The Effect of Glibenclamide on the Pathogenesis of Melioidosis

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The effect of diabetes mellitus and glibenclamide on the pathogenesis of melioidosis. Gavin Christian Koh Kia Wee.

This submission is my own work and contains nothing which is the outcome of work done in collaboration except where specifically where due acknowledgment has been made in the text. This work was conducted at the Wellcome Trust Mahidol-Oxford Tropical Medicine Research Unit, Mahidol University, Bangkok, Thailand (April 2007 to November 2008), the Center for Experimental and Molecular Medicine, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands (March 2009 to December 2010), and the Wellcome Trust Sanger Institute, Cambridge (January 2011 to December 2011) and was supervised by Professor Sharon Jayne Peacock (Department of Medicine, University of Cambridge and the Wellcome Trust Sanger Institute), Professor Nicholas Philip John Day (Mahidol University & University of Oxford), Professor Gordon Dougan (Wellcome Trust Sanger Institute), Dr Willem Joost Wiersinga (University of Amsterdam), and Professor Tom van der Poll (University of Amsterdam).

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The fount is 12 point Georgia, which is a transitional serif typeface designed in 1993 by Matthew Carter and has a larger x-height to increase clarity at smaller fount sizes. A distinctive feature of the fount is its use of text figures.

My father, mother & godmother

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>, greater than.

≥, greater than or equal to.

<, less than.

≤, less than or equal to.

%, per cent.

95%CI, 95 per cent confidence interval.

ALI, acute lung injury. On a spectrum of pulmonary disease that includes ARDS.

AMA, American Medical Association.

AMC, *see* **co-amoxiclav**.

AOR, adjusted odds ratio.

APACHE, acute physiology and chronic health evaluation; a scoring system for predicting mortality in the intensive care setting.

APC, activated protein C.

APhA, American Pharmacists Association,

API, analytical profile index; a set of miniaturised biochemical tests sold commercially for the identification of bacteria.

ARDS, acute respiratory distress syndrome (formerly, adult respiratory distress syndrome). On a spectrum of pulmonary disease that includes ALI.

ASC, an adaptor molecule that forms part of the inflammasome complex. Its formal name is 'apoptosis-associated speck-like protein containing a caspase recruitment domain'.

ATP, adenosine triphosphate.

B. mallei, *Burkholderia mallei.*

B. pseudomallei, *Burkholderia pseudomallei.*

B. thailandensis, *Burkholderia thailandensis.*

BALB/c, an inbred strain of albino mouse commonly used in laboratory research. The strain was developed in 1913 by H. Bragg in New York. There are four major substrains which separated prior to 1940; they are: BALB/cHeAn, BALB/cJ, BALB/cRI and BALB/cWt.

BALF, bronchoalveolar lavage fluid.

bd, *bis die;* twice a day.

BSA, bovine serum albumin.

CRP, C-reactive protein. An acute phase protein released by the liver in response to acute inflammation (specifically interleukin 6 stimulation). Its function is to bind to phosphocholine on the surface of necrotic cells and some bacteria. Binding then activates complement

via the classical pathway. Serum concentrations of CRP are used as a non-specific marker for sepsis.

C3, complement component 3.

C3H/He, a strain of inbred agouti mouse; the strain was developed in 1920 from the DBA mouse by L.C. Strong. There are two major substrains: C3H/HeN (NIH) and C3H/HeJ (Jackson Laboratories).

C4, complement component 4.

C5, complement component 5.

C57Bl/6, a strain of inbred mouse that originated in the early 20th century with the breeder, Abbie Lathrop (1868–1918) who lived in in Granby, Massachusetts. The strain is commonly referred to as just 'Black 6' and is indeed black. There are two important substrains: C57Bl/6J (Jackson Laboratories) and C57Bl/6N (NIH).

C8, complement component 8.

CAP, community-acquired pneumonia.

CASP, caspase.

caspase, cysteine-dependent **asp**artate-directed prote**ase.**

CAZ, ceftazidime.

CD, cluster of differentiation. This is an internally recognised protocol for immunophenotyping cells on the basis of their surface antigens. The CD classification includes signalling molecules and membrane-bound receptors, but the protocol is not designed to capture information about function.

CD11a, cluster of differentiation 11a; also known as integrin α L chain, ITGAL or α_L. It is an adhesion molecule expressed by all leukocytes and also has a role in immune signalling. The CD11a/CD18 complex is known as lymphocyte function-associated antigen 1 (LFA1).

CD11b, cluster of differentiation 11b; also called integrin α M chain, ITGAM or α_M. The CD11b/CD18 complex is known as Macrophage antigen 1 (Mac-1).

CD18, cluster of differentiation 18; also called integrin β 2 chain, ITGB2 or β_2. CD18 forms complexes with **CD11a** and **CD11b**.

CD54, cluster of differentiation 54; *see* **ICAM-1**.

CE, common era (*anno Domini*).

chr., chromosome.

cfu, colony forming units.

CI, confidence interval.

CLED, cysteine lactose electrolyte deficient agar.

co-amoxiclav, amoxicillin-clavulanate.

co-trimoxazole, fixed combination trimethoprim-sulphamethoxazole.

comb. nov., *combinatio nova* (Latin), new combination (marking the first instance of a binomial in the literature).

Cpe, the mouse gene, carboxypeptidase E.

CXCL, a family of cytokines containing a cysteine-x-cysteine motif, where x can be any amino acid. The most prominent member of this family is IL8, also known as CXCL8.

DBA, a strain of inbred brown mouse. This is the oldest strain of inbred mouse in existence and originates from Dr. C.C. Little in 1909. The substrains DBA/1 and DBA2 separated in 1929.

DCCT, Diabetes control and complications trial, was a randomised-controlled trial of 1,441 volunteers with DM1 comparing intensive insulin therapy with standard therapy.[1] It was the largest, most comprehensive study of diabetes ever conducted at the time. DM2 patients were excluded from the study. It was funded by the US NIDDK and subjects were recruited from 29 centres in the US and Canada.

DIC, disseminated intravascular coagulation.

DM, diabetes mellitus.

DM1, diabetes mellitus, type 1.

DM2, diabetes mellitus, type 2.

DMSO, dimethyl sulphoxide.

DNA, deoxyribonucleic acid.

E. coli, *Escherichia coli.*

e.g., *exempli gratia;* for example.

EDTA, ethylenediaminetetraacetic acid.

ELISA, enzyme-linked immunosorbent assay.

FDA, United States Food and Drug Administration.

FDR, false discovery rate. In the statistical analysis of high dimensional biological data, the false discovery rate is the proportion of type I errors out of all the comparisons found to be statistically significant. The Benjamini-Hochberg method and its variants are commonly used to explicitly control the FDR.

FiO_2, the fractional concentration of inspired oxygen. Normal room air is 21% oxygen or 0·21.

g, gram or gramme.

G-CSF, granulocyte colony-stimulating factor.

Gb, glibenclamide (rINN); glyburide (USAN).

GLUT2, glucose transporter 2 (official name now **SLC2A2**).

glinide, meglitinide analogue. A class of insulin secretagogues used

to treat diabetes, that are not structurally related to sulphonylureas, but which have a similar mode of action: examples in current clinical use include glimepiride and repaglinide.

GM-CSF, granulocyte macrophage colony-stimulating factor.

GO, gene ontology; an international project to accurately annotate all genes and gene products.

h, hour(s).

Hb, haemoglobin.

HbA_{1c}, haemoglobin A_{1c} (glycosylated haemoglobin).

HDL, high density lipoprotein. HDL deficiency occurs in Tangier disease.

HES, hydroxyethyl starch.

HLA, human leukocyte antigen. There are two classes. The class I antigens are designated A, B and C. The class II antigens are DP, DM, DOA, DQ and DR.

HRP, horseradish peroxidase.

HUGO, Human Genome Organisation.

ICAM-1, intercellular adhesion molecule 1; also known as CD54. ICAM-1 binds to the integrins CD11a/CD18 and CD11b/CD18.

ICE, interleukin 1 converting enzyme; now known as caspase 1.

i.e., *id est;* that is.

IFA, indirect immunofluorescence.

IFFC, International Federation of Clinical Chemistry.

IFNγ, interferon gamma.

IgG, immunoglobulin G.

IgM, immunoglobulin M.

IHA, indirect haemagglutination test.

IL, interleukin. The interleukins are small messenger proteins that play a role in leukocyte signalling.

IPM, imipenem; a carbapenem antibiotic active against *B. pseudomallei.*

IUPAC, International Union of Pure and Applied Chemistry. The international authority for standards in the naming of the chemical elements and their compounds.

K_{ATP}-channels, ATP-sensitive potassium channels.

KC, keratinocyte-derived cytokine; now called CXCL1.

KEGG, Kyoto Encyclopedia of Genes and Genomes; an online database of protein and pathway information hosted by the Kyoto University.

KIR2DL1, killer cell immunoglobulin-related re;ceptor, 2 domains, long cytoplasmic tail, 1.

kPa, kilopascals; *see* **Pa**.

L, litre.

L. pneumophila, *Legionella pneumophila*.

LAMP, loop-mediated isothermal amplification.

LB, lysogeny broth; also called Luria-Bertani medium, or Luria broth.

LD50, median lethal dose. The dose sufficient to kill half the population tested.

LFA-1, lymphocyte function-associated antigen 1; *see* **CD11a.**

LIX, lipopolysaccharide-induced CXC chemokine. now known as CXCL5.

LPS, lipopolysaccharide; also known as a endotoxin. A component of the outer membrane of all Gram-negative bacteria.

LY96, lymphocyte antigen 96; also known as MD2. LY96 associated with Toll-like receptor 4 and is involved in the recognition of lipopolysaccharide.

M, molar; 1 litre of a 1 molar solution contains 1 mole of solute.

M1, 4-*trans*-hydrocyclohexyl glibenclamide; a metabolite of glibenclamide

M2, now called M2b.

M2a, 4-*cis*-hydrocyclohexyl glibenclamide; a metabolite of glibenclamide.

M2b, 3-*cis*-hydrocyclohexyl glibenclamide; a metabolite of glibenclamide.

M3, 3-*trans*-hydrocyclohexyl glibenclamide; a metabolite of glibenclamide.

M4, 2-*trans*-hydrocyclohexyl glibenclamide; a metabolite of glibenclamide.

M5, ethyl-hydroxy glibenclamide; a metabolite of glibenclamide.

Mac-1, macrophage antigen 1; *see* **CD11b**.

MAQC, MicroArray Quality Control project; an initiative of the FDA to monitor the reproducibility of microarrays, with the view that they will eventually be used for diagnostic applications.

max., maximum.

MD2, *see* **LY96**.

MDP, muramyl dipeptide, a peptidoglycan constituent of both Gram-positive and Gram-negative bacteria.

MEM, meropenem; a carbapenem antibiotic active against *B. pseudomallei.*

MERTH, Melioidosis Eradication Therapy/Thailand; a multicentre randomised controlled trial in Northeast Thailand comparing co-trimoxazole therapy against co-tromoxazole and doxycycline

combination therapy due to report at the end of 2011.

MIAME, Minimum Information About a Microarray Experiment; a published checklist for the minimum information that should be reported for all published microarray experiments.

MIC, minimum inhibitory concentration; the minimum concentration of antibiotic required to inhibit bacterial growth.

MIP-2, macrophage inflammatory protein 2. Now known to consist of two distinct cytokines, CXCL2 and CXCL3.

MLST, multi-locus sequence type.

mM, millimolar. *See* **M**.

mmHg, millimetres of mercury. 1 mmHg = 133.3 Pa; 1 atmosphere = 760 mmHg.

MODY, maturity-onset diabetes of the young.

mol, mole. And SI unit defined as the number of elemental entities in 12 g of carbon-12. This number is called Avogadro's constant, and has a measured value of $6{\cdot}022\ 141\ 79 \times 10^{23}$ per mol.

mRNA, messenger RNA.

MSD, Merck Sharp & Dohme, a pharmaceutical company based in Whitehouse Station, New Jersey, USA. It manufactures and markets imipenem/cilastatin (Primaxin®).

NCBI, National Center for Biotechnology Information; a part of the National Library of Medicine in the United States.

NEQAS, National External Quality Assessment Service. A UK body charged with independently auditing standards in laboratories.

NET, neutrophil extracellular trap.

NF-κB, nuclear factor κB.

NK cell, natural killer cell; a form of cytotoxic lymphocyte.

NLR, Nod-like receptor. A family of cytosolic pattern recognition receptors. Unlike the TLRs, they are not membrane-bound. Since 2008, their new name has been 'nucleotide-binding oligomerization domain, leucine rich repeat', a name chosen to retain the same abbreviation, while adding descriptive information about their molecular properties.

NIDDK, United States National Institute of Diabetes and Digestive and Kidney Diseases.

NIH, National Institutes of Health; a loose federation of government-funded research institutes in Bethesda, Maryland.

Nod, nucleotide-binding and oligomerization domain.

NOD, non-obese diabetic mouse.

OR, odds ratio.

***p*-value,** probability value calculated from a statistical test.

Pa, pascals (SI unit). 1 mmHg = 133.3 Pa; 1 atmosphere = 101.3 kPa.

PAI-1, plasminogen activator inhibitor-1.

PAMP, pathogen-associated molecular patterns; these are highly conserved antigens present on a wide range of pathogens (viruses, bacteria and parasites).

PaO_2, partial pressure of oxygen in arterial blood, usually expressed in mmHg, but also measured in kPa.

PC, protein C.

PBS, phosphate-buffered saline.

PCR, polymerase chain reaction.

PEG, polyethylene glycol; also called poly(ethylene oxide) or polyoxyethylene.

P:F or **P/F ratio,** the ratio of PaO_2 (in mmHg) to FiO_2 (as a fraction). The ratio is used to quantify pulmonary function. In healthy adults, values are usually >380 mmHg; the cut-off for ALI is ≤300 mmHg and the cut-off for ARDS is ≤200 mmHg.

PRR, pattern recognition receptors; the two main families of PRRS are the **TLRs** and the **NLRs**.

PYCARD, old name for **ASC.**

qds, *quater die sumendus;* to be taken four times a day, or every six hours.

qPCR, quantitative reverse-transcriptase polymerase chain reaction; a method for quantifying **DNA**.

qqh, *quaque quarta hora;* to be taken every four hours.

R. argentiventor, *Rattus argentiventor.*

R. exulans, *Rattus exulans.*

R. rattus, *Rattus rattus.*

R. tionamicus jalorensis, *Rattus tionamicus jalorensis.*

RAW264.7, a cell line originating from virus-transformed mouse monocytes. The cell line sheds murine leukaemia virus and should therefore be handled under biosafety II conditions.

rINN, Recommended International Nonproprietary Name. Drug name assigned by the WHO.

RNA, ribonucleic acid.

RNS, reactive nitrogen species; these are ·NO, $\cdot NO_2$, N_2O_3, $ONOO^-$, ONOOH and $ONOOCO_2^-$.

ROS, reactive oxygen species; these are singlet oxygen, ·OH, H_2O_2, $O{=}C(O\cdot)O^-$, O_2^- and O_2^{2-}.

RPMI 1640, Roswell Park Memorial Institute medium 1640. A

commonly used cell culture medium.

rRNA, ribosomal RNA.

S. enterica, *Salmonella enterica*.

***S.* Typhi,** *Salmonella enterica var* Typhi, the causative agent of typhoid fever in humans.

***S.* Typhimurium,** *Salmonella enterica var* Typhimurium. A model organism that causes a typhoid fever-like illness in mice.

S. aureus, *Staphylococus aureus*.

sFLT-1, soluble fms-like tyrosine kinase-1. A marker for endothelial activation.

SLC2A2, solute carrier family 2, member 2 (also called **GLUT2**).

SNK, Student-Newman-Keuls. This is a multiple comparison method and is discussed in the chapter on statistical analyses. Also known as the Newman-Keuls test.

SOP, standard operating procedure.

SPP1, Secreted phosphoprotein 1.

statin, common name for HMG-CoA reductase inhibitors; these are drugs used for the treatment of hypercholesterolaemia.

STZ, streptozocin or streptozotocin.

T-cell, T-lymphocyte; a lymphocyte that originates from the thymus.

tds, *ter die sumendus;* to be taken thrice daily, or every eight hours.

THP-1, a human monocytic leukaemia cell line.

TLR, Toll-like receptor; a class of 13 single membrane-spanning leukocyte receptors that recognise **PAMPs**. Recognition by TLRs results in activation of the innate immune response.

TMB, 3,3′,5,5′-tetramethylbenzadine. A substrate for horseradish peroxidase. TMB is a colourless compound, but is oxidised by horseradish peroxidase to form a blue product that may be detected spectrophotometrically.

TNF, tumour necrosis factor. Usually refers to **TNF*α***, but may refer also to the TNF family of cytokines.

TNF*α*, tumour necrosis factor, alpha; a pro-inflammatory cytokine.

TSA, trypticase soy agar.

TTS, type-three secretion system.

UGDP, University Group Diabetes Program, was a prospective randomised-controlled trial of the treatment of DM2 that ran from 1960 to 1975, and published its final report in 1982. It produced two controversial observations on the safety and efficacy of two oral anti-diabetic treatments: first, that tolbutamide is associated with an excess cardiovascular mortality,[2] and second, that phenformin is

associated with an excess mortality from lactic acidosis.[3]

UKPDS, United Kingdom Prospective Diabetes Study, was a randomised-controlled trial funded by the British Diabetic Association at a cost of £2 million. Starting in 1977, it recruited over 5,000 white patients with recently diagnosed type 2 diabetes and lasted for 20 years.[4]

uPAR, urokinase-type plasminogen activator receptor.

US or **USA,** United States of America.

USAN, United States Adopted Name, assigned by the USAN Council (co-sponsored by the AMA, USP and APhA).

USP, United States Pharmacopeial Convention.

***viz.*,** *videlicet;* namely.

WHO, World Health Organization.

Summary

Melioidosis is an important cause of community-acquired sepsis, endemic to Southeast Asia and Northern Australia. Melioidosis is caused by the soil saprophyte, *Burkholderia pseudomallei,* a motile Gram-negative bacillus, and is associated with a mortality rate that approaches 50% in Northeast Thailand. The most important risk factor for melioidosis is diabetes mellitus, and two-thirds of all adult patients with melioidosis have diabetes as a risk factor. It has been noted previously, however, that patients with diabetes have lower mortality than patients without diabetes. In this dissertation, we look at a cohort of 1160 consecutive adult melioidosis patients presenting to Sappasithiprasong Hospital in Ubon Ratchathani, Thailand, 410 (35%) of whom were diagnosed with diabetes prior to admission. We confirmed previous findings that diabetes protected from mortality in melioidosis, but also found that this protective effect was confined to a smaller subset of patients (208 patients) who were treated with glibenclamide prior to admission. Patients with hyperglycaemia (but no diagnosis of diabetes prior to admission) had the same mortality rate as patients without diabetes. *In vitro* experiments found no inhibitory effect of glibenclamide on bacterial growth, and we therefore looked for evidence of an effect of glibenclamide on the host. We conducted a gene expression study of circulating blood leukocytes in melioidosis patients and compared them to uninfected controls. In this study, we found that glibenclamide was associated with an anti-inflammatory effect on the host response to melioidosis. To further elucidate a mechanism for the action of glibenclamide, we studied the effect of glibenclamide therapy in a mouse model of melioidosis and found that the effect of glibenclamide was specific to interleukin-1β secretion. This reduction in interleukin-1β secretion was associated with reduced cellular influx into the lungs as well as lower bacterial loads in blood, liver and spleen.

1. Introduction

Melioidosis is infection caused by the Gram-negative bacillus, *Burkholderia pseudomallei,*[5] and was first reported in 1912 in a post mortem series of 38 patients seen by Whitmore and Krishnaswami at the Rangoon General Hospital, Burma, the majority of whom were morphine addicts.[6–8]

Whitmore recognised that the causative agent of melioidosis was a novel organism, which he named *Bacillus pseudomallei*.[8] The clinical disease only acquired its name in 1921, being coined from the Greek *melis* (distemper of asses) and *eidos* (resemblance), and refers to its resemblance to glanders, an infection of equids caused by the related organism, *B. mallei*.[9]

The history of melioidosis research is punctuated by waves of activity that coincide with the military interests of the Western powers. British interest in the disease coincided with British imperial interests (specifically Burma for petroleum, Malaya for petroleum and rubber),[10,11] and waned as the empire waned. France reported approximately 100 cases among the 400,000 troops of the French Expeditionary Forces in Indochina between 1948 and 1954.[12] America's interest in (and therefore, research funding for) melioidosis increased and declined proportionate to its involvement in the Vietnam war.[13–18] Following the events of September 11, 2001, funding for melioidosis research in the last ten years has again increased with the realisation that *B. pseudomallei* is easily obtained (it is an environmental organism) and when aerosolised, might see utility as a bioterror agent with a high mortality rate.

1.1 Burkholderia pseudomallei

1.1.1 Taxonomy

B. pseudomallei was for a long time placed in the genus *Pseudomonas* on the basis of its Gram-stain appearance and biochemistry[19] (it is a non-lactose fermenting oxidase-positive Gram-negative bacillus[20]), but the organism was moved to a new genus, *Burkholderia,* in 1992 on the basis of 16S ribosomal RNA (rRNA) sequence.[21]

The causative organism of melioidosis and its characteristics were described very shortly after the disease was first described.[8] Initially named

Bacillus pseudomallei by its discoverer,[6] it was later named *Bacterium whitmori* (Stanton & Fletcher 1921) for its discoverer, by which appellation it is still known to French researchers (*bacille de Whitmore* or Whitmore's bacillus).[22]

In common with many other non-fermenting Gram-negative bacteria, the organism has struggled to find a taxonomic home. The 5th edition of Bergey's Manual placed it in the genus *Malleomyces*[23] due to the many characteristics it shares with the causative agent of glanders (*B. mallei* was type species for the now-defunct genus Malleomyces).[24,25] It was, for a time, called *Pfeifferella whitmori*.[26] It was suggested also that the bacterium be housed in the genus *Loefflerella*.[27] The organism was transferred to the genus *Pseudomonas* in the 6th edition of Bergey's Manual,[19] a decision supported by the best morphological, phenotypic and biochemical evidence available at the time.[28] The organism was finally transferred to the genus *Burkholderia* in 1992 (as *Burkholderia pseudomallei* comb. nov.) on evidence from its 16S ribosomal subunit sequence:[21] *B. pseudomallei* and *P. aeruginosa* are now no longer even in the same class, let alone the same genus, family or order. *B. mallei* was also moved to the same genus using the same criteria,[21] and has been shown to have evolved from *B. pseudomallei* by specialisation to an equine host and a process of genome reduction.[29] Indeed, *B. mallei* may be considered a subspecies of *B. pseudomallei* by multilocus sequence typing.[30] Although *B. mallei* can survive in tap water for up to four weeks (and horses can become infected from contaminated drinking water),[20] *B. mallei* has lost the ability to persist indefinitely in the environment.

In environmental isolates, the main differential for *B. pseudomallei* is *B. thailandensis*. One puzzle of the early 1990's was that environmental surveys of *B. pseudomallei* seemed to show a much larger endemic area than was clinically evident.[31,32] Many of those environmental isolates were later shown to be due to a separate species, *B. thailandensis*, which is distinguished from *B. pseudomallei* on the basis of its ability to assimilate arabinose and its avirulence in mice and hamsters.[32–37] In 1998, *B. thailandensis* was given status as a separate species on the basis of evidence from its 16S ribosomal subunit sequence.[38]

Phylogenetic studies combined with geographical data support an Australian origin for *B. pseudomallei* with a single introduction event into Southeast Asia hypothesised to have occurred during a recent glacial period. *B. pseudomallei* isolates may be resolved into two distinct populations divided by Wallace's Line[39] (a deep-water channel that forms a physical boundary separating Australian flora and fauna from those of Asia). Wallace's Line has separated Australia and Asia for a period in excess of 50 million years, even during glacial advances (when sea levels dropped by up to 120 metres from current levels). It runs along the Lombok Straits, separating Bali (to the west) and Lombok (to the east). Further north, Wallace's Line runs between Borneo (west) and Sulawesi (east).

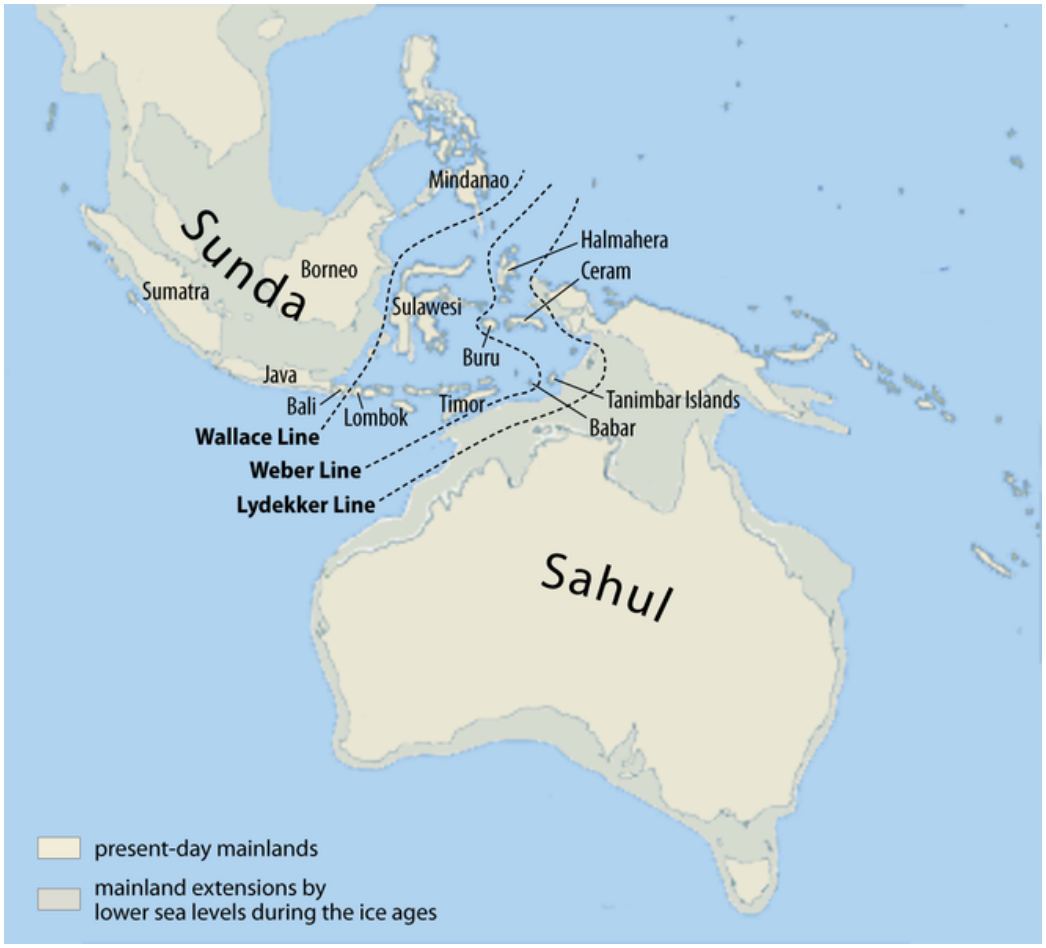

Figure 1. Wallace's Line.

Wallace's Line marks the end of the Sunda Shelf, an area of shallow ocean linking the islands of Sumatra, Java, Bali and Borneo to the continent of Asia. Lydekker's Line marks the limit of the Sahul Shelf, which links Australia and New Guinea. Weber's line marks the transitional area between the two. Image ©2007 Maximilian Dörrbecker.

1.1.2 Laboratory identification of B. pseudomallei

The clinical features of melioidosis are non-specific, and the diagnosis of melioidosis therefore depends on finding *B. pseudomallei* in a clinical specimen.[40,41] It is recommended that *B. pseudomallei* be handled in biosafety level 3 facilities[42] and this recommendation is motivated by the fact that it may be acquired by inhalation and mortality following infection is high. It has been argued that level 2 facilities offer adequate protection as long as samples are centrifuged in sealed cups and opened only in a biosafety cabinet.[43,44]

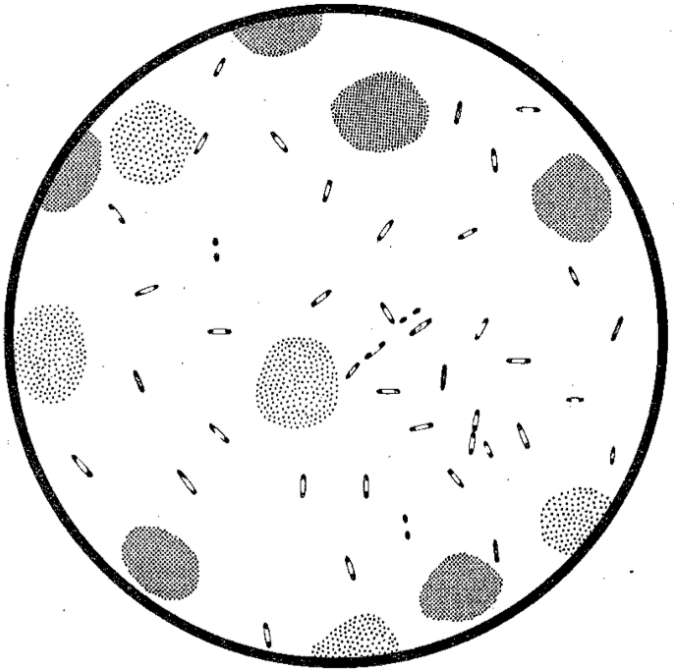

Figure 2. Microscopic appearance of *B. pseudomallei*.

Drawing of *B. pseudomallei* in a pus from an abscess in a guinea pig. The figure is taken from Stanton & Fletcher's 1932 monograph and emphasises the safety-pin appearance of the bacterium.

B. pseudomallei is a motile, non-lactose fermenting, oxidase-positive, Gram-negative bacillus.[19] Although initially believed to be a strict aerobe,[20] *B. pseudomallei* will grow anaerobically if nitrate is present as a terminal electron acceptor.[45,46] The optimum temperature for *B. pseudomallei* is 24–32°C, but it will grow at temperatures up to 42°C.[13,47] Clinical samples are usually incubated at 37°C in 5% carbon dioxide.

B. pseudomallei is not fastidious and grows readily on a wide variety of media, including blood agar, tryptic soy broth, LB, CLED and MacConkey. It will even survive for extended periods in nutrient deficient media. Stanton & Fletcher (1932) reported that *B. pseudomallei* would survive for 44 days in tap water, Miller *et al.* (1948) reported 8 weeks.[20] Wuthiekanun has found that *B. pseudomallei* can survive in distilled water for 16 years and possibly longer.[48–50]

In culture, the *B. pseudomallei* has a characteristic bipolar ('safety pin') appearance when stained for light microscopy (Figure 2), which was first noted by Whitmore,[8] and has been ascribed to the presence of large lipid vacuoles in the centre of the cell that do not stain.[20] Although characteristic,[17] this safety pin appearance is not sufficiently specific as to be diagnostic[51] and does not occur frequently in clinical specimens.[52] *B. pseudomallei* does not form spores.[8] Cultured bacteria are ~0·8 × 1·5 μm, but bacteria found in clinical samples may also form long tangled filaments (Figure 3).[41,52]

Recognition of *B. pseudomallei* is seldom a problem for the microbiologist when the specimen has been taken from an otherwise sterile site such as blood, or pus from a deep collection. The main technical problem is that the doubling time for *B. pseudomallei* is roughly two hours, which is longer than many other bacteria. In specimens taken from non-sterile sites (*e.g.,* catheter urine, sputum and throat swabs) colonies of *B. pseudomallei* are easily overgrown.[53] For this reason, selective media should be used to isolate *B. pseudomallei* from these specimens.[53]

Ashdown agar is made from a base of trypticase soy agar (TSA): it contains crystal violet to inhibit the growth of Gram-positive bacteria and gentamicin to inhibit the growth of other Gram-negatives.[53] *B. pseudomallei* colonies absorb neutral red, which acts as an indicator and helps to distinguish *B. pseudomallei* from other bacteria. The addition of glycerol causes the colonies to wrinkle (colonies are smooth on TSA) and the appearance of *B. pseudomallei* colonies on Ashdown agar has been likened to the appearance of cornflowers (Figure 3). Colonial morphology is extremely variable, with seven morphotypes described, and it is common for pure cultures of a single strain to exhibit multiple morphologies.[54]

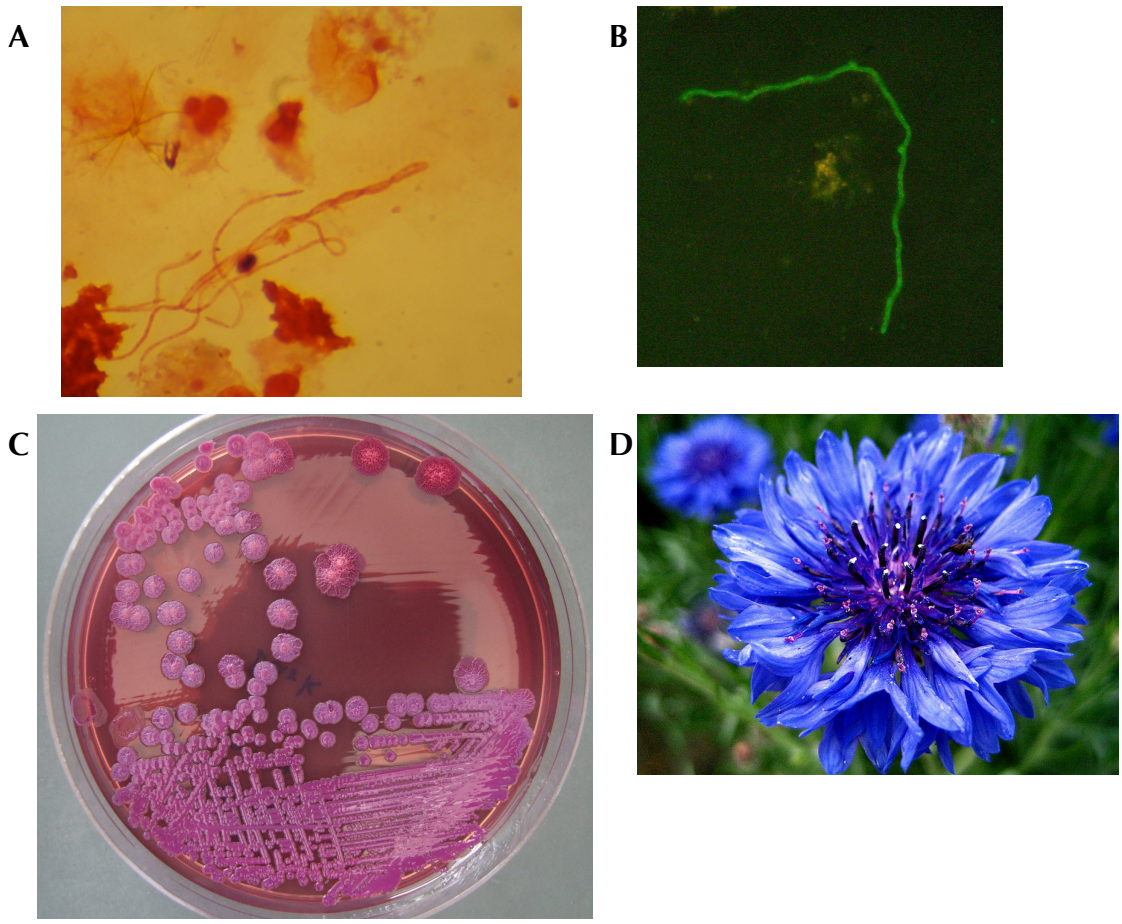

Figure 3. Appearance of *B. pseudomallei* in clinical specimens.

Note.—Both microscope specimens are from the urine of a 71-year-old woman with *B. pseudomallei* septic arthritis of the right knee. She had no urinary symptoms and she had previously had melioidosis two years previous to this presentation. Long filamentous bacteria were seen on Gram stain of urine (**A**, magnification ×1000) and immunofluorescence staining showed these to be *B. pseudomallei* (**B,** magnification ×1000). The cells visible in the background are a combination of leukocytes and epithelial cells. Culture of this sample confirmed *B. pseudomallei* (**C**, Ashdown agar). The lavender colonies take on their characteristic wrinkled appearance after four-days' incubation and are said to resemble cornflowers (**D**). Images A, B, C © 2008 Gavin Koh. Image D © 2007 J Stegman.

Ashdown agar is cheap and easily prepared using readily available ingredients. However, many laboratories in the West have moved away from preparing their own media to using commercially prepared media, and Ashdown agar is not commercially available. For these laboratories,

commercially prepared *Burkholderia cepacia* agar or *Pseudomonas cepacia* agar are suitable alternatives.[55,56]

Diagnosis may be achieved with biochemical methods. Using the API 20E system (bioMérieux, Marcy l'Etoile, France), most *B. pseudomallei* isolates have one of the following seven digit profiles: 2006727, 2206706, 2206707 and 2206727.[36] Profiles on API 20NE are 1156576, 1156577, 1556576 and 1556577.[57] Of the automated systems available commercially, VITEK 1 (bioMérieux) correctly identifies 99% of isolates.[58] Performance on VITEK 2 (bioMérieux) has been uniformly poor by contrast. In an initial assessment performed in 2002, VITEK 2 correctly identified only 19% of isolates.[58] In 2006, using a new colorimetric GN card, this had improved to only 63–81%.[59] Common misidentifications include *Burkholderia cepacia, Pseudomonas aeruginosa, Chromobacterium violaceum* and other non-lactose fermenting Gram-negative bacilli.

Variation in the API profile occurs primarily in citrate utilisation, and in sucrose and amygdalin assimilation. The antibiotic susceptibility pattern for *B. pseudomallei* is also helpful in allowing it to be differentiated from *P. aeruginosa,* because *B. pseudomallei* is invariably resistant to gentamicin and colistin, but is usually susceptible to co-amoxiclav,[36,40,60] a resistance pattern that does not occur in *P. aeruginosa*.

The ability of patient sera to agglutinate suspensions of *B. pseudomallei* was first reported in 1932, first by the English physicians, Stanton and Fletcher, and by the Dutch physicians, de Moor and van der Walle. Both these groups evaluated this technique as a means of diagnosing melioidosis serologically. It was Miller in 1947,[20] who first had the insight that the ability of serum from infected rabbits to agglutinate unknown bacteria obtained from cultures might be used as a means to identify *B. pseudomallei*. Unfortunately, Miller's slide agglutination test never entered routine clinical use.

The sensitivity of slide agglutination test may be increased by conjugating the antibody to latex beads, so that even bacteria from a single colony may form a visible precipitate. A non-commercial latex agglutination test based on a monoclonal antibody to the exopolysaccharide of *B. pseudomallei* has been clinically evaluated and has a sensitivity of 99·5%. The test has been available in Thailand since 2002 (Chulaborn Research Institute, Bangkok, Thailand).[57,61] As

with previous tests based on serological methods (including Miller's test[20]), the test cannot distinguish *B. mallei* from *B. pseudomallei*.

Immunofluorescence microscopy is useful in samples with a high bacterial load[62] (≥10^4 cfu/ml; *e.g.,* sputum, urine or pus from a normally sterile site) and is capable of giving same-day results with >99% specificity.[63,64] Bacterial loads in blood are too low for immunofluorescence microscopy,[65,62] which means that immunofluorescence is not useful in patients who are bacteraemic but with no identifiable focus. The sensitivity is reported to be 66–73% in an idealised research setting,[63,64] but is probably lower in routine clinical practice.

Molecular techniques have been attempted, but are not routinely available in clinical practice. Targets for polymerase chain reaction (PCR) have included the 16S ribosomal subunit,[66,67] the *gltB* and *narK* genes[68] (which are also part of the MLST scheme); *orf11*, *orf13* and *BpSCU2* (components of the type 3 secretion system),[69] *fliC* (which codes for flagellin),[67] and repetitive elements of non-protein-coding DNA.[70] Few of these have been validated in the clinical setting, and the need for equipment capable of PCR make the majority of these methods impractical in the field. On top of this, mere amplification of genes may not be sufficient and additional tests such as gene sequencing may be required.[71]

Loop-mediated isothermal amplification (LAMP) appears to have a number of characteristics that make it preferable to PCR for nucleic acid amplification in resource-poor settings. The method employs a DNA polymerase and a set of four specially designed primers that recognise a total of six distinct sequences on the target DNA. The reaction is simple to set up and requires much less training than PCR, and the initial capital outlay is minimal (the only equipment required is a water bath or heat block capable of maintaining a constant temperature of 60–65°C). A LAMP assay was developed targeting the BPSS 1406 hypothetical protein, situated within the TTS1 gene cluster of *B. pseudomallei*.[72] The lower limit of detection was 38 genomic copies per reaction, which is still too high to reliably detect *B. pseudomallei* in blood.[62,65] Performance was better in other sample types including sputum, urine and pus (sensitivity 45·8–75%, specificity ≥97·9%). No direct comparison was made against immunofluorescence, but these results seem comparable to that

technique,[63,64] which means LAMP is unlikely to supplant immunofluorescence as a method for rapid antigen detection.

Techniques for rapid diagnosis are urgently needed, because culture-based methods entail a minimum turnaround time of 48 hours or more. Immunofluorescence microscopy currently fulfils that function, but is insufficiently sensitive.

B. thailandensis has a biochemical phenotype very similar to *B. pseudomallei,* but *B. thailandensis* almost never causes disease in humans. There are only two reported cases of human *B. thailandensis* infection in the literature[73,74] and the need for a clinical laboratory to distinguish between the two species therefore almost never arises. If necessary, *B. thailandensis* can be differentiated from *B. pseudomallei* by its ability to assimilate arabinose, which *B. pseudomallei* cannot do.[34,38] A definitive distinction between the two species may be made using MLST.[30]

B. mallei (the causative agent of glanders) is morphologically and biochemically indistinguishable from *B. pseudomallei*, except that the bacterium is immotile. Like *B. pseudomallei,* automated laboratory methods may misidentify it as other non-lactose fermenting Gram-negative bacilli.[75] For a clinical laboratory on the 'front lines', the first indication that the isolate is *B. mallei* and not *B. pseudomallei* is the susceptibility profile: ~80% of *B. mallei* isolates are susceptible to gentamicin and may also be susceptible to some macrolides.[76,77] Some isolates may also be susceptible to ampicillin, which *B. pseudomallei* never is.[76] A definitive diagnosis may require 16S RNA sequencing or MLST, but this issue is unlikely to arise, except in a biothreat setting, since glanders has been largely eradicated and the last naturally occurring infection reported in the English-language literature was in 1949.[75] Outbreaks continue to occur in susceptible equids, the most recent of which was an outbreak in dromedaries in Bahrain.[78]

1.1.3 A brief survey of virulence factors

B. pseudomallei is a facultative intracellular pathogen[79–81] and much of the literature on host-pathogen interactions centres on, or needs to be interpreted in light of, this fact.

