GENOTYPIC AND PHENOTYPIC RELATIONSHIP IN *BURKHOLDERIA PSEUDOMALLEI* INDICATES COLONIZATION WITH CLOSELY RELATED ISOLATES

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Abstract. Seven isolates of *Burkholderia pseudomallei* from cases of melioidosis in human (2 isolates) and animal (2 isolates), cat (one isolate) and from soil samples (2 isolates) were examined for *in vitro* sensitivity to 14 antimicrobial agents and for presence of plasmid DNA. Randomly amplified polymorphic DNA (RAPD) analysis was used to type the isolates, using two arbitrary primers. All isolates were sensitive to chloramphenicol, kanamycin, carbenicillin, rifampicin, enrofloxacin, tetracycline and sulfamethoxazole-trimethoprim. No plasmid was detected in all the isolates tested. RADP fingerprinting demonstrated genomic relationship between isolates, which provides an effective method to study the epidemiology of the isolates examined.

INTRODUCTION

Burkholderia pseudomallei (previously known as *Pseudomonas pseudomallei*), is the causative agent of melioidosis in humans and a large variety of animals (Supputtamongkol *et al*, 1994). The major areas where melioidosis is endemic are Southeast Asia and Northern Australia (Dance, 1991). In Malaysia the disease has been reported in humans and animals (Embi *et al*, 1992; Idris *et al*, 1998; Heng *et al*, 1998). The spectrum of melioidosis ranges from subclinical disease to chronic pulmonary infection to fulminant septicemia with metastatic abscesses. Improve therapy has decrease the mortality in severe meliodosis from 80% to 40-50% (Supputtamongkol *et al*, 1994). The epidemiology and pathogenesis of melioidosis remain unclear. Because of the lack of a sensitive enough method that allows two isolates to be defined as identical strains, no correlation between isolates of soil, human and animal sources has been proven (Lew and Desmarchelier, 1993). Randomly amplified polymorphic DNA (RAPD) analysis is a PCR-based method using a single short random primer. This method is now widely used for the study of population genetics in a large variety of species (Kersulyte *et al*, 1992; Haase *et al*, 1995). In the work reported here, *B. pseudomallei* isolated from human, animals and soil were examined for their antibiotic resistance, plasmid profiles and random amplification of polymorphic DNA analysis.

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MATERIALS AND METHODS

Bacterial strains

The seven strains of *B. pseudomallei* had been identified by the forwarding laboratory (Regional Veterinary Laboratory, Kuantan, Pahang) by standard tests. In this case study, two isolates each were isolated from a child admitted to the hospital with melioidosis, a dead goat reared by the child's family and soil samples from the surrounding compound of the family's house in a rural village. A single unrelated isolate from a cat was also included.

Sensitivity to antimicrobial agents

Antimicrobial sensitivity tests were performed by the disc diffusion method according to National Committee for Clinical Laboratory Standard (1997). The *B. pseudomallei* strains were tested against the following antibiotics discs (BBL, Becton Dickinson Microbiology Systems, Cockeysville, Maryland, USA) on Mueller Hinton agar; ampicillin (10 μg), carbenicillin (100 μg), cephalexin (30 μg), chloramphenicol (30 μg), clindamycin (2 μg), enrofloxacin (5 μg), kanamycin (30 μg), latamoxef (30 μg), furazolidone (100 μg), penicillin (10 μg), rifampicin (30 μg), streptomycin (15 μg), sulfamethoxazole-trimethoprim

(25 µg) and tetracycline (30 µg). The control strain was *E. coli* ATCC 25922.

Extraction of genomic and plasmid DNA

Genomic DNA isolation was conducted by using a commercial genomic DNA purification kit (Wizard®, Promega, USA). The isolation method mentioned by the manufacturer was followed. Plasmid profile analysis were performed on all *B. pseudomallei* isolates according to the method of Birnboim and Doly (1979). The plasmid was separated on a 0.7% agarose gel and visualized by ethidium bromide staining. The approximate molecular mass of plasmid was determined by comparison with plasmids of known molecular mass of *E. coli* V517 (Macrina *et al*, 1978).

