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Relationships among *Pseudomonas pseudomallei* Isolates from Patients with Recurrent Melioidosis

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Patients with melioidosis may present with recurrent infections after clinical resolution of their primary illness. Because there has been no satisfactory typing scheme for *Pseudomonas pseudomallei*, recrudescence could not be distinguished from reinfection. We determined the strain identity of primary and relapse isolates of *P. pseudomallei* from 25 patients with culture-proven melioidosis to answer whether secondary infections were due to the initial infecting strain or to the acquisition of a new strain. Fifty-four isolates were compared by the patterns of *Bam*HI restriction digests produced after hybridization with a cDNA copy of *Escherichia coli* rRNA. Twenty-three patients had primary and relapse isolates with identical or highly similar ribotype patterns. The patterns of isolates from two patients were different; the primary and relapse isolates differed by a single fragment for one, and the other had identical primary and first-relapse isolates while the second-relapse isolate was markedly different. The results indicated that recurrent infection probably resulted from endogenous relapse in most of the melioidosis patients studied, although reinfection from an exogenous source was also possible in two cases.

Pseudomonas pseudomallei is a free-living saprophyte of soil and water which causes the disease melioidosis in animals and humans (2). This disease has a major endemic focus in southeast Asia and northern Australia, although sporadic cases have been reported worldwide, often among patients or animals with a history of prior residence in endemic areas (2, 3). Clinical manifestations of human melioidosis vary greatly from subclinical infections to fulminant septicemias with high mortality rates (2). *P. pseudomallei* may remain latent, causing disease years after the likely exposure of the patient to contaminated environments (8, 14). Melioidosis cases have also been reported to relapse after apparent clinical resolution of the primary infection (9, 10, 15). Such infections have been assumed to be a recrudescence of the primary infection, in the latter case because of a failure to eliminate the initial infecting organism. In the absence of a typing system for *P. pseudomallei* which would allow the differentiation of strains, it has not been possible to determine whether these infections result from reactivation of an endogenous focus of *P. pseudomallei* or reinfection from an exogenous source.

From June 1986 to July 1991, a series of 602 patients with culture-proven melioidosis in Ubon Ratchatani, northeast Thailand, were monitored prospectively from their initial diagnoses. Twenty-seven of these patients were observed to relapse after clinical resolution of their infections. This offered the opportunity to study the identity of primary and relapse isolates of *P. pseudomallei* from 25 of these patients. The clinical features of these patients and an analysis of risk factors for relapse will be reported elsewhere. We report here an analysis of these strains, comparing the patterns of restriction fragment length polymorphisms in their chromosomal genes encoding rRNA.

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MATERIALS AND METHODS

Bacterial strains. The details of the clinical isolates are presented in Table 1. Stock cultures were stored at −70°C and subcultured onto nutrient agar at 37°C when required. Isolates had been subcultured a maximum of three times before typing. All procedures involving viable cells were performed in a category 3 containment laboratory (1). The identities of stock cultures were confirmed by their oxidative metabolism of glucose, nitrate reduction, production of arginine dihydrolase, and pattern of utilization of sugars in ammonium-salt-based media (7).

Isolation of genomic DNA and analysis of rRNA gene restriction patterns. Genomic DNA was prepared, by using a rapid extraction procedure with guanidium thiocyanate (16), from a "rice grain" of growth harvested from nutrient agar cultures incubated at 37°C overnight. After ethanol precipitation, the DNA was dissolved in 100 μl of Tris-EDTA buffer and stored at 4°C (5). Genomic DNA was digested with the restriction enzyme *Bam*HI (Bethesda Research Laboratories) according to the manufacturer's instructions, and restriction fragments were separated by agarose gel electrophoresis. To allow comparison of isolates from an individual patient, all isolates from a patient were run in adjacent lanes on a gel. The restriction fragments were Southern blotted onto a nylon membrane and hybridized with a biotin-labelled cDNA probe as previously described (5). A biotinylated *Hin*dIII digest of bacteriophage lambda (Bethesda Research Laboratories) was included in each gel. The molecular weights of the restriction fragment bands were calculated with a computer program donated by J. Hernandez, Valencia, Spain.

