BACTERIAL CHARACTERISATION

Biochemical characteristics of clinical and environmental isolates of *Burkholderia pseudomallei*

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The biochemical characteristics of 213 isolates of *Burkholderia pseudomallei* from patients with melioidosis and 140 isolates from the soil in central and northeastern Thailand were compared. Whereas the biochemical profiles of all the clinical isolates were similar, all soil isolates from the central area and 25% of isolates from northeastern Thailand comprised a different phenotype. This was characterised by the ability to assimilate L-arabinose (100%), adonitol (100%), 5-keto-gluconate (90%) and D-xylose (84%), but failure to assimilate dulcitol (0%), erythritol (0%) and trehalose (10%). Compared with clinical isolates, these organisms had similar antibiotic susceptibility profiles and were also recognised by a specific polyclonal antibody against *B. pseudomallei*. As melioidosis is rare in central Thailand, but common in the northeast, this raises the possibility that this biochemical phenotype may be less virulent, or may even represent a different species.

Introduction

Burkholderia (formerly *Pseudomonas*) *pseudomallei* is an environmental saprophyte capable of causing devastating systemic infections in many species of mammals and birds. Although *B. pseudomallei* has been isolated from environmental samples across the tropical world, the disease in man is confined to the endemic areas of eastern Asia and northern Australia [1]. In northeastern Thailand *B. pseudomallei* is a major cause of morbidity and mortality, causing one-fifth of all community-acquired septicaemias [2]. However, *B. pseudomallei* is also isolated readily from soil samples in the southern and central regions of Thailand, where clinical disease is rare [3, 4]. To investigate this discrepancy between the distribution of disease and the distribution of the organism in the environment, this study compared the biochemical and antigenic features, and antibiotic susceptibilities, of clinical and environmental isolates of *B. pseudomallei*.

Materials and methods

Bacterial isolates

Clinical and laboratory studies on melioidosis have been conducted in Ubon Ratchatani, northeast Thailand, since 1986. More than 1100 patients with culture-proven melioidosis have been seen during this time. Single isolates of *B. pseudomallei* from 213 of these patients were used for this study.

The soil isolates of *B. pseudomallei* were selected from those collected in a survey of rice paddies in northeastern Thailand [5] and in a quantitative comparison of the isolation of the organism from soil in central and northeastern Thailand [4]. A maximum of two isolates was selected from each site sampled, giving a total of 140 isolates, 115 from the northeast and 25 from the central region.

Organism identification

All isolates were identified initially by their characteristic colonial morphology on a differential agar medium [6], positive oxidase reaction, and resistance to colistin and gentamicin [7].

Biochemical identification was obtained with API 20NE (bioMérieux) following the manufacturer's instructions, as detailed previously [7].

Extended biochemical characteristics

For 100 strains of *B. pseudomallei* further biochemical profiles were obtained with API 50CH (bioMérieux) following the manufacturer's guidelines. The 49 individual carbohydrates tested are given in Table 2. The medium used was a sterile minimal salts solution (ammonium chloride 20 g, ammonium nitrate 4 g, anhydrous sodium sulphate 8 g, dipotassium hydrogen orthophosphate 12 g, potassium dihydrogen orthophosphate 4 g, magnesium sulphate 0.4 g, all from Sigma, and distilled water to 1 L) to which agar 0.15% was added. The ability to assimilate a single carbohydrate was recorded if there was any growth after incubation for 48 h at 37°C.

Arabinose agar

A single carbohydrate agar was used for L-arabinose and glucose assimilation with a larger number of isolates of *B. pseudomallei*. This consisted of minimal salts solution (25 ml), agar 2% solution (75 ml) and carbohydrate (L-arabinose or glucose; Sigma) 10% solution (2 ml). Each solution was sterilised separately and then mixed together. The agar plates were spotted with 3 µl of a 1 in 10 dilution of an overnight bacterial suspension adjusted to a 0.5 MacFarland standard. Results were recorded as growth after incubation for 48 h at 37°C. The glucose agar was used as a growth control for all isolates.

Antigenicity

The antigenic similarity of clinical and soil isolates was checked with a latex agglutination test for the rapid identification of *B. pseudomallei*, as described previously [8]. The latex particles were coated with a rabbit antiserum to boiled whole cells of a clinical isolate (NCTC 1688) of *B. pseudomallei*.

Antibiotic susceptibilities

Tests for susceptibility to ceftazidime, chloramphenicol, co-amoxiclav and doxycycline were performed by disk diffusion [9].

Statistical analysis

Proportions were compared with the χ^2 -squared test or Fisher's exact test.