RAPD-PCR

Ten 10-mer primers (Genosys Biotechnologies Inc, USA) were tested for their ability to amplify DNA fragments in the genome of *B. pseudomallei*. The two primers (GEN16006: 5′-CGTCGTTACC-3′ and GEN16010: 5′-GCAGACTGAG-3′) that gave positive reactions were used to characterized the isolates. Amplification reactions were performed in a final volume of 25 µl containing 3 mM of $MgCl_2$, 1 µM (final concentration) each of dNTP, 1U of Taq DNA polymerase, 2 µM primer, 2.5 µl 10x reaction buffer (Promega, USA), and 20-30 ng of genomic DNA. Amplifications were carried out in a thermal cycler (Perkin Elmer Cetus 2400) for 1 cycle at 96°C for 2 minutes, followed by 40 cycles consisting of 1 minute at 96°C, 1 minute at 36°C and 1 minute at 72°C. This was followed by a final extension of 10 minutes at 72°C. A negative control without DNA was included in each RAPD run. Amplification products were analyzed by electrophoresis in 1.2% agarose gels and detected by staining with ethidium bromide. DNA ladder (Promega, USA) was used as DNA size marker.

RESULTS

In susceptibility testing, all the isolates showed identical antibiotic resistance pattern. All the isolates were sensitive to ampicillin, carbenicillin, chloramphenicol, enrofloxacin, kanamycin, rifampicin, sulfamethoxazole-trimethoprim and tetracycline. However, all were resistant to ampicillin, cephalexin, clindamycin, latamoxef, furazolidone, penicillin and streptomycin. Repeated plasmid analysis showed that none of the *B. pseudomallei* isolates were found to harbor plasmid DNA. The RAPD results showed that the two primers used successfully amplified polymorphic DNA fragments from all the isolates tested. The fingerprints obtained with each primer were distinctive and reproducible (Fig 1). In RAPD analysis with primer GEN16006 (Fig 1, lanes 9-15), isolates BP1 and BP5 showed identical pattern. However each of the other isolates tested (BP2, BP3, BP4, BP6 and BP7) showed unique individual RAPD fingerprinting profiles. With primer GEN16010 (Fig 1, lanes 1-7); isolates BP1 and BP5, isolates BP2 and BP4, and isolates BP3 and BP7 showed identical RAPD patterns, respectively. Isolate BP7 was different. When the results of the RAPD patterns from the two primers were combined in a dendrogram, the *B. pseudomallei* isolates were differentiated into three clusters (Fig 2). Cluster 1 consist of isolates BP1, BP5 and BP4, cluster 2 consist of isolates BP3, BP6 and BP7, and cluster 3 consist of only isolate BP2.

DISCUSSION

The *in vitro* susceptibilities of the seven *B. pseudomallei* isolates to 14 antimicrobial agents revealed that all were resistant to 50% of the antimicrobial agents tested. In addition all the isolates were found to be plasmidless. Thus the antibiotic resistance observed was not associated with plasmids. The percentage of resistance to the conventionally used antimicrobial was lower in a previous study compared to the results of this study. Furthermore, in the previous study, it has been reported that fully virulent *B. pseudomallei* strains were resistant to chloramphenicol, and frequently showed cross-resistance to tetracycline and sulphamethoxazole -trimethoprim (Dance *et al*, 1989). In contrast, our study showed that the isolates tested were resistant to chloramphenicol, tetracycline and sulphamethoxazole. Elsewhere the *in vitro* and *in vivo* resistance of *B. pseudomallei* isolates to antimicrobial agents has been reported (Dance *et al*, 1989; Yamamoto *et al*, 1990). Such differences in the frequency of resistance may well be related in particular to the frequency and type of antimicrobial agents prescribed for treating diseases caused by *B. pseudomallei* infection in different geographical zone. Silbermann *et al* (1997) have reported that despite the long-term antimicrobial treatment of patient infected with melioidosis, the patients had a relapse after cessation of therapy. Thus treatment of infection caused by this microorganism should be carefully monitored to detect susceptibility alterations to the chosen therapy.

