

BIOLOGICAL AGENTS

Melioidosis: Pathogenesis, Epidemiology, Treatment and Detection¹

by
S Shahin

MELIOIDOSIS, AN INFECTION CAUSED BY the bacillus *Burkholderia pseudomallei*, is endemic tropical and subtropical areas. In humans, the disease is caused by ingestion or by contact with contaminated water through skin lesions. Clinical diagnosis of the disease is difficult because the symptoms are variable. Since the organism is resistant to a number of commonly used antibiotics, immediate effective treatment is dependent on a correct diagnosis. The detection methods currently in use are time-consuming and identification based on amplifying specific sequences of *B. pseudomallei* DNA appears promising.

INTRODUCTION

The etiologic bacterium of the disease, melioidosis, *Burkholderia pseudomallei*, is closely related to *B. mallei*, the causative organism of glanders, with which it was formerly classified in the genus *Pseudomonas*. The species are aerobic, non-spore forming, Gram-negative rods. They are motile due to the presence of one or more polar flagella and can be recovered on most aerobically incubated isolation media used in the clinical laboratory. *B. pseudomallei* has been termed the "unbeatable foe" for several reasons:

- under-recognition,
- high case mortality,
- unacceptable relapse rate even after long-term antibiotic treatment and
- a "time bomb" for sero-positive patients, the frequency of whom is high in endemic areas.

Melioidosis is a serious infection with a high mortality rate. The pathogenesis of melioidosis is poorly understood. The symptoms of the disease are variable but in general, four categories of infection are recognised:

- acute fulminant septicaemia,
- subacute illness,
- chronic infection and
- subclinical infection.

The disease may be either localised or disseminated, and any organ system may be affected: lungs, skin, bones, joints, liver, spleen, pancreas, kidneys, bladder, prostate, genital organs, brain and meninges, lymph nodes and pericardium¹. Depending on whether infection is acute or chronic, melioidosis can mimic other common bacterial infections such as typhoid fever, malaria and tuberculosis, which makes rapid and correct diagnosis difficult.

The acute septicemic form, usually seen in endemic areas, often results in death within a few days of exposure. Mortality rates for this form of melioidosis are high (up to 75% in some countries) despite intensive antibiotic therapy. Most cases of melioidosis seen in non-endemic areas are of the subacute variety. This form may be either focal or disseminated, with abscesses in many organs, and clinical symptoms can last for years. Likewise, chronic melioidosis can present as a localised infection of almost any organ of the body or can remain asymptomatic for many years. Subclinical infection with *B. pseudomallei* produces minimal or no symptoms. Clinical disease does not develop, probably due to the suppression of the infection by the immune system. However, subclinical infection may intensify to an acute form of the disease when the host is immunocompromised, for example in such illnesses as diabetes mellitus, renal failure, cirrhosis or alcoholism².

EPIDEMIOLOGY

Burkholderia pseudomallei has a limited geographical distribution and is found primarily in tropical and subtropical areas. Melioidosis is endemic in humans in Southeast Asia (particularly prevalent in the rice-growing regions due to the high concentrations of the pathogen in rice paddy surface water) and Northern Australia³ and has been found in many animals including sheep, horses, pigs and dolphins⁶. In humans, infection is usually caused by direct contact with contaminated soil or water, usually through abrasions in the skin, and less commonly by inhalation or ingestion of contaminated soil or water. Incubation can be as short as 3 days, but usually the disease is latent, becoming evident months to years later.

Melioidosis was first described in Australia as an outbreak in sheep in 1949 in North Queensland, and a year later the first case of human melioidosis was documented in Townsville. Melioidosis is endemic in the Northern Territory where it is the most common cause of fatal community-acquired septicaemic pneumonia. During the past 9 years there have been 206 culture-confirmed cases of melioidosis at the Royal Darwin Hospital and the disease has been detected in the north of Western Australia and North Queensland including the Torres Strait Islands'. Melioidosis in temperate regions of Australia has been attributed to animals imported from the north since some soil isolates were molecularly identical to epidemiologically related animal and human isolates. Molecular typing has also implicated the water supply in a couple of outbreaks in remote aboriginal communities in Northern Australia⁷.

Although the organism and its infections are not well known in temperate regions, there have been occasional outbreaks in Europe and the United States. The cases from non-endemic regions are those displaying latency where contraction of the disease occurred in endemic locations. Most of the clinical cases reported in the United States have occurred in people such as military personnel who have travelled or lived in endemic areas. A serologic study of US Army personnel who had been stationed in Vietnam found that 1-2% had significant antibody titres to *B. pseudomallei*. However, more and more cases are now being reported among tourists who have visited infected areas.

