Characterisation and molecular typing of *Burkholderia pseudomallei*: Are disease presentations of melioidosis clonally related?

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Abstract

Eighteen cases of culture positive melioidosis caused by *Burkholderia pseudomallei*, were seen in four geographically separate communities in North Queensland, Australia. The genetic inter-relatedness of the clinical isolates were compared utilising random amplification of polymorphic DNA (RAPD) and multilocus enzyme electrophoresis (MEE). The isolates segregated into two groups that correlated with clinical presentation rather than geographical location. This is the first described association between the varied clinical presentations of this condition and specific molecular type. If proven on larger studies, this may further our understanding of the pathogenesis of this important condition. © 1998 Federation of European Microbiological Societies. Published by Elsevier Science B.V.

Keywords: Melioidosis; *Burkholderia pseudomallei*; Molecular typing

1. Introduction

*Burkholderia pseudomallei*, the causative agent of melioidosis is a saprophyte found in the soil in endemic areas. Both man and animals are thought to contract the infection by inoculation, inhalation or ingestion of this environmental organism in contaminated soil or water [1,2]. Regional endemic foci of this disease are largely confined to South-East Asia and tropical Australia between latitudes 20°S and 20°N, although the organism has been isolated in sub-tropical South-West Australia [3,4]. In many endemic areas, the frequency of the disease varies greatly with time. It is hypothesised that the increased incidence of melioidosis after heavy rains is a consequence of the organism being brought up to the surface by the rising water-table from the deeper moist layers of clay where it is thought to persist during the dry season [5].
people exposed to the organism do not develop clinical disease. The organism may however remain dormant intracellularly for long periods and give rise to disease after a long latent period. This occurrence was well described in US veterans who returned home after the Vietnam war [6]. While recognised risk factors for clinical disease include diabetes, alcohol abuse, and immuno-compromised patients, it should be noted that fulminant infection may occur in previously healthy individuals.

A classification system for melioidosis was proposed with five categories. These are disseminated septicemic, non-disseminated septicemic, localised, transient bacteraemic and probable infection. This last category is based on serological findings. The disseminated septicemic group account for 45% of cases with a mortality of 87%. Non-disseminated septicemic disease is seen in 12% of cases with a mortality of 17% and localised disease accounts for 42% of cases with a mortality of 9% [7].

The clinical presentations of patients with melioidosis usually fall into one of the categories described. There is the chronic asymptomatic infection referred to above, characterised by abnormal serology and which can occasionally progress. In endemic areas, patients may present with an acute localised skin infection at the site of inoculation of the organism usually with an accompanying regional lymphadenitis. The most dramatic presentation is of an acute septicemic illness which may be associated with one or more foci of visceral involvement, pneumonia being the most frequent. This septicemic, pneumonic presentation has been specifically reported in one study to have a fatality rate of up to 75% [1]. Finally patients may present with chronic suppuration and abscess formation. A wide number of organs and tissues including lung, pancreas, spleen, prostate, testis, kidney, brain and bone, may be involved in melioidosis. This may occur either acutely with a septicemic presentation, or chronically after inapparent infection or as a relapse after a previous septicemic presentation. The mechanisms underlying the different manifestations of this disease and the factors that determine which tissues and organs are involved are poorly understood.

2. Materials and methods

This paper is based on isolates from eighteen cases of culture proven melioidosis which were diagnosed through the laboratory at Townsville General Hos-