Virulence factor	Function	References
BimA	Actin polymerization, intracytoplasmic movement and cell-to-cell spread	Kespichayawattana 2000,[82] Breitbach 2003,[83] Stevens 2005,[84] French 2011[81]
BPSS1823	Intracellular survival, swarming motility, resistance to low pH, protease production	Norville 2011[85]
Capsular polysaccharide	Resistance to serum lysis; complement resistance	Reckseidler 2001,[86] Atkins 2002,[87] Reckseidler-Zenteno 2005,[88] Warawa 2009,[89] Wikraiphat 2009,[90] Reckseidler-Zenteno 2010[91]
Flagellin	Motility; cell invasion	Brett 1994,[92] DeShazer 1997,[93] Chua 2003,[94] Inglis 2003,[95] Chuaygud 2008[96]
Lipopolysaccharide	Complement resistance; endotoxin	Matsuura 1996,[97]Luc[1 DeShazer 1998,[98] Wikraiphat 2009,[90] Novem 2009[99]
Quorum sensing	Downstream virulence factors as yet unidentified	Ulrich 2004,[100] Juhas 2004,[101] Valade 2004,[102] Song 2005,[103] Diggle 2006,[104] Lumjiaktase 2006[105]
Superoxide dismutase	Resistance to killing by superoxide	Vanaporn 2011[106]
Type IV pilin	Adhesion to respiratory epithelium	Essex-Lopresti 2005[107]
Type III secretion systems	Injecting effector proteins into the host cell; escape from endocytic vacuoles	Stevens 2002,[108] Stevens 2004,[109] Warawa 2005,[110] Pilatz 2006,[111] Burtnick 2008,[112] Sun 2010,[113] French 2011[81]
Type VI secretion systems	Injecting effector proteins into the host cell	Schell 2007,[114] Shalom 2007,[115] Burtnick 2011,[116] French 2011[81]

Table 1. Summary of *B. pseudomallei* virulence factors.

In 1990, Pruksachartvuthi *et al.* showed by electron microscopy that *B. pseudomallei* was able to persist within both neutrophils and monocytes collected from healthy human volunteers.[79] That the intracellular bacteria were viable and capable of replication was confirmed by culture.[79] *B. pseudomallei* is also capable of parasitizing a number of phagocytic and non-phagocytic cell lines, including A549 (human adenocarcinomic basal epithelial lung cells),[80,85] CHO (*Cricetulus griseus* ovary cells), HeLa (human cervical epithelial cells),[80] J774.1A1 (*Mus musculus* BALB/c peritoneal macrophage),[117] Vero

(*Cercopithecus aethiops* kidney epithelial cells),[80] and U937 cells (human monocyte line).[80] It will also invade rat alveolar macrophages[80] and *Acanthamoeba* species.[118]

After entry into the cell, *B. pseudomallei* is able to evade intracellular killing,[85,119] lyse the endosome membrane and enter the cytoplasm.[81] In the cytoplasm, it is able to mobilise by polymerising actin at one pole of the bacterial cell to form membrane protrusions, which allow it to invade adjacent cells.[82–84] Virulence factors of *B. pseudomallei* are summarised in Table 1.

1.2 Epidemiology

1.2.1 Geographical distribution of melioidosis

Descriptions of the epidemiology of melioidosis are hampered by the lack of clinical awareness and laboratory facilities in areas where it is likely to be endemic.[120,121] Without the ready availability of microbiological diagnosis, melioidosis may go completely unrecognised even in areas where it is highly endemic.[120] The geographical distribution of *B. pseudomallei* should therefore be regarded as poorly defined.

The first evidence for melioidosis in Thailand was from a 1930 case report of a Russian man, normally resident in Bangkok, who presented in a delirious state to police in Phnom Penh, Kampuchea (now Cambodia). Diagnosis was made *post mortem*.[122] The first case of melioidosis reported in Thailand itself was in 1947, in a Dutch soldier who had been held a prisoner-of-war by the Japanese.[123] The first reported case in a native Thai had to wait until 1955.[124] It was not until the expansion of microbiological services that occurred in the 1970's and 80's that it became clear melioidosis was highly endemic in Thailand and probably always had been.[125,126]

The opposite has occurred in Burma. In 1917, Krishnaswami reported over a hundred cases of melioidosis in Rangoon just five years after first describing the disease.[127] Yet, no case of indigenous melioidosis was reported in Burma between 1945[40] and 2000[128] despite the fact that Burma continued to export cases of melioidosis throughout that period[129,130] and despite a high seroprevalence in the native population.[131]

The global distribution of melioidosis has been described in detail by Currie, Dance and Cheng,[132,133] and their map is reproduced in Figure 4.

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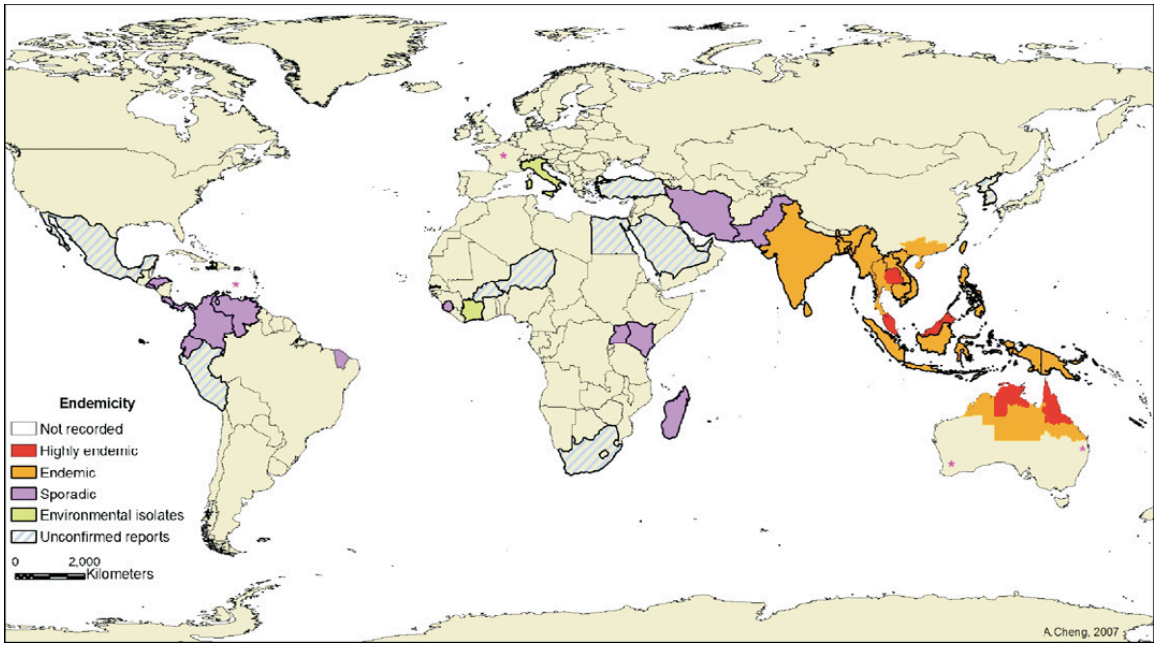


Figure 4. Map of the global distribution of melioidosis.

Note.—Pink asterisks show the location of three outbreaks that occurred in temperate regions: one in France, and two in Australia. Figure taken from Cheng and Currie *Clin Microbiol Rev* 2005;18:383–416.

Melioidosis is a disease of the tropics and appears to be confined to a band roughly 20° north of the equator and 20° south. The endemic area is focused around southeast Asia and northern Australia; its northernmost extent is southern China, Hong Kong and Taiwan; its southernmost extent is northern Australia. Melioidosis is almost certainly endemic to India[134–136] and probably always has been. There are sporadic cases from Africa and Central America,[132,133,137,138] but the lack of microbiological facilities in much of these areas means the true extent of melioidosis is unknown.

1.2.2 Melioidosis as a zoonosis

A zoonosis is a disease where infection occurs by transmission from a vertebrate animal to human (World Health Organization, Geneva, Switzerland). In 1925, Stanton and Fletcher described melioidosis as a zoonosis based on

observations that rodents succumb to the disease.[10] However, in that same paper, Stanton and Fletcher also noted that melioidosis is fatal to rodents, which makes it very unlikely that rodents could be an efficient reservoir or vector. In their 1932 monograph, they revised this assertion, stating, 'The way in which man contracts melioidosis is a problem as yet unsolved,' but continued to assert that they 'believe that infection usually occurs…through the consumption of food which has been contaminated by the excreta of infected rodents.'

In 1948, Harries *et al.* noted that they were unable to find melioidosis in any of >500 rats examined in Rangoon,[129] and in 1969 Strauss *et al.* performed a similar survey of 422 wild rats (*Rattus tionamicus jalorensis, R. argentiventor, R. exulans* and *R. rattus*) in a survey of Carey Island, Selangor, Malaysia,[140] and expressed doubt that rats are a reservoir for the disease. In the 1955, Chambon *et al.* showed that *B. pseudomallei* is primarily environmental,[141] and transmission from animals to humans has not been shown to be an important cause of infection.[132]

The disease is dealt with by the Bacterial Zoonoses Branch of the US Centers for Disease Control and melioidosis continues to be listed with the zoonoses by textbooks.[142] Some books published as recently as 2002 continue to assert erroneously that animals are a reservoir for disease.[143]

Melioidosis infects a large numbers of animals and birds.[144] Sheep and goats are particularly susceptible, resulting in the absolute requirement for pasteurisation of tropical commercial goat's milk.[144] Gorillas are also extremely susceptible, so much so that extraordinary measures are needed to isolate them from the environment in a melioidosis-endemic area.[145] Four male animals imported to the Singapore Zoological Gardens died within months of arriving from Rotterdam, and the one surviving animal was returned to Rotterdam for treatment. On outbreak at the Paris zoo in the 1970's («L'affaire du jardin des plantes») was attributed to an imported panda.[146] Horses were initially reported to be immune, as demonstrated by attempts at experimental inoculation,[10] but cases of naturally occurring infection have subsequently been reported.[146–149] There appears to be no basis for the oft-repeated[150] claim that water buffalos are immune.

Rats, mice and hamsters are all susceptible and are important small animal models of melioidosis.[151–153] Of the three, Syrian golden hamsters are the

most susceptible.[154,155] In fact, such is the susceptibility of hamsters to *B. pseudomallei,* that Miller *et al.* developed a method of purifying *B. pseudomallei* from mixed cultures by infecting hamsters with the contaminated sample and recovering the pure organism from the hearts blood of the animals after they succumbed to the disease.[20]

Strauss' environmental surveys of the 1960's used hundreds of hamsters to purify samples. Filtered samples of soil or surface water were rendered isotonic with saline then injected intraperitoneally into hamsters to yield pure cultures of *B. pseudomallei* when they died five days later of melioidosis.[140] Hamsters are not susceptible to *B. thailandensis*,[37] and it is interesting to speculate whether *B. thailandensis* would have been discovered had hamsters continued to be used in environmental surveys.

1.2.3 Melioidosis is an environmental organism

In 1955, Chambon demonstrated that *B. pseudomallei* could be isolated from soil and water in South Vietnam (including rice fields), that the isolated organism was virulent, and that it produced the characteristic histological features of melioidosis in guinea pigs, rabbits and mice.[141] These findings were confirmed and extended by a series of detailed epidemiological studies published in 1969 by Strauss.[140,156,157] Strauss was able to culture *B. pseudomallei* from 14·6% of the samples taken from rice paddy fields, whereas samples taken from land used for all other purposes had isolation rates <10%.[156] In two seroprevalence surveys of army recruits from states where the principal crop was rice, the proportion with haemagglutinating antibody titres ≥1:40 was 22·9%. By contrast, on Pulau Carey, where agricultural activity was confined to rubber, oil palm, coconut palm, and tea cultivation, only 3·4% of army recruits had haemagglutinating antibody.[157,140] Strauss also surveyed 422 wild rats and found no evidence of carriage.[140] Based on this evidence, Strauss concluded that *B. pseudomallei* is a normal inhabitant of the soil and of water (particularly rice paddy fields), and that there was no evidence that rodents or animals of any kind were required for maintenance of the organism. Subsequent surveys have confirmed the association of *B. pseudomallei* with soil in Thailand[33,158] and ground water in Australia.[159] Environmental concentrations correlate with the incidence of clinical melioidosis.[158,160,]Table 2

The distribution of *B. pseudomallei* in soil is extremely irregular, and a site with no detectable bacteria may lie as little as five metres from a site with >10,000 cfu/g soil.[161] The reasons for this are unknown, but competition with other *Burkholderia* species has been suggested as a reason.[162] Kaestli found that in sites undisturbed by human activity, *B. pseudomallei* was more commonly found in areas rich in grasses. In environmentally disturbed sites, *B. pseudomallei* was associated with the presence of livestock animals and lower soil pH.[163] It has been suggested that water-logged, clay soils may be better at harbouring the bacterium than sandy, well-drained soils.[47,164] In common with other Burkholderia species,[165] *B. pseudomallei* is able to survive and replicate intracellularly within *Acanthamoeba* species *in vitro*.[118,166]

Region of Thailand	No. of positive sites	Infection rate per 100,000 inpatients
Central	24·5%	13·4
North	13·8%	18
Northeast	50·1%	137·9
South	18·4%	14·4

Table 2. Isolation rate of *B. pseudomallei* from soil compared to incidence of melioidosis by region of Thailand.

Adapted from Vuddhakul *et al. Am J Trop Med Hyg* 1999;60:458–61.

1.2.4 Melioidosis and rainfall

The association between melioidosis and rainfall was first noted by Strauss in 1969, who found that he was unable to isolate *B. pseudomallei* from his environmental samples when the weather was dry, but that samples became positive after each rainfall.[140] Studies of human melioidosis cases in Australia, Thailand and Singapore show a clear association with heavy rainfall in the 7 days preceding onset of the illness or the 14 days preceding admission to hospital, and there is a positive correlation year-by-year between the amount of rain and the number of cases of melioidosis.[167–171] There is certainly a correlation with extreme weather events such as hurricanes.[172,173]

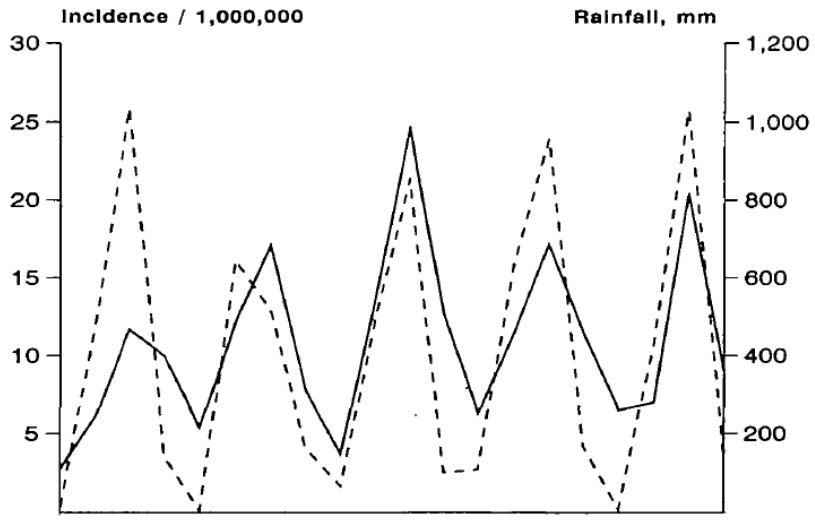


Figure 5. The relationship between rainfall and melioidosis cases in Ubon Ratchathani, Thailand.

Note.—This graph shows the quarterly incidence of melioidosis (solid line) over five years (1987 to 1991), with the rainfall (interrupted line) in each quarter superimposed. Figure from Suputtamongkol *et al. Int J Epidemiol* 1994;23:1082–90.

1.2.5 Human factors

B. pseudomallei is an organism of soil and surface water and a history of contact with soil or surface water is therefore common, and a history of residence in or travel to an endemic area is almost invariable.[169,174–177] In Northeast Thailand, rice farming is an important part of the local economy. Rice fields are flooded for much of the growing season and farmers spend much time bare foot in paddy fields. Rice farming is therefore an important risk factor for melioidosis.[169]

Patients with melioidosis are more likely to be male. The ratio of males to females is 1·41:1 in Thailand,[170] 2·21:1 in Australia,[176] and 2·8–7·2:1 in Singapore.[168] The statistics in Singapore are skewed by the fact that only males are called up for military service, which involves high levels of exposure to contaminated soil, and adjusting for this fact reduces the sex ratio to 1·3:1.[174] Harries *et al.* reported five cases of melioidosis in West African troops (numbering approximately 30,000 in total) stationed in Burma,[129] and

marvelled that no cases were reported among Indian and Burmese troops stationed at the same place. In Australia, aboriginals are overrepresented (24% of the population, but 50% of melioidosis cases).[177] In Singapore, Indians and Malays are at greater risk of melioidosis than the Chinese,[174,178] possibly related to higher rates of diabetes in those two races.[179]

Figure 6. Farming barefoot in northeast Thailand.

Note.—Farmers in Northeast Thailand walk through flooded paddy fields barefoot for practical reasons. Images courtesy of Andrew Taylor © 2008 (left) and Vanaporn Wuthiekanun © 2006 (right).

Diabetes mellitus is the most frequently identified risk factor in patients with melioidosis: 39% of patients in Australia,[176] 60% of patients in Thailand[175] and 76% in India have diabetes.[135] In Northeast Thailand, patients with diabetes have 21·4-times greater risk of having melioidosis compared to people without diabetes.[170] Heavy alcohol use is a risk factor in 12–39%, renal disease (chronic renal failure or kidney stones) in 12–20·1%. Glucocorticoids are present in many herbal preparations (called variously *ya tom, yam mor* or *ya chut*) which patients use to self-medicate, and use of herbal medicines is an important cause of immunosuppression in northeast Thailand. Other important risk factors include thalassaemia and malignancy.[135,170,175,176] Patients with cystic fibrosis may be particularly susceptibility; fortuitously, the endemic ranges for the two diseases do not overlap, except in northern Australia.[52,137] HIV does not appear to be a risk factor.[180]

1.2.6 Routes of acquisition

The route of entry into the body is unknown. In Thailand, rice farmers wade knee-deep in the muddy water of rice paddy fields, and rice farming is an important risk factor for melioidosis.[175] By contrast, melioidosis is not a large problem in rice-farming areas of Malaysia, where the process of farming has been largely mechanised, and the direct exposure of farmers to soil and surface water is limited. It has been suggested that the organism may gain entry via scratches and small skin-breaks in the feet and legs, and there is one reported case of inoculation following a snake bite.[40,125] In this hypothetical scenario, pneumonia is explained by haematogenous spread. Against this hypothesis is the fact that the majority of cases in Singapore (whose population is entirely urban) have no history of contact with soil or surface water, despite a clear association with rainfall.[168]

The evidence supporting an inhalational route for melioidosis is largely circumstantial. There is a clear relationship between rainfall and melioidosis pneumonia,[167,169,170] and during the Vietnam War, an epidemiological link was found between the helicopter crewmen and melioidosis infection,[13] of whom many developed melioidosis pneumonia. This led to the conjecture that the infection may have been due to inhalation of dust generated by the helicopter rotors and that inhalation of aerosolized bacteria may be an important mode of infection.[17,181] Data from animal models also appear to support this: the size of inoculum needed to produce an equivalent effect in mice is four orders of magnitude smaller than that needed to infect the same inbred strains subcutaneously.[182] There is one case of laboratory-acquired melioidosis reported in the literature due to inhalation of aerosolized bacteria,[183] so human infection by this route is possible and viable bacteria have been found to be aerosolized by severe weather events such as typhoons.[184]

A third possible route of infection is via ingestion. Melioidosis has been transmitted from mother to child via infected breast milk[185] and it is possible to infect mice by oral gavage, but the doses required are of the order of 10^8 cfu,[186] which is an order of magnitude higher than that required for subcutaneous infection, and at least six orders of magnitude higher than that required for inhalation. Much of the population in Northeast Thailand draw their water from wells or other untreated water-sources that may become contaminated with soil

during the rainy season. Two outbreaks in Northern Australia were associated with contamination of drinking water,[187–189] but this does not necessarily mean that the main mode of transmission in those outbreaks was ingestion, as potable water was also used for washing and bathing.

The tsunami of Boxing Day, 2004, was associated with a spike in the incidence of melioidosis in affected areas.[190–196] Cases of human-to-human transmission have been described, but are exceptionally rare.[52,185,197,198] Vertical transmission has been described but is extremely rare.[199]

1.3 Clinical features of melioidosis

A definitive diagnosis of melioidosis may only be made on the basis of microbiology (please refer to section 0 below),[40,41] and the main limitation to diagnosis is the availability of adequate laboratory facilities.

Melioidosis has two main presentations: acute and chronic. Paediatric disease is discussed in a separate section below.

1.3.1 Acute melioidosis

Acute melioidosis has no specific clinical features and should be suspected in any septic patient with a history of residence in or travel to an endemic area. The presence of a recognised risk factor (particularly diabetes) is helpful, but the absence of risk factors does not exclude melioidosis. Where an inoculating event has been noted, the mean incubation period is 9 days.[177]

In Northeast Thailand, the median duration of symptoms prior to presentation is 10 days.[200] Acute melioidosis is often disseminated, with multiple sites being involved: 59–62% have bacteraemia and 49–56% have pneumonia[200,201] (appearances on the chest radiograph range from lobar pneumonia to widespread cotton wool patches); 28–52% have abscesses of the spleen and/or liver[200–202] and 23–27% have skin and/or soft tissue infections (principally wound infections and skin abscesses; cellulitis and erysipelas are rare).[200,201] Other infections include those of the urinary tract (ranging from pyelonephritis to prostatitis to asymptomatic bacteriuria), osteomyelitis,[203] septic arthritis,[203] myositis,[203] keratitis,[204] endophthalmitis,[205,206] orchitis,[207,208] and mycotic aneurysms.[209] Mortality is 42·6%.[170]

Melioidosis in Australia presents in a similar manner to Thailand, with some notable exceptions. Mortality is much lower (14%), which may be related

to differences in supportive care.[176] In Australia, 20% of male patients have prostatic abscesses,[176] whereas this presentation is rare in Thailand. It is not certain whether this is a true difference or whether it represents differences in the availability of imaging (computed tomography of the pelvis is not routinely performed in northeast Thailand).

There exists in Australia a syndrome of encephalomyelitis (2·6% of cases), which manifests as a constellation of cerebellar signs, brainstem features and cranial nerve palsies.[176] Few abnormalities are visible on CT, but dramatic changes are visible on MRI.[210] By contrast, neurological melioidosis in Southeast Asia (1·6% of cases in Northeast Thailand[211]) presents with cerebral abscesses (which may be multiple). These usually present with seizures and/or focal neurological deficits and are clearly evident on CT.[212,211,213] In Singapore, four of five cases of cerebral melioidosis were linked to sinusitis.[213] Primary meningitis is extremely rare and there appears to be only one case reported in the literature,[214] although meningitis has been reported to occur secondary to rupture of a cerebral abscess.[40]

1.3.2 Chronic melioidosis

Chronic melioidosis (defined as disease with onset ≥2 months prior to presentation[215]) comprises 12% of all adult melioidosis cases.[215] It usually presents as localised disease, the majority of which are chronic non-healing localised skin or soft tissue lesions, osteomyelitis, or pulmonary abscesses. It often resembles tuberculosis clinically and is well described in the literature.[26,216–220] Survival is the norm: of 30 cases of chronic melioidosis reported in Northern Australia, all survived.[221]

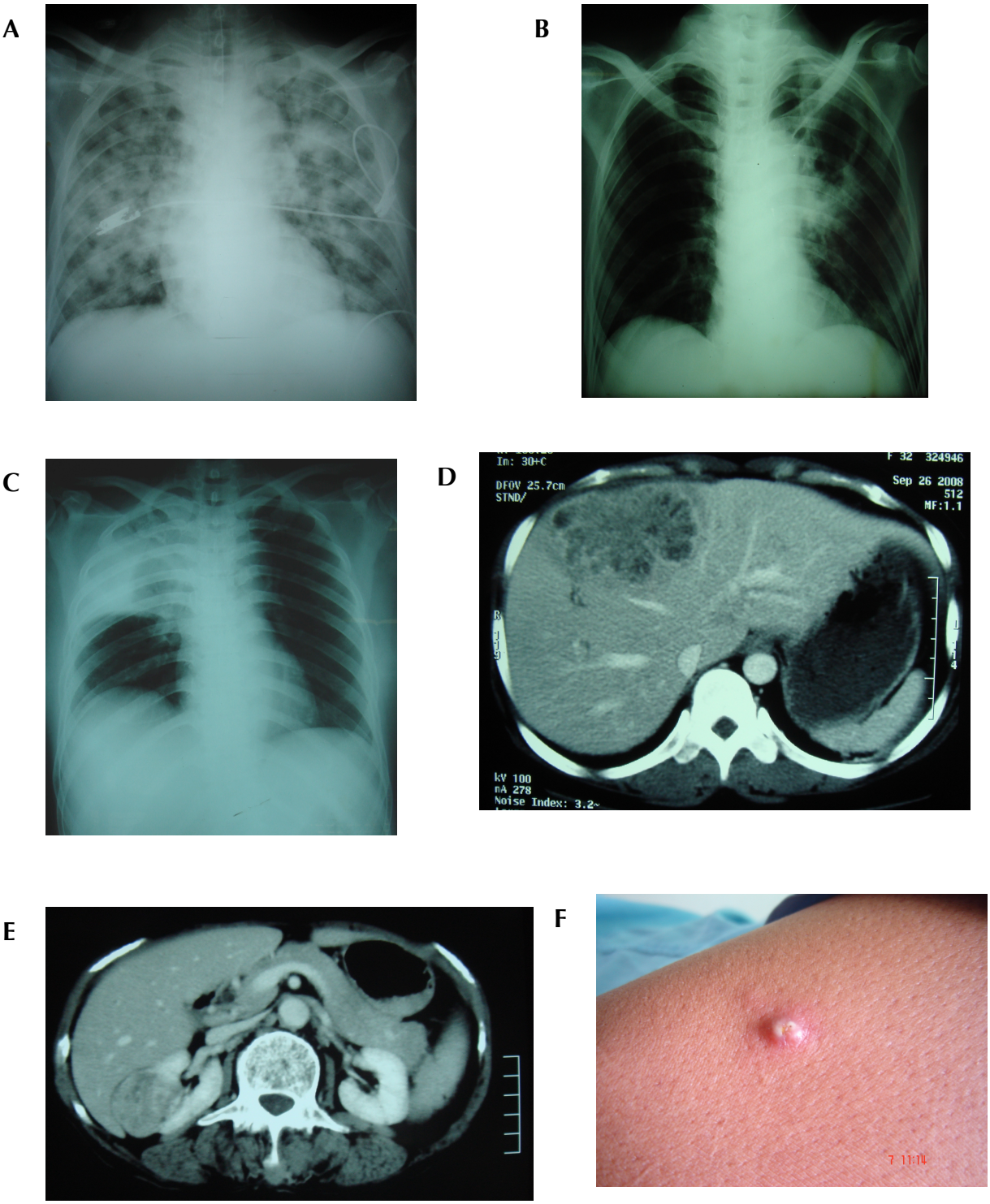


Figure 7. Clinical manifestations of melioidosis.

Note.—Pulmonary melioidosis has a wide range of presentations: **A.** Multiple bilateral cotton wool patches. **B.** Left hilar lung abscess. **C.** Lobar pneumonia of the right upper lobe. **D.** Large liver abscess in a 32-year-old female. **E.** Right renal abscess in a 63-year-old female. **F.** Cutaneous abscess on the right arm of a 47-year-old male.

The majority of early cases reported were acute and fulminant, but it was recognised early on that in a small number of patients, the presentation could have an indolent onset and chronic course. The first two cases of chronic melioidosis were reported by Stanton & Fletcher in 1932, both with osteomyelitis. Both patients survived to discharge, although one patient continued to have discharging sinuses in his feet two years later. Grant and Barwell describe a case of melioidosis in a 27-year-old soldier who present initially in 1938 with septic arthritis of the right hip and ankle (initially diagnosed as gonorrhoea), who went on to develop chronic pneumonia and osteomyelitis of the frontal bone over the next five years. Mayer and Finlayson describe the case of a 33-year-old soldier who developed bilateral lumbosacral abscesses while stationed in Singapore in 1940. A year later, he developed a chronic right lower lobe pneumonia and the lumbosacral abscesses were recognised to have arisen from osteomyelitis of the lower thoracic spine. He was initially diagnosed with tuberculosis, but the diagnosis was revised two years after presentation in the light of *B. pseudomallei* being cultured from the pus. The patient had been unwell for 4 years at the time the report was published.[216]

1.3.3 Paediatric melioidosis

In Thailand, there are two age peaks: one in early childhood (≤10 years, mean age 6·8 years) and one in middle age (≥45 years).[169,170,222] Paediatric melioidosis in Thailand is largely a benign condition. Two-thirds of cases present with localised disease[222] (38–40% of paediatric cases present with a unilateral parotid abscess) and have a good prognosis.[223,224] A third present with pneumonia or bacteraemia and have a presentation and prognosis similar to adult disease.[222] Preliminary reports from Cambodia suggest that the spectrum of paediatric disease there appears similar to that found in Thailand.[225]

Figure 8. A 2-year-old child with unilateral parotitis at Sappasithiprasong Hospital in Ubon Ratchathani, Thailand.

This 2-year-old male child developed swelling of his left cheek one week after playing in a freshwater lake. The abscess was drained surgically and the child was discharged on oral co-amoxiclav. Image © 2008 Gavin Koh. Signed consent obtained from parents.

In contrast to Thailand, Australia and Malaysia report no paediatric peak, and when melioidosis does occur in children, the presentation and prognosis is similar to when it occurs in adults.[215,226–228,227] Paediatric melioidosis in Malaysia is associated with immunocompromise (particularly haematological malignancy),[226] while paediatric melioidosis in Australia is associated with cystic fibrosis.[227,229] Cultural differences may play a role: children in Northeast Thailand are accustomed to playing in flooded paddy fields (splashing around and catching the fish and crabs that grow there: both an important part of the diet of Northeast Thailand). Their exposure to contaminated water is therefore much greater than in elsewhere, and the syndrome of unilateral parotitis in paediatric patients appears confined to Thailand.[215,226]

1.3.4 Incubation period and latent melioidosis

The only systematic attempt to measure the incubation period in melioidosis was made by Currie *et al.* in 2000: of 52 patients who reported an

inoculating event, the mean time to onset of symptoms was 9 days (range 1 to 21 days).[177]

However, the incubation period for melioidosis may be much longer. There were fewer than 300 cases of melioidosis among American soldiers during the Vietnam war, but additional cases continued to present many years after the war's end,[230–232] earning it the name, 'Vietnamese time bomb'.[233] Incubation periods of 18 years[234] and 28 years[235] have been reported in a Vietnam veteran and a World War II veteran respectively. The longest reported incubation period is 62 years, in an American man who had been taken prisoner by the Japanese in 1942, who had worked building railroads in Singapore, Malaysia, Burma and Thailand. After the war, he returned to Texas and did not subsequently travel to a melioidosis endemic area. He presented in 2004 with a non-healing ulcer on his right hand following a dog bite and wound culture grew *B. pseudomallei*.

Latent melioidosis describes persistent clinically silent infection that may activate many years after initial exposure or infection, and is named by analogy to latent tuberculosis. We do not know how to diagnose latent melioidosis, except retrospectively after reactivation. There is no information as to whether serology for *B. pseudomallei* can identify latent melioidosis, or even whether negative serology for *B. pseudomallei* excludes latent melioidosis.

Latent melioidosis seems to be uncommon and Currie *et al.* estimate that latent disease accounts for no more than 4% of all cases.[177] There has not been an explosion of melioidosis cases in America despite the fact that an estimated 225,000 soldiers returning from Vietnam were seropositive.[236] The overwhelming majority of melioidosis cases are reported in endemic areas, are of seasonal onset, and occur during the rainy season, all of which suggest that recent acquisition and short incubation periods are the norm.[169,177] The available evidence supports the conclusion that unlike latent tuberculosis, the majority of patients with evidence of exposure to *B. pseudomallei* do not go on to develop clinical melioidosis, even if they do have silent infection. The only appropriate recommendation therefore is clinical vigilance.

Asymptomatic carriage of *B. pseudomallei* is rare. Currie *et al.* describe a single case who was persistently sputum culture-positive for 12 months after stopping antimicrobial chemotherapy.[221] A single case of asymptomatic throat

carriage in 58-year-old Vietnamese female rice farmer was reported by Phung *et al.*,[221,237,238] but Wuthiekanun found no instances of throat carriage on screening 3,524 Thai subjects.[239] Patients with cystic fibrosis may harbour *B. pseudomallei* in their sputum even after appropriate treatment.[240,241]

For all practical purposes, asymptomatic carriage of *B. pseudomallei* is very rare. *B. pseudomallei* cannot be considered part of the normal human flora, and the presence of even a single colony should be considered diagnostic of melioidosis.

1.3.5 Seroprevalence

Melioidosis seroprevalence is defined as the prevalence in the general population of people with a serum titre for anti-*B. pseudomallei* antibodies within the diagnostic range for melioidosis.

As early as 1932, Dutch and English workers both dismissed serodiagnosis as useless in the diagnosis of melioidosis, because titres in healthy controls were so high.[11,242] Post World War II, Brygoo working in Vietnam (1953) and Nigg working in Thailand (1963), both rediscovered the fact that healthy persons may carry specific antibodies to *B. pseudomallei* in high titre. While evaluating serology as a method for diagnosing melioidosis, Brygoo noted that 9·4% (24 of 255) Vietnamese controls had titres ≥1:40.[243] Nigg (apparently unaware of Brygoo's work) made her observations as part of a trial of a killed *B. pseudomallei* vaccine conducted by the US military in Thailand. She found that a proportion of the healthy Thai volunteers for her study had elevated titres of antibody (as high as 1:5,120) even prior to immunisation.

B. pseudomallei is ubiquitously present in the soil in endemic areas, but seroprevalence varies greatly and survey results are hard to compare because of differences in study population (hospitalised patients versus community-based studies) and in the cut-off titre selected (Table 3). The highest seroprevalence recorded is in Northeast Thailand (Figure 9),[244] but seroprevalence throughout Southeast Asia is high (Table 3). Positive serology is therefore extremely common in otherwise healthy people in endemic areas such as Northeast Thailand and this severely limits the utility of serological tests during the investigation of suspected melioidosis.

Seroprevalence (cut-off)	Study population	Location	Reference
7·3% (1:40)	1,592 adults	Malaysia	Strauss 1969[157]
47·1% (1:40)	227 blood donors	Thailand	Khupulsup 1986[244]
0·73% (1:16)	683 soldiers	Singapore	Yap 1991[245]
83% (1:10), 22% (1:80)	295 children	Thailand	Charoenwong 1992[246]
21% (1:40)	406 adults	Vietnam	Phung 1993[237]
26·5% (1:40)	200 blood donors	Malaysia	Norazah 1996[247]
8·7% (1:40)	160 adults	Australia	O'Brien 2004[248]
38% (1:40), 7% (1:160)	968 adults	Burma	Wuthiekanun 2006[131]
8·2% (1:40)	747 children	Papua New Guinea	Warner 2007[249]
2·5% (1:40)	1,500 blood donors	Australia	Lazzaroni 2008[250]
16·4% (any reaction), 6·5% (1:160)	968 children	Cambodia	Wuthiekanun 2008[251]

Table 3. Summary of seroprevalence studies conducted using IHA.

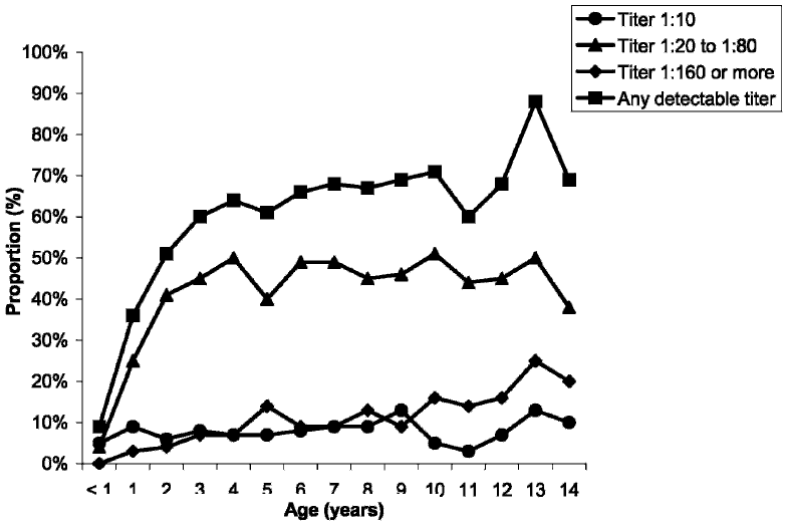


Figure 9. Seroprevalence rises with age in Northeast Thailand.

The graph shows the distribution of IHA titres by age in 2,214 children from Northeast Thailand. The proportion of children with any detectable titre rises rapidly in the first four years of life then plateaus at 60–71%. By contrast, there is a gradual rise in the proportion of children with a titre of ≥1:160, and by age 13, a fifth of children have an antibody titre over 1:160. Figure taken from Wuthiekanun *Am J Trop Med Hyg* 2006;74:1074–5.

There is conflicting evidence whether the high seroprevalence seen in endemic areas is due only to exposure to *B. pseudomallei,* or whether there is cross-reactivity with *B. thailandensis*. Tiyawisutsri *et al.* examined 117 Thai adult patients with culture-proven melioidosis and found that their sera did not cross-react with *B. thailandensis*.[252] Gilmore *et al.* performed a similar study of 25 Australian patients with culture-proven melioidosis and found that their sera did cross-react with *B. thailandensis*.[253] The methods used were similar except that different strains were used in each study, but it is not clear whether this is sufficient to explain the difference in results obtained.

1.4 Diagnosis

1.4.1 Microbiology

'*...there are, indeed, no cardinal symptoms upon which a trustworthy diagnosis can be based, and the true nature of the disease can be determined only by the cultivation of the causative organism...*'

— A. T. Stanton & W. Fletcher, 1932

An early complaint (first made in 1932) was that melioidosis has no cardinal symptoms or signs on which to base a diagnosis.[11] Indeed, the clinical manifestations of melioidosis are protean and melioidosis most frequently presents as undifferentiated sepsis. Infection has been described in almost every organ in the human body, except that melioidosis seldom, if ever, causes primary meningitis or endocarditis.[40]

A definitive diagnosis of melioidosis is made by isolating *B. pseudomallei* from any clinical specimen, because *B. pseudomallei* is not part of the normal human flora.[40,41] Stanton & Fletcher's 1932 monograph advised that a medical officer suspecting melioidosis in a patient, but who had no access to a laboratory, should inoculate guinea pigs with blood or pus, then send the infected animals to the nearest laboratory for diagnosis.[11]

The identification of even a single colony may clinch the diagnosis. Every patient with suspected melioidosis should have a set of blood cultures, urine specimen and a throat swab taken. Sputum, swabs from skin lesions, pus/fluid from collections, etc. should be collected as indicated clinically.[41,239] Bone

marrow aspirates offer no benefits over blood culture.[254] Throat swabs may be culture-positive regardless of the presence or absence of respiratory symptoms.[239] Urine may be culture-positive even when there are no urinary symptoms. Throat swabs and urine should therefore always be collected regardless of symptoms or signs.

1.4.2 Serological diagnosis

Serological diagnosis is the identification of antibodies directed against *B. pseudomallei* in the serum of blood from patients with melioidosis. Antibody reactions to *B. pseudomallei* are common following environmental exposure, are not protective,[250] and high titres may be found in patients with melioidosis. Serological tests have therefore been the focus of the development of rapid diagnostic tests for melioidosis.

The earliest reports measured the ability of patient serum to agglutinate suspensions of whole bacteria. In 1932, De Moor *et al.,* reporting from Batavia, Dutch East Indies (modern day Jakarta, Indonesia), considered the use of serology to diagnose melioidosis, but dismissed it as having little value,[242] because sera from normal controls agglutinated *B. pseudomallei* at titres ≥1:400. They therefore suggested that in the absence of positive cultures, only titres ≥1:1000 could be considered diagnostic. Harries *et al.* (1948) suggested that a titre of 1:80 be reported as 'significant' and that a titre of 1:160 was 'diagnostic', based on the observation that the majority of 56 control sera taken from patients with other infections (principally syphilis) had no detectable titre and none had titres >1:20.[129] The difference between de Moor's and Harries' results appears to be accounted for by the fact that the controls used by de Moor were drawn from the native population (who would be expected to have a high background exposure to *B. pseudomallei*), whereas the controls Harries used were mainly British soldiers (who came from a non-endemic area and would therefore not have had any exposure). Serological diagnosis based on agglutinating whole bacteria continued to be reported sporadically until 1958.[25] Complement fixation tests have also been used.[255,256]

The only validated serological test for melioidosis is the indirect haemagglutination test (IHA).[257] IHA in its current form was described by Strauss *et al.* in 1968[256] (although he was not the first to use IHA in melioidosis:

Nigg had described a version using human blood cells 5 years earlier[258]). Sheep erythrocytes are 'sensitised' with *B. pseudomallei* antigen derived from broth cultures (called 'melioidin' by analogy to tuberculin) and the result reported is the maximum dilution of patient serum that causes the sensitised erythrocytes to agglutinate. The use of crude antigen in IHA means that the strain (or mix of strains) used may result in differences in performance, and results from studies performed using different strains may not be comparable.