Relatively little is known about the incidence of melioidosis in other parts of the world. This is due to both the lack of awareness by the medical fraternity and the lack of resources necessary for the isolation and identification of the organism. However, there is increasing evidence that the disease is endemic in the Indian sub-continent and the Caribbean, and there have been reports of recent cases in South Africa and the Middle East⁹.

MECHANISM OF ACTION

Understanding of the pathogenicity of *B. pseudomallei* is limited. A number of secreted products including protease, haemolysin, lipase and lectinase have been linked to virulence¹⁰. The mechanism

by which these are secreted appears to be the type II secretion pathway¹¹. However, in recent studies, mutants unable to secrete protease, lipase and lecthinase were as virulent as the wild type in the Syrian hamster model, suggesting that these factors may only have a limited role in the virulence of *B. pseudomallei*. Winstanley *et al*¹³ identified, cloned and sequenced *B. pseudomallei* genomic DNA with homology to type III secretion-associated genes. Type III secretion is triggered by host cell proximity and virulence factors are delivered directly to the host cells. Such clusters of virulence genes (pathogenicity islands), have been identified in several other bacterial pathogens including *Y. pestis* and *Paeruginosa*. The principal function of type III secretion is thought to be protein translocation into host cells.

The mechanism by which *B. pseudomallei* is able to survive and remain quiescent in a host, for as long as 26 years in some cases, is not known. Dharakul *et al*¹⁵ used various cell lines in tissue culture to study the intracellular behaviour of the organism and found that intracellular location and replication occurs consistently in the mouse alveolar macrophage. Another possible residential area may be within damaged tissue with poor circulation, where the organisms may form viable but non-replicating microcolonies. Knowledge of how and where quiescent organisms remain viable is an important issue in determining the effectiveness of the antimicrobials used in treatment. For example, if quiescent colonies are viable but non-replicating, antimicrobials that kill susceptible bacteria in the stationary phase should be used.

B. pseudomallei biotypes

Two biotypes of *B. pseudomallei* can be differentiated biochemically based on differences in arabinose (Ara) assimilation. Since the discovery of distinct Ara+ and Ara- biotypes, other differences have also been described. High endemicity of melioidosis appears to correlate with high numbers of the Ara- biotype in the environment¹⁶. The biotypes differ in their virulence in animal models; the Ara+ biotype is non-virulent whereas the Ara- is highly virulent in several animal models. Differences also exist between the biotypes in the production of proteases, lecithinases and lipase, lipopolysaccharide composition, and in the DNA sequences of 11agellin and 16S rRNA-encoding genes¹⁷. The Ara- biotype has two chromosomes of approximately 3.7 and 2.3 x 10⁶ bases whereas the Ara+ biotype has two smaller chromosomes and the two strains can be distinguished by Nco1 restriction enzyme digestion¹⁸. In addition, antigenic differences were demonstrated by Sirisinha *et al* who raised a monoclonal antibody against a 200 kDa surface antigen that specifically recognised the Ara-strain.

ANTIMICROBIAL SUSCEPTIBILITY AND TREATMENT

B. pseudomallei is usually susceptible to the antipseudomonal penicillins (ticarcillin-clavulanic acid, piperacillin, piperacillin-tazobactam and mezlocillin), ceftazidime, ampicillin-sulbactam, cefoperazone, chloramphenicol, amoxicillin-clavulanic acid, and tetracyclines. Susceptibility to the fluoroquinolones is variable and most isolates are resistant to the aminoglycosides and trimethoprim-sulfamethoxazole. The organism is also resistant to antistaphylococcal penicillins, ampicillin, rifampin, and narrow- and broad-spectrum cephalosporins.²⁰

The current antibiotic treatment of melioidosis involves prolonged oral administration of combinations of chloramphenicol, doxycycline and trimethoprim-sulfamethoxazole, or ceftazidime in various combinations with chloramphenicol, cotrimoxazole, doxycycline and/or Amoxicillin/potassium clavulanate". In some clinical cases, regimens involving carbapenems such as imipenem have been successful. Recent studies^{25,26} have also demonstrated the effectiveness of imipenem, meropenem, and imipenem/azithromycin against acute melioidosis with high-dose intravenous imipenem possessing similar efficacy to the historic drug combination treatment. Other combinations such as ciprofloxacin/

clarithromycin and ciprofloxacin/ azithromycin warrant further clinical trials because of the intracellular killing effect and therefore the potential for their use in maintenance therapy.

