Burkholderia pseudomallei: another emerging pathogen in cystic fibrosis

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Background: Burkholderia pseudomallei is an important cause of acute fulminant pneumonia and sepsicaemia in tropical regions of northern Australia and south east Asia. Subacute and chronic forms of the disease also occur. There have been three recent reports of adults with cystic fibrosis (CF) who presumably acquired B pseudomallei infection during extended vacations or residence in either Thailand or northern Australia.

Methods: The clinical course, molecular characteristics, serology and response to treatment are described in four adult CF patients infected with B pseudomallei. Polymerase chain reaction (PCR) based methods were used to confirm B pseudomallei and exclude B cepacia complex. Genotyping was performed using randomly amplified polymorphic DNA (RAPD) PCR and pulsed field gel electrophoresis (PFGE).

Results: Four patients are described with a mean duration of infection of 32 months. All but one patient lived in tropical Queensland. Two patients (with the longest duration of infection) deteriorated clinically and one subsequently died of respiratory failure. Both responded to intravenous treatment specifically targeting B pseudomallei. Another patient suffered two severe episodes of acute bronchopneumonia following acquisition of B pseudomallei. Eradication of the organism was not possible in any of the cases. PFGE of a sample isolate from each patient revealed the strains to be unique and RAPD analysis showed retention of the same strain within an individual over time.

Conclusions: These findings support a potential pathogenic role for B pseudomallei in CF lung disease, producing both chronic infection and possibly acute bronchopneumonia. Identical isolates are retained over time and are unique, consistent with likely environmental acquisition and not person to person spread. B pseudomallei is emerging as a significant pathogen for patients with CF residing and holidaying in the tropics.
Group Ltd, Merseyside, UK), and mannitol salt agar (Oxoid Ltd). Phenotypic identification of *B. pseudomallei* was based on biochemical reactions, a positive oxidase test, and the API 20NE. Isolates were stored in liquid nitrogen using PROTECT bacterial preservers (Technical Service Consultants Ltd, Heywood, UK).

**Antibiotic sensitivity**

Antibiotic sensitivity testing was conducted according to the National Committee for Clinical Laboratory Standards (NCCLS). Disc diffusion was employed using Mueller Hinton agar and Oxoid antibiotic discs (Oxoid Ltd). The following antibiotics (disc concentration) were tested: tetracycline (30 μg/ml), gentamicin (10 μg/ml), sulphamethoxazole/trimethoprim (SXT) (25 μg/ml), tobramycin (10 μg/ml), amikacin (30 μg/ml), ceftazidime (30 μg/ml), piperacillin (100 μg/ml), ticarcillin/clavulanic acid (75/10 μg/ml), ciprofloxacin (5 μg/ml), aztreonam (30 μg/ml), meropenem (10 μg/ml), imipenem (10 μg/ml), and colistin sulphate (10 μg/ml). SXT sensitivities were confirmed using E tests according to NCCLS broth dilution break points.

**Multiple combination bactericidal testing (MCBT)**

MCBT was carried out to identify bactericidal or antagonistic combinations of antibiotics. The procedure was performed in triplicate in 96 well round bottom microtitre plates (Nunc Inc, Roskilde, Denmark) as described by Aaron et al. The following antibiotics were used: tobramycin (Sigma Chemicals) 10 μg/ml and 200 μg/ml (the latter suitable for nebulised use), meropenem (Astra Zeneca) 32 μg/ml, amikacin and cefepime (Bristol-Myers Squibb) both 32 μg/ml, ceftazidime and ticarcillin/clavulanic acid (Glaxo Smith Kline) 32 μg/ml and 32/2 μg/ml respectively, ciprofloxacin (Bayer) 2 μg/ml, chloramphenicol, SXT and doxycycline (all Roche Laboratories, Canada) 20 μg/ml, 10/2 μg/ml and 4 μg/ml, respectively. Reference strains (*P. aeruginosa* ATCC 27853, *E. coli* ATCC 25922, and *E. coli* ATCC 35218) were used to control for the efficacy of the antibiotics.

