Isolates of *Burkholderia pseudomallei* from Northern Australia Are Distinct by Multilocus Sequence Typing, but Strain Types Do Not Correlate with Clinical Presentation

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Melioidosis is the disease caused by the saprophytic organism *Burkholderia pseudomallei*. Previous studies have suggested some strain tropism and differential virulence. In this study, we defined strains by multilocus sequence typing (MLST) of isolates taken from the Top End of Australia's Northern Territory and compared the results with those of other strains typed worldwide. We specifically sought clinical and geographical correlates of strain types. Among 87 Australian isolates, 48 sequence types were defined. None of the sequence types in this study has been found elsewhere in the world. Strains were distributed widely throughout the region, and the different presentations of disease, including neurological and prostatic infection, were associated with many different strains. There was excellent congruence between pulsed-field gel electrophoresis and MLST, and the two typing methods had a similar level of strain discrimination. The work suggests that host and environmental factors may be more important in determining disease presentation than infecting strain type. It is possible that the distinct but diverse strain types found in this study reflect Australia's geographical isolation over many millions of years.

Melioidosis, the disease due to the environmental gram-negative bacillus *Burkholderia pseudomallei*, is an important disease in southeast Asia and northern Australia. It may affect humans and many animal species with a spectrum of severity ranging from acute fulminant sepsis to more indolent presentations (26).

The most severe manifestations of melioidosis are pneumonia and severe sepsis. There are regional variations in disease presentation; for example, northern Australia has a high rate of genitourinary disease, particularly prostate disease, and a distinct encephalomyelitis syndrome (3, 26). Parotid abscesses are a common presentation of melioidosis in Thailand (4), but only one case has been documented in Australia (8). The reasons for these differences, whether host, bacterial, or environmental factors, have not been well characterized. Previous work has hinted at differential virulence and tropism in *B. pseudomallei*, but conclusions have been difficult to draw from relatively small sample sizes (16, 25).

Multilocus sequence typing (MLST) is based on sequence variation in seven housekeeping genes; the slow rate of change in these alleles and the lack of ambiguity in assigning alleles make this an ideal method to compare strains within and between laboratories. MLST has been used to define relationships between bacterial clones and track the global spread and antibiotic resistance in *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Staphylococcus aureus*, and other organisms (6, 7, 19). For *B. pseudomallei*, an MLST method has recently been described and has noted the overlap

between *B. mallei* and *B. pseudomallei* and the clear resolution of these isolates from those of the less virulent *B. thailandensis* (10).

In this study, we explored the possibility of geographic localization and variation in strain tropism and virulence in *B. pseudomallei* with MLST and compared the results of MLST with those of pulsed-field gel electrophoresis (PFGE) typing.

MATERIALS AND METHODS

Since 1989, a collection of *B. pseudomallei* isolates has been maintained, encompassing isolates from all human cases seen in the Northern Territory of Australia. Isolates had been stored at -70°C in Todd Hewitt broth (Oxoid Australia, Melbourne, Australia) with 20% glycerol. A sample of 87 isolates, representing the full range of clinical and geographical diversity, was typed by multilocus sequence typing (MLST). We selected only a single isolate from each patient to preserve the assumption of independence of observations. Clinical classification of human cases was done as previously described (3); severe sepsis was defined according to standard criteria (1). The geographical location was taken from the address at which the patient resided at the estimated time of infection.

To examine the resolving power of MLST, the results of MLST were compared with those of PFGE on a subset of 17 isolates. This subset included two groups, with five and eight isolates, respectively, where isolates were not linked epidemiologically but had identical PFGE banding patterns. A third cluster was derived from a small remote community with four cases over an 11-month period but where isolates were not all related on PFGE typing.

Bacteria were cultured on chocolate agar (Oxoid Australia) and subcultured in Todd Hewitt broth, and DNA was extracted with a DNeasy tissue kit (Qiagen, Hilden, Germany). MLST was performed at the Imperial College London as recently reported (10). The alleles at each of the seven loci were assigned by comparing the sequences to those at the *B. pseudomallei* MLST website (<http://bpseudomallei.mlst.net/>). Novel sequences were assigned new allele numbers and deposited in the MLST allele database. The allele numbers at each locus provide the allelic profile of each strain, and each distinct allelic profile is assigned as a sequence type (designated by the prefix ST). A list of currently described sequence types is detailed at the MLST website; the 47 novel sequence types from this study have been submitted to this database.

