLR) 4 may not play a major role in immunity, as shown by experiments using TLR4-deficient mice; TLR4-deficient mice are equivalent to wild-type mice in terms of bacterial growth and survival \cite{82}, as distinct from other infections \cite{83}. Interestingly, C3H/HeJ mice, which carry a loss-of-function mutation in the TLR4 gene, are resistant to extremely high doses of \textit{B. pseudomallei} LPS \cite{81}. In murine melioidosis, CD14 and TLR2-deficient mice display survival advantages associated with decreased bacterial loads, and lower cytokine responses \cite{82,84}.

The function of cytokines and lymphocytes in protection against \textit{B. pseudomallei} has been studied in several mouse models. In experiments based on a Taylor outbred mouse model, they demonstrated the central role of IFN-\(\gamma\) in early resistance to \textit{B. pseudomallei} infection \cite{79}. However, while IFN-\(\gamma\) is essential for innate resistance in Taylor outbred mice, the innate susceptibility of BALB/c mice is not due to a lack of, or delayed, production of IFN-\(\gamma\) as noted elsewhere \cite{37}. In fact, BALB/c mice express more IFN-\(\gamma\), compared to C57BL/6, which correlates with severe disease \cite{28,37} and parallels what is seen in patients with severe melioidosis \cite{85}. Hyperproduction of IFN-\(\gamma\) in BALB/c mice may be due to an excess production of interleukin (IL)-18 \cite{86}. NK cells and bystander CD8 T cells rapidly make IFN-\(\gamma\) in response to \textit{B. pseudomallei} in mice \cite{87}, and the T cell response is biphasic and essential for resistance \cite{46}. Macrophage-depleted mice are unable to contain infection \cite{88} and IFN-\(\gamma\) primes macrophages to kill \textit{B. pseudomallei}, which probably contributes to host resistance \cite{89,90}. CD4+ T cells appear particularly important in innate and adaptive immunity \cite{46,91} and any future vaccines will likely aim to generate IFN-\(\gamma\)-secreting T cells \cite{46,92}. Neutralisation of IL-12 and IL-18, which are major inducers of IFN-\(\gamma\), leads to increased mortality in mice \cite{46,79}, showing their vital role in protection \cite{93}.

Different strains of \textit{B. pseudomallei} induce variable levels of protection against subsequent challenge in mice \cite{44,73}. In a model of \textit{B. pseudomallei} infection in Syrian golden hamsters Brett et al. \cite{23} performed notable immunisation studies and identified potential vaccine targets \cite{49}. Immunisation of BALB/c mice with LPS or capsular polysaccharide (CPS) induces antibodies \cite{94}, and CPS subunit vaccines, including one comprised of the O-polysaccharide of LPS linked to flagellin \cite{95} provide partial protection in mice \cite{96}. In the diabetic infant rat model, antibodies to flagellin, LPS, and a tetanus toxin-polysaccharide glycoconjugate also provide passive protection \cite{49,95,96}. Conjugate DNA has also been tested in BALB/c mice as a possible immunisation strategy, and CpG-modified plasmin DNA encoding flagellin was shown to improve immunogenicity \cite{97,98}. Mutant \textit{B. pseudomallei} made auxotrophic for branched-chain amino acids were also shown to protect against a lethal dose of wild-type \textit{B. pseudomallei} in BALB/c mice \cite{99}. Finally, mice inoculated with a \textit{B. pseudomallei} BipD mutant were partially protected against subsequent challenge \cite{64}. Dendritic cells pulsed with \textit{B. pseudomallei} (Section VII.5) have been studied in mice as a possible vaccine-delivery vector since they provide heightened cellular responses to \textit{B. pseudomallei} \cite{92,100}. 
3.5. Small animal models for studying antimicrobial therapy

Antimicrobial therapy of melioidosis is of variable efficacy and combinations of drugs are often administered over a prolonged period [101]. Small animal models provide an ideal means with which to analyse antibiotic efficacy, as well as trial novel antimicrobial therapy strategies. The first study of antibiotic therapy for melioidosis in small animals used BALB/c mice and demonstrated a comparable level of efficacy of the first-line antibiotic ceftazidime versus cefpirome in combination with cotrimoxazole or chloramphenicol for systemic infection [102]. Modification of dosing in mice, as performed in such studies, is particularly useful to maintain plasma antibiotic levels at relevant concentrations for small animals [2].

Another model of systemic infection was subsequently used to compare outcomes between BALB/c mice given ceftazidime alone, or in combination with the potential immunotherapy adjuvant granulocyte colony stimulating factor (G-CSF), but failed to show any benefit from G-CSF [103]. More recently, tigecycline was used in BALB/c mice and was the most efficacious treatment combination when administered with ceftazidime [104]. These studies are good examples of the usefulness and limitations of small animal models for studies aimed at analysing antimicrobial therapy in *B. pseudomallei* infection.

4. Large animal models

There are few reports of experimental models of melioidosis in horses, cows and sheep. This Section focusses on goats, pigs and NHP.

4.1. Goats

Naturally occurring melioidosis in goats has been reviewed by Sprague and Neubauer [16]. Goats are considered to be susceptible to melioidosis and exhibit clinical signs including fever, anorexia, progressive emaciation, nasal discharge and paresis of the hind legs. The earliest report of the susceptibility of goats to experimental melioidosis was in 1925; however, little detail is provided [14]. The first comprehensive study compared the susceptibility of goats to subcutaneous (s.c.) or i.p. challenge with $6.5 \times 10^7$ or $6.5 \times 10^9$ colony forming units (CFU) of a goat derived strain of *B. pseudomallei* and involved observation for up to 23 days [105]. Differing disease progression occurred, depending on the route of infection: i.p. infection caused a septicemic disease with a short duration and scattered microabscesses throughout the body; s.c. infection caused chronic infection with no clinical signs, fewer fatalities and large local lesions in the spleen and lungs on post mortem. However, bacteria were recovered from at least one organ from all animals. Contrastingly, s.c. challenge with doses of greater than 500 CFU of another goat isolate of *B. pseudomallei* caused acute fatal disease [106]. More variable disease progression (acute or chronic) was observed in goats when doses lower than 500 CFU were used.

4.2. Pigs

Pigs are generally considered to be resistant to infection with *B. pseudomallei* [16]; however there are widespread reports of melioidosis in pigs in various endemic
regions [16,107]. In these studies, pigs present with one of two forms of the disease (acute or chronic), with young pigs generally being more susceptible to infection. Generally, the lungs are the most frequently affected organ, with the presence of either nodules or areas of consolidation being observed [16]. Lesions on the spleen or liver were either encapsulated with a thick, caseous material or coalescent, respectively [16].

Early experimental infection studies in pigs deemed them to be relatively resistant to the disease by a variety of routes, including oral, s.c., i.m. or i.n. [16,52]. More recent studies showed pigs to be susceptible to infection by the intratracheal (i.t.) and i.v. routes [16]. The i.t. studies compared the susceptibility of pigs experimentally immunosuppressed with cyclophosphamide and untreated pigs [16]. The animals were infected with $5 \times 10^8$ CFU of a porcine strain of B. pseudomallei and studied for up to 84 days. All but one pig had an increased temperature after 24 hours with the majority of the temperatures returning to normal by day 13 post-infection. The majority of the animal showed no overt clinical signs but 18/48 had respiratory distress and one animal had vomiting. Both acute or chronic disease were observed and confirmed by lung pathology, with animals suffering acute disease showing more evidence of respiratory distress. Immunosuppression did not significantly affect clinical signs, histopathology or bacteriology. However, all immunosuppressed animals were affected and were more likely to have acute disease.

Intravenous infection of pigs with $5 \times 10^9$ CFU of B. pseudomallei strain 2796 caused a generalised chronic infection [16]. No overt clinical signs were apparent but all animals were hypothermic on day 1 post-infection, though this gradually decreased with time. Clinical examinations revealed the presence of an increased erythrocyte sedimentation rate, tachypnoea, and leucopenia. Bacteria were recovered from the lungs of animals during the course of the study, with recovery from spleen, mesenteric lymph nodes and liver during the early phase of the disease.

4.3. Non-human primates

There are very few published studies on experimental infection of NHP with B. pseudomallei. However, naturally occurring infections have also been reported. As with all animal models, the susceptibility of NHPs to melioidosis is dependent on the route of infection, the challenge dose and the strain of B. pseudomallei. Cases of melioidosis occur in NHP in endemic regions, and reports of infections in monkeys imported from such regions are summarised by Sprague and Neubauer [16]. Melioidosis in imported monkeys has been reported in the United Kingdom and the United States of America, and naturally occurring melioidosis has been reported in captive animals in several regions, including Malaysia and Australia [16]. Generally, these monkeys present with a broad spectrum of clinical manifestations similar to human infection. Clinical findings include a variety of skin abscesses and organ lesions with granulomatous inflammation and necrosis with infiltration of neutrophils, macrophages and lymphocytes [16]. Incubation times vary between six months and 10 years [16].

The first reports of experimental infection of melioidosis in NHP were in 1925 involving a monkey which had B. pseudomallei mixed in with its food; the animal
survived but no details of any clinical presentation were published [14]. Subsequently, the same group infected a *Macacus cynomolgus* orally, via a pipette, with a culture of *B. pseudomallei* from a patient who had died of melioidosis. This animal remained healthy during the two-month observation period.

During later studies, three *Macaca mulata* were infected s.c. with three different doses of *B. pseudomallei* strain W294. Only the monkey receiving the highest concentration of bacteria (\(1.5 \times 10^6\) CFU) showed any clinical signs, and all animals survived the two-month observation period. The animal that had been injected with the highest dose had an abscess at the site of inoculation that drained after four days and complete healing was observed within two weeks. Elevated temperature and an increase in white blood cell count were also observed which subsided with the draining of the abscess. Eight days post-infection, the animal tested positive using a specific agglutination and complement fixation tests [17].

More recently, there has been an increase in the development of NHP models of melioidosis, including the use of the common marmoset (*Callithrix jacchus*) to study inhalational infection (Nelson M., personal observation). Marmosets were highly susceptible to infection, with few inhaled bacteria (<10 CFU) causing lethal infection. The course of the illness in the marmoset was acute (onset usually within 24 hours), with rapid progression and widespread bacterial dissemination. Characteristic histopathological changes were evident in the lung, liver, spleen and lymph nodes. The spread of the disease and clinical manifestations broadly concur with murine and human data [108,109]. However, the short time to death observed in mice and marmosets is not necessarily a feature of human disease and may be a factor that may primarily depend on the route of infection.

5. Conclusions

The animal models so far developed for human melioidosis have been invaluable to study susceptibility, pathogenesis, immunity, and treatment of disease. Collectively, the data derived to date from all of the studies on distinct animal models suggest that different aspects of human disease are mimicked more closely in some models than in others [21]. Data derived from animal models should therefore be viewed with appropriate consideration of the influence of the model itself and the nature of the model compared to human infection. Nevertheless, the animal models as described herein will continue to be valued by research groups conducting studies on melioidosis, with the goal of improving our understanding of this enigmatic disease.

References


Initiation of *Burkholderia pseudomallei* infection

Yunn Hwen Gan\(^a\), T. Eoin West\(^b\), Stitaya Sirisinha\(^c\)

\(^a\)Department of Biochemistry, Yong Loo Lin School of Medicine, National University of Singapore, Singapore
\(^b\)Department of Medicine, University of Washington School of Medicine, Seattle, WA, USA
\(^c\)Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand

Abstract

*Burkholderia pseudomallei* overcomes mucosal and epidermal defences to initiate human infection after inhalation, percutaneous inoculation, or ingestion. Several different bacterial metabolic pathways are upregulated to facilitate survival of *B. pseudomallei* within the mammalian host. Adhesion and invasion of *B. pseudomallei* to non-phagocytic cells may be attributable to putative bacterial virulence factors, including the capsule, lipopolysaccharide (LPS), flagella, pili, and type III secretion system (T3SS), but susceptibility to infection also varies among different host cell types. In phagocytes, *B. pseudomallei* rapidly escapes from the phagosome in a partly T3SS-dependent manner. In both non-phagocytic cells and in phagocytes, *B. pseudomallei* flourishes within the cytoplasm, suppressing key innate host defences. The role of T3SS in intracellular replication and survival remains controversial. *B. pseudomallei* further ensures successful initiation of infection by engaging in intercellular spread, avoiding components of the host immune system. This may be a type VI secretion system (T6SS)-mediated process involving the formation of actin-based membrane protrusions extending to neighbouring cells.

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1. Routes of infection

Melioidosis occurs upon exposure to a contaminated environment. The two main routes of infection commonly proposed in community-acquired disease are via the respiratory tract and percutaneous inoculation. Support for respiratory tract-initiated infection following aerosolisation of *B. pseudomallei* comes from studies showing a greater frequency of pneumonia and severe disease during the rainy season or after monsoon rains and winds [1,2]. In addition, disease in helicopter crewmen during the USA–Vietnam conflict also suggests that inhalation of bacteria results in disease [3]. Aspiration of bacteria into the respiratory tract is the presumed aetiology of melioidosis complicating near drowning events [4–6]. Percutaneous inoculation of abraded skin is also plausible, as disease incidence increases during the rainy season when rice farmers, frequently working...
barefoot, have greater contact with water and soil. Melioidosis following contamination of burns with soil has been reported [7]. Two Australian epidemiological studies raise questions about the frequency of community acquired infection by this route, however. In one study of patients presenting with melioidosis, only 25% recalled an inoculating event [8]. In a separate case–control study, there was no association between soil exposure and disease [9]. Another possible route of infection is via the gastrointestinal tract, such as following consumption of contaminated water. This is corroborated by the identification of genetically matched strains in domestic water supplies and in clinical isolates in clusters of disease, and by a report of mother-to-child transmission via breast milk [10–13]. Vertical transmission of melioidosis has also been attributed to transplacental spread or perinatal infection [14,15]. Thus, numerous routes of infection may be possible in community acquired disease. In two cases of disease occurring in laboratory settings, infection was thought to have occurred by the inhalation route, although, in one case, percutaneous inoculation could not be ruled out [16]. Animal models of disease have been established using methods of inhalation and oral ingestion, as well as intranasal, subcutaneous, intravenous and intraperitoneal inoculation [17–19].

Further complicating an understanding of the route of infection are the myriad of clinical features of melioidosis, as well as the highly variable incubation period and time course of clinically evident disease. The most common site of infection is the lung, but skin and soft tissue infection, solid organ abscesses, osteomyelitis, septic arthritis, peritonitis and central nervous system (CNS) involvement are all well documented presentations [8,20,21]. In Southeast Asia, parotitis is a frequent manifestation of disease in children [22]. Besides direct invasion of adjoining tissues, haematogenous spread is a clear manner of bacterial dissemination. Bacterial spread within nervous tissues may contribute to the CNS infections observed [8,23]. In humans, it has not yet been possible to link route of infection with clinical presentation in any reliable manner.

Reports of prolonged duration between exposure and development of apparent disease (up to 60 years in one case) suggest colonisation or subclinical infection with B. pseudomallei [24,25]. The variability of presenting clinical features and lack of knowledge about the route of infection greatly limits identification of potential sites of colonisation or early infection. Potential clues can be gleaned from studies documenting relapse in about 10% of melioidosis survivors [26]. Relapse episodes occur in similar organ distributions as the original episode [27], suggesting that colonisation or subclinical infection occurs at the site of initial infection, possibly within granulomas [28]. Notably, re-infection with a different bacterial strain also occurs in a similar distribution as the initial infection, emphasising the importance of host factors in disease [27].

2. Cellular entry and invasion

Given the proposed routes of infection, it is logical to suspect that host defences at mucosal surfaces and epithelium would dictate, at least in part, the outcome of the disease. Of course, the magnitude of bacterial load, together with inherent virulence of the organisms, would also influence the disease progression during the primary infection. Although innate immunity at the two most common sites of infection (respiratory tract
and skin), is important in determining the clinical outcome of initial infection, different lines of evidence, from both human infection and different animal models of experimental melioidosis, indicate that adaptive immunity still plays a significant role, particularly in recovery from primary infection and resistance to re-infection [29].

3. Overview of *Burkholderia pseudomallei* interactions with innate host defence

3.1. The respiratory tract

Prior to reaching their cellular targets, the pathogens entering via this route are exposed to a number of different antimicrobial components in the respiratory tract, such as lysozyme, complement proteins, defensins and cathelicidins. However, *B. pseudomallei* is a hardy organism and known to be resistant to the killing action of defensins and complement, although it is susceptible to cathelicidin LL-37 [30]. There are conflicting reports about the susceptibility of *B. pseudomallei* to defensins in the absence of LPS or a capsule. The presence of an iron-binding protein transferrin in the respiratory fluid may also interfere with *Burkholderia* infection. Transferrin can reduce the capacity of *B. cenocepacia* to invade a respiratory epithelial cell line and, although there is little information about the effect of transferrin on *B. pseudomallei*, the organism is rather resistant to the action of lactoferrin peptides [30]. If the pathogen survives these luminal host defences, contact then ensues with cellular targets, such as airway and alveolar epithelial cells and alveolar macrophages. On occasion, the pathogen may also encounter dendrites of lamina propria dendritic cells (DC) that protrude out into the respiratory tract lumen. It has been observed that these DC can be attracted by cytokines produced by activated airway epithelial cells. The DC can extend their dendrites between the tight junctions of epithelial cells. After sampling the luminal content, the dendrites retract, allowing the activated DC loaded with luminal content to migrate to regional lymph nodes to induce an adaptive response. Nasal-associated lymphoid tissue (NALT) also serves as a site of infection. A recent report showed that, following intranasal inoculation of *B. pseudomallei*, the organism could readily colonise and replicate in the nasal cavity [23]. In fact, these investigators detected the organisms in the respiratory epithelium, olfactory epithelium and associated nerve bundles which could represent a route for *B. pseudomallei* to reach the brain. In humans, *B. pseudomallei* has also been isolated and identified from throat swab [31]. Infection of upper respiratory tract lymphoid tissues is an promising area for future investigation, as it may provide some insights regarding the frequent finding of parotitis in Thai children [22], or explain some of the manifestations of CNS infection.

3.2. The cutaneous tissue

Much less is known about skin antimicrobial components, compared to the respiratory tract. In addition to an acidic, high salt environment characterised by diverse types of volatile organic acids, defensins and cathelicidin LL-37 are found in skin secretions, particularly in the setting of injury or inflammation. After overcoming these host elements, *B. pseudomallei* would most likely contact keratinocytes and epidermal Langerhans cells. If the organism penetrates below the epidermis, several other innate immune cells – such
as dermal DC, plasmacytoid dendritic cells (pDC), macrophages, NK cells and endothelial cells – would be encountered in the dermis. Several types of non-immune cells like fibroblasts may also influence the outcome of interaction between the pathogens and cells of the innate immune system. To the best of our knowledge, invasion of and survival of *B. pseudomallei* in keratinocytes or epidermal Langerhans cells has not been previously reported.

Keratinocytes themselves possess a number of innate immune receptors, such as pathogen recognition receptors (PRR), on their surface. In addition to the well-known surface receptors like Toll-like receptors (TLR), intracytoplasmic pathogen sensors like the Nod-like receptors (NLR) are also present, and they may act collaboratively to initiate a host response. Activation of receptors on the keratinocytes can lead to production of proinflammatory cytokines (e.g. IL-1, IL-6, IL-12 and TNF-α) and also type 1 interferon. Keratinocytes also produce antimicrobial peptides, like defensins and cathelicidin LL-37. Some of these mediators have chemotactic activity, attracting neutrophils and monocytes to the affected site.

Epidermal Langerhans cells are another cell type likely to be encountered by *B. pseudomallei* invading via the cutaneous route. Unlike the keratinocytes, these cells can dampen the inflammatory response and predominantly induce Th2-type cytokine production (e.g. IL-10 and IL-13). Therefore, their role in protection against microbial infections is rather limited and remains to be reevaluated. In addition, other DC subpopulation, like dermal DC and pDC in the dermis, can be attracted to the site of infection and induced to produce several mediators, such as, inducible nitric oxide synthase (iNOS) and TNF-α.

4. Interaction of *Burkholderia pseudomallei* with non-phagocytic respiratory cells

*Burkholderia pseudomallei* adheres to a number of animal and human epithelial cell lines, including those derived from alveolar, bronchial, laryngeal and oral tissues. Adhesion and invasion of *B. pseudomallei* to non-phagocytic cells has been attributed to the presence of a number of putative virulence factors such as LPS, capsule, flagella, T3SS proteins, pili, adhesins and many secretory products (reviewed in [32]). However, the molecular basis for adherence of *B. pseudomallei* to mammalian host cells is not known. The in vitro interactions of *B. pseudomallei* with cultured respiratory cell lines have been studied most extensively by several groups of investigators [33–35]. In one such study, it was found that a pili-defective mutant showed decreased adherence to cultured epithelial cells [34]. However, adherence and microcolony formation varied between strains of *B. pseudomallei* used [36]. In the latter report, these investigators demonstrated further that pilA expression, microcolony formation and host cell adhesion were temperature dependent (all were enhanced at a low temperature of 27ºC compared to 37ºC). By extrapolating the latter observation, it is not unrealistic to predict that acquisition of the infection by inhalation of *B. pseudomallei* present in its environmental niche can be highly efficient as the organisms propagating at low environmental temperature (below 37ºC) can readily adhere to their cellular target. Asialogangliosides GM1 and GM2 appear to be part of the host cell receptor complex [37].
We studied the interactions of \textit{B. pseudomallei} with the A549 respiratory epithelial cell line using LPS, capsule and flagellin mutants, and compared the results with those obtained with the wild-type strain in an attempt to identify potential component(s) responsible for adhesion to, and invasion of, non-phagocytic cells. Our unpublished observations showed that LPS was essential for both adhesion and invasion of this respiratory epithelial cell line. Flagella were needed for adhesion because the adhesion of flagellin mutant could be corrected by centrifugation, which facilitated initial contact. The adhesion to, and invasion of, A549 by the capsule mutant were indistinguishable from those exhibited by the wild-type bacteria.

5. Interaction of \textit{Burkholderia pseudomallei} with non-phagocytic epithelial and endothelial cells

Because there is no published information regarding the susceptibility of human keratinocytes to \textit{B. pseudomallei} infection, we used a human epidermal (keratinocyte) cell line (HaCat) as a model to study the invasiveness and survival of \textit{B. pseudomallei} \cite{38}. Comparisons were made with two other human epithelial cell lines, namely KB (oral) and NEC (oesophageal), and also with a human endothelial cell line (ECV340). The results presented in Table VII.1 show that, in general, all four epithelial cell lines are rather resistant to the infection by \textit{B. pseudomallei}, compared with our previous report using the A549 cell line. The endothelial cell, on the other hand, is more susceptible than HaCat, KB and NEC cell lines. It is logical to speculate that the ECV340 endothelial cells could represent a potential \textit{in vivo} target for the organisms during the natural course of infection, and possibly contribute to bacterial dissemination and cytokine storm noted in sepsis in some patients with severe melioidosis. This speculation is in accordance with our finding that, in addition to the direct infection of the ECV cells by \textit{B. pseudomallei}, these cells could also acquire the infection indirectly from infected macrophages, via cell-to-cell spread through cytoplasmic protrusion and multinucleated giant cell (MNGC) formation \cite{39,40}. In fact, in this study, it was noted that the keratinocytes could

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% Internalisation(^a) Mean</th>
<th>SE</th>
<th>Intracellular multiplication</th>
<th>Time when cytoplasmic protrusions appear (h)</th>
<th>MNGC formation</th>
</tr>
</thead>
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<tr>
<td>HaCat</td>
<td>0.0169</td>
<td>0.0051</td>
<td>Yes</td>
<td>14</td>
<td>Yes</td>
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<tr>
<td>ECV340</td>
<td>1.1457(^b)</td>
<td>0.4231</td>
<td>Yes</td>
<td>10</td>
<td>Yes</td>
</tr>
<tr>
<td>KB</td>
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<td>0.0100</td>
<td>Yes</td>
<td>24</td>
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</tr>
<tr>
<td>EC</td>
<td>0.0014</td>
<td>0.0004</td>
<td>Yes</td>
<td>36</td>
<td>Yes</td>
</tr>
</tbody>
</table>

\(^a\) Calculated from experiments performed with 4 strains of \textit{B. pseudomallei} (modified from Rattanachetkul et al. \cite{38}).

\(^b\) Significantly different from other cell lines (p < 0.001); HaCat, keratinocyte; ECV340, endothelial; KB, oral epithelial; NEC, oesophageal epithelial (all were of human origin).
also become indirectly infected from infected macrophages. Extrapolating from the fact that activated keratinocytes can secrete a number of cytokines and chemokines, these *B. pseudomallei*-infected keratinocytes could potentially attract neutrophils and monocytes to the site of infection to help modulate and combat the infection.

6. Interaction of *Burkholderia pseudomallei* with phagocytic cells

Very little is known about the initial steps following the interaction of *B. pseudomallei* with human phagocytic cells. There is some evidence suggesting that the presence of a capsule interferes with the uptake of *B. pseudomallei* by human neutrophils [41]. Although the role of other components like LPS and flagella may be important in the initial interaction and adherence to non-phagocytic cells, these components seem to be less important with the phagocytic cells [30]. Host cell receptors responsible for phagocytosis of *B. pseudomallei* have never been well characterised, although the receptor for the complement protein C3b on neutrophils may be involved [41]. We previously failed to detect, for example, the involvement of DC-SIGN on human DC that phagocytosed *B. pseudomallei* [42]. Several groups of investigators have reported on the interaction of *B. pseudomallei* with both primary macrophages [43] and macrophage cell lines [32,44,45]. In particular, significant information has been obtained from using the mouse macrophage cell line, RAW 264.7. These cells phagocytose *B. pseudomallei*, although internalised bacteria readily escape from phagocytic vacuoles. They flourish and multiply in the cytoplasm, and eventually induce cytoplasmic protrusions, thus enabling them to spread to neighbouring cells [39]. The bacteria survive and multiply as readily in the DC as in human macrophages [43]. However, both cell types exhibit enhanced killing activity and production of proinflammatory cytokines when they are pre-activated with interferon gamma (IFN-γ), pointing to the importance of this cytokine in host protection.

7. Survival and maintenance inside host cells

7.1. Bacterial gene reprogramming upon encounter with host

*Burkholderia pseudomallei* is a saprophyte that is capable of surviving in soil and water. When the environmental bacterium first encounters a mammalian host, it must reengineer itself drastically to enter and survive in this completely different setting. An array of different metabolic pathways must be engaged to ensure survival as well as to initiate and establish infection. Gene expression studies indicate that relevant pathways include those governing catabolism of D-glucosamine and inositol – both of which could serve as alternative carbon sources for carbohydrate metabolism in the host – as well as novel metabolic pathways for amino acid metabolism, and alternative energy metabolism pathways, such as ubiquinol oxidases [46]. Downregulation of other genes *in vivo*, such as flagellin, has been observed. This would be consistent with the hypothesis that flagellin expression is beneficial only in mobilising the bacteria in initial stages of infection and, perhaps, for spreading from one tissue site to another. Genes implicated in bacterial virulence in the mammalian host include those encoding capsular polysaccharide (CPS)
biosynthesis, DNA replication and repair, a putative oxidoreductase, ABC transporters, a lipoprotein and several amino acid and aromatic compound auxotrophs [47].

Several studies have profiled gene expression of *B. pseudomallei* under low iron conditions, as this is postulated to resemble the host environment. The induction of the siderophore pyochelin biosynthesis gene (*BPSS0588*) in livers of infected hamsters seems to support this hypothesis [46]. Bacteria grown under low iron conditions *in vitro* show upregulation of hydroxamate siderophore biosynthesis and transport genes (*BPSL1776–1774*) and heme-hemin receptor and transport genes (*BPSS0244, BPSS0243*) [48]. Under low iron conditions *in vitro* *BPSL1776* (*mbaA*) and *BPSL1774* (*mbaF*) have been identified as the biosynthetic genes of siderophore malleobactin, and *BPSL1775* (*fmtA*) as the transporter [49].

With the exception of the CPS genes, virulence factors that are known to directly contribute to bacterial virulence or to interference with host defence have not been identified, although two of the T3SS genes *bprA* and *bapC* are highly induced only in the livers and not in other organs of infected hamsters [46]. This is likely due to the low sensitivity of detection in organs. Since *B. pseudomallei* would be present as a mixture of extracellular and intracellular bacteria in the host, those genes which are induced only upon initiation of an intracellular life cycle might be overlooked. *In vivo* expression technology demonstrates that T6SS locus 5 (T6SS5) is highly induced upon bacterial invasion of macrophages [50]. A recent study in *Vibrio cholerae* showed that secretion of T6SS effectors was induced in endosomes of host cells [51]. Another prominent cluster of genes known to be induced upon host cell contact in other Gram-negative pathogens is the T3SS. We found that the genes in T3SS locus 3 (T3SS3) in *B. pseudomallei* were highly induced upon host cell contact when compared to other housekeeping genes [Gan YH et al., unpublished data]. Thus, both T3SS3 and T6SS5 facilitate the initiation of successful *B. pseudomallei* intracellular infection.

### 7.2. Escaping from the phagosome after cellular entry

Upon successful invasion of host cells, *B. pseudomallei* escapes from endocytic vesicles into the host cell cytosol as early as 15 minutes after internalisation by phagocytes [52,53]. This escape is only partially controlled by T3SS3, as mutants in T3SS genes, such as *bsaZ* and *bipD*, remained trapped in vesicles at earlier infection time points [54], but at 8 and 12 hours after infection, the *bsaQ* and *bsaZ* mutants, respectively, are able to escape into the cytosol [55,56]. Since all these T3SS3 mutants are very likely to have defective needle formation due to disruption in structural or translocon genes, the actual T3SS effector(s) responsible for the escape remains unidentified.

### 7.3. Maintaining a cytosolic lifestyle

The requirement of T3SS for bacterial intracellular replication and survival is debatable. Two studies found no differences in the intracellular bacterial counts between independently derived *bsaQ* mutants compared to wild-type bacteria [56,57]. A *bsaZ* mutant has lower intracellular bacterial loads at earlier time points but, at later time points, the bacterial load is comparable to wild-type bacteria, corresponding to the delayed
escape of the mutant from endosomes [55]. It is likely that the reported role of T3SS3 in intracellular replication [54] is due to the indirect consequence of bacteria being trapped within endosomes at early time points. It is also possible that a contribution to bacterial intracellular survival is mediated by the T3SS3 effector BopA under specific circumstances, as BopA enhances intracellular survival by evading cellular autophagy [58]. A transposon-insertion bsaU and a tssK (BPSS1509) mutant do not have any intracellular survival defects in host cells [59]. A clpV1 mutant (BPSS1502) also has normal intracellular replication and survival [50]. In B. mallei, a T6SS mutant (tssE) has significant growth defects in RAW 264.7 cells [60]. Although tssK and clpV1 both belong to the T6SS5 gene cluster, it remains to be seen whether other T6SS5 components contribute to intracellular replication and survival in B. pseudomallei. The alternative sigma factor RpoE contributes to survival in macrophages J774A.1 [61], although this may occur via indirect regulation of other unidentified genes necessary for intracellular survival.

The ability of the bacteria to remain alive intracellularly and to replicate in the hostile host environment attests to their successful evasion of cellular killing strategies. Some of these mechanisms include suppression of induced nitric oxide synthase (iNOS), nitric oxide and proinflammatory cytokine production [44]. In comparison with other Gram-negative bacteria, such as Salmonella enteritica serovar Typhi, some of these suppressive effects are attributable to the minimal induction of beta-interferon (IFN-β) by B. pseudomallei [45]. In addition, B. pseudomallei enhances the production of a number of negative regulators (e.g. SOCS3 and CIS2), which may interfere with the host cell response to infection [62]. Recently, TssM has been identified to be the bacterial effector responsible for downregulating NFκB activation and type I interferon signalling pathway in macrophages infected with B. pseudomallei, through the deubiquitination of key signalling intermediates, such as TRAF3, TRAF6 and IκBα. Mice acutely infected with the tssM mutant showed more inflammation and succumbed to infection with earlier kinetics [63].

8. Intracellular spreading and dissemination

The initiation of a successful infection not only requires survival of the bacteria within the host cell but also necessitates the dissemination of bacteria into new cells as infected cells die. B. pseudomallei is able to form membrane protrusions that extend to neighbouring cells via actin-mediated motility to facilitate intracellular spreading [39,64]. This ability has been attributed to BimA, a protein encoded by a gene located near the T6SS5 locus that is able to initiate actin polymerisation [40]. In B. mallei, bimA expression is regulated by virAG, a two-component regulatory system located in the T6SS5 locus [65]. It is thus possible that actin-mediated intracellular spreading mediated by BimA in B. pseudomallei is also under the control of virAG located in T6SS5.

Intracellular motility is postulated to facilitate intracellular spreading between cells while avoiding the host immune system. It is generally assumed that this spreading would result in cell fusion and the formation of MNGC [39]. However, currently there is no evidence to show that the two events are linked and triggered by the same bacterial factor,
although, in B. mallei, a T6SS mutant (tssE) was not only defective in forming MNGC but also in initiating actin polymerisation, even though it could escape the endosomes [60]. The previously reported absence of MNGC formation by the bipB mutant [66] would likely be due to the indirect consequence of the delayed endosomal escape phenotype. It would be instructive to determine whether components of T6SS5 in B. pseudomallei control MNGC formation. It is also unclear how MNGC formation contributes to disease pathogenesis in vivo, as this would require the identification of a mutant with a defect only in MNGC formation and not other aspects of cellular survival and host interaction.

References


Section VII.3

Host–pathogen interactions in melioidosis

W. Joost Wiersinga\textsuperscript{a,b}, Tassili Weehuizen\textsuperscript{a,b}, Katrin Breitbach\textsuperscript{c}, Ivo Steinmetz\textsuperscript{c}

\textsuperscript{a}Center for Infection and Immunity Amsterdam (CINIMA), Center for Experimental and Molecular Medicine,\textsuperscript{b}Department of Medicine, Academic Medical Center, Amsterdam, the Netherlands\textsuperscript{c}Friedrich Loeffler Institute of Medical Microbiology, Ernst Moritz Arndt University Greifswald, Greifswald, Germany

Abstract

This Section summarises current understanding on host–pathogen interactions in melioidosis, including new insights into the putative bacterial virulence factors, the intriguing intracellular life cycle of \textit{Burkholderia pseudomallei}, host recognition and innate immune responses.

The \textit{B. pseudomallei} genome has been described, as well as a whole range of putative virulence factors, including structural surface components, such as lipopolysaccharide (LPS), other surface polysaccharides and flagella. The proinflammatory immune response, initiated by pathogen recognition receptors has been further dissected. The clinical manifestations of melioidosis probably can be seen as the consequences of a pathogen recognition receptor-mediated dysregulation of the immune response to invading \textit{B. pseudomallei}. Studies on host–pathogen interactions in melioidosis have identified potential new treatment targets.

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1. Introduction

Despite a century of research on melioidosis and its causative agent \textit{B. pseudomallei}, a facultative intracellular pathogen, the burden of this disease remains unacceptably high \cite{1,2}. In endemic regions, such as northeast Thailand, infections caused by the aerobic Gram-negative \textit{B. pseudomallei} are still responsible for 20\% of all community-acquired septicaemias, and up to 40\% of sepsis related mortality. In the last decade, however, the research field has seen a large increase in groups working on the pathogenesis of melioidosis, in part because of increased interest in the pathogenesis of \textit{B. pseudomallei} following its classification as a potential biological threat weapon by the US Centers for Disease Control and Prevention \cite{1,2}. As a result, and in addition to the growing insights from animal models that more closely resemble clinical melioidosis, current knowledge of host–pathogen interactions and their consequences in melioidosis have increased tremendously. The subsequent identification of new potential treatment targets
can hopefully lower the mortality associated with melioidosis in the not too distant future. Here we summarise current knowledge on host–pathogen interactions during melioidosis, and the ensuing opportunities of potential therapeutics. This Section is partly an update of an earlier review [3].