**Serological tests**

*B. pseudomallei* antibodies were measured in each patient using serum collected after the first identification of *B. pseudomallei* in sputum. Stored serum was available for retrospective testing in one case. Total antibody titre was determined using an indirect haemagglutination assay and IgM and IgG subclasses were measured using an enzyme linked immunoassay.

**Molecular identification**

**16S rRNA polymerase chain reaction (PCR) for *B. pseudomallei***

Isolates from each patient were confirmed as *B. pseudomallei* using a modification of the method described by Dharakul et al. The primers used were Bps16S–42L (sense) and Bps16S–266R (antisense) or Bps16S–42L (sense) and Bps16S–427R (antisense) (two separate PCR reactions). DNA amplification was performed in an AB2700 Thermal Cycler (Applied Biosystems, Foster City, USA) with an initial hot start step of 95°C for 15 minutes, followed by 35 cycles of 1 minute at 95°C, 1 minute at 60°C, 1 minute at 72°C, and final extension for 10 minutes at 72°C. The presence of amplified DNA product was detected in 1.5% (w/v) agarose gel stained with 2.5 μl 10 mg/ml ethidium bromide. Bands at 243 bp and 405 bp were interpreted as being consistent with PCR amplification of arabino negative (Ara⁻) *B. pseudomallei* DNA (environmental Ara⁺ isolates of *B. pseudomallei* are seldom encountered in clinical specimens and exhibit minimal virulence in animal models).

**recA PCR for *B. cepacia* complex**

*B. cepacia* was excluded using PCR amplification of the recA gene based on methods previously described. DNA extracted from each isolate was used as a template.

**RFLP analysis of 16S rDNA**

The 16S rDNA-RFLP method as described by Segonds et al. was performed on each isolate and the results compared with a known strain of *B. pseudomallei*. This method has been shown to discriminate effectively between members of the *B. cepacia* complex and other *Burkholderia* species. Restriction endonuclease digestion was performed on each PCR product separately using the enzymes DdeI and MspI. Each individual RFLP profile was assigned an identifying uppercase letter.

**Genotyping**

Genotyping was performed on available isolates from each patient collected over at least several months using randomly amplified polymorphic DNA (RAPD) and pulsed field gel electrophoresis (PFGE). RAPD analysis was performed using a modification of the method previously described by Corney et al. Strains were considered identical if their banding patterns were identical for all five primers. PFGE was performed according to the method of Currie et al using SpeI restriction endonuclease with the modification of adding thiouroea to the running buffer. The results were visually compared.

Clinical and microbiological review of patients attending The Prince Charles Hospital has been approved by the human research and ethics committee as part of the provision of patient data for the Australia and New Zealand Cystic Fibrosis Data Registry.

**RESULTS**

**Clinical description**

**Case 1**

A 23 year old man with CF (AF508/AF508) living in tropical Queensland had *B. pseudomallei* initially identified in his sputum in May 2000. He had severe bronchiectasis (forced expiratory volume in 1 second (FEV₁) 40–45% predicted), CF related diabetes requiring insulin, and chronic *P. aeruginosa* and MRSA infection. Before identification of *B. pseudomallei* he required two to four admissions per year for parenteral antibiotic treatment. Shortly after the acquisition of *B. pseudomallei* he deteriorated significantly with increasingly frequent pulmonary exacerbations, which were characterised by marked constitutional symptoms and increased circulating inflammatory markers. His FEV₁ fell from 40% predicted to 25% predicted over an 18 month period and he developed hypercapnic respiratory failure requiring acute non-invasive ventilatory support during one admission. The patient’s clinical course appeared to stabilise in February 2002 when long term treatment with high dose SXT was commenced (800/160 mg twice daily). He subsequently deteriorated further despite the SXT and then stabilised again with the addition of amoxycillin/clavulanic acid (875/125 mg twice daily). He was listed for lung transplantation from March 2002 but progressive bronchial sepsis occurred and the patient died in November 2002 as a result of respiratory failure.