Results

Arabinose assimilation

The API 20NE results of 213 clinical isolates and 69 soil isolates of *B. pseudomallei* are shown in Table 1. There was a highly significant difference between the two groups in their ability to assimilate arabinose. None of the clinical isolates could assimilate arabinose whereas 52% of the soil isolates did so. Within the soil group there was significant geographical variation; all 25 central region isolates assimilated arabinose but only 11 (25%) of the 44 northeastern isolates ($p < 0.00001$). There was also a small difference in the assimilation of maltose (7.5% versus 0% of clinical

Table 1. Comparison of API 20NE biochemical reactions of clinical and soil isolates of *B. pseudomallei*

Substrate	Percentage positive		p value
	Clinical isolates (n = 213)	Soil isolates (n = 69)	
Nitrate	100	100	
Tryptophan	100	100	
Glucose (acidification)	0	0	
Arginine	100	100	
Urea	0	0	
Aesculin	22.1	27.5	0.35
Gelatin	100	100	
PNPG	0	0	
Assimilation of:			
Glucose	100	100	
Arabinose	0	52.2	< 0.00001
Mannose	100	100	
Mannitol	100	100	
N-acetylglucosamine	99.6	100	
Maltose	7.5	0	0.01
Gluconate	99.2	100	
Caprate	100	100	
Adipate	100	100	
Malate	100	100	
Citrate	92.9	97.1	
Phenylacetate	96.7	92.8	

and soil isolates, respectively) but all other reactions gave very similar results.

Assimilation of arabinose in the API 20NE system was then compared with growth on L-arabinose agar for 58 of the soil isolates (half of which assimilated arabinose in API 20NE). The results were identical. Isolates with the ability to assimilate arabinose in API 20NE or grow on L-arabinose agar are designated ara⁺ and isolates without this ability are designated ara⁻.

Subsequently, growth on L-arabinose agar was assessed for another 71 isolates. Eleven isolates were assessed by API 20NE only. The results of L-arabinose assimilation by agar and API were then combined. Of the northeastern isolates, 29 (25%) of 115 were ara⁺ and 86 (75%) were ara⁻. In contrast, all 25 central region isolates were ara⁺ ($p < 0.00001$).

In northeastern Thailand both ara⁺ and ara⁻ soil isolates of *B. pseudomallei* may be found in the same location. During a previous environmental survey [5], a detailed study of one rice farm was performed. Of 89 isolates with API 20NE results, 69 (77.5%) were ara⁺ and 20 (22.5%) were ara⁻.

Extended biochemical characteristics

Extended biochemical profiles were obtained for 100 isolates of *B. pseudomallei* by the API 50CH system. Fifty of these isolates did not assimilate arabinose in

API 20NE (ara⁻; 25 clinical isolates and 25 soil isolates from the northeastern region) and 50 isolates assimilated arabinose in API 20NE (ara⁺; 25 soil isolates from the central region and 25 isolates from the northeastern region). The results are shown in Table 2. The most significant differences were that ara⁺ isolates of *B. pseudomallei* assimilated L-arabinose (100%), adonitol (100%), 5-keto-gluconate (90%) and D-xylose (84%), but failed to assimilate dulcitol (0%), erythritol (0%) and trehalose (10%). Smaller, but still significant, differences were found in the ability to assimilate aesculin, D-arabinose, L-arabitol, glycerol, glycogen, starch, sucrose and xylitol. Within the ara⁻ group, there were no significant differences between clinical and soil isolates. Comparing soil ara⁺ isolates, there were small but significant differences between central and northeastern isolates in their ability to assimilate sucrose (8% versus 36%; $p = 0.04$) and 5 keto-gluconate (80% versus 100%; $p = 0.025$).

Antigenicity

All 140 soil isolates of *B. pseudomallei* (86 ara⁻ and 54 ara⁺) gave a positive reaction in the latex agglutination test. The majority of clinical isolates included in this study were not tested for latex agglutination. However, the latex agglutination test was evaluated initially with 52 clinical isolates of *B. pseudomallei* [8] and no false positive reactions were recorded or have been seen subsequently in 4 years of use in clinical studies.

Table 2. Extended biochemical reactions of *B. pseudomallei* to compare ara⁻ clinical or soil isolates with ara⁺ soil isolates tested in API 50CH

Substrate*	API 20 NE arabinose assimilation		p value
	Negative (%) (n = 50)	Positive (%) (n = 50)	
Glycerol	80	98	0.01
Erythritol	88	0	< 0.00001
D-Arabinose	36	0	0.00001
L-Arabinose	0	100	< 0.00001
Ribose	2	0	
D-Xylose	0	84	< 0.00001
L-Xylose	22	14	0.3
Adonitol	4	100	< 0.00001
L-Sorbose	2	0	
Rhamnose	2	0	
Dulcitol	98	0	< 0.00001
N-acetylglucosamine	98	98	
Aesculin	0	30	0.0001
Sucrose	2	22	0.006
Trehalose	98	10	< 0.00001
Starch	46	98	0.00001
Glycogen	44	100	< 0.00001
Xylitol	94	72	0.008
L-Fucose	94	96	
L-Arabitol	82	100	0.002
5-Keto-gluconate	0	90	< 0.00001

*Carbohydrates assimilated by all 100 isolates of *B. pseudomallei*: galactose, D-glucose, D-fructose, D-mannose, inositol, mannitol, sorbitol, D-arabitol, gluconate and 2-keto-gluconate. Carbohydrates not assimilated by any isolate of *B. pseudomallei*: β -methyl-D-xyloside, α -methyl-D-mannoside, α -methyl-D-glucoside, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, inulin, melezitose, D-raffinose, β -gentiobiose, D-turanose, D-lyxose, D-tagatose and D-fucose.