Strauss collected sera from 1,592 healthy persons resident in Malaysia and compared them to sera collected from 200 healthy persons resident in the US. He set a 1:40 cut-off because none of the subjects in the US cohort had titres greater than 1:20. He then showed that 7·3% of subjects in the Malaysian cohort had titres ≥1:40. Alexander *et al.* validated the 1:40 cut-off for IHA on 169 melioidosis patients, using as controls, 201 US soldiers serving in Vietnam and Thailand and 200 control sera collected in the US,[256] and concluded that the 1:40 cut-off was reliably specific for melioidosis.

A limitation of IHA clearly evident in Alexander's study was the high number of false negatives encountered in the first week of disease onset: only 38–45% were positive at the 1:40 cut-off during the first seven days. This is a problem, because around half of all the patients who die of acute melioidosis die within the first 48 hours of presentation.[199,200,259] In the study performed by Alexander *et al.*, the number of sera with reactions above the cut-off increased to 88% in the second week, then plateaued at 89–100% in the third week. Alexander was not the first to make this observation: de Moor reported this in 1932, and one of his arguments against the use of serodiagnosis was that antibodies took too long to appear and serology was therefore useless in the majority of patients.[242]

This low sensitivity of IHA in acute melioidosis has been confirmed by subsequent studies, with studies in Thailand tending to report slightly higher sensitivities than elsewhere. Teparrugkul, in Ubon Ratchathani, reported a sensitivity of 86% when using the 1:40 cut-off and 70% when the cut-off was 1:160.[260] In Australia, only 56% of patients with culture-confirmed melioidosis had positive IHA results at admission (cut-off 1:40),[261] but most patients with negative titres at admission subsequently seroconverted. An application for IHA may therefore be in the diagnosis of patients with subacute or chronic disease

who are culture-negative, but in whom seroconversion or a rising titre can be demonstrated.

The specificity of a test for melioidosis is defined as the percentage of healthy people without melioidosis who test negative for melioidosis. The specificity of IHA is therefore a function of the local seroprevalence for melioidosis (speaking imprecisely, it is 100%–seroprevalence). Specificity of IHA is therefore low in areas of high seroprevalence, because the number of false positives is high.

Increasing the cut-off used increases the specificity of IHA at the expense of decreasing the sensitivity.[262] In 1990, Appassakij *et al.* suggested a cut-off of 1:160 and evaluated this in 373 healthy blood donors, 65 cord blood samples and 30 melioidosis patients.[263] He found that at this cut-off, sensitivity of the test was 77% and specificity was 92%. Consistent with earlier studies, the test was less likely to be positive in acute, fulminant melioidosis.

Not all studies from Thailand have been able to achieve such high specificities. In the cohort reported by Teparrugkul (325 patients and 177 controls), a 1:40 cut-off gave a sensitivity of 86% and specificity of 38%; raising the cut-off to 1:160 decreased sensitivity to 70%, but raised the specificity to only 67%,[260] with similar results obtained from a later cohort at the same centre.[264] In the cohort reported by Puapermpoonsiri (47 cases, 318 controls), a 1:40 cut-off gave a sensitivity of 83% and specificity of 90%, but increasing the cut-off to 1:160 dropped the sensitivity to only 49% (specificity 97%).[265]

The performance of IHA has been even poorer outside of Thailand. In Malaysia, a cut-off of 1:40 for IHA resulted in a sensitivity of 36.0% and specificity of 73·5%.[247] Raising the cut-off to 1:80 dropped the sensitivity to 29·0%, but increased the specificity to 80·5%. In Australia, Cheng *et al.* reported a sensitivity of only 56% in 275 patients with culture-confirmed melioidosis.[261]

An important exception is the island of Singapore, where melioidosis is endemic but the background seroprevalence is low: only 0·73% of 683 men in national service had IHA titres ≥1:16, sera from 50 healthy blood donors and 50 sewerage workers were all unreactive.[245] This means that cut-offs as low as 1:16 may be used without sacrificing specificity. The low seroprevalence seen in Singapore may be explained by the fact that the population of Singapore is

100% urbanised.[266] Although the organism is present in the environment, the majority of the population have little or no contact with soil or surface water.

The clinical significance of a positive IHA test in someone who is otherwise well is not certain, but has been regarded as a marker for previous exposure to *B. pseudomallei*. Its relationship to latent melioidosis is unknown. The specificity of IHA for *B. pseudomallei* exposure has been disputed and there is conflicting evidence whether exposure to the non-virulent bacterium, *B. thailandensis,* might also be responsible for high IHA titres.[253,267,252]

A number of other serological tests have been evaluated including an indirect fluorescent antibody test for IgM specific antibody,[244] enzyme-linked immunosorbant assays (ELISA) for *B. pseudomallei* lipopolysaccharide (LPS)[269] and for *B. pseudomallei* glycolipid,[270] as well as a commercial immunochromogenic cassette (PanBio, Queensland, Australia).[264,248,270–272,262] None have so far supplanted IHA.[257]

The role of serological methods has been revised in a recent study comparing culture against four serological tests. Using Bayesian latent class models, which allow for the fact that culture results are an imperfect gold standard, the sensitivity of culture for diagnosing melioidosis has been estimated to be only 60·2%.[274] By contrast, LPS-based ELISAs have reasonable sensitivity (80·2%) and specificity (95·0%), and may be good candidates to take forward for further development.[262]

1.5 Therapy

1.5.1 Historical perspective

The majority of reports of melioidosis in the pre-antibiotic era were *post mortem*. The mainstay of treatment was largely supportive, with drainage of clinically evident collections and debridement of infected tissue. Non-chemotherapeutic therapies tried include autogenous vaccination (isolating the organism, culturing it in a guinea pig, and then repeatedly inoculating the patient with the cultured bacterium).[275]

The first reported attempt using modern antimicrobial chemotherapy to treat melioidosis was in 1943, when Grant and Barwell attempted to treat melioidosis osteomyelitis in a soldier with sulphadiazine. Although a good response was achieved, the patient relapsed each time the treatment was

stopped.[26] Harries *et al.* noted that sulphonamide monotherapy was ineffective in disseminated disease.[129]

It was discovered early on that penicillin was ineffective against *B. pseudomallei in vitro*,[275–277] as was the other wonder drug, streptomycin.[278] However, chloramphenicol[279–281] and the tetracyclines[281,282] were active. In 1952, Dunlop reported the successful treatment of a woman with melioidosis of the ovary with surgical drainage and chlortetracycline.[283] However, any initial enthusiasm for the tetracyclines was tempered by the realisation that resistance develops quickly both *in vitro* and *in vivo*.[284]

B. pseudomallei is intrinsically resistant to a wide variety of antimicrobial agents, including all penicillins (except the anti-pseudomonal penicillins),[280,285] all macrolides,[286] all first and second-generation cephalosporins[280] and polymyxins.[287] It is intrinsically resistant to all aminoglycosides, except that some strains are sensitive to kanamycin *in vitro*.[288,77,280] However, the aminoglycosides, as a class, have no intracellular penetration[286] and are therefore ineffective clinically. There is one reported case of a woman who was successfully treated with kanamycin,[183] but this was in combination with tetracycline, to which the organism was susceptible.

Until 1989, in Thailand, the conventional treatment for melioidosis was combination therapy with co-trimoxazole, doxycycline and chloramphenicol.[259]

1.5.2 Current recommendations

In its most serious manifestation, melioidosis causes severe sepsis associated with hypotension and multiple organ failure, requiring intensive supportive therapy (fluid management, invasive monitoring and artificial ventilation). These measures likely play as great a role in the treatment of melioidosis as do antibiotics.[289] Human-to-human transmission is very rare and patients may be safely accommodated on the open ward.[102-105]

Antimicrobial therapy for melioidosis is divided into two phases: an *intensive phase* (parenteral inpatient therapy) and an *eradication phase* (oral outpatient therapy).[41] Current treatment recommendations are summarised in Table 4.

1.5.3 Intensive phase

Antibiotic therapy should be initiated as soon as possible, as there is evidence from other causes of sepsis that delay in initiating appropriate antimicrobial therapy increases mortality.[290,291] Without access to antimicrobial therapy, mortality from the bacteraemic form of melioidosis approaches 100% and in the pre-antibiotic era, almost all case series reported in the literature were *post mortem*.[127,9]

Ceftazidime treatment was introduced in 1989, after it was shown that it halved mortality compared to conventional therapy (co-trimoxazole, doxycycline and chloramphenicol).[259] The first-line treatment for melioidosis is high-dose intravenous ceftazidime[41,259] for ≥10 days, continued until there are clear signs of clinical improvement (principally defervescence). The median fever clearance time is 10 days despite adequate antimicrobial chemotherapy.[200] Failure to defervesce is not therefore itself an indication to change treatment and it is not uncommon for patients to require over a month of intravenous therapy.

Carbapenems (principally, meropenem and imipenem) are routinely used in Australia.[132] Carbapenems may be preferred to ceftazidime because the minimum inhibitory concentrations (MIC) for imipenem and meropenem against *B. pseudomallei* are lower than those for ceftazidime,[288,292,77] and carbapenem therapy is associated with lower levels of antibiotic-induced endotoxin release.[293] However, a head-to-head comparison of imipenem versus ceftazidime found no difference between the regimens, but the trial was stopped early owing to withdrawal of drug company support.[200] A multi-centre double-blind randomized-controlled trial comparing meropenem against ceftazidime started recruiting in 2007 (ClinicalTrials.gov NCT00579956).

The addition of co-trimoxazole to standard ceftazidime therapy provides no benefit,[294,295] despite evidence from animal models to support the practice.[296] However, co-trimoxazole has excellent tissue penetration into brain and prostate, so there is a theoretical benefit to adjunctive co-trimoxazole when treating these infections.[41] Co-amoxiclav is an alternative to ceftazidime if none of the first-line drugs are available:[297] mortality is the same, but the risk of treatment failure is higher. Co-amoxiclav may be useful in resource-poor settings where first-line drugs are not immediately available.

High intensive parenteral[a]	**Typical dose**[b]	**Duration**
ceftazidime 40 mg/kg (max. 2 g) tds,[c] or	2 g every 8 h[c]	≥10 days, or until clear clinical
meropenem 25 mg/kg (max. 1 g) tds, or	1 g every 8 h	improvement, which ever is longer[d]
imipenem 20 mg/kg (max. 1 g) tds	1 g every 8 h	
Alternatively,		
co-amoxiclav 20/4 mg/kg (max. 1000/200 mg) qqh[e]	1000/200 mg every 4 h	
Eradication oral phase (Thailand)		
co-trimoxazole (<40kg: 160/800mg bd; 40–60kg: 240/1200mg bd; >60kg: 320/1600mg bd) +	320/1600 mg every 12 h 100 mg every 12 h	12–20 weeks
doxycycline 2 mg/kg (max. 100 mg) bd		
Alternatively,		
co-amoxiclav 60/15 mg/kg (max. 1500/375) tds[f]	1500/375 mg every 8 h	
Eradication oral phase (Australia)		
co-trimoxazole (<40kg: 160/800mg bd; 40–60kg: 240/1200mg bd; >60kg: 320/1600mg bd)	320/1600 mg every 12 h	3–6 months

Table 4. Summary of antimicrobial chemotherapy of melioidosis.

Note.— bd = *bis die;* h = hours; max. = maximum; qds = *quater die sumendus;* qqh = *quaque quarta hora;* tds = *ter die sumendus*. The regimen quoted for Thailand is that used at Sappasithiprasong Hospital, Ubon Ratchathani. The regimen quoted for Australia is that used at the Royal Darwin Hospital, Northern Territory. All doses require adjustment for renal function.

[a] In Australia, oral co-trimoxazole is routinely added to parenteral therapy for infection of the central nervous system and for prostatitis, because of the excellent penetration of co-trimoxazole to these sites.

[b] Typical dose for a 70 kg adult with normal renal function.

[c] The Royal Darwin Hospital uses 50 mg/kg (maximum 2 g) qds. A typical dose is therefore 2 g every 6 hours.

[d] Brain abscesses are routinely treated with ≥4 weeks of parenteral antibiotics. Other deep seated abscess that are not amenable to drainage may also require prolonged parenteral therapy.

[e] There is no parenteral preparation of co-amoxiclav available in Australia. Ampicillin-sulbactam is available in a parenteral formulation, but there is no clinical experience of the use of this drug in melioidosis.

[f] If co-amoxiclav preparations are only available in a 2:1 ratio, then additional amoxicillin should be prescribed to achieve the correct ratio of amoxicillin to clavulanate. Co-amoxiclav is routinely used in children and pregnant women in Thailand, because doxycycline is relatively contra-indicated.

Primary resistant to first-line agents is unusual (Table 5) and there is no evidence that these rates are changing. *B. pseudomallei* is an environmental organism, with no human-to-human transmission, and is largely unaffected therefore by antibiotic pressure in healthcare settings. Rarely, resistance can arise during the course of treatment,[298,299] and patients who remain febrile on treatment should have cultures repeated regularly to look for this.[299]

Cefoperazone-sulbactam has been shown to be equivalent to ceftazidime in a randomised controlled trial.[201] There are no obvious circumstances under which cefoperazone-sulbactam would be preferred to ceftazidime or a carbapenem, and the drug has not found a home in current guidelines. There is *in vitro* evidence for biapenem,[292] doripenem,[300] piperacillin-tazobactam[184,301] and tigecycline,[302–304] but no clinical experience to support their use.

CAZ	IPM	MEM	AMC	Region	Reference
99.5%	100%	–	100%	Thailand	Dance 1989
99%	100%	100%	100%	Thailand	Smith 1996[292]
100%	100%	100%	99.4%	Australia	Jenney 2001
100%	100%	100%	100%	Hong Kong	Ho 2002[184]
98%[a]	100%[a]	–	98%[a]	Multiple	Thibault 2004[77]
–	–	–	100%	Singapore	Sivalingam 2006[305]
–	100%	100%	–	Malaysia	Karunakaran 2007[306]
99·4%	99·4%	–	97·9%	Singapore	Tan 2008[285]

Table 5. Susceptibility of *B. pseudomallei* to first-line parenteral agents.

Note.—CAZ = ceftazidime; IPM = imipenem; MEM = meropenem; AMC = co-amoxiclav. These results are for primary isolates and do not include data from resistance acquired during the course of treatment. A dash indicates that that agent was not tested. The breakpoints used are those defined by the Clinical Laboratory Standards Institute in the US (CAZ ≤8 mg/l; IMP ≤4; MEM ≤4; AMC ≤8/2).

[a]Breakpoints defined by Comité de l'Antibiogramme de la Société Française de Microbiologie, France (CAZ ≤32 mg/l; IMP ≤8; AMC ≤16/2).

1.5.4 Eradication phase

Thirteen per cent of patients who survive a first episode of melioidosis go on to experience a recurrence of their disease:[221,307] 75% of these are due to relapse with the same strain and 25% are due to re-infection.[307] The aim of eradication therapy is to reduce the risk of relapse.

Eradication therapy is based on oral medication and is usually conducted on an outpatient basis. Patients are not started on oral eradication therapy until they are clinically well and ready for discharge home. The occasional patient with a solitary abscess that is easily drained, who is afebrile, and who has no systemic signs or symptoms, may avoid the intensive phase and go straight onto oral eradication therapy.

Relapse is more likely in patients who have multifocal disease or bacteraemia at their first presentation.[307] Regimens containing co-trimoxazole have the lowest relapse rate. Ciprofloxacin monotherapy, ciprofloxacin/azithromycin combination therapy and doxycycline monotherapy are associated with higher relapse rates and cannot be recommended.[307–310]

Unfortunately, none of the first-line treatments (ceftazidime, meropenem, imipenem) may be administered orally as they are not absorbed.

Standard therapy in Thailand is combination therapy with trimethoprim-sulphamethoxazole and doxycycline for 12–20 weeks;[311] standard therapy in Australia is co-trimoxazole alone.[132] It is not clear that one therapy is superior to the other, and a double-blind randomised placebo-controlled trial to compare the two regimens, MERTH (*M*elioidosis *E*radication *TH*erapy/*TH*ailand) has just been completed. The study finished recruitment in 2009 and completed follow-up in 2010 (trial registration ISRCTN86140460). A report is expected at the end of 2011.[312]

One potential benefit of co-trimoxazole monotherapy is that co-trimoxazole and doxycycline are antagonistic *in vitro*.[313] It is also anticipated that a simplified regimen would be better tolerated, because those effects due to doxycycline would be eliminated (specifically, oesophageal ulceration and photosensitivity, but also non-specific gastrointestinal effects such as nausea and vomiting) and because of the smaller pill burden. The dose of co-trimoxazole used in Thailand was revised upwards in 2008 on the basis of evidence from pharmacokinetic modelling to be in line with that used in Australia.[314]

1.5.5 Vaccination

There are currently no licensed vaccines for melioidosis. Currently, the best vaccine candidates are live attenuated vaccines, but none of these provide sterilising immunity and there are no vaccines currently in clinical trials.[315,316]

1.5.6 Post-exposure prophylaxis

Most cases of accidental exposure are likely to occur in a clinical laboratory processing a specimen not known or suspected to have *B. pseudomallei*. Given the lack of other evidence, recommendations for post-exposure prophylaxis are based on current treatment recommendations. Following a thorough risk assessment, post exposure prophylaxis with three weeks' co-trimoxazole may be offered.[317] Workers intolerant of co-trimoxazole may be offered co-amoxiclav instead. Workers who seroconvert may be offered an extended course of therapy (12 weeks) and careful monitoring for the development of clinical disease.

There are not currently any published recommendations for travellers with risk factors such as diabetes or cystic fibrosis who travel to endemic areas.

1.5.7 Antisepsis

B. pseudomallei is susceptible to 70% ethanol, 1% hypochlorite, 7% tincture of iodine, 0·05% benzalkonium chloride and 1% potassium permanganate.[20,318] The commercial agents, Perasafe™ (DuPont, active ingredient peracetic acid) and Virkon® (DuPont, active ingredient commercial confidential, but known to include peracetic acid) are also bactericidal for *B. pseudomallei*.[318]

Five per cent phenol does not reliably disinfect *B. pseudomallei*.[20] Outbreaks of melioidosis have been associated with contaminated hand wash.[319,320]

Chlorination effectively treats drinking water: free chlorine concentrations normally used by water purification plants in the West are ~1 mg/l, which is sufficient for the majority of strains of *B. pseudomallei*, but some Australian strains require ≥30 mg/l.[166,321–323]

1.6 Adjunctive therapies

1.6.1 Granulocyte-colony stimulating factor

The only adjunctive treatment ever studied in the specific context of melioidosis was granulocyte colony stimulating factor (GCSF).[324] GCSF is a 20 kDa glycoprotein growth factor produced principally by endothelium and macrophages,

that stimulates the proliferation and differentiation of granulocyte precursors in the bone marrow.[325] Its principal medical application is to reduce the duration of neutropenia that occurs in cancer chemotherapy and HIV. As a drug, its generic name is filgrastim, and is sold in the UK under the trade names Neupogen®, Ratiograstim® and Zarzio®.

The neutrophil response is critically important in the pathogenesis of melioidosis,[326] and the introduction of adjunctive therapy with GCSF coincided with a reduction in melioidosis mortality from 95% to 10% at one Australian centre.[327] Unfortunately, a single-centre randomised-controlled trial found no evidence of benefit.[324]

1.6.2 Intensive glucose management

On the basis that plasma glucose levels are often high in sepsis and that high levels correlate with mortality, it has been proposed that intensive insulin therapy to tightly control glucose levels in the intensive care setting should decrease mortality in critically ill patients (not just patients with sepsis).[328] In a study of 1,548 surgical intensive care patients in Belgium, intensive insulin therapy (target range 4·4–6·1 mM) reduced mortality from 8·0% to 4·6%.[221] This is potentially relevant, because 57% of patients with melioidosis have diabetes or present with hyperglycaemia,[329] and, insulin/ glucose monitoring are routinely available at provincial hospitals in Thailand.

The van den Berghe study[221] was criticised on a number of counts: principally, that this was a single centre study, most of the patients were admitted post-surgery, the study was open label, and the feeding regimen was unusual (all patients received a 200–300 g bolus of glucose at recruitment and total parenteral nutrition or enteral feeding was started within 24 hour of admission).[330] A repeat study by the same investigators in the medical intensive care was unable to replicate this finding.[331] Studies of intensive glucose control in the context of myocardial infarction[332] and trauma[333] have also found no benefit. Conversely, a multi-centre trial of 6104 patients by other investigators found that intensive insulin therapy (target 4·5–6·0 mM) in critically ill patients increased mortality.[334] A meta-analysis of 26 trials (13,567 patients, including the data from NICE-SUGAR)[335] found that intensive insulin therapy increases the risk of hypoglycaemia without conferring any over mortality benefit to critically ill

patients, and the only subgroup of patients found to benefit were surgical patients (pooled relative risk 0·63, 95%CI 0·44–0·91).[335]

1.6.3 Immunosuppressants

A recurring theme in the sepsis literature is that the inflammatory response is essential, yet high levels of inflammation correlate with mortality in observational studies of human sepsis.[336] The prevailing theory has been that mortality from sepsis is due to an uncontrolled inflammatory response:[336] the 'cytokine storm'.[337] This view has been supported by data from animal models of sepsis,[338–344] and that has in turn driven the clinical development of anti-inflammatory drugs as adjunctive therapies in sepsis.

Multiple trials looking for a benefit from high-dose corticosteroids in severe sepsis and septic shock have not found any benefit, with some studies finding a detrimental effect.[345–348] Evidence for the use of low-dose corticosteroids in sepsis is mixed: a single multi-centre, placebo-controlled, randomised, double-blind trial of 300 intensive care patients with septic shock and relative adrenal insufficiency (as measured by a cortocotrophin test) found a mortality benefit from low-dose corticosteroid and fludrocortisone,[349] but this result has not been possible to replicate: one trial found that low-dose corticosteroid reduced time on vasopressors but not mortality,[350] another multi-centre randomised placebo-controlled trial of 248 patients performed by the same investigators found no difference in mortality[351] and a further trial in patients with liver cirrhosis found that the patients in the hydrocortisone arm had an increased incidence in relapse of shock.[352]

The results for anti-tumour necrosis factor-alpha (TNFα) therapy have been disappointing. Despite evidence from primate models that anti-TNFα has a dramatic effect on survival from *Escherichia coli*[353,354,338] and *Staphylococcus aureus* bacteraemia,[339] the result from multiple clinical trials is that anti-TNFα therapy provides no benefit in sepsis.[355–359] In any case, results from mouse models of melioidosis would predict TNFα blockade to be detrimental.[360] Trials of interleukin-1β beta blockade have been similarly disappointing,[361–363] despite promising results from animal models.[340–344]

1.6.4 Activated protein C

Activated protein C (APC) is a serine protease that is known primarily as an anticoagulant. Inactive protein C circulates in the plasma and is cleaved to its active form by activated thrombin. As a drug, its generic name is drotrecogin alfa (activated), and its trade name is Xigris®.

APC is the first (and currently, the only) US Food and Drug Administration (FDA)-approved adjunctive treatment for severe sepsis.[364,365] Its approval was based on the results of a Phase III multi-centre double-blind placebo-controlled trial involving 1,690 sepsis patients in 164 centres in 11 countries (the PROWESS trial).[366] The trial results were published in 2001 and reported a 6% reduction in the absolute risk of death, accompanied by a 1·5% increase in the risk of serious bleeding events.

Levels of protein C are low in melioidosis,[367,368] and we may speculate that melioidosis patients with severe sepsis may benefit from APC therapy. No trials of activated protein C therapy are currently planned in melioidosis.

1.6.5 Gamma interferon

Gamma interferon (IFNγ) is necessary for the normal host defence against *B. pseudomallei* infection and IFNγ levels are indeed elevated in acute melioidosis.[369] Small doses of IFNγ greatly potentiate the effect of ceftazidime *in vitro* and mouse models.[370] IFNγ supplementation is already used in the management of multi-drug resistant tuberculosis, and it seems reasonable to hypothesise that patients with other infections caused by intracellular bacteria may benefit.[371] Using a mouse model of melioidosis, Propst *et al.* found that IFNγ supplementation reduced 20-day mortality in ceftazidime-treated animals from 100% to 30% ($p<0{\cdot}001$);[370] however, the dose of ceftazidime given in that study was sub-therapeutic, which complicates interpretation.[372]

The role of IFNγ in the host response to melioidosis is described in greater detail below.

1.7 The host response to melioidosis

Much of what we know about the host response to melioidosis comes from a limited number of human studies and a larger number of animal studies, primarily in mice (both outbred[373] and inbred[360]), rats,[80] and hamsters.[116] Findings made in

a model organism do not necessarily translate to the clinical situation, and findings made in one model may not necessarily correlate with findings made in another model. Interpretation of the literature is therefore an exercise akin to joining dots to form a picture, except that the dots are unnumbered, some are optional, and many are not even printed on the same page.

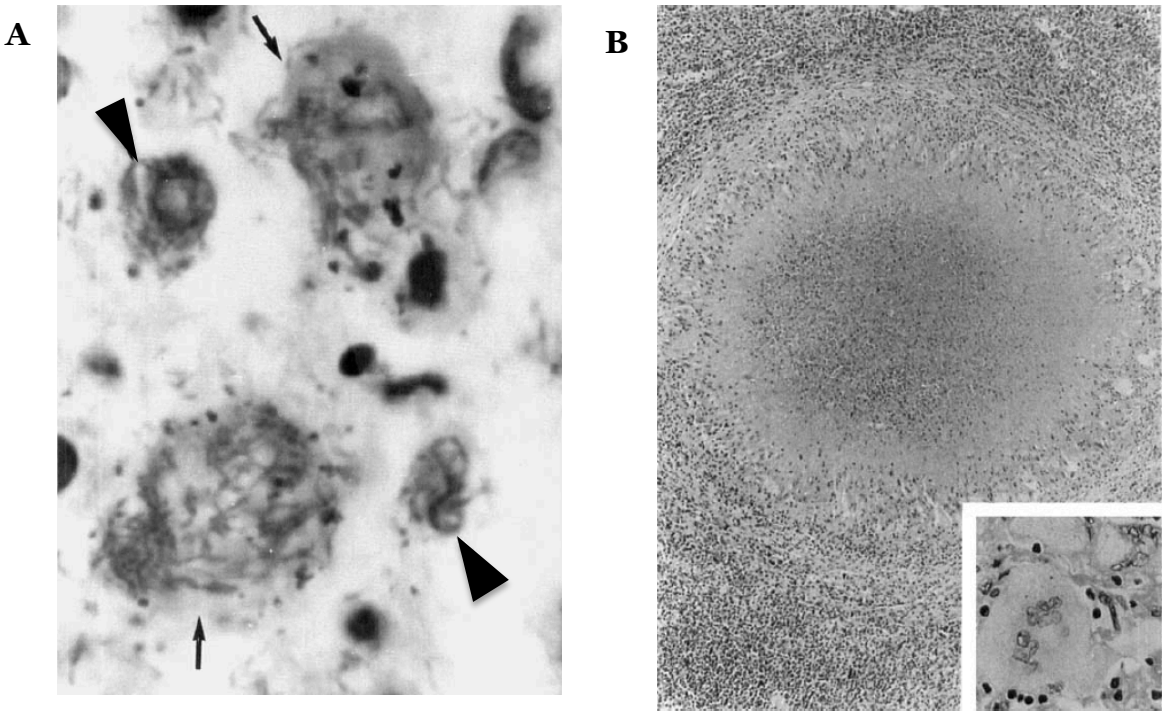


Figure 10. Histological features of human melioidosis.

Note.—**A** Lung section taken *post mortem* from an 18-year-old female. Tangles of extracellular bacteria are indicated by large arrow heads. Tangles of intracellular bacteria within macrophages and giant cells are indicated by thin arrows (magnification ×1000). **B** A splenic granuloma with central necrosis (magnification ×100) surrounded by foamy macrophages and multinucleate giant cells (inset, magnification ×400). Figures adapted from Wong *Histopathology* 1995;26:51.

1.7.1 Histology

There are few histological descriptions of human melioidosis from Thailand because of strong cultural barriers to the collection of tissue specimens. The most recent descriptions come from a series of five *post mortem* cases and 14 biopsy specimens from Malaysia,[52] although older descriptions exist from the Vietnam War[17,374,375] and from before World War II.[11]

In the Malaysian series, Wong *et al.* describe areas of necrotising inflammation containing mixtures of neutrophils, lymphocytes, macrophages and multinucleate giant cells. Numerous bacteria are seen within these lesions and may occur both intra- and extracellularly (Figure 10). When they occur intracellularly in macrophages, the bacteria may be so numerous as to form globi. The lesions may sometimes form granulomas with central necrosis and surrounding foamy macrophages, which cause them to resemble tuberculosis or cat-scratch fever.

1.7.2 The gamma interferon response in melioidosis

B. pseudomallei is a facultative intracellular pathogen,[79–81] and the host cytokine response to melioidosis may be appropriately summarised as an IFNγ response to an intracellular pathogen.[369,376,377] While IFNγ elevation is not a general feature of other causes of sepsis,[378,379] it is a feature of infection caused by other intracellular organisms, such as *Brucella* species,[380] *Mycobaterium tuberculosis,*[381,382] *Neisseria meningitidis,*[383–385] and *Salmonella* Typhi.[386] This is to be expected, given that a primary function of IFNγ is to stimulate the killing of intracellular pathogens.[387–389]

The interferons were initially discovered in 1947 by Henle and Henle as a substance produced in the allantois of chick embryos infected with influenza that 'interfered' with viral replication.[390] This substance was given the name *interferon* by Isaacs and Lindeman in 1957,[391] but in the mid-1970's, it was realised that interferon was not one protein but a mixture of proteins.[392,393] In 1979, Grob and Chadha separated human 'interferon' into three fractions, later designated alpha, beta and gamma.[394] Macrophage activating factor was first isolated in 1973 from guinea pig lymphocytes and found to activate the intracellular killing effects of macrophages,[395] but it was cloning of the IFNγ gene (IFNG) in 1982[396,397] that brought the realisation that IFNγ and MAF are the same molecule.[398–402]

In 1991, Brown *et al.* studied 65 melioidosis patients and found that serum concentrations of IFNγ at admission were elevated in melioidosis, and that the higher the serum concentration of IFNγ, the more likely the patient was to die.[369] There are two possible interpretations for this finding: the first is that mortality is due to an inappropriate and excessive inflammatory response.[403] The second is that the inflammatory response is appropriately high, that it correlates with higher bacterial loads, disease severity, and hence, mortality.[376] The distinction is an

important one: if the first is true, then suppressing IFNγ would be expected to decrease mortality; if the second is true, then suppressing IFNγ would be expected to increase mortality. Similar arguments may be made for other cytokines.

Evidence for the first hypothesis comes from murine models of endotoxic shock (a model of septic shock induced with intravenous LPS).[404,405] Blocking antibodies directed against IFNγ prevented mortality from endotoxic shock[404] and IFNγ receptor knockout mice were also able to tolerate 100 to 1000-times more LPS than wild type mice.[405] However, the clinical relevance of data obtained from endotoxic shock models has been questioned repeatedly.[406–409]

Evidence in favour of the second hypothesis was obtained by Lauw *et al.* in her survey of 62 consecutive melioidosis patients.[376,377] Although she did not quantify bacterial loads, she found that bacteraemic patients had higher levels of IFNγ.[376] In murine models of melioidosis, blocking IFNγ increased susceptibility in Taylor outbred mice, lowering the intraperitoneal LD50 from 5×10^5 cfu to 2 cfu,[373] whereas exogenous IFNγ supplementation improved survival.[370] Taken together, the experimental and clinical evidence support the conclusion that IFNγ levels are appropriately elevated in melioidosis in response to the intracellular nature of the pathogen and the severity of the disease.

In mice, the primary source of the IFNγ are natural killer (NK) cells and memory $CD8^+$ T-lymphocytes,[360,410] and this IFNγ response appears to be secondary to the synergistic action of IL12 and IL18.[360,410] Ablating either IL12 or IL18 ablates the IFNγ response and worsens mortality.[360,411,412] This is supported by the clinical data, which shows elevations of IL18 in plasma[376] as well as elevations of IL18 mRNA in blood mononuclear cells[412] in melioidosis patients.

Cytokine	Primary source(s)	Summary of functions	References
CXCL9	macrophage, various	Secreted in response to IFNγ stimulation. Chemoattractant for T cells and NK cells.	Lauw 2000[377]
CXCL10	macrophage, various	Secreted in response to IFNγ stimulation. Chemoattractant for T cells and NK cells.	Lauw 2000[377]
IFNγ	NK, CD8, (macrophage)	Secreted in response to IL12 and IL18, Stimulate macrophages and killing of intracellular bacteria.	Santinarand 1999,[373] Lauw 1999[376]
IL6	macrophage	Downstream of TLR activation. Stimulates monocyte differentiation to macrophage. Inhibits IL1β and TNFα.	Friedland 1992[413]
IL8	macrophages, endothelium	Downstream of TLR activation. Recruits neutrophils.	Friedland 1992[413]
IL12	monocytes, macrophages[414,415]	Downstream of TLR activation. Stimulate IFNγ production.[416]	Lertmemongkolchai 2001,[360] Koo 2006[410]
IL15	monocytes, macrophages	Stimulates proliferation of NK cells.[416]	Lauw 1999[376]
IL18	monocytes, macrophages	Produced by inflammasome activation. Stimulate IFNγ production.[360,411]	Lertmemongkolchai 2001,[360] Koo 2006[410]
TNFα	macrophages	Downstream of TLR pathways. Amplifies IFNγ production.	Santinarand 1999,[373] West 2008[417]

Table 6. Cytokines and chemokines involved in clinical melioidosis.

Note.—CD = cluster of differentiation; CD8 = CD8-positive T-lymphocytes; IFN = interferon; IL = interleukin; NK = natural killer cells; TLR = Toll-like receptor; TNF = tumour necrosis factor. This table is restricted to those cytokines and chemokines that have been measured in clinical melioidosis. This table is not intended to be a comprehensive list of cytokines known to play a role in the host response to melioidosis.

TNFα levels were measured in a clinical study of 55 melioidosis patients, and the presence of detectable plasma TNFα at admission correlated with mortality.[418] In mice, ablating TNFα reduces the magnitude of the IFNγ response by up to 30%, but does not ablate it.[360] The relationship of TNFα to IFNγ is circular, with TNFα being both upstream[373] as well as downstream of IFNγ secretion.[419,420] One interpretation consistent with these data is that the role of TNFα in melioidosis is to amplify the IFNγ response, but not to initiate it.

Downstream of IFNγ, CXCL9 (formerly called MIG) and CXCL10 (formerly IP-10) are secreted by a large variety of cell types in response to IFNγ stimulation. Plasma concentrations of both CXCL9 and CXCL10 are elevated in patients with melioidosis and levels were higher in patients with bacteraemia.[377] CXCL9 and CXCL10 are T-cell and NK cell chemoattractants[421] and probably work to amplify the IFNγ response.

In clinical studies of melioidosis, plasma concentrations of IL6, IL8 and IL10 have also been found to be elevated and to correlate with mortality (Table 6).[413,422]

1.7.3 Pattern recognition receptors

Pattern recognition receptors (PPRs) are a set of evolutionarily-conserved receptors present on leukocytes to identify pathogen-associated molecular patterns (PAMPs), *e.g.*, the lipopolysaccharide of Gram-negative bacteria, lipoteichoic acid of Gram-positive bacteria and viral RNA. In evolutionary terms, these receptors predate the development of adaptive immunity and allow the innate host response to identify antigens that are not 'self'.

The best studied of the pattern recognition receptors are the Toll-like receptors (TLRs) and the Nod-like receptors (NLRs).

1.7.4 Toll-like receptors and nuclear factor kappa B

There are 13 human and mouse Toll-like receptors (TLR) described to date.[423–425] The TLRs are membrane-bound glycoprotein receptors and are members of the interleukin-1 receptor superfamily.[424] The TLRs are one of the more evolutionarily conserved parts of the innate immune system and may have originated in the common ancestor of all bilaterian animals.[426,427] TLRs signalling (with the exception of TLR3) proceeds via the adaptor molecule,

MyD88,[424] and the importance of the TLRs in the host response to *B. pseudomallei* infection has been demonstrated by the fact that *Myd88* knockout mice are extremely susceptible to *B. pseudomallei* infection.[428] Mouse gene expression studies have also found upregulation of *Tlr2, Tlr4* and *Tlr7* in experimental melioidosis.[429]

A major downstream effect of TLR signalling is nuclear factor kappa B (NF-κB) activation. NF-κB is a family of constitutively expressed protein complexes that function as DNA transcription factors. These complexes are homo- or heterodimers of a family of five proteins, RELA (also called p65), RELB, c-REL, NF-κB1 (also called p50 and p105) and NF-κB2 (also called p52 and p100).

NF-κB is activated by a wide range of stimuli, including cytokines (notably TNFα and IL1[430]), growth factors, mitogens, oxidative stress, chemical stress, bacterial and viral antigens. Activation of NF-κB causes it to move from the cytoplasm to the nucleus, where it regulates the expression of several hundred genes, including several cytokines and chemokines (*e.g.,* IL2, IL8, IL12, GM-CSF and CD40 ligand).[431,432] IL8 secretion is often used as a marker for TLR activation, because it is produced in very large quantities.[152] NF-κB is a rapidly acting transcription factor, due to the fact that it is constitutively expressed in the cytoplasm of cells and does not require new protein synthesis for its activation.

Aside from cytokine secretion, NF-κB activation also promotes cell survival and proliferation.[433,434] Although TLR activation was initially believed to result in a non-specific NF-κB response, it is now known that specific responses do occur: for example, TLR4 and 9 activation produces a Th1-type response, but TLR2 activation results in T regulatory cell activation.[435] The picture is also complicated by the fact that there also exists extensive crosstalk both within the TLR family and between TLRs and other PRRs.[436,437]

The best described TLRs are members 1 to 9. Of these, TLRs 1, 2, 4, 5 and 6 are located on the cell membrane where they form dimers. TLR2/TLR1 and TLR2/TLR6 form heterodimers that recognises triacyl lipopeptide and diacyl lipopeptide respectively, and both will recognise lipoteichoic acid.[427,438] TLR4 and TLR5 form heterodimers that classically recognise lipopolysaccharide and

flagellin respectively.[427] TLRs 3, 7, 8 and 9 are found intracellularly on the endosomal membrane, where they recognise single and double stranded RNA, and unmethylated CpG oligodeoxynucleotides.[427]

In clinical melioidosis, Wiersinga *et al.* found increased expression of TLR1, TLR2, TLR4, TLR5, and TLR8 in circulating blood leukocytes,[152] while levels of TLR3 and TLR7 were no different from controls. The lack of differential expression of TLR3 is unsurprising, as this TLR is restricted to dendritic cells, which do not circulate; this is reflected in the low absolute expression values reported.[439] TLR7 is constitutively expressed by human plasmacytoid dendritic cells (PDC) and B lymphocytes: upon stimulation, expression of TLR7 increases in PDCs but decreases in B lymphocytes.[440] Wiersinga's study did not distinguish between types of mononuclear cells and was therefore not designed to detect this type of differential expression.

The increased expression of TLR4 accords with the fact that *B. pseudomallei* is a Gram-negative organism and, as such, expresses the classical TLR4 ligand, LPS.[99,441,442] Other proteins in the endotoxin receptor complex, CD14 and LY96 (=MD2), were also upregulated in melioidosis patients[443] and it has also been reported that TLR4 polymorphisms are associated with an increased susceptibility to melioidosis,[444] however, the evidence for a role of TLR4 in melioidosis is contradictory. TLR4 appears to have no role in the innate host response to *B. pseudomallei* as evidenced by the fact that *Tlr4*-knockout mice had no phenotype when compared to wild-type mice on a C57Bl/6 background.[152] Supporting evidence was provided by two studies using the closely-related organism, *B. thailandensis,* in *Tlr4*-knockout mice as well as the *Tlr4*-deficient C3H/HeJ mouse strain, both of which showed that TLR4 is not a major player in sepsis caused by *B. pseudomallei*.[445,446] A possible explanation for the lack of a TLR4 response may be due to structural differences in the lipid A moiety of *B. pseudomallei,* which make it less immunoreactive.[447,99]

Although the ability of TLR2 to recognise lipoteichoic acid has led to it being described as a receptor for the recognition of Gram-positive organisms,[438] TLR2 will also recognise *E. coli* LPS when associated with lymphocyte antigen 96 (LY96).[448] Wiersinga reported that TLR2 appears also to recognise *B. pseudomallei* LPS in transfected HEK293 cells,[152] but West *et al.* were unable

to replicated this finding using a similar system.[417] There are no clear reasons to explain the conflicting results, but possibilities include strain differences in LPS structure[442] and the way in which NF-κB activation was assessed (IL8 production[152] versus a dual-luciferase reporter system[417]).

Nevertheless, if TLR2 is able to recognise *B. pseudomallei* LPS, that interaction is not beneficial to the host, because TLR2 knockout mice have reduced end-organ damage and improved survival,[152,446] which is contrary to what would be expected given the results of other mouse models of Gram-negative infection.[449–452] The reason why *Tlr2*-knockouts are protected remains an open question.

1.7.5 Nod-like receptors

The Nod-like receptors (NLR) are cytosolic receptors named for the nucleotide-binding oligomerization domain (or *Nod*) that they all possess. Unlike the TLRs, the NLRs are not membrane-bound: instead, they recognise antigens present in the cytosol.

The NLRs are divided into two major subfamilies, the NLRCs (NLRC1 to 5) which possess a caspase recruitment domain and the NLRPs (NLRP1 to 14) which possess a pyrin domain.[453] A further three genes do not fall into either of the two major families (NLRA, NLRB1, NLRX1).[453] Like the TLRs, the NLRs are evolutionarily conserved and are present in species as distantly related as sea urchins[454] and zebra fish,[455] although they are not present in insects or worms.[455]

The use of animal models to study NLRs is complicated by the fact that there is not a one-to-one correspondence between human and mouse NLRs: instead, there are 22 named human NLRs but 34 named mouse *Nlr*s.[453] For example, the NLRB family has only one human member (NLRB1) but seven mouse members (*Nlrb1a* to *f*). Similar discrepancies occur in all the families: so, although there is a human NLRP11, there does not exist a corresponding mouse *Nlrp11*. Many of the NLRs remain to be characterised, but are believed to function as pattern-recognition receptors.