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Age/Sex</th>
<th>Clinical summary</th>
<th>Isolation site</th>
<th>Place of residence</th>
<th>Risk factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6 yr/M</td>
<td>Brain stem encephalitis</td>
<td>Post-mortem brain and CSF</td>
<td>Community A</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>8 yr/M</td>
<td>Brain stem encephalitis</td>
<td>Blood</td>
<td>Community B</td>
<td>None</td>
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<tr>
<td>3</td>
<td>55 yr/M</td>
<td>Septicaemic illness</td>
<td>Blood</td>
<td>Community C</td>
<td>Diabetes, alcohol intake</td>
</tr>
<tr>
<td>4</td>
<td>55 yr/M</td>
<td>Pneumonia</td>
<td>Blood</td>
<td>Community C</td>
<td>Diabetes, alcohol intake</td>
</tr>
<tr>
<td>5</td>
<td>45 yr/M</td>
<td>Pneumonia</td>
<td>Blood</td>
<td>Community C</td>
<td>Diabetes, steroids</td>
</tr>
<tr>
<td>6</td>
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<td>Sputum</td>
<td>Community C</td>
<td>Diabetes, alcohol intake</td>
</tr>
<tr>
<td>7</td>
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<td>Pneumonia</td>
<td>Sputum</td>
<td>Community C</td>
<td>Diabetes, alcohol intake</td>
</tr>
<tr>
<td>8</td>
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<td>Pneumonia</td>
<td>Pleural fluid</td>
<td>Community D</td>
<td>Alcohol intake</td>
</tr>
<tr>
<td>9</td>
<td>43 yr/M</td>
<td>Pneumonia</td>
<td>Blood</td>
<td>Community D</td>
<td>Alcohol intake</td>
</tr>
<tr>
<td>10</td>
<td>48 yr/F</td>
<td>Septic arthritis</td>
<td>Blood, synovial fluid</td>
<td>Community D</td>
<td>Diabetes</td>
</tr>
<tr>
<td>11</td>
<td>47 yr/F</td>
<td>Lung abscess</td>
<td>Lung aspirate</td>
<td>Community D</td>
<td>Diabetes</td>
</tr>
<tr>
<td>12</td>
<td>58 yr/M</td>
<td>Scrotal abscess</td>
<td>Pus</td>
<td>Community D</td>
<td>Alcohol intake</td>
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<tr>
<td>13</td>
<td>35 yr/M</td>
<td>Hepatosplenic and pancreatic abscesses</td>
<td>Blood</td>
<td>Community C</td>
<td>Diabetes, alcohol intake</td>
</tr>
<tr>
<td>14</td>
<td>45 yr/M</td>
<td>Osteomyelitis</td>
<td>Pus</td>
<td>Community C</td>
<td>Diabetes, alcohol intake</td>
</tr>
<tr>
<td>15</td>
<td>46 yr/M</td>
<td>Prostatic/pelvic abscess</td>
<td>Blood and pus</td>
<td>Community D</td>
<td>Alcohol intake</td>
</tr>
<tr>
<td>16</td>
<td>45 yr/M</td>
<td>Septic arthritis</td>
<td>Blood and synovial fluid</td>
<td>Community B</td>
<td>Alcohol intake</td>
</tr>
<tr>
<td>17</td>
<td>53 yr/F</td>
<td>Splenic and renal abscesses</td>
<td>Blood</td>
<td>Community C</td>
<td>Diabetes</td>
</tr>
<tr>
<td>18</td>
<td>30 yr/F</td>
<td>Splenic and hepatic abscesses</td>
<td>Blood</td>
<td>Community C</td>
<td>Diabetes, alcohol intake</td>
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</table>
hospital. The majority of these were admitted to the hospital which serves as a tertiary referral centre for North Queensland. They demonstrate the diversity of clinical presentations of this condition. These cases occurred in four geographically distinct communities in North Queensland. All cases came from four communities (A-D). These communities were up to 1000 km apart, one being an island community from which eight of the cases came. Details are provided in Table 1.

The appearance of these cases over a six-month period coincided with a period of heavy rainfall in the region. For three years before, during which time there had been failures of the wet season, no acute cases of melioidosis were seen in the hospital. The initial aim of the study was to explore whether there was any evidence of relatedness between strains isolated from patients living in the different geographical areas. Isolates were typed by random amplification of polymorphic DNA (RAPD) and multi-locus enzyme electrophoresis (MEE).

2.1. Cases

A case was defined as one in which *B. pseudomallei* was isolated from clinical material, e.g. blood, sputum, pus, pleural fluid and post-mortem brain. All eighteen cases demonstrated clinical evidence of sepsis. There were seven cases of acute septicaemia with pneumonia, two of brain stem encephalitis, four with pancreatic or hepatosplenic abscesses, three with septic arthritis or osteomyelitis, one with a soft tissue infection and one septicaemic illness of unknown origin.

2.2. Bacterial isolation and identification

All isolates were presumptively identified by their characteristic colonial morphology, growth on Ashdown’s agar (Oxoid) and gentamicin resistance. These were confirmed by the API-20 NE (Bio-Merieux).