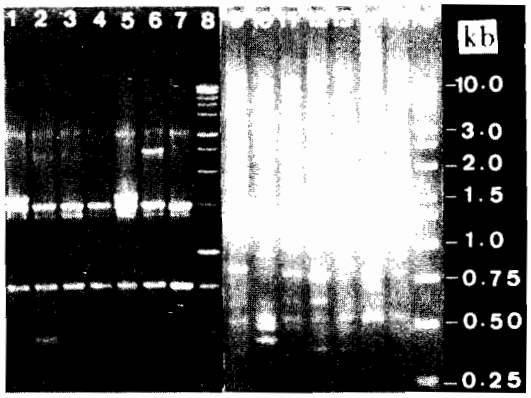

Fig 1–Genomic fingerprinting of *B. pseudomallei* isolates obtained by RAPD-PCR with primers GEN16010 (lanes: 1, BP1; 2, BP2; 3, BP3; 4, BP4; 5, BP5; 6, BP6; 7, BP7) and GEN16006 (lanes: 9, BP1; 10, BP2; 11, BP3; 12, BP4; 13, BP5; 14, BP6; 15, BP7). Lanes 8 and 16 contain DNA molecular mass marker in kilobase pairs (kb).

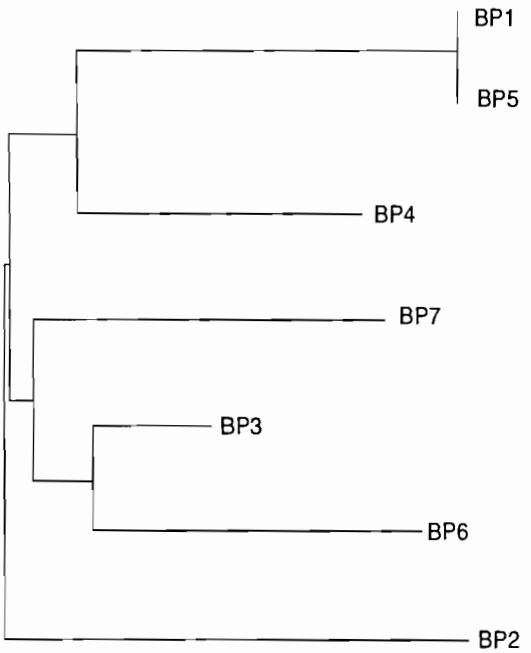

Fig 2–The dendrogram for RAPD profile of *Burkholderia pseudomallei* isolates using the RAPDistance package. The branch length represents the distance between isolates in each cluster.

In order to determine the relatedness of the *B. pseudomallei* isolated in this case study, we examined the utility of phenotypic (antibiotypes) and genotypic (plasmid and RAPD analysis) methods. Both antibiotype and plasmid profiling revealed a single pattern for all seven isolates as all were resistant to the same antibiotics and as well as being plasmidless. Thus, in this study these two methods could not provide type differentiation among the *B. pseudomallei* isolates, and are of limited interest. RAPD patterns analysis obtained by two arbitrary primers (GEN16006 and GEN16010) revealed six and four different genotypes among the *B. pseudomallei* isolates. The identical patterns of the strains isolated from human, animals and soil indicated that these sources were colonized by the same genotype with the same phenotypes. However, the interpretation of RAPD results suffers from some limitations, as strains with identical patterns generated by a primer might be different when investigated with additional primers or primer combination (Rath *et al*, 1995), as evidenced in this study. Fortunately, the combination of the two primers were successful in generating reproducible patterns in the PCR.

When dendrographic analysis was attempted using the combined results of the RAPD patterns obtained from the two primers, the resulting dendrogram appeared as a tree with three main clusters, indicative of the sufficient information in this method with which to analyze stable genetic relationships. In this case study, cluster 1 in Fig 1 showed that the child, goat and soil were colonized with closely related *B. pseudomallei* strains (BP1, BP5 and BP4). The colonization with identical or closely related strains is most probably explained by cross-contamination. The environment has been suggested as an important source for acquisition of *B. pseudomallei* (Van Phung *et al*, 1993; Wuthiekanun *et al*, 1995), and this is supported by the finding of similar strain genotypes in isolates from human, animal and soil sources in this study. In conclusion, the RAPD typing confirmed that the human and animal melioidosis were closely related and were acquired from the same environment (soil) sources. Antibiotyping and plasmid analysis used in this study were insufficient for fingerprinting the *B. pseudomallei* isolates. Hence, highly discriminatory methods of strain characterization are therefore necessary for epidemiological investigations, and global studies require standardized reproducible methods. RAPD analysis, because of its characteristics, will be especially useful in the investigation of pathogenesis, carrier state, and transmission.

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