NOD1 and NOD2 (also called NLRC1 and NLRC2) were the first NLRs to be described[456] and will activate NF-κB directly.[457] Stimulation of RAW264.7 cells (transformed mouse monocytes) with *B. pseudomallei* causes upregulation

of both *Nod1* and *Nod2*.[458] However, no clinical or animal studies of NOD1 or NOD2 have been reported to date, so their role in melioidosis is as yet undescribed.

1.7.6 NLRs, caspases, and the inflammasome

The *inflammasome* is a large macromolecular multiprotein complex first described in 2002 by Martinon *et al.* in THP-1 cell lysates (a human monocytic leukaemia line).[459] The inflammasome was named by analogy to the apoptosome, the multiprotein complex that drives caspase-9 activation.[460] The structure of the inflammasome has been confirmed by purifying and assembling the individual components *in vitro*.[461]

The inflammasome is a complex of three proteins: an NLR receptor, an adaptor protein, ASC, and caspase-1. Three NLRs are known to initiate inflammasome assembly: NLRP1, NLRP3 and NLRC4,[460] and all three NLRs associate with caspase-1 via ASC.[459,462–467]

NLRP1 (also known as NALP1 and CARD7) was the first caspase-1 activating receptor identified. NLRP1 recognises muramyl dipeptide (MDP), which causes a conformational change in NLRP1 that allows it to bind nucleotides and form oligomers, thus initiating inflammasome assembly.[460] However, the mechanism by which it does so is unknown, because NLRP1 does not bind MDP.[460] NLRP3 (also known as cryopyrin and NALP3) is stimulated by LPS, MDP, and bacterial RNA, but assembly of the NALP3 inflammasome requires a second signal, such as extracellular ATP acting on the P2X7 potassium channel[460] (other second signals include bacterial pore-forming toxins, silica and asbestos[460]). NLRP3 will also recognise silica, asbestos and urate.[460] Neither NLRP1 nor NLRP3 have been studied in the context of melioidosis.

NLRC4 (also called IPAF and CARD12) will recognise flagellin as well as the basal body rod component of the *B. pseudomallei* type 3 secretion system.[468] *Salmonella enterica serovar* Typhimurium, *Legionella pneumophila* and *Pseudomonas aeruginosa* appear to induce assembly of the NLRC4 inflammasome via the cytosolic delivery of flagellin, which may explain why a functioning type III or type IV secretion system is necessary for NLRC4 activation.[466,467,469–471]

Nlrc4-knockout mice have an increased susceptibility to *B. pseudomallei* (F. Re, unpublished data[472]). Unlike NLRP1 and NLRP3 activation, NLRC4 activation is associated with a special type of cell death called pyroptosis, and pyroptosis is a feature of *B. pseudomallei* infection of macrophages.[473,474] Taken together, these data suggest that the NLRC4 inflammasome may be more important than the NLRP1 or NLRP3 inflammasomes in melioidosis, but this hypothesis has not yet been addressed experimentally. Pyroptosis is discussed in more detail below.

Caspase-1 was identified in the late 1980's and early 1990's as a cysteine protease expressed only in monocytes and macrophages,[475–480] and was initially given the name *interleukin 1 converting enzyme* or ICE. When the enzyme was cloned and sequenced in 1992,[480] it was found to be unrelated to any other known protein, but by 1996, a further nine human and mouse homologues had been found.[481] In 1996, ICE was renamed *caspase-1*[482] in recognition of the fact that it was the first member of this new family to be discovered. There are currently 12 caspases recognised in humans and mice,[483] but caspase-12 is inactive in 64% of humans.[484]

The caspases were first studied in relation to their role in apoptosis,[481] but three caspases (human caspase-1, -4, and -5; mouse caspase-1, -11 and -12)[485] have roles in the processing and secretion of pro-inflammatory molecules. The only caspase for which we have detailed information in melioidosis is caspase-1.[473,474]

Caspase-1 is constitutively present in the cytosol as inactive pro-caspase-1.[479,480] Activated caspase-1 is responsible for converting pro-IL1β[480] and pro-IL18[486–489] to their active forms, and will also cleave pro-caspase-7.[490] It is also responsible for a type of programmed cell death called *pyroptosis,* which occurs only in the context of inflammation.[491,492]

Breitbach *et al.* studied experimental *B. pseudomallei* infection (100 cfu of intranasal *B. pseudomallei*) in *Casp1*-knockout mice on a C57Bl/6 background.[474] In this model, all *Casp1*-knockouts died ≤72 h after inoculation, whereas wild-type mice survived ≥6 days. Bacterial loads in lung, liver and spleen were also higher at 48 h in *Casp1*-knockouts compared to wild-type controls. This was confirmed by *in vitro* experiments, which showed that intracellular burdens of *B. pseudomallei* were 10-times higher in *Casp1*-

knockout macrophages. Serum concentrations of IL18 and IFNγ were both reduced in *Casp1*-knockout mice.

It has recently been discovered that the most commonly used strain of *Casp1*-knockout mouse is also caspase-11 deficient,[492] implying that work done to date with this mouse may require re-evaluation.

Caspase-3 is the only other caspase that has been studied in melioidosis. Breitbach reported that caspase-3 was activated only when *Casp1* was knocked out.[474] This is consistent with what we already know: *i.e., B. pseudomallei*-infected macrophages die by pyroptosis and not by apoptosis, and the function of caspase-3 is primarily the induction of apoptosis.[494–496] Nothing further is known of the role of caspase-3 in melioidosis. Other caspases (caspase-2, -4, and -8) have also been reported to be upregulated in a BALB/c murine model of melioidosis,[429] but have not otherwise been studied.

1.7.7 Caspase-1 substrates: IL1β, IL18 and caspase-7

Interleukin 1 was first identified by Gery in 1972 as a soluble factor produced by mouse peritoneal exudate cells that was capable of stimulating T lymphocyte proliferation.[497–499] Based on this property, Gery proposed the name *lymphocyte-activating factor* or LAF. Dinarello rediscovered the same cytokine in 1977 and called it *leukocytic pyrogen*. LAF and leukocytic pyrogen were shown to be the same in 1979, and renamed interleukin 1.[500] Interleukin 1 was successfully cloned in 1985, which led to the realisation that the IL1 consisted of two distinct but closely related proteins, IL1α and IL1β.[501]

IL1β is secreted primarily by monocytes and macrophages and has myriad functions.[502] Its systemic effects are fever, headache, nausea, myalgia and fatigue. It has endocrine effects (up-regulation of cortisol and ACTH secretion, but depression of insulin secretion) and inflammatory effects, including the up-regulation of chemokines and cytokines (TNFα, IL6, IL8, G-CSF and GM-CSF), adhesion molecules (*e.g.,* ICAM-1) and the activation of $CD4^+$ T lymphocytes, B lymphocytes and NK cells.[502]

Secretion of IL1β is a two-step process.[460] Pro-IL1β is not present in the cell in significant quantities, so pro-IL1β must first be transcribed and translated. Pro-IL1β induction is mediated by NF-κB, and therefore requires either TLR or NOD2 activation.[465] The second step is inflammasome assembly

and cleavage of pro-caspase-1 to its active form, which in turn leads to the conversion of pro-IL1β to mature IL1β.

In contrast to IL1β, preformed pro-IL18 is present in the cell in significant quantities, so secretion of IL18 is essentially a one-step process: *viz.,* the cleavage of pro-IL18 to mature IL18 by caspase-1.[503] This means that the IL18 response always precedes the IL1β responses. Although pro-IL18 is expressed by a wide variety of cell types, the caspase-1 inflammasome is only assembled in monocytes or macrophages,[477,486,487] which are therefore the only source of mature IL18.

Caspase-7 has been called an *executioner* or *effector* caspase,[494] because it cleaves a large set of substrates that ultimately result in the hallmarks of apopotosis, which are DNA fragmentation and mitochondrial damage.[495,496] Caspase-7 is upregulated in murine melioidosis,[429] but there is currently no published clinical data on caspase-7 in melioidosis. It seems unlikely that the primary role of caspase-7 in melioidosis should be apoptosis-related since Sun *et al.* have already shown that in the context of *B. pseudomallei* infection, the predominant mode of cell death is pyroptosis. In *Legionella pneumophila* infection,[504] caspase-7 has been shown to have a role in delivering endocytosed bacteria to the lysosome. Future studies on caspase-7 using *B. pseudomallei* may prove equally illuminating.

1.7.8 Caspase-1, pyroptosis, and cytokine-independent killing

Caspase-1 is responsible for inflammation-related programmed cell death called pyroptosis,[491,492] which is distinct from apoptosis. Apoptosis is an energy-dependent process involving the orderly dismantling of a cell by DNA fragmentation and blebbing, with minimal inflammation. By contrast, pyroptosis is not energy-dependent: instead, it involves perforation of the cell membrane, osmotic cell lysis and an accompanying burst of pro-inflammatory cytokines.[491] Pyroptosis is mediated by a macromolecular complex called the *pyroptosome,* which is similar to, but distinct from, the inflammasome. The pyroptosome is an oligomer, composed of multiple ASC dimers that rapidly recruit and activate caspase-1.[492,505] A cell only ever assembles a single pyroptosome.

Pyroptosis was originally described in *S.* Typhimurium infections of mouse macrophages.[491,492] Miao *et al.* found that although pyroptosis destroys the macrophage, this causes the intracellular organisms to spill out into the extracellular milieu where they are killed. Although Miao's primary observations were of *S.* Typhimurium, he also studied *B. thailandensis* (which is closely related to *B. pseudomallei*) and found that IL1β/IL18 double knockouts were still capable of controlling infection, but that caspase-1 knockouts were not. This lead him to conclude that caspase-1 is able to kill intracellular pathogens by a cytokine-independent mechanism, most likely pyroptosis.[506]

Sun *et al.* showed that *B. pseudomallei* induces caspase-1-dependent macrophage death, which is then accompanied by the release of IL1β and IL18.[473] Although the study predates the first description of the pyroptosome by two years, Sun found that the process was dependent on the presence of a functional type 3 secretion system and membrane pore formation, before proceeding to cell swelling and lysis.[473] These are all characteristic of pyroptosis, even though Sun was not able to apply that term to his observations. It should be noted that the precise mechanism by which pyroptosis destroys intracellular bacteria is unclear: Miao presented data suggesting that the bacteria are subsequently destroyed by neutrophils, but Breitbach's *in vitro* system still demonstrated an effect despite containing no neutrophils.

The data from cytokine and pattern recognition studies may be summarised thus: the story begins with *B. pseudomallei* infecting an immature monocyte. The infected monocyte is not able to kill the intracellular bacteria using reactive oxygen species (ROS) or reactive nitrogen species (RNS) because it has not been stimulated by IFNγ. The monocyte is, however, able to assemble inflammasomes (and a pyroptosome) under NLRC4 stimulation. The infected monocyte sends out a cytokine distress signal (TLR-mediated IL12 and NLR-mediated IL18) before destroying itself and the bacteria by pyroptosis. The distress signal is picked up by NK and $CD8^+$ cells, which generate and amplify the critical IFNγ signal. The IFNγ signal then activates intracellular killing mechanisms in other cells.

1.7.9 Monocytes and macrophages

Circulating monocytes may be stimulated to differentiate into any one of three cells types: dendritic cells, Langerhans cells, or macrophages.[507] Monocyte differentiation is directed by exposure to different environmental factors encountered as the monocyte progresses from the peripheral circulation to the tissues. Exposure to IL4 and granulocyte-macrophage colony stimulating factor (GM-CSF) causes monocytes to become dendritic cells;[508–510] exposure to IL15 plus GM-CSF causes monocytes to become Langerhans cells;[511] while exposure to IL6,[512,513] IL10[514] and macrophage colony stimulating factor (M-CSF)[513] causes them to mature into macrophages. Concentrations of M-CSF have never been measured in acute melioidosis, but plasma levels of IL6 and IL10 are high,[413,422] which supports a role for macrophages in melioidosis. IFNγ levels are also high in acute melioidosis,[369,376] and IFNγ is able to direct monocytes to differentiate into macrophages by stimulating autocrine IL6 and M-CSF production.[507]

In melioidosis, macrophages are the principal orchestrators of the host cytokine response. They are the generals and neutrophils are the foot soldiers. Although capable of killing *B. pseudomallei,* their primary function is not to kill, but to initiate the host cytokine response (by secreting IL12, IL18 and TNFα),[360,373,411] in inflammasome activation,[474] and in recruiting neutrophils to the site of inflammation (by secreting IL1β[515,516*] and chemoattractants such as IL8).

B. pseudomallei is a facultative intracellular pathogen, and monocytes are an important target.[52,79,80] Macrophages are susceptible to being parasitized by *B. pseudomallei* and may be subverted to become incubators for bacterial replication in human tissues.[52] The effective host response therefore depends on IFNγ stimulation. Macrophages stimulated by IFNγ are thought to kill intracellular pathogens by producing a burst of reactive nitrogen intermediates (RNI) (principally the production of nitric oxide from L-arginine, but also nitrogen dioxide and nitrous acid) and reactive oxygen species (ROI) (*e.g.,* superoxide anion, peroxide, hydroxyl radical and singlet oxygen).[517–520] RNIs

[*] IL1β is not chemoattractant *per se*, but it induces the secretion of chemoattractants and the expression of cell adhesion molecules on the endothelium.

and ROIs both appear to have a role in killing *B. pseudomallei,*[521,522] with the role of RNIs possible more important than that of ROIs,[521] but they are not the only mechanism by which stimulated macrophages are able to kill *B. pseudomallei*[519,523] and lysosomal killing is probably important.[524]

1.7.10 Lymphocytes

Host defences to melioidosis are dependent on macrophage-lymphocyte interactions,[525] but, unusually, T cell help does not predominate in this relationship.

In mouse models of melioidosis, the primary source of IFNγ is natural killer (NK) cells and $CD8^+$ T lymphocytes (at least in the earliest stages of the host response).[360,373,411] This stands in marked contrast to other pathogens such as *Leishmania major*[526,527] and *Mycobacterium tuberculosis,*[528,529] where IFNγ is produced primarily by $CD4^+$ T lymphocytes. The lack of a role for helper T cells in the initial host response to melioidosis means that it lies outside the Th1/Th2 paradigm within which host responses are usually defined.[530] This may also explain why clinical epidemiological studies have not found HIV infection to be a risk factor for melioidosis[180] (unlike other intracellular infections like leishmaniasis[531,532] and tuberculosis[533,534]), because HIV depletes $CD4^+$ cells without depleting NK or $CD8^+$ cells.[535]

A role has been found for $CD4^+$ cells in later stages of melioidosis in mouse models,[411] which leads one to speculate that melioidosis patients co-infected with HIV may have an increased risk of recurrence, but the small number of patients co-infected with HIV and melioidosis makes it difficult to collect enough epidemiological data to examine this question adequately.[180,307,536]

1.7.11 Neutrophils

The neutrophil chemoattractant, IL8 (also called CXCL8), is present in elevated concentrations in clinical melioidosis.[413] Mice do not have IL8, but do express the related chemokines, CXCL1, CXCL2, and CXCL5, all of which are elevated in experimental melioidosis.[242] Unsurprisingly, leukocytosis is a feature of acute clinical melioidosis and this leucocytosis is predominantly a neutrophilia.[242]

The role of neutrophils in experimental *B. pseudomallei* infection has been demonstrated convincingly in two mouse studies.[326,537] Bacteria were detectable

by flow cytometry ≥24 hours after infection, and the only cell type with which bacteria were measurably associated were neutrophils, not monocytes or macrophages. The bacteria were confirmed to be intracellular by confocal microscopy, but the viability of these bacteria was not confirmed.[537] The ability of *B. pseudomallei* to invade and replicate within neutrophils has been confirmed by multiple independent teams,[79,80,538,539] but the question of whether neutrophils are a site for *B. pseudomallei* survival and replication *in vivo* remains to be answered.

Neutrophils are an important part of the host defence against melioidosis, and animals in whom neutrophil function is compromised do worse at controlling the infection. Easton *et al.* ablated neutrophils using a monoclonal antibody against *Ly6g* (formerly called *Gr1*) and found that bacterial loads were much higher after four days than in untreated mice.[326] *Secreted phosphoprotein 1* (SPP1, formerly called osteopontin) is a 33 kDa extracellular matrix protein that is cleaved by thrombin during inflammation to reveal a cryptic sequence that is recognised by integrin receptors present on neutrophils.[540] SPP1 therefore plays a role in the neutrophil recruitment during melioidosis. In agreement with Easton's results, van der Windt *et al.* found that *Spp1*-knockout mice had fewer neutrophils at the site of infection, had higher bacterial loads and worse survival.[242] Plasminogen activator, urokinase receptor (*Plaur,* formerly called uPAR or CD87) is expressed by neutrophils and has also been shown to be necessary for neutrophil recruitment, probably via an effect on integrin expression.[242] Again, *Plaur*-knockout mice had a reduced ability to recruit neutrophils to an area of infection and had a reduced ability to clear *B. pseudomallei,* although this time, survival was marginally better in *Plaur* knockout mice.[541] This apparent contradiction might be explained by the fact that *Plaur* also promotes phagocytosis[541] and *B. pseudomallei* is capable of subverting leukocyte endocytosis to further its own survival.

Part of the difficulty with studying neutrophils has been that freshly isolated neutrophils die within hours of stimulation with live bacteria. It now appears that this self-destruction is due to a newly described form of neutrophil-mediated killing, in which neutrophils expel their own chromatin to form extracellular traps (NET).[542] The role of NETs in the host response to

B. pseudomallei remains to be explored, but seem likely to be an important part of the host response to melioidosis.

1.8 Diabetes and infection

Diabetes mellitus is a heterogenous group of disorders characterized by chronic elevation of serum glucose levels. The majority of diabetes cases are primary and idiopathic, but a minority of cases occur secondary to other conditions. Primary diabetes is divided into types 1 and 2, with type 1 diabetes being primarily a disease of insulin-deficiency with onset in childhood, resulting from the loss of insulin-producing beta-islet cells of the pancreas. Type 2 diabetes is associated with obesity,[543,544] increasing age[544–546] and family history.[547] The primary lesion is not insulin deficiency, but insulin resistance, with the tissues requiring ever higher concentrations of insulin to achieve the same effect. Diabetes may also occur secondary to pregnancy,[548] corticosteroid use,[549–552] Cushing's disease,[553] pancreatitis,[554] pancreatic resection,[554] cystic fibrosis,[555] haemochromatosis (both primary[556] and secondary[557]) and acromegaly.[558,559]

The majority of cases that occur worldwide are due to type 2 diabetes and there is a problem of under diagnosis, even in developed countries,[560] because patients often feel well in the early stages of type 2 diabetes and because screening programmes are not often in place. In 2010, there were an estimated 347 million adults worldwide with diabetes and that number is projected to increase.[546] Although age standardised rates of diabetes are not increasing in Southeast Asia, the number of patients with diabetes is increasing due to population growth and aging.[546] However, the epidemiology of diabetes in Thailand is complicated by the fact that diabetes in Thailand is associated with relative insulin deficiency, not just insulin resistance.[775] The reasons for this are not known.

1.8.1 Diabetes and infection

Patients with diabetes mellitus have an increased risk of developing infections and sepsis,[561,562] and constitute 20·1–22·7% of all sepsis patients.[334,563] This association was first observed a thousand years ago by Avicenna (980–1027 CE), who noted that diabetes was frequently complicated by tuberculosis.[564] In the pre-insulin era, Joslin noted in a series of 1000 cases

that diabetic coma was usually precipitated by infection.[565] Even today, infection remains an important cause of death in diabetics.[566] Much of the literature does not distinguish between types of diabetes and regards all complications as secondary to hyperglycaemia and as independent of diabetes aetiology.

A small number of conditions are strongly associated with diabetes, including malignant otitis externa,[567–569] emphysematous pyelonephritis,[570–573] emphysematous cholecystitis,[574,575] *Klebsiella* liver abscesses,[576] and rhinocerebral mucormycosis,[577,578] so much so, that when the diagnosis is made in someone who is not known to have diabetes, the clinician should consider testing for diabetes.

Most of the aforementioned infections are unusual. Instead, most infections in patients with diabetes are those that occur also in the general population. Two population-based studies have proved pivotal to our understanding of the susceptibilities of patients with diabetes:[561,562] a study of 523,749 Canadians with diabetes and an equal number of matched controls [562] found diabetes increased the risk for cystitis (risk ratio 1·39–1·43), pneumonia (1·46–1·48), cellulitis (1·81–1·85) and tuberculosis (1·12–1·21). A study of 7,417 Dutch patients with diabetes found a higher incidence of lower respiratory tract infection (adjusted odds ratios 1·42 for type 1 diabetes and 1·32 for type 2), urinary tract infection (1·96 and 1·24), and skin and mucous membrane infection (1·59 and 1·33).[561] The association between diabetes and tuberculosis was re-confirmed by a recent meta-analysis of 16 studies spanning a 50-year period.[579]

Although diabetes mellitus is implicated in susceptibility to infection, its influence on the subsequent clinical course and outcome is less clear. Some studies have shown an association with increased mortality,[580–583] others found no effect,[563,584–592] while still others found improved survival.[574,575,593] The largest of these (12·5 million sepsis cases)[574] found that patients with diabetes were less likely to develop acute respiratory failure and linked this to two previous studies that found diabetics seem protected from acute lung injury.[594,595] The largest single study to show an adverse effect of diabetes on mortality in sepsis was conducted in 29,900 Danish patients with community-

acquired pneumonia and found that patients with diabetes had a higher risk of mortality (OR 1·2).[582]

The reasons for different outcomes between these studies are unclear, but may relate to differences in study population, varying outcome measures, differences in statistical analysis and in diabetes drug prescription habits between countries.[329] Population-based studies are less prone to selection bias compared to hospital-based studies, but more detailed clinical information is usually available in hospital-based studies. In terms of outcome measures, studies with outcomes at longer time points (*e.g.*, 6 months versus 28-day mortality) are more likely to find informative differences, but are much more difficult to conduct.[596] Observational studies often make use of multivariable regression techniques to correct for confounders (a common, but incorrect, approach to model-building is to include all measured parameters and then remove parameters on the basis of *p*-value). Overadjustment or unnecessary adjustment for variables that are not confounders can produce biased or spurious results (these issues are further discussed in Chapter 2).[597] Patients with diabetes also have multiple co-morbidities that may worsen outcomes: it is debatable whether these co-morbidities should be adjusted for, since many are caused by diabetes itself and therefore, by definition, are not confounders.[598] Nevertheless, a number of studies have adjusted their results for these comorbidities, regardless of the validity of these adjustments.[581,582,584]

1.8.2 Diabetes and the host response

In 1904, Lassar suggested that high levels of glucose may drive infection by serving as a nutrient source for bacteria,[599] but in 1911 Handmann showed that glucose supplementation did not enhance bacterial growth,[600] and proposed instead a defect in immune function, which Da Costa and Beardsley demonstrated in 1907.[601] The subsequent literature on this topic is complicated by the fact that different techniques have been used over the years, and gaps of a decade or more may separate experiments, making it difficult to compare results. Most studies have shown defects in neutrophil function, with good evidence for abnormalities in adhesion, chemotaxis and intracellular killing, but evidence for a phagocytosis defect are contradictory. The evidence that neutrophil defects are solely responsible for the increased susceptibility of

diabetics to infection is equivocal.[602] There is good evidence that humoral responses in diabetics are poorer, and may play a larger role than previously recognized.

1.8.3 Diabetes and general markers of inflammation

Diabetes is associated with elevations in CRP,[603] TNFα,[604] IL6[603] and IL8,[605] but no differences are seen in circulating cell surface markers or coagulation markers between patients with and without diabetes in the context of sepsis.

In a cohort of 1,799 patients with community-acquired pneumonia (CAP),[606] concentrations of pro-inflammatory cytokines (TNFα, IL6 and IL10), coagulation (antithrombin, Factor IX and thrombin-antithrombin complexes) and fibrinolysis (PAI-1 and D-dimer) biomarkers were similar in subjects with and without diabetes at presentation and in the first week of hospitalization.[606] In addition, monocyte expression of CD120a, CD120b, HLA-DR, TLR4 and TLR2 on monocytes was not different between the groups.[606] These results are consistent with a cohort study of 830 sepsis patients, in whom plasma concentrations of IL6 and TNFα were elevated to the same extent in patients with and without diabetes, both at admission and at follow-up.[563] In this second study, diabetes was not found to exacerbate the known procoagulant response seen in sepsis.[563] Since sepsis and diabetes both induce a proinflammatory and procoagulant state and since both interfere with the host response, the lack of a strong influence of diabetes on the proinflammatory and coagulation pathways during sepsis is remarkable. Preclinical studies in healthy volunteers have shown that acute hyperglycæmia and insulin resistance may both directly influence inflammation and coagulation,[607,608] but these changes may not be detectable on the background of the much larger abnormalities attributable to sepsis. There is also evidence that local responses may be impaired in diabetes: *e.g.*, levels of urinary IL6 and IL8 are lower in diabetic women with bacteriuria.[609] Endothelial activation has been implicated in the pathogenesis of sepsis[610] and diabetes is itself known to activate endothelium. A recent study of 207 sepsis patients (of whom 30% had diabetes) showed that markers of endothelial cell activation (plasma E-selectin and sFLT-1) were higher in diabetes.[611]

1.8.4 Diabetes and neutrophil adhesion

Recruitment of neutrophils to a site of inflammation requires endothelial adhesion followed by transmigration and exit from the circulation, a process requiring the expression by neutrophils of integrins (*e.g.*, CD11a/CD18 and CD11b/CD18),[612,613] which then bind to endothelial cell adhesion molecules (*e.g.*, intercellular adhesion molecule 1, or ICAM-1[614–616]).

A study in which neutrophils were harvested from 26 patients with diabetes and an equal number of controls demonstrated that adhesion to bovine aortic endothelium was increased for neutrophils from diabetics, but only if the endothelium was also incubated with plasma from patients with diabetes.[617] Increased adhesion appears to be due to both an increase in expression of integrins by diabetic neutrophils and of adhesion molecules by endothelium. Diabetic neutrophils have increased expression of CD11b and CD11c,[618] and glucose itself appears able to stimulate the expression of ICAM-1 by endothelial cells,[614–616,619–621] possibly via an osmotic effect.[620,621]

1.8.5 Diabetes and neutrophil chemotaxis

Chemotaxis is the ability of neutrophils to detect and move towards a chemical stimulus. Studies of chemotaxis may be divided by technique: those using the two-chamber Boyden technique[622] have produced conflicting results,[623,624] but the subagarose technique[625] (which includes a negative control, which Boyden's technique lacks) have reproducibly shown a defect in diabetes.[618,626]

1.8.6 Diabetes and neutrophil phagocytosis

Phagocytosis is the engulfment and ingestion of foreign bodies by a cell, allowing neutrophils to remove and destroy pathogens.

The evidence for a defect in phagocytosis in diabetes is contradictory, with some reporting a defect,[627–630] but others not.[618,631] These inconsistencies may be attributed to differences in methodology: neutrophils will not phagocytose unopsonised particles, so bacteria and cells need first to be incubated with serum containing C3b or IgG. Many studies have used autologous serum,[627–630] but those that have used a standard serum or opsonin have found no defect.[618,631] In 1976, Bagdade found that phagocytosis of *Streptococcus pneumoniae* was reduced in neutrophils recovered from eight patients with

poorly-controlled diabetes, but this defect improved with diabetes treatment.[629] Notably, control neutrophils incubated with serum taken from patients with diabetes also demonstrated a defect in phagocytosis, implying that the defect is in fact due to defective opsonisation and not to a deficit in neutrophil phagocytosis *per se*: in other words, the defect is humoral. In 1984, Davidson *et al.* studied ingestion of *Candida guilliermondii* by neutrophils from 11 patients with diabetes and found that phagocytosis was reduced. However, if pre-opsonized yeast cells were used, then phagocytosis was no different from controls, again suggesting that a humoral defect must exist.[631] Delamaire *et al.* used a single control serum for all samples to remove the possibility of a difference in opsonisation,[618] convincingly demonstrating that no phagocytosis defect exists in diabetic neutrophils.

1.8.7 Diabetes and neutrophil-mediated killing

Neutrophils have two distinct mechanisms for killing bacteria, intracellular and extracellular. Phagocytozed bacteria are killed by superoxide anions and other oxygen-derived species. Culture-based methods have demonstrated a defect in intracellular killing of *Staphylococcus aureus,*[626,632,633] *Streptococcus pneumoniæ,*[628,634] and *Candida albicans*.[635] More recent studies have confirmed this finding used chemiluminescence methods,[636–639] a superior method compared to culture, because it separates the effect of phagocytosis from that of intracellular killing. The killing defect cannot be corrected by incubation with normal serum,[633] suggesting that it is cellular in origin. The defect improves with glycaemic control.[638]

Neutrophils are also able to kill bacteria extracellularly by expelling chromatin, which combines with granule proteins to form NETs.[542] Interestingly, β-hydroxybutyrate (a ketone body present in diabetic ketoacidosis) has been shown to inhibit the formation of NETs,[640] but the relevance of this finding to patients remains to be demonstrated.

1.8.8 Diabetes and monocyte function

Monocytes in diabetes have been less well studied than neutrophils, but also appear to have defects of chemotaxis[641] and phagocytosis.[642,643] Adhesion to endothelium is also enhanced.[644,645] In contrast to neutrophils, intracellular killing seems to be enhanced.[646] Monocytes obtained from 24 diabetic patients

produced similar amounts of TNF-α when compared to healthy controls when stimulated with lipopolysaccharide (LPS), but IL-6 levels were higher in patients with type 1 diabetes.[647]

1.8.9 Diabetes and lymphocyte function

Few studies have looked at the effect of diabetes on lymphocyte function. One measure of lymphocyte function is transformation in response to a mitogen or bacterial antigen. Studies containing acidotic patients appear to find that responses are diminished[648,649] and that correction of the acidosis leads to prompt resolution of the defect,[649] but more recent studies have found deficient proliferative T-cell responses even in treated patients.[639,650] Diabetic T-cells express higher levels of CD152, a down regulator of the immune response.[651] Three other studies failed to find a defect in lymphocyte function.[624,652,653]

1.8.10 Humoral defects

In 1907, Da Costa and Beardsley found that sera from diabetes patients were less able to opsonize *S. aureus* compared to sera from controls.[601,654] In 1973, Farid and Anderson surveyed 46 patients and found that IgG levels were lower in insulin-treated diabetics, but not in patients on oral treatments or diet alone. More recently, a study of 66 patients with type 1 diabetes demonstrated that total IgG levels were lower in patients with uncontrolled diabetes as measured by HbA1c.[655] Also, the apparent defect in neutrophil phagocytosis appear to be humoral and not cellular in origin (see above).

The best evidence for a humoral defect in diabetic patients comes from vaccine studies. It was described as early as 1930 that deficient agglutinin responses are seen in diabetic patients after subcutaneous typhoid vaccination.[656,657] Multiple studies have shown that patients with diabetes are less likely to mount a protective antibody response to hepatitis B vaccination,[658–661] leading some authorities to recommend routinely adding a booster dose to the standard regimen for patients with diabetes.[658,662] The literature on influenza vaccination is more mixed (reviewed by Brydak and Machala[663]). Pozzilli *et al.* looked at 52 diabetic patients and found fewer activated lymphocytes in patients with type 2 diabetes following influenza vaccination, but no differences in antibody responses.[664] Muszkat *et al.,*

studying a more elderly population, found lower antibody responses in patients with type 2 diabetes.[665]

Diabetes is also associated with a waning in the duration of protection afforded by tetanus vaccination although the initial response appears to be normal.[666,667] Patients with diabetes appear to respond well to pneumococcal polysaccharide vaccine,[668] although there are no studies studying duration of protection in diabetic patients. There are also no studies specifically linking humoral responses in sepsis to diabetes.

1.8.11 Complement abnormalities

Inherited deficiencies of component 4 (C4) have been implicated in the pathogenesis of type 1 diabetes,[669–671] but whether this contributes to susceptibility to infection in type 1 diabetics is not known. By contrast, obesity and elevated insulin levels (as occur in type 2 diabetes) appear to be associated with elevations in C3.[672] Karlsson *et al.*, looking for biomarkers for maturity-onset diabetes of the young (MODY), found that complement C5 and C8 are both elevated in diabetes regardless of etiology,[673] a possible mechanism for these abnormalities being that complement activation can be driven by glycated immunoglobulins.[674] One explanation for why diabetic sera are less able to opsonize bacteria may be that glucose attacks the thiolester bond of complement C3, thus preventing it from binding to the bacterial surface.[675]

1.9 Diabetes and melioidosis

1.9.1 B. pseudomallei *and the diabetic neutrophil*

Diabetes is the most important risk factor for melioidosis, and the epidemiological evidence for this has been reviewed above. We also know that the host response to melioidosis is critically dependent on neutrophils,[326] and diabetes is known to cause defects in neutrophil function, as reviewed above.

The effect of diabetes on the neutrophil response to *B. pseudomallei* infection has been explored by Chanchamroen *et al.*[539] Neutrophils were collected from 56 patients with diabetes who were otherwise healthy and compared to 36 healthy controls. Cells were then infected with *B. pseudomallei* K96243. All study subjects were from Northeast Thailand.

Chanchamroen demonstrated that *B. pseudomallei* is able to persist intracellularly in neutrophils. She also reported impairment of phagocytosis, chemotaxis and an increased rate of neutrophil apoptosis. Her results are difficult to interpret due to a number of limitations in the study design as follows:–

Chemotaxis was studied using the two-chamber method,[622] which is inferior to the subagarose technique[625] because it has no negative control and cannot distinguish between specific and non-specific migration. Despite this, she was still able to show a chemotactic defect in diabetic neutrophils.

Phagocytosis was measured using autologous serum, a method which we now know to be severely confounded by humoral effects. The study appeared to show increased intracellular killing by diabetic neutrophils, which is a totally unexpected finding. The intracellular killing assay is severely confounded by the fact that phagocytosis was poorer in diabetic neutrophils, which means the initial number of intracellular bacteria was smaller in the diabetic neutrophils. This was not corrected for in the analysis, which in turns means that no meaningful comparison between diabetic and non-diabetic neutrophils was possible. The same study demonstrated that *B. pseudomallei* appeared to delay apoptosis in healthy neutrophils but not in diabetic neutrophils: however, interpretation of this result is also problematic due to the same confounding factors.

1.9.2 B. pseudomallei *and the diabetic macrophage*

Williams *et al.* studied peritoneal macrophages and bone marrow-derived dendritic cells harvested from C57Bl/6 mice made hyperglycaemic by treatment with low-dose streptozocin (STZ).[676] In this model, mice were treated with 55 mg/kg STZ intraperitoneally for six days; control mice were treated with buffer only. Mice were considered diabetic if their blood glucose levels were ≥13 mM for 9 days. Mice in the 'acute diabetes' group were sacrificed 19 days after the first STZ injection, while mice in the 'chronic diabetes' group were sacrificed 70 days after the first infection.

Dentritic cells were harvested by culturing bone marrow for 10 days with GM-CSF. Peritoneal macrophages were harvested after injection of the inflammatory agent, Brewer's thioglycollate. Phagocytosis was measured using

the ability to internalise fluorescent beads. Intracellular killing was measured by lysing the cells with 0·1% Triton-X, then culturing the lysate on Ashdown's agar. Colony counts at 18 hours were expressed as ratios of the number of bacteria internalised at 4 hours, thus adjusting for differences in phagocytosis. Cytokine expression was measured by quantitative PCR.

Williams found no differences in cells collected from the acute diabetes group when compared to the controls, but in cells collected from the chronic diabetes group, intracellular survival of *B. pseudomallei* was greater than in controls. She also found that expression of IL11β, IL12, IL18 and TNFα were depressed in the chronic diabetes group compared to the control group.

1.9.3 Melioidosis in murine models of diabetes

Hodgson has published a murine model of melioidosis in type 2 diabetes, using the BKS.Cg-*Dock7*^{m} +/+ *Lepr*^{db}/J mouse (also called the *db/db* mouse).[677] This inbred strain was originally created in 1966 from a C57Bl/KsJ background.[678] Homozygotes for the spontaneous leptin receptor mutation, *Lepr*^{db}, become hyperinsulinaemic at 10–14 days of age, obese at 3–4 weeks, hyperglycaemic at 4–8 weeks and die at around 10 months.[679] Non-diabetic litter mates heterozygous for the *Lepr*^{db} mutation were used as controls.

Diabetic mice were more susceptible to *B. pseudomallei* infection than age and sex-matched controls: following a subcutaneous challenge of 4·6 × 10^6 cfu, all mice in the diabetic group were dead by day 4, whereas the control mice all survived until day 11. In this study, no differences in neutrophil oxidative burst were found between diabetics and controls, but no other test of neutrophil function was performed. This is in contrast to another study by a different group using *S. aureus* that showed impaired oxidative burst in *db/db* neutrophils.[680]

Hodgson found no differences in bacterial loads in liver or spleen, but did find higher loads in subcutaneous tissue. Concentrations of TNFα and IL1β mRNA in the spleens of *db/db* mice were higher than controls. *In vitro* studies of peritoneal macrophages harvested from *db/db* mice showed that *B. pseudomallei* was better able to survive in *db/db* macrophages and that *db/db* macrophages produced lower levels of nitric oxide. Hodgson therefore attributed the increased susceptibility of diabetic mice to a defect in macrophage responses.

1.9.4 Melioidosis and diabetes in the clinic

It has been recognised that once melioidosis has been acquired, patients with diabetes paradoxically seem to do better. In a prospective observational study of 755 melioidosis patients, diabetes was independently associated with survival (adjusted odds ratio for mortality 0·36, 95% confidence interval 0·25–0·50).[681] This is at odds with what we know about the effect of diabetes on the host response, which would lead us to predict that outcomes should be poorer in diabetes. This finding is not unique to melioidosis, and the conflicting data in the literature on the influence of diabetes on sepsis outcomes has been discussed above.

1.10 Outline of the work

In this dissertation, the link between diabetes and melioidosis is explored using classical epidemiological techniques (Chapter 2), and it is shown that this link is attributable to the use of glibenclamide prior to hospital admission for melioidosis and not to diabetes *per se*. The host response to melioidosis is explored by studying gene expression in peripheral blood leukocytes (Chapter 3), and glibenclamide is shown to have an anti-inflammatory effect in patients with melioidosis. The effect of glibenclamide is then described in a mouse model of melioidosis (Chapter 4), in which it is shown that glibenclamide reduces interleukin-1β secretion.

2 Glibenclamide, not diabetes, protects from mortality in melioidosis

2.1 Introduction

2.1.1 The melioidosis cohort at Sappasithiprasong Hospital

The Wellcome Trust was founded in 1936 under the will of Sir Henry Wellcome, co-founder of the pharmaceutical firm, Borroughs-Wellcome, who established tropical medicine research laboratories in London and Khartoum following visits to Ecuador and The Sudan. The Wellcome Trust Unit in Bangkok was started in 1979 as a long term collaboration between Oxford University and Mahidol University by Professor Sir David Weatherall, Professor David Warrell, Professor Emeritus Chamlong Harinasuta and Professor Emeritus Khunying Tranakchit Harinasuta with support from the then director of the Wellcome Trust, Dr Peter Williams.

The Unit's involvement in melioidosis research began when the then director of the Wellcome Trust Unit, Professor Nicholas White, was approached by a physician from Ubon Ratchathani, Professor Wipada Chaowagul, who had noticed increasing numbers of melioidosis cases in her practice. The laboratory at Sappasithiprasong Hospital (Figure 11) was established by David Dance and Nicholas White in 1986.

Ubon Ratchathani is a province in Northeast Thailand (Figure 12), 629 km to the east of Bangkok. It is bordered by Laos to the east (from which it is separated by the Mekong River) and Cambodia to the South. Ubon Ratchathani was founded in 1786 as a vassal state of Siam and became a province of Thailand in 1882. It was the largest province in Thailand until Yasothon (1972) and Amnat Charoen (1993) gained status as independent provinces.

Figure 11. Sappasithiprasong Hospital, Ubon Ratchathani.

Note.—The front façade of Sappasithiprasong Hospital, Ubon Ratchathani. Sappasithiprasong is a 1000-bedded tertiary referral centre for the province of Ubon Ratchathani. ©2008 GCKW Koh.

Ubon Ratchathani province has an area of 16,112 square kilometres and a population of 1·8 million (2009 data). It is subdivided into 25 districts (called amphoe) and is further subdivided into 219 subdistricts (thambon). The largest city is Ubon Ratchathani city (population 85,000), followed by Warin Chamrap (population 31,000). The main industry is rice farming. The climate is tropical, with a rainy season that runs roughly from June to October, and a dry season that runs roughly from November to May.

Sappasithiprasong Hospital is a 1000-bedded provincial hospital. It is the largest hospital in the region and a tertiary referral centre for 25 community hospitals. Data from all patients who were culture-positive for *B. pseudomallei* have been collected prospectively from the establishment of the Wellcome Trust laboratory at Sappasithiprasong until 2007. The data from this cohort has been an important source of information on the epidemiology of melioidosis.[125,169,170]

Figure 12. Ubon Ratchathani province.

Note.—Ubon Ratchathai province is marked in red. The province is bordered by the Mekong River to the east, which separates it from Laos, and Cambodia to the south. Its internal boundaries are formed by the provinces of Yasothon and Amnat Charoen to the north and Srisaket to the west. ©2009 NordNordWest, Wikimedia Commons.

It has previously been observed in Ubon Ratchathani, that although patients with diabetes are more likely to develop melioidosis, they are also less likely to die from it.[681] This finding was made incidentally in a study of the prognostic value of semi-quantitative urine cultures in melioidosis and diabetes was not the primary exposure of interest. We sought to confirm this association between diabetes and survival in a separate cohort of patients from the same hospital using causal reasoning to identify possible confounders, and then to adjust for these confounders using multivariable logistic regression models. We hoped our methods would be rigorous enough either to confirm convincingly the effect of diabetes, or else to identify alternative explanations for the observed effect.