Despite several attempts at eradication, *B. pseudomallei* continued to be isolated from the sputum (moderate to heavy growth), MRSA and *P. aeruginosa* were also usually but not invariably identified in the sputum and *Stenotrophomonas maltophilia* had been cultured infrequently. Parenteral antibiotics to attempt eradication of *B. pseudomallei* have included ceftazidime (up to 15 g/day) and meropenem (up to 6 g/day) for as long as 8 weeks. These have been used in combination with high dose oral SXT. Oral tetracycline and ciprofloxacin
have been used individually and in combination with SXT with little benefit.

Case 2
A 36 year old man with CF (ΔF508/ΔF508) living in tropical Queensland had *B pseudomallei* first identified in the sputum in 1994. Previously the patient had been well with mild bronchiectasis (FEV₁ 65–75% predicted) and had only previously required parenteral antibiotics on two occasions. Ten years previously the patient’s mother had been successfully treated for acute melioidosis (severe pneumonia). They shared accommodation before the episode of pneumonia and for several years after recovery, and she remains well. Subsequent to the identification of *B pseudomallei*, he gradually deteriorated. The patient’s FEV₁ is currently 33% predicted. Pulmonary exacerbations have become increasingly frequent and are typically accompanied by systemic symptoms. On two occasions the patient received 2 weeks of high dose intravenous cefazidime, meropenem and oral SXT which resulted in improved spirometry; however, *B pseudomallei* persisted in the sputum. The patient continues to require up to four courses of intravenous antibiotics per year, including either cefazidime or meropenem and oral SXT, for pulmonary exacerbations with accompanying clinical response. Between exacerbations he is maintained on SXT or tetracycline. Most sputum samples have a pure light to heavy growth of *B pseudomallei*. *P aeruginosa* is also cultured infrequently.

Case 3
A 15 year old boy with CF (ΔF508/G542X) from tropical far north Queensland had *B pseudomallei* first isolated in the sputum in December 2001. The patient has mild bronchiectasis with two previous admissions (aged 8 and 14 years) for parenteral treatment of pulmonary exacerbation. Before December 2001 *Staphylococcus aureus* and *P aeruginosa* had been isolated in the sputum and *B pseudomallei* has continued to be isolated from the sputum subsequently (scant to light growth). The patient has remained well and has not received any specific treatment targeting *B pseudomallei*. The patient had an older sibling with CF who had chronic *B cepacia* (genovar IIb) infection and died of respiratory failure in April 2001.

Case 4
A 24 year old woman with CF (ΔF508/ΔF508), moderate bronchiectasis (FEV₁ 65% predicted), and chronic *B cepacia* (genovar IIb) infection (Australian epidemic strain, LMG18830) relocated to subtropical Queensland from a temperate region in New South Wales in 1998. She typically had required four courses of parenteral antibiotic treatment for pulmonary exacerbations per year. Sputum intermittently cultured *P aeruginosa* and *S aureus* in addition to *B cepacia*. In a separate analysis to determine whether *B pseudomallei* antibodies were positive in CF patients infected with *B cepacia*, we identified very high titres in this patient in April 2002. The patient had neither resided nor visited tropical regions within Australia or south east Asia. Subsequent analysis of stored serum from March 2000 and July 2001 also revealed positive *B pseudomallei* antibodies suggesting exposure prior to 2000. Furthermore, there was an eightfold rise in antibody titre between March 2000 and July 2001. No other patient with *B cepacia* treated in the unit (eight patients) had positive *B pseudomallei* antibodies. Subsequent re-examination of sputum revealed *B pseudomallei* in addition to *B cepacia*. Sputum now consistently grows both *B pseudomallei* (scant to heavy growth) and *B cepacia*. In June 2001 the microbiology laboratory identified an organism from the sputum atypical for *B cepacia* which had an antibiogram identical to the subsequently identified *B pseudomallei*. The patient has had two severe exacerbations complicated by bronchopneumonia in July 2001 and June 2002, both of which responded to 3 week courses of intravenous cefazidime, meropenem, and tobramycin.