Antibiotic susceptibilities

All 140 soil isolates of *B. pseudomallei* (86 ara⁻ and 54 ara⁺) were susceptible, according to the NCCLS definitions [9], to the four antibiotics tested. Of the 213 clinical isolates of *B. pseudomallei*, 202 were susceptible to all four antibiotics. Ten patients had isolates that were resistant to chloramphenicol alone; nine were resistant when the patient was admitted to hospital and one developed resistance during treatment. The remaining patient's isolate was resistant to co-amoxiclav, but this also developed during treatment.

Discussion

Clinical and environmental isolates of *B. pseudomallei* are morphologically similar and antigenically indistinguishable. The immunodominant antigen of *B. pseudomallei* is the cell wall lipopolysaccharide [10]. This is remarkably invariant among different isolates, and is sufficiently similar in clinical and environmental isolates for all to be recognised by the polyclonal antibody used for the latex agglutination test. The antibiotic susceptibility profile of clinical isolates of *B. pseudomallei*, characterised by aminoglycoside resistance and susceptibility to ceftazidime, co-amoxiclav, chloramphenicol and tetracyclines was also found in the soil isolates.

In terms of biochemical reactions there were significant differences between environmental and clinical isolates. Obviously organisms causing clinical melioidosis originate from the soil, and organisms were found in the soil of northeastern Thailand that were identical in all respects with those causing disease. However, another discrete biotype was also present. This biotype is characterised by assimilation of L-arabinose (100%), adonitol (100%), 5-keto-gluconate (90%) and D-xylose (84%), but failure to assimilate dulcitol (0%), erythritol (0%) and trehalose (10%). This soil biotype (designated ara⁺) was found together with the clinical biotype (designated ara⁻) in rice-paddies of the northeast, but was the only type isolated in the central area of Thailand. This corresponds with the clinical distribution of melioidosis. The infection is common in the northeast, where it causes c. 20% of community-acquired septicaemias, but is rare in the central region, yet rice-farming practices (the major occupational risk factor for the disease) are common to both regions. This suggests that the ara⁺ biotype may be considerably less virulent than the ara⁻ biotype.

This difference in biochemical reactivity, and possible difference in virulence raises the possibility that these two biotypes are sufficiently far apart to constitute different species. Ara⁺ soil isolates of *B. pseudomallei* are significantly different from other L-arabinose-positive environmental *Burkholderia* species described by Gillis *et al.* [11] and Yabuuchi *et al.* [12]. *B.*

cepacia, *B. gladioli* and *B. pickettii* are arginine dihydrolase-negative and have significant differences in the assimilation of nine or more of the carbohydrates. *B. andropogonis*, *B. caryophylli*, *B. glumae* and *B. plantarii* differ from ara⁺ *B. pseudomallei* in the assimilation of seven or eight carbohydrates. *B. cocovenenans* and *B. phenazinium* differ by assimilation of five and four carbohydrates respectively. Compared with ara⁺ *B. pseudomallei*, *B. vandii* [13] differs in the ability to assimilate adonitol, lactose and starch, but only one isolate and few carbohydrates were tested. However, *B. vandii* is susceptible to gentamicin. Recently a new species of *Burkholderia*, *B. vietnamensis*, was isolated from rice rhizospheres in southern Vietnam [11]. This species also differs from the ara⁺ isolates of *B. pseudomallei* in the present study in that the former does not produce arginine dihydrolase and fails to assimilate adonitol, L-arabitol, glycogen and starch. Also, *B. vietnamensis* assimilates D-arabinose, cellobiose, dulcitol, gentiobiose, D-lyxose and D-fucose.

The results for ara⁻ isolates of *B. pseudomallei* differ significantly from those of Redfearn *et al.* [14] in the ability to assimilate cellobiose, D-fucose, maltose, salicin and sucrose, but this may reflect differences in methodology and geographical distribution of the isolates.

The absence of disease caused by the ara⁺ biotype of *B. pseudomallei* should now be confirmed by a comparison of virulence in an animal challenge. If lack of virulence is confirmed this would also raise the possibility that an apparently immunologically similar organism might be a vaccine candidate for the prevention of melioidosis, and might provide an important insight into the mechanisms of virulence in *B. pseudomallei*.

It is also possible that the ara⁺ biotype represents a new species of *Burkholderia* which is morphologically and antigenically similar to *B. pseudomallei*. Therefore further study and comparison of the genetic structure of the two biotypes is needed.

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