2.1.2 Logistic regression

Logistic regression has two common uses. The first is to estimate the probability of a particular outcome given some set of risk factors. An example of this first use is the APACHE score, which combines multiple clinical and laboratory parameters to provide an estimate for ICU mortality. A second use is to adjust for confounding in an estimate of the effect of an exposure on outcome. It is for this second use that we employed logistic regression in the cohort study.

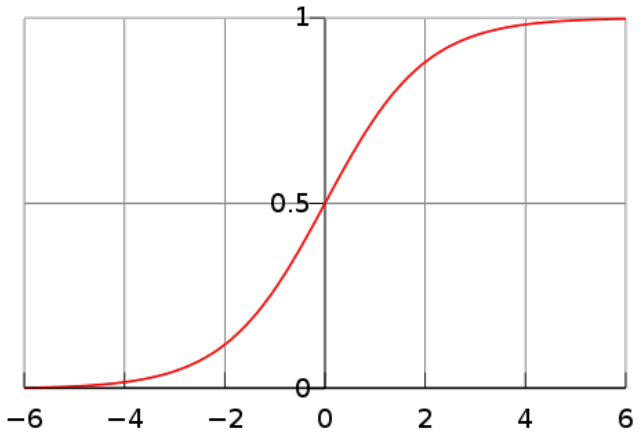


Figure 13. The logistic function.

Note.—The logistic function can take any value as its input, but its output is constrained to lie between 0 and 1. More formally, the domain of the logistic function is (−∞, +∞), and its co-domain is (0, 1). ©2008 Qef, WikiMedia Commons.

The logistic equation may be defined as,

$$y = \frac{1}{1+e^{-z}}, \qquad \textbf{Equation 1}$$

where

$$z = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \cdots + \beta_n x_n. \qquad \textbf{Equation 2}$$

The main value of the logistic function is that it may take as its input, x, any value for x from $-\infty$ to $+\infty$, but its output, y, is constrained to lie between 0 and 1 (Figure 13). This is why the function is given the name, 'logistic': it is useful for circumstances where the outcome may take only two values, e.g., 1/0, yes/no, true/false. The value of y may then be interpreted as the probability of the outcome being 'yes' or 'true', given a particular value for x.

The logistic function may take any number of inputs, x, (in the equation above, these inputs are numbered, $x_1, x_2, \ldots, x_n$), however, there can be only one output, y. The variables, x and y, may be called by a variety of names, depending on the context (Table 7).

x	***y***
input	output
abscissa	ordinate
independent variable	dependent variable
co-variate	outcome
exposure	disease
risk factor	

Table 7. Alternative names for *x* and *y* in multivariable regression models.

Note.—The variables x and y can take a great many names depending on the context. The terms input/output and abscissa/ordinate are used mainly be mathematicians. Statisticians tend to speak of independent/dependent variables, while epidemiologists talk of exposure/outcome or risk factor/disease. The term 'disease' is very inexact, because depending on the context, different diseases may appear as *x* or *y* variables.

The logistic equation has been presented here in a form that emphasises its link to linear regression. Equation 2 is in fact the linear regression model. In Nelder and Wedderburn's method of generalised linear models,[682] logistic regression is related to linear regression via equation 1, which is the link function.

The logistic function, in its simplest form, was first described by a Belgian mathematician, Pierre-François Verhulst, in 1838,[683] as the exact solution to the first order differential equation,[†] $\frac{dy}{dx} = \beta y(1-y)$.

[†] Here is a simple demonstration that $y = \frac{1}{1+e^{-x}}$ is a solution for $\frac{dy}{dx} = y(1-y)$.

Note that β may be any constant, so for the purposes of this analysis we may set it to 1 without affecting the validity of the results.

The paper of 1838 was brief, but a fuller account was given in 1845,[684] in which, for the first time, Verhulst calls the solution, *la courbe logistique* or 'the logistic curve'. Verhulst applied the equation to model population growth, in the situation where that growth is initially exponential, but then encounters resistance (that is, the exhaustion of resources). Berkson was the first to advocate its use in bioassays[685] (a variation of this curve is commonly used to analyse ELISA results) and other catalytic processes.[686] Berkson also introduced the term 'logit' (by analogy to 'probit').[686] The logistic function is therefore also a good model for the market penetration of iPhones, which are also a catalytic process: growth is initially exponential, but then slows as the market becomes saturated.[686]

The use of the logistic function in case-control studies arose naturally. If e^z is defined as the odds, then z is the log odds, and the linking function (Equation 1) is then mathematically identical to the risk. The application of the logistic function to binary data may be attributed to Cox, who published a series of papers in the 1960's, culminating in an influential textbook on the subject in 1969.[687] Two events allowed the expansion of logistic regression in epidemiological research during the 1990's: the first was the availability of computer software packages that could fit the models automatically (fitting curves by hand is laborious and takes weeks of tedious calculation). The second was the publication of a textbook by Hosmer and Lemeshow in 1989,[688] which provided guidance on building models while omitting mathematical details that could be left to the computer.

Mathematically, the minimum number of precisely specified data points needed to fit a logit model is one plus the number of inputs to be estimated. In Verhulst's original 1838 paper, he fit curves for the populations France, England and Russia based on three points only each. The naturally occurring variation in clinical data means this is never possible in practice. Instead, the number of inputs that may be included in a model is limited by the amount of data available to fit the model.

There is no consensus on how much data is 'enough'. Computer simulations suggest that for n variables, a minimum of $10n$ data points are

---

Starting with $y = \frac{1}{1+e^{-x}} = \frac{e^x}{1+e^x}$, multiplying out the fractions yields $y + ye^x = e^x$.
Differentiating, gives us , $\frac{dy}{dx} + e^x\frac{dy}{dx} + ye^x = e^x$. Re-arranging, $\frac{dy}{dx} = \left(\frac{e^x}{1+e^x}\right)(1-y) = y(1-y)$.

needed for each input variable.[689] Some authorities have suggested that is too stringent;[690] but some have suggested instead much larger numbers, of the order of 4^n.[691] If the figure of $10n$ is right, then a model with ten variables only needs a study of 50 patients; using a figure of 4^n, the same study needs 1024 patients. These uncertainties mean that sample size calculations for multivariable models are often done simply for the univariable analysis relating exposure and outcome, and not for the multivariable analysis.

2.1.3 Adjusting for confounding

Logistic regression provides a powerful method for adjusting for confounding. In a cohort study, where the natural metric for risk is the risk ratio, odds ratios are still frequently reported, because these can be adjusted for confounders using logistic regression.

The classical method of adjusting for confounding is the Mantel-Haenszel method. Briefly, one stratifies the data by each confounder, calculates the estimate for each stratum, then combines the stratified estimates into a single summary estimate.

The method is best illustrated using a hypothetical example. Consider the effect of diabetes on mortality (Figure 14). A worked example of stratification using invented data is presented in Table 8.

The Mantel-Haenszel method has two major problems: the first is that it is not good at handling adjustment for several confounders. The second is that the method will only allow categorical confounders, so continuous data first needs to be transformed into categorical data before it can be used, which in turns leads to inefficiencies (in that one has to discard information before performing the analysis). Using a logistic regression model to adjust for confounding addresses many of these concerns. The model easily accepts continuous variables as inputs and is more forgiving of sparse data.

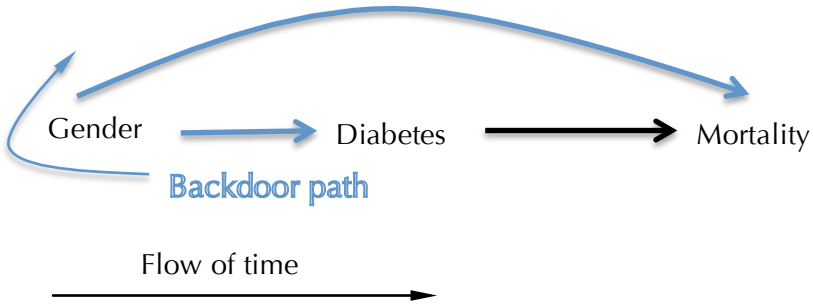


Figure 14. The influence of diabetes on mortality is confounded by gender.

Note.—The effect of diabetes on mortality is confounded by the effect of gender, because gender is associated with both diabetes and with mortality and does not lie on the causal pathway between diabetes and mortality.

The thick arrows in the graph indicate the flow of causation and must always follow the direction of time, because effect always follows cause. Confounding occurs when one is able to follow a path from exposure to outcome that goes against the direction of causation at some point (*i.e.*, against the flow of time). This is known as a backdoor path. In this example, the effect of interest is the effect of diabetes on mortality (black arrow); there is however, a backdoor path linking diabetes and mortality that runs through gender (blue arrows). Gender is therefore a confounder for the effect of diabetes on mortality.

One cannot infer causation from data alone, in part, because the data contain no information about time sequence. Information about time ordering must be added to the analysis by the investigator. In epidemiological studies, the person with this information is the epidemiologist, not the statistician. It is therefore never the statistician's responsibility to identify confounding.

A. Fictional population

	Male		Female	
	Diabetic	Not diabetic	Diabetic	Not diabetic
Died	450	10	200	360
Total	900,000	100,000	100,000	900,000

B1. Matched case control study: unstratified

	Diabetic	Not diabetic
Died	650	370
Alive	470	550

B2. Matched case-control study: stratified by gender

	Male		Female	
	Diabetic	Not diabetic	Diabetic	Not diabetic
Died	450	10	200	360
Alive	414	46	56	504

Table 8. A hypothetical study population.

Note.—**Table A** presents data from a purely hypothetical population categorised by three parameters: gender (male/female), diabetic (yes/no) and mortality (died/survived). **Table B** shows data from a case-control study drawn from that hypothetical population. The study investigators in our idealised study were able to recruit every patient who died, and the controls are perfectly frequency matched by gender.

A: the odds ratio for a person dying if he or she has diabetes is 5.0 = (450×100,000)/(10×900,000) = (200×900,000)/(360×100,000).

B: In B1, the odds ratio as estimated from the unstratified data is (650×550)/(370×470) = 2·1. This value is clearly incorrect, because in this hypothetical example, we know that the true population odds ratio is 5·0, because that is what we set it to be. In this case, we know that the estimate is confounded by gender. In B2, the same data has been divided into two strata, Male or Female. This removes the effect of confounding, because every person in each stratum has the same gender (either Male or Female). In the Male stratum, the odds ratio is (450×46)/(414×10) = 5·0; In the Female stratum, the odds ratio is (200×504)/(56×360) = 5·0. Combining the odds ratios in this simple example yields a summary odds ratio of 5·0, which is the correct value.

Aside from demonstrating how to adjust for confounding, this example demonstrates that matching on a confounder in a case-control study is not sufficient to remove the confounding. The final estimates from a matched case-control study must still be adjusted for all confounders that were matched, and failure to do so will produce erroneous results. It is even possible to demonstrate that matching for a variable that is *not* a confounder in a case-control study is capable of *introducing* confounding into the results. Many authors have therefore argued against matching in case-control studies, and many case-control study designs are now unmatched. This example is adapted from Rothman 1986.[692]

To adjust for more confounders, the data must be divided into strata for every possible combination of variables. For example, to adjust for the effect of obesity as well as a the effect of gender, a minimum of four strata would be needed: obese/male, obese/female, non-obese/male, non-obese/female. The number of strata required therefore increases exponentially with the number of confounders. If there are too many confounders to adjust for, there will not be enough data to fill every stratum and some strata will be empty or partly empty. The calculation becomes impossible for empty strata, because the estimated odds ratios will be either zero or undefined (division by zero).

The statistical package, Stata (StataCorp, College Station, Texas), fits logistic regression models using maximum likelihood estimation, which is better able to cope with sparse data. The command `logistic` reports odds ratios, while `logit` reports log odds ratios: the models produced by both commands are identical.

Logistic regression can be extended to accommodate outputs with more than two levels (multinomial or multivariate logit modelling), or situations where the inputs are not independent (conditional random field modelling). These are implemented in Stata as `blogit` and `glogit`, respectively.

2.1.4 Identification of confounders and causal reasoning

Informally, a confounder is one that accounts for all or part of an apparent association between the exposure and the outcome and is not itself affected by the exposure. One definition of a confounder is that a confounder is any parameter associated with both exposure and mortality, but which does not lie

on the causal pathway between exposure and mortality.[598] The identification of confounders is the business of the epidemiologist and not the statistician, because it requires knowledge about the data that cannot be inferred from the data itself.[693] There is no statistical test for confounding, so any method based solely on *p*-values is incorrect.[694]

The greatest challenge facing the model-builder is the correct identification of confounders to include in the model. There exists a popular misconception that adjusting for more variables is a good thing. Few data sets are large enough to allow adjustment for every possible confounder and unnecessary adjustment wastes power: in other words, a true effect can be made to spuriously disappear by adjusting unnecessarily for something that is not a confounder.[597,688,695]

There is an even more serious problem with unnecessary adjustment. Adjusting for a parameter which is not a confounder may *introduce* confounding and hence produce biased results.[597,693,696]

When logistic regression models were first introduced into clinical research in the 1970's and 80's, model fitting had to be performed by hand. Starting in the early 1990's, computer software packages became available that would fit logistic regression models in milliseconds, which has led to an over-dependence on computer-led methods of model building, principally, the step-wise selection of variables.

Generally speaking, step-up procedures add variables one by one on the basis of *p*-value (smallest *p*-value first), while step-down procedures throw all the variables into the model and then remove them one by one according to *p*-value (largest *p*-value first).[697] The attraction of these data-driven approaches is that they remove the necessity for careful thought and will invariably produce models with excellent fit. Unfortunately, it has been repeatedly shown that models produced in this way give overoptimistic estimates for *p*-values and seldom give reproducible results[688,695] (a problem known as 'over-fitting').

A more serious problem with stepwise methods is their validity. The *p*-value reported (whether calculated by the Wald test or by the likelihood ratio test) measures the value of that risk factor in predicting the outcome. Stepwise methods are therefore only valid (if they are *ever* valid) for the purpose of building a predictive model. When models are built to adjust for confounding,

the *p*-values are irrelevant, because the hypothesis they test is unrelated to whether the variable is or is not a confounder.[698]

The selection of variables for inclusion in a model is always dependent on external information,[693,699] and is only weakly dependent on information contained in the data itself. An important example of this external information is a knowledge of the temporal relationship between variables. In fact, this is probably the single most important piece of information available to the investigator, for the single reason that effect must always follow cause. If variable A follows variable B in time, then variable A can *never* be a confounder for variable B, because confounders must *always* precede in time, or at least be roughly contemporaneous with the variables they confound (thus allowing for the *possibility* that variable A might precede variable B).

The simplest tool for identifying confounders is the conceptual hierarchy[700] (Figure 15). In the hierarchical conceptual framework that we built for this cohort study, time flows from the top of the chart to the bottom. In our cohort study, the exposure of interest was diabetes (Figure 15, level 2) and the outcome of interest was death (Figure 15, level 6). Any one factor can be confounded only by other factors in the same level or in the levels above. Factors in lower levels must be either in the causal pathway or be common effects: they cannot be confounders.

The question then arises: What is the effect of adjusting for variables on the causal pathway? All diabetes drugs must lie on the causal pathway between diabetes and death, the reason being that they are only prescribed after the a diagnosis of diabetes is made and are only prescribed in the context of diabetes. However, the choice of drug may be manipulated by the clinician. Adjusting for drug therapy allows us to ask the question, “what if we did not prescribe this drug?” It provides an elaboration of what is going on and it permits us to look for possible mechanisms. It is important to note that while this operation is mathematically identical to adjustment for confounding, the interpretation of the result is completely different from adjusting for a confounder.

1	gender	age	access to healthcare	
2	diabetes mellitus	chronic kidney disease	corticosteroid use	
3	glibenclamide	metformin	insulin	admission antimicrobials
4	Melioidosis			
5	bacteraemia	respiratory failure	hypotension	
6	Death			

Flow of time

Figure 15. Conceptual hierarchical framework of risk factors for melioidosis mortality.

Note.—Gender, age and access to healthcare occupy the highest level in the hierarchy because they are not dependent on any other factors. Factors in each level are dependent on factors in the level above and factors in lower levels cannot confound the effect of factors in higher levels because they occur later in time. Factors in level 5 are immediate proximate causes of death. Other pre-existing medical conditions were omitted from level 2 (e.g., malignancy, thalassaemia, etc.) because they do not confound diabetes or its treatment. We used occupation (rice farming) as a proxy for access to healthcare.

Measures of disease severity must also lie on the causal pathway. Why then is it possible to adjust for drug therapy and not disease severity, when both must lie on the causal pathway? The answer is that any exposure which changes mortality must automatically change severity. It is implausible that any effective intervention might reduce mortality while having no effect on clinical severity. To adjust for severity in the analysis is an uninterpretable exercise, because the clinician cannot modify severity independent of mortality.

2.2 Methods

We prospectively identified all patients aged 15 years or more presenting to Sappasithiprasong Hospital, Ubon Ratchathani, northeast Thailand with culture-confirmed melioidosis between 1st January 2002 and 31st December 2006. Patients on their first admission for culture-confirmed melioidosis were

eligible, but patients under the age of 15 were excluded because paediatric cases have a different clinical presentation and prognosis. There were no other exclusion criteria.

Patients were classified into three groups according to diabetes status at presentation: known diabetes, hyperglycaemia or no diabetes. Patients with a pre-existing diagnosis of diabetes mellitus were classified as having known diabetes. The hyperglycaemia group were patients not previously known to have diabetes, who had either a blood glucose >200 mg/dl (11·1 mmol/l) at any point during the admission or new diabetes diagnosed after recovery as defined by WHO criteria. We did not sub-classify this group further for the following reasons: melioidosis is a common first presentation of type 2 diabetes in this region[175] and an associated mortality of 50% (half of which occurs in the first 48 hours) means that a new diagnosis of diabetes cannot be made for a significant proportion of cases, and hyperglycaemic patients who die are more likely to be classed as having sepsis-induced hyperglycaemia while cases who survive are more likely to be classed as having a new diagnosis of diabetes. Patients who did not fall into the known diabetes or hyperglycaemia groups were classified as having no diabetes. Hb A_{1c} values were not available for patients in this cohort.

A full history and clinical examination were recorded from culture-confirmed cases of melioidosis, and each patient was seen daily until discharge or death. Blood samples were taken on admission for a complete blood count, capillary glucose, blood urea nitrogen, creatinine, electrolytes and liver function tests. Intravenous ceftazidime, imipenem or meropenem was started as soon as melioidosis was suspected and continued for a minimum of 10–14 days. Clinical data were recorded on a password-protected computer database.

Details of pre-admission drug treatment for diabetes (glibenclamide, metformin and insulin) and duration of infection-related symptoms prior to admission were obtained by questioning the patient or nearest relative. We recorded whether effective admission antimicrobial therapy (parenteral ceftazidime, imipenem, meropenem, co-amoxiclav, or cefoperazone-sulbactam) was given within 24 hours of admission.

Sepsis was defined, using a simplified version of the 1992 joint American College of Chest Physicians and Society of Critical Care Medicine Consensus Conference Committee definition, as two or more of (1) temperature <36°C or

>38°C; (2) heart rate >90 beats per minute; (3) respiratory rate >20 breaths per minute; or (4) total white cell count >12 × 10^9 cells/l. *Bacteraemia* was defined as one or more blood cultures positive for *B. pseudomallei*. The extent of infection was based on the number of organs or anatomical sites involved in the infective process: *Single organ disease* meant involvement of a single site, and *multiorgan disease* meant infection of more than one site. Blood culture and throat swab positivity were excluded from the organ count. *Pneumonia* was defined as the presence of clinical features consistent with pneumonia, plus radiographic changes or sputum culture positive for *B. pseudomallei*. *Duration of symptoms prior to admission* was recorded in days (but entered into the analysis as fractions of weeks). A history was taken for *chronic kidney disease, thalassemia, malignancy* and *chronic liver disease* (chronic hepatitis B or C infection, or chronic alcoholic liver disease). Patients with nephrolithiasis were identified from either the history or calculi visible on a plain abdominal radiograph or ultrasonography.

The primary study outcome was in-hospital mortality, but we also pre-defined two secondary outcomes: hypotension (a systolic blood pressure of less than 90 mmHg at any point during admission), and respiratory failure (hypoxia judged clinically to require mechanical ventilation; arterial blood gases are not taken routinely in our setting).

2.2.1 *Statistical analysis*

Analyses were performed using Stata/SE 9 (StataCorp, College Station, Texas). Differences between the three patient groups were compared using the Fisher's exact test for categorical variables and the Mann-Whitney U test for continuous variables. Time to death (to a maximum of 28 days) was analysed using the Kaplan-Meier method; patients discharged alive from hospital within 28 days were assumed to have survived, but patients who self-discharged against medical advice were censored on the day of discharge.

To inform the selection of parameters used in the logistic regression models, we created a conceptual hierarchical framework[700] of risk factors to explore interactions between a number of variables (Figure 15). Parameters were chosen on the basis of whether they were possible confounders for the

effect of diabetes on mortality (Model A, Table 10);[90] *p*-values reported are for the likelihood-ratio test, but were not used to inform parameter selection.

In Model A (Table 10), we adjusted for age and sex as possible confounders. We also adjusted for corticosteroid use because corticosteroids are available without a prescription in northeast Thailand, are commonly abused, and may cause diabetes. It is possible that patients in the known diabetes group had increased access to healthcare, so we adjusted for this by using the occupation of rice farmer as a proxy since this is associated with poverty, increased physical distance from hospitals and clinics and poorer transport links. It is impossible for parameters occurring below diabetes in the framework to confound the effect of diabetes (e.g., treatment, pneumonia, severity, organ failure).[700]

A second regression model was developed to explore explanations for the survival advantage in known diabetics (Model B, Table 10) by examining modifiable parameters lying on the causal pathway between diabetes and mortality. This included analysis of treatment for diabetes prior to admission, and administration of appropriate admission antimicrobial therapy within 24 hours of admission. We adjusted for chronic kidney disease in this model because metformin is contra-indicated in renal failure, and renal failure is associated with mortality. Additional models were developed to evaluate the effect of diabetes on our secondary outcomes (hypotension and respiratory failure: Table 11, Models C & E), and to explore factors that could explain the difference in outcomes observed in patients with diabetes (Table 11, Models D & F).

The result of the Hosmer-Lemeshow test for goodness-of-fit for Model A was p=0·41, B was p=0·53; C was p=0·22; D was p=0·28; E was p=0·51; and F was p=0·47.

2.2.2 Glibenclamide inhibition study

Plates of Müller-Hinton agar were inoculated with a lawn of *B. pseudomallei* (0·5 MacFarland standard). A single glibenclamide tablet was crushed and dissolved in 1 ml DMSO, with 100 µl of the solution (~100 µg of glibenclamide) dropped onto the centre of the agar plate. The same volume of

DMSO was used for control plates. The plates were incubated at 37°C for 48 hours and the plates examined for a zone of inhibition.

2.3 Results

2.3.1 Diabetes and mortality

We identified 1384 patients with culture-positive melioidosis, of whom 224 were removed from the final analysis for reasons shown in Figure 16. Of the remaining 1160 patients, 410 (35%) had known diabetes, 250 (22%) had hyperglycaemia and 500 (43%) had no diabetes. Patient characteristics, clinical features of melioidosis and primary and secondary outcomes were compared between the three groups, using the no diabetes group as the comparator for the two other groups (Table 9). In-hospital mortality was lower in patients with known diabetes compared with non-diabetics (p=0·04), with no difference observed between non-diabetics and patients with hyperglycaemia. These findings were reproduced in the survival analysis (Figure 17A).

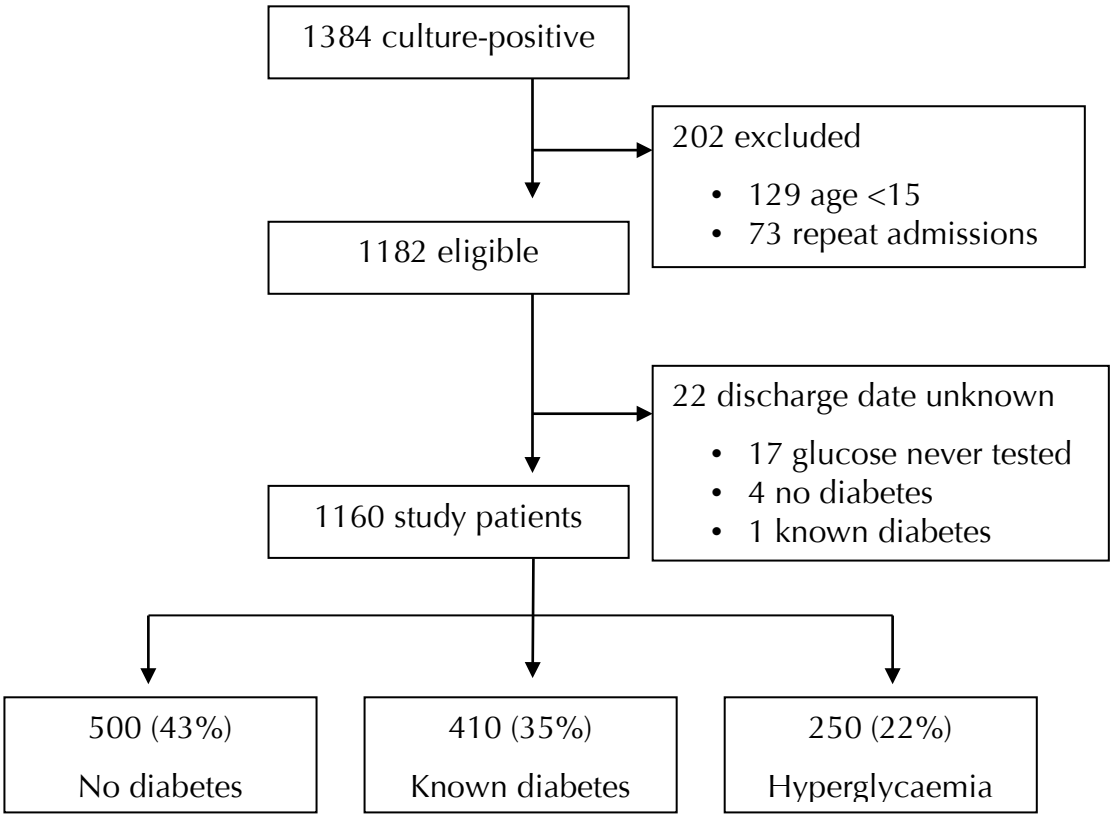


Figure 16. Summary of patient recruitment for cohort study.

	No diabetes (*n*=500)	Known diabetes (*n*=410)		Hyperglycaemia (*n*=250)		Total (*n*=1160)
	number (%)	number (%)	*p*-value[a]	number (%)	*p*-value[a]	row total (%)
Female	165 (33)	219 (53)	–	114 (46)	–	498 (43)
Male	335 (67)	191 (47)	<0.001	136 (54)	0.001	662 (57)
Median age (yrs, IQR)	52 (39–63)	51 (42–59)	0.40[b]	51 (42–59)	0.22[b]	–
Rice farmer	367 (73)	326 (80)	0.04	212 (85)	<0.001	905 (79)
Median (IQR) days of infective symptoms prior to presentation	7 (4–15)	10 (5–21)	0.02[b]	8 (5–15)	0.03[b]	7 (5–20)
Diabetes treatment[c]						
Glibenclamide	–	208 (51)		–		208 (18)
Metformin	–	51 (12)		–		50 (4)
Insulin	–	81 (20)		–		81 (7)
Other sulphonylurea	–	10 (2)		–		10 (0.9)
Unknown oral drug	–	51 (12)		–		51 (4)
No medication	–	53 (13)		–		53 (5)
Risk factors for melioidosis						
Chronic kidney disease	54 (11)	36 (9)	0.32	9 (4)	0.001	99 (9)
Nephrolithiasis	35 (7)	15 (4)	0.03	6 (2)	0.01	56 (5)
Corticosteroid use	21 (4)	19 (5)	0.75	9 (4)	0.84	49 (4)
Thalassaemia	11 (2)	4 (1)	0.19	2 (0.8)	0.24	17 (1)
Malignancy	9 (2)	1 (0.2)	0.03	1 (0.4)	0.18	11 (1)
Chronic liver disease	6 (1)	5 (1)	1.00	1 (0.4)	0.43	12 (1)
Organ involvement						
Pneumonia	190 (38)	154 (38)	0.95	117 (47)	0.02	461 (40)
Skin or soft tissue	86 (17)	94 (23)	0.04	42 (17)	0.92	222 (19)
Urinary tract	78 (16)	48 (12)	0.10	15 (6.0)	<0.001	141 (12)
Liver abscess(es)	33 (7)	48 (12)	0.01	27 (11)	0.06	108 (9)
Spleen abscess(es)	45 (9)	45 (11)	0.37	35 (14)	0.04	125 (11)
Septic arthritis	24 (5)	40 (10)	0.004	24 (10)	0.02	88 (8)

Distribution of disease						
Bacteraemia	274 (55)	238 (58)	0.35	164 (66)	0.005	676 (58)
Single organ disease	291 (58)	240 (59)	0.95	146 (58)	1.00	677 (58)
Multiorgan disease	110 (22)	102 (25)	0.31	58 (23)	0.71	270 (23)
Complications						
Hypotension	187 (37)	135 (33)	0.16	99 (39)	0.58	421 (36)
Respiratory failure	179 (36)	117 (29)	0.02	101 (40)	0.23	397 (34)
Sepsis[d]	392 (78)	334 (81)	0.28	209 (84)	0.10	935 (81)
Antibiotic treatment and in-hospital mortality						
Effective antibiotic treatment within 24 hours of admission	367 (73)	359 (88)	<0.001	212 (85)	<0.001	938 (81)
Died	225 (45)	157 (38)	0.04[f]	117 (47)	0.64[f]	499 (43)
Discharged well	254 (51)	245 (60)	–	128 (51)	–	627(54)
Outcome unknown (Self-discharged)	21 (4)	8 (2)	–	5 (2)	–	34 (3)

Table 9. Patient characteristics, clinical features of melioidosis and outcome.

Note.—IQR = interquartile range. Anatomical sites for which there were less than 20 cases in the 5-year period are omitted from the table. Variables recording organ involvement at ten sites are not shown here, because there were fewer than 20 events recorded: these were parotitis, pleural disease, central nervous system disease, peritonitis, pericarditis, osteomyelitis, prostatitis, thyroiditis, ophthalmitis and cholecystitis. They were, however, taken into account when counting the number of organs involved.

[a]Fisher's exact test, except where indicated.

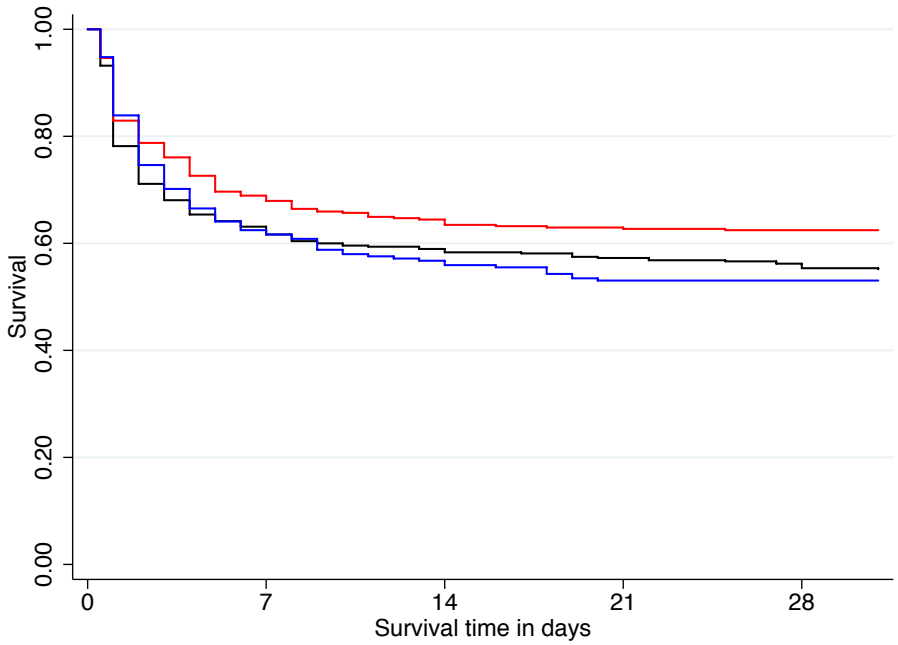
[b]Mann-Whitney U test with *No diabetes* as the comparator group, because the data was non-normal and could not be transformed to normal.

[c]Numbers do not add up to 410 (100%) because some patients were taking more than one type of medication.

[d]Sepsis defined as temperature >38ºC or <36ºC, heart rate >90 beats per minute, respiratory rate >20 breaths per minute, or total leucocyte count $>12 \times 10^9$ cells/L.

[f]Patients who took their own discharge were counted as alive.

Figure 17A



Numbers at risk on	day 0	day 7	day 14	day 21	day 28
No diabetes	500	304	281	271	265
Known diabetes	410	279	259	252	251
Hyperglycaemia	250	154	138	130	130

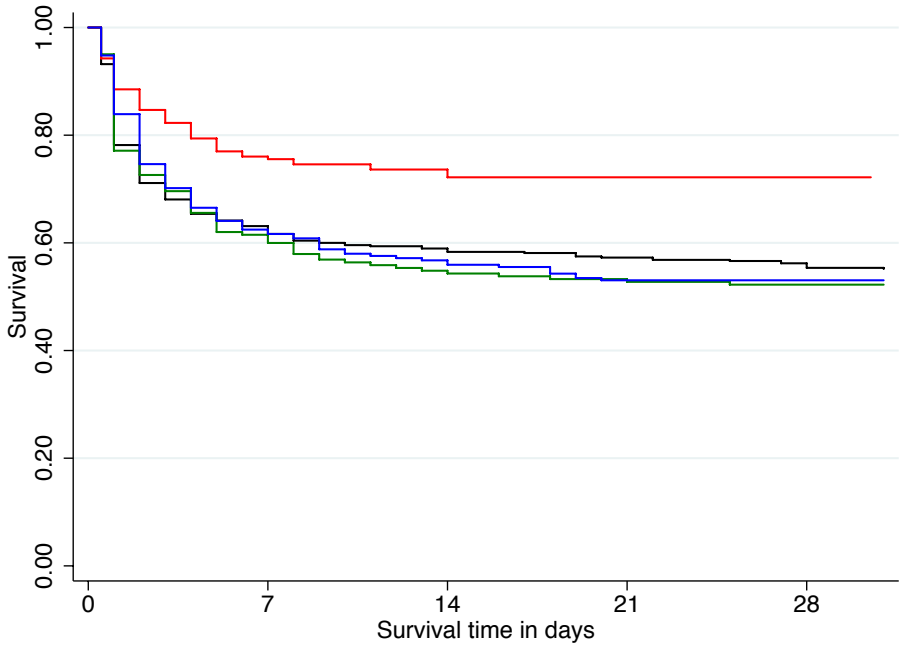
No diabetes Known diabetes Hyperglycaemia

Known diabetes *v.* No diabetes (p = 0·03)

Hyperglycaemia *v.* No diabetes (p = 0·91)

Known diabetes *v.* Hyperglycaemia (p = 0·05)

Figure 17B



Numbers at risk on	day 0	day 7	day 14	day 21	day 28
No diabetes	500	304	281	271	265
Known diabetics on glibenclamide	209	158	153	153	149
Known diabetics not on glibenclamide	201	121	106	103	102
Hyperglycaemia	250	154	138	130	130

—— No diabetes group —— Known diabetes on glibenclamide

—— Known diabetes not on glibenclamide —— Hyperglycaemia group

Known diabetes on glibenclamide *v*. No diabetes (p<0·001)

Known diabetes on glibenclamide *v*. Hyperglycaemia (p<0·001)

Known diabetes on glibenclamide *v*. Known diabetes not on glibenclamide (p<0·001)

Known diabetes not on glibenclamide *v*. No diabetes (p = 0·59)

Hyperglycaemia *v*. no diabetes (p = 0·91)

Figure 17. Kaplan-Meier survival curves of 1160 patients with melioidosis.

Note.—The survival curves in **A** show that patients with diabetes have a survival advantage after the development of melioidosis, but the survival curves in **B** indicate that this effect was seen only in the patient group taking glibenclamide. The *p*-values reported are for the log-rank test. Median duration of follow-up was 6·5 days and total follow-up was 11,845 patient days.

A previous study conducted at the same hospital but in a different (earlier) patient cohort reported an inverse association between diabetes and mortality (unadjusted odds ratio [OR] 0·49).[681] We confirmed this observation in our independent cohort (OR 0·76; adjusted odds ratio [AOR] 0·78, 95% confidence interval [CI] 0·59 to 1·0, p=0·07), and extended previous observations that patients in the hyperglycaemia group had no survival advantage (Model A, Table 10).

2.3.2 Glibenclamide and mortality

We hypothesised that the association between diabetes and a reduced risk of death was due to the treatment received for diabetes and/or melioidosis. This was tested in a second logistic regression model (Table 10, Model B). We considered drugs prescribed for diabetes (glibenclamide, metformin, insulin) prior to admission, noting that more than half of known diabetics were receiving glibenclamide (Table 9). We adjusted for chronic renal failure, because metformin is avoided in renal failure, because of the risk of lactic acidosis, and because renal failure is associated with mortality. We included the administration of appropriate admission antimicrobial therapy because the association between diabetes and melioidosis is well-described and could lead clinicians to prescribe these at an earlier stage in diabetic patients.

The adjusted odds ratio for death was lower for patients who received glibenclamide therapy prior to admission (AOR 0·47, 95%CI 0·28–0·74, p=0·005) and for those receiving effective antimicrobial chemotherapy at admission (AOR 0·23, 95%CI 0·17–0·34, p<0·001) (Table 10, Model B). Neither metformin nor insulin treatment were associated with survival in any analysis. The unadjusted survival curve for patients in the glibenclamide group is shown in Figure 17B.

	univariable analysis		logistic regression **model A** (n = 1160)		logistic regression **model B** (n = 1109)[c]	
	OR	(95% CI)	AOR	(95% CI)	AOR	(95% CI)
No diabetes[a]	1·0	—	1·0	—	1·0	—
Known diabetes	**0·76**	**(0·58–0·99)**	**0·78**	**(0·59–1·0)**	**1·4**	**(0·89–2·3)**
Hyperglycaemia	1·1	(0·79–1·5)	1·1	(0·81–1·5)	1·4	(0·98–1·9)
Age[b]	1·1	(1·0–1·2)	1·1	(1·0–1·2)	1·1	(0·99–1·2)
Male sex	1·2	(0·94–1·5)	1·1	(0·89–1·5)	1·0	(0·78–1·3)
Rice farming	0·97	(0·74–1·3)	1·0	(0·75–1·3)	1·2	(0·86–1·6)
Corticosteroid use	1·5	(0·86–2·7)	1·6	(0·87–2·8)	1·0	(0·62–2·2)
Chronic kidney disease	2·8	(1·8–4·3)			2·2	(1·6–4·0)
Glibenclamide treatment[c]	**0·48**	**(0·35–0·67)**			**0·47**	**(0·28–0·74)**
Metformin treatment[c]	0·61	(0·33–1·1)			1·1	(0·62–2·4)
Insulin treatment[c]	0·79	(0·49–1·3)			0·65	(0·37–1·2)
Effective admission antibiotic treatment[c]	**0·22**	**(0·16–0·31)**			**0·23**	**(0·17–0·34)**

Table 10. The effect of diabetes on mortality.

Note.—OR = odds ratio (not adjusted); AOR = adjusted odds ratio; CI = confidence interval. An odds ratio below 1 indicates association with survival, whereas an odds ratio above 1 indicates association with mortality. The first column describes the contribution of each factor in isolation. The second column (model A) attempts to explain the effect of diabetes by adjusting for several possible confounders for diabetes simultaneously; the third column (model B) adjusts additionally for the effect of diabetes treatment and post-admission antibiotics.

When considered in isolation, diabetes (OR 0·76), glibenclamide treatment (OR 0·48) and effective admission antibiotic treatment (OR 0.22) were associated with survival. The effect of diabetes persisted after correcting for confounders for diabetes (AOR 0·78, model A). When the effect of glibenclamide treatment and effective admission antibiotic were taken into account, diabetes was no longer associated with survival (AOR 1·4, model B). **Sensitivity analysis.** We conducted a sensitivity analysis to examine the impact of the missing data. Model A was constructed by

assigning the patients who self-discharged as "alive"; but if these patients were instead assigned as "dead", then the AOR for known diabetes in model A became 0·71 (0·54–0·93, p = 0·01). In model B, assigning the patients who self-discharged to "dead", caused the AOR for known diabetes to become 1·3 (0·79–2·1, p = 0·31), glibenclamide treatment AOR 0·48 (0·28–0·83, p = 0·008), and effective admission antibiotic treatment AOR 0·18 (0·13–0·27, p<0·001). Putting the patients on an unknown oral diabetes medication into the glibenclamide group (n = 1160) in model B, meant the AOR for glibenclamide treatment rose to 0·59 (0·36–0·97, p = 0·04) and effective admission antibiotic treatment became 0·24 (0·17–0·34, p<0·001). When the patients on an unknown oral diabetes medication are put into the metformin group (n = 1160), the AOR for glibenclamide treatment becomes 0·49 (0·30–0·78, p = 0·003) and effective admission antibiotic treatment becomes 0·25 (0·18–0·34, p<0·001).

[a]Comparator group.

[b]Number of decades above age 15 years.

[c]The patients on unknown oral diabetes medication were omitted (n = 1109) in model B and in the unadjusted OR for treatment variables.

