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Subdivision of *Burkholderia pseudomallei* Ribotypes into Multiple Types by Random Amplified Polymorphic DNA Analysis Provides New Insights into Epidemiology

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Ribotyping has previously been used for epidemiological studies of *Burkholderia pseudomallei* (previously *Pseudomonas pseudomallei*). We show here that random amplified polymorphic DNA (RAPD) analysis allows subdivision of strains of the same ribotype. With five different primers, no two epidemiologically unrelated isolates of any single ribotype in this study of 102 isolates from humans, goats, cats, and soil had identical RAPD patterns. Conversely, RAPD analysis showed clonality for isolates from each of two animal outbreaks of melioidosis and from a nontropical focus of animal and human melioidosis spanning 25 years. Some soil isolates were identical to epidemiologically related animal and human isolates as determined by RAPD typing. There was no evidence that the clinical outcome of melioidosis was related to RAPD patterns.

Burkholderia pseudomallei (previously *Pseudomonas pseudomallei*), the causative agent of melioidosis, is a natural saprophytic organism of soil and water in tropical areas. The major areas where melioidosis is endemic are in southeast Asia and northern Australia (6). Melioidosis is an infection of humans and a large variety of animals. The organism enters the body mainly through wounds but also by inhalation or ingestion. The spectrum of melioidosis ranges from subclinical disease to chronic pulmonary infection to fulminant septicemia with metastatic abscesses. Improved therapy has decreased the mortality rate in severe melioidosis from around 80% to 40 to 50% (20).

B. pseudomallei is the most commonly recognized cause of fatal community-acquired pneumonia at Royal Darwin Hospital, Casuarina, in the Northern Territory of Australia (2). In the 5 years since October 1989, there have been 106 cases of melioidosis in the tropical region of the Northern Territory, with 27 deaths (25%) (2a).

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 soil isolates and human or animal isolates has been proven (13). The conventional typing method was unable to distinguish strains in a sensible manner (7). Ribotyping allowed grouping of strains into 22 different ribotypes (13, 17). However, more than 60% of all isolates from the region of high endemicity in the Northern Territory of Australia fell into only three ribotypes (4), so this method was still unable to clarify the means of transmission, as it does not allow strain identity between isolates to be proven. Greater discrimination is required to investigate possible epidemics of melioidosis, such as the 1990 to 1991 Darwin melioidosis outbreak (14) and cases in regions where melioidosis is not endemic (4).

Random amplified polymorphic DNA (RAPD) analysis is a PCR-based method using a single short random primer which, under low-stringency conditions, gives rise to amplification products wherever the primer binds on opposite strands within an easily amplifiable distance. This method is now widely used for the study of population genetics in a large variety of species (10, 22, 23, 25).

We have employed RAPD analysis as a new, faster and more sensitive typing method for studying epidemiological problems, such as clonality of outbreaks of melioidosis, mechanisms of disease transmission, and possible predictability of clinical outcome of the infection with a given strain.

MATERIALS AND METHODS

Bacterial isolates. Human *B. pseudomallei* isolates were obtained from blood, sputum, urine, or wound specimens. Animal isolates were collected by the Department of Primary Industry and Fisheries from goats involved in an outbreak of melioidosis at a single goat farm and from cats involved in a veterinary nosocomial outbreak. Multiple soil samples from the goat farm paddock were collected on four occasions spanning the wet and dry seasons. Soil isolates were cultured by a modified Ashdown's procedure. A 30-ml volume of Ashdown's broth was inoculated with 7 g of soil and incubated outside at approximately 30°C ambient temperature. The broth was cultured on Ashdown's plates after 2 and 7 days of incubation, and the plates were kept at 37°C for 2 days. Suspected *B. pseudomallei* colonies were replated onto horse blood agar plates for purity. All isolates were confirmed by API 20E (Biomerieux, Marcy l'Etoile, France) and Microbact 24E (Oxoid, Heidelberg West, Australia) systems.

DNA preparation. A loopful of colonies was resuspended in 50 μl of 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 of 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 of lysozyme per ml, 2 μl of a 10-mg/ml RNase stock solution) was added and the mixture was incubated at 37°C overnight. The buffer was then replaced with 150 μl of proteinase K buffer (0.5 M EDTA, 1% sarcosyl, 1 mg of proteinase K per ml) at 55°C overnight. After this, the blocks were soaked in Tris-EDTA buffer three times, 500 μl of water was added, and the plugs were melted at 65°C.