Laboratory identification

Microbiology

Each of the isolates induced a pink colouration when grown on MAST *B cepacia* medium. Colonies on the selective medium were similar to that of *B cepacia* but could be distinguished by a slightly drier appearance. Older cultures (>3 days) exhibited the characteristic wrinkled colonial morphology of this organism. A distinct “earthy” odour emanated from each of the isolates when cultured onto solid media. Each isolate was identified as *B pseudomallei* based on the API20NE with cases 1, 2 and 4 sharing the same profile (1156577, 99.9% *B pseudomallei*). The API20NE profile for case 3 was 1556577 (99.8% *B pseudomallei*). Co-pathogens included *P aeruginosa* (three cases), *B cepacia* (one patient), *S aureus* (two patients), and MRSA (one patient). In two cases *B pseudomallei* was the dominant pathogen cultured in the sputum based on it being the sole organism cultured in the majority of sputum samples (cases 1 and 2).

Antibiotic sensitivity

All isolates were resistant to all classes of aminoglycosides and colistin. All but one of the isolates was sensitive to cefazidime and all were sensitive to the carbapenem antibiotics, meropenem and imipenem. Three of the four isolates were sensitive to tetracycline and SXT, but only one was sensitive to ciprofloxacin.

MCBT

All isolates were tested against 11 single, 47 double, and 34 triple antibiotic combinations. High dose tobramycin (200 µg/ml) and ticarcillin/clavulanic acid were the most effective single agents, being bactericidal for all four isolates, while meropenem, cefazidime, and cepefime were bactericidal for three of the four isolates. High dose tobramycin in combination with all single antibiotics except chloramphenicol was bactericidal for all four isolates; high dose tobramycin with chloramphenicol was bactericidal for three of the four isolates. Among dual combinations suitable for intravenous or oral use, meropenem with SXT or tobramycin (10 µg/ml) in combination with meropenem or cefazidime or cepefime were most effective being bactericidal for all four isolates. The addition of a third antibiotic to these low dose tobramycin combinations was counterproductive in two of the four cases. Some combinations including cefazidime and meropenem were bactericidal for three of the isolates but antagonistic for the fourth isolate.

Serological tests

All patients had positive serum *B pseudomallei* antibodies which, in three cases (1–3), were performed after the initial identification of *B pseudomallei* in sputum. Case 4 had positive antibodies before the first laboratory identification of *B pseudomallei* in sputum, with seroconversion prior to July 2001. All cases had positive *B pseudomallei* total antibody and IgG, but only cases 1 and 4 had positive IgM antibodies.

Molecular identification

16S rRNA PCR for *B pseudomallei*

The *B pseudomallei* 16S rRNA PCR was performed on DNA extracted from each isolate, producing bands at 243 bp and 405 bp in each case, consistent with Ara”B pseudomallei.”
in four patients with CF, three of whom were children who had no deleterious effects associated with *B. pseudomallei* infection. The fourth patient suffered two severe episodes of bronchopneumonia following the acquisition of *B. pseudomallei*. During the first episode of bronchopneumonia in June 2001 an unidentified organism with an antibiogram consistent with *B. pseudomallei* was cultured from the sputum. Retrospective analysis of stored serum revealed an eightfold rise in the titre of melioid antibodies between March 2000 and July 2001. A specific immune response to *B. pseudomallei* was also evident in each of the other cases. Taken together, these findings support a potential pathogenic role for *B. pseudomallei* in CF lung disease, producing both chronic infection and possibly acute bronchopneumonia. Attempts to eradicate *B. pseudomallei* have so far been unsuccessful. The one previous report describing eradication in a patient with CF required 36 weeks of continuous antibiotic treatment. Prolonged courses of antibiotics specifically targeting *B. pseudomallei* in three of the four cases have been administered and eradication was not possible. The most effective antibiotics in terms of clinical response were cefazidine and meropenem when used in combination. Most (but not all) of the patients had *B. pseudomallei* isolates that were sensitive to these antibiotics. Continuous high dose SXT resulted in stabilisation in case 1 which had proved difficult to achieve despite multiple courses of triple parenteral antibiotics. While the effectiveness of MCBT has not yet been validated in clinical trials, the results of this study suggest that it may have a role in the management of patients with *B. pseudomallei*. The combination of cefazidine and meropenem, commonly used in the treatment of this organism and clinically useful in three of the four cases reported here, was also bactericidal by MCBT. MCBT may also provide new options for strategies to treat and potentially eradicate *B. pseudomallei*. Several combinations not usually associated with the treatment of this organism were found to be bactericidal for the majority of isolates. Among these were combinations involving tobramycin tested at high concentration reported to be achievable in the airways following nebulisation. A recent Cochrane review of interventions for treating melioidosis recommended that regimens for the acute phase of the illness should contain cefazidine and meropenem. The same review suggested that oral maintenance therapy after the acute phase should consist of a three drug regimen containing chloramphenicol, doxycycline, and SXT. Our clinical experience is consistent with these recommendations. Interestingly, combinations of chloramphenicol with doxycycline or doxycycline with SXT were not bactericidal for any of the isolates, but MCBT was not performed on the triple combination of chloramphenicol, doxycycline, and SXT.