Antibiotic resistance

The successful treatment of melioidosis patients is difficult because *B. pseudomallei* is resistant to a variety of antibiotics, including beta-lactams, aminoglycosides, macrolides and polymyxins. Initial experiments designed to identify genes involved in aminoglycoside resistance resulted in the isolation of two transposon mutants demonstrating increased susceptibility to the aminoglycosides streptomycin, gentamicin, neomycin, tobramycin, kanamycin and spectinomycin²⁷. Further experiments revealed that the mutants were also susceptible to the macrolides erythromycin and clarithromycin but not to the lincosamide clindamycin. Sequencing of DNA flanking the transposon insertions revealed an operon whose gene products were homologous to multidrug efl1ux systems found in a variety of Gram-negative bacteria²⁸. These include a membrane fusion protein (amrA), an RND-type transporter (amrB), an outer membrane protein (oprA) and a divergently transcribed regulator protein (amrR). In addition to the presence of an efl1ux system, both mutants accumulated [3H] streptomycin, whereas the parent strain did not²⁷.

B. pseudomallei also demonstrates high intrinsic resistance to the action of cationic antimicrobial peptides including human neutrophil peptides, melittin, polymyxins, protamine sulphate, magainins and poly-L-lysine²⁹. In susceptible Gram-negative bacteria, cationic antimicrobial agents cause cell death by permeabilising the inner and outer membranes. Using a virulent clinical isolate capable of replication in medium containing high concentrations of polymyxin B and a Tn5-OT182 mutagenesis system, Burtnick and Woods isolated several polymyxin B-susceptible mutants. Two of these mutants (PMB7 and PMB20) contained transposon integrations in genes involved in LPS core synthesis and include a rfaF gene homolog encoding a putative heptosyl transferase and a gene whose product encodes a putative UDP-glucose dehydrogenase. These mutants demonstrated altered lipopolysaccharide and outer membrane profiles. Another mutant, PMB4, had a transposon insertion in a locus whose product shows homology to the lytB gene product of *E. coli*. In *E. coli*, LytB regulates relA, which is involved in the stringent response. This mutant also has an altered outer membrane profile. Characterisation of these three loci and their products should lead to a more thorough understanding of polymyxin resistance in *B. pseudomallei*.

VACCINES

There is currently no licensed vaccine available for the immunoprophylaxis against melioidosis. To develop a suitable vaccine construct, Brett and Woods³⁰ employed a combination of molecular, biochemical and immunological techniques to identify and characterise antigens expressed by *B. pseudomallei* isolates. These studies demonstrated that both polyclonal and monoclonal antisera raised against *B. pseudomallei* flagellin proteins and lipopolysaccharide and O-polysaccharide (PS) moieties derived from endotoxin expressed by *B. pseudomallei* isolates provided passive protection against bacterial challenge using the diabetic infant rat infection model^{31,32}. Since the flagellin proteins and PS moieties are structurally and antigenically conserved throughout the species, these investigators are assessing the viability of a flagellin-PS conjugate as a vaccine candidate.

DETECTION

Burkholderia species can be recovered on most aerobically incubated primary isolation media used in the clinical laboratory to isolate enteric Gram-negative bacilli. Grown on Ashdown medium, *B. pseudomallei* colonies are typically dry, wrinkled and violet-purple with a pungent, earthy odour. These

characteristics are sufficiently distinct to allow a presumptive identification of the organism, particularly in specimens containing mixed bacterial flora. The key biochemical characteristics of *B. pseudomallei* are positive oxidase reaction, production of gas from nitrate, arginine dihydrolase and gelatinase activities and oxidation of a wide variety of carbohydrates²⁰.

Culture and identification of *B. pseudomallei* from clinical specimens can take many days, requires selective/ enrichment media, and in mixed cultures the organism may be overlooked³³. Many patients with suspected melioidosis remain culture negative. Attempts to make an early diagnosis of melioidosis without relying on cultures have centred on methods to detect specific antibodies or antigen. *B. pseudomallei* specific IgM antibody can be detected by immunofluorescence and enzyme immunoassay⁵. However, serological testing is of limited use because background seropositivity rates are high in endemic areas and a definitive diagnosis of melioidosis requires isolation of the organism. In endemic areas as many as 80% of the children had antibodies by the age of four years.