TABLE 1. Characteristics and ribotype patterns of *P. pseudomallei* strains isolated from primary and relapse infections of melioidosis

Patient	Strain no.	Site of isolation	Date of isolation (mo/day/yr)	Relapse period (mo)[a]	Ribotype pattern
A	1	Blood	10/22/86	—[b]	1
	2	Blood	5/15/87	7	1
	3	Blood	10/8/89	29	2
B	4	Pus	12/19/86	—	3
	5	Blood	4/28/87	4	3
C	6	Blood	10/21/87	—	4
	7	Splenic pus	8/24/88	10	4
D	8	Sputum	1/21/88	—	5
	9	Sputum	8/14/90	31	6
E	10	Sputum	7/5/88	2	3
	11	Blood	5/8/88	—	3
	12	Sputum	1/2/90	18	3
F	13	Blood	2/6/90	7	7
	14	Blood	7/17/88	—	7
G	15	Blood	7/21/88	—	3
	16	Blood	11/16/88	4	3
H	17	Blood	3/15/89	5	8
	18	Blood	10/4/88	—	8
I	19	Pus	2/7/89	4	9
	20	Sputum	10/18/88	—	9
J	21	Blood	10/22/88	—	10
	22	Blood	1/24/89	3	10
K	23	Blood	11/1/88	—	11
	24	Splenic pus	5/31/89	6	11
L	25	Sputum	6/23/89	6	12
	26	Pleural fluid	12/10/88	—	12
M	27	Sputum	6/8/89	—	13
	28	Sputum	10/6/89	4	13
N	29	Blood	7/16/89	—	14
	30	Blood	9/24/89	2	14
O	31	Blood	2/28/90	5	15
	32	Blood	9/14/89	—	15
P	33	Blood	10/27/89	—	16
	34	Pus	3/7/90	5	16
Q	35	Pus	7/5/89	—	17
	36	Pus	7/12/90	12	17
R	37	Pus	6/7/89	—	18
	38	Pleural fluid	9/2/91	27	18
S	39	Blood	9/19/89	—	19
	40	Blood	12/30/90	15	19
	41	Pus	5/8/91	5	19
T	42	Splenic pus	6/14/90	—	20
	43	Liver pus	6/16/91	12	20
U	44	Blood	6/10/90	—	21
	45	Blood	3/23/91	9	21
V	46	Rectum	9/15/90	—	22
	47	Pus	3/22/91	6	22
W	48	Pus	1/5/91	—	23
	49	Sputum	5/1/91	4	23
	50	Pleural fluid	6/6/91	1	23
X	51	Throat swab	6/18/88	—	24
	52	Blood	9/4/91	33	24
Y	53	Pus	3/23/91	—	17
	54	Pleural fluid	8/20/91	5	17

[a] Period since the last culture-proven clinical diagnosis of melioidosis.
[b] —, primary diagnosis of melioidosis.