2. Structural surface components as virulence factors and regulators of virulence

Recent work has provided new insights on how \( B. \) pseudomallei is capable of inflicting its dreadful consequences. Two studies, aimed to identify \( B. \) pseudomallei target proteins that elicit the humoral response in infected humans, identified an immunome of both surface and non-surface proteins which are upregulated during infection [4,5]. Whether these immunogens are also virulence factors, remains to be elucidated. \( B. \) pseudomallei has a high degree of phenotypic plasticity, and recent data suggest that complex changes in the phenotype of \( B. \) pseudomallei are associated with altered interactions with the host [6]. Switching between isogenic morphotypes occurred in a mouse model of melioidosis, and different morphotypes were characterised by different virulence in vivo [6].

2.1. Quorum sensing

Quorum sensing (QS) is a cell-density-dependent communication system in Gram-negative bacteria that uses \( N \)-acyl-homoserine lactones (AHL) for the coordination of gene expression [2]. The extracellular secretion of these AHL is highly dependent on the \( B. \) pseudomallei BpeAB-OprB efflux pump [7]. This efflux pump (Section VI) is notorious for being responsible for conferring antimicrobial resistance to aminoglycosides and macrolides as well as biofilm formation [2,8]. \( B. \) pseudomallei mutants lacking QS systems were demonstrated to have reduced virulence both in vitro, using diverse cell lines, and in vivo, in various animal models [2,9,10]. The \( B. \) pseudomallei QS system, called BpsIR, is composed of five bpsR genes and three bpsI genes [2,11].

2.2. Surface polysaccharides

\( Burkholderia \) pseudomallei expresses several surface polysaccharides. Next to capsular polysaccharide (CPS) and LPS, two other surface polysaccharides, termed type III and IV O-PS, have been identified [12]. Mutants defective in CPS and type IV O-PS significantly attenuate \( B. \) pseudomallei virulence in mice [12–14]. CPS might act as a barrier, blocking access of the complement receptor-1 to C3 [15]. Recently, three different LPS types among 1327 isolates from different geographic and clinical origins were identified by using gel electrophoresis (SDS-PAGE); two smooth LPS serotypes (A and B) possessing different ladder profiles, and a rough LPS without ladder appearance [16]. Of interest, the two less common types (smooth type B and rough type; together compromising 3% of isolates) were found more in clinical isolates, and the rough type had a higher capacity to produce biofilm [16]. In vitro studies in mouse macrophage cell lines using a LPS mutant suggested a possible role for the O-antigenic polysaccharide moiety of LPS in internalisation and intracellular survival of \( B. \) pseudomallei [17]. Compared to \( E. \) coli LPS, \( B. \) pseudomallei LPS is a less potent activator of macrophages [18,19]. Recently it
was found that both murine and human macrophages produced lower levels of TNF-α and IL-6 in response to *B. pseudomallei* LPS compared to *B. thailandensis* LPS [20]. This was explained by the authors that syntheses of lipid A species with different fatty acyl chains (long-chain FA C14:0(2-OH) and Ara4N-modified phosphate groups) could allow it to induce weaker immunological activities and thus evade early host defences [20]. The fact that *B. pseudomallei* is apparently less capable of activating immune cells might at least in part explain why Toll-like receptor 4 (TLR4), which is regarded as the LPS receptor, did not have a role in host defence against experimentally induced melioidosis in mice [21].

2.3. Flagella

Flagella are essential for *B. pseudomallei* motility [22–25]. *In vitro*, flagella clearly act as an immuno-stimulatory molecule causing upregulation of proinflammatory cytokines in both mononuclear cells and T cells [23,25]. In addition, flagella have been implicated in cell invasion of phagocytic and non-phagocytic cells [24]. Furthermore, in a mouse model of systemic infection, flagella-deficient *B. pseudomallei* has been shown to be less virulent compared to the wild-type strain [26]. Of interest, in a study, CpG-modified plasmid DNA encoding flagellin improved immunogenicity and provided partial protection against murine *B. pseudomallei* infection [22]. Of note, however, in a model of septic melioidosis using BALB/c mice in which mutant *B. pseudomallei* strains were compared with respect to virulence. It was observed that LPS and capsule mutants showed a marked reduction in virulence, while a flagellin mutant was only slightly less virulent than the parent strain, suggesting that LPS and capsule are more important virulence factors than flagella [27].

3. The intracellular life cycle of *Burkholderia pseudomallei* and corresponding virulence factors

*Burkholderia pseudomallei* can invade a range of phagocytic and non-phagocytic cells, and there is experimental evidence from *in vivo* models that the intact intracellular lifestyle is crucial to cause disease [28,29]. After invasion, this bacterium belongs to the few pathogens which are able to escape from the vacuole and start to replicate within the host cell cytosol. In this compartment, *B. pseudomallei* can induce actin rearrangement initiated at one pole of the bacterium, leading to intracellular actin tail formation and, finally, intercellular spreading [30]. In addition, *B. pseudomallei* exhibits the striking and, for a Gram-negative bacterium, unique ability to induce multinucleated giant cell formation (MNGC) [30,31]. In recent years, several genes have been identified that have been shown to play a role in survival of the pathogen in its intracellular niche. *B. pseudomallei* harbours a variety of distinct secretion systems, including three type III secretion systems (T3SS) [32,33]: T3SS1 and T3SS2 share similarities to the T3SS of the plant pathogen *Ralstonia solanacearum* [34], and have recently been shown to be involved in pathogenesis in a plant infection model [35], whereas T3SS3 resembles the inv/mxi/spa T3SS of *Salmonella* and *Shigella* and was shown to be essential for causing disease in mammalian models of infection [36].
Mutants affecting components of the T3SS3 secretion and translocation apparatus (bsa) were impaired in escaping from the vacuole, intracellular replication and the induction of MNGC [29,36−38]. A mutant lacking the apparatus component, bsaQ, showed defects in secreting the effector protein, BopE, suggesting that impaired secretion of effector components might contribute to the observed phenotypes [37]. BopE shares homologies with the *Salmonella* SopE/E2 protein, and was reported to exhibit guanine nucleotide exchange factor activities and contribute to the ability of *B. pseudomallei* to invade HeLa cells [39]. Another effector protein, BopA, was shown to be involved in the evasion of autophagy, thereby protecting the pathogen from innate bactericidal mechanisms of the host [40].

Beside three T3SS, *B. pseudomallei* additionally harbours six type VI secretion systems, of which at least one has been implicated in macrophages invasion and *in vivo* virulence [29,41]. Once inside the cytosol, *B. pseudomallei* is capable of inducing actin tails that enable the bacterium to move inside the host cell and, eventually, lead to the formation of membrane protrusions and direct cell-to-cell spread [30,42]. The gene which is essential for this process has recently been identified and termed *bimA* [43]. *B. pseudomallei* actin tail formation is independent of N-WASP and Ena/VASP proteins [42], but the exact role of the Arp2/3 complex, a key factor for actin tail formation in other pathogens such as *Shigella* and *Listeria*, is still unclear. BimA could not be shown to induce actin polymerisation in an Arp2/3-dependent manner *in vitro*, although several subunits of the Arp2/3 complex are present throughout the actin tails [42,43]. Thus, it is likely that additional co-factors on either the host, and/or the bacterial side, are involved in *B. pseudomallei*-mediated actin tail formation that also lead to the recruitment of the Arp2/3 complex to initiate actin polymerisation.

4. Innate immune defences towards *Burkholderia pseudomallei* infection

Innate and adaptive immune mechanisms are critical in determining the outcome of bacterial infection (Section VII.4). The compartmentalised gene expression profiles of important inflammatory molecules have been described in murine and human melioidosis [44,45]. Regarding essential innate immune mechanisms that contribute to resistance against melioidosis, it was shown that IFN-γ has a key role, with additionally vital roles played by TNF-α, IL-12 and IL-18 [2,46−49]. The proinflammatory caspase-1 was shown to contribute to resistance, possibly by promoting IFN-γ [50]. Depletion experiments in murine studies revealed that neutrophils are crucial for resistance against the pathogen [49]. Activated neutrophils are rapidly recruited to the primary site of infection after *B. pseudomallei* challenge [49,51−53] and myeloid-differentiation-primary-response-gene-88 (MyD88), the key signalling adaptor for most TLR, the IL-1 receptor and IL-18 receptor, is crucially involved in protective neutrophil recruitment [53]. In a randomised, placebo-controlled trial involving 60 patients with severe melioidosis, granulocyte colony stimulating factor treatment, which increases neutrophil production, had no effect on mortality, but was associated with a slightly longer duration of survival [54]. Of note, macrophage depletion also led to highly increased susceptibility in mice [55]. The IFN-γ-mediated effector molecule, nitric oxide (NO),
does not seem to be crucial for resistance. However, NADPH-oxidase, which plays a role in both neutrophils and macrophages to eliminate bacterial pathogens, is important for resistance [55]. Interestingly, caspase-1 seems to have an important function in macrophages in the prevention of rapid intracellular growth of *B. pseudomallei* by a still unknown mechanism [50]. Murine studies, in which the CD4+ and CD8+ cells were depleted, have shown that T cells, in particular CD4+ T cells, are important in both innate and adaptive immunity against *B. pseudomallei* infection [46,56]. Of importance, however, is that no association has been observed between HIV infection and melioidosis [57]. Antibody formation is important for protection against *B. pseudomallei* infection, but it does not seem to be essential for primary resistance [46,58,59]. Lastly, macrophage migration inhibitory factor (MIF) has emerged as a pivotal mediator of innate immunity and has been shown to be an important effector molecule in severe sepsis. MIF concentrations are markedly elevated during clinical melioidosis and correlate with patients’ outcomes [60]. In experimental melioidosis, MIF seems to modestly impair antibacterial defence [60].

### 4.1. Recognition of *Burkholderia pseudomallei*

The TLRs, together with other pattern recognition receptors (PRR), are the first to detect host invasion by pathogens, initiate immune responses and form the crucial link between innate and adaptive immunity (Figures VII.1, VII.2) [61]. PRR recognise conserved mo-

![Fig. VII.1. Encounter between *B. pseudomallei* (arrows) and a mouse monocyte (scanning electron microscope picture; J. van Marle, Cell Biology and Histology, AMC, Amsterdam).](image-url)
Host–pathogen interactions in *B. pseudomallei* infection: where bacterial virulence meets the immune system. Proposed scheme of the first encounter between *B. pseudomallei* and the immune system. Putative virulence factors on the bacterial cell surface include quorum sensing, type III secretion system (T3SS), lipopolysaccharide (LPS), capsular polysaccharides, flagella, bacterial DNA (CpG), actin tail formation and virulence factors essential for the intracellular life cycle of *B. pseudomallei* and other putative virulence factors. Mononuclear cells are probably the most important immune cells in early infection. The Toll-like receptors (TLR) work together in recognizing the pathogen-associated-molecular-patterns (PAMP) among which are they virulence factors on *B. pseudomallei*. TREM-1, of which the natural ligand is still unknown, causes amplification of the TLR initiated inflammatory response. The complement receptors CR1, CR3 and possibly FcγR mediate opsonin-dependent phagocytosis. Recognition of *B. pseudomallei* will cause activation of proinflammatory genes via nuclear-factor-κB (NF-κB) and will lead to the activation of the immune response via the release of pro-inflammatory cytokines. This will lead to activation of the innate and adaptive immunity together with activation of the complement- and coagulation system. Immunosuppression and apoptosis of immune cells will follow in order to counteract on the initiated overwhelming proinflammatory immune response. IL-1R-associated-kinase-M (IRAK-M) is a negative regulator of the TLR cascade.

**Fig. VII.2.** Host–pathogen interactions in *B. pseudomallei* infection: where bacterial virulence meets the immune system. Proposed scheme of the first encounter between *B. pseudomallei* and the immune system. Putative virulence factors on the bacterial cell surface include quorum sensing, type III secretion system (T3SS), lipopolysaccharide (LPS), capsular polysaccharides, flagella, bacterial DNA (CpG), actin tail formation and virulence factors essential for the intracellular life cycle of *B. pseudomallei* and other putative virulence factors. Mononuclear cells are probably the most important immune cells in early infection. The Toll-like receptors (TLR) work together in recognizing the pathogen-associated-molecular-patterns (PAMP) among which are they virulence factors on *B. pseudomallei*. TREM-1, of which the natural ligand is still unknown, causes amplification of the TLR initiated inflammatory response. The complement receptors CR1, CR3 and possibly FcγR mediate opsonin-dependent phagocytosis. Recognition of *B. pseudomallei* will cause activation of proinflammatory genes via nuclear-factor-κB (NF-κB) and will lead to the activation of the immune response via the release of pro-inflammatory cytokines. This will lead to activation of the innate and adaptive immunity together with activation of the complement- and coagulation system. Immunosuppression and apoptosis of immune cells will follow in order to counteract on the initiated overwhelming proinflammatory immune response. IL-1R-associated-kinase-M (IRAK-M) is a negative regulator of the TLR cascade.

**Burkholderia pseudomallei** expresses various potential PAMPs, including LPS, lipid A, peptidoglycan, flagella, T3SS and DNA. Different components of one microorganism are recognised by different TLR and related molecules such as CD14 and MD-2. Not surprisingly, an upregulation of CD14, MD-2, TLR1, TLR2, TLR3, TLR4, TLR5, TLR8, and TLR10 is seen in patients with melioidosis [21]. The finding that mice lacking MyD88, the key TLR adaptor protein, demonstrate a strongly accelerated mortality during experimental melioidosis further supports a critical role for TLR in disease pathogenesis [53]. Furthermore, MyD88 regulates TNF-α production in response to *B. pseudomallei* [18,53]. *B. pseudomallei* can activate nuclear-factor-κB (NF-κB) via TLR2 (probably in combination with TLR1 or TLR6), TLR4, and TLR5 [18,21,62]. Whether the LPS of *B. pseudomallei* signals via TLR2, such as the LPS of *Legionella pneumophila*, *Pseudomonas aeruginosa* and *Leptospira interrogans* [21,63,64], or via TLR4 as with all other Gram-negative bacteria [21,61], remains the subject of intense study. Methods and strains used, and the possibility of contamination with other LPS or lipoproteins, should be carefully monitored in these studies. Surprisingly, during experimental melioidosis, CD14- and TLR2-deficient mice display a markedly improved host defence, as reflected by strong survival advantages, together with decreased bacterial loads, reduced proinflammatory cytokine levels and reduced organ injury [21,65]. In contrast, TLR4-deficient mice are indistinguishable from wild-type mice with respect to bacterial outgrowth and survival in experimentally induced melioidosis [21]. This undermines the paradigm in immunology that TLR4 is the main receptor for Gram-negative bacteria.

**Burkholderia pseudomallei** is capable of activating NF-κB without the TLRs [62]. Using HEK cells, no contribution towards *B. pseudomallei* infection of the intracellular PRRs NOD1/2 was seen in vitro [62]. In the same cell system, however, mitogen-activated-protein-kinase (MAPK) inhibitors cause impaired IL-8 induction by *B. pseudomallei* and reduced bacterial internalisation, pointing towards activation of...
the MAPK pathway [62]. Interestingly, this TLR-independent IL-8 induction required a functional T3SS. The inflammasome may play an important role in *B. pseudomallei* infection. Inflammasome is a large multiprotein complex formed, among others, by NOD-like receptors whose assembly can lead to the activation of caspase-1, which promotes the maturation of proinflammatory cytokines IL-1β, IL-18 and IL-33 [66]. *B. pseudomallei* was demonstrated to induce rapid cell death (pyroptosis) in macrophages in a caspase-1-dependent manner [67], and a recent report identified the T3SS apparatus component BsaK to be a ligand for the NLRC4 inflammasome [68]. Beside caspase-1-mediated pyroptosis, *B. pseudomallei* is also considered to induce “classical” apoptosis in macrophages [30,38]. Macrophages lacking caspase-1 have been shown to undergo caspase-3-dependent apoptosis [50].

### 4.2. Regulation of the innate immune response in melioidosis

Various mechanisms do exist that regulate the immune response following the recognition of *B. pseudomallei*. Triggering-receptor-expressed-on-myeloid-cells (TREM)-1, which is known to be an amplifier of TLRs, is upregulated during both human and murine melioidosis [69]. Purified human TREM-1 negative granulocytes showed reduced responsiveness to *B. pseudomallei* relative to TREM-1-positive granulocytes [69]. Next to amplification, negative regulators of the TLR also exist [61]. For instance, IL-1R-associated-kinase-M (IRAK-M) is a negative regulator of the TLR-cascade and has been found to be upregulated in melioidosis and to be associated with an immunosuppressed state (Wiersinga WI, et al., unpublished observations). Another mechanism that limits collateral damage is apoptosis, a physiological process by which cells are eliminated in a controlled manner. Intriguingly, *B. pseudomallei* is also able to induce apoptosis by itself [2,67]. Autophagy, a process by which intracellular bacteria are sequestered into double-membraned vesicles which are subsequently eliminated, suppresses the intracellular survival of *B. pseudomallei* [40]. The bacterium attempts to suppress autophagy via a T3SS protein, named *B. pseudomallei* BopA [40].

### 4.3. Coagulation and fibrinolysis during melioidosis

Accumulating evidence demonstrates a bidirectional interaction between coagulation and inflammation during severe bacterial infection [70]. The proinflammatory cytokines, TNF-α, IL-1, IL-6 and IL-12, all of which have been shown to be upregulated during melioidosis [2,44], are capable of activating the coagulation system *in vivo*. Indeed, the coagulation system is strongly activated in severe melioidosis, involving all three major pathways, with concurrent enhancement of procoagulant and impairment of anticoagulant and fibrinolytic mechanisms [71]. The clinical importance of these findings is underscored by the notion that the extent of coagulation activation correlates with mortality, shedding light on one of the possible underlying mechanisms responsible for the high mortality observed in melioidosis [71]. Urokinase receptor (uPAR, CD87), a glycosylphosphatidylinositol-anchored protein, is considered to play an important role in inflammation and fibrinolysis. Recently, we found that uPAR deficiency does not influence the haemostatic and fibrinolytic responses during melioidosis. However, uPAR
is involved in the host defence in melioidosis by facilitating the migration of neutrophils towards the primary site of infection and, subsequently, facilitating the phagocytosis of \textit{B. pseudomallei} [72]. These insights provide a new avenue for research focussing on the interplay between coagulation and inflammation. Recently, for the first time a patient with severe melioidosis was reported to have been successfully treated with activated protein C [73]. Since patients with melioidosis have low circulating levels of protein C which correspond with poor outcome this could be an effective therapeutic strategy [71,74].

5. Conclusions

\textit{Burkholderia pseudomallei} is a facultative intracellular pathogen that can invade host cells, escape from endocytic vesicles, multiply intracellularly, induce actin-tail formation and membrane protrusions, leading to direct cell-to-cell spreading. A number of new virulence genes have been identified which are essential for the intracellular life cycle of \textit{B. pseudomallei} and its pathogenicity. Melioidosis can be described as the clinical manifestation of a TLR-orchestrated dysregulation of the immune response to invading \textit{B. pseudomallei}. However, despite the major advances in our understanding of important virulence factors, and the host response during melioidosis, large gaps remain in our understanding of the pathogenesis of this bewildering infection. Nonetheless, studies on immunity to \textit{B. pseudomallei} have identified many new attractive possible therapeutic targets.

Future approaches to therapeutics should target virulence factors of \textit{B. pseudomallei} and involve immunomodulatory agents directed against the host response. For instance, QS systems and inhibition of the BpeAB-OprB efflux pump are attractive targets for the development of novel antimicrobials for the treatment of \textit{B. pseudomallei} infection [7,75]. With respect to host responses, it was shown in a murine model that treatment with IFN-\(\gamma\) and ceftazidime elicited strong synergistic inhibition of \textit{B. pseudomallei} growth, demonstrating that immunotherapy with IFN-\(\gamma\) could significantly increase the effectiveness of conventional antimicrobial therapy for treatment of acute \textit{B. pseudomallei} infection [76]. Granulocyte colony stimulating factor (G-CSF) increases neutrophil counts and stimulates neutrophil function and has been considered as an adjunct for treatment of melioidosis [54]. Given the findings that CD14- and TLR2-deficient mice are partially protected against a lethal dose of \textit{B. pseudomallei} one could postulate that inhibition of CD14 or TLR2, in addition to antibiotics, could be advantageous [21,65]. Lastly, the role of coagulation and fibrinolysis in melioidosis is a new and exciting research area in which new treatment targets are being evaluated in preclinical models. One hopes, but also anticipates, that over the next 100 years, new approaches will be developed that will efficiently control this infection.

References


Melioidosis – A Century of Observation and Research


Section VII.4
Development of protection

Jodie L. Morris, Kelly A. Hodgson, Natkunam Ketheesan
Microbiology and Immunology, School of Veterinary and Biomedical Sciences,
James Cook University, Townsville, Australia

Abstract
The immune system plays a pivotal role in orchestrating the host response that dictates the outcome of Burkholderia pseudomallei infection. Despite a century of research, there remain many deficits in our understanding of the host immune responses underlying susceptibility and resistance to this bacterium. In this Section, the current understanding of why certain individuals are more susceptible to this disease, and the possible impact of selected risk factors on the host immune response to the pathogen, is described. An outline of how early host–pathogen interactions set the stage for activation of adaptive immune responses and resolution of the infection is provided. Potential avenues for filling the important gaps in our current understanding of host–pathogen interactions are briefly discussed.

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1. Host susceptibility to Burkholderia pseudomallei infection

Burkholderia pseudomallei causes a spectrum of disease symptoms across a wide host range [1]. Several studies have demonstrated the influence of host genetics and environmental factors on susceptibility to this tropical infection [2–8]. Melioidosis is more prevalent in males, presumably due to a higher risk of occupational exposure for this gender [3]. The main co-morbid conditions that are associated with increased risk for B. pseudomallei infection are diabetes, high alcohol intake, renal disease, lung disease, liver disease, cancer, or steroid therapy [8]. Recent analysis of 540 cases from the 20-year Darwin Prospective Melioidosis Study demonstrated that, compared to individuals with no risk factors, those with diabetes are more likely to present with acute melioidosis pneumonia, progressing rapidly to septic shock and fatal outcome [8]. One or more identified risk factors were associated with all fatal melioidosis cases, with diabetes being the most frequent co-morbid condition [8]. A common feature of the risk factors for melioidosis is their association with functional defects in cells of the immune system, such as impaired phagocytic and migrational response of polymorphonuclear cells (PMN) [9]. Defects in PMN function in diabetes, excess alcohol intake and renal disease are well
described and were the basis for trialling therapy with granulocyte colony stimulating factor (G-CSF) in melioidosis [10]. However, much remains to be discovered concerning the specific immunological mechanisms influencing host susceptibility to *B. pseudomallei* infection.

Toll-like receptors (TLR) play a central role in initiating and regulating innate immune responses, thereby influencing disease progression (Sections VII.2, VII.3). Recently, West et al. demonstrated a significant association between TLR polymorphisms and susceptibility to melioidosis [11]. Whilst protection from melioidosis was associated with the TLR4<sup>1196C>T</sup> variant, additional TLR4 variants were associated with susceptibility to *B. pseudomallei* infection, as were polymorphisms for TLR1 [11]. Furthermore, diabetes–melioidosis comorbidity was linked to TLR6<sup>−1−10</sup> region variants. The study by West and colleagues highlights the significant role TLRs play in host susceptibility to melioidosis, and will no doubt stimulate great interest over the next decade into investigation of TLR signalling pathways responsible for contrasting immune responses and disease progression following infection with *B. pseudomallei* [11].

Major histocompatibility complexes (MHC) in humans, human leucocyte antigens (HLA), are another group of highly polymorphic cell-surface molecules that control activation of antigen-specific T cells. Therefore, polymorphisms in these alleles have important effects on initiating adaptive cellular immunity to pathogens, with potential to divide individuals into ‘resistant’ and ‘susceptible’ groups. In tuberculosis and leprosy, protective immunity and clinical manifestations of infection are governed by HLA alleles [12]. Similarly, Dharakul et al. demonstrated an association between septicemic and relapsing melioidosis, and certain HLA alleles in a Thai population [13]. Additional larger studies, particularly in other regions endemic for melioidosis, will be important to expand on the significance of this finding.

Studies in both patients and animal models of melioidosis have demonstrated a link between severity of disease and the levels of cytokines and chemokines in serum, including TNF-α and IFN-γ [14–17]. Polymorphisms of the TNF-α gene directly influence TNF-α production and secretion, with individuals with the TNF2 allele producing higher levels of TNF-α in response to various stimuli, than individuals with the TNF1 allele. A single study, conducted in northeastern Thailand, demonstrated a significantly higher frequency of the TNF2 allele for patients with severe septic melioidosis compared to healthy, uninfected controls [18]. No association was found between TNF-β allelic variations and clinical manifestations of *B. pseudomallei* infection [18].

There remains much opportunity for further investigation of host factors contributing to susceptibility and outcome from *B. pseudomallei* infection. Given recent evidence of the importance of early cellular immune responses in controlling *B. pseudomallei* dissemination [19], and findings from DNA polymorphism studies [11,13,18], future investigations into allelic variations associated with innate receptors (e.g. mannose binding proteins, TLR, NK cell receptors), macrophage response genes and IFN-γ and TNF-α signalling pathways are warranted. This is likely to be an expanding field of research over the next decade.
2. Regulation of adaptive immune responses by cytokines and cells of the innate immune system

Macrophages and PMN are pivotal in bacterial containment at infection sites. They also play a central role in triggering subsequent adaptive immune responses. *B. pseudomallei* is able to invade and persist within many host cells, including macrophages and PMN [20] (Figure VII.3). We are only beginning to appreciate how the complex interaction between this bacterium and cells of the innate immune response subsequently influences later immune responses and determines disease progression. Whilst inflammation is necessary for early bacterial containment, uncontrolled amplification of this process can result in bystander damage to tissues. The mechanisms controlling excessive inflammatory responses in melioidosis are poorly understood. Animal infection models play an important role for investigations into the pathogenic mechanisms of melioidosis. The BALB/c and C57BL/6 mouse models mimics the acute and chronic forms of human melioidosis, respectively. Compared to BALB/c mice, C57BL/6 mice are less susceptible to *B. pseudomallei* infection, but not completely resistant [21]. This differential susceptibility is maintained regardless of the route of *B. pseudomallei* infection [21–26]. Hyper-production of proinflammatory cytokines in BALB/c mice parallel increased inflammatory cytokine responses described in human melioidosis, and is associated with bacterial dissemination and poor prognosis [16,25]. Resistance of C57BL/6 mice to *B. pseudomallei* appears to be related to more efficient control of bacterial growth at the site of infection, particularly by tissue macrophages [23].

Disparate expression of mRNA for several chemokines and colony stimulating factors in murine models of acute and chronic melioidosis is associated with differences in cellular infiltrates to sites of infection [27]. Compared to C57BL/6 mice, sites of inflammation in BALB/c mice contain a higher ratio of neutrophils to mononuclear

Fig. VII.3. *Burkholderia pseudomallei* (arrow) is able to invade, persist and replicate within human macrophages and polymorphonuclear cells (Giemsa stain, magnification 100×, J. Morris, James Cook University, Australia).
cells, suggesting there may be an inherent failure to attract and activate the “correct” immune cell types during early stages of infection [17,26,27]. The activation of innate immune cells via pattern recognition receptors signals the production of inflammatory cytokines and chemokines, particularly IFN-γ and TNF-α. The kinetics and concentration of various cytokines are critical in determining the balance between resistance to infection or resultant immunopathology [14–16,22,23,25,26,28,29]. Studies in both patients and animal models of melioidosis have demonstrated a link between severity of disease and the levels of cytokines and chemokines in serum [14,16]. While pro-inflammatory cytokines are essential for protection against B. pseudomallei infection, hyperproduction of pro-inflammatory cytokines, such as IFN-γ, TNF-α, IL-1β and IL-6, correlates with rapid bacterial dissemination and multiplication in acute melioidosis, with mortality occurring within 96 hours [14–16,23,25,26,28,29].

Diabetes is the most common risk factor associated with melioidosis. Although defects in the early immune responses are considered to be important factors responsible for susceptibility to B. pseudomallei infection in diabetics, the precise mechanisms remain unclear (Figure VII.4). Recently, the first animal model of type 2 diabetes and melioidosis comorbidity was characterised [30]. Mice with type 2 diabetes are more susceptible
to subcutaneous infection with *B. pseudomallei* than non-diabetic mice. The increased severity of infection is associated with higher expression of proinflammatory cytokines and decreased blood glucose, responses frequently described in sepsis. Poor outcome following *B. pseudomallei* infection in diabetic mice corresponds to decreased phagocytic and antimicrobial capacity of macrophages, highlighting the importance of early immune responses in controlling bacterial dissemination [30].

In experimental melioidosis, failure to produce IFN-γ in the first 24 hours of infection results in overwhelming septicaemia and death of the host [31]. This is supported by studies in diabetic mice, where lower mRNA levels of IFN-γ at the infection site at 24 h after exposure to *B. pseudomallei*, precedes bacteraemia and mortality in these mice, when compared to non-diabetic littermates (Hodgson KA, et al., unpublished data). IFN-γ, a potent activator of macrophages, is produced predominantly by NK and CD8+ T cells [25,32]. TNF-α, produced predominantly by macrophages, neutrophils and T cells, is also important in conferring resistance to *B. pseudomallei* infection [17,33]. The biological response to TNF-α is mediated by two forms of cell surface receptors, TNF receptor-1 and TNF receptor-2, both of which are essential for mediating the effects of TNF-α in melioidosis [17]. Continued production of TNF-α from macrophages and neutrophils at sites of infection enhances their activation and, through the release of chemokines, triggers the recruitment of dendritic cells (DC) and other inflammatory cells. In the early stages of the innate immune response, TNF-α works synergistically with IFN-γ to induce macrophage activation.

Macrophages are often the first host immune cells to infiltrate sites of infection, playing a dual role in bacterial clearance by interacting directly with bacteria and releasing potent chemokines and cytokines, including TNF-α. Electron microscopy studies demonstrated increased uptake of *B. pseudomallei* and bactericidal activity in macrophages from healthy individuals compared to macrophages derived from patients with melioidosis, although bacterial replication and macrophage rupture was eventually observed in macrophages from both groups [34]. Peritoneal elicited cells (PEC), derived from C57BL/6 mice, are more efficient at inhibiting *B. pseudomallei* proliferation than BALB/c-derived PEC [21]. Similarly, while uptake of *B. pseudomallei* by bone marrow-derived macrophages (BMDM) is equivalent for susceptible BALB/c and partially resistant C57BL/6 mice, intracellular bacterial killing is higher in IFN-γ-stimulated BMDM derived from C57BL/6 mice [33]. The central role of macrophages in orchestrating an effective immune response to this bacterium is highlighted by the observation that in vivo depletion of macrophages or TNF-α in partially resistant C57BL/6 mice increases their susceptibility to *B. pseudomallei* infection [17,33].

Antimicrobial mediators produced by macrophages in response to *B. pseudomallei* include reactive nitrogen intermediates (RNI), reactive oxygen intermediates (ROI) and inducible nitric oxide synthase (iNOS). Intracellular growth of *B. pseudomallei* is suppressed following IFN-γ activation of macrophages and increased expression of inducible iNOS [35]. However, *B. pseudomallei* has been shown to invade and replicate within both unstimulated and stimulated macrophages, without activating iNOS [33,35–37]. Investigation of the mechanisms employed by *B. pseudomallei* to evade macrophage killing led to the identification of RpoS, a global regulator found in
Gram-negative bacteria. RpoS is considered to be involved in regulating multinuclear giant cell (MNGC) formation, interfering with macrophage iNOS expression and inducing apoptotic cell death in macrophages [38,39]. In addition, there is recent evidence that B. pseudomallei is able to evade lysosomal killing within macrophages by escaping to the cytosol and resisting uptake by autophagosomes [40]. Continued elucidation of additional mechanisms used by B. pseudomallei to persist within phagocytes will provide further insight into the immune response pathways important in control and clearance of this pathogen.

There have been limited studies investigating the role of dendritic cells (DC) during B. pseudomallei infection, despite the importance of DC as antigen-presenting cells in other bacterial infections. Recently, it was shown that BALB/c-derived DC mature in the presence of B. pseudomallei, although the uptake and survival of the bacteria in BALB/c DC differs significantly from that in DC isolated from C57BL/6 mice [41]. The role of DC as potent stimulators of T cell activation has been exploited as potential vectors for immunisation [42,43], with partial protection provided to immunised mice (Section VII.5). However, a major impediment to vaccine studies using DC has been the lack of basic information regarding the interaction between DC and B. pseudomallei, and the role this has, if any, in providing protection against B. pseudomallei.

In intracellular bacterial infections, the successful elimination of the pathogen is highly dependent on an efficient interaction between antigen-presenting cells and antigen-specific T cells. The inflammatory mechanisms of innate resistance set the stage for T cell differentiation. Further characterisation of cellular infiltrates within sites of B. pseudomallei infection, and the interaction between PMN, macrophages, DC and T cells within the susceptible host phenotype, will be valuable to our understanding of which immune cells and mechanisms are involved in the development of protective, adaptive immune responses. Also, the interaction of individual bacterial products with the host immune system will be an important avenue for future melioidosis research to identify potential candidate molecules for inclusion into subunit vaccines, or improvements to diagnostic assays.

3. Cellular immune responses to Burkholderia pseudomallei

Infection with B. pseudomallei can manifest across a spectrum of disease states ranging from acute, to chronic, to asymptomatic states, with potential for reactivation of clinically apparent disease after long periods of ‘dormancy’ and protective seropositivity [44]. The immunopathogenesis of B. pseudomallei infection, and how it relates to disease outcome, remains poorly characterised. Innate immune responses are responsible for the initial containment of infection. However, successful elimination of intracellular pathogens, such as B. pseudomallei, is crucially dependent on the activation of cell-mediated immune (CMI) responses, since they must either be prevented from invading host cells, or detected within them and eliminated [45]. Control of infections caused by intracellular pathogens can only be achieved with a CMI response governed by Th1 cells. Th1-type cells characteristically secrete IL-2 and IFN-γ, resulting in potent activation of phagocyte bactericidal activity. In contrast, Th2 cells are predominantly
involved in stimulating B cells and produce anti-inflammatory cytokines, such as IL-4 and IL-10 [46]. Strong Th1 immunity is believed to contribute to the partial resistance of C57BL/6 mice to *B. pseudomallei* infection, in contrast to susceptible BALB/c mice which are prone to Th2-mediated responses [21,22]. However, human [15,47,48] and animal [16,49] studies have demonstrated upregulation of both proinflammatory (Th1) and anti-inflammatory (Th2) cytokines following *B. pseudomallei* infection, providing evidence that a delicate balance in involvement of both pathways is important for control of bacterial dissemination and regulation of excessive host inflammatory responses, respectively.

Within hours of infection, *B. pseudomallei* induces the production of IFN-γ by NK cells, together with CD44<sup>high</sup>CD8<sup>+</sup> T cells, a response which is essential for the early containment of the bacterium [32,50]. IFN-γ production by CD44<sup>high</sup>CD8<sup>+</sup> memory T cells occurs via antigen-independent activation, through a process mediated by macrophage-derived IL-12 and IL-18 [32,50]. Consistent with observations in mouse models of *B. pseudomallei* infection, several human studies have shown that NK and memory CD8<sup>+</sup> cells from sero-positive healthy individuals and survivors of melioidosis are primed and able to produce IFN-γ after stimulation with *B. pseudomallei* or its antigens in vitro [51]. In addition to their important role in IFN-γ production, *B. pseudomallei*-specific CD8<sup>+</sup> T cells are able to lyse infected host cells through the expression of granzymes, Fas-L, granulysin and perforin, thus facilitating elimination of intracellular *B. pseudomallei* [52]. As well as stimulating activation of phagocyte bactericidal mechanisms, IFN-γ and IL-12 production subsequently drives the development of a Th1-type response [53].