Table 11

Hypotension

	univariable analysis		logistic regression **Model C** (n = 1160)		logistic regression **Model D** (n = 1109)[a]	
	OR[b]	(95% CI[c])	AOR[d]	(95% CI[c])	AOR[d]	(95% CI[c])
No diabetes[e]	1·0	—	1·0	—	1·0	—
Known diabetes	**0·80**	**(0·62–1·0)**	**0·84**	**(0·63–1·1)**	**1·3**	**(0·79–2·0)**
Hyperglycaemia	1·2	(0·90–1·6)	1·1	(0·83–1·6)	1.3	(0·91–1·7)
Age[f]	1·2	(1·1–1·3)	1·1	(1·0–1·3)	1·1	(1·0–1·2)
Male sex	1·1	(0·87–1·4)	1·1	(0·87–1·4)	1·0	(0·77–1·3)
Rice farming	1·0	(0·75–1·4)	1·0	(0·76–1·4)	1·1	(0·78–1·5)
Corticosteroid use	2·9	(1·6–5·2)	3·0	(1·6–5·4)	2·4	(1·3–4·5)
Chronic kidney disease	2·0	(1·3–3·0)			1·8	(0·92–3·5)
Glibenclamide treatment[a]	**0·56**	**(0·40–0·78)**			**0·48**	**(0·30–0·78)**
Metformin treatment[a]	1·1	(0·60–1·9)			1·8	(0·92–3·5)
Insulin treatment[a]	0·69	(0·42–1·2)			0·60	(0·33–1·1)
Effective admission antimicrobial therapy[a]	**0·46**	**(0·34–0·62)**			**0·51**	**(0·37–0·70)**

	Respiratory failure					
	univariable analysis		logistic regression **Model E** (n = 1160)		logistic regression **Model F** (n = 1109)[a]	
	OR[b]	**(95% CI[c])**	**AOR[d]**	**(95% CI[c])**	**AOR[d]**	**(95% CI[c])**
No diabetes[e]	1·0	—	1·0	—	1·0	—
Known diabetes	**0·72**	**(0·54–0·95)**	**0·73**	**(0·54–0·97)**	**1·2**	**(0·74–1·9)**
Hyperglycaemia	1·2	(0·89–1·7)	1·2	(0·90–1·7)	1·4	(1·0–2·0)
Age[f]	1·0	(0·94–1·1)	1·0	(0·93–1·1)	0·99	(0·92–1·1)
Male sex	1·2	(0·94–1·5)	1·2	(0·91–1·5)	1·1	(0·82–1·4)
Rice farming	0·98	(0·73–1·3)	0·99	(0·73–1·3)	1·0	(0·76–1·4)
Corticosteroid use	1·6	(0·90–2·8)	1·7	(0·92–3·0)	1·2	(0·62–2·2)
Chronic kidney disease	2·2	(1·5–3·3)			2·0	(1·2–3·1)
Glibenclamide treatment[a]	**0·44**	**(0·31–0·64)**			**0·50**	**(0·28–0·86)**
Metformin treatment[a]	0·52	(0·26–1·0)			1·0	(0·48–2·1)
Insulin treatment[a]	0·80	(0·49–1·3)			0·75	(0·39–1·4)
Effective admission antimicrobial therapy[a]	**0·40**	**(0·29–0·54)**			**0·42**	**(0·31–0·58)**

Table 11. Effect of diabetes on hypotension and respiratory failure.

Note.—

[a]Patients on unknown oral diabetes medication were omitted (n = 1109) in Models D & F and in the unadjusted OR for treatment variables.

[b]Odds ratio (unadjusted).

[c]Confidence interval.

[d]Adjusted odds ratio.

[e]Comparator group.

[f]Number of decades above age 15 years.

Patients in the known diabetes group were more likely to receive effective antimicrobial therapy on admission compared to patients without diabetes (88% versus 73%, $p<0{\cdot}001$), an association that was seen in each of the treatment groups (glibenclamide 92%, $p<0{\cdot}001$; metformin 98%, $p<0{\cdot}001$; insulin 90%, $p=0{\cdot}02$), but the association between glibenclamide therapy and survival was independent of admission antimicrobial therapy (Table 10).

We used logistic regression to evaluate the relationship between diabetes and the secondary outcome measures. We found that diabetes was negatively associated with both hypotension and respiratory failure (AOR 0·84, Model C, Table 11) and AOR 0·73, Model E, Table 11), but that this association did not persist after adjustment for treatment (antimicrobials and anti-diabetic treatment) (Models D & F, Table 11). However, glibenclamide was negatively associated with both hypotension (AOR 0·48, 95%CI 0·30–0·78, $p=0{\cdot}007$) and respiratory failure (AOR 0·50, 95%CI 0·28–0·86, $p=0{\cdot}01$), as was effective antimicrobial therapy within 24 hours of admission (AOR 0·51, 95%CI 0·37–0·70, $p<0{\cdot}001$ and AOR 0·42, 95%CI 0·31–0·58, $p<0{\cdot}001$ respectively) (Models D & F, Table 11). In a sensitivity analysis, if patients taking an unknown oral drug were placed in the glibenclamide group, then the associations between glibenclamide and hypotension (AOR 0·65, 95%CI 0·39–1·1, $p=0{\cdot}09$) and between glibenclamide and respiratory failure (AOR 0·60, 95%CI 0·36–1·0, $p=0{\cdot}05$) were no longer significant statistically.

2.3.3 Glibenclamide does not inhibit growth of B. pseudomallei

We observed that glibenclamide did not inhibit the growth of *B. pseudomallei* at the concentrations used.

2.4 Discussion

We confirmed the finding that diabetes patients were more likely to survive, and that this was associated with a reduction in the number of cases of respiratory failure. These results are consistent with those of the three observational studies cited in the introduction.[574,594,595]

We further noted that this difference for mortality was attributable to glibenclamide treatment and the fact that diabetes patients were more likely to receive appropriate antimicrobial chemotherapy at admission. Diabetes mellitus had no independent effect on mortality from melioidosis after adjustment for

these two factors, but none of the previously-cited studies[574,594,595] looked for an effect of diabetes treatment.

The effect of antimicrobial chemotherapy is unsurprising, and is consistent with what we know from other causes of sepsis.[701] The effect of glibenclamide was unexpected and has not previously been described.

2.4.1 Glibenclamide

Glibenclamide rINN (=glyburide USAN, Figure 18) is a second-generation sulphonylurea widely used to treat type 2 diabetes and acts by inhibiting ATP-sensitive[699] (K_{ATP}-channels) in pancreatic β-cells,[95] leading to stimulation of insulin secretion.

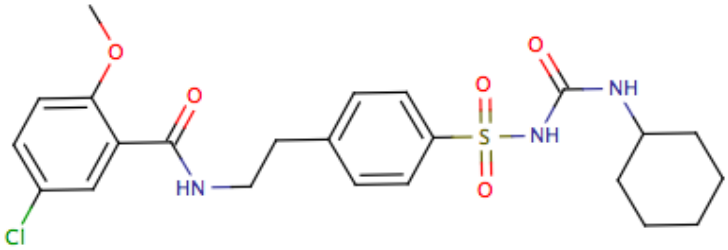


Figure 18. Glibenclamide rINN or glyburide USAN.

Note.— IUPAC name, N-[2-[4-(cyclohexylcarbamoylsulfamoyl)phenyl]ethyl]-2-methoxybenzamide. Molecular weight 494·0 g/mol. Daonil® (formerly Hoechst, now Aventis); Eugluclon® (Roussel); Sigma-Aldrich catalogue no. G0639.

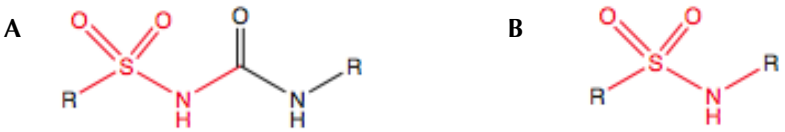


Figure 19. General structure of sulphonylureas and sulphonamides.

Note.—H = hydrogen; N = nitrogen; O = oxygen; R = general alkyl group, S = sulphur. The generalised structure on the left is a sulphonylurea (**A**); the structure on the right is a sulphonamide (**B**). The part of the structure highlighted in red is the sulphonamide moiety, which is clearly present in the sulphonylurea structure as well.

The sulphonylureas were first described in the 1942 by Janbon *et al.* while searching for novel antimicrobials. From a chemical point of view, the sulphonylureas are also sulphonamides (Figure 19) and we initially hypothesised that glibenclamide was acting via an antimicrobial action on *B. pseudomallei*. However, we were unable to show that glibenclamide inhibits growth of *B. pseudomallei*, thus excluding an antimicrobial effect of glibenclamide on *B. pseudomallei*.

2.4.2 Glibenclamide and vascular smooth muscle

K_{ATP}-channels are also expressed in the heart and on vascular smooth muscle, and excessive opening of vascular K_{ATP}-channels in sepsis (due to a fall in the ATP/ADP ratio, pH or oxygen tension, or a rise in intracellular lactate) leads to hyperpolarization of the cell membrane, a blocking of the calcium influx and loss of vascular tone (contributing to the 'vascular paresis' of septic shock). By blocking vascular smooth muscle K_{ATP}-channels, glibenclamide is able to protect from shock in animal models of sepsis,[702] although this did not translate into a detectable effect on cardiovascular parameters in two small clinical trials in which glibenclamide was given at doses normally used to treat diabetes.[703,704] Neither study was designed to find an effect on mortality, and neither looked at end points such as respiratory failure or inflammation.

2.4.3 Limitations of this study

In seeking confounders for our study, we considered whether glibenclamide treatment was simply a marker for less advanced diabetes and that this was associated with a survival benefit compared with patients on insulin which is reserved for patients who fail to achieve adequate diabetic control on oral medication. However, only glibenclamide treatment and not metformin was associated with an improvement in mortality. We also considered whether thiazolidinediones and statins (which are anti-inflammatory and may be required more commonly in diabetics) was a confounder, but found that although available, these are very rarely used in our setting. This is an observational study in which glibenclamide was given for a specific indication, namely, the treatment of type 2 diabetes and it is not possible to exclude unequivocally the possibility of confounding.[705]

3 Glibenclamide is associated with reduced inflammation in melioidosis

3.1 Introduction

Having observed an association between glibenclamide and survival, we initially postulated that glibenclamide was acting as an antimicrobial drug based on its structural homology with the sulphonamides (to which *B. pseudomallei* is susceptible). However, a solution of glibenclamide in DMSO has no inhibitory effect *B. pseudomallei* grown in LB or on tryptic soy agar. We therefore hypothesized that glibenclamide was having an effect on the host response to melioidosis.

Glibenclamide is a broad-spectrum ABC transporter inhibitor that alters responses of macrophages to a range of stimuli *in vitro* and *in vivo*.[706–708] We used peripheral leukocyte gene expression as a screening tool to identify global trends in gene function that might be ascribed to the action of glibenclamide. We compared gene expression profiles in 10 diabetic patients who were taking glibenclamide at the time of presentation with melioidosis against 10 diabetic melioidosis patients who were not taking this or another sulfonylurea, and repeated the comparison in healthy controls.

3.1.1 DNA microarrays

DNA microarrays are a multiplex assay, consisting of two-dimensional arrays of DNA fragments immobilized on a solid substrate. The first DNA microarray had 378 probes, but current commercial arrays may contain tens of thousands of probes and are able to interrogate across an entire eukaryotic genome. When investigating gene expression, messenger RNA is first purified from lysed white blood cells and then converted to cDNA. The cDNA is then amplified by transcription to RNA by an RNA polymerase, which is preferable to PCR amplification because the amplification proceeds in a linear fashion that preserves the relative proportions of each mRNA transcript. A fluorescent label is attached to the RNA, and the RNA is then hybridised with a DNA microarray, where each RNA fragment will bind to matching DNA probes on the chip. The

fluorescence read out gives an estimate of the amount of RNA bound to each probe.

Microarrays are a refinement of the Southern blot. DNA blotting was first described by Edwin Southern in 1975 at the University of Edinburgh.[709] Southern described a method for identifying specific sequences by separating DNA fragments electrophoretically by size on an agarose gel, blotting the DNA onto nitrocellulose filters, then detecting the sequences of interest using complementary radiolabelled or fluorescent-labelled DNA probes.[709]

Microarrays arose from the need for an assay that was able to simultaneously detect the presence or absence of multiple sequences. In 1982, at the Sloan-Kettering Institute in New York, Augenlicht and Kobrin realised that it was not necessary to separate the DNA fragments in the sample, but that similar results could be obtained by spatially separating and immobilising the probes in an array instead. Their first array consisted of 378 probes immobilised on nitrocellulose membranes, on which they were able to semi-quantitatively describe differences in gene expression in a mouse tumour.[710] Five years later, the same team published a protocol for computerised scanning and image processing that allowed quantitative analysis of 4000 human sequences simultaneously.[711]

Affymetrix was founded by Stephen Fodor in 1992 and has its headquarters in Santa Clara, California. Affymetrix began marketing the first commercially viable DNA microarrays (under the trademark, GeneChip®) in 1994, produced using semiconductor manufacturing techniques, and this made microarray technology widely available to researchers.

3.1.2 Illumina BeadChip

Illumina was founded in 1998 to develop and manufacture integrated systems for the analysis of genetic variation of function. The company was founded to exploit the BeadArray technology developed at Tufts University and has its headquarters in San Diego, California. The first BeadArray application was to genotype single-nucleotide polymorphisms, but has expanded to include copy number variation analyses, DNA methylation and gene expression profiling.

In a BeadArray, each microarray consists of hundreds of thousands of 3 µm silica beads randomly assembled in 3 µm microwells on silica glass slides, and each silica bead is coated with hundreds of thousands of copies of a single complementary DNA (cDNA) probe. The arrays are self-assembling, with each bead bound to its well by van der Waals forces alone.

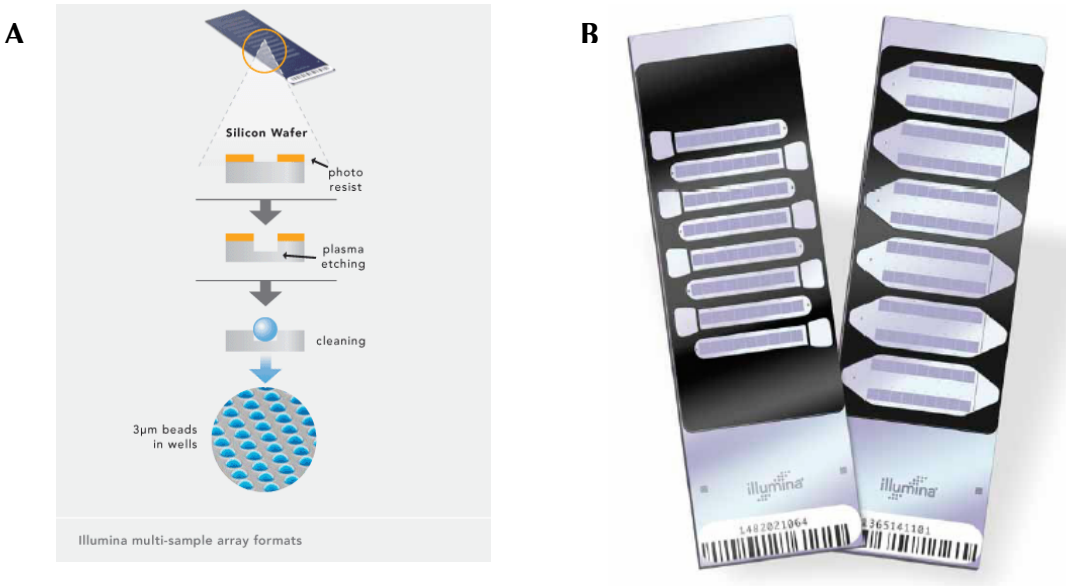


Figure 20. Illumina BeadArray.

Notes.—**A** Illumina BeadArrays are distinguished from other microarrays principally by their method of manufacture. Conventional microarrays are created by printing spots onto slides or membranes: BeadArrays are created from randomly self-assembling beads. First, 3 µm microwells are etched into glass slides, then a mixture of glass beads, each coated with hundreds of copies of a single probe are poured onto the slide. The 3 µm beads slot randomly into wells and are held there by van der Waals forces. Excess beads are then washed off. Each probe is represented by ~30 beads distributed randomly across the array. **B** The glass slide on the right is the Illumina HumanWG-6 v.3 BeadChip used for this project. Each BeadChip contains twelve strips (two strips per array, one sample per array). In contrast, Affymetrix GeneChips will only take a single sample per chip. Images taken from Illumina product literature.

Each probe is replicated by ~30 beads per strip (the actual number of beads varies randomly from ~15 to ~60) and the final position of each bead is completely random. Each probe consists of a 50-mer probe sequence and a 29-mer address sequence. The address sequence is decoded at the factory to reveal

the probe sequence at each position. Each chip is shipped with this information and this information is unique for each chip. The sequence of these address tags is commercial confidential.

This method of manufacture has a number of advantages. Conventional arrays are printed onto slides or membranes using pins. Small defects in individual pins result in systematic errors that propagate across the entire array, because every spots printed by the same pin will carry the same defect. The BeadArray technology relies on random self-assembly of the array, which means that this kind of systematic error cannot occur. A further advantage of the BeadArray is the high degree of replication, which means that Illumina BeadArrays are robust to spatial defects. As much as three-quarters of each strip could be destroyed with only minimal loss of information. By contrast, Affymetrix GeneChips each contain only one copy of each probe at a constant location on every chip: GeneChips are therefore extremely susceptible to spatial artefacts, with no way of recovering from these defects. A final advantage is that BeadArrays are cheaper to manufacture than conventional spotted arrays and cheaper to use. At the time the project was planned. Affymetrix GeneChips would only take a single sample per GeneChip, whereas a BeadChip could take 6 or 8 samples per chip.

The microarray used in this project was the HumanWG-6 v3.0,[712] which contains 12 strips per glass slide, or six samples (two strips per array, one array per sample). The array covers 48,804 probes or 25,440 manually curated genes from the NCBI RefSeq[713] (build 36.2, release 22) and UniGene[714] databases (build 199). The genes included cover the whole human genome and are not restricted to well-characterised genes, but also includes gene candidates and splice variants.[712] The platform is single colour (Cy3, which fluoresces green), which avoids the problems associated with ozone degradation from which two-colour platforms suffer,[715] and the need for complicated experimental designs involving dye swaps.

3.1.3 Analysis of microarray data

The output of a microarray experiment consists of a fluorescence value per probe per sample. Fluorescence values are proportional to absolute expression values, but are measured in arbitrary units. They therefore gain meaning only in

comparisons between groups (treated *versus* untreated, wild-type *versus* knockout, *etc.*). This also means that it is very hard to compare raw data acquired by different scanners. The most commonly used statistical test is the Student *t*-test (or a modified version thereof, such as a moderated *t*-test or B-statistic).

The primary difficulty in the statistical analysis of microarray results is the large number of statistical tests that are carried out, often with small sample sizes. Should one set a *p*-value cut-off of 5% then one expects 5% of the tests to have a *p*-value <5% (because the distribution of *p*-values should be uniform). With 48,804 probes, this means 48,804 *t*-tests, with an expected 2440 'hits', even in the case when there is actually no difference between the groups. When a statistical test calls a gene differentially expressed when there is in fact no difference in expression, then a type 1 error is said to have occurred (i.e., a false positive).

There are a number of methods available to deal with this problem and they all involve manipulations of the *p*-values or its cut-off (these are mathematically equivalent procedures). The simplest (and most conservative) of these is the Bonferroni correction.[716] If n tests are performed, then the *p*-value cut-off is divided by n. If 48,804 tests are performed, then the new *p*-value cut-off is 0·05/48,804 = 0·00000102. In the case where there is truly no difference between groups, the new cut-off reduces the expected number of false positives to <1.

The Bonferroni correction follows naturally from Boole's inequality,

$$\sum_{i=1}^{n} P(E_i) \geq P\left(\bigcup_{i=1}^{n} E_i\right).$$

The inequality states that the sum of the individual probabilities of each of the events E_1 to E_n occurring is greater than or equal to the probability that one or more of the events occurs. Other frequently used corrections include the Dunn-Šidák[717] and Holm-Bonferroni.[718] These all work by modifying ('correcting') the *p*-values, or by defining a lower cut-off, but are all less conservative.

A slightly different approach is taken by Benjamini and Hochberg,[719] who define a quantity called the 'false-discovery rate', which is the proportion of statistically significant tests that are in fact false; or, put another way, the proportion of 'hits' that are due to type 1 error. It may be considered a less

conservative method than family-wise error correction methods (which are the Bonferroni method and its relatives), but in essence, the method also works by changing the *p*-value threshold to achieve the desired false discovery rate.

The major criticism of these methods this is that they all simultaneously increase the type 2 error rate while reducing the type 1 error rate.[720] A type 2 error occurs when a true difference is missed by a statistical test (*i.e.,* a false negative) and it has been argued that type 2 errors are as important as type 1 errors.[720] The assumptions underlying these methods have also been questioned: if each test is independent, then why should the result of each test be corrected for the number of tests performed? The number of tests performed depends on the investigator and is not a characteristic of the test, so it seem irrational to address the problem by modifying the test[720] (in slightly more technical terms: these are mathematically rigorous methods for maintaining the family-wise error rate, but there are no strong arguments why maintaining the family-wise error rate should be desirable in the first place). It should also be noted that all *p*-value cut-offs are arbitrary (including the 'conventional' threshold of 0·05), because all *p*-values exist on a continuum from 0 to 1, and therefore the decision to set any threshold (whether 0·001 or 0·1) may be criticised.[721] It is also worth mentioning that even in classical significance testing, the decision is not merely to accept or reject the null hypothesis. Keppel has pointed out that under certain circumstances, a third option may be more appropriate, which is to suspend judgement.[722]

The strongest single argument against adjusting for multiple comparisons is that blind reliance on stringent statistical criteria gives results that cannot be reproduced.[723] The MicroArray Quality Control project (MAQC) is an initiative of the US Food & Drug Administration, the aim of which is to assess the reproducibility of microarray experiments and the comparability of results across different platforms. In a study published in 2006, two distinct, high-quality RNA samples were assayed at 137 different sites on seven different microarray platforms across the continental USA. A major finding of that study was that reproducibility falls as statistical stringency increases, leading the investigators to recommend that non-stringent *p*-value cutoffs be used for microarray experiments.[723]

A second (complementary) method of dealing with the gene lists generated by microarray experiments is to group them by function. The rationale behind this is that genes that are truly differentially regulated are likely to share the same function. In contrast, the false positives in the gene list are likely to come from any part of the genome and are less likely to form functional groupings.[724–726] This group of methods is commonly known as gene set enrichment and is a necessary part of the interpretation of any gene set.

Broadly speaking, there are two main functional groupings: the first is pathway and the second is ontology.

Pathways may be defined most generally as a sequence of biochemical interactions within a cell. Metabolic pathways are easy to define and circumscribe, because they follow the serial modifications of a single molecule, where the product of one reaction is the substrate for another. For example, in glycolysis (the first metabolic pathway to be described), glucose (substrate) is phosphorylated to glucose-6-phosphate (product) by the enzyme, hexokinase. Glucose-6-phosphate is the substrate for phosphoglucose isomerase, and so forth down the pathway, ending with pyruvate or acetyl-CoA. In signal transduction, there is not usually a single molecule that can be followed, but instead the trail follows a notional 'signal' that is passed from protein to protein. The first signalling pathway to be described was glucagon signalling by Martin Rodbell (who first described G-proteins, for which he won the 1994 Nobel Prize for Medicine).[727,728] Signalling pathways follow a series of activation steps and secondary messengers. Unlike metabolic pathways, there is usually no single molecule that follows the course of the entire pathway. Although signalling pathways are usually defined as the events downstream of an initiating signal molecule (*e.g.*, insulin or adrenalin), that molecule seldom progresses along the entire pathway. Pathways often interact, multiple signalling molecules may initiate identical events in the same pathway, and some pathways may require multiple signals. The 'canonical' or textbook description of any signalling pathway is therefore a subjective construct, its boundaries defined by ease of interpretation and not by biological function. The descriptions of many pathways will therefore vary from textbook to textbook and from database to database, with no clear reasons why some proteins are included and some omitted. The pathways for coagulation and inflammation have multiple

initiators and are even harder to describe clearly or consistently because of multiple overlaps and interactions, with some molecules having apparently contradictory roles (for example, IL10 is said to be both pro-inflammatory and anti-inflammatory). There are two currently two large systematic efforts to develop canonical databases of known pathways: Reactome[729] and KEGG (the Kyoto Encyclopedia of Genes and Genomes).[730]

Gene ontology is an even more nebulous concept and the dictionary definition is 'the branch of metaphysics dealing with the nature of being'. A more helpful definition is that gene ontology is accurate and succinct description of genes. There are a number of projects that do this, the largest of which is the Gene Ontology project.[731] The GO project is developing and maintains three separate ontologies (controlled, structured vocabularies) for the description of gene products. These are (i) cellular component (e.g., nucleus or membrane or organelle), (ii) biological process (e.g., inflammation or small molecule transport or DNA replication), and (iii) molecular function (e.g., protein kinase or transmembrane transporter or oxidoreductase).

We elected to use Innate DB for our analysis because of its focus on manually curating pathways related to innate immunity.[725] It is also linked to other protein interaction databases and uses standard nomenclature such as those developed by HUGO and GO. The database takes lists of genes and clusters them by pathway or by ontology, the statistical test being the hypergeometric test.

The techniques for analysing microarray data (and high throughput data generally) are still an active area for research. There is therefore no definitive 'best practice' defined, but the literature support an approach that uses a relatively lax statistical criteria, so that both type 1 and type 2 error rates are reasonably controlled, then use functional analysis to eliminate false positives and aid interpretation.

The main barrier to exchanging microarray data and to reproducing microarray experiments is the complete lack of standardisation of fabrication, assay protocols or analysis methods. Many of these details of fabrication are commercial confidential, and assay protocols are usually specific to platform. However, in theory, analyses should be reproducible. The MIAME checklist (minimum information about a microarray experiment) defines the minimum

level of detail that should be reported for all microarray experiments, and has been adopted by many journals as a requirement for the submission of papers that incorporate microarray results,[732] with the aim of improving the reproducibility of microarray experiments.[733]

3.2 Methods

3.2.1 Study subjects

We compared peripheral white blood cell gene expression in (i) diabetics who were taking glibenclamide (case) or were not taking this or another sulfonylurea (control) at the time of admission to Sappasithiprasong Hospital with culture-confirmed melioidosis and sepsis, and (ii) otherwise healthy diabetics attending a routine out-patient clinic who were taking glibenclamide (case) or were not taking this or another sulfonylurea (control). Eligible cases for both studies were persons aged between 18 and 75 years. A total of 40 patients (10 in each group) were recruited in the period 31 Jan 2008 to 31 Oct 2008. Diabetes was defined as an abnormal Hb A_{1c} at enrolment (7.8% or greater,[734] Bio-Rad® D-10 fully-automated Hb A_{1c} system, Bio-Rad Laboratories) or a previous diagnosis of diabetes. The Hb A_{1c} concentration allowed us to identify patients with previously unrecognized diabetes who went on to die before we could make a diagnosis of diabetes by WHO criteria.

We obtained approval from the Oxford Tropical Research Ethics Committee and the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University for the gene expression study. Written informed consent was obtained from all subjects by a native Thai speaker. All procedures performed were in accordance with the Helsinki Declaration of 1975 (revised 1983).

3.2.2 RNA extraction and microarray analysis

A 3 ml blood sample was collected from each subject in a PaxGene™ Blood RNA tube (PreAnalytiX) and stored at −70°C prior to mRNA extraction according to the manufacturer's instructions. At study completion, blood samples collected in PaxGene™ Blood RNA tubes were transported in one shipment on dry ice to the Wellcome Trust Sanger Institute, Cambridge, where

RNA was extracted using the PaxGene™ Blood RNA Purification Kit (PreAnalytix, GmbH) according to the manufacturer's instructions.

RNA purity was measured using the ratio of absorbance at 260 and 280 nm and all samples had ratios between 1·7 and 2·1. RNA integrity was evaluated using the Bioanalyzer (Agilent) and all samples had RNA integrity numbers greater than 6. The RNA was amplified using the Illumina® TotalPrep RNA Amplification Kit (Applied Biosystems, Carlsbad, California) and assayed using the Illumina® HumanWG-6 v3.0 Expression BeadChip (Illumina, Cambridge, UK), which probes 48,804 transcripts from across the human genome. We examined all genes with ≥1·5-fold difference in expression with the false-discovery rate set to 10% (Student's *t*-test with Benjamini-Hochberg correction;[719] GeneSpring GX version 10.0, Agilent Technologies, California).

3.2.3 Quantitative reverse-transcriptase polymerase chain reaction

Results were confirmed by quantitative reverse-transcriptase polymerase chain reaction (qPCR): 500 ng of sample RNA was reverse transcribed using the QuantiTect Reverse Transcription kit (Qiagen 205313) according to the manufacturer's instructions. Primers were ordered from Qiagen gene globe, as follows: IL18R1 catalogue number QT00082922; IL8, QT00000322; TNF, QT01079561; IFNG, QT00000525; ACTB1, QT00095431. Real time PCR was performed in triplicate using the QuantiTect SYBR Green PCR kit (Qiagen 204145) according to the manufacturer's instructions on a StepOne Plus (Applied Biosystems) for 40 cycles. Melting curve analyses were performed for all PCR products. The β-actin housekeeping gene was selected as loading control because of the stability of its expression in the microarray analysis. Fold change was calculated using the comparative Ct method.

Gene ontology over-representation analysis was performed using InnateDB[725] (http://www.innatedb.ca/) and the *p*-values reported are for the hypergeometric test. Gene lists were supplemented by manual literature searches for genes not curated by InnateDB. Microarray data have been deposited at ArrayExpress, EMBL-EBI (accession number E-TABM-852-n).

3.2.4 Neutrophil stimulation study

Neutrophils from three healthy male caucasian volunteers were isolated using Polymorphprep™ (Axis-Shield, Oslo, Norway) according to the

manufacturer's instructions, then resuspended (final concentration $1{\cdot}0 \times 10^6$ cells/ml) in RPMI 1640 (Gibco, Invitrogen, Paisley, UK) + L-glutamine + 15% heat-inactivated fetal bovine serum (Lonza BioWhittaker, Verviers, Belgium). Purity was >99% by flow cytometry (Becton Dickinson FACSCalibur; monocytes and neutrophils identified by their characteristic scatter profile and CD14 staining). Cells were incubated with medium or 1.0 mM glibenclamide (comparable to the peak human plasma concentration achieved following a 20 mg oral dose) for 60 minutes in a 96-well plate, then stimulated with medium or heat-killed *B. pseudomallei* 1026b (5×10^7 cfu/ml) for 4 hours at 37°C in 5% carbon dioxide. The cells were centrifuged ($300 \times g$, 5 minutes) and the supernatant removed. The pellet was resuspended (5% bovine serum albumin w/v, 350 μM EDTA, 0·01% sodium azide w/v in phosphate-buffered saline) and stained for CD14 and CD11b (BD Pharmingen, Erembodegem-Aalst, Belgium). IL1β (Invitrogen™, Camarillo, California), IL8 (Arcus Biologicals, Modena, Italy) and TNFα (Arcus) were measured by enzyme-linked immunosorbent assay according to the manufacturers' instructions. Paired t-tests were performed using Prism 5.0c for Mac (GraphPad Software, San Diego, California).

3.3 Results

There were no significant differences in any of the clinical parameters recorded for the two patient groups (Table 12). The expression of 205 probes (representing 186 distinct genes), were significantly different in patients taking glibenclamide compared to those who were not (Figure 21). Sixty-three immune-related genes were differentially expressed in melioidosis patients on glibenclamide (p=0·001), the net effect of which would be postulated to be anti-inflammatory (Appendix A). Prominent among the immune-related genes include those implicated in neutrophil activation, endothelial adhesion and transmigration. There was no statistical difference in neutrophil, lymphocyte or monocytes counts between the two groups (Table 12), indicating that this was not merely an effect of differences in cellular blood constituents. In the microarray analysis, tumor necrosis factor (TNF)-α, interleukin (IL)-18R1 and IL-8 expression were all down-regulated in glibenclamide-treated patients; interferon (IFN)-γ showed a modest trend to down-regulation. Quantitative

reverse-transcriptase PCR corroborated the TNFα and IL18R1 results; IFNγ was down-regulated by this methodology, but the IL8 results were not confirmed (Table 13).

We sought further evidence for an effect of glibenclamide on gene expression profiles in otherwise healthy diabetics who were taking glibenclamide (n=10) or who were not taking a sulfonylurea drug (n=10). There were no significant differences in baseline parameters between the two groups (Table 12). We found that 31 probes (29 distinct genes) were significantly different in patients taking glibenclamide compared to those not, 13 of which were immune related (Appendix A), again suggesting an anti-inflammatory effect. Of note, genes associated with neutrophil function were down-regulated in the glibenclamide group (p<0.001).

We then looked for a direct effect of glibenclamide on neutrophil function. Neutrophils harvested from healthy human volunteers were pre-treated with glibenclamide, then stimulated with LPS or heat-killed *B. pseudomallei*. CD11b expression and IL8 secretion were used to measure neutrophil stimulation. Concentrations of IL1β and TNFα were below the limit of detection. We were unable to find any direct anti-inflammatory effect of glibenclamide on neutrophils *in vitro* (Figure 22).

Diabetes patients with melioidosis			
Parameter	**Taking glibenclamide**	**Not taking glibenclamide**	***p*-value**
Male gender	6 of 10	5 of 10	1·00[a]
Age (years)	60±7	51±9	0·02[b]
Glucose (mg dL^{-1})	218±64	256±111	0·37[b]
Hb A_{1c} (%)	10·8±3.2	11·1±3.3	0·83[c]
Neutrophils ($\times 10^9$ L^{-1})	10·8±8·5	8·8±4.6	0·52[b]
Lymphocytes ($\times 10^9$ L^{-1})	1·6±0.9	1·5±1.4	0·93[b]
Monocytes ($\times 10^9$ L^{-1})	0·8±0·3	0·5±0·4	0·05[b]
Eosinophils ($\times 10^9$ L^{-1})	0·1±0·1	0·1±0·1	0·82[c]
Mortality	2 of 10	5 of 10§	0·41[a]
Diabetes patients who were otherwise healthy			
Parameter	**Taking glibenclamide**	**Not taking glibenclamide**	***p*-value**
Male gender	3 of 10	5 of 10	0·65[a]
Age (years)	54±11	56±11	0·66[b]
Glucose (mg dL^{-1})	126±31	124±48	0·93[b]
Hb A_{1c} (%)	9·0±2·1	9·0±2·2	0·98[c]
Neutrophils ($\times 10^9$ L^{-1})	4·7±1·3	5·1±2·1	0·63[c]
Lymphocytes ($\times 10^9$ L^{-1})	4·4±1·4	5·1±2·1	0·40[b]
Monocytes ($\times 10^9$ L^{-1})	0·4±0·2	0·6±0·3	0·19[b]
Eosinophils ($\times 10^9$ L^{-1})	0·7±0·4	0·8±1·3	0·22[c]

Table 12. Comparison of gene expression study patient characteristics.

Note.—Values are reported as means, except where stated. Errors reported are standard deviations.

[a]Fisher exact test.

[b]Welch *t*-test.

[c]Student *t*-test.

[d]One patient in this group was lost to follow-up following discharge from hospital, and was counted as having survived to discharge.

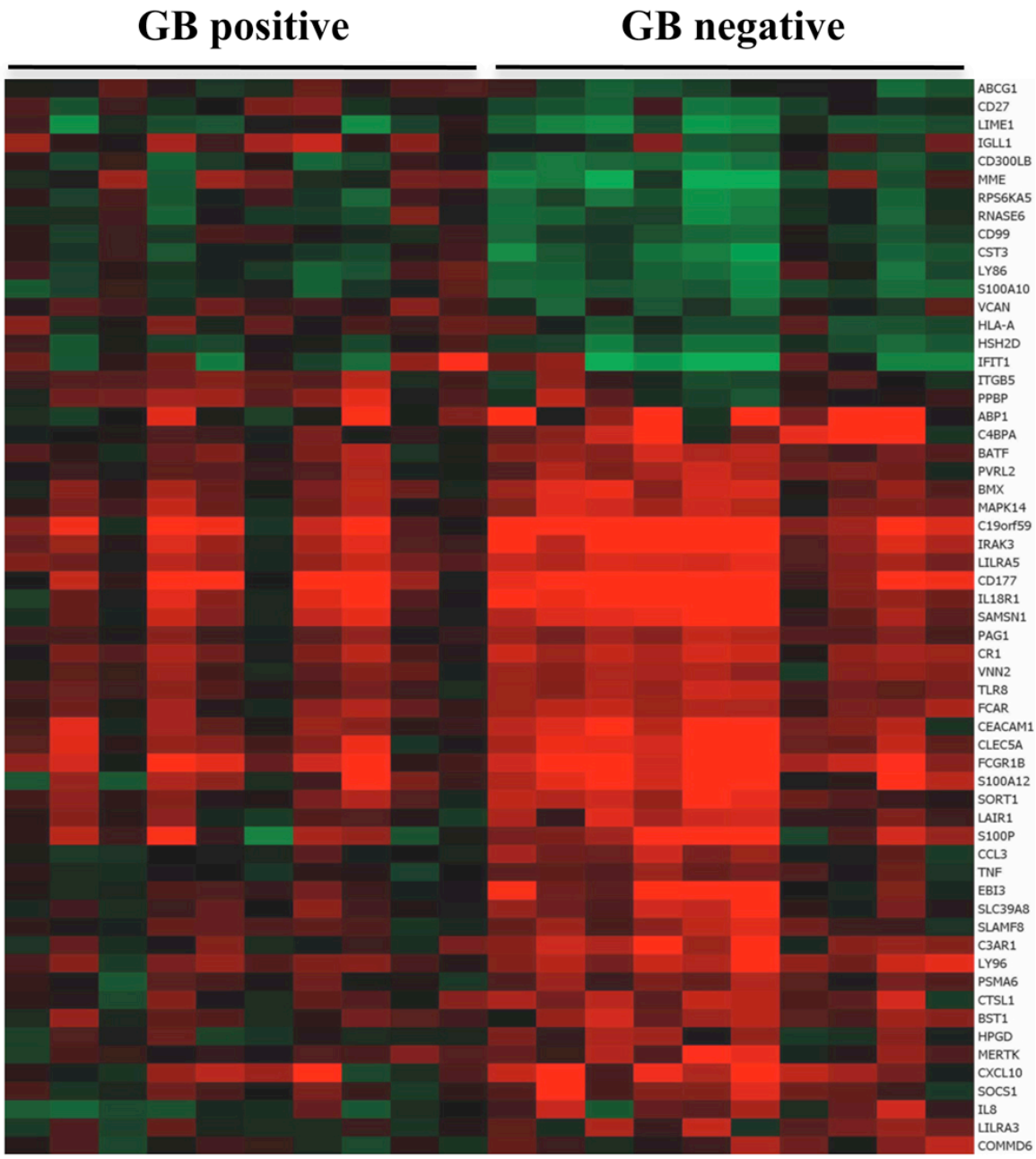


Figure 21. Differential expression of inflammation-associated genes in diabetic patients with acute melioidosis with or without glibenclamide.

Note.—Gb = glibenclamide. Genes that are up-regulated are shown in red, down-regulated in green; genes in black are not differentially expressed. The gene symbols used are those assigned by the HUGO gene nomenclature committee.

Gene	Fold change attributable to glibenclamide therapy in melioidosis patients
IFNγ	–2·32
IL8	+4·23
IL18R1	–1·39
TNFα	–15·2

Table 13. Results of quantitative reverse-transcriptase polymerase chain reaction.

Note.— IFNγ= gamma interferon; IL8 = interleukin 8; IL18R1 = interleukin 18 receptor 1; TNFα = tumour necrosis factor alpha.

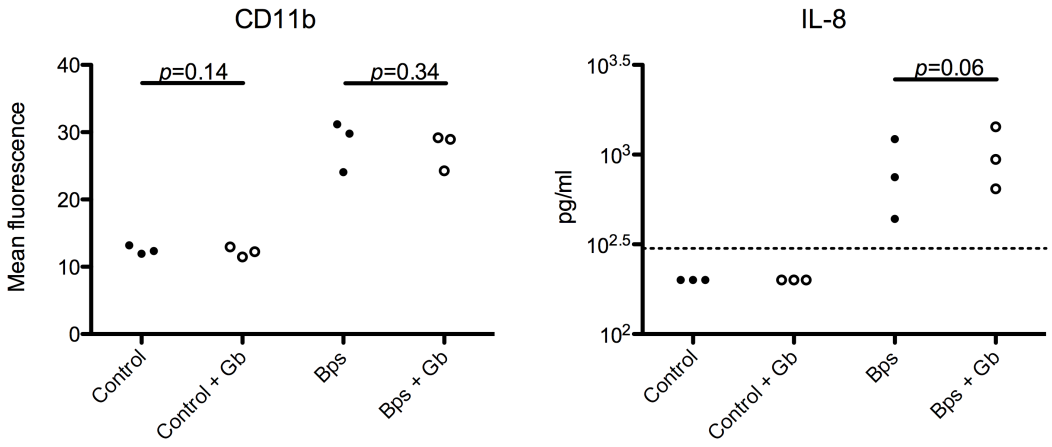


Figure 22. Effect of glibenclamide pre-treatment on neutrophil stimulation

Note.—Bps = heat-killed *Burkholderia pseudomallei*; Gb = glibenclamide, IL = interleukin. The interrupted line marks the limit of detection. Black dots represent control samples; open circles are samples pre-treated with glibenclamide. Isolated human neutrophils ($1{\cdot}0 \times 10^6$ cells/ml, >99% purity) were treated for 60 minutes with 1·0 mM glibenclamide then stimulated with heat-killed *B. pseudomallei* (5×10^7 cfu/ml). The cells were incubated at 37°C for four hours in 5% carbon dioxide, then CD11b expression was measured by flow cytometry. IL8 concentrations were measured in supernatant by enzyme-linked immunosorbent assay. IL1β and TNFα concentrations were below the limit of detection. The *p*-values reported are for paired t-tests.