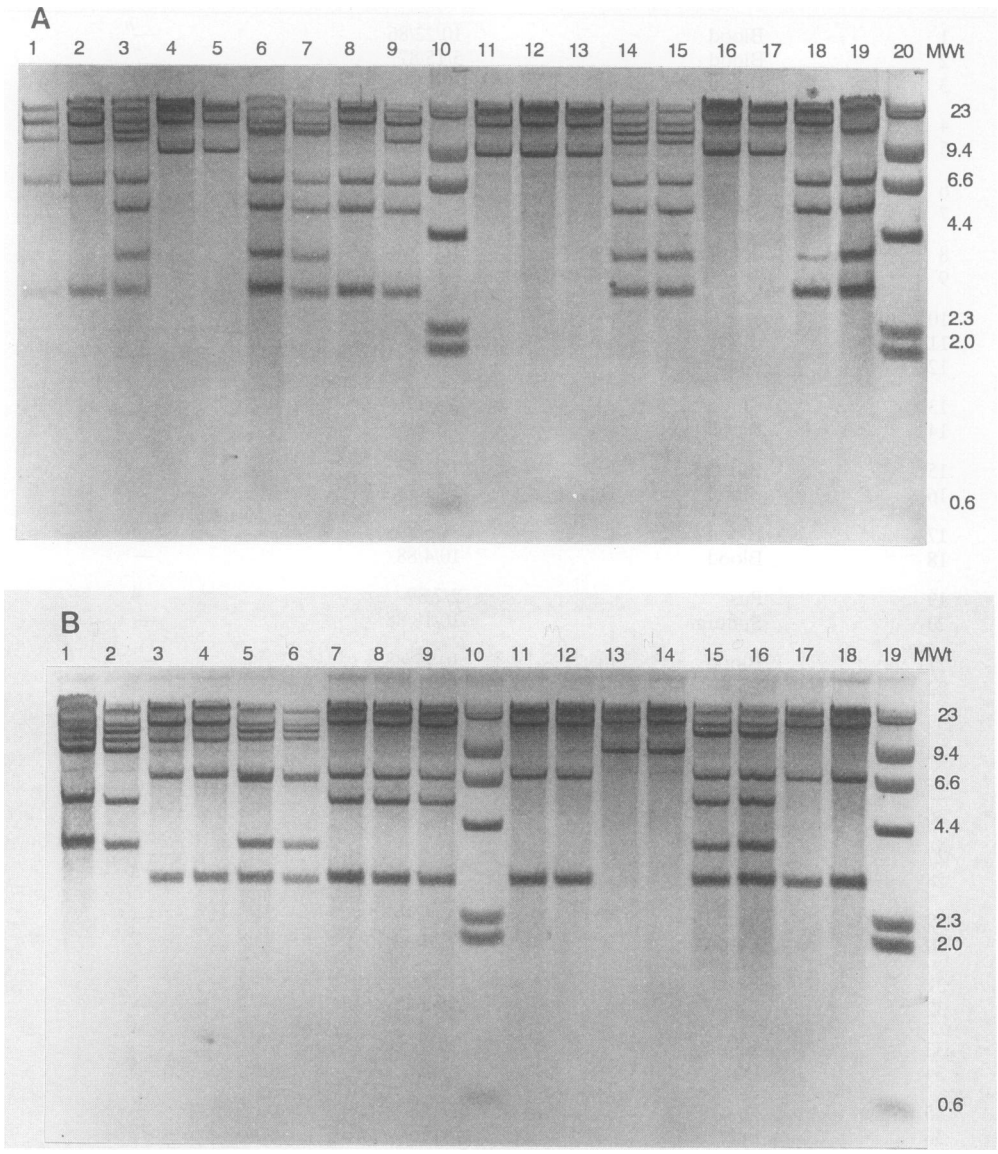

FIG. 1. rRNA gene restriction patterns for *Bam*HI digests of total chromosomal DNA from *P. pseudomallei* isolates from recurrent cases of melioidosis. (A) Lanes: 1 through 3, patient A strains 1 through 3; 4 and 5, patient B strains 4 and 5; 6 and 7, patient C strains 6 and 7; 8 and 9, patient D strains 8 and 9; 10, molecular mass (MWt) markers; 11 through 13, patient E strains 10 through 12; 14 and 15, patient F strains 13 and 14; 16 and 17, patient G strains 15 and 16; 18 and 19, patient H strains 17 and 18; 20, molecular mass markers. (B) Lanes: 1 and 2, patient I strains 19 and 20; 3 and 4, patient J strains 21 and 22; 5 and 6, patient K strains 23 and 24; 7 through 9, patient L strains 25 (mucoid), 25 (rough), and 26; 10, molecular mass markers; 11 and 12, patient M strains 27 and 28; 13 and 14, patient N strains 29 and 30; 15 and 16, patient O strains 31 and 32; 17 and 18, patient P strains 33 and 34; 19, molecular mass markers. Molecular masses are given in kilobase pairs.

RESULTS

Preliminary experiments with *Eco*RI, *Hin*dIII, and *Bam*HI (11), as well as with *Dra*I, *Xba*I, and *Bgl*I in this study, showed that *Bam*HI provided the largest number of bands and optimal discrimination between strains after hybridization with the rRNA probe. Three to nine bands were detected per strain. The patterns of restriction fragment molecular weights observed among the 54 isolates when digested with *Bam*HI have been arbitrarily numbered 1 through 24 for the purposes of this study (Table 1). Examples of some restriction fragment patterns are presented in Fig. 1. Patterns of isolates from individual patients were compared and considered to be distinct if they differed in the numbers and/or the molecular weights of the bands.