During subsequent stages of infection, host resistance is dependent on *B. pseudomallei*-specific priming of Th1 cells [54]. In addition to facilitating elimination of intracellular *B. pseudomallei* through activation of macrophage bactericidal functions, Th1 cells promote antigen presentation by upregulating expression of co-stimulatory molecules together with MHC II, thereby propagating the expansion of antigen-specific T cells [55]. High-throughput protein microarrays have been used to screen for immunogenic peptides of *B. pseudomallei* using sera from individuals with a history of exposure to the bacterium [56,57]. Several reactive peptides were subsequently shown to evoke distinct T cell activation patterns in vitro, using cells from patients who had recovered from clinical melioidosis or from healthy, seropositive individuals [51,57] (Section VII.5). Bioinformatic approaches are also being utilised in an attempt to characterise CD4<sup>+</sup> T cell epitopes of *B. pseudomallei* [58]. In addition to classical T cell activation pathways, Ye et al. demonstrated that *B. pseudomallei* is able to directly co-stimulate CD4<sup>+</sup> and CD8<sup>+</sup> T cells resulting in IL-2 and IFN-γ production [59], perhaps explaining the bystander activation of NK and T cells that is observed in the early stages of infection [32]. Whether direct co-stimulation of T cells by pathogens, such as *B. pseudomallei*, serves a protective or detrimental host response to infection remains to be seen.

Dendritic cells are key regulators in the initiation of T cell responses to infection. Whilst several studies have investigated T cell responses to recombinant proteins of *B. pseudomallei*, studies focussing on *B. pseudomallei* antigen processing within DC and
subsequent influence on antigen-specific T cell immunity are only beginning to emerge. Following uptake and presentation by human DC, several ‘virulence-associated’ proteins of *B. pseudomallei* were recently shown to stimulate recall responses *in vitro* in CD4+ and, to a lesser extent, CD8+ T cells derived from individuals with a previous history of exposure to *B. pseudomallei* [60]. Such studies will be essential for identifying key immunogenic antigens of *B. pseudomallei* that are responsible for driving the development of protective cell-mediated immunity, if progress is to be made in the development of a vaccine for melioidosis. Previous investigations have also identified a predominant role for CD4+ T cells in host protection against *B. pseudomallei* [47,54,61]. Patients who recover from infection with *B. pseudomallei* develop an adaptive cell-mediated immune response characterised by activation of antigen-specific CD4+ and CD8+ T cells following secondary antigenic challenge [47]. The presence of specific cell-mediated immunity to *B. pseudomallei* in survivors of melioidosis and healthy individuals provides merit to the importance of strong Th1-type immune responses in host protection [61], and progression to clinically apparent infection [62]. However, a central protective role for CD4+ T cells in *B. pseudomallei* infection appears to be contradicted by the lack of evidence that HIV infection increases the risk for severe melioidosis. Perhaps this enigma will be explained in the coming decade as our understanding of the mechanisms underlying the development of protective immunity to *B. pseudomallei* continues to grow. Certainly, the contribution of other T cell subsets, such as Th17 cells, regulatory T cells (Treg) and gamma delta (γδ) T cells, to the immunopathogenesis of *B. pseudomallei* infection is yet to be described.

4. Humoral immune responses to *Burkholderia pseudomallei*

Whilst the development of adaptive cellular immune responses is essential for defence against *B. pseudomallei* infection, they do not solely account for protection. Humoral immune responses are also considered to contribute significantly to the development of host protection to melioidosis. Indeed, high titres of *B. pseudomallei*-specific immunoglobulins (Ig) correlate with improved clinical outcome [63,64]. This is consistent with mouse models of immunisation against melioidosis, in which survival following infection is heightened following immunisation regimes that trigger both strong Ig production and cell-mediated immunity [42]. *B. pseudomallei*-specific Ig have also been demonstrated in healthy individuals with no clinical history of melioidosis residing in regions endemic for this disease [65,66], providing possible support for a role for humoral immunity in limiting disease progression.

In patients with melioidosis, IgG is the predominant isotype detectable in sera, with more than 65% of subclass IgG1 [67]. Regardless of outcome, IgG was the predominant isotype in all acutely infected patients, as well as those with a presumptive diagnosis of melioidosis [65,67,68]. Consistent with the critical role of Th1-type response in protection against *B. pseudomallei*, closer examination of the IgG isotype response to *B. pseudomallei* revealed a predominance towards IgG1 and, subsequently, IgG2 subclasses [67]. Interestingly, IgG3 was detectable only in survivors of septicemic infection [65]. These three IgG subclasses activate the classical complement pathway,
and IgG1 and IgG3, in particular, have high opsonic potential, binding strongly to mononuclear cells, a major component of the cell types involved in \( B.\) pseudomallei clearance \([65,67]\). This supports an important role for humoral immunity in host protection to melioidosis through antibody-dependent bacterial uptake and killing by phagocytic cells.

High levels of IgM and IgA to \( B.\) pseudomallei antigens have also been reported \([67,69−71]\). The highest levels of IgA and IgM were detected in patients with localised, soft-tissue infections \([65,67]\). It has been suggested that long term persistence of high \( B.\) pseudomallei-specific antibody titres may reflect continuous B cell priming due to sequestration of intracellular bacterial antigens, perhaps necessitating continued antimicrobial therapy \([72]\). Further studies aimed at correlating Ig isotype switching with disease progression and outcome will provide important data that could facilitate therapeutic management of melioidosis patients.

Both B and T cells are involved in the production of antibodies in response to protein antigens, and such antigens are, therefore, regarded as T-dependent antigens \([73]\). Protein microarray studies have begun to identify a large number of \( B.\) pseudomallei protein targets of human antibody responses, highlighting the complexity of the immunopathogenesis of this disease \([56,57]\). In contrast, antibody responses to polysaccharide and lipid antigens, or T-independent antigens, do not require the help of T cells. T-independent antigens stimulate the rapid production of antibody, mainly IgM isotype, within 48 hours of infection. Despite their inability to specifically activate Th cells, many polysaccharide antigens can induce long-lived protective immunity, presumably due to the persistence of polysaccharide in lymphoid tissues causing the continued stimulation of maturing B cells \([74]\). To date, the majority of the literature concerning Ig against \( B.\) pseudomallei describe antibodies that are directed against polysaccharide antigens, outer membrane protein and flagellin \([65,75−80]\). Antibodies against \( B.\) pseudomallei polysaccharides have properties that promote phagocytosis, and are likely to be among the first produced in the initial clearance of the bacterium \([65]\). Whilst passive transfer of antibodies against a range of \( B.\) pseudomallei antigens has been shown to partially protect naive mice from \( B.\) pseudomallei infection \([77,81−85]\), protection from lethal infection after high-dose challenges with \( B.\) pseudomallei is not complete \([77,86]\). Studies focussing on identification of T-dependent and T-independent antigens of \( B.\) pseudomallei will be an important avenue to pursue to identify promising targets for preventative strategies against melioidosis, and to improve current diagnostic assays.

5. Persistence of \( Burkholderia\) pseudomallei in the host

Following initial infection with \( B.\) pseudomallei, the adaptive immune response is often unable to achieve complete eradication of the pathogen, allowing it to persist in the host either subclinically or as a chronic infection. The mechanisms underlying \( B.\) pseudomallei persistence are poorly understood. Chronic melioidosis is characterised by a persistent, localised infection that is difficult to eradicate and often associated with relapse \([87]\). Low-dose exposure of relatively resistant C57BL/6 mice towards \( B.\) pseudomallei has been used as a model for chronic melioidosis, providing
evidence of histopathological changes that resemble granulomatous lesions described in tuberculosis [21,88]. *B. pseudomallei* is also able to cause a subclinical, latent form of melioidosis, whereby infection only becomes evident decades after initial exposure to the bacterium, typically following an immunosuppressive event [87,89–93]. Reactivation in individuals with latent melioidosis has been documented up to 62 years following exposure during World War II [93]. In northern Australia, 4% of patients with melioidosis have a clinical history suggestive of reactivation of *B. pseudomallei* infection from a latent focus [87].

The indirect haemagglutination assay (IHA) is still considered the gold standard for serological studies for melioidosis. However, it is not known whether a positive IHA result in samples from apparently healthy individuals is indicative of exposure and clearance of *B. pseudomallei* infection, or of persistence of asymptomatic, latent infection [94]. Up to 80% of children over the age of four years have *B. pseudomallei*-specific antibodies in northern Thailand [95]. In northern Australia, serological studies for melioidosis report 2.5–10% of residents are seropositive for antibodies toward *B. pseudomallei* [90,96]. The ability of *B. pseudomallei* to remain dormant for extended periods in the host has potential implications for individuals living within endemic regions who have been exposed to the bacterium, and may go on to develop diabetes or require immunosuppressive treatment. Intracellular survival of the bacterium also impedes the success of antimicrobial therapy.

Though latency is an important feature of melioidosis, there have been limited investigations into this form of the disease. The site of persistent *B. pseudomallei* infection within the host is unknown. Mechanisms responsible for the association between disease relapse and a failure of the host immune system to contain *B. pseudomallei* infection are also unclear. Apparent reactivation of latent *B. pseudomallei* infection in an animal model was first described by Steward et al. [97]. However, the primary focus of the study was the treatment of melioidosis, so latent infection was neither defined nor demonstrated prior to immunosuppression. Subsequently, Titball et al. reported that TO mice, which are relatively resistant to *B. pseudomallei* infection, remain asymptomatic for up to nine months following intraperitoneal infection, at which time the infection spontaneously reactivates [1]. Reactivation was also induced in TO mice following neutralisation of IFN-γ, as evidenced by increased animal mortality [1].

*Burkholderia pseudomallei* is an intracellular bacterium that possesses several immune evasion strategies to allow it to survive inside host cells for extended lengths of time. Resistance against the bactericidal actions of various compounds present in activated macrophages or neutrophils, including the cationic peptide protamine and purified human defensin, respectively, contribute to bacterial persistence [98]. Following activation, immune cells can be eliminated by apoptosis so as to limit their potential for tissue injury. *B. pseudomallei* was demonstrated to induce caspase-1-dependent cell death, or pyroptosis [99], and is also able to avoid killing by autophagy, at least in murine cell lines [100]. Chanchamroen et al. demonstrated *B. pseudomallei* interferes with spontaneous apoptosis of neutrophils from healthy individuals, but not diabetic individuals, suggesting another mechanism by which this bacterium is able to persist in the host [9]. However, the implications of these evasion strategies in the pathogenesis of chronic and latent infection are yet to be elucidated.
Burkholderia pseudomallei is a facultative intracellular bacterium. In vitro studies and electron microscopy have demonstrated that B. pseudomallei is capable of survival within membrane-bound vacuoles and the cytoplasm of host cells. Electron microscopic images obtained from mice i.v. infected with B. pseudomallei. Liver was removed at 72 h post infection. Under electron microscopy, B. pseudomallei can be observed within vacuoles of host cells. (J. Morris, James Cook University, Australia.)

Burkholderia pseudomallei actively promotes internalisation by non-phagocytic cells [98,101–103], and endosome escape within phagocytes [98,103–105] (Figure VII.5). Escape of B. pseudomallei into the cytoplasm of the host cell can occur prior to the fusion of the phagosome and lysosome [103,105], and may be facilitated by the type 3 secretion system (T3SS) [103,106,107], and the more recently identified T6SS [108]. Once within the cytosol, B. pseudomallei induces actin-based membrane protrusions which facilitate cell to cell spread [102,109] (Figure VII.6), and MNGC formation [102,110]. While MNGC and granuloma formation have been reported in human melioidosis [110], their role in the pathogenesis of B. pseudomallei infection, particularly latent infection, has not yet been described. Certainly, in studies of Mycobacterium tuberculosis,
granuloma formation controls dissemination of the pathogen, and also circumscribes toxic environments to protect surrounding tissue [111]. Generation of a hypoxic environment within a granuloma may promote the formation of dormant state in bacteria [111]. Upon encountering an hypoxic environment, M. tuberculosis shifts into a state of non-replicating persistence, in which the bacilli are metabolically active, but there is no growth [112]. Once in this state, the pathogen can remain there for years, until a shift in the control by the immune system occurs and the bacilli reanimate [113].

Observations that B. pseudomallei can survive for extended periods in a dormant, non-replicating state in vitro [114,115] provide evidence to support hypotheses that this pathogen is also able to adapt to protracted survival in vivo. Additional evidence was provided by Chantratita et al. who demonstrated association between colony morphology switching in B. pseudomallei isolates, and persistence in a murine model [116]. In addition, using a rat model of acute and chronic pulmonary melioidosis, van Schaik et al. identified differential gene regulation patterns that suggest persistence of B. pseudomallei in the host is facilitated by restructuring of outer membrane proteins [117]. The authors elegantly demonstrated that isocitrate lyase (ICL), an enzyme of the glyoxylate shunt, is essential for establishment of chronic infection, and inhibition of ICL results in increased virulence of B. pseudomallei and a shift to overwhelming acute infection [118].

Once B. pseudomallei becomes intracellular, CMI responses become essential for clearance of B. pseudomallei from the host [47,50,61,119]. Many immunopathogenesis studies have focussed on the initiation of immune responses in the acute stages of B. pseudomallei infection [17,49,50,118]. The mechanisms of immune control in later stages of chronic, and in latent, B. pseudomallei infection have not been studied. Development of a suitable animal model of latent B. pseudomallei infection is critical so that basic information regarding how B. pseudomallei is able to persist in the host and what factors are involved in activation of a latent infection becomes available.

6. Conclusions and future perspectives

Most of our understanding regarding host immune responses to B. pseudomallei has been derived from studies on other pathogens. However, in the last decade significant inroads have been made using animal models to correlate some of the key observations regarding host responses observed in clinical melioidosis. While the role of macrophages in melioidosis has been investigated, very little is known about the mechanisms involving PMN and DC interactions with the pathogen in the early phase of infection, and how this may influence subsequent adaptive immune responses. Although the importance of an adequate adaptive immune response in protection and survival has been recognised, the role of antibodies is less well understood. There is also very little in the literature regarding persistence of the pathogen in the host, and the immune responses, or lack thereof, associated with relapse and activation of latent melioidosis. A deeper understanding of the immune mechanisms that confer resistance to B. pseudomallei is critical to be able to develop tools for clinical use; for both early detection and for effective treatment of acute infection. Such studies will also provide information essential for the development of potential vaccines.
References


Abstract

Burkholderia pseudomallei is the causative agent of melioidosis, a serious disease of humans and animals which is endemic in Southeast Asia, northern Australia, parts of Africa, South and Central America. There is currently no licensed vaccine against melioidosis. B. pseudomallei is considered to be a potential biowarfare agent and this has also driven research to develop a vaccine. There has been progress in characterising the immune responses in individuals exposed to B. pseudomallei and mechanisms of resistance to infection have been studied in mice. Antibody and CD4+ T cells play an important role in protection from disease but the role of CD8+ T cells is less clear. A number of approaches to vaccine development have been proposed, including the use of live attenuated mutants, killed cells and sub-units. In murine models the most effective immunogens are live attenuated mutants, but these may not ultimately be suitable for use in humans.

1. Introduction

In recent years there has been significant interest in the possibility that vaccines could be developed to protect against melioidosis. This interest is largely, but not exclusively, driven by an awareness that B. pseudomallei could be used as a bioterrorism or biowarfare agent. A melioidosis vaccine would be especially valuable because of the inherent resistance of the organism to many classes of antibiotics. Clearly, were such a vaccine to be developed, it could have value for the immunisation of at-risk populations in areas of the world where the disease is endemic.

The ease with which such a vaccine could be devised has been the subject of much discussion and debate. Most individuals in melioidosis endemic areas of the world have antibodies which react with B. pseudomallei, and relapsing disease is common. These
observations suggest that prior exposure to this bacterium does not induce protective immunity. However, a contrasting point of view is that in endemic areas it is clear that most individuals do develop immune responses to \textit{B. pseudomallei}, yet few develop disease. This suggests that most individuals are able to mount a protective immune response. Work to devise a vaccine against melioidosis is still in its infancy, but in recent years significant progress has been made, as summarised in this Section.

2. Immune responses in humans exposed to \textit{Burkholderia pseudomallei}

Both humoral and cellular immune responses have been reported in individuals who have recovered from melioidosis. It is likely that many of the antibodies are directed against cell surface polysaccharides. However, a number of studies have identified immunoreactive proteins in convalescent sera. Many of these studies have used two-dimensional electrophoresis to separate proteins which are then reacted with serum. However, an alternative approach involved the use of a \textit{B. pseudomallei} protein microarray, where individual proteins are produced in a cell-free transcription–translation system and then immobilised onto glass slides [1]. The arrays were then probed with 747 melioidosis-positive sera from individuals in northeast Thailand or Singapore, or with melioidosis-negative sera from individuals from different parts of the world [1]. A group of 49 proteins that reacted with sera from patients with melioidosis was identified using this approach (Table VII.2).

It is also clear that individuals infected with \textit{B. pseudomallei} develop strong cellular responses to \textit{B. pseudomallei} antigens [2]. Studies to identify these antigens are less comprehensive than those which had identified antibody-reactive proteins. Nevertheless, CD4$^+$ responses, and to a lesser extent CD8$^+$ responses, to ABC transporter proteins LolC, PotF and OppA have been reported [2].

3. Protective immune responses

There is limited information available on the nature of protective immune response to \textit{B. pseudomallei} infection in humans, and this indicates an involvement of both humoral and cellular immunity. The involvement of antibodies in protective immunity is well established. In humans, the antibody titre to bacteria is broadly correlated with disease severity [3]. In small animal models of infection (diabetic rats or mice), the passive transfer of antibodies against capsular polysaccharide (CPS) or lipopolysaccharide (LPS) provides protection against subsequent challenge [4]. In addition, the immunisation of mice with purified CPS or LPS provides protection against a parenterally delivered \textit{B. pseudomallei} challenge [4]. A common feature of these studies is the difficulty of establishing sterile immunity against anything other than low challenge doses. Rather, the onset of overt disease is delayed in immunised animals. The precise roles of antibodies against CPS or LPS are not known. However, it is known that the CPS limits the deposition of the C3b component of complement on the surface of the bacteria and, therefore, reduces phagocytosis of the bacteria [5]. The O-antigen component of LPS appears to play a role...
Table VII.2
Antigens which react with antibodies in convalescent sera identified using a proteome array [1]

<table>
<thead>
<tr>
<th>Gene</th>
<th>Annotated function of gene product</th>
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<tbody>
<tr>
<td>BPSL2697</td>
<td>60 kDa chaperonin</td>
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<tr>
<td>BPSS1532</td>
<td>Putative cell invasion protein</td>
</tr>
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<td>BPSS0477</td>
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</tr>
<tr>
<td>BPSS1512</td>
<td>Putative membrane protein</td>
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<tr>
<td>BPSL3319</td>
<td>Flagellin</td>
</tr>
<tr>
<td>BPSS1492</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>BPSS1599</td>
<td>Putative type IV pilus biosynthesis protein</td>
</tr>
<tr>
<td>BPSS0280</td>
<td>Putative flagellar hook-associated protein</td>
</tr>
<tr>
<td>BPSS1913</td>
<td>Putative exported protein</td>
</tr>
<tr>
<td>BPSS2520</td>
<td>Putative exported protein</td>
</tr>
<tr>
<td>BPSS1385</td>
<td>Putative ATP/GTP binding protein</td>
</tr>
<tr>
<td>BPSS1722</td>
<td>Malate dehydrogenase</td>
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<tr>
<td>BPSS2522</td>
<td>Outer membrane protein A precursor</td>
</tr>
<tr>
<td>BPSS3247</td>
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<tr>
<td>BPSS2141</td>
<td>Putative periplasmic oligopeptide-binding protein precursor</td>
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<td>Putative type III secretion protein</td>
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<td>BPSS0752</td>
<td>Putative lipoprotein</td>
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<td>BPSS2030</td>
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<td>BPSS2017</td>
<td>Di-haem cytochrome C peroxidise</td>
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<tr>
<td>BPSS2160</td>
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</tr>
<tr>
<td>BPSS0421</td>
<td>Lipopolysaccharide biosynthesis protein (O-antigen-related)</td>
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<tr>
<td>BPSS0739</td>
<td>Hypothetical protein</td>
</tr>
<tr>
<td>BPSS2052</td>
<td>Putative activator</td>
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</table>

continued on next page
Table VII.2, continued

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<th>Annotated function of gene product</th>
</tr>
</thead>
<tbody>
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<td>BPSS0122</td>
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</tr>
<tr>
<td>BPSS1434</td>
<td>Putative membrane-anchored cell surface protein</td>
</tr>
<tr>
<td>BPSS1727</td>
<td>Putative hemagglutinin related protein</td>
</tr>
<tr>
<td>BPSS1652</td>
<td>Putative patatin-like protein</td>
</tr>
<tr>
<td>BPSS0065</td>
<td>Putative exported protein</td>
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<td>BPSS0708</td>
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<tr>
<td>BPSS2136</td>
<td>Family S43 non-peptidase homologue</td>
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<tr>
<td>BPSL0093</td>
<td>Putative lipoprotein</td>
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<tr>
<td>BPSL1661</td>
<td>Putative hemolysin-related protein</td>
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<tr>
<td>BPSS1649</td>
<td>Putative sugar-binding protein</td>
</tr>
<tr>
<td>BPSS2053</td>
<td>Putative cell surface protein</td>
</tr>
</tbody>
</table>

in promoting survival within macrophages [6]. It seems likely that antibodies to CPS or LPS are opsonising, though this needs to be tested experimentally.

The role of antibodies to proteins in protective immunity is generally less clear. Antibodies to flagellin have been shown to inhibit the motility of *B. pseudomallei* [7]. However, a flagellin mutant of *B. pseudomallei* shows minimal reduction in virulence in BALB/c mice (10^4 colony forming units (CFU) to 3 × 10^4 CFU) [8], suggesting that the loss of motility is not responsible for the protective efficacy of antibodies to flagellin.

Studies in murine models of disease also indicate a role for cellular immune responses in protection against disease. *B. pseudomallei* is adapted to live within host cells and thus requires a cell-mediated immune response for elimination. The bacterium grows within host macrophages, unless they are activated by IFN-γ [9], and in other cell types, such as epithelial cells [10] which have little intrinsic killing capacity and therefore may rely on lysis by cytotoxic T cells for elimination. Yet our understanding of how T cell-mediated immune responses are initiated against *B. pseudomallei*, and their role in natural or vaccine-mediated resistance to this organism, remains rudimentary. Few vaccination models have specifically targeted cell-mediated responses for protection, although it is generally agreed that both antibody and cellular responses will be required for any vaccine to prevent infection and generate sterilising immunity to this organism.

Insight into the cell-mediated responses required for protection has been gained from studies with different attenuated strains and mutants of *B. pseudomallei* which are able to induce protective immunity. Immunisation of mice with an *ilvI* auxotroph allowed the generation of CD4^+^ and CD8^+^ T cells, which secreted IFN-γ upon restimulation *ex vivo* [11]. The adoptive transfer of purified T cells from vaccinated donors into SCID mice showed the direct protective effects of T cells in the absence of immunoglobulins. Interestingly, despite the presence of vaccine-induced CD4^+^ and CD8^+^ T cells, protection was abolished by antibody depletion of CD4^+^, but not CD8^+^, cells.
prior to challenge. Thus *B. pseudomallei*-reactive CD4\(^+\) T cells can provide significant protection, presumably via their production of IFN-\(\gamma\), but the potential for CD8\(^+\) T cell-mediated protection requires further study.

A key event in the generation of T cell responses is the uptake of vaccine antigens by dendritic cells (DC), and their activation to express costimulatory molecules and cytokines. Healey and colleagues explored this by pulsing murine bone marrow-derived DC with killed intact *B. pseudomallei*, and immunising mice prior to challenge [12]. Uptake of bacteria triggered increased expression of MHC Class II, CD80, CD86 and CD54, and these DC induced proliferation of naive T cells \textit{in vitro}. Immunisation of mice with pulsed DC generated even stronger T cell proliferative responses. Protection against challenge was best when mice received additional boosting doses, and generated both antibody and T cell responses. Further improvement by adding immunostimulatory CpGs resulted in priming of IFN-\(\gamma\)- and, to a lesser extent, IL-4-producing T cells, and gave protection against challenge with both homologous and heterologous *B. pseudomallei* strains [13].

Vaccination with purified proteins of *B. pseudomallei* in adjuvant, or encoded in a DNA vaccine, also induces significant T cell responses and can be protective. Chen et al. used this approach to express *B. pseudomallei* flagellin, demonstrating induction of primed T cells capable of proliferation and IFN-\(\gamma\) secretion upon restimulation \textit{in vitro} [14]. Importantly, they showed that culturing primed spleen cells with naive peritoneal exudate cells (which are predominantly macrophages) increased the opsonic killing of live *B. pseudomallei*, one of the few experiments to directly prove the functional importance of the response. It also is reported that direct infection by *B. pseudomallei* can co-stimulate T cells for production of IL-2 and IFN-\(\gamma\), which is mediated, at least in part, by the actions of flagellin [15].

4. Live attenuated vaccines

In 1958, Levine and Maurer first reported that an attenuated mutant of *B. pseudomallei* was able to induce protective immunity in mice [16]. The mutant tested was auxotrophic for purines and was generated following the exposure of bacteria to ultraviolet radiation. The use of radiation as a mutagen raises the possibility that this strain possessed multiple mutations. Nevertheless, this study established the feasibility of protecting against melioidosis after immunisation with live attenuated mutants. Like other pathogens, the nature of the attenuating lesion is important for the induction of protective immunity. For example, mutants of *B. pseudomallei* which fail to produce CPS are markedly attenuated in mice but exposure to these mutants does not induce protective immunity [17].

More recently a number of workers have identified a range of genetically defined (rationally attenuated) mutants which are able to induce protective immunity in mice (Table VII.3). Some of the pathways interrupted in these mutants are similar to the pathways used to rationally attenuate other bacterial species. For example, *Salmonella enterica* vaccine strains have been constructed with mutations in the aromatic amino acid biosynthesis (shikimate), or in the Spi2 type III system. Purine auxotrophs of *Brucella melitensis*
### Table VII.3

<table>
<thead>
<tr>
<th>Pathway/Region</th>
<th>Gene</th>
<th>Immunising dose (CFU),route, mouse strain</th>
<th>Challenge strain, dose (CFU) and route</th>
<th>Protection reported</th>
</tr>
</thead>
<tbody>
<tr>
<td>Branched chain amino acid biosynthesis</td>
<td>ilvI</td>
<td>10⁶,i.p., BALB/c</td>
<td>576, 10⁴, i.p.</td>
<td>8/10 survival</td>
</tr>
<tr>
<td>Serine biosynthesis</td>
<td>serC</td>
<td>10⁵,i.p., BALB/c</td>
<td>576, 10⁴, i.p.</td>
<td>8/10 survival</td>
</tr>
<tr>
<td>Aromatic amino acid biosynthesis</td>
<td>aroB</td>
<td>10⁶,i.n., BALB/c</td>
<td>K96243, 10⁷,i.p.</td>
<td>Survival time increased from 4.5 to 7 days</td>
</tr>
<tr>
<td></td>
<td>aroC</td>
<td>3−3.5 × 10⁷ (2 doses), i.p., BALB/c</td>
<td>K96243, 10³,i.p., C57Bl/6</td>
<td>Survival time increased from 4.5 to 7 days</td>
</tr>
<tr>
<td>Purine biosynthesis</td>
<td>purN</td>
<td>10⁵,i.p., BALB/c</td>
<td>K96243, 10⁶,i.n., BALB/c</td>
<td>8/8 survival</td>
</tr>
<tr>
<td></td>
<td>purM</td>
<td>5 × 10^3,i.n., BALB/c</td>
<td>E8, 10⁵,i.p.</td>
<td>Survival time extended</td>
</tr>
<tr>
<td>Hypothetical protein</td>
<td>BPSS1509</td>
<td>10⁶,i.p., BALB/c</td>
<td>E8, 10⁵,i.p.</td>
<td>3/4 survival</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>lipB</td>
<td>2 × 10^5,i.p., BALB/c</td>
<td>E8, 10⁵,i.p.</td>
<td>3/4 survival</td>
</tr>
<tr>
<td>p-Aminobenzoate biosynthesis</td>
<td>pabB</td>
<td>2 × 10^5,i.p., BALB/c</td>
<td>E8, 10⁵,i.p.</td>
<td>3/4 survival</td>
</tr>
<tr>
<td>Type III secretion</td>
<td>BopD</td>
<td>10⁶,i.p., BALB/c</td>
<td>K96243, 262, 10⁴,i.p., BALB/c</td>
<td>576, 10⁴,i.p.</td>
</tr>
</tbody>
</table>

Reported LD₅₀/MLD doses of challenge strains by the i.p. route: strain 576, 80 CFU; strain K96243, 262 CFU; strain E8, 14 CFU.
have been proposed as vaccines, and branched chain biosynthesis pathway mutants of *Mycobacterium tuberculosis* and *M. bovis* have been shown to have vaccine potential.

The most extensive studies in mice have been carried out with a branched chain amino acid (*ilvI*) mutant of *B. pseudomallei*. The immunisation of mice with this mutant provided protection against a subsequent challenge with $1.25 \times 10^4$ median lethal doses (MLD) of *B. pseudomallei* strain 576, or challenge with $3.3 \times 10^5$ MLD of strain BRI. Therefore, immunisation with this auxotroph can provide protection against strains producing typical (BRI) or atypical (576) forms of the LPS O-antigen [18]. Many of the challenged mice in this study did eventually succumb to infection, and those that received two doses of the auxotroph did not have greater protection [11]. A more detailed analysis of splenic colonisation after the challenge of immunised mice with wild-type *B. pseudomallei* indicated two clear patterns of colonisation. By day 13, some mice had low or undetectable bacterial load, whilst the remainder had splenic abscesses containing over $10^7$ bacteria. It is possible that, given sufficient time, all mice would have developed abscesses or some mice would have been able to resolve the infection.

Parallel studies would be required to establish which of the *B. pseudomallei* mutants is the most effective as an immunogen, and, to our knowledge, none of the attenuated mutants reported to date have progressed beyond proof of principle studies in mice. Indeed, it may be argued that, because of the potential for *B. pseudomallei* to establish persistent or latent disease, an attenuated mutant would not be licensable as a vaccine for use in humans. Nevertheless, immunisation with these mutants is able to provide high levels of protection against disease and this has allowed an understanding of the nature of protective immunity to melioidosis.

5. Killed whole-cell vaccines

There are only a few reports in the open literature on the efficacy of heat-inactivated *B. pseudomallei* as vaccines. In an early study, Razak et al. immunised mice with $5 \times 10^5$ bacteria by the intraperitoneal (i.p.) route, followed by two boosters at 4 day intervals; and 5 days later mice were challenged with wild-type bacteria at $1 \times 10^6$ cells/ml [19]. All of the immunised mice survived up to 7 days post challenge, but survival data beyond this time was not reported. A more recent study compared heat-inactivated cells and live attenuated *B. pseudomallei* as immunogens in BALB/c mice [20]. In both groups, 290 CFU of bacteria were used to immunise mice by the subcutaneous route, followed by boosters on days 10 and 30. On day 45 animals were challenged with $2 \times \text{LD}_{50}$ of wild-type *B. pseudomallei* by the intravenous (i.v.) route and observed for 40 days. Mice immunised with heat-inactivated cells were not protected, although there was 80% survival in the group which had been immunised with live *B. pseudomallei*. *B. pseudomallei* is particularly virulent by the i.v. route, with an LD$_{50}$ of approximately 10, and this may explain the lack of protection seen after immunisation with killed cells.

The more encouraging results reported by Sarkar-Tyson et al. [21] might well reflect the use of a challenge route which results in a less aggressive disease. In this study, either killed *B. pseudomallei*, *B. mallei* or *B. thailandensis* cells were used as immunogens in BALB/c mice [21]. Groups of mice were immunised i.p. with $1 \times 10^8$ CFU of killed
bacteria with boosters at days 14 and 28, followed by an i.p. challenge four weeks later with 40 MLD of wild-type *B. pseudomallei* K96243. At the end of this experiment (day 45), 60%, 70% or 60% respective survival was reported. Furthermore, immunisation with killed bacteria resulted in a significant increase in time to death following an aerosol challenge with 18 MLD of *B. pseudomallei* [21]. These results support the finding by Ilyukhin et al. that immunisation with *B. thailandensis* resulted in approximately 50% protection against 200 LD<sub>50</sub> doses of *B. pseudomallei* in a guinea pig model of disease [22].

Sterile immunity following immunisation with killed cells is difficult to achieve. Challenged animals often show a delayed time to death, or at post mortem, show evidence of significant bacterial colonisation of organs [21]. The level of protection appears to be dependent on the amount of immunogen used, the route of immunisation, challenge dose and route of challenge. It is clear that there is scope for an inactivated vaccine which could be based on either *B. pseudomallei* or closely related species. However, further investigation is required to refine the immunogen and immunisation regime to obtain the highest levels of protection.

6. **Sub-unit vaccines**

The immunisation of mice with CPS (deoxy-β-D-manno-heptopyranose, also known as type I O-PS) or the typical form of LPS (also known as type II O-PS) [(−3)]β-D-glucopyranose-(1−3)-6-deoxy-α-L-talopyranose-(1−)] has been shown to provide protection against experimental melioidosis [4]. However, it must be mentioned that a minority of strains of *B. pseudomallei* produce LPS with an immunologically distinct O-antigen. The ability of CPS or LPS to protect against strains which produce the variant O-antigen is not known. Neither has the potential for variant form of LPS to induce protective immune responses been tested.

Immunisation with either CPS or LPS fails to provide complete protection against disease or sterile immunity, with immunised animals typically showing a delayed time to death. This finding is broadly similar to the findings with other vaccine candidates, outlined above. In addition, it is important to note that the immunisation of mice with either CPS or LPS is not reported to result in significant protection against respiratory infection [4].

A number of protein antigens have been identified which, when used to immunise mice, induce significant but not complete protection against experimental melioidosis. The most promising candidate reported to date is LolC, an inner membrane protein which forms part of a lipoprotein export system. Immunisation with recombinant LolC protein, in combination with the molecular adjuvants ISCOMS and CpG oligodeoxynucleotide 10103, protected five of six mice from death until the end of the study at 42 days after an i.p. challenge with approximately 250 MLD of strain K96243 [23]. Immunisation of mice with similarly adjuvanted LolC protected 30% of mice against death following a challenge with 7,500 MLD of *B. pseudomallei* strain 576. Since strains K96243 and 576 produce typical and variant forms of the O-antigen, this suggests that the LolC might be useful as a protective immunogen against all strains. Other candidate protective
antigens include recombinant outer membrane proteins BPSL2522 or BPSL2765 which have been shown to protect two of four animals from death after i.p. challenge with 10 LD50 doses of *B. pseudomallei* strain D283 [24]. The immunisation of BALB/c mice with recombinant flagellin provided a broadly similar level of protection [14] to that reported after immunisation with BPSL2522 or BPSL2765 [24]. However, flagellin-immunised mice were challenged by the i.v. route, making meaningful comparisons with the degree of protection afforded by BPSL2522 or BPSL2765 (or LolC) difficult.

A common feature of the immune responses induced by BPSL2522, BPSL2765 or LolC is the dominance of IgG2a subclass antibodies, which is indicative of a Th1-type immune response. The use of ISCOMS and CpG as adjuvants for LolC, or Freund's adjuvant for outer membrane proteins, would promote a Th1-type response. In addition, the immunisation of mice with flagellin, delivered using a mammalian expression vector, provided enhanced protection compared to protection afforded after immunisation with recombinant flagellin [14]. Importantly, the antibody titre after immunisation with naked DNA expressing flagellin was lower than the response after immunisation with recombinant flagellin, but was dominated by IgG2a antibodies [14]. This finding also suggests the need for a Th1 immune response for protection. Therefore, at this stage, and unlike the protective response induced after immunisation with polysaccharides, the role of antibodies against proteins in protection is not clear. Indeed, the available evidence would suggest the requirement for cellular immunity for protection.