3.4 Discussion

Glibenclamide is known to have a wide-range of anti-inflammatory effects, one of which is an inhibitory effect on inflammasome assembly.[735] The inflammasome is an intracellular protein complex present in macrophages that activates caspase 1 and converts pre-formed IL1β and IL18 to their active forms when presented with an appropriate inflammatory stimulus.[459] IL1β and IL18 levels are high in bronchoalveolar lavage fluid from patients with acute lung injury/acute respiratory distress syndrome (ALI/ARDS) and correlate with mortality,[736,737] which leads us to hypothesize that inhibition of inflammasome assembly may be a mechanism for our finding that glibenclamide therapy and respiratory failure are inversely associated. It is also unsurprising that fewer genes were found to be differentially expressed in the otherwise healthy diabetic controls than in the patients with melioidosis, as the inflammasome is only assembled as part of the acute inflammatory response and so we expect to see little effect of glibenclamide when there is no active inflammation.

Neutrophil recruitment to an area of tissue inflammation or infection can also be linked to the inflammasome through the action of IL1β. While IL1β is not itself a chemoattractant for neutrophils,[738] IL1β up-regulates the expression of endothelial intercellular adhesion molecules essential for the recruit of neutrophils to an area of inflammation.[515,516] Neutrophils are a critical component of the host response to *B. pseudomallei in vivo,*[326] but may also drive tissue injury and organ damage.[739–741] The bronchoalveolar fluid of patients with ALI/ARDS is neutrophil-rich and some animal models of ARDS are neutrophil-dependent.[739] Glibenclamide has been shown to prevent neutrophil extravasation and accumulation in animal models,[740,741] and this may represent indirect evidence that glibenclamide was responsible for reducing the incidence of respiratory failure in study patients taking this drug. We speculate that conflicting evidence from previous observational studies of the impact of diabetes on outcomes from bacterial sepsis may be explained in part by differences in the management of type 2 diabetes between Europe (metformin has been available in Britain since 1958 and has traditionally been used first-line) and the US (where sulfonylureas have often been used first-line[742] and metformin has only been available since 1995).

Glibenclamide has been said to inhibit the inflammasome by blocking K_{ATP}-channels,[743] but this seems unlikely, since the K_{ATP}-channel blockers glipizide and tolbutamide do not have this effect.[744] The two K_{ATP}-channels known to be targeted by the sulphonylureas, ABCC8 (formerly SUR1) and ABCC9 (formerly SUR2). Neither of these proteins are expressed by macrophages.[744] The actual target therefore remains to be elucidated.[735,745]

The $P2X_7$ receptor has been suggested as a possible target,[746,747] but the target appears to be downstream of $P2X_7$[735] and there is no sequence or structural homology between $P2X_7$ and any of the known targets for glibenclamide. The development of inflammasome-inhibitors that do not have the hypoglycaemic effects of glibenclamide may depend on unravelling this pathway. However, enthusiasm for this approach should be tempered by the finding of one multi-centre trial that direct IL1β blockade does not improve survival in all-cause sepsis.[363] Our study is also unable to provide guidance on whether any benefit would be seen were glibenclamide started following the onset of infection in non-diabetic patients.

Inflammasome function is not restricted to IL1β activation and inflammasome inhibition is not the only action of glibenclamide. Glibenclamide is a broad-spectrum inhibitor of ABC-transporters[706–708] and many members of this family have been implicated in regulating immune function. Glibenclamide also has a direct effect on neutrophils chemotaxis when administered at a dose 100-times the concentration achievable in humans,[748] although we found no direct effect of glibenclamide on neutrophils *in vitro*.

4 Glibenclamide reduces interleukin 1β secretion in a mouse model of melioidosis

4.1 Introduction

Having found an anti-inflammatory effect of glibenclamide in patients with melioidosis, we sought to replicate this finding in an experimental model. The effect we found in the clinic was a general effect on inflammation, but without one pathway predominating. We therefore hoped than an experimental model might provide insight into mechanisms underlying the effect of glibenclamide.

4.1.1 Animal models of melioidosis

The model organism most commonly used to study melioidosis is the mouse (*Mus musculus*). The first report of experimental *B. pseudomallei* infection in mice was by de Moor *et al.* in 1932, using a strain described only as *weiss* or 'white'.[242] An important advantage of using a mouse model is the wide range of tools and reagents available commercially (monoclonal antibodies, cytokine assays, bioinformatics, and so forth). Indeed, the largest library of gene knockouts available is in mice,[749,750] and a decision not to use mice means to forego access to these tools. European legislation requires that the model used be the least sentient model possible, and there exist established models of both melioidosis and diabetes in the mouse: this was therefore a consideration in the selection of a suitable model for this part of the project.

A number of mouse models of melioidosis have been described, but current published work uses only one of two inbred strains: BALB/c and C57Bl/6.[751,752] Other strains that have been used include the C3H mouse,[752] the DBA/2 mouse[752] and the Taylor outbred mouse.[373] Older mouse studies refer to no specific mouse strain and refer only to colour (white, black or agouti), if at all.

BALB/c and C57Bl/6 inbred mouse strains form contrasting models of melioidosis. In 1999, Hoppe *et al.* surveyed four mouse species (C57Bl/6N,

BALB/c, C3H/HeN and DBA/2).[752] He found that BALB/c mice were relatively susceptible to *B. pseudomallei* infection, while C57Bl/6N mice were relatively resistant. Susceptibility of the C3H/HeN and DBA/2 strains were intermediate. The contrasting susceptibilities of BALB/c and C57Bl/6 has been independently replicated by other groups.[751,182] The BALB/c mouse is more susceptible to *B. pseudomallei* infection, with an LD50 of ~5 cfu. By comparison, the LD50 for C57Bl/6 mice is consistently two to four orders of magnitude higher, depending on route of infection.[751,752,182] In C57Bl/6 mice, smaller inocula (*e.g.,* 100 cfu of *B. pseudomallei* 576) produce a chronic version of melioidosis.[753]

Hoppe found that in BALB/c mice, bacterial loads increased faster in the first 24 hours than in C57Bl/6 mice. Hoppe also found a higher IgG2a:IgG1 ratio in C57Bl/6 mice compared to BALB/c mice, which is suggestive of a Th1-type immune response,[752] because IFNγ promotes IgG2a isotype switching.

This dichotomy between BALB/c and C57Bl/6 has been found in other infections, *e.g., Leishmania major*,[754] *Mycobacterium tuberculosis*,[755] and *Yersinia enterocolitica*,[756] where in each instance, BALB/c mice are relatively susceptible, but C57Bl/6 mice are resistant.[*] The susceptibility of BALB/c mice to *Leishmania major*[526,527] and *Y. enterocolitica*[756] correlates with a deficiency in the IFNγ response (traditionally considered a Th1 cytokine). Indeed, it was initially thought that the contrasting BALB/c and C57Bl/6 models of melioidosis paralleled the Th1/Th2 paradigm developed for *Leishmania*.[751]

Ullett *et al.* found that contrary to there being an inadequate IFNγ-response in BALB/c mice compared to C57Bl/6 mice, IFNγ mRNA levels in liver and spleen were higher in BALB/c mice than in C57Bl/6 mice.[759] This finding was confirmed by Koo and Gan using direct measurements of IFNγ as well as measurements of IFNγ mRNA.[410] Koo and Gan found that splenocytes from BALB/c mice produced higher concentrations of IL18 and TNFα compared to splenocytes from C57Bl/6 mice (IL12 concentrations were not different), which in turn resulted in high concentrations of IFNγ. They hypothesised that the elevated cytokine response in BALB/c mice is explained by a reduced responsiveness of phagocytes to IFNγ. This hyporesponsiveness manifested as a

[*] There exist infections where the converse is true. The C57Bl/6 mouse is more susceptible to *Helicobacter felis*[757] and *Pseudomonas aeruginosa*[758] compared to the BALB/c mouse.

failure of BALB/c phagocytes to kill intracellular bacteria despite adequate IFNγ stimulation. They felt that the continued presence of intracellular bacteria in macrophages was responsible for the continued secretion of IL18 and TNFα by macrophages, which in turn was driving IFNγ secretion. That there is no defect in the cytokine response was confirmed by Barnes *et al.,* who found that cytokines downstream of IFNγ, such as CXCL9 (formerly called Mig) and CXCL10 (formerly called IP-10), were expressed earlier and at higher levels in BALB/c mice compared to C57Bl/6 mice.[760] Recruitment of neutrophils to sites of infection in the liver and spleen also occur earlier and in greater numbers in BALB/c mice compared to C57Bl/6 mice, again consistent with an inability of BALB/c mice to kill the bacteria, despite apparently adequate cytokine and cellular responses.

The mechanism underlying the reduced ability of BALB/c mice to kill intracellular *B. pseudomallei* is as yet unknown. Miyagi *et al.* showed that RNS, and to a lesser extent, ROS were important for intracellular killing in the J774.1 macrophage-like cell line[521] (which is BALB/c derived[761]). However, subsequent studies have found that differences in intracellular killing between BALB/c and C57Bl/6 strains are not explained by differences in RNS and ROS generation.[519,523]

Watanabe *et al.* have reported that BALB/c macrophages expressed lower levels of the lysosomal enzyme, β-glucuronidase, in response to macrophage-activating lipopeptide-2 (a synthetic TLR2 ligand) and to *E. coli* LPS when compared to C57Bl/6 macrophages.[762] This is an attractive explanation for the susceptibility of BALB/c mouse, but the role of lysozymal enzymes has not been explored in the context of the host response to melioidosis. In humans, β-glucuronidase deficiency manifests as Sly syndrome, or mucopolysaccharidosis type VII. The potential association of the BALB/c mouse with an inherited human disease might prompt caution in the interpretation of experiments conducted using this strain.

One final comment on the Th1/Th2 balance in melioidosis may be made. In the context of melioidosis, IFNγ is produced predominantly by NK cells and $CD8^+$ T lymphocytes, not by $CD4^+$ T helper cells.[360,411] In fact, T helper cells seem to play little or no role in the initial host response to melioidosis, although

they do have a role at later stages in the infection.[411] This picture does not fit neatly into the conventional Th1/Th2 paradigm,[763] in which T helper cells (Th) are the primary orchestrators of the cytokine response, even though the cytokine profile in melioidosis is consistent with the classical Th1 response.

A variety of *B. pseudomallei* strains are used in animal models, which hinders the comparison of results from different groups, since different strains likely have different virulence. Ivo Steinmetz's group in Greifswald uses the E8 environmental strain of *B. pseudomallei* collected from Northeast Thailand in 1996 by Andrew Simpson.[32,83,519,752] Despite being an environmental strain, E8 is more virulent than NCTC 7431, which is a clinical isolate.[752] Work published by Greg Bancroft's group in London uses the 708a strain,[360,373] which is a clinical isolate from Thailand. Rick Titball's group at the Defence Science and Technology Laboratory (now at Exeter University) uses the 576[764] and K96243[107] strains. We are not aware of any published studies directly comparing the virulence of these strains.

The work described here uses the C57Bl/6 model of melioidosis first described by Wiersinga *et al.* in 2007.[152,412,765] In this model, 10–14 week-old animals are inoculated with 500 cfu of *B. pseudomallei* intranasally. Animals develop a severe pneumonia, bacteraemia and microabscesses in the liver and spleen, and median survival is four days.[152] The strain used was *B. pseudomallei* 1026b, which was originally collected by David Dance in 1993 from a 29-year-old Thai female rice farmer with bacteraemia, soft tissue, cutaneous lesions, septic arthritis and splenic abscesses.[93] The strain was donated to Don Woods' group in Calgary, Canada, in 1996[93] and subsequently transferred from Canada to Tom van der Poll's group in Amsterdam, The Netherlands, in 2006, where it has since been maintained by serial passage in mice.

A variety of other animals have been used to model human melioidosis. In Whitmore's 1912 description of the disease, Koch's postulates were established using guinea pigs,[8] while Stanton & Fletcher and de Moor *et al.* both described rat models of melioidosis. Rats are relatively resistant when inoculated intraperitoneally (LD_{50} >10^8 cfu), unless made susceptible by inducing diabetes with streptozocin (LD_{50} 10^3–10^4 cfu).[151] Like mice, susceptibility is also increased by administering the inoculum directly into the respiratory tract (<10^2 to 4·0×10^4 cfu).[766] Hamsters (*Mesocricetus auratus*) are extremely susceptible

to *B. pseudomallei* infection,[35,155] which has led to their use in the study of bacterial virulence factors[86,88,91] and for the purification of mixed cultures.[140,156] However, some bacterial mutants that are attenuated in mice are not attenuated in hamsters,[86,88,93,94,767] and it is unclear which model better identifies virulence factors important to clinical melioidosis.

The first primate model of melioidosis was reported by Miller in 1948 using macaques.[155] More recently, a marmoset model of melioidosis has become available at the UK Defence Science and Technology Laboratory.[768] An NIH grant has also been awarded to Don Estes at the University of Texas, Galveston, to develop a marmoset model of melioidosis (grant no. 5U01AI082103-02 dated 29 Jul 2010). Other models reported in the literature include baboons (*Simia hamadryas*)[769] ferrets (*Mustela putorius furo*),[155] goats (*Capra aegagrus hircus*),[770] guinea pigs (*Cavia porcellus*),[769] pigs (*Sus scrofa domestica* Landrace),[771] and roundworms (*Caenorhabditis elegans*).[772,773]

The development of large animal models of melioidosis is critical, because in the evaluation of a new drug, both the US Food and Drug Administration and the European Medicines Agency require data from at least two animal models (small and large) as a pre-condition for the commencement of phase I clinical trials.

4.1.2 Mouse models of diabetes

Animals have had a prominent role in diabetes research for over a hundred years. In 1889, Oskar Minkowski removed the pancreas from a dog and found that it became diabetic,[774] leading the way to Banting and Best's discovery of insulin in 1922.[775]

We hypothesized that the effect of glibenclamide was independent of its effect on glucose control. Although the majority of cases of diabetes in Thailand have type 2 diabetes, we elected to use a model of diabetes in which glucose concentrations were not responsive to glibenclamide, because alterations to plasma glucose and insulin concentrations are themselves associated with changes to the immune response (as reviewed in Chapter 1 of this dissertation). This review is therefore confined to mouse models of type 1 diabetes.

The best-characterised and most widely-used mouse model of spontaneous type 1 diabetes is the inbred non-obese diabetic (NOD) mouse,

developed in 1974 by Makino at Shionogi Research Laboratories, Japan, from outbred Swiss mice.[776,777] Most mice of this strain develop some degree of insulitis (inflammation and destruction of β-islet cells), but progression to diabetes takes up to 24 weeks and depends on sex and environmental factors. Under specific pathogen-free (SPF) conditions,[778] 80–90% of females are diabetic by 24 weeks of age; by contrast, only 40% of males are diabetes by 30 weeks of age. The development of diabetes in this strain may be synchronised by a single dose of intraperitoneal cyclophosphamide 200 mg/kg,[779] which makes the mice easier to use.

The selection of an appropriate control for the NOD mouse is difficult because the NOD mouse was developed from an outbred strain, not an inbred strain, and because the defect is polygenic in origin and poorly defined.[780,781] Both C57Bl/6 mice and BALB/c mice have been used as control strains despite the fact that neither strain is Swiss-derived. Multiple Swiss-derived control strains have been developed for the NOD mouse, including the non-obese non-diabetic (NON) mouse (also developed by Makino), SJL/J, outcrosses of NOD with diabetes-resistant strains and various NOD congenic strains. The NON mouse is a particularly troublesome control, because despite its name, it is prone to obesity and develops impaired glucose tolerance.

Two other mouse models are worth mentioning briefly: the C57Bl/6NJCL-*Insulin2*^{Akita} and the C57BLKS/J-*Cpe*^{fat} mice.

C57Bl/6NJCL-*Insulin2*^{Akita} heterozygous males develop diabetes at weaning (four weeks of age) and the mouse was described in 1997 as a model for maturity onset diabetes of the young.[782] Histological examination showed that the mice had atrophic pancreatic islets deficient in β-cells and the mouse is therefore a model of type 1 diabetes. The defect is a missense mutation in the insulin 2 gene (*Ins2*) on chromosome 7, that renders it non-functional. Note that *Mus musculus* possesses two equally-expressed insulin genes: *Ins1* is located on chromosome 19.[783] These mice are insulin-responsive, and non-diabetic littermates may be used as controls. This mouse model has been proposed as a replacement for the STZ model.[784]

The C57BLKS/J-*Cpe*^{fat} mouse homozygous males are severely obese by 8 weeks of age, and this is associated with very high circulating insulin levels and hyperglycaemia, but not severe diabetes.[785] The model was therefore initially

described as a model for type 2 diabetes. The defect is a point mutation in carboxypeptidase E (*Cpe*) on chromosome 8, which results in abnormal proinsulin processing. The mouse is therefore insulin-responsive and not insulin-resistant. The C57BLKS/J background has problems with dental malocclusion and polycystic kidney lesions which the Jackson Laboratories solved in 2005 by moving the *Cpe*^{fat} mutation to a C57Bl/6 background.

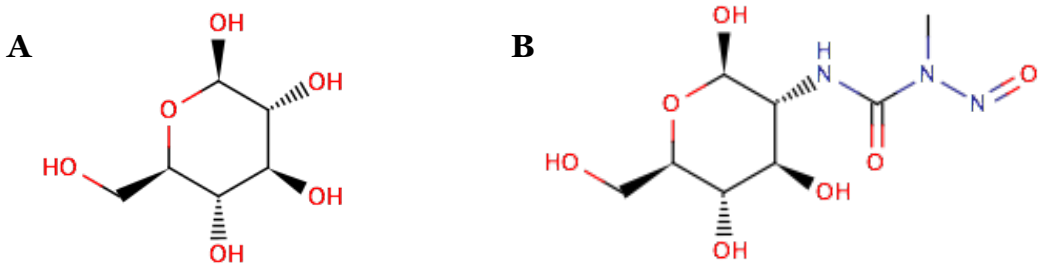


Figure 23. A. D-Glucose. B. Streptozocin.

Note.—H = hydrogen; N = nitrogen; O = oxygen. The glucose and streptozocin (STZ) molecules are oriented to emphasise their structural similarity.

Streptozocin (STZ) was first identified in the late 1950's by scientists at Upjohn (U.S. patent 3,027,300 granted 1961) and is a true antibiotic produced by *Streptomyces achromogenes* (an actinomycete isolated from soil collected in Blue Rapids, Kansas).[786]

STZ is a glucosamine-nitrosourea compound and, like other nitrosoureas, it exerts its cytotoxic effects by alkylating DNA.[787] The glucose transporter, SLC2A2 (previously called GLUT2), is highly expressed by β-cells. The structural homology between glucose and the glucosamine moiety of STZ (Figure 23) allows STZ to be preferentially taken up by the β-cell,[788] which then induces a cytotoxic inflammatory response that destroys the cell.[789] This explains the specificity of STZ for pancreatic β-cells and STZ therefore has a clinical application as treatment for β-islet-cell tumours.[790]

STZ-treated animals were first described in 1963 by Rakieten *et al.*[791] as a model for diabetes and the model is similar to the older alloxan model of

diabetes (first described 1943).[792] Both STZ and alloxan are taken up by SLC2A2, but alloxan kills the cell by producing reactive oxygen species instead of by DNA alkylation. The main advantage of STZ over alloxan is its (relative) chemical stability, which makes it easier to use.[788] The main disadvantage of both chemical models of diabetes is that SLC2A2 is also expressed in the renal tubule, which means both STZ and alloxan are potentially nephrotoxic. This nephrotoxicity may be avoided by using multiple smaller doses of STZ (*e.g.,* 60 mg/kg over five days) instead of fewer large doses (*e.g.,* two doses of 150 mg/kg).[793] In the STZ model of diabetes, glucose levels start to rise a week after initiating STZ treatment and plateau roughly two weeks after the last dose of STZ. Only male mice are susceptible to the effect of STZ, which may seem unusual, but in fact, all mouse models of diabetes exhibit some sexual dichotomy. The reason for this is not known.

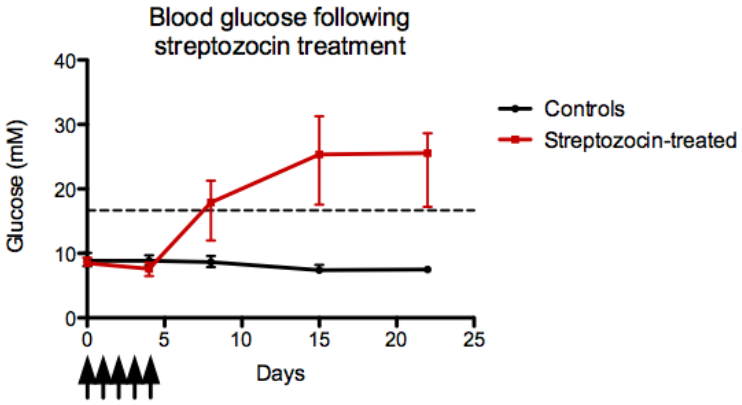


Figure 24. Development of hyperglycaemia in mice treated with STZ.

Sixty mice were injected with STZ 60 mg/kg daily for five days (marked by arrows on horizontal axis) and compared to 60 controls (treated with citrate buffer pH 4). STZ-treated mice are shown in red, control mice are shown in black. The horizontal interrupted line is the 16·7 mM cutoff we used to define diabetes in mice. This number of mice was chosen to allow for losses due to mice not becoming diabetic or mice dying from hyperglycaemia prior to inoculation.

An STZ model of melioidosis has previously been described by Woods *et al.,* who used an infant rat model of melioidosis.[151] In Woods' report, STZ-treated infant rats were more susceptible to *B. pseudomallei* infection than untreated animals (LD_{50} ~10^3–10^4 cfu compared to >10^8 cfu).

STZ is stable in solution at pH 4 and rapidly degrades at higher or lower pH.[791] STZ solutions are therefore made up with cold sodium citrate buffer 50–100 mM.[793,794] Higher concentrations of sodium citrate are more irritant. Solutions are not stable for longer than 12 h and must be made up freshly each day. Woods' model uses intraperitoneal streptozotocin 80 mg/kg on two consecutive days,[151] with controls given phosphate buffered saline. It might be argued that Woods should have used citrate buffer for controls instead of phosphate-buffered saline in his experiments. Another idiosyncracy of Woods' report is that the STZ dose he used is not attested elsewhere in the literature: he references the STZ rat model of Tancrède *et al.,* but Tancrède does not describe a 80 mg/kg dose.[795]

Since starting this project, we have become aware of two other groups working on STZ-models of melioidosis. The first group is based in Townsville, Australia, and used a BKS.Cg-*Dock7*$^{m+/+}$*Lepr*^{db}/J mouse model of diabetes.[677] This model has a spontaneously arising autosomal recessive mutation in the leptin receptor (*Lepr*^{db}) that arose in the C57BLKS/J (BKS) colony at The Jackson Laboratories in 1966 and is considered a model of type 2 diabetes.[796] This model is characterized by obesity, hyperglycaemia and insulin insensitivity. Using this model, Hodgson *et al.* found that diabetic mice had decreased survival following subcutaneous *B. pseudomallei* infection, with 100% succumbing to the disease in the first four days post-infection, compared to non-diabetic mice, all of which survived ≥10 days. The group found no defect of neutrophil or dendritic cell function. The second group is in Kuala Lumpur and is run by Sheila Nathan at the Universiti Kebangsaan Malaysia; their data is awaiting publication and was not available at time of writing.

Of the available models for type 1 diabetes, we elected to use the STZ model of diabetes for practical reasons. The model is easy to use: the phenotype may be induced on any background, at any time, to produce a cohort of mice that become diabetic synchronously. At the time the experiments were designed, we considered the possibility that we might need to use knockout mice to elucidate mechanisms and the knockouts available to us were all on a C57Bl/6 background. Furthermore, the murine melioidosis model in Amsterdam was already established in C57Bl/6 mice.

The C57Bl/6 inbred strain separated from the parent C57Bl outbred strain in 1937 and the colony was moved to the Jackson Laboratories in 1948. The C57Bl/6J substrain male mouse (Jackson Laboratories) is a model for diet-induced type 2 diabetes. This is due to a spontaneous 17·8 kb deletion in the nicotinamide nucleotide transhydrogenase gene, *Nnt*, that appeared in the Jackson Laboratories colony some time between 1951 and 1970.[797,798] The C57Bl/6N substrain (National Institutes of Health, Bethesda, Maryland) separated from the Jackson Laboratories colony in 1951 and was distributed to Charles River (Wilmington, Massachusetts) in 1974 (designated C57Bl/6NCrl). The C57Bl/6N substrain has an intact *Nnt* gene[799] and is the substrain used for all mouse experiments described here. The C57Bl/6N substrain is also the background used by the Knockout Mouse Project.[749,750] This is important, because there are published studies that have inadvertently used wild-type C57Bl/6J mice as non-diabetic controls, not realising that this substrain is a model for diabetes.

4.2 Methods

4.2.1 Mouse experiments

Specified pathogen-free 10-week-old C57Bl/6NCrl mice (Charles River) were given STZ 60 mg/kg daily for five days.[793] We chose to use a low-dose regimen because at this dose, STZ does not have a direct toxic effect on the kidneys,[793] which might otherwise confound our results. STZ 6 mg/ml (Sigma-Aldrich, Zwijndrecht, The Netherlands) was prepared fresh every day in citrate buffer (pH 4), then passed through a 0·2 μm polyethersulphone filter (VWR 514-0073) and administered intraperitoneally within 60 minutes of preparation. Losses were around 20% with this regimen, either from complications of the hyperglycaemia or because the animals failed to become diabetic and had to be sacrificed.

Animals were allowed *ad libitum* access to food (801733 CRM, Tecnilab-BMI) and water, and were group-housed in polycarbonate cages with 12-hour light/dark cycles, temperature 18–22°C, humidity 40–65%. Plasma glucose was checked weekly by cheek puncture[800] (Bayer Contour meter). For 10-week-old mice not treated with STZ, glucose was 8·75±1·52 mM (mean±standard

deviation) and for 16-week-old mice, 9·30±1·64 mM. We defined diabetes as a plasma glucose ≥16·7 mM.[793]

Mice were infected with ~6×10^2 *Burkholderia pseudomallei* intranasally 4–5 weeks after STZ treatment. In order to mimic the clinical situation seen in our cohort study, we treated all animals with full-dose antibiotics. Ceftazidime 600 mg/kg (GlaxoSmithKline, Brentford, England) was administered intraperitoneally starting 24h after inoculation and continued twice daily until sacrifice.[296]

For glibenclamide experiments, glibenclamide 50 mg/kg (Sigma-Aldrich) in 20% dimethylsulphoxide (DMSO) was administered intraperitoneally starting 7 days before inoculation and continued until sacrifice; control mice were given 20% DMSO. This dose of glibenclamide was chosen because it is equivalent to the highest human dose (20 mg daily to a 50 kg Thai male) after taking into account differences in pharmacokinetics (human $t_{½} \approx 8$ hours, mouse $t_{½} \approx 1$ hour).

Eight animals from each group were sacrificed 48, 72 and 96 hours after inoculation. Mice were sedated with ketamine/medetomidine intraperitoneally (12·5 mg/20 μg per 100 g weight), then exsanguinated by cardiac puncture. Death was confirmed by cutting the diaphragm. Glucose measurements were obtained prior to sedation as medetomidine itself elevates glucose concentrations.

The lungs were lavaged with three 300 μl aliquots of sterile 0·9% saline then collected for culture. Lung, liver and spleen were homogenized in four volumes of sterile saline. Organ homogenates, blood and bronchoalveolar lavage fluid (BALF) were plated onto blood agar in serial dilutions to quantify bacterial burdens. The remaining lung homogenate was incubated for 30 minutes with Greenberger lysis buffer then centrifuged at $650 \times g$ for 10 minutes. The supernatant was passed through a 0·2 μm filter to remove viable bacteria, then stored at –20°C for cytokine measurements. Heparinized blood was centrifuged at $700 \times g$ for 10 minutes; the plasma was filtered and stored at –20°C pending analysis.

In lung homogenates and BALF, interleukin(IL) 1β, IL6, IL10, CXCL5 (LIX) and tumor necrosis factor-α (TNFα) were measured by sandwich ELISA (R&D Systems, Oxford, England). Cytokine levels in plasma were measured by

particle immunoassay (mouse inflammation cytometric bead array, BD Biosciences, San Jose, California) because of limited sample quantity. Protein concentrations were measured using the DC™ protein assay (Bio-Rad, Hercules, California).

Statistical analyses were performed on Stata 11 (StataCorp, College Station, Texas). Bacterial loads and cytokine concentrations were log-normal. Linear regression was used in preference to ANOVA because it reports not just a *p*-value for each group, but also estimates size of difference and permits interaction testing. Models were fit by the method of variance-weighted least-squares, because the method does not assume homoscedasticity and because all explanatory variables could be treated as categorical. Responses were not assumed to vary linearly with time. Separate *p*-values were reported for each time point only when justified by a test for interaction.

Mouse experiments were performed at the Academic Medical Center, University of Amsterdam and were approved by the animal experimentation committee there (DIX101725).

4.3 Results

4.3.1 Glibenclamide does not reduce glucose concentrations in the STZ model of diabetes

The host response is altered by changes in glucose concentration. The primary action of glibenclamide in diabetes is to reduce blood glucose concentrations by stimulating insulin secretion by the β-islet cells of the pancreas. STZ treatment destroys the β-cells of the pancreas, making the animals hyperglycaemic and unresponsive to the action of glibenclamide. This allows us to dissect out the separate effects of glibenclamide on glucose and on the inflammatory response. Blood glucose measurements taken at sacrifice confirmed that in the STZ model of diabetes, blood glucose concentrations are not altered by glibenclamide treatment (Figure 25).

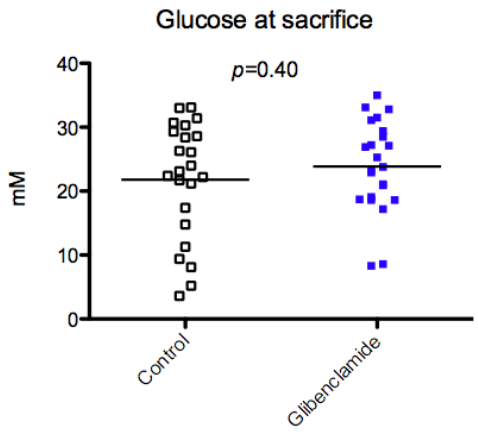


Figure 25. Glucose concentrations do not fall with glibenclamide treatment in strepztozocin-treated mice.

The graph shows plasma glucose at sacrifice in mice with STZ-induced diabetes and compares mice treated with glibenclamide against control mice treated with vehicle alone. Glibenclamide 50 mg/kg once daily was started 7 days prior to inoculation and continued until sacrifice; controls were given vehicle alone. All mice were infected with *B. pseudomallei* 48, 72 or 96 hours prior to sacrifice.

4.3.2 Glibenclamide does not alter bacterial loads in the lungs

Glibenclamide is structurally related to the sulphonamides, which are active against *B. pseudomallei*. If glibenclamide inhibits the growth of *B. pseudomallei,* then any differences in the inflammatory response might be explained by a direct effect on the bacterium and not by an effect on the host response.

In this model of melioidosis pneumonia, the primary site of infection is the lungs. We found no effect of glibenclamide on bacterial loads in either lung tissue or in bronchoalveolar lavage fluid at any of the three time points measured.

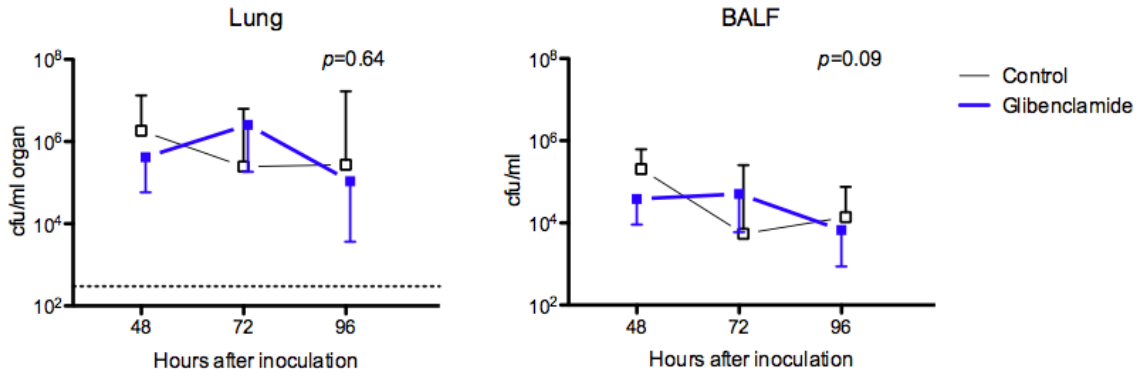


Figure 26. Bacterial loads in the lungs and BALF are unaffected by glibenclamide therapy.

Note.— BALF = bronchoalveolar lavage fluid.

Mice were treated with glibenclamide or vehicle for 7 days prior to inoculation with 650 cfu *B. pseudomallei*. All mice were treated with ceftazidime 600 mg/kg twice daily started 24 h after inoculation and continued until sacrifice at 48, 72 or 96 h (n = 8 per group per time point). Results for glibenclamide-treated animals are indicated in blue, while results from control animals are indicated in black. Error bars indicate standard deviations. Bacterial loads were determined by serial dilution on horse blood agar. The horizontal interrupted line marks the limit of detection for the assay.

4.3.3 Glibenclamide is associated with lower IL1β concentrations

Glibenclamide has previously been reported to inhibit inflammasome assembly[735] and one of the roles of the NLRP3 inflammasome is the activation and secretion of interleukin(IL)-1β. We found that in glibenclamide-treated animals, concentrations of IL1β were 73·3% lower in lung tissue 48 hours after inoculation compared to controls ($p < 0{\cdot}001$, Figure 27).

In lung tissue, IL1β is present in both its mature and immature forms. Only mature IL1β is secreted into the alveolar space, and IL1β in bronchoalveolar lavage fluid (BALF) should therefore be a better measure of inflammasome activation than lung tissue. We found that IL1β concentrations in BALF were 81·7% lower than controls ($p < 0{\cdot}001$) at 48 hours.

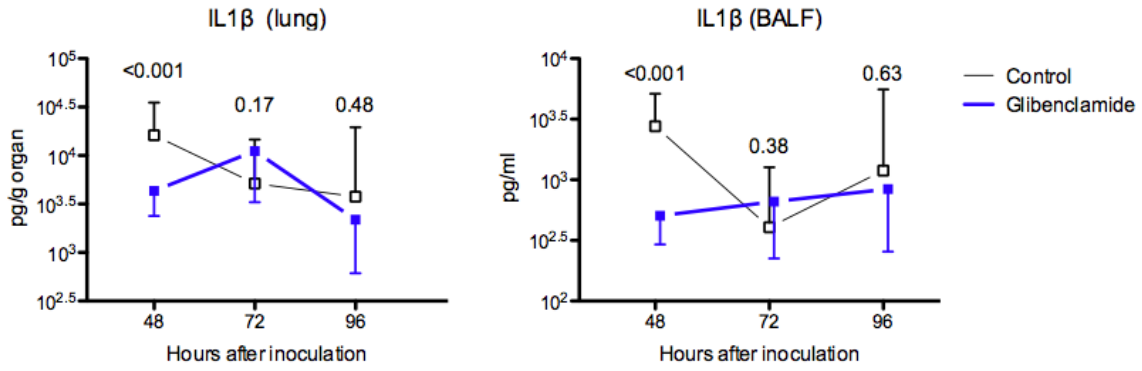


Figure 27. Cytokine levels in glibenclamide-treated mice (inflammasome related).

Note.— BALF = bronchoalveolar lavage fluid; IL = interleukin; IFNγ = interferon γ. Mice were treated with glibenclamide or vehicle for 7 days prior to inoculation with 650 cfu *B. pseudomallei*. All mice were treated with ceftazidime 600 mg/kg twice daily started 24 h after inoculation and continued until sacrifice at 48, 72 or 96 h (n = 8 per group per time point). Results for glibenclamide-treated animals are indicated in blue, while results from control animals are indicated in black. Error bars indicate standard deviations. A single *p*-value is reported for each cytokine unless there is evidence from a test of interaction that effects at each time point are different. The horizontal interrupted line marks the limit of detection for the assay.

4.3.4 Glibenclamide is associated with a reduced cellular influx into the lungs

Melioidosis pneumonia is characterised by a florid influx of cells into the lungs, and these cells are predominantly neutrophils.[765] Although neutrophils form a critical part of the innate host response to melioidosis,[326] neutrophils may also contribute to mortality by causing damage to the respiratory tract and have been implicated in the pathogenesis of the acute respiratory distress syndrome.[739] IL1β has been implicated in the recruitment of neutrophils to the inflamed lung, and we hypothesised that glibenclamide might function by limiting this influx of cells.[515,738]

In this study, we found that glibenclamide did not influence the wet weight of the inflamed lungs or the amount of protein in BALF (both are markers for capillary leakage) (Figure 28). We did however find that the total number of

cells was reduced in glibenclamide-treated animals compared to controls. The majority of cells in this inflammatory infiltrate were neutrophils, but macrophages were also present in significant numbers. This difference in cell counts was most apparent at the 48 h time point (Figure 28).

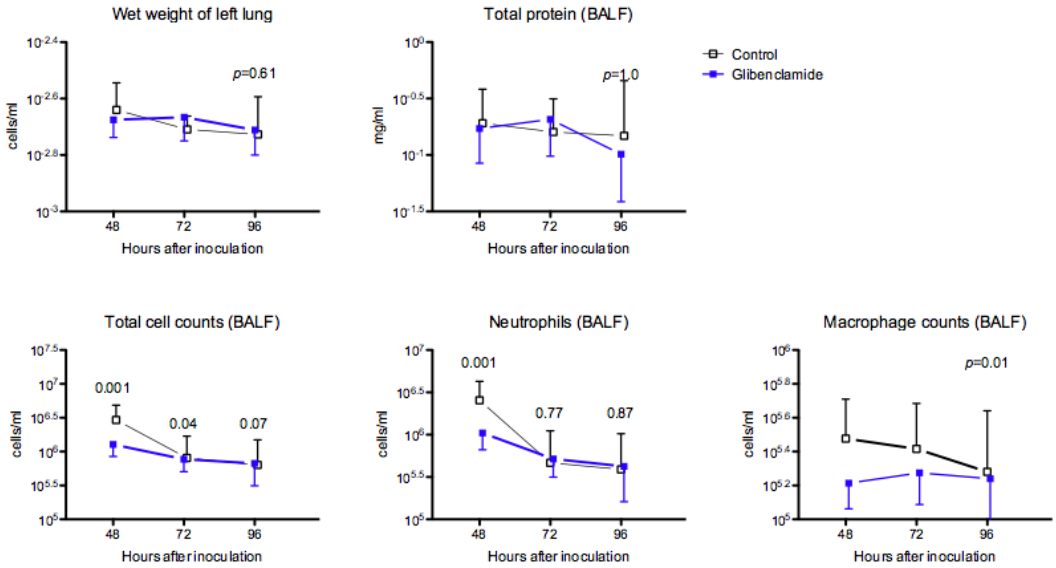


Figure 28. Lung infiltration in glibenclamide-treated mice.

Note.—BALF = bronchoalveolar lavage fluid. Mice were treated with glibenclamide or vehicle for 7 days prior to inoculation with 650 cfu *B. pseudomallei*. All mice were treated with ceftazidime 600 mg/kg twice daily started 24 h after inoculation and continued until sacrifice at 48, 72 or 96 h (n = 8 per group per time point). Results for glibenclamide-treated animals are indicated in blue, while results from control animals are indicated in black. Error bars indicate standard deviations. A single *p*-value is reported for each cytokine unless there is evidence from a test of interaction that effects at each time point are different.

4.3.5 Glibenclamide is associated with reduced dissemination of B. pseudomallei *from the lungs*

Melioidosis is characterised by haematogenous spread from the primary site of infection to distant organs such as a liver and spleen.[765] *B. pseudomallei* is a facultative intracellular organism with an ability to parasitise leukocytes. We hypothesised that a reduction in leukocyte recruitment to the site of primary

infection might therefore hinder the spread of infection to distant organs. We therefore measured bacterial loads in liver and spleen and found that bacterial loads were lower at all three time points measured (Figure 29). Consistent with this, bacterial burdens were also lower in blood, but this is less easy to interpret statistically, as a large proportion of points were below the limit of detection.

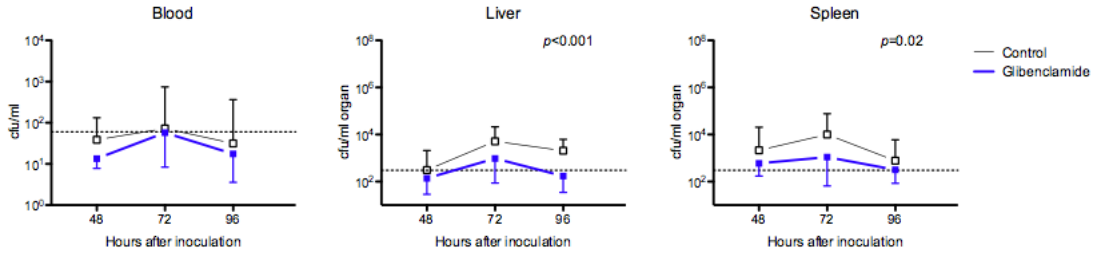


Figure 29. Bacterial loads in blood, liver and spleen are lower in glibenclamide-treated mice.

Note.—Mice were treated with glibenclamide or vehicle for 7 days prior to inoculation with 650 cfu *B. pseudomallei*. All mice were treated with ceftazidime 600 mg/kg twice daily started 24 h after inoculation and continued until sacrifice at 48, 72 or 96 h (n = 8 per group per time point). Results for glibenclamide-treated animals are indicated in blue, while results from control animals are indicated in black. P-values reported are for variance-weight least squares regression. Error bars indicate standard deviations. Bacterial loads were determined by serial dilution on horse blood agar. The horizontal interrupted line marks the limit of detection for the assay, which is 3 cfu/50 µl of sample (corrected for dilution), which is the smallest concentration of bacteria that may be distinguished from zero with 95% confidence. It was not possible to report a *p*-value for blood as >50% of data points were below the limit of detection.