2.3. Typing of bacterial isolates

A total of eighteen isolates were typed by RAPD PCR analysis and 12 isolates were typed by multi-locus enzyme electrophoresis (MEE).

2.4. Chromosomal DNA preparation for RAPD typing

All bacteria were cultured on Ashdown’s agar and sub-cultured in flasks containing 100 ml tryptone soy broth at 37°C overnight in a shaking incubator. The cultures were centrifuged in small aliquots at 10,000×g for 60 min and the cell pellets were resuspended in 570 µl TE buffer, pH 8.0, containing 3 µl of 20 mg ml⁻¹ Proteinase K and 30 µl of 10% (w/v) SDS. The samples were incubated at 37°C for 60 min, and 100 µl of 5 M NaCl was added, and the contents mixed thoroughly. A volume of 80 µl of 10% hexadecl trimethyl ammonium bromide (CTAB)/0.7 M NaCl solution was added, the contents were mixed and incubated at 65°C for 30 min. An equal volume of chloroform/isoamyl alcohol (24:1) was added, the contents mixed and microcentrifuged for 5 min. The supernatant fluid was transferred to a fresh tube and an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added. The tubes were again centrifuged for 5 min and the supernatant fluid transferred to a fresh tube. Isopropanol to a final concentration of 0.6 total volume was added, the tubes centrifuged, and the pellets were washed, dried, and resuspended in 50 µl TE buffer. All DNA samples were stored at 4°C until analysed.

2.5. Selection of primers

A commercially available Arbitrary Priming Fingerprinting kit containing a set of 20 random 10-base oligonucleotide primers was used for RAPD typing (Bresatec, Adelaide, Australia). Each primer was tested singly with the 18 DNA samples and eight primers found to produce the best discrimination between samples were selected for all further studies.

2.6. RAPD PCR

Amplification was performed in a total volume of 25 µl containing 50 pmol of each primer, 2.5 mM MgCl₂, 200 µM each dNTP, 2 U *Taq* polymerase in PCR buffer (Bresatec, Adelaide). The reaction mix was overlaid with 20 µl mineral oil, and incubated for 5 min at 94°C. The reaction profile performed in a programmable thermal cycler (Brestaece, Adelaide)
consisted of 5 cycles of 1 min at 95°C, 2 min at 35°C, and 2 min at 72°C. This was followed by a further 35 cycles of 1 min at 95°C, 1 min at 35°C, and 1 min at 72°C. A final extension step of 5 min at 72°C was included, and the tubes were stored at 4°C until further analysis.

2.7. Detection of amplified products

The products were visualised by ethidium bromide staining following electrophoresis on a 1.5% (w/v) agarose gel in 0.5×TBE buffer (45 mM Tris-borate/1 mM EDTA buffer at 5 V cm⁻¹. In addition, products were electrophoresed on a 8% (w/v) polyacrylamide gel at 3 V cm⁻¹ and detected by staining with ethidium bromide and UV transillumination.

2.8. Analysis of banding profiles

All banding profiles were converted into matrices where ‘1’ represented the presence of a particular band, and ‘0’ represented its absence. One matrix was produced for each set of samples with each of the 8 selected primers. The data generated from these tables was analysed using the RAPDistance computer software package (Armstrong et al., Australian National University, Canberra, Australia).

2.9. Sample preparation for MEE

Isolates 1–12 were streaked onto tryptone soy agar containing yeast extract to enable isolation of single colonies. After incubation at 37°C for 24 h, a single colony was selected and inoculated into 100 ml brain heart infusion broth. The flasks were incubated with shaking at 37°C for 24 h, at which time they were centrifuged at 5000 × g for 30 min. The supernatant fluid was discarded, and the pellets were resuspended in 1.2 ml of breaking buffer (10 mM Tris, pH 6.8, 1 mM EDTA, 0.5 mM NADP). The suspensions were frozen and ground with a mortar and pestle on dry ice, until a fine white powder resulted. The powder was allowed to liquefy at room temperature, and then centrifuged at 9000 × g for 5 min at 4°C in a microfuge. The supernatant fluid (clarified cell lysate) was removed from the pellet (whole cells and cell wall fractions) and stored in 60 μl aliquots at −20°C until required.