7. Glycoconjugate vaccines

Bacterial polysaccharides often make excellent vaccines, evidenced by the number of capsule-based vaccines that are currently licensed for humans, including those to combat *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Neisseria meningitidis* or *Salmonella typhi* infections. However, polysaccharides alone are poor immunogens that do not generate an anamnestic response because of the lack of T cell involvement in the generation of immunity. To elicit a more favourable T cell-dependent response, polysaccharides are often conjugated to proteins.

Previously *B. pseudomallei* LPS has been conjugated to tetanus toxoid to produce a glycoconjugate. The passive transfer of antisera against this glycoconjugate protected diabetic rats against *B. pseudomallei* strain 316c [25]. Subsequently, a conjugate consisting of *B. pseudomallei* flagellin and O-antigen has been described [7]. This conjugate induced IgG responses in rabbits. The passive transfer of antiserum into diabetic rats provided protection, evidenced as a 10^2-fold increase in LD50 dose, when challenged with *B. pseudomallei*. Neither of these conjugates have been reported to have been tested in an active immunisation study, and, considering the possible role of Th1 immune responses in protection against protein antigens, this type of study would appear to be a priority.

8. Conclusions

Clearly a number of studies have demonstrated the feasibility of inducing protective immunity against *B. pseudomallei* infection in mice. However, it is also clear that a
vaccine suitable for use in humans is still some way from licensing. Protection against an i.p. challenge can be elicited after immunisation with live, killed or sub-unit vaccines. This protective response typically evidences as an increase in time to death of challenged animals rather than sterile immunity. Protection against i.v. or inhalational challenges, both of which result in a more acute form of disease, is more difficult to achieve. There is some evidence that protection against i.v. or inhalational challenges may be dependent on route of immunisation. The possibility merits further attention.

Central to the challenge of developing a \textit{B. pseudomallei} vaccine is an understanding of the immune responses required to protect against infection. Both humoral and cellular immunity appear to be important for protection, but the precise roles of these different arms of the immune system are not known. The importance of cytokines other than IFN-\(\gamma\), the role of cytotoxic CD8\(^+\) T cells, and the range of potential antigens that could be targets for T cell-directed vaccines remain to be determined.

Finally, we return to the utility of a melioidosis vaccine. The findings to date suggest that it will be difficult to achieve sterile immunity after immunisation. However, it is possible that vaccines would be used in conjunction with antibiotics or other therapeutics to provide the necessary levels of protection. A possibility that merits particular attention is the use of antibiotics and vaccination to treat melioidosis patients, with a view towards reducing the likelihood of relapse following the completion of chemoprophylaxis.

\textbf{References}


VIII
Melioidosis in animals
Many terrestrial and aquatic mammals, birds, reptiles and fish can be affected by melioidosis. The clinical presentation of melioidosis in animals is diverse and somewhat similar to the human disease ranging from subclinical infection, local infection, subacute illness, latent infection, chronic infection to acute fulminate septicaemia with haematogenous dissemination and high mortality.

Melioidosis in animals occurs in similar areas to that of human disease i.e. between latitudes 20°N and 20°S although sporadic cases have been reported outside the endemic zone primarily associated with the importation of infected animals. Host susceptibility, prevalence and disease manifestations vary greatly between species: subclinical or chronic infections occur in many affected animals; some animals develop melioidosis only if immunocompromised, whilst severe acute disease may occur in some individuals. Animals usually acquire melioidosis from *Burkholderia pseudomallei* in the environment and infection can occur by inhalation, ingestion, percutaneous inoculation or through wounds or abrasions. Infected animals can excrete the organism in saliva, respiratory secretions, milk, wound exudates, urine and faeces, leading to contamination of the environment.

The aim of this Section is to summarise the animal species affected by melioidosis, clinical signs and pathological findings in animals, epidemiological aspects of disease and current diagnostic, prevention and treatment methods for animal melioidosis.
Melioidosis — A Century of Observation and Research, pp. 313–336
Edited by N. Ketheesan
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Section VIII.1

Melioidosis in animals

Catherine M. Rush\textsuperscript{a}, Annette D. Thomas\textsuperscript{b}

\textsuperscript{a} Microbiology and Immunology, School of Veterinary and Biomedical Sciences, James Cook University, Townsville, Australia
\textsuperscript{b} Biosecurity Queensland Tropical and Aquatic Animal Health Laboratory, Department of Employment, Economic Development and Innovation, Townsville, Australia

1. Introduction

Stanton and Fletcher [1] described a 1913 outbreak of a septicaemic disease in a guinea pig colony at the Institute for Medical Research in Kuala Lumpur, Malaya. They isolated an infectious agent from diseased animals that was indistinguishable from Whitmore’s bacillus (at that time named \textit{Bacillus whitmorii}). They named the disease “melioidosis”. Over the next decades, they and others in Southeast Asia documented numerous human cases and several cases in wild and domesticated animals. Early experimental infection studies by Stanton and Fletcher [2] demonstrated the relative susceptibility of rodents and goats, compared to other animals such as horses and monkeys. These initial investigations also showed that susceptible animals can be infected with the organism by ingestion, by contamination of mucous membranes or abraded skin or by injection by any route. Stanton and Fletcher [2] also pioneered the serological tests used for diagnosing the disease over the subsequent decades.

2. Animal species affected

A wide variety of animal species have now been shown to be susceptible to melioidosis, including sheep [3–5], goats [6–9], cattle [10], water buffalo [11], pigs [12,13], camels [14,15], alpaca [16], horses [17], deer [18], kangaroos and wallabies [19–21], koalas [22], cats [23], dogs [24–26], monkeys [27–32], birds [33–37], crocodiles [38] and aquatic animals [39]. A comprehensive review of cases of animal melioidosis in the last century is given by Sprague and Neubauer [40].

Melioidosis in most species is sporadic and occurs as isolated cases. However, epidemics have been described for sheep (Australia [41]), pigs (Australia [12]), sheep, goats and pigs (Aruba [5]), pigs (Vietnam [42]), horses (France [43]), primates (United Kingdom [31]) and dolphins (Hong Kong, cited in [39]). Epizootic outbreaks in zoological gardens tend to occur among animals imported from areas of endemicity. An
outbreak in a Paris zoo in the 1970s resulted in the spread of the disease to other zoos and equestrian clubs throughout France and to the deaths of at least two humans and a number of animals including horses and buffalo. This outbreak, referred to as “l’affaire du Jardin des Plantes” was thought to be due to either the importation of horses from Iran or an infected panda donated by Mao Tse-Tung [43–46]. Another outbreak was identified in 13 of a batch of 50 feral cynomolgus monkeys (Macaca fascicularis) imported to Britain from the Philippines in the 1990s, and three other cases were identified in monkeys imported from Indonesia [31]. These outbreaks are a reminder of the potential for the establishment of environmental foci of melioidosis in countries outside the endemic zone.

In tropical Australia, cases in introduced livestock, particularly sheep, goats and pigs, are most frequent and these animals appear to be highly susceptible to disease; camels and alpaca also appear to be vulnerable to severe disease. A retrospective estimation of the incidence of culture-proven melioidosis in animals in Thailand for the years 2006 to 2010 [38] showed that the highest incidence was in goats, followed by pigs and cattle. Interestingly in this study the estimated incidence of melioidosis in humans in a given region paralleled that of melioidosis in goats. In the Sabah region of Malaysia, cases in animals that lived in forests including monkeys, orang-utans, goats and deer were more frequent than in livestock [47]. Cattle, water buffalo, birds and crocodiles are generally considered to be relatively resistant to melioidosis despite their constant exposure to mud [48] although a number of fatal cases in both birds and cattle have been reported [35,36]. The underlying causes for increased disease susceptibility in some animal species are not known, although some of the factors that have been suggested include stress (e.g. weaning, caging, long distance transport), fatigue (e.g. horses), change of climate or environmental conditions, malnutrition and pregnancy. Furthermore it is unclear why particular animal species are affected in some geographical regions but less so in others.

3. Epidemiology of animal melioidosis

3.1. Geographical distribution

Melioidosis in animals occurs in similar areas to that of human disease although cases have been reported outside the endemic zone primarily associated with the importation of affected animals. Cases in diverse animal species have been reported from countries in endemic regions, including Australia [10,12,13], Papua New Guinea [19], southern China [49–51], Malaysia [18,47,52–54], Vietnam [42], Philippines [31], Hong Kong [39], Thailand [38,55], Sri Lanka [56], Singapore [37] and Taiwan [57]. Sporadic cases of animal melioidosis have also been reported from Iran [58], Saudi Arabia [59], the United Arab Emirates [60], Aruba [5], Chad [61], Madagascar [62], South Africa [9], Turkey [63] and Brazil [64,65]. Numerous animal and human cases have occurred outside the traditional endemic zone (i.e. 20ºS) in Australia [10,13,66]. A cluster of animal cases and one human case occurred over a 25 year period in southwest Western Australia (31ºS) [66]. Burkholderia pseudomallei
has been cultured from both animals and soil in this region, suggesting that at least in Australia pockets of endemicity are now found through temperate Australia. As discussed previously, disease outbreaks in zoological gardens associated with imported animals have been described in numerous countries far removed from endemic zones, including the United States of America [32], the United Kingdom [31], France [46,67] and Aruba [5]. Readers are referred to Sprague and Neubauer [40] for a comprehensive review of cases of animal melioidosis and their geographical locations.

3.2. Prevalence and incidence

In most countries, the true prevalence, incidence and geographic distribution of melioidosis in animals is not known. Several small-scale surveys in individual animal species, in focussed locations in endemic areas have been conducted [21,47,48]. However as infections in many animal species are asymptomatic, subclinical or chronic, and necropsies may not be performed on animals that die from apparently natural causes, a diagnosis is unlikely to be made in many instances. Furthermore, as melioidosis is “the great imitator” and clinical presentation is so variable in animal species, misdiagnoses are likely to have been made, thus grossly underestimating the scale of disease in animal populations.

Thomas et al. [21] conducted a melioidosis survey of farms in north Queensland, Australia, in which 21.8% of samples submitted for testing for suspect melioidosis were culture positive. These included 30/110 (23.5%) of pigs tested, 9/39 (23.1%) sheep tested, 5/10 (50%) goats tested and infrequent cases in birds (2/8), horses (1/8) and tree kangaroos (1/1). Choy et al. [48] reviewed culture-confirmed animal cases in the Northern Territory, Australia from 1992 and 1997 and showed that the highest number of cases had occurred in goats (43 cases), followed by sheep (14 cases) and pigs (11 cases), with less than five cases in each of camels, alpacas, deer, cats, dogs, birds and cattle. Data from the Animal Disease Research Centre (ADRC) records for Sabah, Malaysia for the period 1994 to 2003 showed 341 animal melioidosis cases were identified from 12,139 cases examined by post-mortem at this facility [47]. The most frequently affected animals lived in forests and included monkeys (11.11%), orangutans (8.92%), goats (8.01%) and deer (5.19%); captive animals such as cattle (0.23%), buffalo (0.39%), horse (0.63%), pig (0.72%) and birds (0.22%) were less often affected.

Serological studies in various animal species have been used to survey endemic areas for the presence of B. pseudomallei, i.e., exposure of animals to the organism in the environment (Table VIII.1). For example, 29.6% of buffalo, 18.2% of dairy cattle and 12% of beef cattle in seven areas of the Khon Kaen province in north eastern Thailand were seropositive using the indirect haemagglutination assay (IHA) [71]. Although it is not clear if the same assays were used, this contrasts with the relatively low seropositivity in cattle in other regions of Thailand (e.g. Chiang Mai and Chiang Rai) [55,68]. Serosurveys in cattle, pigs and horses in the southern provinces of China demonstrate variable and in some cases high B. pseudomallei exposure and/or infection status. In these regions the highest seroprevalence correlated with sites in which B. pseudomallei was also isolated from the soil. Goat (n = 1885) and pig (n = 3219) sera, submitted
Table VIII.1
Seroprevalence for *B. pseudomallei* in various animal species in endemic countries

<table>
<thead>
<tr>
<th>Animal</th>
<th>Location</th>
<th>Test, cutoff</th>
<th>Seroprevalence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dairy cattle</td>
<td>Chiang Mai, Northern Thailand</td>
<td>IHA, &gt;1: 40</td>
<td>2%, n = 253</td>
<td>[55]</td>
</tr>
<tr>
<td></td>
<td>Chiang Rai, Northern Thailand</td>
<td>IHA, &gt;1:80</td>
<td>7.29%, n = 384</td>
<td>[68]</td>
</tr>
<tr>
<td></td>
<td>Northeast Thailand</td>
<td></td>
<td>22%</td>
<td>[69]</td>
</tr>
<tr>
<td></td>
<td>18 provinces, Thailand</td>
<td>IHA, &gt;1:160</td>
<td>2.56%, n = 78</td>
<td>[70]</td>
</tr>
<tr>
<td></td>
<td>Khon Kaen province, Thailand</td>
<td>IHA</td>
<td>18.2%</td>
<td>[71]</td>
</tr>
<tr>
<td>Cattle</td>
<td>China</td>
<td>IHA</td>
<td></td>
<td>[49]</td>
</tr>
<tr>
<td></td>
<td>Xinglong*</td>
<td>IHA</td>
<td>33.3%, n = 22/66</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tonghu*</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zhaojiang*</td>
<td>IHA</td>
<td>6.7%, n = 2/30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nanning*</td>
<td></td>
<td>34%, n = 17/50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yingde*</td>
<td>IHA</td>
<td>0%, n = 0/30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Chenzhour*</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beef cattle</td>
<td>Khon Kaen province, Thailand</td>
<td>IHA</td>
<td>12%</td>
<td>[71]</td>
</tr>
<tr>
<td>Buffalo</td>
<td>Khon Kaen province, Thailand</td>
<td>IHA</td>
<td>29.6%</td>
<td>[71]</td>
</tr>
<tr>
<td>Oxen</td>
<td>Qiong, Yue and Gui. Provinces, China</td>
<td>n/a</td>
<td>9.1–18.4%</td>
<td>[50]</td>
</tr>
<tr>
<td>Goats</td>
<td>Queensland, Australia</td>
<td>IHA, &gt;1:40</td>
<td>5.9%, n = 34/577</td>
<td>[72]</td>
</tr>
<tr>
<td></td>
<td>18 provinces, Thailand</td>
<td>IHA, &gt;1:160</td>
<td>0.33%, n = 6576</td>
<td>[70]</td>
</tr>
<tr>
<td></td>
<td>5 provinces, southern border Thailand</td>
<td>IHA, &gt;1:160</td>
<td>0.4%, n = 3546</td>
<td>[73]</td>
</tr>
<tr>
<td></td>
<td>Queensland, Australia</td>
<td>IHA &gt;1:40</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cooktown-Cairns and district</td>
<td></td>
<td>1.6%, n = 6/369</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Townsville and district</td>
<td></td>
<td>7.1%, n = 46/650</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mackay and district</td>
<td></td>
<td>2.1%, n = 1/48</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mt.Isa</td>
<td></td>
<td>4%, n = 7/176</td>
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<tr>
<td></td>
<td>Gayndah-Mundubbera district</td>
<td></td>
<td>0.8%, n = 5/642</td>
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<tr>
<td>Sheep</td>
<td>18 provinces, Thailand</td>
<td>IHA, &gt;1:160</td>
<td>6.83%, n = 366</td>
<td>[70]</td>
</tr>
<tr>
<td>Pigs</td>
<td>China</td>
<td>IHA</td>
<td></td>
<td>[49]</td>
</tr>
<tr>
<td></td>
<td>Xinglong*</td>
<td>IHA</td>
<td>22.7%, n = 34/150</td>
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<tr>
<td></td>
<td>Tonghu*</td>
<td>IHA</td>
<td>26.7%, n = 8/30</td>
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<td></td>
<td>Zhaojiang*</td>
<td>IHA</td>
<td>13.4%, n = 9/67</td>
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<td></td>
<td>Nanning*</td>
<td>IHA</td>
<td>15%, n = 9/60</td>
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<td></td>
<td>Yingde*</td>
<td>IHA</td>
<td>n/a</td>
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<tr>
<td></td>
<td>Chenzhour*</td>
<td>IHA</td>
<td>0%, n = 0/30</td>
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<tr>
<td></td>
<td>Qiong, Yue and Gui. Provinces, China</td>
<td>n/a</td>
<td>6.6–33.0%</td>
<td>[50]</td>
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<tr>
<td></td>
<td>18 provinces, Thailand</td>
<td>IHA, &gt;1:160</td>
<td>7.23%, n = 1050</td>
<td>[70]</td>
</tr>
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*continued on next page*
for all causes, from various regions in Queensland, Australia were evaluated by IHA at the Oonoonba Veterinary Laboratory, Department of Primary Industry, Townsville, Australia [74]. The greatest prevalence of seropositive pigs and goats originated from Townsville and surrounding districts, the Gulf of Carpentaria and the northern extremity of Cape York Peninsula, particularly the Torres Strait Islands (Table VIII.1). In this study seroprevalence in pigs was higher than in goats (4.9% versus 3.5%).

3.3. Seasonal variation in cases

As is the case for human melioidosis, animal melioidosis is a “wet season” disease and the number of cases of infection and symptomatic disease increases with rainfall. Studies surveying the incidence in animals in northern Queensland, Australia between January 1975 and December 1979, revealed that the majority of isolates were cultured between November to March [21] which coincides with the most significant rainfall period in tropical Australia. Similar studies in the Northern Territory, Australia showed a distinct pattern during the wet seasons, although increased incidence in some drier months (April to October) correlated with known outbreaks and increased disease surveillance.
A retrospective estimation of the incidence of culture-proven melioidosis in animals in Thailand during 2006 to 2010 [38] showed that 31 (61%) of the 49 cases were identified during the rainy season (June to November). This phenomenon can be explained by animals coming into contact with muddy water and soil particles carrying bacteria from deeper soil layers [75,76]. This is also true for infections after flooding [10].

3.4. Transmission

Infection is thought to be opportunistic and primarily a result of transmission from the environment rather than from animal to animal. The bacteria can be shed from an animal via its urine, faeces, milk or wound and respiratory secretions. The most common routes of infection in animals are via percutaneous inoculation [77], contamination of wounds [75], ingestion of soil, contaminated feed [77] or carcasses, or by inhalation [13]. Transplacental infection resulting in abortion has been reported in goats [78] and pigs. The organism has been isolated from normal and mastitic goat’s milk suggesting that transmission may also occur via this route [6,7]. Sexual transmission and other means of host to host transmission are possible but not documented. Nosocomial transmission has been reported in a Darwin veterinary hospital where four cats had melioidosis [48]. Arthropods can transmit the disease experimentally. Blanc and Baltazard [79] showed that the bloodsucking rat flea *Xenopsylla cheopis* and the mosquito *Aedes aegypti* could transmit the organism from one experimental guinea pig to another and they suggested that other biting arthropods may also act as vectors. There have been several reports of suspected transmission of melioidosis by insects [80,81].

Outbreaks in both animals and humans have also been associated with earth moving works in river beds or ponds, with erosion, geological events and the use of drinking water or untreated water for spray-cooling of animals kept under intensive management when there was heavy contamination with soil particles [13,21,75,82]. In China animal melioidosis correlates with positive soil sites [49]. In northern Queensland, Australia, 64 *B. pseudomallei* isolates were obtained from 1158 soil samples collected from sheep paddocks in an endemic area over a five-year period (1984 to 1988) [74]. Most isolates came from around shelters and water troughs in these paddocks and in most cases isolation was correlated with periods of high rainfall. Sixty-eight of the 115 sheep (59%) that grazed in these paddocks were seropositive and during this study period 34 sheep were killed because of suspect melioidosis. *B. pseudomallei* was not isolated from any of 4837 insects (midges, flies and mosquitoes) also collected as part of this study.

3.5. Animals as a reservoir of human disease

There is little evidence of a major animal reservoir of human infection, or to implicate particular occupational or recreational contact with animals as a risk factor for human infection [37,48,83]. Nevertheless some reports of possible animal to human transmission do exist and include an abattoir worker, a goat farmer, a sheep farmer and a veterinarian [40,48,83]. It is also plausible that in these cases both human and animal were exposed to the same environmental sources. Ribotyping of goat and human isolates from the Northern Territory, Australia showed that the ribotypes from infected goats and
one human case in Western Australia were indistinguishable [84]. More recently, Sim et al. [85], using microarray analysis of genomic DNA from diverse human, animal and environmental isolates, showed that human clinical isolates tended to cluster together and were divergent from animal and environmental isolates. This analysis therefore suggests that strains associated with human melioidosis may be distinct from many animal and environmental isolates.

It is clear that *B. pseudomallei* is endemic in the environment and can infect livestock, companion animals, or feral or native animal species that are often in close proximity to humans. Until such time as the zoonotic potential of the bacterial strains in animals is known, animal melioidosis cases may be indicative of both the environmental distribution of *B. pseudomallei* and potential human disease risk.

4. Clinical and pathological findings in affected animals

The spectrum of disease in animal melioidosis is similar to that in man. Once *B. pseudomallei* enters the body, the infection can either fail to spread, cause insignificant illness detected only serologically, or progress to peracute, acute, disseminated, chronic or latent forms. The majority of infections in animals are asymptomatic or subclinical and are often only diagnosed using serological assays. As is the case in human melioidosis, the incubation period in animals is variable, ranging from a few days to many years, and the early stage of melioidosis may have no notable signs. Upon dissemination of the organism and infiltration of organs, signs of disease may become apparent. Clinical signs vary depending on the location of lesions in the animal and whether the presentation is acute or chronic. The acute form can be septicemic or pneumonic and onset may present as fever, prostration, respiratory distress, gastroenteritis or diarrhea. Fatalities often occur in association with acute fulminating infections or when vital organs are affected. Chronic disease may be localised or systemic and often there may be only minimal, if any, visible changes to the general health of the animal although some animals may suffer from lethargy, fever, weight loss, lameness and arthritis. Chronic disease in animals is often only diagnosed at slaughter (livestock) or necropsy.

Pathological findings in man and animals are similar and primarily depend on whether the disease is acute or chronic. Infection may be associated with single or multiple suppurative or caseous nodules and abscesses, which can be located in any organ of the body. In acute cases where disease is of short duration, lesions are often microscopic (<3 mm) and typically contain a central necrotic core of neutrophils surrounded by fibrous tissue, macrophages, lymphocytes and plasma cells. The presence of a fibrous tissue capsule surrounding these small lesions is variable [4,41,86]. Chronic lesions are more often seen in animals than man and are grossly visible as widespread and multiple nodules and abscesses. The lesions may measure up to 100 mm. The purulent, necrotic core is surrounded by epithelioid cells and polymorphonuclear cells (PMN), in turn surrounded by a layer of lymphocytes, plasma cells, epithelioid cells, macrophages, PMN and sometimes multinucleated giant cells. A thick, fibrous tissue capsule encloses the lesion [7,41,86].
Choy et al. [48] reviewed lesion sites in infected animals in the Northern Territory, Australia, and showed that lung abscesses were found in 100% and 63% respectively of infected sheep and goats. Other common lesion sites were spleen (goats [60%], sheep [36%] and pigs [73%]) and liver (goats [44%], sheep [43%] and pigs [36%]). Other abscess sites varied according to the species but included lymph nodes, kidney, mammary glands, aorta, joints, bone and the central nervous system (CNS). The gastrointestinal tract is rarely affected although small areas of ulceration of the intestinal mucosa have been described [43,86,87]. Testicular lesions and epididymitis have been described in rams [88]. Case histories, clinical presentation and pathological findings for selected animals are described below.

4.1. Sheep

The first report of melioidosis (animal or human) in Australia was in 1949 to 1950 when “Malleomyces pseudomallei” was isolated from the lung abscesses of sheep during an outbreak of disease near Winton in northwestern Queensland [3,41]. This was the first description of melioidosis in sheep, although natural infections in domestic mammals (including guinea pigs, rabbits, rats, dogs and cats, cattle, horses, and pigs) had previously been described in Malaya (Malaysia), Ceylon (Sri Lanka) and the Dutch East Indies (Indonesia) in the 1920s to 1940s.

The original description of natural infection in sheep [41] reported that “the only symptoms observed were staggering and when driven the affected sheep soon became too weak to travel further. They became recumbent and died in one to seven days. The different age groups in the flock – five, six and eight years – had all sustained losses.” The manifestations of disease in these sheep varied and included: (a) abscesses in the lungs, spleen, liver and lymph nodes resembling classical melioidosis in humans and other animals; (b) suppuration and ulceration of the nasal mucosa similar to equine glands; (c) suppurative polyarthritis and (d) meningoencephalitis [41]. Culture of pus from lung abscesses yielded pure cultures of *B. pseudomallei*. The isolated bacterium caused disease in guinea pigs, mice and sheep when inoculated via various routes.

Both acute and chronic forms of disease are now recognised in sheep. Melioidosis in sheep is typically indicated by lameness (hind quarters), arthritis and respiratory distress. Death can occur in two weeks to two to three months. In acute disease the most commonly seen characteristic is pneumonia and respiratory distress with caseous abscesses in the lungs at necropsy. However, a high fever, anorexia, cough and a profuse ocular and/or nasal mucopurulent yellow discharge may also be present. If the infection is disseminated arthritis or encephalitis may also be seen. Central nervous system involvement may manifest as lameness, paralysis of the hindquarters, walking in circles, convulsions and blindness. In rams, orchitis and testicular nodules can be seen [88]. Cutaneous abscesses may also be present. Histological images of the lung and brain of a sheep presenting with purulent encephalitis and bronchopneumonia are provided in Figure VIII.1. Melioidosis was the definitive diagnosis.

Early experimental studies by Stanton and Fletcher (1932) [77] demonstrated the relative susceptibility of sheep to disease compared to other animals like pigs, horses,
Fig. VIII.1. Histological images taken from lung and brain of a sheep presenting with neurological symptoms which rapidly progressed to paralysis within 24 hours. The animal had a percutaneous wound three weeks prior to onset of neurological signs. *B. pseudomallei* was cultured from the pleural fluid. (a–c) The animal had purulent bronchopneumonia as evidenced by the congested lung tissue and marked neutrophil infiltration combined with hyperplasia and hypertrophy of the bronchial epithelium. (d) Multiple abscesses were present in the pons and medulla oblongata with marked neutrophil infiltration consistent with purulent encephalitis. Melioidosis was the definitive diagnosis. (a), (b) scale bar = 20 µm; (c), (d) scale bar = 10 µm; (K. Hodgson and L. Reilly, School of Veterinary and Biomedical Sciences, James Cook University, Townsville, Australia.)

monkeys and birds which were then, and are still, considered relatively resistant to severe disease. Stanton and Fletcher injected large doses of bacteria into sheep and caused death in six days, with nodules and abscesses seen in the liver and spleen. Similarly, Cottew [3] and Cottew et al. [41] infected sheep experimentally resulting in rapid death and clinical signs that recapitulated the presentation of natural infection (i.e. anorexia, dyspnoea, fever, lameness, nasal and ocular discharge and neurological signs).

4.2. Goats

The clinical picture of melioidosis in goats is somewhat similar to that in sheep; progressive emaciation, fever, anorexia, lameness and hindleg paresis and cutaneous abscesses have been reported [5–9,48,86,89]. Disease in older goats tends to be chronic with acute cases less frequent. Respiratory disease, including nasal discharge, coughing and pneumonia, is usually less severe in goats than in sheep, although lung abscesses
Melioidosis may be present [6,7,88]. Mastitis with palpable abscesses in the udder and lymph nodes appears to be particularly common in this species, as are abortions and aortic aneurysms [5,8,9,48,86,89]. Central nervous system disorders may also be present. Choy et al. [48] reported that lesions in culture-positive goats (n = 43) in the Northern Territory, Australia, were found in lungs (63%) > spleen (60%) > liver (44%) > lymph node (42%) > mammary gland (35%) > kidney (30%) > aorta (21%) > joint/bone (19%) > CNS (9%).

Goats are highly susceptible to experimental infection [78]. High doses (≥500 bacilli) caused acute, fatal infections. Lower doses (90 to 225 bacilli) caused acute or chronic disease when infection became established. In experimentally infected goats, clinical signs included undulating fever, wasting, anorexia, paresis of the hind legs, severe mastitis and abortion, mirroring natural disease presentation. At necropsy, abscesses were found predominantly in the spleen, lungs, subcutaneous injection site and the draining lymph node.

4.3. Pigs

Infection in adult pigs is often chronic or asymptomatic and various studies have shown that pigs are relatively resistant to melioidosis if nutrition and husbandry are satisfactory. Affected slaughter pigs usually show no visible abnormality until slaughter, when abscesses are found during routine meat inspection. Abscesses can occur in internal organs and are commonly found in the spleen, liver, lung and lymph nodes of the lung. If signs of disease are apparent they are usually mild but may include: skin ulcers, paresis of the hind legs, or joint problems causing lameness, anorexia, wasting and occasionally respiratory distress and nasal discharge. Orchitis may be observed in boars. Young animals may develop acute septicemia with fever, ocular and nasal discharge or coughing [5,12,86,90–92]. In one investigation [93], *B. pseudomallei* was isolated from piglets’ dermal ulcers, parotid lymph node, tonsil, lung and a superficial inguinal lymph node; a water sample from the unchlorinated bore supplying the property was confirmed positive for the organism. Molecular typing of isolates from three pigs using pulsed-field gel electrophoresis (PGFE) of DNA showed all the pig isolates to be identical supporting a point source (clonal) outbreak. The bore water isolate showed a different, although related, strain on molecular typing to those isolated from the pigs.

Most cases in pigs occur if the animals are raised in wet muddy conditions; however outbreaks can occur in intensive piggeries if drinking water is sourced from contaminated water supplies. During the early 1980s, the disease was diagnosed in the sub-tropical Central Burnett region of Queensland, Australia, in several intensive piggeries [12]. All the affected piggeries sourced water from the Burnett River. Accordingly, piggery owners using Burnett River water are advised to chlorinate the water in an effort to prevent infection. Affected pigs are condemned as unfit for human consumption following laboratory confirmation of the disease.

Pigs can be experimentally infected with high doses of bacteria [91]. Experimental infection of a single dose of $5 \times 10^8$ bacteria by intratracheal injection resulted in acute (21 cases) or chronic (19 cases) melioidosis in 40 of 48 pigs. Fifteen (10 acute
and 5 chronic) had been immunosuppressed by cyclophosphamide before inoculation. The major clinical signs were initial fever, marked neutrophilia and in the acute cases, respiratory distress. There were no signs of the nasal and ocular discharge, paresis or diarrhoea seen in acute cases in Southeast Asia. *B. pseudomallei* was isolated predominantly from the spleen, lungs and the injection site. Gross pathology of organs taken from experimentally infected animals showed abscesses in the trachea, lungs and spleen (Figures VIII.2a–c). This study suggested that Australian strains of *B. pseudomallei* may not be as virulent as the Southeast Asian isolates in pigs. A serological study of feral pigs in north Queensland revealed that 66% of animals tested (41/61) were seropositive for *B. pseudomallei* [94].

### 4.4. Horses

Stanton and Fletcher (1921) working in Malaya in the 1920s and 1930s introduced the term “melioidosis” to describe a condition resembling glanders which at the time was well known in horses. They and others however recognised that the aetiological agent causing disease in humans and animal in the early years of the 20th century could be

![Gross pathology of organs taken from animals with melioidosis.](image)
readily differentiated from the glanders bacillus. At this time the glanders-like disease melioidosis had been recognised in guinea pigs, rodents and humans but not in horses. Stanton and Fletcher [95] experimentally infected horses and ponies with large numbers of bacteria (10⁹ to 10¹⁰ organisms) and due to the lack of disease signs they concluded that "horses are apparently immune to infection with melioidosis". However subsequent investigations identified B. whitmorii as the aetiological agent responsible for respiratory illness in a race horse (named Elkins) imported to Malaya from Australia in 1925. B. whitmorii was repeatedly isolated from the horse and the isolated bacteria caused disease in laboratory animals. Furthermore, the blood of the animal agglutinated the standard strain of B. whitmorii in high dilutions, hence representing some of the earliest serological investigations of the disease.

Cases of melioidosis in horses are relatively uncommon with various disease syndromes described: (a) peracute with high fever, septicemia, limb oedema, diarrhoea and death within 24 hours; (b) acute with limb oedema, slight colic and intestinal hypermotility; (c) subacute to chronic (most common) which lasts three weeks to three months with no loss of appetite. Other signs of disease in horses include weakness, emaciation, anorexia, dyspnea, fever, mild cough, CNS involvement and coughing or purulent discharge from the nose and mouth [17,86]. Multiple abscesses in the lung are common and spleen, liver, kidney and brain abscesses and meningoencephalitis have also been described [4].

4.5. Cattle

Cattle appear to have significant resistance to B. pseudomallei [41] and cases are rarely reported; if reported, disease tends to be chronic and may include the following signs: cutaneous abscesses, fever, aggressive behaviour, panting respiration, continuous profuse salivation and a staggering gait. Acute arthritis may also be present. At necropsy splenic [56,96] and adrenal abscesses and meningoencephalitis and meningomyelitis [4] have been described.

In two animals in southeastern Queensland, Australia (27ºS), acute fatal infection with B. pseudomallei caused pneumonia, placentitis and endometritis in a pregnant cow, while a more chronic disease with encapsulated caseous lesions in the lung, and arthritis, occurred in a bull on the same property [10]. Nutritional and physical stress caused by a recent flood were thought to be predisposing factors for the disease occurring in this region which is far south of the melioidosis endemic area (i.e. 20ºN to 20ºS).

Experimental inoculation of cattle may result in local abscess formation and resolution although in general animals are quite resistant to experimental infection [4,56]. Serological studies in dairy and beef cattle and water buffalo in various provinces in Thailand demonstrate that up to 30% of animals in some areas had been exposed to B. pseudomallei [49,55,68–71].

4.6. Dogs and cats

Canine melioidosis is rarely seen; however, seven cases have been reported in United States military dogs in Vietnam [24,25,97,98]. Clinical signs in these dogs included fever, dermal abscesses, epididymitis, myalgia, lameness and leg swelling with acute
cases characterised by septicaemia with fever, severe diarrhoea and fulminating pneumonia. Subacute disease lasted from days to several months and manifested as skin lesions with development of lymphangitis and lymphadenitis resulting in septicaemia. Pulmonary involvement with subsequent septicaemia has also been observed. Postmortem lesions in affected dogs consisted of multiple abscesses of the skin, lungs, liver and epididymitis.

Melioidosis in cats is also very rare and necropsy findings such as abscesses in liver, spleen, and lymph nodes have been described although the clinical presentation and pathology can be diverse. O’Brien et al. [23] described the clinical presentation and pathological findings of two cats diagnosed with melioidosis at necropsy; one presenting with jaundice and anaemia, the other presenting with a wound infection, lameness and neurological signs. Both animals died and culture-positive splenic and hepatic abscesses were found at necropsy. Furthermore, the spinal cord, brain stem and spinal meninges of the second cat showed severe, focal necrosis with purulent inflammation. The cats had been recently relocated from Malaysia and northern Australia respectively; it was believed that disease may have represented reactivation of a latent infection brought on by the stress of relocation.

More recently two cats presented in Darwin, Northern Territory, Australia with sudden onset of a ‘red eye’ and eyelid spasm which progressed to an enlarged, firm globe with the loss of pupillary light reflexes and vision [99]. In both cats the affected eye was surgically removed and vitreal culture revealed a pure growth of \textit{B. pseudomallei}. The first case was still alive approximately one year later whilst the second cat was euthanased when a localised abscess developed on the same side of the face as the healed surgical incision. Both cases were caused by the same multilocus sequence type (MLST; ST 116) of \textit{B. pseudomallei}, which had only been isolated previously from two human patients, both living in the same isolated geographical area as the cats of this report. No epidemiological links were evident between the two cats and/or the two people.

