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RAPD analysis of isolates of *Burkholderia pseudomallei* from patients with recurrent melioidosis

A. HAASE, A. MELDER, H. SMITH-VAUGHAN, D. KEMP AND B. CURRIE

Menzies School of Health Research, P.O. Box 41096, Casuarina NT 0811, Australia

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SUMMARY

Twenty-seven isolates of *Burholderia pseudomallei* (formerly *Pseudomonas pseudomallei*) from ten patients with recurrent melioidosis were analysed by RAPD. In two cases RAPD patterns in recurrent isolates differed from the original isolates; one was considered a likely reinfection while the other may represent relapse from one of two strains initially infecting the patient. In two cases where a change in antibiotic resistance had occurred between original and relapse isolates, slight changes in RAPD patterns were found with one of the four primers used. In the other six cases the relapse was clearly due to the original strain re-emerging unchanged, with identical RAPD patterns with all four primers.

INTRODUCTION

Melioidosis is caused by *Burkholderia pseudomallei* (formerly *Pseudomonas pseudomallei*), a natural organism of the soil. It is endemic in tropical regions in Southeast Asia and northern Australia where it is an important cause of septicaemia and pneumonia [1, 2]. In the tropical 'top end' of the Northern Territory of Australia it is the commonest recognized cause of fatal community-acquired pneumonia [3]. Since October 1989 there have been 106 cases of melioidosis in the 'top end' with 27 deaths (B. Currie, unpublished data).

Acute infection can lead to septicaemia and death within 48 h of onset of symptoms. Severe disease is usually associated with risk factors such as diabetes, high alcohol consumption and renal disease. The organism can also remain latent for many years, presumably surviving within macrophages, particularly in the lung [4, 5]. Many cases have been reported in US veterans of the Vietnam war as well as in visitors returning from endemic areas [6–9].

The longest latency period described is 26 years [10]. In addition to latent infection the disease often relapses within weeks or months of antibiotic treatment for acute disease, especially if the recommended maintenance therapy of at least 3 months is not adhered to [7, 11, 12]. However, it has been difficult to prove that relapses are really due to re-emergence of the original strain, as opposed to reinfection with a new strain, which is possible, especially if the patient has risk factors for melioidosis. In addition, it is possible that occasionally the initial infection may be with more than one strain or clone of *B. pseudomallei*, with only one strain later relapsing.

An earlier study based on ribotyping found the same ribotype for original and relapse isolates in the majority of cases [4]. However, in 2 of 25 cases the results suggested either reinfection or possible initial infection with two different strains. An additional possibility for one of the cases was strain variation over time, with a minor ribotype change occurring with the relapse isolate due to rearrangements in rRNA genes. Ribotyping has had limited discriminating ability for Northern Territory *B. pseudomallei* isolates, with over 60% of isolates falling into only three BamHI ribotypes [13].

RAPD (randomly amplified polymorphic DNA) [14, 15] is a new, quick and sensitive PCR based typing method. It uses random primers to amplify parts of the genome where a particular random primer binds on opposite strands within an easily amplifiable distance. There are numerous examples to show the capacity and sensitivity of this method [16, 17]. We have found, unlike with ribotyping, that RAPD has distinguished all epidemiologically unrelated isolates of *B. pseudomallei* tested [18] and we have used it to analyse initial and recurrent isolates.

METHODS

Bacterial isolates

B. pseudomallei isolates were obtained from patients referred to the Royal Darwin Hospital for clinical management. They were cultured from sputum, tissue lesions or blood (see Table 1) on HBA plates and confirmed by API20E (Biomerieux, Marcy d'Etoile, France) and Microbact 24E (Oxoid, Heidelberg West, Australia).

DNA preparation

A loopful of colonies was resuspended in 50 μl PIV (10 mM Tris-HCl pH 7·6, 1 M-NaCl)/1·6% low melting point agarose in Eppendorf tubes. After setting of the agarose plug 150 μl lysis buffer (6 mM Tris-HCl pH 7·6, 1 M-NaCl, 100 mM EDTA, 0·5% Brij-58, 0·2% deoxycholate, 0·5% sarcosyl, 1 mg/ml lysozume, 2 μl of a 10 mg/ml RNAse stock solution) were added and incubated at 37 °C overnight. The buffer was then replaced with 150 μl proteinase K buffer (0·5 M EDTA, 1% sarcosyl, 1 mg/ml Proteinase K) at 55 °C overnight. After this the blocks were soaked in TE buffer three times, 500 μl water were added and the plugs melted at 65 °C.