2.10. Electrophoresis and gel staining

For each of the 12 cell lysates, the electrophoretic mobility of 10 different enzymes was determined by the procedure of Selander et al. [8], and modified by Sutherland and Porritt [9]. Catalase activity was assayed by the method of Harris and Hopkinson [10]. Cell lysates were subjected to horizontal electrophoresis at 130 V in 11.4% (w/v) starch gels containing either Tris/citrate buffer, pH 8.0, or Tris/maleate buffer, pH 8.2. Electrophoresis was continued for 4 h or until a marker (0.1% bromophenol blue) had migrated to approximately 1 cm from the anodal end of the gel. Each gel was sliced horizontally, to yield 4 slices, each approximately 2 mm thick. The slices were stained separately for specific enzymatic activity.

2.11. Analysis of MEE data

Analysis of the data was carried out with a computer program described by Selander et al. [8], and obtained from the author, T.S. Whittam (Penn. State University). The program calculates the genetic diversity for each enzyme locus among the electrophoretic types (ETs), and the genetic distance between ETs is expressed as a proportion of the loci at which dissimilar ‘alleles’ occurred, with the contribution of each locus weighted by the genetic diversity at the locus. The clustering of the ETs was produced using the average-linkage method from a matrix of coefficients of pair-wise genetic distances.

3. Results

3.1. RAPD analysis

A total of 20 arbitrary primers were tested with each of the 18 DNA samples. All were found to give some degree of discrimination between samples. A series of 8 primers found to produce the highest number of discriminatory bands, were used to produce the banding profiles subsequently analysed in a RAPD distance program. All isolates showed different banding patterns with this set of primers. Samples 11 and 12 which were indistinguishable on the
basis of MEE data, were able to be separated using the RAPD technique (Fig. 1).

The clinical isolates were compared with respect to both geographical location and clinical outcome. Fig. 1 depicts two major clusters occurring within the isolates. These isolates do not appear to be grouped according to the geographical location of the isolates, although the majority of one cluster contains isolates from an island community, while the other cluster contains the majority of the isolates obtained from rural Queensland centres. If the RAPD results are analysed on the basis of disease

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**Clinical Presentation** | **Community**
--- | ---
Neurological | A
Pneumonia | B
Soft Tissue Abscess | C

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Fig. 1. *B. pseudomallei* isolates: dendrogram demonstrating relatedness by RAPD analysis. Isolates obtained from cases 1–18. Individual case details are given in Table 1.
state, patients presenting with pneumonia symptoms cluster together, as do patients presenting with soft tissue abscesses. Isolates 1 and 2 which cluster together were geographically isolated, however both cases suffered from neurological disease and were fatal.

3.2. Multilocus enzyme electrophoresis (MEE) analysis

A total of 10 enzymes as listed in Table 2 were used to type the *B. pseudomallei* isolates. These produced nine distinct electrophoretic types, with samples 11 and 12 being indistinguishable. Three clusters could be distinguished with MEE, which related to the distribution patterns observed with the RAPD data.

4. Discussion

In common with most infective organisms, the pathogenicity of *B. pseudomallei* is related to both host and organism factors. Host risk factors for clinical disease in Northern Australia have been well described and include male sex, Aboriginality, diabetes mellitus, alcohol abuse and an outdoor occupation [11,12]. A number of features of the organism have been shown to correlate with pathogenicity. One of these is toxin production. Cytolethal toxin (CLT) has been identified from culture filtrates of *B. pseudomallei* [13]. Its mechanism of action is unknown although its rapidity of action would suggest that it could act directly on the cell membrane. Soil isolates have been found to be least cytolethal while those from severely ill patients with encephalitis caused by *B. pseudomallei* were the most active. Up-regulation of this toxin may occur during passage through animals and humans [13].

A recent study looking at differences between clinical and environmental isolates showed that two groups of ribotype patterns were found. Group I consisted of both clinical and environmental isolates while Group II had only environmental strains. It was further demonstrated that all but one of the Group II isolates were able to utilise L-arabinose. This was in contrast to the Group I isolates of which only 3% could utilise this sugar as their sole energy source [14]. The significance of this finding is unknown but it may be a potential marker of pathogenicity.

Epidemiological investigations are highly dependent on the reliability and reproducibility of typing systems able to differentiate between isolates, and isolates from different sources. No single typing method is adequate for typing organisms. Ideally, two or three methods should be used, producing comparable results. The choice of a typing method is dictated by cost, ease of use and reproducibility.