Ribotyping. DNA was isolated by the guanidinium-thiocyanate method (15). It was digested with *Bam*HI, fractionated by electrophoresis on a 0.8% agarose gel in 1× TAE (Tris-acetate-EDTA) buffer for 16 h at 1.1 V/cm, and blotted onto Hybond N Plus (Amersham) in 0.4 N NaOH. The prehybridization mixture was 0.3 M NaCl–20 mM NaH_2PO_4–2 mM EDTA–1% sodium dodecyl sulfate–0.5% nonfat skim milk powder–0.5 mg of herring sperm DNA per ml. The probe was a 7-kb insert from plasmid pKK3535 bearing an *Escherichia coli* rRNA operon. Hybridization was done in the solution described above at 65°C. For ribotype identification, we used the *Bam*HI ribotype numbering scheme of Lew and Desmarchelier (13).

RAPD PCR. The following primers were eventually chosen out of 20 primers

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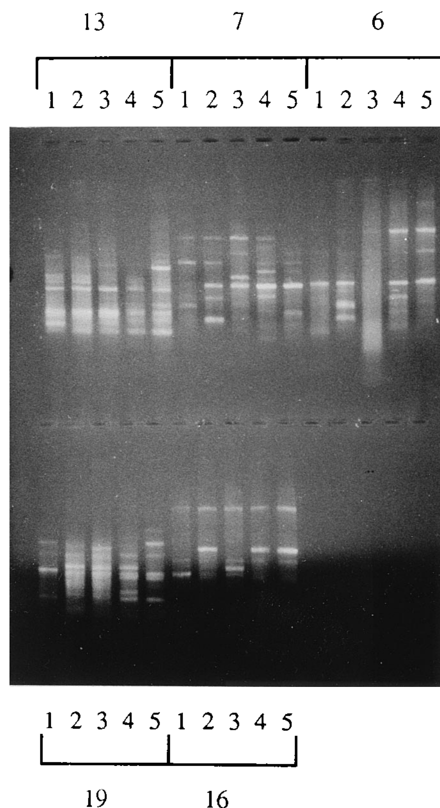

FIG. 1. RAPD patterns with primers 6, 7, 13, 16, and 19 of five unrelated human ribotype 11 isolates. Lanes: 1 and 2, Darwin isolates from 1991; 3, Darwin isolate from 1992; 4 and 5, isolates from Papua New Guinea.

from 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; and primer 19, GTC CGT ACT G. Each 25-μl reaction mixture contained 50 pmol of primer, 1 μl of the DNA preparation described above, 2 mM $MgCl_2$, and 2 U of *Taq* polymerase (Bresatec). Reactions were performed in a Corbett machine using 1 min at 95°C, 2 min at 35°C, and 2 min at 72°C for the first five cycles and then 1 min each at 95, 35, and 72°C for another 35 cycles. PCR products were analyzed on a 1% agarose gel in Tris-borate-EDTA buffer.

RESULTS

RAPD analysis as a tool for subdividing ribotypes. Out of 20 random primers, the 5 finally chosen gave the clearest and most distinctive banding patterns for any one ribotype tested. Strains were considered identical if their RAPD patterns were identical with all five primers. In most cases, two or three primers were informative enough to show differences between strains, but all strains were typed with at least four primers. Primer 19 was the least discriminatory.

In order to examine if the method was discriminatory enough to distinguish between strains of the same ribotype, we chose five geographically and temporally unrelated human ribotype 11 isolates from patients with melioidosis. Every isolate showed a different RAPD pattern, in most cases with each of the five primers (Fig. 1). In order to confirm that this was also true for other ribotypes, we tested 26 ribotype 1 and 13 ribotype 2 isolates, which are the most common isolates from humans in the Northern Territory of Australia. Again, unrelated isolates showed different RAPD patterns, usually with more than one primer (examples are shown in Fig. 2). In striking contrast, in some situations in which we examined considerable numbers of independent but epidemiologically related isolates, the patterns were found to be indistinguishable (Fig. 3); this is considered in more detail below, but here it serves to illustrate the reproducibility of the RAPD patterns.