Ribotyping

DNA was isolated using the guanidinium-thiocyanate method [19]. It was digested with BamHI, fractionated by electrophoresis on a 0·8% agarose gel in 1XTAE buffer for 16 h at 1·1 V/cm and blotted to Hybond N Plus (Amersham) in 0·4 N-NaOH. The pre-hybridization mixture was 0·3 M-NaCl, 20 mM-NaH_2PO_4, 2 mM EDTA, 1% SDS, 0·5% non-fat skim milk powder and 0·5 mg/ml herring sperm DNA. The probe was a 7 kb insert from plasmid pKK3535 bearing an *Escherichia coli* ribosomal RNA operon. Hybridization was in the above solution at 65 °C.

For ribotype identification we used the BamHI ribotype numbering scheme of Lew and Desmarchelier [20].

Table 1. *Isolates used in this study*

Isolate	BamHI ribotype (rtp.)	Remarks
Case A, original	rtp. 33	
Case A, relapse	rtp. 33	
Case B, original	rtp. 5	Amoxycillin/clavulanate and ceftazidime sensitive
Case B, 1st relapse	rtp. 5	Amoxycillin/clavulanate resistant
Case B, 2nd relapse	rtp. 5	Amoxycillin/clavulanate and ceftazidime resistant
Case C, original	rtp. 16	
Case C, relapse	rtp. 16	
Case D, original	rtp. 27	
Case D, relapse	rtp. 27	
Case E, original	rtp. 1	
Case E, reinfection	rtp. ?19	Change in ribotype
Case F, original	+*	
Case F, relapse	+	Ribotype identical
Case G, original	rtp. 3	Doxycycline sensitive
Case G, relapse, joint	rtp. 3	Doxycycline resistant
Case G, relapse, blood	rtp. 3	Doxycycline sensitive
Case H, original	rtp. 14	
Case H, relapse	rtp. 14	
Case I, original	+	
Case I, relapse	+	Change in ribotype
Case J, original	ribotype not determined	
Case J, relapse	Ribotype not determined	

* +, Ribotype not in BamHI numbering scheme of Lew and Desmarchelier [20].

RAPD PCR

The following primers were chosen out of a kit from Operon technologies Inc.: primer 6, GAG ACG CAC A; primer 7, CAG CCC AGA G; primer 13, AGC GTC ACT C; primer 16, AAG CGA CCT G.

Each reaction of 25 μl contained 50 pmol random primer, 1 μl of the above DNA preparation, 2 mM-$MgCl_2$ and 2 U Taq polymerase (Bresatec). Reactions were performed in a Corbett machine using 1 min 95 °C, 2 min 35 °C, 2 min 72 °C for the first 5 cycles; then 1 min each for 95 °C, 35 °C, 72 °C, for another 35 cycles, PCR products were analysed on a 1% agarose gel.

We have previously demonstrated reproducibility of this RAPD protocol (paper submitted for publication).

RESULTS AND DISCUSSION

Ten cases of relapsed melioidosis occurring between 1990 and 1994 were studied. Two cases showed a different ribotype with recurrent infection and the two strains showed different RAPD patterns. One (case, see Table 1) was a likely reinfection, re-presenting with melioidosis 14 months after his initial infection. The other patient (case I, see Table 1), a 22-year-old woman with no risk factors for melioidosis, may represent an unusual scenario of relapse from one of two different strains of *B. pseudomallei* she was initially infected with. The patient was not compliant with maintenance antibiotics and she re-presented 8 months after

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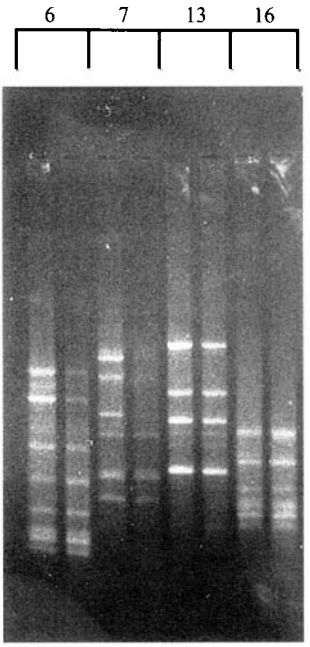

Fig. 1. RAPD analysis of case A, original and relapse isolate with primers 6, 7, 13, 16; example of both isolates being the same strain.

initial diagnosis with chronic osteomyelitis, thought to be from bacterial seeding during her initial illness with melioidosis pneumonia. Re-infection was unlikely as the osteomyelitis was clinically suspected from not bacteriologically proven when she initially presented and her two presentations spanned the 'low risk' dry season.