Identification of *B. pseudomallei* in previously reported cases has proved difficult.2,3 Even in a specialist laboratory dealing with CF microbiology, *B. pseudomallei* can be mistaken for *B. cepacia* when using commercially available identification systems.22 A high index of suspicion is needed, not only in clinics that provide care for patients living in tropical areas but also in those clinics caring for patients travelling to endemic areas. Laboratories outside endemic areas should be aware of the various characteristics and identification procedures that may be used if *B. pseudomallei* is suspected. The organism can be readily grown on most respiratory solid culture media and is characteristically colistin resistant and therefore easily grown on *B. cepacia* medium. Although similar to *B. cepacia* complex, *B. pseudomallei* is a distinct species. Its colonies exhibit a slightly drier appearance and earthy odour, and older cultures have a characteristic wrinkled appearance. These subtle phenotypic differences underscore the need for appropriate molecular confirmation of *B. pseudomallei* and exclusion of *B. cepacia* complex. We have shown that...
B pseudomallei produces an identical RFLP fingerprint to that of B cepacia, B cenocepacia, and B antha using 16S rDNA RFLP analysis, which again highlights the importance of the two specific PCR assays to differentiate B pseudomallei from B cepacia complex.16

Most cases of infection with B pseudomallei are presumed to occur via direct inoculation from contaminated soil or water. Four cases of person to person transmission have been described12–14 and one of these involved two siblings with CF.17 Each of these four cases involved close contact with the index case, with either prolonged exposure or direct exposure to open wounds. None of the patients in our series had overlapping admissions or social contact. The B pseudomallei strain from each individual patient was genetically distinct using molecular techniques, suggesting environmental acquisition and not person to person spread. Furthermore, each of the patients retained identical B pseudomallei strains over prolonged periods. The uniqueness of B pseudomallei strains in our patients is consistent with the known epidemiology of this organism, with clonal outbreaks being unusual.18 None of the cases had excessive exposure to soil or water and, while case 2 had lived with his mother at the time that she contracted melioidosis, a decade had elapsed before he developed the first signs of B pseudomallei infection. It therefore seems unlikely that case 2 acquired B pseudomallei infection from his mother. The potential transmissibility of B pseudomallei raises infection control issues. The cases of person to person transmission described to date have mostly involved prolonged close contact with patients harbouring identifiable risk factors for acquisition.14–21 Our approach has been to manage these patients in the same manner as those infected with B cepacia. They attend clinic on a separate day, are managed in a separate ward as inpatients, and are advised to avoid close contact with other CF patients. Staff are asked to follow standard universal precautions and we have not imposed any other specific infection control measures. The evidence thus far would suggest that patients suffering from melioidosis do not pose a significant risk to otherwise well healthcare workers.

At a time when subjects with CF are undertaking international travel more than ever before, B pseudomallei is emerging as a significant pathogen for those holidaying in the tropics. A recent editorial has highlighted the importance of careful planning for air travel in patients with CF.14 Patients should be advised to avoid soil and exposure to fresh water supplies such as swimming lagoons when visiting endemic areas, particularly during the wet season. They need to have an awareness of B pseudomallei and the potential risk it poses for the traveller with CF.

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