Several studies have compared different typing methods for *Listeria monocytogenes* [15], *Enterobacter cloacae* [16] and *Neisseria meningitidis* [17]. The investigators carrying out these studies have differed in their opinion of which particular typing method is the more discriminatory. It may be that different typing methods are more suited to some organisms than others. The results of this study indicate that although both MEE and RAPD assays

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Classification</th>
<th>Buffer system</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase</td>
<td>EC 1.11.1.6</td>
<td>Tris-maleate, pH 8.2</td>
</tr>
<tr>
<td>Alanine dehydrogenase</td>
<td>EC 1.4.1.1</td>
<td>Tris-citrate, pH 8.0</td>
</tr>
<tr>
<td>α-Esterase</td>
<td>EC 3.1.1.1</td>
<td>Tris-maleate, pH 8.2</td>
</tr>
<tr>
<td>Fumarase</td>
<td>EC 4.2.1.2</td>
<td>Tris-maleate, pH 8.2</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>EC 1.15.1.1</td>
<td>Tris-maleate, pH 8.2</td>
</tr>
<tr>
<td>Phosphoglucoisomerase</td>
<td>EC 5.3.1.9</td>
<td>Tris-citrate, pH 8.0</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>EC 1.1.1.49</td>
<td>Tris-citrate, pH 8.0</td>
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<tr>
<td>Adenylate kinase</td>
<td>EC 1.7.4.3</td>
<td>Agar overlay</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>EC 3.1.3.2</td>
<td>Tris-maleate, pH 8.2</td>
</tr>
<tr>
<td>Phosphoglucomutase</td>
<td>EC 1.7.5.1</td>
<td>Tris-maleate, pH 8.2</td>
</tr>
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</table>

Table 2
Enzymes and the buffer systems used for multilocus enzyme electrophoresis typing of *B. pseudomallei* isolates

FEMSIM 829 5-2-98
were highly discriminatory for B. pseudomallei isolates. RAPD was able to differentiate between two isolates found to be identical with MEE (cases 11 and 12). Although the stimulus to this study was to investigate the possibility that there was an epidemiological link between the cases of melioidosis which occurred at the same period of time in four different locations in North Queensland after some years of quiescence in the incidence of the disease, the results from this typing study indicated that the isolates tested, clustered on the basis of disease presentation, rather than by geographical location. Two broad groups are seen based on the relatedness of the isolates as defined by the RAPD and MEE analysis. Cases 1–9 which formed the first group consist mainly of patients with respiratory disease. It also includes two children who had brain stem encephalitis (cases 1 and 2) and case 3 in whom no clinical source of sepsis could be determined. The second group (cases 10–18) consist of isolates from patients with abscesses in viscera other than lung or brain, and from patients with bone and joint disease.

This finding represents an interesting development not previously described. These results suggest that B. pseudomallei may carry genetic regions capable of influencing tissue tropism of the infecting strain and thus contributing to the particular manifestation of melioidosis in the patient infected with that particular strain. The contribution of genetically defined microbial virulence factors to the manifestation of infection with organisms that vary in their expression of virulence factors, is well recognised in infectious diseases. Examples include the variable expression of exotoxin by the group A streptococcus and its role in generating streptococcal toxic shock syndrome, the presence of the lysogenic beta prophage in strains of Corynebacterium diphtheriae that cause clinical diphtheria and the contribution of various genetically defined virulence determinants in strains of Escherichia coli to the differences in the forms of gastroenteritis caused by this bacterial species. The variation in the presentation of melioidosis is well recognised though the determinants of this have not as yet been identified. They would logically include both host and microbial factors. This study suggests differences in the patterns of disease caused by genetically clustered isolates of B. pseudomallei, and may point the way to identifying microbial factors that contribute to how infection with this organism expresses itself. These findings clearly need to be examined in a prospective study with a larger group of clinical isolates. A comparison of clonality between clinical isolates and environmental isolates obtained during the same period, would also help in the development of this clonal hypothesis.

The use of other typing methods such as pulsed field gel electrophoresis (PFGE) along with the techniques used in this study would add greater reliability and discrimination. If clonality with B. pseudomallei is shown to be related to disease process, it would be a significant advance in our understanding of the pathogenesis of melioidosis.

Acknowledgments

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References