Each of the isolates from 23 of the 25 patients had identical or highly similar ribotype patterns (Table 1). Isolates from patients A and D differed in the numbers and molecular weights of some of the restriction fragments detected. Patient A presented with culture-proven melioidosis on three occasions. The primary isolate and that isolated during the relapse 9 months later were identical, while the isolate from the relapse 29 months after the second relapse had a distinct ribotype pattern (Fig. 1). Patient D presented with culture-proven melioidosis on two occasions. The two isolates from

this patient were different. The second strain was isolated 31 months after clinical resolution of the primary infection and had an additional 13.4-kb restriction fragment (Fig. 1). Isolates from patient I had the same number of bands, although the molecular masses of three bands were different. In a separate *P. pseudomallei* ribotyping study (11), the standard deviation of fragment sizes 2,000 to 9,000 bp was approximately 0.1 to 1.0%, and for those larger than 9,000 bp the standard deviation was approximately 5%. The differences observed in the patterns of isolates from patient I were at the extremes of these tolerances, and the isolates have been given the same pattern designation. The culture from the primary infection of patient L produced both rough and mucoid colonies on nutrient agar. Both primary colony types and the isolate from the subsequent infection had the same ribotype pattern.

DISCUSSION

A prerequisite to understanding the problem of recurrent infections with *P. pseudomallei* is to know whether isolates from primary and subsequent infections are identical. In the past, it has not been possible to study the relationships among isolates because of the lack of a discriminatory typing method. Serological typing of somatic and capsular antigens (4) and analysis of lipopolysaccharide antigens (17) of *P. pseudomallei* have shown little diversity among strains and do not provide useful epidemiological information. Recently, rRNA typing or ribotyping of a collection of *P. pseudomallei* isolates in Australia was shown to be a useful technique for discrimination among strains (11). In this study, we observed sufficient differences between ribotype patterns among individual patients to allow us to conclude that isolates of similar patterns from a single patient represented a single strain.

The isolates from primary melioidosis and from subsequent *P. pseudomallei* infections were clonally related for 23 of the 25 patients studied. The results show that in each of these patients, a primary infecting strain had persisted after the resolution of clinical symptoms and the development of symptoms subsequently was a relapse of infection from the same endogenous source. The ribotype patterns of primary and relapse isolates from patients A and D, respectively, differed in the numbers of bands present. The primary isolate from patient D differed from the relapse isolate only by the absence of a 13.4-kb fragment. While this may indicate the presence of a different strain, it may equally be the result of rearrangements in rRNA genes of the persisting strain. Such rearrangements resulting in deletions, transpositions, and inversions in the chromosome have been demonstrated in *Escherichia coli* (6). Similarly, Woods et al. (18) found the ribotype patterns of isolates of *Neisseria meningitidis* from the blood and cerebrospinal fluid of a single patient to differ in the positions of two of their respective four bands. In contrast, the differences observed in the second-relapse isolate from patient A, compared with the primary and first-relapse isolates, are too extensive for such an explanation.

Three of the patients with identical isolates suffered two relapses, and the remainder relapsed once during the study period. Relapse infections have been reported in the literature after periods of 9 (13) to 26 (12) years. During this 5-year study, relapses occurred 2 to 33 months apart. The relapse periods for patients A and D, the two patients with nonidentical isolates, were two of the three longest relapse periods recorded in this study, 29 and 31 months, respectively. Patient A was shown to have a persistent infection resulting in the first relapse at 7 months; however, the second relapse after 29 months was due to a different strain. It is possible that the patient was simultaneously infected with two strains and that only one was detected at each clinical presentation. A sweep of colonies was taken from the isolation plates in an attempt to avoid this, but single colonies were subsequently used for typing, so that selection of a single strain may have occurred. Alternatively, as the period after the primary infection increases, the opportunity for reinfection from an exogenous source and the possibility of a genetic event resulting in polymorphisms would be expected to increase simultaneously. Consequently, it is not possible to say which of these explanations accounts for the differences observed between initial and relapse strains.

In conclusion, the strain identities of *P. pseudomallei* isolates from primary and relapse infections of melioidosis in this study provide evidence that, in the majority of cases, relapse resulted from reactivation of a persistent endogenous source of infection, although in two cases reinfection from an exogenous source could not be excluded.

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