Pongsunk et al produced a monoclonal antibody specific to the 30 kDa protein of *B. pseudomallei* by in vivo immunisation of BALB/c mice with a crude culture filtrate antigen. This antibody recognised over 200 clinical isolates of *B. pseudomallei* but no other Gram- negative bacteria. However, it did cross-react with gram- positive *Bacillus* species and *Streptococcus pyogenes*. A combination of blood culture and an agglutination assay with the 30 kDa monoclonal antibody increased the specificity and reduced the time of detection by 2 days over that of culture alone. Another latex agglutination test based on Bps-LI monoclonal antibody, which recognises the lipopolysaccharide antigen of *B. pseudomallei* clinical isolates, was 100 % effective in rapid identification of the bacteria in blood cultures in areas endemic for melioidosis³⁷.

An alternative method for early detection is an amplification of specific DNA sequences by the polymerase chain reaction which requires short, specific fragments of DNA to act as primers. Recently a number of polymerase chain reaction (PCR) techniques for the detection of *B. pseudomallei* DNA have been described. Lew and Desmarchelier³⁸ used primers selected from homologous 23S rRNA sequences of *B. cepacia* and *P. aeruginosa*. An 18 - base oligonucleotide probe was produced by partial sequencing of *B. pseudomallei* 23S rRNA and selecting the portion with the greatest sequence variation compared to *B. cepacia*. However, DNA from *B. mallei* was detected as well. Kunakom and Mark ham combined a semi-nested PCR and EIA to detect the conserved ribosomal regulatory region of *B. pseudomallei* and Dharakul et al.¹⁵ developed a nested PCR system that amplified a part of the 16S rRNA gene. However, although neither of these studies included *B. mallei* in the bacterial strains tested, it is likely to be detected because the DNA sequences in the chosen region are very similar. In addition, even though the nested PCR assay using the 16S rRNA derived primers in clinical samples had a sensitivity approaching 100%⁴⁰ some samples from patients with other diagnoses were also positive. Recently, Rattanathongkom et al developed a PCR technique capable of detecting a single bacterial cell inoculated into uninfected blood. This detected all of the *B. pseudomallei* isolates but none of the other bacterial strains tested. Wajanarogana et al⁴² also developed a PCR-based method capable of differentiating between the arabinose assimilation biotypes using primers covering a 15-bp deletion region in the flagellin gene. Zysk et al compiled a comprehensive review of the diagnostic techniques used in the past decade.

THE *B. PSEUDOMALLEI* GENOME

The Sanger Centre in Cambridgeshire, a genome research centre founded by the Wellcome Trust and the Medical Research Council in the United Kingdom, has been funded by Beowulf Genomics to sequence the genome of *B. pseudomallei* in collaboration with Dr Rick Titball of the Defence

Evaluation Research Agency, Porton Down, and Dr Ty Pitt of the Central Public Health Laboratory, UK. The Beowulf Genomic Initiative was established by the Wellcome Trust, UK, in 1998, to ensure that the genomic sequences of microbial pathogens were freely available.

The *B. pseudomallei* genome is approximately 6×10^9 bases in size with two chromosomes of 3.5×10^6 and 2.5×10^6 bases and a G+C content of approximately 65%. They are sequencing strain K96243, a clinical isolate from Thailand and have to date approximately 58,859 unedited single reads in the database corresponding to 26.574×10^6 bases, a theoretical coverage of 98.3% of the genome.

CONCLUSION

Melioidosis is a serious infection which is endemic in humans in tropical and subtropical regions and, alarmingly increasing numbers are being diagnosed in temperate areas, especially amongst tourists. A particular concern about the disease is that there are several levels of infection. The serious septicemic form is fatal if left untreated. Alternatively, a healthy individual may become infected while residing in an endemic area, but not develop disease symptoms until later. Other areas of concern include the absence of an effective vaccine and lack of rapid diagnostic assays. In addition, initial symptoms can be similar to those of common infections such as influenza. Therefore, rapid diagnosis is difficult and, furthermore, the organism is inherently resistant to the common antibiotics used in the treatment of these infections. There is also an incomplete understanding of the mechanism of infection. Specific virulence factors need to be identified before effective prophylactic measures can be developed. Research directed towards assessing the suitability of a number of antigens for the development of a vaccine, elucidating the mechanism of action, and devising rapid diagnostic assays are being actively pursued in the research community.

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