Isolates from northern Australia were compared to other isolates in the MLST

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database. Many of the isolates in the MLST database were from imported infections in countries where the disease is not endemic, and these were removed from the comparator group. Other isolates from Australia were also removed because it was unknown whether these were from imported infections or those acquired in areas of Australia where the disease is endemic. The comparator groups included 62 sequence types where the country of origin was known; a subset of this group included the 25 sequence types recovered from countries of southeast Asia.

The relatedness among isolates was displayed as a dendrogram by the unweighted pair group method with arithmetic averages (UPGMA) method with the matrix of pairwise differences between the allelic profiles of the isolates (18). The sequences of the seven loci from the isolates characterized here and from the comparator group from the *B. pseudomallei* MLST database were joined in-frame to produce a concatenated sequence of 3,399 bp, and a minimum evolution tree was constructed by means of the Kimura two-parameter method for estimated pairwise genetic distances with the MEGA 2.1 program (Molecular Evolutionary Genetics Analysis software, Arizona State University, Tempe, Ariz.). The significance of the nodes on the tree was evaluated with the bootstrap technique with 1,000 resamplings from the data set.

PFGE was performed at the Menzies School of Health Research as previously described with SpeI (9a); DNA macrorestriction patterns were analyzed for similarity with the Dice coefficient. A dendrogram was formed with the UPGMA method. Band similarities were assessed with the Tenover criteria closely related (one to three band difference) or identical (no band differences) (22).

Ethical approval for this study was obtained from the Human Research Ethics Committee of the Department of Health and Community Services and the Menzies School of Health Research.

RESULTS

Eighty-seven isolates were typed by MLST. There were 48 sequence types defined in this study, of which only one (ST36, where the isolate already in the MLST database was from elsewhere in Australia) had been described previously. Figure 1 shows the clustering of the sequence types on an UPGMA tree obtained with differences in the allelic profiles and their relatedness to the 62 sequence types in the MLST database from other areas where the disease is endemic.

The majority of sequence types from the Northern Territory differed from all other sequence types at more than one locus (linkage distance of >0.14); four pairs of sequence types differed at only one of the seven loci (single-locus variants), and one group of three sequence types were also closely related, with two sequence types being different single-locus variants of the third sequence type. Although none of the isolates was identical in sequence type to isolates from other countries in the MLST database, in a few cases there were closely related strains in the database. For example, ST110 was a single-locus variant of ST23, previously sourced from both Australia and Thailand, and ST105 was a single-locus variant of ST18, sourced from Kenya.

Geographical distribution of sequence types. Genetic diversity was evident at each of the three geographical regions of the tropical Top End of the Northern Territory. In the Darwin region, incorporating the surrounding rural areas, nine sequence types were seen in 13 isolates. In the Katherine rural region, five sequence types were demonstrated in five isolates, and in the East Arnhem region, 16 sequence types were seen in 21 isolates.

Since the sequence types of the isolates from northern Australia were distinct from those described from other regions where the disease is endemic, we further explored whether these isolates clustered apart from the 25 sequence types of southeast Asia. On a dendrogram constructed with the concatenated sequences, the Australian isolates in this study ap-

peared clustered together and were distinct from those from countries of southeast Asia (Fig. 2). However, isolates of *B. pseudomallei* are very uniform at housekeeping loci (10), and the nodes on the minimum evolution tree therefore had very poor bootstrap support.

Strain tropism and virulence. There was no evidence of strain tropism or differential virulence. Presentations with severe sepsis ($n = 14$) were caused by strains of 13 different sequence types. Skin and soft tissue infections ($n = 5$) were caused by strains of five different sequence types, melioidosis encephalomyelitis ($n = 9$) was caused by eight different sequence types, and prostate infections ($n = 11$) were caused by eight different sequence types. Individual sequence types were associated with multiple sites of infection, such as ST109, which was associated with prostatic and neurological presentations. There was also no evidence of associations between disease presentation and the clustering of isolates on the minimum evolution tree obtained with the concatenated sequences (data not shown).