4.7. Laboratory animals

Melioidosis was originally described as a natural disease of rodents [52]. In 1913 an outbreak occurred in laboratory rabbits, guinea pigs and rats housed at the Institute for Medical Research, Malaya; every case was fatal. Stanton and Fletcher described the clinical presentation and pathology in these animals:

The earliest symptom in rabbits and guinea pigs was a white milky discharge from the eyes and nose; within a few days the breathing became difficult and the animal died . . . . The appearance found postmortem depended very largely upon the duration of the illness. In animals which had died very quickly from acute septicaemia, the only visible signs of disease, apart from the milky discharge about the eyes and nose, were congestion of the nasal mucous membrane, trachea and lungs, and a few, yellow, miliary nodules on the nasal septum. When the course of the disease was chronic the lungs contained minute, caseous nodules, like miliary tubercles which consisted of a bronchiole and its surrounding alveoli filled with nuclear debris . . . . In the two rats, where the course of the disease was far less acute than in rabbits and guinea pigs, the miliary nodules had spread and coalesced until the thorax was filled with a caseous mass firmly adherent to the thoracic walls. Very little lung tissue remained which was not caseous; in the caseous mass the heart was embedded, and the
oesophagus behind it was studded with minute caseating nodules . . . . *B. whitmorii* could be cultivated from them in whatever situation they occurred and it was usually found in films made from the smaller nodules though not often present in very large numbers . . . . The spleens of our naturally infected laboratory animals were always enlarged.

Excerpt from Stanton and Fletcher [2]

Throughout the 1930s to 1980s it was generally accepted that rats were the main animal reservoir for human infection, with transmission to man occurring via eating food contaminated with rat excreta or by direct contact with infected animals. This was despite accumulating evidence through the 1930s to 1960s describing the regular isolation of the organism from soil and water and its infrequent isolation from healthy field rats. There were however occasional reports of the isolation of *B. pseudomallei* and seropositivity of feral rats. In one report 12 of 136 rodents had complement fixing antibody for *B. pseudomallei*; 10 of these animals were from the endemic Townsville area in northern Australia [100].

A variety of animals have been used as experimental models, including inbred mouse strains [101], chickens [102] and rats [103], hamsters [104] and guinea pigs [105,106]. Rats are now considered to be relatively resistant to *B. pseudomallei* infection although the infection of infant diabetic rats with *B. pseudomallei* typically results in acute septicaemic melioidosis and death, usually within seven days. Guinea pigs and Syrian hamsters have been shown to be exquisitely susceptible to infection and develop rapid fatal disease when as few as 10 organisms are injected intraperitoneally.

Most recently, the susceptible BALB/c and more resistant C57BL/6 inbred mouse strains have been used extensively in studies of host responses to *B. pseudomallei* [107,108]. BALB/c mice die of septicaemic disease with overwhelming bacterial loads in blood and organs, accompanied by organ inflammation and necrosis 24 to 72 hours after infection, reflecting a failure of the host innate immune response to contain the infection. A more comprehensive review is provided in Section VII.I.

### 4.8. Marsupials

Signs of melioidosis in marsupials are diverse and often mimic other diseases. Disease may be acute or chronic with subclinical infection being most common in these animals. Single or multiple purulent nodules/abscesses may be present and the organs most commonly affected include the lungs, spleen, liver and associated lymph nodes.

Ladds et al. [22] reported the postmortem findings of a koala (*Phascolatus cinereus*) that had been captured for routine chlamydial serological testing but that later died after release. They reported large necrotic lesions in the liver and spleen, haemorrhages of serosal surfaces including kidney and bladder and thorax and marked congestion and haemorrhage in the lung, kidney, adrenal glands and lymph nodes. Both *B. pseudomallei* and *Escherichia coli* were isolated from the spleen and liver lesions. Interestingly serological analysis of this animal was complement fixation test (CFT) negative for *B. pseudomallei*, whereas other healthy animals in the colony were CFT positive (8/50 animals).

*Burkholderia pseudomallei* has been responsible for the deaths of captive and free-ranging tree kangaroos in northern Queensland, Australia [21], a captive tree kangaroo
in Papua New Guinea [19] and numerous imported wallabies in Malaysia [109]. Presentation in kangaroos includes nasal discharge and posterior paralysis with abscess formation in the liver and spleen. Necropsy of wallabies revealed congestion of the liver, spleen, kidneys and lungs and very small necrotic foci throughout the liver and spleen, but no large abscesses. Figure VIII.2d shows the enlarged spleen of a wallaby with small necrotic foci.

4.9. Camels

Until the early 1990s melioidosis had not been recorded in camels, possibly because the organism is rare, or even absent, in the camel’s natural arid environment. Camels appear to be particularly at risk of contracting melioidosis when they are moved from their natural habitat into moist tropical areas [14,15]. In Australia camel farms have been established in higher rainfall tropical areas in recent years, subjecting animals to conditions not previously encountered. Although this is a rare occurrence in camels, affected animals presented with a severe purulent pneumonia, ataxia of the hind limbs, pyrexia and anorexia. Time to death was rapid in the reported cases suggesting that these animals are highly susceptible. Histologic examination of lungs revealed extensive neutrophil infiltration.

4.10. Marine mammals

Reports of melioidosis in cetaceans are infrequent, however multiple deaths in an oceanarium (Ocean Park) in Hong Kong, occurring during the annual typhoon season, have been reported. Twenty-four dolphins in 1975 and 27/32 dolphins in 1976 were the first reports of marine mammals with melioidosis and since this time there have been sporadic mortalities of large numbers of marine and terrestrial mammals and birds at this zoological park [39]. The clinical presentation and pathological findings in 25 marine mammals [seven false killer whales (Pseudorca crassidens), six aduncas-type bottlenosed dolphins (Tursiops truncatus), five gill-type bottlenosed dolphins (Tursiops truncatus), four Pacific white-sided dolphins (Lagenorhynchus obliquidens), one killer whale (Orcinus orca), one Californian sea lion (Zalophus californianus), and one grey seal (Halichoerus grypus)] that died in this oceanarium over an 11-year period (1982 to 1993) and from which B. pseudomallei was isolated have been described in detail elsewhere [39]. The clinical presentation in the majority of these animals was that of acute septicaemia. In summary, affected animals suffered from chronic and acute manifestations. Inappetence, anorexia, lethargy, pyrexia, enteric disease with diarrhoea and respiratory distress were also observed prior to death. B. pseudomallei was isolated from blood, lung, liver and kidney samples. Lesions of varying severity were present in the organs of all animals. Oedema, haemorrhage and white or yellow nodules were present throughout the lungs and were the most frequent abnormality. Small abscesses were present within the liver and hepatomegaly was seen in about 60% of cases. Splenic abscesses were uncommon and individual animals showed bone and mammary gland abscesses or focal necrosis in other tissues. The precise mode, mechanism and source of infection in this oceanarium is still unresolved.
In response to mortalities at Ocean Park an experimental acellular *B. pseudomallei* vaccine was developed and evaluated in captive cetaceans [110]. The antibody response to the vaccine was evaluated by ELISA. The vaccine preparation consisted of a protein–polysaccharide (1:3) mixture. An initial dosage of 500 μg carbohydrate for newly arrived dolphins and false killer whales and 1 mg carbohydrate for other long term resident animals followed by 500 μg boosters at six to ten week intervals for all animals maintained high specific antibody levels. Mortality due to melioidosis was reduced 45% to less than 1% over a five-year period. Antibiotic and water filtration programmes were also implemented to control disease in this facility.

### 4.11. Non-human primates

A relatively large number of cases of melioidosis have been described in primate species including chimpanzee, gorilla, orang-utan, marmosets, spider monkey, gibbon, banded leaf monkey and various macaques; the details of these cases are listed elsewhere [40]. All cases have occurred in either captive zoo or research facility animals in Southeast Asia, Australia, the United States of America or the United Kingdom with cases in the latter three countries occurring in (presumably) latently infected animals imported from melioidosis-endemic countries in Southeast Asia. It was assumed that a lowered resistance to disease in these animals due to transportation, handling and enclosure stress resulted in the reactivation of a latent infection. In the outbreak in a zoo in the United Kingdom, Dance et al. [31] noted that most of the infected animals had no clinical signs despite extensive splenic and hepatic abscesses and some soft tissue and skin infection and in this case the presence of infection was only suspected when they were shown to have serum antibodies to *B. pseudomallei* by ELISA. Others have reported that signs of melioidosis in primates include wasting, listlessness, general weakness, intermittent cough, nasal discharge, and mild respiratory disease that can lead to acute fulminating bronchopneumonia. Tissue swelling with the formation of multiple abscesses, typically in the lung, spleen, liver and occasionally bone has been noted, as has occasional nerve damage and paralysis [32,37,111,112]. The common marmoset (*Callithrix jaccus*) has been used to study inhalational melioidosis [113]. Disease progression in the common marmoset appears to be consistent with human infection in terms of bacterial spread, pathology and physiology.

### 4.12. Birds

Birds have generally been regarded as resistant to infection with *B. pseudomallei* [77,114]. Melioidosis is uncommon in wild birds but occurs in captive or exotic birds in melioidosis-endemic locations [35,36,102] or in birds imported from these regions. Asymptomatic carriage of *B. pseudomallei* can occur in wild birds but appears to be unusual [33]. The bird species affected are listed in Table VIII.2. There are few reports of the disease presentation in birds, although inappetence, lethargy, anorexia and diarrhoea leading to death have been described [35].

Recently, Hampton et al. [33] conducted a survey to determine *B. pseudomallei* carriage in wild native finches and doves from the melioidosis-endemic Darwin region,
Table VIII.2
Burkholderia pseudomallei isolation in birds

<table>
<thead>
<tr>
<th>Common name, species</th>
<th>Origin of bird species</th>
<th>Residence of diseased bird</th>
<th>Captive status</th>
<th>B. pseudomallei isolation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amazon parrots, Amazona albilorsus</td>
<td>n/a</td>
<td>Malaysia</td>
<td>Captive</td>
<td>n/a</td>
<td>[115]</td>
</tr>
<tr>
<td>Sulfur-crested cockatoo, Cacatua galerita</td>
<td>Australia</td>
<td>Townsville, Australia</td>
<td>Captive</td>
<td>Necropsy</td>
<td>[36]</td>
</tr>
<tr>
<td>Galah, Eolophus roseicapillus</td>
<td>Australia</td>
<td>Townsville, Australia</td>
<td>Captive</td>
<td>Necropsy</td>
<td>[35]</td>
</tr>
<tr>
<td>Macaroni penguin, Eudyptes chrysolophus</td>
<td>Macquarie Island</td>
<td>Hong Kong</td>
<td>Captive</td>
<td>Necropsy</td>
<td>[34]</td>
</tr>
<tr>
<td>Casowary, Casuarus sp.</td>
<td>Australia</td>
<td>Singapore</td>
<td>Captive</td>
<td>n/a</td>
<td>[37]</td>
</tr>
<tr>
<td>Palm cockatoo, Probosciger aterrimus</td>
<td>Australia, New Guinea</td>
<td>Singapore</td>
<td>Captive</td>
<td>n/a</td>
<td>[37]</td>
</tr>
<tr>
<td>Crowned pigeon, Goura sp.</td>
<td>New Guinea</td>
<td>Singapore</td>
<td>Captive</td>
<td>n/a</td>
<td>[37]</td>
</tr>
<tr>
<td>Chicken, Gallus gallus</td>
<td>Southeast Asia</td>
<td>France</td>
<td>Captive</td>
<td>Experimental injection/necropsy</td>
<td>[102]</td>
</tr>
<tr>
<td></td>
<td>Southeast Asia</td>
<td>Darwin, Australia</td>
<td>Captive</td>
<td>Skin lesions</td>
<td>[33]</td>
</tr>
<tr>
<td>Scarlet macaw, Ara macao</td>
<td>Central America</td>
<td>Hong Kong</td>
<td>Captive</td>
<td>n/a</td>
<td>[116]</td>
</tr>
<tr>
<td>Zebra dove, Geopelia striata</td>
<td>Southeast Asia</td>
<td>Hong Kong</td>
<td>Captive</td>
<td>n/a</td>
<td>[116]</td>
</tr>
<tr>
<td>Unknown bird</td>
<td>n/a</td>
<td>Malaysia</td>
<td>Wild</td>
<td>n/a</td>
<td>[47]</td>
</tr>
<tr>
<td>African grey parrot, Psittacus erithacus</td>
<td>Africa</td>
<td>Darwin, Australia</td>
<td>Captive</td>
<td>Necropsy</td>
<td>[33]</td>
</tr>
<tr>
<td>Yellow-bibbed lorikeet, Loriculus chlorocercus</td>
<td>Solomon Islands</td>
<td>Darwin, Australia</td>
<td>Captive</td>
<td>Necropsy</td>
<td>[33]</td>
</tr>
<tr>
<td>Peaceful dove, Geopelia phasian</td>
<td>Australia</td>
<td>Darwin, Australia</td>
<td>Wild</td>
<td>Beak swab</td>
<td>[33]</td>
</tr>
<tr>
<td>Emu, Dromaius novaehollandiae</td>
<td>Australia</td>
<td>Darwin, Australia</td>
<td>Captive</td>
<td>Brain</td>
<td>[33]</td>
</tr>
</tbody>
</table>

Adapted from Hampton et al. [33]; n/a, no data available.
Northern Territory, Australia. Swab specimens from beaks, feet, cloacae and faeces were cultured for *B. pseudomallei* and one healthy (normal physical appearance, weight, and hematocrit) native peaceful dove (*Geopelia placida*) was found to carry *B. pseudomallei* in its beak. Using MLST, the *B. pseudomallei* isolate was identified as sequence type 144, which had previously been found in soil and associated with human cases within 30 km of the site of bird capture. This survey confirmed that native birds are not very susceptible to infection with *B. pseudomallei* and disease but the finding of an asymptomatic native bird with *B. pseudomallei* in its beak suggests that dispersal of bacteria by birds from melioidosis-endemic regions to previously uncontaminated areas may be feasible. These authors also report a 2007 outbreak of melioidosis in an aviary in which two imported exotic yellow-bibbed lorikeets (*Lorius chlorocercus*) died within months of arriving in Darwin from a non-endemic area in southern Australia. At necropsy the birds showed nodules throughout the spleen and liver and *B. pseudomallei* was cultured from the liver, spleen, crop, beak, and rectum. Interestingly, *B. pseudomallei* was also found in water from sprinklers, the water bore head, soil next to the bore, and the drain of the aviary. The unchlorinated sprinkler system used to cool the aviary was identified as the likely source of infection.

5. Laboratory diagnosis of animal melioidosis

5.1. Isolation of organisms

Isolation and identification of the organism are required for the definitive diagnosis of melioidosis in animals. The organism can be isolated from lesions and discharges and sampling techniques used for humans are considered suitable for diseased animals. Pus, blood, urine and postmortem samples, including abscess material, milk (for mastitis cases), sputum and faeces (diarrhoea cases) may all be cultured for isolation of *B. pseudomallei*. The organism is readily cultured on routine diagnostic media and has a characteristic wrinkled colony form and odour in Ashdown’s media. In chronic conditions *B. pseudomallei* may be found in very small numbers and regressing or dry lesions are often sterile [7]. Optimal isolation is more likely from acute lesions with semi-solid to caseous contents. Direct Gram-stained smears of pus or other exudates can sometimes identify the bipolar “safety-pin”-shaped Gram-negative rods that are characteristic of *B. pseudomallei*.

5.2. Serological assays

Although bacterial isolation provides a definitive diagnosis, serological tests including CFT and IHA are often used to ascertain the infection status in animals and as a tool for herd surveillance [8,49,55,68,70]. Positive serological tests are not necessarily indicative of current infection but rather give an indication that there has been some contact with the organism with subsequent induction of an immune response. Rising antibody titres in paired or serial samples is more conclusive of a current or recent infection. Other serological tests that have been used in the diagnosis of melioidosis in animals include the immunofluorescent antibody test (IFA), serum agglutination tests (SAT) or microtitre agglutination test (MAT) and ELISA. To date, none of these tests are commercially
available. Cross-reactions may occur in serological tests with *Burkholderia mallei*, the causative agent of glanders.

From the early 1970s the IHA has been the serological test of choice for the diagnosis of human melioidosis, especially in endemic areas [117]. This test is also widely used for diagnosis and/or surveillance in diverse animal species. The titre of a positive IHA test is variably reported with titres ranging from $\geq 1/40$ to $\geq 1/160$ (Table VIII.1). The sensitivity and specificity of the IHA test is dependent on the antigen preparation used in the assay and this may account for the different titre cut-offs used by different investigators. IHA titres have no prognostic value and can persist for many years [117]. In acute cases, IHA titres greater than 1/1280 have been reported [8,32,98]. The availability of standard positive and negative sera across testing facilities may allow for the standardised reporting and a true comparison of IHA results within regions, countries and in different animal species.

Thomas et al. [8] evaluated the CFT, two IHA (IHA-A, IHA-L) tests which differed in antigen preparation and technique and a MAT for the serodiagnosis of melioidosis in goats. One hundred and eighteen experimental serum samples and 3143 field serum samples from goats in endemic and non-endemic areas of north Queensland, Australia, were tested. *B. pseudomallei* was isolated from 112 goats providing evidence of infection in this region. In this study the IHA-A test was the most sensitive whereas the CFT was the most specific. These authors recommended that the IHA-A be used as a screening test followed by the CFT for confirmation of active melioidosis. In this study the IHA-A test alone was a superior indicator of past infection. Similar studies in pigs [118] suggested that the IHA developed by Alexander [117] used in combination with a confirmatory CFT test is a sensitive and specific diagnostic strategy for porcine melioidosis. ELISA has been reported to be of similar sensitivity and specificity as the IHA test for the diagnosis of caprine melioidosis [74].

Serology for melioidosis diagnosis is largely restricted to a limited number of expert laboratories worldwide. The development of a standardised sensitive and specific serological test is urgently required both to understand the true prevalence of animal melioidosis in endemic regions and to adequately manage the introduction and dissemination of *B. pseudomallei* into non-endemic regions.

5.3. Other methods

In addition to serological methods, molecular methods including rapid PCR-based tests have been developed and are being used increasingly for organism identification and typing of isolates in cases of animal melioidosis. These assays can be designed to readily differentiate *B. pseudomallei* from the related organism *B. mallei* and other *Burkholderia* species [119,120].

6. Treatment and prevention

Treatment of animals with melioidosis is rarely attempted as it requires long term, expensive antibiotic regimes. If a treatment regime is pursued, ceftazidime and carbapenems, the antibiotics of choice for human melioidosis, may be used. More
conventional antibiotics, including tetracycline and cotrimoxazole are often still used due to their lower costs and greater accessibility to veterinarians.

Vaccines are not currently available for human or animal melioidosis and thus infection is typically dealt with by prevention and control measures. Animals are typically exposed to the organism in the environment and thus disease incidence may be reduced by removing animals from the contaminating source. This may include minimising contact with soil (e.g. using raised, concrete or paved flooring), providing drainage to prevent surface water accumulation, providing safe, clean drinking water (e.g. chlorinated) and the thorough cleaning and disinfection of animal areas [74]. Any animals showing signs of illness should be promptly isolated and a veterinarian consulted.

7. Concluding statement

Melioidosis has been a recurring problem in diverse animals in endemic regions since it was first reported in animals in 1913. Despite the increasing number of field and epizootic case reports there are still many deficiencies in our understanding of animal melioidosis, in particular host–pathogen interactions in different animal species, epidemiology of disease in endemic regions, zoonotic potential, optimal methods of diagnosis and treatment and prevention strategies. Exciting basic science research continues to elucidate much about the bacterium responsible for this disease and its interactions with its host. It is essential that existing and future collaborative networks facilitate the application and translation of this fundamental knowledge to answer some of the key questions surrounding this fascinating disease which continues to afflict both man and animals.

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IX
*Burkholderia pseudomallei* in the environment
Section IX. *Burkholderia pseudomallei* in the environment

**Editorial overview**

Mirjam Kaestli\(^a\), David M. Wagner\(^b\)

\(^a\) Tropical and Emerging Infectious Diseases Division, Menzies School of Health Research, Darwin, Australia

\(^b\) Department of Biological Sciences, Northern Arizona University, Flagstaff, Arizona, USA

*Burkholderia pseudomallei* is an intriguing organism to study. Despite dedicated efforts by the melioidosis research community there remains a host of unanswered questions. Perhaps some of the most intriguing questions have to do with the genetic diversity, geographic distribution, and environmental associations of *B. pseudomallei*. These exciting topics are the focus of this Section.

As discussed in this Section, examinations of genetic diversity within *B. pseudomallei* have provided important insights into this species and revealed that it is incredibly diverse. This diversity, which is due to both mutations and genetic recombination, provides both opportunities and challenges. Its high mutation rate has allowed researchers to study the evolution of *B. pseudomallei* on small scales, such as the infection of a single patient. The high recombination rates of *B. pseudomallei*, however, present significant challenges for reconstructing phylogenetic patterns within this species. Indeed, the recombination rates for *B. pseudomallei* are higher than those recorded for any other bacterial species. For this reason, it is considered to have an “open genome”, and recent studies employing whole genome comparisons have confirmed that large portions of the genome are variable from strain to strain. Where do these variable regions come from? Given that it is an environmental saprophyte, it seems obvious that the answer to this question will be found, quite literally, in the environment.

The global distribution of *B. pseudomallei* remains poorly understood. Outside of the main endemic regions, it has been reported sporadically from a variety of locations. Some of these have been tracked back to likely infection sources in endemic regions, but not all of them. This suggests that cryptic populations may exist outside of the known endemic regions but go undetected for a variety of reasons. In short, the real global distribution of *B. pseudomallei* remains unknown. This Section provides a discussion regarding tools and research strategies that can be used to address this question by detecting the presence of *B. pseudomallei* in the environment in a systematic and repeatable fashion.

*Burkholderia pseudomallei* is often referred to as a soil saprophyte. However, these bacteria are also commonly found in aquatic habitats, including unchlorinated rural water supplies in northern Australia. The elucidation of the ecological niche of *B. pseudomallei*
in the environment could assist in understanding virulence mechanisms and pathogenesis of these bacteria in humans. Their ability to invade eukaryotic cells likely provides them a selective advantage over other microbes in their natural environment. Indeed, *B. pseudomallei* has been shown to colonise protozoa and fungi and evidence is emerging regarding colonisation of plants. Together, these data show the different habitats in which *B. pseudomallei* is able to thrive and reflect the extensive genetic diversity observed. Genetic exchange with other members of the microbial community in soil, water, or the rhizosphere further strengthens the survival ability of these versatile bacteria.

It is essential that we develop a better understanding of the environmental associations of *B. pseudomallei* as this holds the key to better strategies for control and prevention. Indeed, we must look to the environment to address important questions such as: Why has *B. pseudomallei* become so virulent? What is its natural life cycle? Why is it found in some locations but not others? In addition to understanding the natural environmental associations of *B. pseudomallei*, we also must understand the influence of human activities on its occurrence and abundance. This Section contains detailed discussions on both natural and anthropogenic environmental associations of *B. pseudomallei*. 
Section IX.1

Genetic diversity and geographic distribution of *Burkholderia pseudomallei*

Talima Pearson, Erin P. Price, Apichai Tuanyok, Paul Keim

*Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ, USA*

**Abstract**

Frequent genetic recombination among *Burkholderia pseudomallei* isolates has resulted in impressive genetic diversity. Mutation, on the other hand, occurs frequently at some loci and generates diversity even during an acute infection. Strain variation is linked to geographic location and land use patterns. On small spatial scales, genetic diversity is greatest in Southeast Asia, but on a larger scale, it is greatest in Australia. While endemic to these two regions, *B. pseudomallei* is sporadically found in many equatorial countries, yet the worldwide distribution remains obscure, due to underreporting of melioidosis and increased globalisation that has facilitated dispersal to previously non-endemic regions. Nonetheless, two major populations exist that correlate with Southeast Asian and Australian regions. The Southeast Asian population probably originated following introduction from the ancestral Australian population.

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1. Genetic diversity of *Burkholderia pseudomallei*

Two chromosomes and a diverse set of genes make up the highly plastic genome of *B. pseudomallei*, facilitating survival in a dynamic soil environment and allowing rapid adaptation to a pathogenic lifestyle [1–3]. Genomic diversity is a result of varied genetic acquisitions, leading to a large pan genome of just over 7500 genes [3], with a resultant mosaic composition among these genes. Indeed, the core genome is likely to continue to decrease even below its current size of about 4800 genes [3].

Different regions of the *B. pseudomallei* genome vary in their diversity, due to the contributions of mutation and recombination. Coding sequences for essential functions are more prevalent in the large chromosome (Chromosome I), while sequences coding for accessory functions are more common in the small chromosome (Chromosome II) [1]. It is therefore not surprising that, on the small chromosome, the rate of nonsynonymous nucleotide substitutions is higher [3], and there is a larger proportion of genomic islands (Tuanyok A, personal observation), and the frequency of tandemly repeated regions is greater [4].
Evolutionarily stable molecular markers are needed to determine relatedness among a diverse set of strains. Quickly evolving markers produce confusing results, as unrelated strains may share alleles by chance rather than by descent, a phenomenon known as “homoplasy”. Loci used for multilocus sequence typing (MLST) are selected from essential housekeeping genes, specifically because they are predicted to be evolutionarily stable and thus evolve slowly [5]. The MLST scheme for *B. pseudomallei* includes seven loci on the large chromosome that are conserved among *B. mallei*, *B. thailandensis*, *B. oklahomensis*, and other *Burkholderia* species [6]. A large MLST database is publicly available (http://bpseudomallei.mlst.net), and houses sequence types (STs) from hundreds of isolates. At the species level, these loci provide a robust indication that: *B. mallei* is a monophyletic derivative of *B. pseudomallei*; *B. thailandensis* is a sister group to *B. mallei*/*B. pseudomallei*; an as yet unnamed species is sister to *B. mallei*/*B. pseudomallei*/*B. thailandensis*, and *B. oklahomensis* is sister to all four groups [6–8].

Multilocus sequence typing is not ideal for determining relatedness among *B. pseudomallei* STs, due to high levels of lateral gene transfer (LGT) [8,9]. In fact, the contribution of recombination is between 18 and 30 times greater than that of mutation in generating allelic diversity, far higher than any other bacterial pathogen [8]. Interestingly, despite this high ratio of recombination to mutation, genetic diversity at MLST loci is relatively low compared to other pathogens, indicating that overall levels of both mutation and recombination at these housekeeping genes must be low [8].

Allelic differences at MLST loci are due to single nucleotide polymorphisms (SNPs). SNPs are highly informative evolutionary markers, as they have low mutation rates and few character states. SNPs are not limited to MLST loci, but are extensively distributed throughout the genome and are best discovered through whole genome comparisons. For phylogenetic purposes, assaying a large number of SNPs dispersed across the entire genome can mitigate the effects of LGT, as different portions of the genome are likely to have different evolutionary histories. Vast numbers of SNPs increase both the likelihood that differences will be found among closely related isolates, and that phylogenetic signals will outweigh the noise of convergent evolution. Assaying SNPs from across the genome decreases the ability for individual LGT events to disrupt the phylogeny.

Out our phylogenetic analysis of 23 whole genome sequences (WGS) of *B. pseudomallei* resulted in statistically robust and fully resolved relationships [8]. This work showed that Australian *B. pseudomallei* isolates share a more ancient common ancestor than do isolates from Southeast Asia, consistent with an Australian origin for the species. A rough estimation of divergence dates using molecular clock methods places the origin of the Southeast Asian population at 16,000 to 225,000 years ago. We also estimated the date of the last common *B. pseudomallei* ancestor at 25,000 to 346,000 years ago. It is difficult to compare genetic diversity at SNP level across populations and across species, as similar work only exists for a small number of species and sampling across populations is poor. However, given our results, we predict that genomes from Australian isolates are more genetically diverse than those from Asia.

Genomic islands (GIs) are foreign DNA segments acquired by inter- or intraspecies horizontal gene transfer (HGT). GIs are common in *B. pseudomallei*, *B. thailandensis* and *B. oklahomensis*, but not *B. mallei* [10]. One of the major mechanisms responsible...
for the high genomic diversity within \textit{B. pseudomallei} is DNA mobilisation via bacteriophages [1]. To date, we have described 128 GIs from 11 complete, or nearly complete, \textit{B. pseudomallei} genomes (Tuanyok A, unpublished data). These GIs are found at 42 chromosomal locations, are differentially present among multiple \textit{B. pseudomallei} strains, and are often associated with tRNA gene loci, making tRNA-mediated site-specific recombination a likely mechanism facilitating the HGT of GIs [10]. Functionality of most of these GIs is unknown or unverified. However, these GIs may contain genes that could benefit survival in a host or under extreme environmental conditions.

In addition to SNPs and GIs, variable number tandem repeat (VNTR) regions are valuable for phylogenetic purposes, due to their high mutation rates and also may have a diversifying impact on a genome. VNTRs usually mutate by slipped-strand mispairing, although recombination also contributes to VNTR differences in \textit{B. pseudomallei} [11]. While mutation rates of \textit{B. pseudomallei} VNTRs tend to be similar to other species, these loci are much more frequent in the \textit{B. pseudomallei} genome [4]. The \textit{B. pseudomallei} genome contains an average of 69.9 tandem repeat arrays/Mbp on the large chromosome and 102.1 arrays/Mbp on the small chromosome (Figure IX.1). In contrast, the \textit{Yersinia pestis} CO92 and \textit{Bacillus anthracis} Ames genomes contain only 37.4 arrays/Mbp and 12.6 arrays/Mbp, respectively [4]. Recombination within and between chromosomes also adds to the diversity of VNTRs in \textit{B. pseudomallei}, as approximately 36% of arrays are duplicated in either chromosome [4].

Multiple-locus VNTR analysis (MLVA) provides the ability for fine scale evolutionary resolution that can reveal more detailed epidemiological patterns than SNPs, due to these high mutation rates. Because of the intrinsic speed of evolutionary change at VNTR loci, homoplasy is common and can result in misleading conclusions regarding relatedness of samples. However, the patterns and rates of VNTR mutations can be incorporated into evolutionary models for increased phylogenetic accuracy [8,11].

\textit{Burkholderia pseudomallei} has an exceptional ability to accumulate genetic diversity within a single infection, particularly at fast-evolving GI and VNTR loci (Figure IX.2). This remarkable genetic diversity has been observed by genetically characterising multiple \textit{B. pseudomallei} isolates obtained from within single acute and chronic infections, using MLVA [11,12]. WGS has also been used to compare genomes of \textit{B. pseudomallei} collected from chronic melioidosis patients. The paired isolates, 1106a and 1106b, and 1710a and 1710b, obtained three years apart from two Thai melioidosis patients, differ by five and eight (1106 and 1710, respectively) validated genic SNPs in both patients [3]. WGS is ideal for identifying all mutations between closely related isolates, but has the shortcoming of being impractical for genotyping large numbers of samples.

The epidemiological, evolutionary and therapeutic consequences of the rapid genetic divergence in \textit{B. pseudomallei} are still not completely understood. However, the ability to further increase the genetic repertoire is likely to facilitate rapid adaptation to myriad environments, including mammalian hosts.
Fig IX.1. Linear repeat array distribution of *B. pseudomallei* and *B. anthracis*. Horizontal scale indicates chromosomal location of repeat arrays. The length of repeat arrays is indicated by nucleotide length. Data generated by Tandem Repeats Finder software [4].
2. Geographic distribution of *Burkholderia pseudomallei* and melioidosis

*B. pseudomallei* is endemically found in Southeast Asia and northern Australia. In recent years other tropical and subtropical parts of the world have become recognised as having *B. pseudomallei* sporadically in the environment, or also being endemic for this bacterium (Figure II.2) [13,14]. Cases of confirmed melioidosis have been reported in other tropical and subtropical areas, suggesting a wider *B. pseudomallei* presence than previously thought [14,15]. *B. pseudomallei* has also been documented following clonal introductions into temperate areas such as France, southwest Western Australia, and southeast Queensland [15], but sporadic cases of melioidosis in non-endemic regions are usually associated with travel to regions where *B. pseudomallei* is endemic or with *B. pseudomallei*-contaminated merchandise.

The true extent of geographic distribution of *B. pseudomallei* remains abstruse, as melioidosis is under-reported for several reasons. Firstly, cases are usually associated with certain seasonal factors, such as heavy rains or typhoons, and therefore a lack of cases at other times of the year may lead to under-recognition of endemicity. Secondly, diagnosis and confirmation requires laboratory facilities not always available in many regions where *B. pseudomallei* may occur. Thirdly, diagnostic expertise may be lacking in...
countries where *B. pseudomallei* is not commonly encountered. Therefore, improvements in diagnosis of *B. pseudomallei* and better recognition of melioidosis symptoms, will likely increase our understanding of *B. pseudomallei* distribution and prevalence.

3. Genetic diversity on a small spatial scale

Understanding the population genetic structure and existing biodiversity of *B. pseudomallei* in endemic environments has implications for tracing outbreak sources [16,17], or identifying a deliberate release of *B. pseudomallei* during a bioterrorist event [18]. Characterising *B. pseudomallei* biodiversity in the environment may also provide clues on whether certain genotypes are more likely to cause human or animal infection. Lastly, biodiversity of *B. pseudomallei* may be influenced by the effects of anthropogenic disturbance of the environment, such as agriculture (for example, tilling, pesticide use, fertilisation, planting and flooding), or housing development. In non-endemic regions, genetic diversity is more limited than in endemic regions and follows a pattern similar to an infection outbreak, in which a single clonal introduction and expansion over time results in a relatively homogeneous population [13,19]. Here, we focus only on *B. pseudomallei* population structure in endemic environments.

Relatively great genetic diversity of *B. pseudomallei* in the environment exists on both small (for example, single soil-sampling site) and large spatial scales. Studies of genetic diversity have shown that *B. pseudomallei* is genetically heterogeneous in the environment, such that there is sometimes limited overlap of *B. pseudomallei* genotypes in time and space, even when sampling sites are in close proximity; although dominant genotypes usually coexist with several minority genotypes [18,20,21]. Some differences in *B. pseudomallei* prevalence and genotypes exist between disturbed and undisturbed sites [22,23], although the extent to which these differences are caused by human–environment interaction is not known. In summary, even at a micro-environmental scale, *B. pseudomallei* diversity can be exceptionally high. Population structure is extremely difficult to discern and may remain so even as more intensive sampling is conducted.

4. Genetic diversity on a regional scale

Regional differences between *B. pseudomallei* strains from the two major endemic regions of northern Australia and Southeast Asia have been well established, unlike differences in a small spatial scale. Differences in clinical manifestation between melioidosis in Australia and Southeast Asia suggest genetic differences between isolates from these two geographic regions. Specifically, patients in Thailand are much more likely to exhibit parotid abscesses but much less likely to display brainstem encephalitis and genitourinary infection compared with Australian patients [13,24].

Genetic differences between these two populations have since been identified. For example, approximately 90% of Australian isolates contain a flagellin and chemotaxis gene cluster, whereas 98% of Thai isolates possess a more truncated fimbrial gene cluster (Figure IX.3) [25]. Regional differences have also been demarcated using a population
Fig. IX.3. Regional distribution of flagellin/chemotaxis (group YLF) and fimbrial (group BTFC) gene clusters in *B. pseudomallei* isolates [25].

Fig. IX.4. MLST diversity of *B. pseudomallei* isolates from Australasia (red) and Southeast Asia and elsewhere (black) [8].

A genetic approach with MLST data; this shows two main populations of *B. pseudomallei* that are largely correlated with the geographic regions of Australia or Southeast Asia [8].