Predictability of clinical outcome. We compared human isolates A to L (randomly designated) with regard to the clinical outcome of infection to ascertain whether some strains are more likely to lead to a fatal outcome than others. Among Northern Territory ribotype 1 human isolates, 10 of 21 were associated with fatal cases (referred to as "lethal" isolates). Lethal isolates G and H were identical in RAPD pattern with isolate F, which was from a nonfatal infection. Lethal isolate I was also very similar to isolates G and H, but so were isolates K and L, both from patients who survived. Lethal isolates C and D were identical with nonlethal isolates B and J, respectively (examples in Fig. 2A).

Four of eight patients with ribotype 2 isolates died. Isolates from two fatal cases were similar, showing only slight differences with primers 6 and 16. However, isolate A, from a patient who survived, was equally similar to lethal isolate E, with differences only with primers 6 and 7. These results do not suggest predictability of clinical outcome based on RAPD analysis.

RAPD analysis in investigations of outbreaks. From 1992 to 1993, an outbreak of melioidosis occurred on a goat farm near Darwin, Australia. Fifteen goats were eventually culture positive for *B. pseudomallei*. A total of 26 isolates from 14 goats were ribotype 11, with the other goat having a ribotype 2 isolate. The RAPD patterns of all the ribotype 11 goat isolates were identical (examples in Fig. 3). None was identical to the human ribotype 11 isolates mentioned previously (Fig. 1).

The isolates from four nosocomially infected cats were all ribotype 1. Their RAPD patterns were also identical with all five primers but different from those of the human ribotype 1 isolates (data not shown).

In the 1990 to 1991 outbreak of human melioidosis in the Northern Territory, there were 33 cases and 12 deaths (3). Epidemiological investigations eventually refuted the initial concern of a point source or clonal outbreak (14). RAPD analysis of 14 isolates from the outbreak showed one group of three identical isolates and another of two, with the remaining nine isolates being different from all others.

Melioidosis has occurred over a period of 25 years in a temperate rural area of southwest Western Australia (8). Ten *B. pseudomallei* isolates from the area, spanning 25 years, were recently found to be ribotypically identical (4). We have now demonstrated that the RAPD patterns of all these isolates (two goat and five sheep isolates and one dog, one human, and one soil isolate) were identical with all five primers tested. These results confirm the clonality of isolates in this nontropical focus of melioidosis and also demonstrate strain stability over time.

Are soil isolates infectious? Isolates of *B. pseudomallei* from serial soil samples from the paddock of the goat farm of the 1992 to 1993 goat melioidosis outbreak were ribotyped and analyzed by use of RAPD. Although 14 of 15 goats were infected with ribotype 11, only 3 of 24 soil isolates were ribotype 11. However, these three soil isolates were identical by RAPD patterns and also identical to the goat ribotype 11 isolates. Nine of 24 soil isolates were ribotype 2, and their RAPD patterns were identical and also identical to that of the one goat isolate of ribotype 2. A total of 12 of 24 soil isolates were ribotype 1, and 11 of these were identical as determined by RAPD analysis.

DISCUSSION

The lack of a discriminatory typing method had always been a problem with *B. pseudomallei* until ribotyping was developed. However, the fact that in an area of endemicity, such as the