Comparison with PFGE strain types. All isolates from one PFGE clone (clone 1; $n = 8$) were of the same sequence type (ST109). Isolates from another clone defined on PFGE typing (clone 2; $n = 5$) as identical were assigned to two sequence types (ST132 and ST133), but these were very closely related, as they differed at only one locus (Fig. 3). Both of these clusters contained isolates from patients who were not epidemiologically linked and which were recovered over periods of 8 years (Table 1). A third group of isolates from a remote community over an 11-month period showed concordance of PFGE and MLST results; two of the four isolates were indistinguishable by PFGE and had identical sequence types. The other two isolates had unique genotypes by both PFGE and MLST (Table 2).

DISCUSSION

Previous work on *B. pseudomallei* has defined its genetic relationship to *B. mallei* and *B. thailandensis* and identified three clones responsible for an outbreak in Hong Kong (10). In this study, a broad diversity of strains was seen in isolates from the region of Northern Territory of Australia where the disease is endemic and no clinical correlates of strain types were evident.

The northern Australian isolates examined here appear to be distinct from those described from southeast Asia. No sequence type from isolates in this study has been demonstrated in other countries, and only two previously identified sequence types in the MLST database have been noted from Australia as well as other countries (ST23, found in Australia and Thailand, and ST60, found in Australia and Fiji). However, it is unclear whether the Australian isolates of ST23 and ST60 in the MLST database were acquired in northern Australia where the disease is endemic or were imported infections from other countries to the temperate regions of Australia where the disease is not endemic. With a few exceptions, the Australian isolates appeared to cluster on the minimum evolution tree distinct from those recovered in southeast Asia.

The minimum evolution tree attempts to define the relatedness between all strains, but the validity of these relationships depends on the relative impact of recombination and point mutation to the divergence between strains, which is presently

FIG. 1. Relatedness among isolates displayed as a dendrogram by the UPGMA method with the matrix of pairwise differences between the allelic profiles of the isolates. Clusters and arrows indicate Australian isolates from this study. The number of Australian isolates from this study is indicated in parentheses unless only one was studied.

unknown. Furthermore, *B. pseudomallei* is genetically quite uniform at housekeeping loci (10); the concatenated sequences from many of the isolates differ at only one or a small number of nucleotide sites. Therefore, the nodes on the minimum evolution tree had poor bootstrap support. Further studies with larger numbers of isolates are required to explore the distinctiveness of the genotypes recovered in different areas where the disease is endemic, although the lack of sequence diversity may make it difficult to obtain robust population genetic inferences about the extent and nature of geographic substructure.

What then can be concluded about the origins of *B. pseudomallei* in Australia? Australia has been isolated geographically for many millions of years. However, during that time, ex-

change of flora and fauna through various transient land links with southeast Asia is well recognized. Spread of *B. pseudomallei* can result from contact with humans or animals. Previous outbreaks in countries where the disease is not endemic have implicated imported animals from countries where the disease is endemic (5, 21). Clusters in temperate Australia have also been attributed to animals brought in from the tropical north (2, 14).

Despite its recent description in Australia, the genetic and geographical diversity found does not support theories that melioidosis was introduced by servicemen returning to Australia following the second world war (9). Prior to European contact, human contact between southeast Asian traders and indigenous Australians may have resulted in its introduction to

FIG. 2. Tree constructed from the concatenated sequence of the seven MLST loci from *B. pseudomallei* isolates, illustrating distribution of Australian sequence types (clusters indicated by brackets and unclustered strains by arrows) and those from other areas where the disease is endemic. Nodes in the minimum evolution tree are poorly supported by bootstrap resamplings, reflecting low sequence diversity.

TABLE 1. Isolates not epidemiologically linked but clonal on PFGE: comparison with MLST

Year	Isolate no.	PFGE clone	ST	Assigned allele no. for each gene ^a :						
				<i>ace</i>	<i>gltB</i>	<i>gmhD</i>	<i>lepA</i>	<i>lipA</i>	<i>narK</i>	<i>ndh</i>
1994	257	1	109	1	2	13	4	1	19	1
1997	571	1	109	1	2	13	4	1	19	1
1998	719	1	109	1	2	13	4	1	19	1
1999	786	1	109	1	2	13	4	1	19	1
1999	888	1	109	1	2	13	4	1	19	1
2000	910	1	109	1	2	13	4	1	19	1
2000	1105	1	109	1	2	13	4	1	19	1
2002	1415	1	109	1	2	13	4	1	19	1
1993	207	2	132	1	16	13	4	6	21	1
1994	264	2	132	1	16	13	4	6	21	1
1997	480	2	132	1	16	13	4	6	21	1
1999	767	2	132	1	16	13	4	6	21	1
2001	1128	2	133	1	16	13	4	15	21	1