4.3.6 Glibenclamide is not associated with differences in other cytokines

Another role of the NLRP3 inflammasome is the activation and secretion of IL18. IL18 then stimulates natural killer and $CD8^+$ cells to secrete interferon γ (IFNγ). Inflammasome inhibition would be expected to inhibit the secretion of mature IL18, and in turn reduce IFNγ secretion.

Immature IL18 is constitutively expressed at high concentrations in unstimulated cells.[503] We therefore did not measure IL18 in lung tissue. Instead, IL18 was measured in BALF, which should represent the mature secreted form

of the cytokine only. At the 48-hour timepoint, we found that IL18 concentrations were lower in glibenclamide-treated mice when compared to controls, however, this difference was not statistically significant (Figure 30).

We found no difference in IFNγ concentrations in lung. IFNγ concentrations in blood were lower at 48 hours, but this difference was also not statistically significant (Figure 30). IFNγ was not measured in BALF because previous experiments have shown it to be at or below the limit of detection in this model.

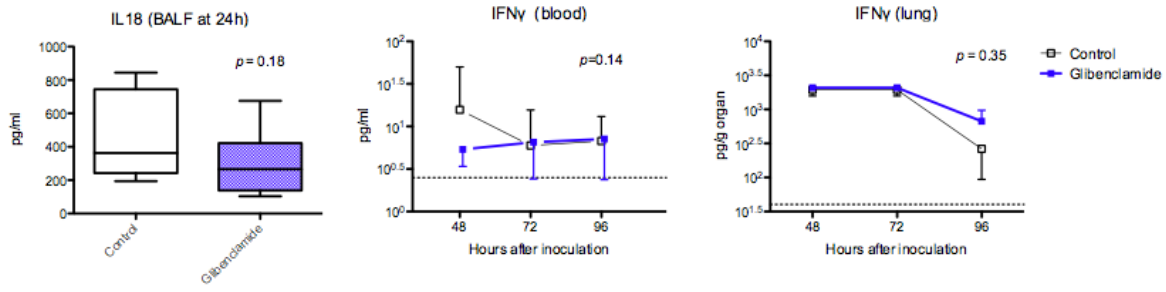


Figure 30. IL18 and IFNγ levels in glibenclamide-treated mice.

Note.—BALF = bronchoalveolar lavage fluid; IL = interleukin; IFN = interferon.
Mice were treated with glibenclamide or vehicle for 7 days prior to inoculation with 650 cfu *B. pseudomallei*. All mice were treated with ceftazidime 600 mg/kg twice daily started 24 h after inoculation and continued until sacrifice at 48, 72 or 96 h (n = 8 per group per time point). Results for glibenclamide-treated animals are indicated in blue, while results from control animals are indicated in black. Error bars indicate standard deviations. The horizontal interrupted line marks the limit of detection for the assay.

4.3.7 Glibenclamide has no effect on cytokines unrelated to the inflammasome

In order to determine if the effect of glibenclamide was isolated to IL1β, or whether it was a general anti-inflammatory effect, we assayed the pro-inflammatory cytokines, IL6, TNFα and CXCL5. The secretion of these three cytokines is unrelated to the inflammasome. We also considered the possibility that glibenclamide might be acting to promote secretion of the anti-inflammatory cytokine, IL10.

Although there were differences in IL6 and TNFα at the 48 h time point, these differences were small and did not reach statistical significance. Overall, we found no evidence of an effect of glibenclamide on the secretion of CXCL5, IL6, IL10 or TNFα (Figure 31).

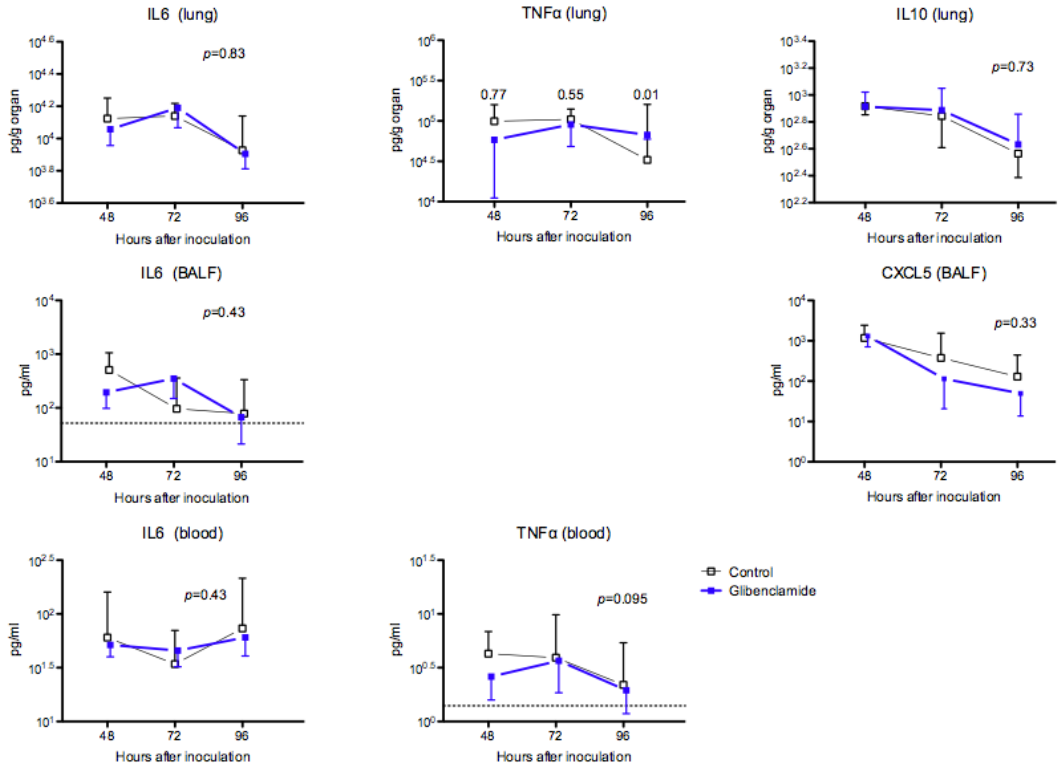


Figure 31. Levels of cytokine unrelated to the inflammasome in glibenclamide-treated mice.

Note.—IL = interleukin; TNF = tumour necrosis factor; CXCL5 = chemokine (CXC-motif) ligand 5. Mice were treated with glibenclamide or vehicle for 7 days prior to inoculation with 650 cfu *B. pseudomallei*. All mice were treated with ceftazidime 600 mg/kg twice daily started 24 h after inoculation and continued until sacrifice at 48, 72 or 96 h (n = 8 per group per time point). Results for glibenclamide-treated animals are indicated in blue, while results from control animals are indicated in black. Error bars indicate standard deviations. A single *p*-value is reported for each cytokine unless there is evidence from a test of interaction that effects at each time point are different. The horizontal interrupted line marks the limit of detection for the assay. In blood, concentrations of IL1β, IL10 and IL12p70 were all below the limit of detection (data not shown).

4.4 Discussion

We have presented evidence for an anti-inflammatory effect of glibenclamide in a diabetic mouse model of melioidosis and this anti-inflammatory effect appears specific to IL1β. This is consistent with the results of previous *in vitro* and *in vivo* reports.[735,801,802] To the best of our knowledge, this is the first time an inhibitory effect of glibenclamide on IL1β secretion has been described in any sepsis model.

A limitation of clinical studies of infection has always been that the interplay between the burden of infection and the inflammatory response has been difficult to tease out. Cytokine levels may be higher because bacterial burdens are higher, or bacterial burdens may be higher because of an ineffective innate host response.[376] Animal studies allow us to control the size of the initial inoculum and therefore the initial burden of infection. In this study, we found that bacterial loads in the lungs (the primary site of infection in our model) were not different between glibenclamide-treated and untreated mice. This excludes the possibility that the differences in IL1β levels in the lungs are due to an effect of glibenclamide on the burden of infection in the lungs.

We found that glibenclamide limits the cellular influx into the lungs, with effects on both neutrophil and monocyte numbers. Neutrophils are a crucial part of the host response to melioidosis,[326] but neutrophils have also been implicated in the pathogenesis of the acute respiratory distress syndrome.[739] Glibenclamide was not associated with a complete ablation of the neutrophil response (neutrophils are not a normal constituent of BALF taken from the healthy lung). Glibenclamide was only associated with a reduction in the magnitude of the neutrophil response and not its complete ablation. This balance between 'enough' and 'too much' is a common theme in the pathogenesis of sepsis and its complications.[803] A reduction in neutrophil influx into the lungs is consistent with our finding that glibenclamide is associated with a lower incidence of respiratory failure in patients with melioidosis (Chapter 2).

It is also worth noting that *B. pseudomallei* is a facultative intracellular pathogen and that it is capable of parasitizing macrophages.[79–81] Bacterial

burdens in blood, liver and spleen were lower in our model, suggesting that glibenclamide reduces bacterial dissemination. The role of neutrophils and macrophages in the dissemination of *B. pseudomallei* is not known, but it is possible to speculate that the reduced influx of leukocytes into the lungs reduces the number of cells parasitized by the bacterium and therefore impairs the ability of *B. pseudomallei* to disseminate.

We found no effect on cytokines unrelated to the inflammasome. Specifically, there was no effect on CXCL5, IL6, IL10 or TNFα secretion, which is again consistent with previous reports in the literature[735,804] and rules out a more general anti-inflammatory effect. In this model, all animals were treated with full-dose ceftazidime in order to simulate the clinical study that motivated this experiment.[329] Under this regimen, all animals showed signs of recovery by 96 hours and the fact that no differences were seen in cytokine responses at later time points is therefore not surprising.

The mechanism by which glibenclamide is able to inhibit IL1β secretion is not known. Lamkanfi *et al.* demonstrated that glibenclamide is able to inhibit assembly of the NLRP3 inflammasome in response to stimulation with lipopolysaccharide (LPS) and adenoside triphosphate (ATP). However, when *S.* Typhimurium was used as a stimulus, glibenclamide was not able to prevent inflammasome assembly, as measured by caspase 1 cleavage (caspase 1 being a critical component of the NLRP3 inflammasome). This suggests that there are alternative pathways which permit the assembly of the NLRP3 inflammasome, and the specific pathway that glibenclamide blocks is bypassed when macrophages are confronted by a complex stimulus such as a whole bacterium.

In our model, glibenclamide-treatment reduced IL1β secretion in BALF, but did not block it completely. This means that there must have been some inflammasome assembly despite glibenclamide treatment, because only mature IL1β is secreted into BALF. Immature IL18 is also cleaved by the inflammasome, so our finding that inflammasome assembly is not completely blocked by glibenclamide in melioidosis is supported by the fact that we observed only a modest reduction in IL18 that was not statistically significant.

Our study does not exclude other mechanisms for the action of glibenclamide. Aside from an effect on the inflammasome, Hamon *et al.* have presented evidence that glibenclamide may block the secretion of mature

IL1β.[804] Glibenclamide has also been shown to have other anti-inflammatory effects, including the prevention of ischaemic reperfusion injury,[740,741] and an enhancement of intracellular killing of *Leishmania* parasites.[805]

In conclusion, we have replicated our clinical finding that glibenclamide is associated with reduced inflammation in melioidosis. Specifically, we found reductions in IL1β secretion, cellular infiltration into the lungs and dissemination to distant organs. Glibenclamide has previously been shown to prevent IL1β maturation and secretion by inhibiting inflammasome assembly, but the presence of detectable IL1β in BALF coupled with smaller-than-expected effects on IL18 and IFNγ means inflammasome inhibition is incomplete, or that this may not be the mechanism by which glibenclamide acts to reduce IL1β in the context of melioidosis.

5 Final discussion and conclusion

We have shown that patients with a pre-existing diagnosis of diabetes have a lower mortality than patients without diabetes and patients with hyperglycaemia or undiagnosed diabetes. There is no protective effect of diabetes *per se:* instead, the mortality benefit seen is associated specifically with glibenclamide therapy. There was no evidence for an effect of diabetes on mortality after adjusting for the effect of glibenclamide. We also found that glibenclamide appears to protect from respiratory failure.

In a gene expression study of total peripheral blood leukocytes in melioidosis patients, glibenclamide was associated with a broad decrease in inflammation, with no one pathway predominating. We therefore used a mouse model to dissect out the effect of glibenclamide on the host response and found that glibenclamide reduces IL1β secretion in melioidosis with no effects on other cytokines. The reduction in IL1β secretion is also associated with a reduced cellular influx into the lungs, which may help explain the clinical association with a reduced incidence of respiratory failure, because neutrophils have been implicated in the pathogenesis of the acute respiratory distress syndrome. There was also an association with decreased bacterial dissemination to distant sites, which we speculate is related to the reduced cellular influx.

The mechanism by which glibenclamide reduces IL1β production by monocytes is not known. In the context of LPS stimulation, glibenclamide has been shown to block inflammasome assembly in isolated macrophages, however, we have only equivocal evidence for an effect of glibenclamide on the inflammasome in the context of melioidosis.

It may be argued that a knowledge of mechanism is not required for a drug to enter therapeutic use. The primary indication for glibenclamide is the treatment of diabetes and glibenclamide stimulates insulin production regardless of plasma glucose concentrations. The administration of glibenclamide to the critically ill but euglycaemic patient may therefore induce potentially fatal hypoglycaemia. While hypoglycaemia is managed with relative ease in the setting of the Western intensive care unit, the use of glibenclamide is

dangerous in a resource poor setting where close monitoring is not so readily available.

5.1 Cardiac effects of glibenclamide

A further problem with the use of glibenclamide is its effect on the heart. In 1970, the University Group Diabetes Program (UGDP) reported that cardiovascular mortality was 2½-times higher in patients treated with the sulphonylureas (principally, tolbutamide) compared to patients treated with placebo and that limb of the trial was terminated early.[2] These findings created controversy for a number of reasons: first, because they were unexpected and, second, because the excess mortality was seen at only three centres. These findings were not unchallenged[806,807] and the criticism surrounding their report was heated. The subsequent debate was governed in part by emotion,[808] but also by commercial interests centred around tolbutamide (Orinase®, then manufactured by Upjohn). Subsequent studies have reported mixed conclusions, with some supporting the effect of sulphonylureas on cardiovascular mortality and some not. The interested reader is referred to the fully referenced record of that debate which has been published elsewhere.[808]

The response to the UGDP results was mixed. Phenformin (the only biguanide then available in the US, and also the only oral alternative at that time) was withdrawn in the mid-1970's, on the back of evidence that it caused fatal lactic acidosis (also from the UGDP). The lack of oral alternatives and mixed evidence from subsequent trials meant a partial return of confidence in the use of sulphonylureas.[809] Doubts have lingered and the FDA mandated package insert for all sulphonylureas sold in the US still contains a bold-print warning about cardiovascular risks.[742]

Follow up studies looking at the effect of sulphonylureas of cardiovascular mortality have found that it is not a class effect, and that in addition to tolbutamide, glibenclamide is a prime offender.[810–812] Other members of the class, such as gliclazide do not appear to have this problem.

Glibenclamide is thought to increase the risk of cardiovascular disease by blocking ischaemic pre-conditioning.[813] Ischaemic pre-conditioning is a phenomenon in heart muscle where brief episodes of ischaemia render the heart more resistant to subsequent ischaemia. Diabetics often have diffuse coronary

disease which means diabetic patients who have a heart attack are likely to have had smaller ischaemic insults that presage the heart attack and later partially protect it from the effect of the larger, more serious event through ischaemic pre-conditioning. Ablation of the ischaemic pre-conditioning response removes this acquired resistance to ischaemia. There is indeed evidence from the warm-up phenomenon (patients with coronary artery disease are given two consecutive exercise tests, the relevant outcomes being symptomatic angina and electrocardiographic changes on the second test)[814,815] and from human angioplasty studies that glibenclamide is able to block ischaemic pre-conditioning.[816–818] The experimental evidence therefore supports the observational evidence that glibenclamide predisposes to cardiovascular mortality.

The effect on ischaemic preconditioning is not seen across the whole family of sulphonylureas. The second-generation sulphonylurea, glipizide, did not block ischaemic preconditioning in rabbits,[819] and the meglitinide analogues ("glinides") were originally marketed on the basis that they have a lower affinity for cardiac receptors (however, the post-marketing evidence for that is mixed: glimepiride appears not to block ischaemic pre-conditioning,[817,818,820] but repaglinide does[821]).

There is a second mechanism by which sulphonylureas might increase cardiovacular mortality, which is through their effects on smooth muscle. The second-generation sulphonylureas, glibenclamide and gliclazide, and (to a lesser extent) the glinide, glimepiride, all reduce coronary artery blood-flow, increase coronary resistance, depress the mechanical activity of the heart and increase myocardial oxygen extraction.[822] It may be noted that this vasopressor effect is precisely that which made glibenclamide an attractive candidate for the adjunctive treatment of sepsis in the 1990's.

These data should give us pause when considering the possibility of using glibenclamide to prevent infection-related mortality in patients with diabetes, because any gain from preventing infection-related mortality might be wiped out by an increase in cardiovascular mortality.

5.2 Separating the anti-inflammatory effects of glibenclamide from its effects on insulin secretion

Purely chemical considerations mean that separating the anti-inflammatory effect of glibenclamide from its hypoglycaemic effect may not be possible. To explain why this is so requires elaborating on the chemical structure of glibenclamide, its metabolites, and the pharmacological properties of the related compound, meglitinide.

The two major metabolites of glibenclamide are 4-*trans*-hydrocyclohexyl glibenclamide (**M1**) and 3-*cis*-hydrocyclohexyl glibenclamide (**M2** or **M2b**)[823] and account for 36% and 15% of the total, respectively (Figure 32). Experiments in rabbits suggested that these two compounds are biologically inactive (they are 1/400th and 1/40th as active, respectively, and data from rats show that 4-*trans*-hydrocyclohexyl glibenclamide is only 1/6·5 as potent as glibenclamide).[824] However, Rydberg *et al.* reported that in healthy human volunteers, glibenclamide, M1 or M2, all have equivalent biological activity.[823] Indeed, glibenclamide is much more likely to cause hypoglycaemia than its half-life would suggest (particularly in patients aged 70 years and older),[825] presumably because of its active metabolites.

Glibenclamide metabolites are excreted 50% in urine and 50% in bile. This is qualitatively different from that of other sulphonylureas, which are excreted primarily in the urine. Other metabolites that have been described are 4-*cis*- (**M2a**), 3-*trans*- (**M3**), 2-*trans*-hydrocyclohexylglibenclamide (**M4**), and ethyl-hydroxyglibenclamide (**M5**) (Figure 32).[826] Although the procedure for synthesising these metabolites has been described,[827] these compounds are not available commercially and their pharmacological and pharmacokinetic properties have not been studied clinically.

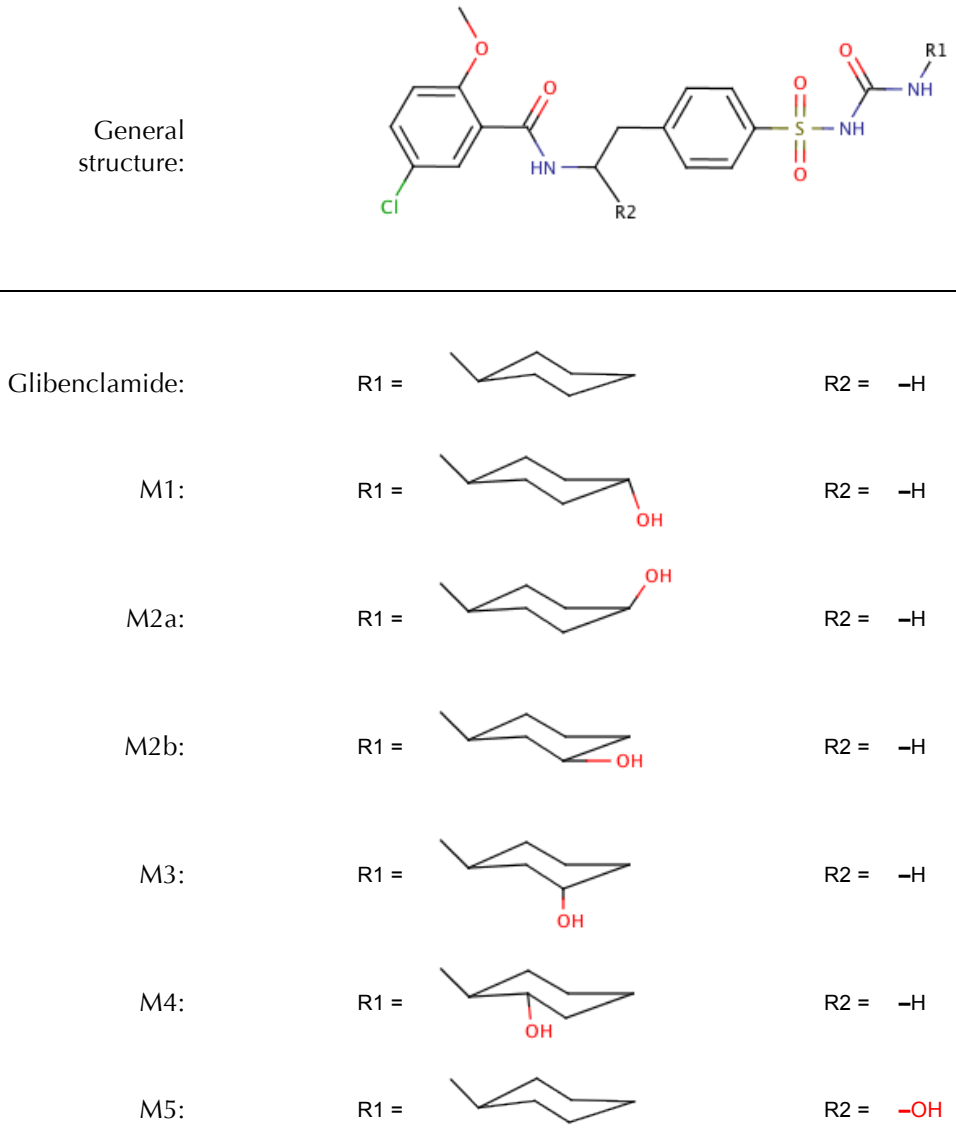


Figure 32. Structure of glibenclamide and its metabolites.

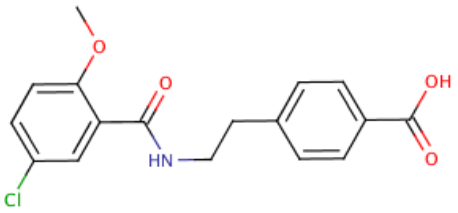


Figure 33. Structure of meglitinide.

The molecule is oriented to emphasise its homology with glibenclamide (above). IUPAC name: 4-[2-[(5-chloro-2-methoxy–benzoyl)amino]ethyl]benzoic acid (molecular weight, 333·8 g/mol). At present, meglitinide is not available commercially or clinically, but the compound was previously available as a gift from Hoechst (now part of Sanofi-Aventis). The patent on meglitinide is still in force, so it is not possible to study this compound without the cooperation of Sanofi-Aventis.

Nevertheless, certain properties may be deduced from the chemical structure of these compounds. Lamkanfi *et al.* have found that inflammasome inhibition by glibenclamide is not dependent on the cyclohexyl ring of glibenclamide.[735] Metabolites M1, M2a, M2b, M3 and M4 (M1–4) only differ from glibenclamide in that the cyclohexyl ring has been hydroxylated. If Lamkani is right, then the five compounds, M1–4, would all be predicted to inhibit inflammasome assembly. M5 differs from glibenclamide in that one of the carbons between the two aromatic rings patients has been hydroxylated and the effect of this modification is unknown.

Meglitinide is an insulin secretagogue that is not a sulphonylurea, but like the sulphonylureas, meglitinide will stimulate pancreatic β-islet cells to secrete insulin (Figure 33).[828] Comparison of the meglitinide with M1–4 reveals that the half of the glibenclamide molecule containing the two aromatic rings is almost identical to meglitinide. This means that metabolites M1–4 should all possess insulin secretagogue properties also, because the meglitinide moiety is unaltered. Evidence that this is true in humans is provided by Rydberg's clinical study, cited above.[823] This means that the anti-inflammatory part of the glibenclamide molecule is probably also responsible for at least part of its hypoglycaemic effect.

5.3 Suggestions for future research

The unwanted effects of glibenclamide (hypoglycaemia and adverse cardiovascular effects) mean that glibenclamide is an unattractive candidate drug for the prevention of melioidosis-related mortality. Further advances will only occur when the precise mechanism underlying the action of glibenclamide has been elucidated, because only then may rational therapies be designed.

IL1β is a product of the macrophage inflammasome and the inflammasome has already been studied in bone marrow-derived macrophage models of *B. pseudomallei* infection.[473,474] Future studies using these systems may help either to confirm or refute the role of the inflammasome in the effect of glibenclamide on the host response to melioidosis.

A Appendix. Microarray results

Appendix Table 1. List of genes differentially expressed in patients with melioidosis on glibenclamide at the time of admission

ProbeID	Symbol	Gene	RefSeq ID	Absolute fold change	Corrected *p*-value	Comment
Immune-related						
5860377	ABCG1	ATP-binding cassette protein G1	NM_016818.2	+1·55	0·03	Regulation of Toll-like receptor expression
5420440	ABP1	Amiloride-sensitive amine oxidase	NM_001091.2	–2·80	0·07	Postulated to be involved in endocytosis
6220195	BATF	Basic leucine zipper transcription factor, ATF-like	NM_006399.2	–1·54	0·05	Transcription factor elevated in B-cells following EBV infection
3140487	BMX	BMX non-receptor tyrosine kinase	NM_001721.4	–1·60	0·07	Regulates TLR-4-mediated IL-6 production.
6270681	BST1	Bone marrow stromal cell antigen 1; CD157	NM_004334.1	–1·56	0·05	?Regulation of B-cell maturation
1070367	C19orf59 or MCEMP1	Mast cell expressed membrane protein 1; chromosome 19 open reading frame 59	NM_174918.2	–2·95	0·04	Hypothesized to be involved in mast cell function or differentiation
840068	C3AR1	C3a anaphylatoxin chemotactic receptor	NM_004054.2	–2·30	0·03	Inflammatory response and cell motility
2000128	C4BPA	Complement component 4 binding protein, α subunit	NM_000715.3	–2·42	0·03	Classical pathway of complement cascade
6590682	CCL3 or MIP1A	Chemokine (C-C motif) ligand 3; *or* macrophage inflammatory protein 1A	NM_002983.1	–1·67	0·03	Neutrophil recruitment and activation
1300465	CD177	Neutrophil-specific antigen 1	NM_020406.2	–2·42	0·07	Neutrophil development and differentiation
1240450	CD27	CD27	NM_001242.4	+1·62	0·04	Generation and long-term T-cell immunity. Regulates B-cell activation and immunoglobulin synthesis
4610110	CD300LB	CD300 molecule-like family member B, TREM5	NM_174892.2	+1·57	0·04	Activating receptor expressed on myeloid cells
4290097	CD99	CD99 protein	NM_002414.3	+1·61	0·03	Cell surface glycoprotein: leukocyte migration, T-cell adhesion & apoptosis
5700753	CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1 (=biliary glycoprotein)	NM_001024912.1	–2·03	0·05	Myeloid cell adhesion to endothelium and angiogenesis
1260228	CLEC5A	C-type lectin domain family 5, member A	NM_013252.2	–1·85	0·06	Myeloid cell activation
4260484	COMMD6	COMM domain containing 6	NM_203497.2	–1·58	0·04	Inhibits NFκB

1050025	CR1	Complement receptor 1	NM_000573.3	–1·67	0·04	C3b and C4b binding
6450543	CST3	Cystatin C	NM_000099.2	+1·84	0·03	Inhibits lysosomal cysteine proteases
4150189	CTSL1	Cathepsin L1	NM_001912.3	–1·73	0·04	Controls neutrophil elastase activity
6270553	CXCL10	Chemokine (C-X-C motif) ligand 10	NM_001565.2	–2·08	0·08	Monocyte, T-cell and NK cell activation
2340220	CD55	Complement decay accelerating factor	NM_000574.2	–1·52	0·07	Complement regulation
3360279	CFH	Complement factor H	NM_001014975.1	–1·55	0·07	Complement cascade regulation
2810767	EBI3	Epstein-Barr virus induced 3	NM_005755.2	–2·33	0·05	Positive regulation of IFN-γ response
5560471	FCAR	Receptor for Fc fragment of IgA	NM_133280.1	–1·58	0·04	IgA receptor
6620209	FCGR1B or CD64	Receptor for Fc fragment of IgG, high affinity Ib	NM_001004340.1	–1·81	0·08	IgG receptor
2350066	HLA-A	Human leukocyte antigen-A	NM_002116.5	+1·61	0·03	MHC class I molecule
4250364	HPGD	15-hydroxy prostaglandin dehydrogenase	NM_000860.3	–1·53	0·06	Prostaglandin metabolism
6040647	HSH2D	Hematopoietic SH2 domain containing	NM_032855.2	+1·61	0·03	Target of T-cell receptor and CD28 activation. Function unknown.
2000148	IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	NM_001548.3	+2·48	0·08	Interferon-mediated innate immune response
4730747	IGLL1	Immunoglobulin lambda-like polypeptide 1	NM_020070.2	+1·74	0·04	Pre-B cell receptor, controls B-cell proliferation and differentiation
3370326	IL18R1	Interleukin-18 receptor 1	NM_003855.2	–2·17	0·08	IFN-γ mediated immune responses
1340743	IL8	Interleukin-8	NM_000584.2	–2·10	0·03	Neutrophil chemotaxis
2360719	IRAK3	Interleukin-1 receptor-associated kinase 3	NM_007199.1	–2·13	0·04	Monocyte tolerance to endotoxin
2490411	ITGB5	Integrin β5	NM_002213.3	+1·52	0·04	Cell adhesion
4150184	LAIR1 or CD305	Leukocyte-associated immunoglobulin-like receptor 1	NM_021706.2	–1·78	0·04	Inhibits NK cells, T-cells and B-cells
6110037	LILRA3	Leukocyte immunoglobulin-like receptor, subfamily A (without TM domain), member 3	NM_006865.2	–1·65	0·04	?Inhibits monocyte and B-cell function
5890095	LILRA5	Leukocyte immunoglobulin-like receptor, subfamily A (with TM domain), member 5	NM_021250.2	–1·59	0·06	Monocyte activation
6420450	LIME1	Lck-interacting transmembrane adaptor 1	NM_017806.1	+1·55	0·09	Expressed on T-cell membranes, and down-modulated on activation
6840408	LY86	Lymphocyte antigen 86	NM_004271.3	+1·55	0·07	Humoral immune response
70167	LY96	Lymphocyte antigen 96	NM_015364.2	–1·92	0·03	Associated with TLR4
5130767	MAPK14	Mitogen-activated protein kinase 14	NM_001315.1	–1·52	0·08	Activated by pro-inflammatory cytokines
7550066	MERTK	c-mer proto-oncogene tyrosine kinase	NM_006343.2	–1·58	0·08	Mediates macrophage phagocytosis of apoptotic cells
5670674	MME	Membrane metallo-endopeptidase	NM_000902.3	+3·33	0·03	Lymphocyte activation; Inactivates peptide hormones

1240228	PAG1	Phosphoprotein associated with glycosphingolipid microdomains 1	NM_018440.3	–1·53	0·03	?regulation of T-cell activation
2230563	PPBP	Platelet basic protein	NM_002704.2	+1·68	0·05	CXC chemokine. Neutrophil chemoattractant and activator.
7040470	PSMA6	Proteasome (prosome, macropain) subunit, α type, 6	NM_002791.1	–1·52	0·04	Processing of MHA class I peptides
4890707	PVRL2 or CD112	Poliovirus receptor-related 2 (herpesvirus entry mediator B)	NM_002856.2	–1·56	0·04	Enhances NK cell-mediated lysis
130681	RNASE6	Ribonuclease, RNase A family, k6	NM_005615.4	+1·56	0·05	Expressed in neutrophils and monocytes
6220438	RPS6KA5	Ribosomal protein S6 kinase, 90kDa, polypeptide 5	NM_004755.2	+1·65	0·04	MAP kinase signaling pathway
2490195	S100A10	S100 calcium binding protein A10	NM_002966.1	+1·66	0·03	Cell cycle progression and differentiation
1410221	S100A12	S100 calcium binding protein A12	NM_005621.1	–2·62	0·05	Myeloid-related protein
1510424	S100P	S100 calcium binding protein P	NM_005980.2	–2·27	0·09	RAGE ligand
1410181	SAMSN1	SAM domain, SH3 domain and nuclear localization signals 1	NM_022136.3	–1·97	0·07	B-cell activation
6040577	SLAMF8	SLAM family member 8	NM_020125.2	–1·50	0·07	Lymphocyte activation
3120543	SLC39A8	Solute carrier family 39 (zinc transporter), member 8	NM_022154.5	–1·69	0·06	Cellular zinc import at onset of inflammation
1990300	SOCS1	Suppressor of cytokine signaling 1	NM_003745.1	–1·67	0·07	Regulation of IFNγ response
160019	SORT1	Sortilin 1	NM_002959.4	–1·62	0·07	Trans-Golgi network transmembrane protein, anti-apoptotic and B-cell survival
3390612	TLR8	Toll-like receptor 8	NM_016610.2	–1·60	0·03	Pathogen recognition
2640301	TNF	Tumor necrosis factor (TNF superfamily, member 2)	NM_000594.2	–1·53	0·03	Proinflammatory cytokine
6350632	TSC22D3	TSC22 domain family, member 3	NM_198057.2	+1·53	0·03	Stimulated by glucocorticoids and IL-10. Anti-inflammatory
5910113	VCAN	Versican core protein	NM_004385.2	+1·62	0·03	Leukocyte aggregation and pro-coagulant
5310754	VNN1	Vanin 1	NM_004666.1	–2·19	0·07	Hematopoeitic cell trafficking
7100161	VNN2	Vanin 2	NM_004665.2	–1·54	0·04	Transendothelial migration, monocyte activation
Coagulation						
2230241	F13A1	Coagulation factor XIII, subunit A	NM_000129.3	+1·89	0·04	Clotting cascade
2340220	CD55	Complement decay accelerating factor	NM_000574.2	–1·52	0·07	Complement regulation
3360279	CFH	Complement factor H	NM_001014975.1	–1·55	0·07	Complement cascade regulation

Carbohydrate metabolism						
4810438	MAN2B2	Mannosidase α, class 2B, member 2	NM_015274.1	+1·54	0·006	Carbohydrate metabolism
290348	POFUT1	GDP-fucose protein O-fucosyltransferase 1	NM_015352.1	+1·55	0·03	Carbohydrate metabolism
3390129	C9orf103	Chromosome 9 open reading frame 103	NM_001001551.1	–1·67	0·05	Probable glucokinase
940274	GK	Glycerol kinase	NM_000167.3	–1·62	0·05	Glycerol phosphorylation
670528	GYG1	Glycogenin 1	NM_004130.2	–1·62	0·08	Regulates glycogen synthesis
3060612	HK3	Hexokinase 3 (white cell)	NM_002115.1	–1·93	0·04	Glucose phosphorylation
6620689	MTHFD2	Methylenetetrahydrofolate dehydrogenase (NADP+ dependent) 2, methenyltetrahydrofolate cyclohydrolase	NM_001040409.1	–1·52	0·04	Mitochondrial bifunctional enzyme
7040181	PFKFB2	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2	NM_001018053.1	–2·30	0·05	Glycolysis/gluconeogenesis
1470601	PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	NM_004566.2	–1·76	0·04	Glycolysis/gluconeogenesis
4830504	PGM2	Phosphoglucomutase 2	NM_018290.2	–1·52	0·04	Glucose and glycogen metabolism
6180465	SLC2A6	Solute carrier family 2 (facilitated glucose transporter), member 6	NM_017585.2	–1·77	0·03	Hexose transport across cell membrane
3420332	SLC37A3	Solute carrier family 37 (glycerol-3-phosphate transporter), member 3	NM_032295.2	–1·50	0·05	Transmembrane sugar transport
Oxidative stress and DNA damage						
130181	ANKRD22	Ankyrin repeat domain 22	NM_144590.2	–2·93	0·06	Mediates protein-protein interactions
4880673	GADD45A	Growth arrest and DNA-damage-inducible, alpha	NM_001924.2	–2·02	0·05	Stress response
2600382	GPX4	Glutathione peroxidase 4	NM_001039847.1	+1·57	0·02	Reduces peroxide
6400392	GSTM1	Glutathione S-transferase μ1	NM_000561.2	+2·35	0·03	Deals with oxidative stress
6130168	GSTM2	Glutathione S-transferase μ2	NM_000848.2	+2·38	0·03	Deals with oxidative stress
150400	LBA1	Lupus brain antigen 1	NM_014831.1	+1·52	0·04	DNA repair
5050537	OPLAH	5-oxoprolinase (ATP-hydrolyzing)	NM_017570.2	–1·75	0·04	Glutathione metabolism
2510551	STK3	Serine/threonine kinase 3 (STE20 homolog, yeast)	NM_006281.2	–1·60	0·03	Stress response

DNA replication, cell cycle regulation, cell differentiation and proliferation

940288	BAZ1A	Bromodomain adjacent to zinc finger domain, 1A	NM_182648.1	–1·60	0·03	DNA replication
7400279	CCNA1	Cyclin-A1	NM_003914.2	–1·84	0·05	Cell cycle regulation
6220674	CCPG1	Cell cycle progression 1	NM_020739.2	–1·54	0·03	Cell cycle regulation
1780564	CDC42	Cell division cycle 42	NM_001039802.1	–1·59	0·03	Regulation of cell cycle progression
6200064	CECR1	Cat eye syndrome chromosome region, candidate 1	NM_177405.1	+1·79	0·06	Adenosine deaminase; putative growth factor
4490528	CKAP4	Cytoskeleton-associated protein 4	NM_006825.2	–1·79	0·05	Cell cycle regulation
6510554	DACH1	Dachshund homolog 1 (Drosophila)	NM_080760.3	–1·76	0·03	Cell fate in eye development
7560288	DCUN1D3	DCN1, defective in cullin neddylation 1, domain containing 3 (*S. cerevisiae*)	NM_173475.1	–1·51	0·03	Cell cycle regulation
4220605	ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2 (avian)	NM_005239.4	–1·68	0·07	Stem cell development
610164	FYN	FYN oncogene	NM_153047.1	+1·50	0·05	Tyrosine kinase oncogene
6180427	G0S2	G0/G1switch 2	NM_015714.2	–1·53	0·06	Cell cycle regulation
6130136	GPR177	G-protein coupled receptor 177	NM_001002292.1	+1·57	0·04	Required for WNT3A expression (cell fate regulation)
5490768	GPR56	G protein-coupled receptor 56	NM_201525.1	+1·61	0·10	Cell fate regulation and neurogenesis
4120750	OBFC1	Oligonucleotide/oligosaccharide-binding fold containing 1	NM_024928.3	–1·56	0·04	Increases DNA-polymerase-template affinity
4260253	OBFC2A	Oligonucleotide/oligosaccharide-binding fold containing 2A	NM_001031716.1	–1·51	0·03	?DNA metabolism
2470520	RBMS1	RNA binding motif, single stranded interacting protein 1	NM_002897.3	–1·83	0·03	DNA/RNA processing
6960022	SEPT5	Septin 5	NM_002688.4	+1·58	0·08	Cell cycle regulation
3830735	UPB1	Ureidopropionase β	NM_016327.2	–1·59	0·07	Pyrimidine degradation
7570673	UPP1	Uridine phosphorylase 1	NM_003364.2	–1·61	0·08	Uridine phosphorylation

Appendix Table 2. List of genes differentially expressed in glibenclamide-treated diabetic patients who are otherwise healthy

ProbeID	Symbol	Gene	RefSeq ID	Absolute fold change	Corrected *p*-value	Comment
Inflammation-related						
2030142	CLC	Charco-Leyden crystal protein	NM_001828.4	+2·01	0·05	Lysophospholipase expressed by eosinophils and basophils
2260731	ERAP2	Endoplasmic reticulum aminopeptidase 2	NM_022350.2	+1·58	0·05	Trimming peptides for MHC class I presentation
940519	GPR44	G protein-coupled receptor 44	NM_004778.2	+1·57	0·05	Chemoattractant receptor expressed on Th2 cells
510079	HLA-DRB4	HLA-DRB1 MHC class II antigen	NM_021983.4	–1·58	0·05	Antigen presentation
620544	HLA-DRB6	HLA-DRB6 MHC class II antigen	NR_001298.1	–2·50	0·05	Antigen presentation
4210458	KIR2DL1	Natural killer cell immunoglobulin-like receptor, 2 domains, long cytoplasmic chain, 1	NM_014218.2	–1·54	0·06	NK cell modulation
Neutrophil function						
1500735	CTSG	Cathepsin G	NM_001911.2	–1·84	0·05	Neutrophil azurophil granule protease
4540239	DEFA1	Neutrophil defensin 1	NM_004084.2	–2·49	0·05	Neutrophil microbicidal peptide
2970747	DEFA3	Neutrophil defensin 3	NM_005217.2	–2·49	0·06	Neutrophil microbicidal peptide
6550164	DEFA4	Neutrophil defensin 4	NM_001925.1	–2·20	0·05	Neutrophil microbicidal peptide
7650497	ELA2	Neutrophil elastase 2	NM_001972.2	–1·93	0·05	Neutrophil azurophil granule protease
7150170	LOC728358	Neutrophil defensin 1 precursor	NM_001042500.1	–2·58	0·05	Neutrophil microbicidal peptide
2470364	PRSS33	Serine protease 33	NM_152891.2	+1·85	0·05	Serine protease expressed in neutrophils and macrophages

Note.—All genes in this table have a fold change in expression greater than 1·5 and a corrected *p*-value less than 0·10 (Benjamini-Hochberg); when more than one probe for the same gene was positive, only the probe with the most significant corrected *p*-value is displayed. Only ontologies with a significance level <0·05 (hypergeometric test, InnateDB) are listed here; ontologies have been supplemented with genes not curated by InnateDB but which were identified on literature searches. Microarray data have been deposited at ArrayExpress, EMBL-EBI (accession number E-TABM-852-n) and are available publicly.

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List of manuscripts

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