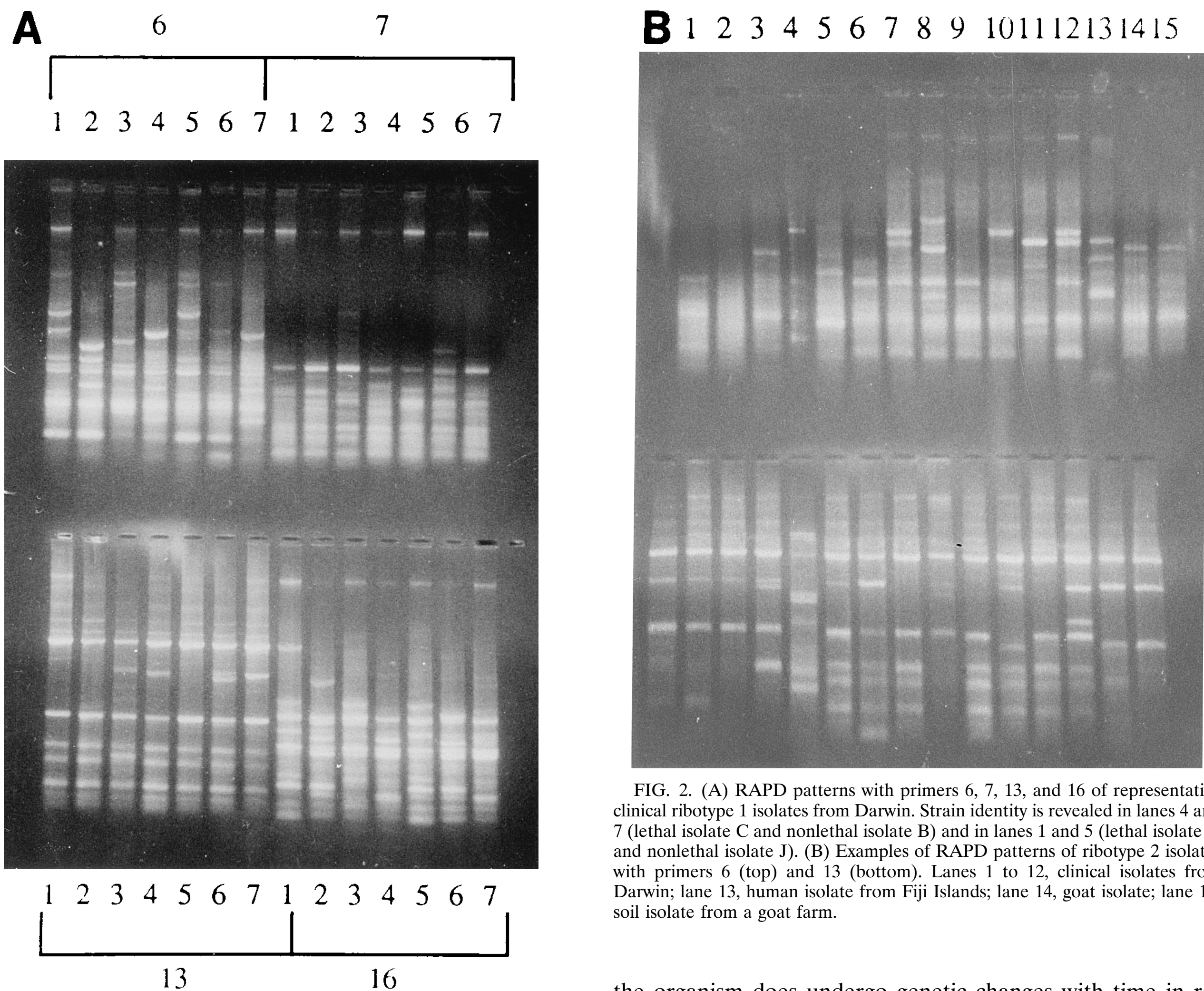

FIG. 2. (A) RAPD patterns with primers 6, 7, 13, and 16 of representative clinical ribotype 1 isolates from Darwin. Strain identity is revealed in lanes 4 and 7 (lethal isolate C and nonlethal isolate B) and in lanes 1 and 5 (lethal isolate D and nonlethal isolate J). (B) Examples of RAPD patterns of ribotype 2 isolates with primers 6 (top) and 13 (bottom). Lanes 1 to 12, clinical isolates from Darwin; lane 13, human isolate from Fiji Islands; lane 14, goat isolate; lane 15, soil isolate from a goat farm.

Northern Territory of Australia, the majority of isolates belong to only three different *Bam*HI ribotypes suggests that this method is not discriminatory enough to confirm that two isolates are the same strain. Ribotyping may therefore not be the ideal tool for detailed epidemiological studies. Our results demonstrate that RAPD analysis is a suitable method for distinguishing between unrelated strains even of the same ribotype when all five primers are used. We were able to distinguish 6 different RAPD patterns for ribotype 11 isolates, 18 for ribotype 1 isolates, and 14 for ribotype 2 isolates. This method is not only much more discriminatory than ribotyping but also much simpler and quicker to carry out. It is therefore a valuable tool for epidemiological studies.

RAPD typing confirms likely clonality of *B. pseudomallei* in the goat farm melioidosis outbreak and the nosocomial cat cases. It also confirms that the 1990 to 1991 Darwin "outbreak" was due to mostly unrelated isolates of *B. pseudomallei* during an exceptionally wet monsoon. Indeed, in 1993 and 1994, a similar situation occurred with another heavy monsoon (unpublished data).