Phylogenetic analyses using WGS which indicate an Australian origin for *B. pseudomallei*, and regional patterns of genetic diversity, reinforce this observation [8]. An ancestral population can be expected to exhibit greater genetic diversity due to its relative age, while derived populations will be subject to founder effects that decrease diversity. Indeed, MLST data show a higher relative allelic diversity in the Australian population, and WGS-based phylogenies suggest that there was a limited ancient introduction from Australia to Southeast Asia [8]. Interestingly, despite the higher allelic diversity in the Australian population, the overall number of STs is lower than in the Southeast Asian population (Figure IX.4). This seemingly contradictory situation is likely to be due to a higher level of LGT in the Southeast Asian population as a result of greater environmental *B. pseudomallei* densities, facilitating greater contact and genetic exchange among Southeast Asian strains compared with their Australian counterparts.
5. Genetic diversity on a global scale

Increased recognition of melioidosis in non-endemic parts of the world will increase our understanding of the global distribution of *B. pseudomallei*. Clinical and environmental samples of *B. pseudomallei* are being found in countries with subtropical and even temperate climes. In most instances an epidemiological link can be made to an endemic region, either through trade or travel. However, in other sporadic cases, there is no obvious epidemiological link. For example, in 2008 a case of melioidosis was reported in Arizona, in which the patient had not travelled to a *B. pseudomallei*-endemic region, and extensive environmental sampling did not produce any *B. pseudomallei* isolates. The source of infection thus remains unclear. Curiously however, an MLST match between the clinical sample and an isolate from Malaysia was found.

Examples where anthropogenic travel and trade resulted in melioidosis cases in non-endemic areas probably represent only a small fraction of novel introductions. Ecological establishment of *B. pseudomallei*, on the other hand, must link persistently favourable environmental conditions with an introduction event. Without the involvement of modern humans, ecological establishment in a novel region is probably rare. Non-anthropogenic mechanisms for the dispersal of *B. pseudomallei* are not well understood, but could be wind- or animal-mediated. The discovery and attribution studies of *B. pseudomallei* outside the tropical regions of Southeast Asia and northern Australia would shed insight on both the distribution and dispersal mechanisms of this organism.

References


Section IX.2

Presence and sampling of *Burkholderia pseudomallei* in soil

Direk Limmathurotsakul\textsuperscript{a,b}, Vanaporn Wuthiekanun\textsuperscript{b}, Apichai Tuanyok\textsuperscript{c}, Sharon J. Peacock\textsuperscript{b,d}

\textsuperscript{a} Department of Tropical Hygiene, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand  
\textsuperscript{b} Mahidol–Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand  
\textsuperscript{c} Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ, USA  
\textsuperscript{d} Department of Medicine, University of Cambridge, Addenbrooke’s Hospital, University of Cambridge, Cambridge, UK

Abstract

*Burkholderia pseudomallei* has been isolated from the environment in Southeast Asia, northern Australia and Brazil. Environmental sampling is influenced by the natural distribution of *B. pseudomallei* in soil, as well as agricultural land use. Sampling commonly utilises culture-based methods, although polymerase chain reaction (PCR) is more sensitive and applicable for studies defining the presence or absence of *B. pseudomallei*. Sampling should be designed to reduce the probability of false negative results. Defining *B. pseudomallei* genotypes in soil samples is complex, requiring culture and genotyping of multiple primary colonies from a single sample and evaluation of multiple independent samples. This is because a single sample may contain multiple genotypes, and genotypes may vary between soil samples just a few metres apart. Approaches that address some of the pitfalls of existing methodology are discussed.

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1. Introduction

Melioidosis was initially thought to be a zoonotic disease with rats acting as the reservoir host [1]. However, subsequent microbiological surveys demonstrated that *B. pseudomallei* was an environmental saprophyte [2]. Publications reporting *B. pseudomallei* detection in the environment, or cases of melioidosis in indigenous, non-mobile populations, have gradually extended the known geographical distribution of *B. pseudomallei*. It has been isolated from the environment in south and east Asia (India, Laos, Thailand, Cambodia, Vietnam, Malaysia, China, Hong Kong, Taiwan and Singapore), areas of Australia (most notably the northern Top End but also the northeast and tropical western regions), and Brazil [3]. As mapping has been ad hoc and piecemeal, the actual global distribution of *B. pseudomallei* is uncertain. In addition, most previous environmental detection
studies utilised culture, a methodology that has not been standardised. Recent studies have highlighted problems with common sampling strategies and proposed the need for more rigorous approaches with relevant geostatistical tools [4–6].

2. Factors associated with the presence of environmental Burkholderia pseudomallei

Environmental B. pseudomallei is predominantly found in tropical or sub-tropical climates. Specific environmental factors associated with its occurrence have been reviewed by Inglis and Sagrianti [7]. Although our understanding of these factors is very incomplete, it is thought that temperature, humidity, rainfall, sunlight, pH, soil composition, aeration, ultraviolet light, type of vegetation and presence of other soil micro-organisms, may all affect B. pseudomallei survival in soil. In a laboratory setting, increased soil water content has been positively associated with increased B. pseudomallei survival, with survival for 70 days when water content was less than 10%, compared to 726 days when water content was greater than 40%. It can be isolated from soil and water at a wide range of pH (2 to 9) but the optimum has been reported to be pH 5 to 8, consistent with most cultivated soils in Southeast Asia. B. pseudomallei has been associated with lower soil pH in northern Australia, as well as different combinations of soil texture and colour and the presence of livestock [8]. Laboratory studies also have demonstrated that B. pseudomallei may interact with fungi, free-living amoeba and nematodes, and become internalised in amoebic cysts. B. pseudomallei is also able to colonise inside spores and hyphae of the arbuscular mycorrhizal fungus, Gigaspora decipiens. It has been postulated that plant roots may serve as reservoirs for environmental B. pseudomallei. Indeed, B. pseudomallei is often associated with rice plants and rubber trees in Thailand and grasses in Australia (Section IX.4).

3. Methods of detection of Burkholderia pseudomallei in soil

The most common methodological approach for B. pseudomallei detection in soil involves culture of soil using selective media, with identification of suspected B. pseudomallei colonies on the surface of solid agar. The protocols used have varied, and overall efficiency of these different techniques has not been compared. Variation among protocols include: soil sampling depth, weights of the starting soil sample, soil/water ratios, use of enrichment media versus water, degree to which the soil/water mixture is shaken prior to sedimentation, length of time the mixture stands prior to removal, and centrifugation of supernatant and culture of the deposit rather than direct plating of supernatant [4,5,8–14]. Most protocols utilise selective enrichment broths, but the specific broth varies among studies, including selective modified Ashdown broth [14], selective enrichment broth consisting of threonine-basal salt plus colistin (TBSS-C broth), and trypticase soy broth containing crystal violet and colistin (CVC broth) [4,5,13]. Selective broths have been compared in both laboratory [15] and field settings [12], with the latter study determining that TBSS-C was the best selective enrichment broth of the three mentioned above. Semi-quantitative counts of environmental B. pseudomallei can be obtained by inoculating
serial dilutions of supernatant onto Ashdown agar, and performing a colony count following a period of incubation [6]. It is assumed that all colonies of a given morphological appearance are \textit{B. pseudomallei}, following the positive identification of several representative colonies on primary culture plates. Where available, the use of a rapid and specific latex agglutination test [16] can assist in colony identification and facilitates testing of numerous colonies, including representatives of a range of colony morphology types.

Polymerase chain reaction is documented to be more sensitive and applicable for studies defining the presence or absence of \textit{B. pseudomallei} in soil. A study in Australia reported that conventional PCR targeting the 16S rRNA gene was more sensitive than culture for detecting \textit{B. pseudomallei} [17]. Similar sensitivity was reproduced for the 16S rRNA assay in a study conducted in Taiwan, along with an assay targeting the flagellin gene [18]. A real-time PCR assay targeting the \textit{B. pseudomallei} type III secretion system has also been developed; this assay was shown to be faster and more sensitive than culture for detection of \textit{B. pseudomallei} in soil collected in northern Australia [14]. This assay has been used to examine the association between the presence of \textit{B. pseudomallei} in soil and environmental factors [8]. Historically, \textit{B. pseudomallei} has also been detected in the environment via intraperitoneal inoculation of supernatant fluid from sedimented suspensions of soil into hamsters, with subsequent culture from animals that die [9] although it is not currently used.

4. Spatial distribution of \textit{Burkholderia pseudomallei} in soil

Environmental sampling to detect and quantify \textit{B. pseudomallei} will be influenced by the natural distribution of the organism in soil, and agricultural land use, which affect bacterial distribution. Design of the most effective sampling strategy can be aided by several geostatistical approaches. The distance (range) over which counts of a given environmental bacterium are related can be defined using a geostatistical tool called the “semivariogram” [19]. Use of this approach has found that the range can vary from micrometres to several metres, depending on the bacterial species. Optimal distance between sampling points (sampling grid spacing) can be determined using the range of spatial autocorrelation over distance between two sampling points (lag distance). The first study to utilise these approaches for \textit{B. pseudomallei} [6] applied a semivariogram to datasets that defined presence and quantity of \textit{B. pseudomallei} in disused land [4] and a rice field [5] in northeast Thailand. Spatial autocorrelation of \textit{B. pseudomallei} was detected: samples from adjacent sampling points were likely to yield the same culture result (i.e. \textit{B. pseudomallei}-positive or -negative). \textit{B. pseudomallei}-positive samples yielded similar \textit{B. pseudomallei} counts (high or low; Figure IX.5) [6]. The range of spatial autocorrelation in quantitative \textit{B. pseudomallei} count was 11.4 metres for disused land, compared with 7.6 metres for the rice field.

5. Genotypes of \textit{Burkholderia pseudomallei} in soil

Genotyping \textit{B. pseudomallei} isolates obtained from environmental samples is of interest for several reasons. The resulting genotypes can be compared to genotypes obtained from
cases of disease in humans and animals to determine likely environmental sources or substances of infection, and to determine whether invasive isolates form a more virulent subset of the overall population. Genotyping *B. pseudomallei* soil isolates would also be important for attribution purposes, following the deliberate release of *B. pseudomallei* associated with bioterrorist activity. The accuracy of such studies depends on detection of all *B. pseudomallei* genotypes present in a given sample (with the exception of those that may fall below the level of detection).

Until recently, the primary approach for defining the genotype of environmental *B. pseudomallei* was to perform random sampling in the geographical area of interest and genotyping a single bacterial colony picked from a primary agar plate culture for each
sample. However, two studies conducted in northeast Thailand [4,5] have demonstrated that this approach is potentially flawed, due to the number of genotypes detected from a single sample. Direct culture of soil sampled from an area of disused land using a fixed interval sampling grid detected *B. pseudomallei* in 77 of 100 samples [4]. Genotyping was undertaken to determine variation both across the sampling site, and within a single soil sample [4]. Genotyping of 200 primary plate colonies from each of three independent sampling points was performed using a combination of pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). Twelve PFGE types and nine sequence types (STs) were identified; the majority of genotypes were present in only a single sample. Two samples contained four STs, and the third contained three STs. Although distances among the three sampling points were small (7.6 to 13.3 metres), only two STs were present in more than one sample. Each of the three samples was characterised by the localised expansion of a single *B. pseudomallei* clone, consisting of a different genotype in each sample, such that one clone represented 51% to 87% of the 200 colonies examined from each sample. These results imply that genotyping one bacterial colony from a single sample is likely to detect the predominant clone in that sample and will not represent all genotypes present. Furthermore, genotyping a single *B. pseudomallei* isolate present in a sample may not be representative of genotypes present in soil just a few metres away [4]. A similar study conducted in a rice field in the same region demonstrated that genetic diversity and structuring of *B. pseudomallei* was present, despite flooding and the physical and chemical effects associated with farming [5].

6. **Sampling strategies**

6.1. **Soil sampling strategies**

Sampling strategies include fixed-interval, random, stratified, adaptive, and multistage sampling [20]. The most appropriate strategy will depend on study objectives, and whether existing information is available for the area to be sampled. “Fixed-interval” sampling is simple and recommended for the generation of semivariograms. “Stratified” sampling, in which the experimental area is divided into zones or strata and unequal numbers of samples are taken from each stratum, may be used where sampling areas differ greatly or prior information indicates that the *B. pseudomallei* prevalence varies across the study area. “Adaptive” sampling may be suitable in an area where the presence and/or distribution of *B. pseudomallei* is unknown, because sampling fewer than 10 points per site may give an overall false negative result for the site, even if the actual probability that an independent sampling point will be positive is as high as 30%. For example, a pilot study could be performed in which 20 random points are sampled and tested for the presence of *B. pseudomallei*. If any point is positive for *B. pseudomallei*, this confirms the presence of the organism at the site. If none are positive, a second round of sampling would be conducted in which a larger number of random points or all possible sampling grid points are sampled in the same area. Limmathurotsakul and colleagues have suggested that, for northeast Thailand, a minimum of 100 sampling points be used in an area of land measuring 30×30 metres in the event that the first round of sampling
is negative [6]. Elsewhere, number of sampling sites and number of sampling points per site should be based on formal sample size calculations [21,22]. A multilevel approach could be used to determine the geographical distribution of *B. pseudomallei* within a wider region, such as a province or country. This sampling method involves considering a primary sample unit, such as a field, and a secondary sample unit, such as a sampling point within a field. Defined areas of land (the primary sample unit) are selected from the entire region, using a sample size calculation. Each experimental area is then sampled using adequate numbers of sampling points, and an initial sampling grid size. Sampling strategies in each field could be based on random, fixed-interval, stratified, or adaptive sampling, as described above.

6.2. Optimal sampling grid size

Optimal sampling grid size could be calculated based on spatial autocorrelation (semivariogram) of *B. pseudomallei* in a given region, with a consideration of the level of precision required [21,22]. The suggested distance between sampling points in northeast Thailand is 2.5 to 3.5 metres [6]. However, variability in *B. pseudomallei* counts from soil within, and among, different countries is well described [11,13,14], and therefore the range of autocorrelation is likely to differ. Thus, the sampling distance proposed above may not hold true in areas where the predicted *B. pseudomallei* count in soil is markedly different. To evaluate the range of spatial autocorrelation across geographic areas, a semivariogram and area-specific spatial autocorrelation values should be calculated from initial sampling of at least 100 to 150 sampling points [23]. Optimal distance between sampling points can then be calculated using half the range of the spatial autocorrelation observed in the semivariogram [22].

6.3. Determination of the presence or absence of *Burkholderia pseudomallei* in the environment

One of the most important reasons for environmental sampling is to define the global distribution of *B. pseudomallei*, and the associated geographic distribution of risk to humans and livestock. With this in mind, the sampling strategy should be designed to have high sensitivity and a low risk of false negatives. This requires consideration of the number of samples that need to be taken before a negative result can be viewed with confidence. In most studies conducted in Southeast Asia and northern Australia, the number and position of sampling points in an area were most likely selected on an *ad hoc* basis, and without reference to sample-size power calculations or type of sampling strategy [10–14]. These studies provide important information for regions where *B. pseudomallei* occurs, and have determined that bacterial load in soil is higher in areas of Southeast Asia, including northeast Thailand and Laos, than in northern Australia. However, use of a small number of sampling points per test site could lead to false negative results and an underestimation of the geographical distribution of positive sites, particularly in areas where environmental *B. pseudomallei* is present at a low count and/or is sparsely distributed.
The risk of a false negative result can be quantified by defining the reliability of a negative culture, based on the 95% binomial confidence interval (CI) [6]. If only 10 independent environmental samples are randomly collected in one area, and all are negative, the exact 95% binomial CI would range from 0% to 30.8%. This means that, even if the probability of a sample being positive for *B. pseudomallei* is actually as high as 30%, it is still not uncommon for all 10 independent samples randomly selected from this site to be negative as the 95% binomial CI includes 30%. For example if 100 samples were taken, the exact 95% binomial CI would be 0% to 3.6%, and if 1000 samples were taken the CI would be 0% to 0.4% (Figure IX.6). In addition, if sampling points are very close to each other, the results are likely to be the same. As a result, they are not independent and the power to detect *B. pseudomallei* in the field decreases.

6.4. Sampling strategies to determine the genotype of environmental Burkholderia pseudomallei

Using binomial sampling statistics, it is possible to calculate that genetic characterisation of approximately 60 individual, primary plate bacterial colonies from a single sample, would provide a 70% probability of detecting a genotype present in that sample, at a frequency of 2%. The frequency of 2% is based on the observation that, when 200 primary plate colonies of *B. pseudomallei* were genotyped from a single sample, the lowest frequency of a single genotype was 9/200 (4.5%), the lower limit of the 95% confidence interval for which is 2% [4]. The temporal stability of genotype distribution is not known,
and it is possible that markedly different genotypic frequencies might be recorded on repeated sampling of the same sampling site. It is also unclear whether the degree of genetic diversity described in the two studies conducted in disused land and a rice field in northeast Thailand is reproducible elsewhere in Thailand and in other endemic countries. A study by Pearson et al., in which genetic diversity was demonstrated by variable-number tandem repeat (VNTR) analysis within a small geographic area of northern Australia [24], suggests that it may prove to be a reproducible finding.

7. Conclusion

A systematic approach to global mapping of *B. pseudomallei* using standardised soil sampling protocols and sampling strategies that take into account recent findings related to the random distribution and spatial correlation of *B. pseudomallei* in soil, and the extent of genetic diversity both within and among soil samples, is required. Further studies are required to determine whether these findings are reproducible beyond Thailand. Molecular methods are likely to become more widely used, since they are rapid and more sensitive than culture. However, current methods are only suitable for studies that need to determine whether *B. pseudomallei* is present or absent. Such an approach is not suitable for the dissection of bacterial genotypes in soil since DNA extraction may contain mixed *B. pseudomallei* genotypes. An understanding of the basis as to why *B. pseudomallei* persists in soil in some regions but not others offers the tantalising prospect of disease control through manipulation of the environment.

References


Section IX.3

Burkholderia pseudomallei in water

Timothy J.J. Inglis\textsuperscript{a,b}, Mark J. Mayo\textsuperscript{c}

\textsuperscript{a} Division of Microbiology and Infectious Diseases, PathWest Laboratory, Nedlands;
\textsuperscript{b} Microbiology and Immunology, School of Biomolecular, Biomedical and Chemical Sciences, University of Western Australia, Australia
\textsuperscript{c} Menzies School of Health Research, Darwin, Australia

Abstract

Burkholderia pseudomallei is well suited to prolonged survival in water and can tolerate a range of physical and chemical stresses. Its distribution in surface water and moist soil ensures contact with free-living aquatic protozoa and other eukaryotes, in which \textit{B. pseudomallei} may find a favourable intracellular habitat that assists long term survival. Water droplets are the presumed vehicle for transmission of \textit{B. pseudomallei} during the rainy season, when the peak incidence of melioidosis occurs. At least three clusters of septicaemic infection have occurred in association with water supplies. Water treatment with chlorine may have been successful in preventing further cases, although some strains of \textit{B. pseudomallei} are chlorine tolerant. \textit{B. pseudomallei} in water is sensitive to broad-wavelength ultraviolet (UV) light, suggesting that the peak incidence of acute septicaemic disease might be due as much to the absence of UV light as to the presence of contaminated rain drops.

1. Introduction

In the introduction to clinical and epidemiological papers, \textit{B. pseudomallei} is often described as a bacterial “soil saprophyte”, underplaying its predilection for an aquatic habitat. \textit{B. pseudomallei} has long been recognised as being capable of prolonged periods of survival in nutrient-free water \cite{1}, but only recently has its behaviour in water been studied as a contributor to the pathogenesis and epidemiology of melioidosis. The properties demonstrated by \textit{B. pseudomallei} during survival, growth and adaptation to life in water suggest that the species should be listed among the water bacteria.

2. Survival in water

Prolonged survival in distilled water, an experiment conducted over a period of years \cite{1}, was originally thought to depend on carbon stores such as the polyhydroxybutyrate (PHB)
granules known to be present in *B. pseudomallei*. However, this view has been challenged by the recent finding that PHB knock-out mutants did not show markedly reduced water survival [2]. Studies, from a variety of sources, into *B. pseudomallei* adaptation in waters at differing temperatures and pH showed morphological changes at extremes of the survival range and may indicate a process of reductive division [3]. In other words, *B. pseudomallei* may be unusually efficient in recycling its own carbon and other essential elements scavenged from effete bacterial cells. The Gram-negative bacillus with which we are familiar may change into a Gram-positive coccus, under the right conditions. Morphological change has possible implications for *B. pseudomallei*’s buoyant density and other aerodynamic properties which, in turn, may affect the potential for bacterial aerosol generation.

3. Interaction with aquatic protozoa

The limits of physical or chemical conditions that allow bacterial survival can be altered by sequestration in a protected micro-habitat, such as in a polymicrobial biofilm or inside a suitable phagocytic eukaryotic cell. Formation of *B. pseudomallei* biofilms has been described under laboratory conditions, but in the natural environment, polymicrobial biofilms containing *B. pseudomallei* are more plausible, forming at solid–liquid interfaces. In these conditions, free living protozoa are likely to graze on bacteria as their nutrient source. A wide range of potentially pathogenic bacteria have been shown to enter free-living amoebae this way and persist in feeding vacuoles, most notably *Legionella* species [4]. *B. pseudomallei* demonstrates a complex repertoire of cellular interactions during co-culture with free-living amoebae [5], the initial engagement depending upon the presence of an intact flagellar mechanism [6]. This bacterial–eukaryotic interaction has been proposed as a forerunner of the intracellular infection known to occur in melioidosis [7]. It remains to be shown whether the formation of respirable particles containing *B. pseudomallei* after amoebic internalisation of bacteria is a precursor to human infection.

4. Water and the weather

Recognition that the peak incidence of acute, septicemic melioidosis occurs within days of the onset of heavy rainfall in northern Australia has led to further consideration of the contribution of extreme weather to human infection [8]. The prevailing view was that the arrival of the rainy season caused an elevation of the water table, so that *B. pseudomallei* buried deeper in the ground would rise to the surface. More recent work in Thailand shows how patchy the distribution of *B. pseudomallei* is, and how the species has a preference for specific physicochemical conditions [9]. It is possible that the bacteria survive at deeper levels where moist soil particles are linked by connecting water, or inside the cysts of amoebae or spores of mycorrhizal fungi [7,10]. The effect of regeneration of these eukaryotic elements of soil on bacterial growth may provide a possible explanation for the short delay between the onset of the rainy season and the
first cases of septicaemic melioidosis. The significance of the rain itself, however, and of the wind that may accompany it, must also be considered.

A common experience at the start of the rainy season is the notable cleansing of the air, due to the early rainfall causing precipitation of dust. During the tropical cyclones, the ferocity of air movement causes considerable mass transfer at ground level. Entrainment of bacteria from surface water collections, or inside cysts or spores from still dry surface soil, is likely to occur during vigorous air movement adjacent to the ground. The finding of a *B. pseudomallei* environmental isolate indistinguishable from the Western Australian outbreak strain NCTC 13177 on a mine site 500 km to the east, eight years after the melioidosis outbreak following a once in a generation severe weather event, highlights the role of severe weather events as a possible long range distributor of the organism [11]. However, *B. pseudomallei* cells dispersed in surface water remain highly susceptible to the disinfecting effect of the ultraviolet (UV) radiation in sunlight [12]. It is thus possible that an additional effect of weather during the rainy season is to block the antibacterial effect of natural UV light, thus increasing the probability of *B. pseudomallei* survival in recently contaminated surface water.

5. Water and sporadic disease

There have been numerous reports of sporadic infection following water inhalation, such as near drowning events, most notably during massive water displacement across a large distance as occurred during the 2004 tsunami [13]. As these patients often lack the co-morbidities associated with increased risk of acute melioidosis, this raises the question of whether inhalation of *B. pseudomallei* in water occurs due to the prior disturbance of mud, contact with a vulnerable portal of entry, such as the cribiform plate, or delivery of an unusually large inoculum to the lungs [14].

6. Water and melioidosis outbreaks

The occurrence of a small cluster of cases of septicaemic melioidosis during the dry season in tropical Western Australia indicated an unusual combination of contributory bacterial, human and environmental factors [15]. It took more than a year to reconstruct the likely course of events leading up to the outbreak, involving interviews, site visits, field investigations, molecular epidemiology and environmental surveillance [16].

A further melioidosis case cluster occurred in a remote community in the Northern Territory, causing nine cases with four deaths [17]. Based on molecular typing using pulsed-field gel electrophoresis (PFGE), the unchlorinated community water supply was likely the source of the outbreak. There followed a three-year environmental surveillance programme into the possible role of potable water supplies as a source of melioidosis in northern Australia. The results indicated that, under normal water management procedures, the potable water to these communities was unlikely to be a major source of exposure to *B. pseudomallei*. Nevertheless, water disinfection procedures for the remote communities were reviewed and tightened up. In 2005, a small cluster of fatal cases occurred in the northeastern state of Ceará in Brazil [18]. These were children who...
had been swimming in an irrigation dam soon after the arrival of the annual rains. Seroepidemiology showed that other people in nearby villages around the dam were seropositive.

On review of the literature, at least two veterinary case clusters, both in pigs, have been linked to dissemination by water [19,20]. One of them occurred after a river burst its banks, while the other was linked to a contaminated unchlorinated bore water supply. Lesions in the parotid glands of the pigs possibly indicate an oral route of infection.

7. *Burkholderia pseudomallei* in bore water of northern Australia

Bore water is ground water pumped to the surface from aquifers underground and used for domestic or farming purposes. Aquifers in the Darwin region in northern Australia are mainly found in dolomite, carbonate rocks or fractured sandstone and siltstone. They are at varying depths usually between 20 to 50 metres [21]. In 1998, an assessment of shallow aquifers in this area found *B. pseudomallei* in 14% (five samples) of 36 bore water samples collected from private bores [21]. An environmental surveillance project in 2001 to 2002 detected *B. pseudomallei* in 11 of 40 bores tested, with 6 of these directly linked to melioidosis cases with bore water the likely source of infection as determined by PFGE and ribotyping [11]. More recent research found 33% (18/55) of water bores in rural Darwin positive for *B. pseudomallei*. Multilocus sequence typing (MLST) produced 15 different sequence types (ST). Twelve of these had previously been isolated from clinical samples from humans and animals as well as from environmental samples from the same area. A study in 2008 on 47 unchlorinated water bores found a strong association between *B. pseudomallei* and physicochemical water parameters such as low pH, low salinity and high iron levels.

The origin of the contamination of bore water with *B. pseudomallei* is unclear. Once bore water is pumped to the surface it can be dispersed directly from the bore head or stored in large tanks. *B. pseudomallei* has been isolated from both the bore head and water storage tanks [7,15,17]. In some cases, the origin of the bore contamination appears likely to be surface water or soil contaminated with *B. pseudomallei* as some *B. pseudomallei*-positive water samples were found to also be rich in coliform bacteria and showed increased turbidity. Recently, a *B. thailandensis*-like bacterium (strain MSMB 43) was isolated from a bore from rural Darwin. This finding is the first report of a *B. thailandensis*-like isolate in Australia and will help elucidating the evolution and phylogeny of *B. pseudomallei* in Australia [22].

8. Water treatment

Following the Western Australian melioidosis outbreak of 1997, the potable water supply was closely monitored by the water authority for a repeat of the soil incursions and chlorination failure that were thought to have contributed to the original outbreak. Environmental surveillance was extended across northern Australia, and several locations found to be culture positive, though none of these were public water supplies [6]. The efficacy of chlorine as a *B. pseudomallei* water disinfectant was studied and the
outbreak strain found to be tolerant of high chlorine concentration [23]. Variable levels of chlorine tolerance have since been independently confirmed [24]. Though these details were not known in the immediate aftermath of the Western Australian melioidosis outbreak, hyperchlorination was used as an environmental control method to remove biofilms and other disinfection inhibiting factors known to assist the persistence of other intracellular water bacteria, such as *Legionella* species [16]. A variety of other water disinfection methods have since been assessed on *B. pseudomallei* [25]. There have been no further melioidosis case clusters in the decade following that incident, confirming that environmental control measures used then have been effective.

9. Climate change and melioidosis

A further complicating factor is the possible role of climate change and its contribution to annual rainfall, which appears to fluctuate. The extreme weather events that occur in Australia during the peak melioidosis season appear to occur with increasing frequency, energy and unpredictability. If they are the consequence of cyclical fluctuations in Indian Ocean surface temperature, we may see a wider distribution of *B. pseudomallei* in the natural environment and the occurrence of human infection. Close monitoring of human

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**Fig. IX.7. Burkholderia pseudomallei in water.** (1) Persistence during dry season as microcolonies in deep moist soil, in amoebic cysts or mycorrhizal fungal spores, or biofilms on solid surfaces. (2) Regeneration of dormant forms with first summer rain, followed by (3) amplification cycle either at nutrient rich air–water interface or by internalising in aquatic protozoa. (4) Direct human encounter with bacteria at air–water interface or as result of extreme weather event. (5) Interruption of natural (sunlight) or intentional (chlorine) water disinfection. (6) Dissemination and distribution via wind-blown aerosol disturbing air–water interface, in rain clouds or via water supply. (7) Encounter of disseminated bacteria with humans by inhalation, inoculation or ingestion.
B. pseudomallei infections, therefore, has possible value as a downstream indicator of the effects of climate change.

10. Conclusion

Burkholderia pseudomallei belongs to a group of bacteria that have adapted to long term survival in aquatic habitats. Its behaviour in water, between soil particles, in rain drops, or in association with the aquatic microbiota, goes some way to explaining its ecology and distribution (Figure IX.7). There are still critical knowledge gaps in the final means of bacterial encounter with humans, and its role in the pathogenesis of melioidosis: how does early summer rainfall in the tropics affect B. pseudomallei; to what extent is it disseminated by weather events in respirable particles; which of inhalation, ingestion or inoculation are the predominant form(s) of water-borne exposure; and, does water act as the final medium of bacterial transfer to the nasal epithelium? Further studies into the behaviour of B. pseudomallei in water are likely to add to our understanding of when, where and how infection occurs.

References

Section IX.4

The association of *Burkholderia pseudomallei* with plants and mycorrhizal fungi

Avram Levy<sup>a</sup>, Anthony Baker<sup>b</sup>

<sup>a</sup>Microbiology and Immunology, School of Biomedical, Biomolecular and Chemical Sciences, University of Western Australia, Nedlands, Australia

<sup>b</sup>Microbiology and Immunology, School of Veterinary and Biomedical Sciences, James Cook University, Townsville, Australia

Abstract

*Burkholderia pseudomallei* share their habitat with the dominant rhizosphere eukaryotes, namely plants and fungi. Other *Burkholderia* species vary in their relationships with plants. The genus includes pathogens and symbionts which colonise and invade plant tissues. *Burkholderia pseudomallei* can be isolated from the rhizosphere of diverse plants, and its distribution has been linked to that of plants. *B. pseudomallei* adheres to and colonises external surfaces of plants and fungi *in vitro*. Penetration of *B. pseudomallei* into fungal cells also occurs *in vitro*, and fungal spores isolated from soils yield *B. pseudomallei* DNA. Recently, *B. pseudomallei* has also been isolated from plant tissues and identified within plant roots. However, little is understood of the specific niche *B. pseudomallei* occupies within the rhizosphere, the nature of its interaction with plants and fungi, and the implications of these interactions for persistence, proliferation and dispersal of *B. pseudomallei* in the environment.

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1. Rhizosphere and mycorrhizal fungi

1.1. The rhizosphere – habitat of bacteria and fungi

The rhizosphere is the narrow zone of soil influenced by a living root. Bacteria are the most common group of rhizosphere organisms and *Burkholderia* species comprise a significant portion of the rhizospheric bacteria [1]. Mycorrhizal fungi are soil fungi that form symbiotic associations with roots of most terrestrial plants. The hyphae of mycorrhizal fungi penetrate soil and increase nutrient uptake, particularly in adverse environmental conditions. The high surface area presented by mycorrhizal hyphae in soil, and their proximity to plant and fungal metabolites, offer valuable sites for bacterial colonisation and interaction with eukaryotes. *Burkholderia* species, in particular, are efficient colonisers of the mycorrhizosphere habitat [2].
1.2. Burkholderia species coexist with plants and soil fungi

Very few specific interactions between B. pseudomallei or B. thailandensis, and either plants or soil fungi, have been documented. However, interactions in the B. cepacia group including plant pathogens and bacterial species which benefit plants through biocontrol, nitrogen fixation, or symbiosis have been described. Other Burkholderia species demonstrate further specialisation for rhizosphere habitats, more evidence of symbiosis, and are less frequently isolated from human tissues. Some Burkholderia species colonise plant roots, spread systemically to aerial plant parts [3], and can be sustained by ‘vertical’ transmission to subsequent generations via seeds [4]. Burkholderia species interactions with plants can therefore be broadly divided into those (a) beneficial to plants, (b) saprophytic, or (c) phytopathogenic. Associations between Burkholderia species and soil fungi lead to a range of outcomes, from mutualism to parasitism. These outcomes are preceded by intracellular and extracellular fungal colonisation, and passage through the eukaryotic host. Members of the B. cepacia group express inhibitory activity against a broad range of fungi, including mycorrhizal fungi [5] and are known to cause disease in some fungi.

2. Evidence of Burkholderia pseudomallei associations with plants

2.1. Molecular markers of rhizosphere niche

The genome of B. pseudomallei encodes three type III secretion systems (T3SS) [6]. While T3SS-3 is homologous to that found in Salmonella and Shigella [7] and a known virulence factor of B. pseudomallei in animal models [8], T3SS-1 and T3SS-2 are homologous to T3SS of the plant pathogen, Ralstonia solanacearum [7], and are less critical for B. pseudomallei virulence in animals [9]. Instead, T3SS-1 and T3SS-2 have been shown to contribute to phytopathogenicity of B. pseudomallei in tomato plants but not rice [10]. Tomato plants are not native to B. pseudomallei-endemic areas, in contrast to rice which might have gained resistance during its coevolution with B. pseudomallei.

Genome analysis of B. pseudomallei has also revealed several antifungal compounds such as mobile, but complete, pyrrolnitrin (PRN) operons [11]. PRN is a broad spectrum antifungal compound produced by some Gram-negative bacteria and active against phytopathogenic fungi common in the rhizosphere. Similarly, homologues of syringolin and related glidobactin have been identified in the B. pseudomallei genome [12,13]. Syringolin was isolated from the phytopathogenic bacterium Pseudomonas syringae and was shown to confer resistance to the fungus Pyricularia oryzae, in rice [14]. The glidobactin gene cluster in B. mallei was found to be non functional due to chromosome rearrangements [12], suggesting these genes were made redundant in mammalian hosts.

2.2. Isolation of Burkholderia pseudomallei from rhizosphere soil

Reports regarding the isolation of B. pseudomallei from rhizosphere soil and plant tissue are limited. Inglis and colleagues recovered B. pseudomallei from the roots of a wattle shrub (Acacia colei) in Western Australia [15], and B. pseudomallei was isolated...
from the roots of native grass species in a melioidosis-endemic region in the Western Province of Papua New Guinea. In northern Australia, a correlation was found between the environmental prevalence of *B. pseudomallei* and roots of native grasses, such as spear grass, but also introduced pasture grasses, such as tully grass (*Brachiaria humidicola*) or mission grass (*Pennisetum pedicellatum*) [16].

### 2.3. In vitro interactions of Burkholderia pseudomallei with plants

Plant roots are the primary source of carbon for the growth of bacteria in the rhizosphere, with adherence to roots securing rich plant exudates for rhizobacteria. *In vitro*, *B. pseudomallei* colonises *Trifolium subterraneum* (clover), *Solanum lycopersicon* (tomato), *Acacia coleí* [17] and *Pennisetum pedicellatum* (mission grass) surfaces (Kaestli M, personal communication). Areas of roots preferentially colonised were the root tip, the zones of mitosis and elongation, and root hairs within the maturation zone. Adherence to these zones is a common feature of rhizobacterial colonisation, reflecting trophic chemotaxis of bacteria to a region rich in exudates. Within these zones, *B. pseudomallei* often colonised the area of longitudinal contact between epidermal cells [17], an area also colonised by another *Burkholderia* strain [3].