For all seven other cases tested, the relapse and initial ribotypes were identical, including one patient who relapsed twice (Fig. 1). However, in two cases a change in antibiotic sensitivity was observed with relapse, although the ribotype remained the same. Antibiotic resistance emerged after incomplete maintenance therapy with amoxycillin/clavulanate in the first case and with doxycycline in the second. In the first patient (case B, see Table 1) the original isolate was sensitive to amoxycillin/clavulanate and ceftazidime. On relapse 3 months later the organism had acquired resistance to amoxycillin/clavulanate. A month later a further relapse occurred and resistance against ceftazidime had emerged. RAPD analysis reveals slight differences between all three isolates, with primer 7 showing a single band changing its molecular weight in the second isolate and then a double band appearing in the third isolate (Fig. 2).

In the other patient (case G, see Table 1) a relapse isolate from joint showed doxycycline resistance not present before. Of interest, a blood isolate during the same relapse was still sensitive to doxycycline. RAPD analysis shows an additional band with primer 16 in the doxycycline resistant isolate when compared with the original and sensitive relapse isolate (Fig. 3). Obviously, the genetic changes in this case are different from the first case. However, the emergence of a single extra band again suggests a chromosomal mutation which has been selected for. In both cases the change in RAPD pattern is unlikely to be

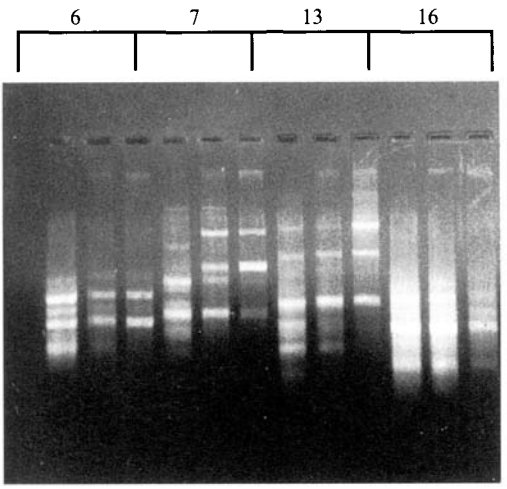

Fig. 2. RAPD analysis of case B with primers 6, 7, 13, 16. Original, 1st and 2nd relapse isolates are in adjacent lanes for each primer. Primer 7 (lanes 3, 4, 5) is showing a slightly different pattern for each isolate.

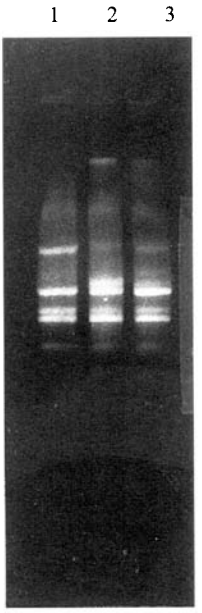

Fig. 3. Change in RAPD pattern in case G with primer 16 between original (lane 1) and a relapse isolate from a joint (lane 2) which had acquired doxycycline resistance. A relapse isolate from blood (lane 3) was still doxycycline sensitive.

directly related to the mechanism of acquisition of drug resistance but nevertheless reflects the sensitivity of RAPD in detecting genetic variation. RAPDs may be useful markers when looking for emergence of antibiotic resistance in longitudinal studies.

The five other patients with isolates identical by ribotyping and another patient with isolates only tested by RAPD (case J, see Table 1) all had initial and relapse isolates which were identical by RAPD for all four primers.

RAPD typing has confirmed that the majority of relapse cases of melioidosis are re-emergence of the initial infecting strain. In addition RAPD patterns generally but not always remain stable during the period of latency. In our experience relapsed melioidosis is almost always associated with failure to complete maintenance therapy. As with tuberculosis [21], emphasis on supervision of and compliance with a standard therapeutic regimen is the primary objective in curing melioidosis. At the Royal Darwin Hospital, after 7–21 days initial intensive management with regimens including ceftazidime, we aim for a minimum of 3 months maintenance monotherapy usually with either doxycycline (200 mg daily, adult dose) or trimethoprim/sulfamethoxazole (320 mg/1600 mg twice daily, adult dose).

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