Strain stability could be demonstrated for isolates from southwest Western Australia, where the organism was most likely introduced as a single strain and persisted as such over a 25-year period. Considering the large variety of *B. pseudomallei* strains in the Northern Territory, it might be expected that the organism does undergo genetic changes with time in regions where it is endemic. Genetic exchange within species has been demonstrated for environmental organisms (18, 24, 26) and for *Streptococcus pyogenes* (1, 9), and genetic exchange between species has been suggested for other streptococci (12). The large number of organisms in tropical countries in which melioidosis is endemic with frequent interaction in the environment and within the human host creates opportunities for genetic exchange, leading to an enhanced evolution of strains.

In our study, identical strains have been found to be associated with both fatal and mild infections. This is in agreement with clinical observations that the outcome of melioidosis is determined largely by the prior condition of the patient and that the presence of risk factors, such as alcoholism and diabetes, is of considerable prognostic value (3, 16, 20). Virulence factors in *B. pseudomallei* remain poorly defined, and further work on soil and human isolates may yet identify strain differences of clinical significance. Preliminary data suggest no relationship between strains of *B. pseudomallei* from patients with neurological melioidosis, a syndrome which is possibly exotoxin related (25a). Many animals appear susceptible to melioidosis (6), and we have shown the same strain to be infective for sheep, goats, dogs, and humans.

The fact that newly acquired infections occur almost exclusively during the wet season has led to the hypothesis that the organism comes up with the rising water table during the wet

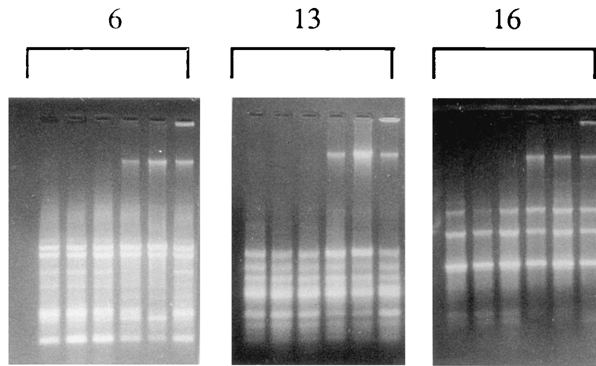

FIG. 3. Examples of RAPD patterns with primers 6, 13, and 16 of three goat (rightmost lanes in each panel) and three soil (leftmost lanes) ribotype 11 isolates from a goat farm near Darwin. The extra high-molecular-weight band in the three goat isolates appeared with every primer and is therefore attributed to a higher DNA concentration than in the soil isolates. Note the difference from Fig. 1, demonstrating the variety of unrelated ribotype 11 isolates.

season, whereas in the dry season it remains in deeper regions of the soil (19, 21). However, analysis of soil isolates from the goat paddock associated with melioidosis shows that the same organisms are present throughout the year, even in superficial layers (data not shown). An alternative suggestion is that the organisms remain in an inactive state during the dry season. However, the goat farm may be unrepresentative of the usual soil situation because of possible heavy contamination with *B. pseudomallei* organisms from infected animals. The fact that among the isolates from various sites at the goat farm we found by RAPD only analysis a single ribotype 11 strain, two ribotype 1 strains, and a single ribotype 2 strain suggests that animals may contribute to the spread of the organism in the soil (5, 13, 21).

There is a paradox in that all goats in the outbreak except one were infected with a ribotype 11 isolate, whereas the most common soil isolate from this paddock was ribotype 2. As ribotyping shows only a single band difference between types 2 and 11, we wished to examine the possibility that the difference has arisen by a very recent mutation. RAPD pattern analysis did not support this, with no suggested relatedness between ribotypes 2 and 11 (Fig. 2) and with identical patterns for all ribotype 2 isolates from soil and goats and for all ribotype 11 isolates. It is possible that ribotype 11 was new to the goat farm, and goat-to-goat spread may have occurred. Alternatively, infection of goats from an external source is possible, as was suggested for an outbreak among pigs in Queensland, Australia (11).

We have confirmed that soil isolates can be identical to epidemiologically related human (southwest Western Australian farms) and animal (goat farm outbreak) isolates. This is also likely to have been the case in the imported melioidosis outbreak in France in the 1970s, which affected zoos, equestrian clubs, and human contacts and resulted in extensive environmental contamination (6).

Further work on melioidosis is needed to unravel the relationships between soil and organism, wet and dry seasons, and animal and human hosts and to elucidate the modes of transmission and virulence.

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