On initial contact with plant surfaces, *B. pseudomallei* showed flagella-tethered motility and end-on adhesion [18], then became irreversibly attached by filaments (Figure IX.8). Filaments between bacteria and plants are common [19] and function to irreversibly bind bacteria to the plant. In the case of *B. pseudomallei*, this led to the formation of biofilms on seedlings within seven days. In addition to surface colonisation, *B. pseudomallei* enters plants, and is capable of colonising xylem vessels of tomato leaves [10]. *B. pseudomallei* was also found inside root hairs of axenically grown mission grass (Kaestli M, personal communication).

### 3. Interactions of Burkholderia pseudomallei with mycorrhizal fungi

An association between *B. pseudomallei* and mycorrhizal fungi exists within the rhizosphere. *B. pseudomallei* DNA has been detected in mycorrhizal spore DNA extracts from the rhizosphere of a range of native and cultivated plants in Australia [20]. *In vitro,*
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Fig. IX.9. Scanning electron micrograph of erosion of Gigaaspera decipiens spore surface by B. pseudomallei.

*B. pseudomallei* adheres to and colonises hyphae and spores of the mycorrhizal fungus, *Gigaaspera decipiens*, with spores alone capable of supporting *B. pseudomallei* for well over a year [21]. A similar process of adhesion was observed on this fungus as described above on plant surfaces, including permanent attachment by microscopic filaments. *B. pseudomallei* also enters *G. decipiens* spores [21]. Entry of *B. pseudomallei* into *G. decipiens* is likely to occur through natural openings or by direct erosion (Figure IX.9). Inside spores, *B. pseudomallei* is rarely bound, but either evenly distributed or in small clumps adjacent to the inner spore wall. Each *G. decipiens* spore contained on average $5 \times 10^5$ bacteria. Mycorrhizal spores present a high-nutrient compartment for proliferation and survival of *B. pseudomallei*.

4. Implications of *Burkholderia pseudomallei* colonisation

Current knowledge of *B. pseudomallei* rhizosphere ecology indicates a potential for movement between plant, soil and water. *B. pseudomallei* adheres to mycorrhizal fungal hyphae and to plant roots, which allows for co-migration of roots and bacteria with hyphae through soil. *B. pseudomallei* enters mycorrhizal fungal cells; a process which may aid bacterial survival in the absence of plants. *B. pseudomallei* possibly travels from spore to hyphae or *vice versa*, representing a means of bi-directional passage between soil and plant. *B. pseudomallei* can also enter plants directly via leaves [10] or root hairs, and then travels to other tissues and possibly seeds. Exit of *B. pseudomallei* from seeds or mycorrhizal spores during germination and reversion to a free-living existence may occur during periods of heavy rainfall, when rhizosphere *Burkholderia* populations proliferate in bulk soil. In addition to migration within the rhizosphere, plant seeds and fungal spores allow long-distance travel of *B. pseudomallei*. While the presence of *B. pseudomallei* within seeds remains to be demonstrated, mycorrhizal spores can contain *B. pseudomallei* and are small enough to be easily carried by wind or water. Both seeds and mycorrhizal spores consumed by various animals, birds and insects could account for dispersal of the bacteria.

Until recently, evidence of *B. pseudomallei* as a rhizosphere dweller has been circumstantial. However, now there is more compelling *in vitro* and environmental
evidence for specific interactions with plants and mycorrhizal fungi in the rhizosphere. Close interaction with eukaryotic cells secures nutrient-rich habitats for \textit{B. pseudomallei} and provides protection from adverse conditions, such as desiccation, radiation, predation and salinity, to which free-living \textit{B. pseudomallei} is vulnerable. Plant species \cite{22} and mycorrhizal fungi \cite{23} affect the population structure of bacteria. The mechanisms by which \textit{B. pseudomallei} populations are influenced by plants are yet unknown. Some plant and fungal species could be preferential companions for \textit{B. pseudomallei} while others may be potentially inhibitory, hostile or indifferent.

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Section IX.5

The influence of anthropogenic environmental changes upon *Burkholderia pseudomallei*

Mirjam Kaestli\textsuperscript{a}, Jeffrey Warner\textsuperscript{b}

\textsuperscript{a} Tropical and Emerging Infectious Diseases Division, Menzies School of Health Research, Darwin, Australia

\textsuperscript{b} Microbiology and Immunology, School of Veterinary and Biomedical Sciences, James Cook University, Townsville, Australia

Abstract

Mankind has been altering the natural environment for thousands of years. Although the influences are most obvious on macroflora and -fauna, microbial communities are also affected. Human activity has also recently been linked to the distribution and dispersal of *Burkholderia pseudomallei*. In endemic regions, the prevalence in soil of the bacteria may be increased due to land-use practices, agriculture, irrigation and the import of pasture grasses. Clear correlations exist with the incidence of melioidosis and rice and livestock farming, and building practices that expose *B. pseudomallei* from deeper levels in the soil. Humans have been involved in the dispersal of the organism, from the ancestral origins of Australia throughout the traditional endemic regions of Asia, as well as to non-endemic areas, a classical example of the latter being “l’affaire du Jardin des Plantes”. Studying human interactions with *B. pseudomallei* is critical to understanding melioidosis epidemiology.

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1. Anthropogenic influence in the dispersal of *Burkholderia pseudomallei*

The influence of human activity in the dispersal of human pathogens is uncontroversial; indeed human migration patterns can be inferred from the phylogenetics of their companion microbes [1]. What is more debatable is the extent to which humans influence the dispersal of non-obligate pathogens. Clearly, humans have affected the environmental prevalence of *B. pseudomallei* through the use of their environment, and this has resulted in increased incidence of melioidosis and point source outbreaks. The question is: has this association affected regional and potentially global dispersal?

Evidence suggests that cosmopolitan dispersal due to air and sea currents may be hindered by the organism’s sensitivity to salt and ultraviolet (UV) light [2,3]. Importation of *B. pseudomallei* into regions not considered endemic have been reported and implicated in stable clonal outbreaks over decades. These have been linked indirectly to human
influence mostly through the importation of infected animals, as observed on a farm in southwest Australia [4] or causing “l’affaire du Jardin des Plantes”, a melioidosis outbreak in Paris zoos in the mid-1970s [5]. Given the wide host range of the organism, dispersal via native and domesticated animals, both ancestral and contemporary, is possible and it is likely that these animals pass the bacteria in their faeces. In northeastern Thailand, *B. pseudomallei* can be recovered from over one third of rectal swabs of patients with melioidosis, suggesting that faecal–oral transmission and seeding into the environment is possible within the human population [6]. Also, given that graminaceous and some other food-bearing plants can be infected by *B. pseudomallei*, their selection and introduction by humans over the millennia may have directly imported or enhanced persistence [7,8]. Although it is possible that human influence may have been responsible for transmission, phylogenetic evidence is required.

Through analysis of the extensive public database recording the phylogeny of nearly 2000 strains of *B. pseudomallei* collected from around the world (MLST, http://bpseudomallei.mlst.net/), evidence is emerging that two populations of *B. pseudomallei* exist, divided by the Wallaceline [9]. These data suggest that *B. pseudomallei* dispersal has been affected by the geographic boundaries and geological history that have influenced the migration of macrofauna, including humans (Section IX.1). Unfortunately, the limitations of MLST-derived sequence data, and the extent of lateral gene transfer in *B. pseudomallei*, have hindered any detailed analysis of the subpopulations to test this hypothesis.

2. Land development leads to increased exposure to *Burkholderia pseudomallei*

Alterations in land management impact on the distribution of *B. pseudomallei* in the environment, and on the level of human contact with these bacteria. Steady population growth within melioidosis-endemic areas, such as experienced in northern Australia due to exploitation of natural resources, would result in increased exposure. Although the mining industry has not yet identified melioidosis as an occupational risk [10], land development with earth and construction work has caused melioidosis among excavation workers [11].

3. Agricultural practices

Agricultural practices substantially change the microbial community structure in soil [12–14], and may be associated with persistence of *B. pseudomallei* due to the following factors:

3.1. Irrigation

An obvious environmental manipulation favourable to the hydrophilic *B. pseudomallei* is irrigation. In tropical Australia for example, during the dry season there is very little rain and *B. pseudomallei* is thought to survive in deeper and moister layers in the soil. However, regular irrigation during the dry season creates conditions facilitating
B. pseudomallei survival in shallow soil layers all year round. In a soil survey in northern Australia, residential gardens in the dry season had a significantly higher proportion of B. pseudomallei-positive soil samples, as compared to other sites, which was in stark contrast to the following wet season where environmentally undisturbed sites showed the highest incidence of B. pseudomallei [7]. Irrigation systems in rural areas of northern Australia are being fed by unchlorinated water supplies and a high proportion of bore water samples tested have been found to be culture-positive for B. pseudomallei (Mayo M, personal communication). This raises the question of whether irrigation systems not only create favourable conditions for B. pseudomallei survival in shallow soil layers, but also whether they contribute to B. pseudomallei distribution by pumping and distributing water from contaminated bores. Irrigation in rice farming is known to be a major risk factor in acquiring melioidosis in Southeast Asia [15,16].

3.2. Fertilisers

The broad use of fertilisers has caused major changes to the global nutrient cycles, with an increase in supply of the otherwise limiting nutrients, nitrogen and phosphates. These changes are likely to influence the dynamics of host–parasite interactions [17]. The use of fertilisers also has been reported to have an effect on the Burkholderia community structure in soil [13]. In studies conducted in vitro, the addition of different fertilisers and fertiliser components to soil, or minimal medium inoculated with B. pseudomallei, were found to substantially increase growth of the organism, compared to controls. Phosphates are a major component of most plant fertilisers and a well known contaminant of soil and water. Polyphosphates are also generated by B. pseudomallei for use in oxidative stress response, motility and biofilm formation [18]. Nitrogen-containing compounds, such as urea, are favourable for the growth of B. pseudomallei. Urea is hydrolysed to ammonia and oxidised to nitrates by nitrifying soil bacteria. Nitrates are reduced by B. pseudomallei as electron acceptors under anaerobic conditions. B. pseudomallei-positive soils have been found to have higher levels of total nitrogen content [19]. Nitrogen-containing fertilisers also are known to acidify the soil in the long term by nitrification processes. Soil pH is a strong driver of the soil bacterial composition [14], and it was found that B. pseudomallei preferred more acidic soil [7,19] and water conditions. Acidic soil conditions also increase the bio-availability of phosphates, by increased activity of acid phosphatases releasing phosphates from organic substrates. Acid phosphatases are abundant in the soil, as most soil bacteria produce these, including B. pseudomallei [20]. Fertilisers also have an indirect effect upon B. pseudomallei, by increasing root development. B. pseudomallei was found to be associated with roots- and grass-rich soil in northern Australia [7] and B. pseudomallei was detected by real-time polymerase chain reaction (PCR) and fluorescent in situ hybridisation (FISH) in the rhizosphere of grasses in northern Australia.

3.3. Livestock

Animal waste products are rich in organic matter and nutrients, such as phosphates and urea, and the isolation of B. pseudomallei in soil was found to be associated with the
presence of animals [7]. It is unknown whether animals serve as reservoirs contributing to the distribution of these bacteria. It is unlikely for some livestock which are more susceptible to melioidosis, such as goats, sheep or pigs, to be able to serve as efficient reservoirs [21].

In summary, data is accumulating that land development supports growth of \textit{B. pseudomallei} and, thus, increases the risk of exposure to these bacteria. This also is supported by the fact that many members of the family Betaproteobacteria, including \textit{B. pseudomallei}, are capable of ammonium oxidation, denitrification and polyphosphate accumulation [14]. This provides an advantage over other bacteria in heavily fertilised, eutrophic ecosystems. A greater relative abundance of Betaproteobacteria was found in sediments of eutrophic reservoirs and agricultural wetland soils, which decreased after restoration [14]. In another study, a shift to \textit{Burkholderia} species was evident after a change from forest to pasture vegetation [12]. Reduced use of fertilisers and restoration of native vegetation requiring less irrigation and fertilisers could be a measure in some endemic areas to contain the spread of the bacteria. \textit{B. pseudomallei} was no longer detected after removal of chemical fertilisers and restoration of vegetation in a study conducted in Western Australia [10].

4. Changes in landscape ecology support spread of \textit{Burkholderia pseudomallei}

In the 20\textsuperscript{th} century, several exotic, mainly African, grass species were introduced to northern Australia as pasture grasses. Some of these have vigorously spread to non-target areas, replacing native vegetation, and have been declared as weeds [22,23]. Several areas in northern Australia with high environmental prevalence of \textit{B. pseudomallei} were found to harbour exotic grasses, such as mission grass (\textit{Pennisetum pedicellatum}), tully grass (\textit{Brachiaria humidicola}), or plicatum grass (\textit{Paspalum plicatum}). Real-time PCR and FISH analysis demonstrated a close association of \textit{B. pseudomallei} with the extensive roots of these grasses. Invasive grasses are suggested to cause major alterations to tropical savanna ecosystems, including changes to biomass, nitrogen cycle [22], or increased persistence of annual soil wetting [23]. This could prove advantageous to the survival of \textit{B. pseudomallei}, increasing the area of suitable habitat. This habitat is also more stable, as many exotic grasses are perennial and, thus, provide \textit{B. pseudomallei} a niche to survive all year round. This is in contrast to many native annual grasses which die during the dry season. Although phylogenetic studies point to an Australian origin of \textit{B. pseudomallei} [9] and it is unlikely that \textit{B. pseudomallei} was originally introduced to northern Australia by import of exotic grasses, it needs to be evaluated whether \textit{B. pseudomallei} could spread with invasive grasses across northern Australia to previously uncontaminated areas.

5. Climate change and \textit{Burkholderia pseudomallei}

Climate change is predicted to lead to an increase in heavy storms and warmer temperatures in the tropics in the future. Extreme natural events, such as monsoonal...
heavy rains or cyclones, have a large impact upon landscapes and soil and such events have been reported to be associated with an increase of melioidosis incidence and have even unmasked melioidosis in areas considerably south of the melioidosis-endemic belt in Australia [24,25]. A spread of *B. pseudomallei* to non-endemic regions beyond the current tropical belt is also likely, if significant changes occur to the environment due to increase in global temperatures.

6. Conclusions

Melioidosis incidence is spatially clustered and linked to environmental prevalence. Evidence is mounting that in regions that are considered endemic, the way humans interact with their landscape influences persistence and availability of *B. pseudomallei*, and therefore is a risk factor for acquiring melioidosis. This association needs to be clarified so that public health measures designed to prevent transmission could be formulated. Further, importation of the organism and climate change could potentially establish new regions of endemicity.

References


X
Other *Burkholderia* species
Section X. Other *Burkholderia* species

**Editorial overview**

David DeShazer

*United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, USA*

*Burkholderia thailandensis* and *B. mallei* are the species most closely related to *B. pseudomallei*. *B. thailandensis* and *B. pseudomallei* diverged from a common ancestor around 47 million years ago (mya). *B. thailandensis* is a soil saprophyte that exhibits biochemical, immunological, and genetic similarities to *B. pseudomallei*. Recent studies have identified a number of important genotypic and phenotypic traits that enable these organisms to be rapidly differentiated from one another. Amongst these are: the ability of *B. thailandensis* to assimilate L-arabinose; the inability of the organism to synthesise a -3)-2-O-acetyl-6-deoxy-β-D-manno-heptopyranose-(1- capsular polysaccharide as well as the relative avirulence of *B. thailandensis* towards humans and animals. While *B. thailandensis* is weakly pathogenic for mammals, its genome harbors putative virulence genes. Numerous comparative studies between *B. thailandensis* and *B. pseudomallei* have been conducted and have revealed *B. pseudomallei* virulence determinants required for pathogenesis in mammalian models of infection. In fact, *B. thailandensis* is considered to represent a tractable model system for studying specific virulence factors involved in the pathogenesis of melioidosis.

*Burkholderia mallei*, the etiologic agent of glanders, is a host-adapted clone of *B. pseudomallei* and an obligate mammalian pathogen. *B. mallei* primarily infects horses, mules and donkeys, although humans and other animals, including dogs and cats, are susceptible hosts. In natural settings, chronically infected horses are considered to be the principal reservoir of this facultative intracellular pathogen. *B. mallei* is transmitted amongst equines by oral, nasal and possibly ocular mucous membrane exposure, inhalation, and ingestion of infected skin exudates and respiratory secretions. Crowded and unsanitary housing conditions are believed to promote the maintenance and spread of the organism within equine populations. Zoonotic transmission of *B. mallei* to humans is rare and typically requires direct contact with infected animals, their secretions or infectious aerosols generated upon nasal exhalation.

*Burkholderia mallei* and *B. pseudomallei* diverged only around 3.5 mya, and their close genetic relationship makes studies conducted with one microbe inherently relevant to the other. For example, common virulence determinants and pathogenic mechanisms have been identified experimentally using both organisms independently. Because of its close
relatedness to *B. pseudomallei*, studies focussing on *B. mallei* are likely to continue to provide insight for certain aspects of melioidosis research. This Section provides an overview of *B. thailandensis* (Section X.1) and *B. mallei* (Section X.2) and sheds new light on these organisms, providing a better understanding of the biology of *B. pseudomallei*. 
Burkholderia thailandensis

Jonathon P. Audia, Mary N. Burtnick, Paul J. Brett

Department of Microbiology and Immunology, College of Medicine, University of South Alabama, Mobile, AL, USA

Abstract

Burkholderia thailandensis is an environmental saprophyte that can be isolated from a variety of sources throughout Southeast Asia and northern Australia. Historically, B. thailandensis isolates were often mistaken for B. pseudomallei strains, due to similarities in their biochemical, morphological, and antigenic profiles. Recently, however, studies have identified a number of important genotypic and phenotypic traits which enable these organisms to be rapidly differentiated from one another. Amongst these are: the ability of B. thailandensis to assimilate L-arabinose; the inability of the organism to synthesise a α-(1-2)-O-acetyl-β-D-manno-heptopyranos-(1-capsular polysaccharide; as well as, the relative avirulence of B. thailandensis towards humans and animals. Interestingly, B. thailandensis appears to produce a number of potential virulence factors also known to be expressed by B. mallei and B. pseudomallei. Because of this, B. thailandensis is considered to represent a tractable model system for studying specific virulence factors involved in the pathogenesis of melioidosis and glanders.

1. Introduction and history

The historical significance of B. thailandensis is intimately tied to that of its closest phylogenetic neighbour, B. pseudomallei. Environmental sampling efforts, and the resulting identification of physiological and biochemical differences between B. pseudomallei-like isolates, helped pave the way for the classification of B. thailandensis as a distinct species. Environmental and clinical isolates from various regions in Thailand that were verified as B. pseudomallei-like by a latex agglutination test varied in their ability to assimilate different carbon sources, most notably L-arabinose (Ara⁺ versus Ara⁻ phenotypes) [1]. Indeed, there was a direct correlation between L-arabinose assimilation and the ability to cause disease [1,2]. All Ara⁻ clinical isolates and Ara⁺ environmental isolates demonstrated virulence in animal models of infection. Conversely, environmental Ara⁺ isolates selected on medium containing L-arabinose as the sole carbon source invariably showed attenuated virulence in animal model studies and were very rarely, if ever, isolated from patients [2,3]. Intriguingly, both Ara⁺/avirulent and Ara⁻/virulent B. pseudomallei-like strains were present in the environment of endemic areas in southeast Thailand whereas only the Ara⁺/avirulent phenotype was present in central Thailand, where cases
of melioidosis were a rarity [1]. This raised the question of the association between virulence and the Ara\(^+\) phenotype in these \(B.\) \textit{pseudomallei}\)-like strains. The answer was revealed in a phylogenetic study of the Ara\(^+\) and Ara\(^-\) \(B.\) \textit{pseudomallei}\)-like isolates, based on sequencing of the 16S rRNA genes which led to the proposal that Ara\(^+\) isolates were a novel species and subsequently termed “\(B.\) \textit{thailandensis}” [3,4]. This distinction enlightened the \textit{Burkholderia} research community and facilitated the development of new diagnostics that, along with existing tests, definitively confirmed \(B.\) \textit{thailandensis} as endemic to northern Australia, in addition to Southeast Asia [5]. Outstanding questions remain regarding \textit{Burkholderia} geographic distribution, associated with the apparent exclusion of \(B.\) \textit{pseudomallei} from the environment in central Thailand, and the dearth of reports of \(B.\) \textit{thailandensis} environmental isolates in North America or other parts of the world where rare occurrences of disease involving \(B.\) \textit{thailandensis} have been reported [6].

2. General characteristics

Several of the salient characteristics of \(B.\) \textit{thailandensis} are summarised in Table X.1. The species name was derived from the origin of the first environmental isolates in Thailand. \(B.\) \textit{thailandensis} is a mesophilic environmental saprophyte that inhabits a range of niches, including soil and water. The organism is typically isolated from environmental samples by selective culture techniques, including plating on modified Ashdown’s medium containing gentamicin and colistin, where \(B.\) \textit{thailandensis} colonies will generally appear smooth and glossy with a pink pigmentation. Biochemical characteristics include: positive oxidase reaction; the production of siderophores, lipases, lecithinases and proteases; the ability to assimilate L-arabinose, adonitol, 5-keto-gluconate, and D-xylose; and the inability to assimilate dulcitol, erythritol, and trehalose [1−4]. \(B.\) \textit{thailandensis} is a Gram-negative, facultative intracellular \(\beta\)-proteobacterium. The bipolar appearance of cells visualised by Gram staining is presumably due to the production and accumulation of the storage polymer, \(\beta\)-polyhydroxybutyrate. Motility is driven by a polar tuft of two-four flagella, and swarming motility has been directly linked to the production of rhamnolipid surfactants via genetic analyses [4]. As mentioned above, \(B.\) \textit{thailandensis} gives a positive reaction in a latex agglutination test used to identify \(B.\) \textit{pseudomallei}, because the O-polysaccharide (OPS) moieties expressed by these species are structurally and antigenically indistinguishable from one another [1,7]. Interestingly, \(B.\) \textit{thailandensis} appears to be devoid of \(\beta\)-hydroxymyristic acid, a useful diagnostic tool for differentiating this species from \(B.\) \textit{pseudomallei} [8]. Most notably, however, \(B.\) \textit{thailandensis} lacks production of a \(-3\)-2-\(O\)-acetyl-6-deoxy-\(\beta\)-D-manno-heptopyranose-(1- capsular polysaccharide which may significantly contribute to its attenuated virulence phenotype [9].

3. Virulence and disease

Despite the fact that \(B.\) \textit{thailandensis} is readily isolated from areas of central Thailand, there is a paucity of documented cases of human infection, which highlights the inefficiency of the organism to cause disease in this host. Attenuated virulence has been
Table X.1
General characteristics of *B. thailandensis*, *B. pseudomallei* and *B. mallei* isolates

<table>
<thead>
<tr>
<th>Genotype/Phenotype</th>
<th><em>B. thailandensis</em>&lt;sup&gt;a&lt;/sup&gt;</th>
<th><em>B. pseudomallei</em>&lt;sup&gt;b&lt;/sup&gt;</th>
<th><em>B. mallei</em>&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Environmental reservoir(s)</td>
<td>Soil and Water</td>
<td>Soil and Water</td>
<td>Soil and Water</td>
</tr>
<tr>
<td>Human pathogen</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Animal pathogen</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Genetically manipulable</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Chromosome I</td>
<td>3.80 Mbp (67% G+C)</td>
<td>4.07 Mbp (67% G+C)</td>
<td>3.51 Mbp (68% G+C)</td>
</tr>
<tr>
<td>Chromosome II</td>
<td>2.91 Mbp (68% G+C)</td>
<td>3.17 Mbp (68% G+C)</td>
<td>2.32 Mbp (69% G+C)</td>
</tr>
<tr>
<td>Protein coding genes</td>
<td>5634</td>
<td>6304</td>
<td>5229</td>
</tr>
<tr>
<td>Colony morphology (MASM agar)</td>
<td>Smooth, Pink</td>
<td>Wrinkled, Purple</td>
<td>No growth</td>
</tr>
<tr>
<td>L-Arabinose assimilation</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Aminoglycoside resistance</td>
<td>High</td>
<td>High</td>
<td>Low</td>
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<tr>
<td>Polymyxin resistance</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Type I capsule</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Type II OPS O-acetylation patterns</td>
<td><em>O-2 &amp; O-4</em></td>
<td><em>O-2 &amp; O-4</em></td>
<td><em>O-2</em></td>
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<td>Flagella</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Type II secretion</td>
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<td>Functional</td>
<td>Non-functional</td>
</tr>
<tr>
<td>Type III secretion systems</td>
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<td>T3SS-2 &amp; -3</td>
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<td>Type VI secretion systems</td>
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<td>T6SS-1, -2, -3, &amp; -4</td>
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<td>Quorum sensing systems</td>
<td>System-1, -2 &amp; -3</td>
<td>System-1, -2 &amp; -3</td>
<td>System-1 &amp; -3</td>
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<td>Intracellular survival/replication</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Escape from endocytic vacuoles</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Actin-based motility</td>
<td>Yes</td>
<td>Yes</td>
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<td>MNGC formation</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
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<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt; − mice</td>
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<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt; − hamsters</td>
<td>High</td>
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</table>

Genotypic traits obtained from<sup>a</sup>*B. thailandensis* E264, <sup>b</sup>*B. pseudomallei* K96243 or <sup>c</sup>*B. mallei* ATCC 23344 whole genome sequence data.

MNGC, multinucleated giant cells.
confirmed using animal models, showing that infection and elicitation of a pathology in small rodents, such as Syrian hamsters, BALB/c, and C57BL/6 mice, requires extremely large bacterial burdens (>10^6 organisms in some reports) [2,3,10]. However, animal models have revealed tremendous variability; in that some B. thailandensis environmental strains are completely avirulent (even at very high doses) for small rodents. Some reported animal infection models, such as C3H/HeJ and C3H/HeN mice, are entirely resistant to B. thailandensis infection [11,12]. To date, there have been only four reported cases of human disease caused by B. thailandensis, two in Southeast Asia and two in the southeastern United States of America, with patients presenting with symptoms similar to melioidosis [11]. Based on the circumstances surrounding these rare human cases, and data from animal infection experiments, it appears as though a massive inoculum is required to establish a B. thailandensis infection. For example, one of the cases involved a two-year old child who had drowned, subsequently was resuscitated, and developed aspiration pneumonia caused by a polymicrobial infection in which B. thailandensis was identified [6]. One of the major questions left facing the research community is the nature of B. thailandensis virulence – is it a case of attenuated virulence, or is the organism avirulent and the rare cases of human disease are in reality the result of a consortium of microbes working together to cause an infection? Addressing this and other important issues will require further studies to understand the link between differences in B. thailandensis- and B. pseudomallei-virulence, and genomic differences between the two, including the role of the polysaccharide capsule.

4. Genomics

Biochemical differences, such as carbohydrate utilisation, exopolysaccharide production, and fatty acid profile, that have been exploited as diagnostics to distinguish B. thailandensis from B. pseudomallei have been augmented by genomic differences that serve as the basis for nucleic acid-based diagnostic assays. The full genome sequence of B. thailandensis E264 is available, and has revealed that the two chromosomes of this organism are predicted to encode over 5600 protein-coding genes (Table X.1). Sequence analysis revealed the presence of at least 15 regions that diverge with respect to GC content, and/or containing sequences with similarity to bacteriophage genes and integrases [13]. In fact, some B. thailandensis strains have been shown to harbour bacteriophage that are shed at low levels, whereas other strains harbour temperate phage that can be induced by exposure of the bacterium to ultraviolet light. These B. thailandensis-derived phage often demonstrate a tropism to exclusively infect other Burkholderia species, which has been touted as having potential diagnostic value [14]. The extent to which phage integration events and lateral gene transfer have undoubtedly shaped genome evolution, and the pathogenic capacity of B. thailandensis, remains to be elucidated.

Whole genome sequencing, and comparison of closely related virulent and avirulent microorganisms, represents a possible avenue to identify genetic factors influencing pathogenesis. The genomes of B. thailandensis and B. pseudomallei have been subjected to such a comparison. Intriguingly, this analysis revealed that B. thailandensis possesses an extensive arsenal of putative secretion system and virulence factor homologues, despite
being attenuated for virulence. This comparison also revealed that the shared core of
B. thailandensis and B. pseudomallei metabolic genes showed much less divergence
than the shared core of putative virulence factors [13]. The extent to which these
differences in the putative virulence play a role in the pathogenic capability of the two
species (for example, diminished virulence factor function) is as yet unknown. Aside
from confirming known differences in exopolysaccharide production and carbohydrate
metabolism, the B. thailandensis and B. pseudomallei genomic comparison confirmed
a lack of pilus/fimbriae production for adhesion, and revealed differences in polyketide
and non-ribosomal peptide synthesis that also could contribute to attenuated virulence of
B. thailandensis [13]. This type of genomic analysis is likely to be critical in determining
the links between gene loss and increased virulence capacity in pathogenic microbes.
For example, the introduction of the B. thailandensis L-arabinose utilisation operon into
B. pseudomallei resulted in a substantial reduction in virulence, most likely due to global
changes in B. pseudomallei gene expression [15]. Studies such as these indicate the
tremendous promise inherent to B. thailandensis and B. pseudomallei comparative studies
in generating testable hypotheses to provide insight into mechanisms of pathogenesis.

5. Genetic manipulation
Several research groups have taken advantage of the genetic tractability of B. thailan-
densis, and used both targeted and random mutagenic approaches to test specific hypotheses
regarding the role(s) of putative virulence genes in growth, survival, and host cell
infection. The use of suicide vectors to generate site-specific insertion mutations by
homologous recombination has been widely successful in this species. In addition,
allelic exchange mutagenesis strategies based upon the use of vectors harbouring
counter-selectable alleles, including rpsL, sacB and pheS have been utilised to construct
various non-polar, markerless mutations [16,17]. Furthermore, the generation of random
mutations suitable for screening or selection-based studies has been accomplished using
transposase systems, such as Tn5 and Himar1 [18,19]. A TnphoA system also has been
described for use in B. thailandensis to screen for secreted proteins [18]. The recent
demonstration that B. thailandensis can be made naturally competent for the uptake
of exogenous DNA has been used to develop strategies using linear, polymerase chain
reaction generated target DNA fragments to generate site-directed mutants by homologous
recombination. Once constructed, the mutant alleles can be moved between strains by
incubating a recipient strain with isolated chromosomal DNA from a mutant strain, which
is taken up by naturally competent bacteria [20]. Finally, there are several examples
of broad host range plasmids that can be used for functional complementation and/or
over-expression studies [16,21]. These approaches used separately, or in combination,
represent powerful tools to genetically manipulate B. thailandensis for use in both in
vitro and in vivo studies.

6. Burkholderia thailandensis as a model system
Several studies have shown that B. pseudomallei and B. mallei strains express a variety
of factors required for full virulence in animal models of infection. Included amongst
these are: quorum sensing systems; the Bsa type III secretion system (T3SS); the VirAG two-component regulatory system; the cluster I type VI secretion system (T6SS-1); and lipopolysaccharide (LPS). Both \textit{B. pseudomallei} and \textit{B. mallei} have been shown to invade, survive and replicate in both phagocytic and non-phagocytic cell lines, as well as polymerise host cell actin and stimulate multinucleated giant cell (MNGC) formation. At present, relatively little is known regarding the molecular mechanisms used by these organisms to persist within phagocytic cells, or how they specifically evade innate and acquired host immune defenses. Because \textit{B. thailandensis} strains produce many of the same virulence factors and protective antigens expressed by these pathogenic species, this non-pathogenic organism represents a tractable model system for providing insight into the pathogenesis of melioidosis and glanders. Specific examples of this are discussed below.

6.1. O-Polysaccharide

Lipopolysaccharides, commonly referred to as “endotoxins”, are a major component of Gram-negative cell envelopes. Bacterial strains expressing a ‘smooth’ phenotype synthesise LPS antigens that are composed of three covalently-linked domains: a lipid A moiety, a core region, and an OPS. Unlike other Gram-negative organisms, \textit{B. thailandensis} and \textit{B. pseudomallei} isolates appear capable of expressing only a restricted repertoire of structurally diverse OPS antigens. It has even been suggested that these species may be defined by as little as two serotypes [7]. At present, the significance of these observations is unknown. Based upon several studies, it is apparent that the predominant OPS serotype expressed by these species is an unbranched heteropolymer, consisting of disaccharide repeats having the structure -(1→3)-\(\beta\)-D-glucopyranose-(1→3)-6-deoxy-\(\alpha\)-L-talopyranose-(1→ which approximately 33% of the 6-deoxy-\(\alpha\)-L-talopyranose (L-6dTalp) residues possess 2-O-methyl and 4-O-acetyl substitutions, while the remainder of the L-6dTalp residues bear only 2-O-acetyl modifications [4,21]. Because of this, \textit{B. thailandensis} represents a convenient model to investigate the biosynthesis of this unique antigen, as well as a viable source of OPS to construct glycoconjugate vaccine candidates for immunisation against melioidosis.

Interestingly, studies have shown that \textit{B. mallei} expresses OPS antigens that are structurally similar to those expressed by \textit{B. pseudomallei} and \textit{B. thailandensis} strains, except that their L-6dTalp residues lack acetyl modifications at the O-4 position [21]. This phenomenon certainly explains the ability to generate monoclonal antibodies specific for \textit{B. pseudomallei} or \textit{B. mallei} OPS antigens, as well as the presence of \textit{B. mallei}-specific bacteriophage recognising smooth and not rough LPS strains [14]. Previous studies have demonstrated that the \textit{wbiA} locus expressed by \textit{B. thailandensis} and \textit{B. pseudomallei} is responsible for the 2-O-acetylation of their OPS antigens [21]. It is anticipated that, once the 4-O-acetyl transferase expressed by these organisms is identified, \textit{B. thailandensis} strains lacking this activity will provide a safe and cost-effective means of producing \textit{B. mallei}-like OPS antigens, for use in the development of novel glanders vaccines.

6.2. Type III secretion

It is well established that T3SSs play key roles during the interactions of bacteria with eukaryotic cells. In numerous Gram-negative pathogens, these specialised protein
translocation systems have been shown to be required for maximal virulence in animal models of infection. Three T3SSs have been identified in the \textit{B. pseudomallei} genome, including two plant pathogen-like systems (T3SS-1 and T3SS-2) and a virulence-associated animal pathogen-like system (T3SS-3 or Bsa T3SS). Similar to \textit{B. mallei}, only the T3SS-2 and T3SS-3 gene clusters are present in the \textit{B. thailandensis} genome [13]. A recent study by Haraga et al. demonstrated that, although T3SS-3 was not required for \textit{B. thailandensis} to invade HeLa cells, this system was critical for vacuolar escape and optimal intracellular replication [16]. This study also showed that an acidic environment was required for the secretion of the T3SS-3 associated effector protein, BopE, and for escape of \textit{B. thailandensis} from endocytic vacuoles into the host cytosol. When assessed in a murine model of \textit{B. thailandensis} infection, T3SS-3 mutants failed to replicate to wild type levels, and were largely restricted to the primary site of inoculation, the lungs [16]. These findings are consistent with the attenuated phenotypes observed for \textit{B. pseudomallei} and \textit{B. mallei} T3SS-3 mutants in animal models of infection, and support the hypothesis that \textit{B. thailandensis} may serve as a suitable model to study specific functions associated with T3SS-3. At present, the role of T3SS-2 in the lifestyle of these organisms remains to be defined.

6.3. Type VI secretion

Type VI secretion (T6S) is a recently-described mechanism for protein translocation in Gram-negative bacteria. Type VI secretion systems (T6SSs) are key virulence determinants in several pathogens, and, similar to T3SSs, appear to be involved in the translocation of bacterial effector proteins into eukaryotic cells. In most bacteria, expression of these systems is highly regulated at the genetic level and commonly involves two-component activator proteins or posttranslational regulation. Six, five and four intact T6SS gene clusters have been identified in \textit{B. pseudomallei}, \textit{B. thailandensis} and \textit{B. mallei}, respectively. Analyses by Schell et al. have revealed that while clusters 1 to 3 are present in all three species, cluster 4 is unique to the pathogenic species, and cluster 6 is unique to \textit{B. pseudomallei} and \textit{B. thailandensis} [22]. Interestingly, cluster 5 is predicted to encode a protein involved in fimbrial biogenesis, and is found in \textit{B. pseudomallei} and \textit{B. thailandensis}, but not \textit{B. mallei} [22]. Of these systems, T6SS-1 is the best characterised, and has been shown to be a major virulence factor in both \textit{B. pseudomallei} and \textit{B. mallei}. In all three species, this gene cluster is adjacent to a two-component regulatory system (\textit{virAG}), the \textit{Burkholderia} intracellular motility (\textit{bim}) locus, and the clan CA cysteine protease encoding gene, \textit{tssM}. In \textit{B. mallei}, T6SS-1 expression can be activated \textit{in vitro} by over-expression of the VirAG two-component system, or the AraC-type regulator, BMAA1517. In addition, expression of T6SS-1 is up-regulated following uptake of \textit{B. mallei} and \textit{B. pseudomallei} by macrophage cell lines. Recent studies have shown that \textit{B. mallei} T6SS-1 mutants exhibit defects in intracellular replication, actin-based motility, and multinucleated giant cells (MNGC) formation in RAW 264.7 cells. Similarly, \textit{B. pseudomallei} T6SS-1 mutants demonstrate cell-to-cell spread defects, as indicated by a reduced ability to form plaques on PtK2 epithelial cell monolayers. Due to the similarity in gene organisation and high degree of homology of T6SS-1 across
these three *Burkholderia* species, it is anticipated that *B. thailandensis* may provide a non-pathogenic model system to facilitate various structure/function analyses of T6SS-1.

6.4. Actin-based motility

Following uptake by host cells (via phagocytosis or invasion), *B. thailandensis* enters into primary phagosomes. During the process of phagosomal maturation, secretion of Bsa T3SS effectors promotes the disruption of vacuolar membranes facilitating escape of bacteria into the host cytosol. Once in the cytosol (Figure X.1), *B. thailandensis* induces its own propulsion by polymerising actin at one bacterial pole [16,23]. Consistent with what is known for *B. pseudomallei*, *B. thailandensis* appears to induce actin polymerisation through a mechanism distinct from those employed by other bacterial species demonstrating this phenotype. In *B. pseudomallei*, actin polymerisation is mediated by the activity of the BmA protein localised at a single pole of the bacterium. Furthermore, actin-based motility of the pathogen in J774.2 cells is abolished by mutation of *bimA*, and can be restored by providing the gene in trans [24]. In addition, it has been shown that BmA binds monomeric actin in vitro, and is able to weakly stimulate actin polymerisation in an Arp2/3-independent manner. Interestingly, the ectopic expression of BmA in eukaryotic cells also results in F-actin clustering. Collectively, these findings suggest that BmA alone might be sufficient for actin-based motility of *B. pseudomallei*, by acting as a nucleation-promoting factor mimic. Similar to *B. pseudomallei*, *B. thailandensis* also appears to exhibit a BmA-dependent intracellular actin-based motility. Although significant sequence variations exist between the BmA antigens expressed by these species, motility defects associated with *B. pseudomallei bimA* mutants can be complemented in trans via the expression of *B. thailandensis bimA* [24].

![Fig. X.1. Interaction of *B. thailandensis* with RAW 264.7 murine macrophages. Multinucleated giant cell formation and actin-based motility phenotypes following infection of RAW 264.7 cells with *B. thailandensis*. Nuclei stained with DRAQ5 appear blue, host cell actin stained with Alexa568 phalloidin appears red and GFP expressing bacteria appear green.](image-url)
6.5. Multinucleated giant cell formation

A unique feature of *B. mallei*, *B. pseudomallei* and *B. thailandensis* is their ability to stimulate host cell fusion leading to the formation of MNGC; a phenomenon that is thought in part to be due to the actin-based motility phenotypes associated with these organisms [23]. MNGC have been observed *in vitro* in both phagocytic and non-phagocytic cell lines, as well as *in vivo* in the infected tissues of melioidosis patients and in glandorous animals. At present, little is known about the specific bacterial and host factors involved in the formation of MNGC. It has been shown, however, that bacterial escape into the host cytosol is critical for the development of MNGC, as *B. pseudomallei* Bsa T3SS mutants demonstrating delayed vacuolar escape phenotypes exhibit delayed MNGC formation in RAW 264.7 cell monolayers. In addition, genetic analyses have implicated the T3SS effector BipB, and the sigma factor RpoS, in *B. pseudomallei*-induced MNGC formation. Interestingly, recent studies have demonstrated that *B. mallei* T6SS-1 mutants exhibit intracellular growth and actin polymerisation defects, and are incapable of inducing MNGC formation in RAW 264.7 cell monolayers suggesting an important role for T6SS-1 in this process. Although the specific role of MNGC formation in the pathogenesis of melioidosis and glanders remains to be defined, it is interesting to speculate that stimulation of host cell fusion may represent a strategy to facilitate cell-to-cell spread, evasion of host immune responses, and persistence of *B. pseudomallei* or *B. mallei* within a host. Due to similarities in the way that the pathogenic and non-pathogenic *Burkholderia* species promote MNGC formation in tissue culture, it is anticipated that *B. thailandensis* could serve as a useful model system to identify specific bacterial factors involved in this process.

6.6. Quorum sensing

Quorum sensing (QS) allows bacteria to sense and respond to extracellular signaling molecules, such as N-acyl-homoserine lactones (AHL), in a cell-density-dependent manner. This process leads to the coordinated modulation of gene expression within entire populations. Multiple complex quorum sensing networks of the LuxIR family have been described in *Burkholderia* species. *B. thailandensis* and *B. pseudomallei* both possess three pairs of luxI-luxR homologues, designated “systems 1”, “2” and “3”, while *B. mallei* possesses only systems 1 and 3. All three species produce and respond to a variety of different AHL. Recent studies have shown that the *B. thailandensis* system 1 (*btaI1-R1*) is required for cell aggregation, and that system 2 (*btaI2-R2*) controls antibiotic synthesis [17,25]. In addition, a *B. thailandensis* *btaI1* mutant, and a triple mutant deficient in all three systems, were both shown to be as virulent as the wild-type strain in a mouse infection model [17]. These results are in contrast to studies demonstrating that mutations in the *B. pseudomallei* and *B. mallei* luxI-luxR homologues resulted in strains with decreased virulence in hamsters and mice. For the most part, the specific virulence-associated genes regulated by these systems remain to be defined. Overall, these findings suggest that although *B. thailandensis* may be a valuable tool for studying QS-mediated gene regulation, it may not be the most appropriate model to determine the role of these systems in virulence, *per se*.
7. Conclusions

When the recognised limitations and caveats are appropriately controlled for, *B. thailandensis* can serve as an attractive model system to elucidate the functional role(s) of homologous virulence factors common to this organism and the virulent *Burkholderia* species. *B. thailandensis* can invade and grow in eukaryotic cells and shares many of the characteristics of its virulent phylogenetic neighbours, *B. pseudomallei* and *B. mallei*, including vacuolar escape, actin motility and MNGC formation. In addition, because *B. thailandensis* is non-pathogenic, it can be grown and handled under standard laboratory conditions and is not subject to the regulatory limitations that are imposed on the pathogenic *Burkholderia* species. Importantly, *B. thailandensis* is a genetically tractable model system that affords the luxury of using a variety of antibiotic resistance markers that are typically restricted for use in Select Agents. Based upon these attributes, it is expected that *B. thailandensis* would continue to serve as a useful tool for accelerating the pace of melioidosis and glanders research.

References


Section X.2

Burkholderia mallei

Deborah M. Ramsey\textsuperscript{a}, Paul J. Brett\textsuperscript{b}, Mary N. Burtnick\textsuperscript{b}

\textsuperscript{a}Institute for Cellular Therapeutics, University of Louisville, Louisville, KY, USA
\textsuperscript{b}Department of Microbiology and Immunology, College of Medicine, University of South Alabama, Mobile, AL, USA

Abstract

Burkholderia mallei, a reductive clone of \textit{B. pseudomallei}, is a host-adapted organism that causes a debilitating disease, known as glanders, in humans and animals. In endemic regions, chronically infected horses are the only known reservoir of this zoonotic pathogen. Human infections are typically acquired in occupational settings, via direct contact with infected animals. The clinical progression of glanders is similar in humans and equines, and may manifest as chronic or acute illnesses. In the absence of appropriate antibiotic treatment, glanders is almost always fatal. Several animal models of glanders have been established and successfully utilised to identify a number of bacterial virulence determinants required for disease progression and evasion of host immune defenses. Immunity against \textit{B. mallei} appears to be complex and to involve both humoral and cell-mediated responses. At present, no licensed vaccines are available for the prevention of equine or human glanders. © 2012 Elsevier B.V. All rights reserved.

1. Historical overview

Glanders is one of the oldest documented infectious diseases. As long ago as 330 B.C., Aristotle wrote of an illness of horses resembling glanders and termed the disease ‘melis’ or ‘malleus’. By about the fourth century, the Greek veterinarian Apsyrtus, and the Roman veterinarian Vegetius, had recognised the contagious nature of the disease referred to as ‘malis’ in horses and recommended isolation of infected animals. It was not until the 19th century, however, that the transmissibility of the disease was definitively proven by the French dermatologist Pierre-Francois-Olive Rayer, who successfully infected a horse using material from a human case of glanders. In Germany, in 1882, Friedrich Loeffler and Wilhelm Schütz isolated the glanders bacillus in pure culture from the liver and spleen of a horse that died from the disease. French researchers also isolated the organism from an infected horse in the same year [1]. In 1886, the glanders bacillus was designated “\textit{Bacillus mallei}” by Flügge. Over the past century, \textit{B. mallei} has been assigned to numerous genera including \textit{Bacillus},
Pfeifferella, Loefflerella, Mycobacterium, Corynebacterium, Malleomyces, Actinobacillus, Pseudomonas and, currently, Burkholderia.

2. Bacteriology and epidemiology

Burkholderia mallei is a non-motile, non-spore forming, Gram-negative bacillus that is closely related to B. pseudomallei. While B. pseudomallei can be readily isolated from tropical soils and waters, B. mallei is an obligate mammalian pathogen that has not been found free-living in the environment. Both organisms can be cultured using standard bacteriological medium; however, B. mallei is somewhat more fastidious, and the addition of glucose or glycerol to culture medium results in improved growth. A key feature that distinguishes B. mallei from B. pseudomallei is its lack of motility. In addition, B. mallei also lacks the distinct earthy odour and wrinkled colony morphology commonly associated with B. pseudomallei. Evidence from laboratory settings indicates that B. mallei is easily aerosolised and is highly infectious via this route, thus, work with this organism requires biosafety level 3 containment [2,3].

Burkholderia mallei primarily infects horses, mules and donkeys, although humans and other animals, including dogs and cats, are susceptible hosts. In natural settings, chronically infected horses are considered to be the principal reservoir of this facultative intracellular pathogen. B. mallei is transmitted amongst equines by oral, nasal, and possibly ocular, mucous membrane exposure, inhalation, and ingestion of infected skin exudates and respiratory secretions. Crowded and unsanitary housing conditions are believed to promote the maintenance and spread of the organism within equine populations. Zoonotic transmission of B. mallei to humans is rare and typically requires direct contact with infected animals, their secretions or infectious aerosols generated upon nasal exhalation. Individuals at highest risk of contracting glanders are those that have occupations involving frequent and close interactions with infected animals [2,3].

Glanders was once distributed globally, and was an important disease due to man’s dependence on horses as a primary means of transportation. However, the implementation of control and eradication programmes in the late 19th century requiring the quarantine and slaughter of animals testing positive by the mallein test has resulted in the eradication of glanders from North America, Western Europe and Australia [1,3]. The disease continues to be excluded from glanders-free countries due to strict quarantine measures and screening requirements for imported animals. Although most developed nations are currently free of glanders, endemic foci of infection still exist in South and Central America, the Middle East, Africa and Asia [3].

3. Clinical features, diagnosis and treatment

In equines, glanders can present as either chronic or acute illnesses. While mules and donkeys typically develop acute glanders, horses tend to become chronically infected. Acute disease is characterised by lung involvement, ulcerative nasal and tracheal lesions, and visceral abscess formation. In most cases, acute glanders results in pneumonia, septicemia and death within weeks. Chronic glanders can manifest as
nasal, pulmonary or cutaneous forms of disease. Nasal symptoms generally include the persistent production of a mucopurulent discharge and, in some cases, nasal septum ulceration. Pulmonary disease is exemplified by the presence of pus-filled nodules in the lungs. The cutaneous form, known as “farcy”, is characterised by lymphatic involvement and the presence of nodules and ulcerative skin lesions on the extremities of infected animals. Chronically infected horses often exhibit an extended course of disease, with periods of improvement followed by the recurrence of symptoms. In some situations, chronic infection can rapidly progress to acute illness and death of the animal. The clinical progression of human glanders is similar to that observed in equines, and may manifest as chronic or acute localised infections, acute pulmonary infections, or fulminating septicaemias. Although the clinical symptoms in humans are variable, both cutaneously and inhalationally acquired glanders typically result in acute febrile illness. In the absence of appropriate diagnosis and treatment this leads to septicaemia and death [2].

Ideally, the isolation and positive identification of *B. mallei* is required for a definitive diagnosis of glanders. In many cases, this is not possible since the organism is not always readily culturable from chronically infected animals. Several diagnostics tests have been developed for the detection and screening of potentially glanderous equines. The mallein diagnostic skin test was first developed in the 1890s and was implemented in the early 1900s as part of glanders control and eradication programs in many countries [1]. The complement fixation test (CFT) became available in 1909 and is still used today for the testing of equine sera. Additional tests, including an indirect haemagglutination test as well as ELISA and PCR based methods, have been developed and can be used for the confirmation of CFT results [3]. Animals that test positive for *B. mallei* are not routinely treated, but instead are culled. In the case of human glanders, diagnosis may be challenging since this organism can easily be confused with *B. pseudomallei*. Due to the rarity of human glanders, standardised treatment regimes using modern antibiotics are not readily available. Fortunately, *B. mallei* is susceptible to a variety of clinically relevant antibiotics and evidence from laboratory acquired infections indicates that human infections are responsive to antibiotic therapy. The most recently reported case of laboratory acquired glanders occurred in 2000, and was successfully treated with intravenous doxycycline and imipenem for one month, followed by oral doxycycline and azithromycin for six months [2]. Based on historical data, it is clear that, without treatment, glanders in humans is inevitably fatal.

4. Genomics

In 2004, the first *B. mallei* genome (strain ATCC 23344) was sequenced and shown to consist of two circular replicons, designated “chromosome I” (~3.5 Mbp) and “chromosome II” (~2.3 Mbp), harbouring a total of approximately 5500 open reading frames (ORF) [4]. Whole genome comparisons, in combination with multilocus sequence type (MLST) analyses, suggest that *B. mallei* has evolved from a single clone of *B. pseudomallei* through the process of genome erosion [4,5]. This is believed to have been a recent event that occurred upon infection of a mammalian host with a single
strain of *B. pseudomallei*, followed by multiple random insertion sequence (IS)-mediated recombination events. MLST analyses of several *B. mallei* strains have identified only two MLST types, that differ by only one nucleotide in a single locus (*gltB*), indicating a lack of genetic diversity between different strains [6].

Similar to *B. pseudomallei*, the *B. mallei* genome has an overall G+C content of approximately 68%, has a large number of simple sequence repeats, and the smaller replicon (chromosome II) is indispensable since it harbors several genes required for essential metabolic functions [4]. In contrast, the *B. mallei* genome is about 1.5 Mb smaller, is missing or variant in around 1200 genes, lacks several genomic islands, and has considerably more IS elements than the *B. pseudomallei* genome [4,6]. Importantly, almost all of the genes that have been retained by *B. mallei* share at least 99% DNA–DNA sequence identity with their *B. pseudomallei* orthologues. The large number of IS elements present in the *B. mallei* genome are believed to be responsible for extensive genome-wide rearrangements, including deletions, insertions and inversion mutations, and have resulted in several pseudogenes [4,6]. Interestingly, all *B. mallei* strains analysed to date exhibit disruptions in *fliP*, a gene involved in flagellar biogenesis, which seemingly explains the non-motile phenotype of this organism [6]. It is likely that the structural flexibility of the *B. mallei* genome accounts for many of the apparent phenotypic differences that distinguish it from *B. pseudomallei*. Recent studies suggest that genome evolution continues in *B. mallei* as this pathogen undergoes further adaptation to a host-restricted lifestyle.

5. Pathogenesis

5.1. Virulence factors

*Burkholderia mallei* expresses several major virulence determinants that are required for disease in animal models of infection. These include a capsular polysaccharide (CPS), lipopolysaccharide (LPS), a complex quorum sensing network, an animal pathogen-like type III secretion system (T3SSAP), the type VI secretion system cluster-1 (T6SS-1), and the VirAG two-component regulatory system. Disruption of any of these factors results in significantly attenuated virulence in vivo.

Polysaccharides can protect bacteria from antimicrobial peptides that form a natural line of defense against inhaled pathogens in the airways, as well as modulate bacterial interactions with host cells in the upper and lower respiratory tracts. *B. mallei* expresses a CPS whose synthesis locus is 99% similar to the *B. pseudomallei* CPS-I biosynthesis gene cluster that encodes for a capsule consisting of an unbranched homopolymer with the structure -3)-2-O-acetyl-6-deoxy-D-manno-heptopyranose-(1- [7]. The CPS may provide a singular advantage to the bacteria by preventing phagocytosis at early stages of infection, as well as conferring resistance in the harsh environment of the phagolysosome until the bacteria are able to escape. It has been shown that CPS-I of *B. pseudomallei* interferes with the activation of the complement cascade by reducing the deposition of complement factor C3b on the surface of bacteria, and thereby promotes evasion of innate immune responses. It is likely that the *B. mallei* capsule functions in a similar manner.
Similar to the CPS, the genes that encode the biosynthetic enzymes for LPS O-antigen of \textit{B. mallei} and \textit{B. pseudomallei} are 99% identical [8]. Compared to \textit{B. pseudomallei}, differences exist in the O-acetylation pattern of the \textit{B. mallei} l-talose residues, which indicates that the structure of \textit{B. mallei} LPS O-antigen is likely \(-3)\beta\-d-glucopyranose-(1,3)-6-deoxy-\alpha\-l-talopyranose-(1\-), where the talose residues only contain 2-O-methyl, or 2-O-acetyl, modifications. LPS O-antigen is an important virulence determinant for \textit{B. mallei}, since strains deficient in O-antigen are killed by 30% normal human serum [8]. In addition, studies conducted in 1925 by Stanton and Fletcher demonstrated that \textit{B. mallei} NCTC 120, now recognised as a rough isolate, was avirulent in both equine and rabbit models of infection [3].

Quorum sensing is a process whereby N-acyl-homoserine lactone (AHL) signaling molecules are released by bacteria to monitor their own population density, leading to coordinated modulations in the expression of virulence factors over an entire population of organisms. The quorum sensing system in \textit{B. mallei} is complex and comprised of multiple \textit{luxIR} homologues that modulate the expression of multiple virulence factor(s) contributing to pathogenicity [9]. Current research has shown that the type of animal model used to study \textit{luxIR} homologue mutants may be as important as the bacterial strains themselves. While 70\% of BALB/c mice survived an aerosol challenge with a \textit{bmaR1} mutant, the same mutant was as lethal as wild-type \textit{B. mallei} in hamsters [9]. Further research is needed to determine which virulence factors are regulated by AHL signaling molecules, and how these molecules affect intra- and intercellular spread.

Both type III and type VI secretion systems contribute significantly to the ability of \textit{B. mallei} to survive within eukaryotic cells. \textit{B. mallei} expresses both the T3SS\textsubscript{AP} and the T6SS-1 during infection, and both systems are required for virulence in hamsters [10,11]. In order for intracellular replication to occur, the T3SS\textsubscript{AP} must be expressed so that bacteria can escape from the endocytic vacuoles and polymerise actin for intra- and intercellular actin-based motility. The environmental cue for T6SS-1 expression occurs prior to disruption of the plasma membrane of the endocytic vacuole. The T6SS-1 is required for optimal growth and actin-based motility, thereby contributing to intra- and intercellular spreading [12]. Together, these two secretion systems modulate interactions with host cells in such a way that the host defense mechanisms are subverted, and the bacteria are able to survive and replicate in their intracellular niche.

The VirAG two-component system is an important regulator of virulence gene expression in \textit{B. mallei} and is required for virulence in hamsters [13]. Approximately 60 genes are positively regulated by VirAG, including genes encoding actin-based intracellular motility and T6SS-1 [11]. VirAG also controls the expression of \textit{tssM}, which encodes a putative deubiquitinase that is expressed shortly after intracellular uptake and provides the bacteria with an enzymatic tool that can potentially regulate multiple eukaryotic cell processes [14]. The specific signal that triggers \textit{virAG} expression \textit{in vivo} remains to be defined.
5.2. Interactions with host cells

As an intracellular pathogen, survival for B. mallei involves binding to and invading eukaryotic cells, successfully escaping the phagolysosomal compartment, and growing and multiplying in the cytosol. Little is known regarding how B. mallei binds to cells, or what surface receptors are being targeted by the bacteria. B. mallei adhere poorly to, and do not efficiently invade, A549 and LA-4 respiratory epithelial cell lines; however, they readily invade several phagocytic cell lines, including J774.2 and RAW 264.7 murine macrophages [13,15]. These observations suggest that aerosolised B. mallei may preferentially target a specific cell type in the lower respiratory tract, and bind to those cells by a yet undefined mechanism. Since a rise in anti-pilin antibody titres in infected mice have been observed, there is some evidence that B. mallei may express type IV pili in vivo. However, the role of pili in adhesion of B. mallei to host cells has not been determined.

Once B. mallei is bound to the host cell surface, the bacteria are capable of invading cells and using the T3SS to escape endocytic vacuoles. Upon escape, the bacteria can polymerise host cell actin and use actin-based motility to move about the cell. Following intracellular multiplication, the bacteria induce cell-to-cell fusion and the formation of multinucleated giant cells (MNGC), a phenomenon that is thought to be linked to actin-based motility and intracellular spread [13]. MNGC may provide a protective niche and metabolic resources for the bacteria, but little is known about the bacterial or host factors that are required for MNGC formation. Recent evidence points to expression of the T6SS-1 as a requirement for efficient intra- and intercellular spread in B. mallei (Figure X.2) [12], and BipB and RpoS have been implicated in MNGC formation by B. pseudomallei and are likely targets for future studies in B. mallei.

5.3. Animal models of infection

The natural hosts of B. mallei include horses, mules and donkeys, and a horse model of infection has been established to mimic the natural development of glanders. Several small animal models also have been used for studying B. mallei pathogenesis, including Syrian golden hamsters, mice (C57BL/6 and BALB/c), guinea pigs, monkeys, and wax moth larvae (Galleria mellonella) (Table X.2).

Equines are the most physiologically relevant animal model, since this species is the most common reservoir for B. mallei in nature. Horses tend to develop chronic glanders and exhibit a long disease course, with periods of improvement and relapse. A recent study using intratracheally inoculated horses found that bacilli-positive lesions formed in the nasal septum, but lesions were largely absent from other tissues [16]. This differs somewhat from the clinical pathology observed during the natural disease course, where fibrous lesions are found in the nasal septum, as well as the lungs, livers and spleens, of horses naturally infected with B. mallei. The applicability of this animal model to B. mallei vaccine research is unquestionable, although maintenance and facility costs are high and could potentially limit its widespread use.

Syrian golden hamsters are an excellent small animal model for studying acute B. mallei pathogenesis due to their extreme susceptibility to infection (LD<sub>50</sub> <10 colony
Following uptake into primary phagosomes, the intracellular signal sensed by the VirAG two-component regulatory system stimulates expression of T6SS-1. During the process of phagosomal maturation, T3SS$_{AP}$ facilitates escape of B. mallei into the cytoplasm by promoting the disruption of vacuolar membranes. At this point, mutants incapable of undergoing vacuolar escape are rapidly killed by the macrophages. Once free in the host cytosol, T6SS-1 then appears to influence the ability of B. mallei to efficiently grow, spread both intra- and intercellularly via actin-based motility, and induce MNGC formation. PM, Plasma membrane.
Table X.2
Infection models for investigation of *B. mallei* pathogenesis

<table>
<thead>
<tr>
<th>Model</th>
<th>Route</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse</td>
<td>Intratracheal</td>
<td>Mimics the natural development of chronic glanders in its native host</td>
</tr>
<tr>
<td>Rhesus monkeys</td>
<td>Subcutaneous</td>
<td>Developed subcutaneous lesions that healed after three weeks; no evidence of chronic or acute infection</td>
</tr>
<tr>
<td>Syrian golden hamster</td>
<td>Intraperitoneal</td>
<td>Develops acute infection; bacteria are transported to the mediastinal lymph nodes and seeded to other tissues, forming lesions in the spleen as early as one day post-inoculation; rapid disease course, with death occurring around six days</td>
</tr>
<tr>
<td>Guineapig</td>
<td>Subcutaneous, Intraperitoneal</td>
<td>Develops acute infection; bacteria are transported to inguinal and axillary lymph nodes (subcutaneous), or mediastinal and mesenteric lymph nodes (intraperitoneal), at early time points post-inoculation</td>
</tr>
<tr>
<td>BALB/c mouse</td>
<td>Intranasal, Intraperitoneal</td>
<td>Commonly-used model due to low cost, high susceptibility to <em>B mallei</em> and a well-documented disease pathology; more resistant to <em>B mallei</em> when delivered intraperitoneally; acute infection specific for <em>B pseudomallei</em></td>
</tr>
<tr>
<td>C57BL/6 mouse</td>
<td>Intranasal Intraperitoneal</td>
<td>Similar to BALB/c, in that mice are more resistant to <em>B mallei</em> when delivered intraperitoneally; chronic infection specific for <em>B pseudomallei</em></td>
</tr>
<tr>
<td>Wax moth larva</td>
<td>Injection into the haemocoel</td>
<td>Used to screen putative virulence mutants; between 3–200 CFU of wild-type <em>B. mallei</em> leads to &gt;90% killing within six days</td>
</tr>
<tr>
<td><em>Galleria mellonella</em></td>
<td></td>
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</tr>
</tbody>
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forming units (CFU). When injected intraperitoneally with B. mallei, granulomas develop in the spleens of hamsters at early time points post-inoculation, and in the lungs at late stages of disease. B. mallei appears to preferentially target reticuloendothelial tissues, and glanders-related lesions are detected in this model in liver, bone marrow and mediastinal and mesenteric lymph nodes as early as two days post-inoculation [17]. Although the sensitivity of hamsters to B. mallei makes them useful for studying acute disease, chronic glanders exhibits a milder symptomology that is unattainable in this model. Vaccine development studies tend to use a more resistant model than hamsters to examine vaccine efficacy in both chronic and acute disease states.

BALB/c mice are the most commonly used strain of mouse in B. mallei research. This strain is more resistant to B. mallei infection than hamsters, with an LD<sub>50</sub> of approximately 10<sup>3</sup> CFU by aerosol route, or 10<sup>6</sup> CFU delivered intraperitoneally. BALB/c mice exhibit a disease pathology that is well documented and most closely simulates human infection. When infected intraperitoneally, inflammation is localised at early time points to reticuloendothelial tissues of the mediastinal lymph nodes, and fibrotic lesions develop later in spleen, liver, peripheral lymph nodes, and bone marrow [18]. With an aerosolised inoculum, severe acute inflammation is observed in the nasal cavity, and fibrotic lesions are detected in the nasal septum, trachea, lung, spleen, liver, submandibular lymph node and brain. Aerosolisation of B. mallei represents a primary route of inoculation for humans, and the acute inflammatory response observed in the nasal septum of BALB/c mice after aerosol initiated infection mimics what is seen in human disease. Because of this similarity and lower maintenance costs, BALB/c mice are frequently used in vaccine studies as a clinically relevant small animal model for acute glanders.

6. Host immunity

6.1. Immune responses

Burkholderia mallei can establish chronic or acute infections in multiple species, which suggests that the native immune response is unable to eradicate wild-type organisms upon primary challenge in these hosts. Cell-mediated immune mechanisms, as well as cytokine and chemokine expression, contribute to clearance of B. mallei. Maximising the effectiveness of these responses would limit the duration of infection and reduce disease pathology. An understanding on how innate and adaptive immune responses fall short of completely clearing the bacteria from the host should provide clues for designing effective vaccination strategies.

Burkholderia mallei is capable of infecting many cell types, including macrophages. Macrophages have been shown to be important for reducing the susceptibility of wild type C57BL/6 mice to pneumonic B. mallei infection, and several published reports describe the uptake and intracellular spread of the organism in phagocytic cell lines, such as J774.2 and RAW 264.7 macrophages. Various subsets of macrophages exist in vivo, and demonstrate different functional properties in vitro, but the role of specific macrophage subsets in B. mallei pathogenesis has not been explored.
Host resistance to intracellular pathogens is often linked to the rapid and significant production of pro-inflammatory and phagocyte-activating cytokines and chemokines. IFN-γ is a powerful cytokine that controls the rate of bacterial growth by regulating cell-mediated immune responses. The pro-inflammatory cytokines IL-12 and IL-18 induce IFN-γ production by T cells and NK cells at early time points post-infection. Previous studies by Rowland et al. [19] have shown that the expression of IL-12 and IL-18 mRNAs in the spleens of B. mallei infected BALB/c mice peaked at five hours post intraperitoneal inoculation. In addition, it was shown that IFN-γ, IL-6, and the macrophage chemoattractant protein 1 (MCP-1) were elevated in the sera of the infected animals at five hours, and reached maximal levels at 24 hours post inoculation. By three days post inoculation, however, cytokine and chemokine levels in the sera of B. mallei-infected mice were equivalent to values obtained from uninfected controls. Interestingly, the high levels of MCP-1 observed at 24 hours correlated with macrophage influx into the spleens of the infected animals. Results of these studies also demonstrated that NK cells and CD8+ T cells were the predominant populations secreting IFN-γ in response to B. mallei [19]. While the initial burst of IFN-γ was able to control bacterial replication, clearance of B. mallei was not achieved.

Although cytokines, such as IFN-γ, play an important role in macrophage activation, LPS and other microbial products can activate macrophages through Toll-like receptors (TLR). LPS signals through TLR4, and the signal is subsequently relayed through MyD88-dependent or -independent pathways. Purified B. mallei LPS is a strong activator of human TLR4 complexes, and stimulates production of TNF-α, IL-6, and RANTES in human antigen presenting cells (APC) [20]. The expression of these cytokines and chemokines by APC reflects signaling through both MyD88-dependent (TNF-α and IL-6) and -independent (RANTES) pathways. Upon exposure to Escherichia coli LPS, B. mallei infected RAW 264.7 macrophages upregulated expression of genes encoding inducible nitric oxide synthase (iNOS), and iNOS activity led to the generation of reactive nitrogen oxide species and clearance of intracellular B. mallei [20]. These studies illustrate the importance of TLR4 signaling in macrophage activation and clearance of intracellular B. mallei.

6.2. Vaccine candidates

At present, there are no licensed vaccines for protection of humans or animals against glanders. To date, most vaccination strategies have targeted B. mallei-specific antigens alone, or in conjunction with immunotherapies. Although several virulence factors have been studied as potential vaccine candidates, attempts to generate sterilising immunity in vaccinated animals have been met with limited success. These preliminary vaccine studies highlight the importance of generating both humoral and cell-mediated immune responses to combat B. mallei infection, but much work is left to be done toward identifying viable vaccine targets and determining the host factors required for resistance.

Vaccination studies using killed, whole-cell vaccines or live attenuated strains, have generated mixed Th1/Th2 immune responses with Th2-like antibodies predominating, and this immune response is not protective upon challenge with B. mallei [21,22].
When BALB/c mice were challenged with heat-killed or irradiated, or an irradiated non-encapsulated strain, splenocytes isolated from infected animals expressed IFN-\(\gamma\), IL-4, and IL-10 [22]. Antibody titres from infected animals reflected higher levels of IgG1 than IgG2a, and only the addition of IL-12 in a later study was able to enhance the amount of IgG2a in the sera and induce a more Th1-like humoral response [21,23]. Although following the administration of an aerosolised non-encapsulated live attenuated strain of \(B. mallei\) to BALB/c mice and subsequent challenge with \(B. mallei\) ATCC 23344 resulted in the production of higher levels of IgG1 antibodies, none of the animals survived [22]. In the same study, a live attenuated branched-chain amino acid auxotroph was also administered by aerosolisation to mice. The subsequent challenge with \(B. mallei\) produced higher levels of IgG2a circulating antibodies with the survival of 25% of the animals. However, the spleens of surviving animals were infected with bacteria, indicating that Th1-like humoral responses alone may not be sufficient to achieve sterilising immunity. In addition to inducing Th1/Th2 antibodies through vaccination, passive immunotherapy using monoclonal antibodies to \(B. mallei\) LPS was successful in preventing death in BALB/c mice up to 14 days after aerosol challenge. However, passive transfer of antibodies failed to achieve sterilising immunity [24].

Activation of cell-mediated immune responses for efficient clearance of intracellular bacteria is also a goal in \(B. mallei\) vaccine design. BALB/c mice pretreated with CpG oligodeoxynucleotides (ODN) that signal through TLR9 exhibited elevated levels of IL-6, IL-12 and IFN-\(\gamma\) following aerosol challenge with \(B. mallei\), resulting in lower numbers of bacteria in the lungs and spleen [25]. Such observations suggest that CpG ODN may be a promising addition to \(B. mallei\) vaccine preparations. Several immunogenic antigens are currently under investigation as components of future \(B. mallei\) vaccine candidates, including surface polysaccharides (CPS and O-antigen) and other cell surface exposed antigens, such as outer membrane proteins and various components from the T3SSs and T6SSs. As additional protective antigens are identified and the host responses to \(B. mallei\) are further understood, developing a protective vaccine against \(B. mallei\) would become possible.

7. Conclusions

Although glanders has been recognised for centuries, the biology of \(B. mallei\) has remained relatively understudied. Over the past decade, however, there has been a resurgence of interest in this pathogen primarily due to its classification as a Select Agent by the Centers for Disease Control and Prevention (CDC). Several recent studies have defined important virulence factors and putative protective antigens expressed by \(B. mallei\), as well as provided new information regarding the nature of host responses against this organism. Future studies will undoubtedly lead to a better understanding of the pathogenesis of glanders, and will be important for the design of future vaccines and therapeutics. Because of its close relatedness to \(B. pseudomallei\), studies focussing on \(B. mallei\) are likely to also provide insight into certain aspects of \(B. pseudomallei\) infection.
References


Since the description of melioidosis a century ago, this is the first monograph in this field. Authored by leaders in research and clinical aspects of melioidosis, this comprehensive monograph is a compendium of multi-author reviews included within specific sections, with commentaries provided by section editors.

The monograph includes sections on: history, epidemiology, microbiology, clinical manifestations, laboratory diagnosis and detection, treatment, pathogenesis and development of protection, veterinary infections, environmental aspects, in addition to a section on other closely related *Burkholderia* species.

Authored by an international team, this monograph provides an insight into historical milestones, the current state of knowledge and future directions in key areas. Each section highlights important aspects for clinicians and research scientists, microbiologists, epidemiologists and immunologists.

Above all for those investigators commencing work in the growing field of melioidosis and *Burkholderia pseudomallei* research, it encapsulates the published literature of a century of discoveries.