^a *ace*, acetyl coenzyme A reductase; *gltB*, glutamate synthase; *gmhD*, ADP glycerol-mannoheptose epimerase; *lepA*, GTP-binding elongation factor; *lipA*, lipoleic acid synthetase; *narK*, nitrite extrusion protein; *ndh*, NADH dehydrogenase.

or from northern Australia; however, the distinct nature of the Australian isolates does not support repeated exchange of *B. pseudomallei* strains between these regions. Alternatively, *B. pseudomallei* is a soil saprophyte, and intercontinental wind dispersal has been noted for a wide variety of organisms (11, 15). However, it is likely that this mechanism of dispersal is restricted to fungi and spore-forming bacteria; although *B. pseudomallei* can survive in waterless soil for 30 days, it is sensitive to UV light exposure, and thus long-range dispersal seems unlikely (23).

We therefore hypothesize that the diverse but distinct phylogeny of strains in both southeast Asia and Australia, including the lack of *B. thailandensis* and *B. mallei* in Australia, may reflect geographical isolation over a longer period of time. It is possible that *B. pseudomallei* originated in Australia or southeast Asia and was propagated through animal migration during the Miocene period around 15 million years ago, when southeast Asia was linked to the Australia-New Guinea continent through a land bridge. Movements of flora and fauna along this route have been implicated in the paleogeography of plants, including rice (*Oryza sativa*) (13, 24), and animals, including Australia's earliest native rodents (12, 20).

Other sequence types are common among various southeast Asian countries; this may suggest that migration of humans and animals may have resulted in their subsequent dissemination and shared phylogenies. The widespread distribution of ST40 (*B. mallei*) is likely to represent later successful adaptation and restriction to a particular niche (a mobile equine host)

and subsequent dissemination through trading routes through the Middle East to Europe, Africa, and South America. Occasional reports of melioidosis or environmental isolates of *B. pseudomallei* from Africa and South America may reflect even more ancient origins of *B. pseudomallei* or may be related to more recent importation from the major regions where the disease is endemic.

Strain tropism from geographically localized sequence types may have been an explanation for the distinct clinical syndromes seen in Australia, in particular the more frequent neurological and prostatic disease compared to other countries where the disease is endemic (3, 26) and the near absence of parotid disease. Previous studies of small numbers of isolates have suggested that strains may have demonstrated tissue tropism; one study, with multilocus enzyme electrophoresis and random amplified polymorphic DNA analysis, defined one cluster associated with pneumonia ($n = 9$) and another associated with neurological or soft tissue infection (16). Another study with ribotyping suggested that certain ribotypes were correlated with fatal outcomes and others with relapse (17). Furthermore, considerable and consistent differential virulence of *B. pseudomallei* strains has been demonstrated in a mouse model (25). However, in this study with a more precise and unambiguous method of strain characterization and of determining the genetic relatedness between isolates, we have not found evidence of strain tropism or differential virulence in isolates from northern Australia.

As a tool for epidemiological investigation, MLST and PFGE appear to provide similar levels of discrimination, whether based on XbaI (10) or SpeI restriction. Isolates of two clones defined by PFGE derived from isolates that were not epidemiologically linked were closely related or identical on MLST. Conversely, isolates from a case cluster that was polyclonal on PFGE were also polyclonal on MLST. Although MLST provides results that are comparable between laboratories, PFGE typing is quicker and cheaper in our context to define the molecular epidemiology of specific case clusters. Precise assignment of the genotype can then be made by characterizing one isolate of each cluster by MLST.

We conclude that, in contrast to previous studies, there is no evidence to support strain tropism of *B. pseudomallei*; differences in the mode of acquisition, inoculating dose, and host factors are more likely to result in differences in presentations and outcomes of melioidosis. Within the Top End of the Northern Territory, *B. pseudomallei* strains are distributed widely throughout remote locations. These strains are distinct from those found in other areas where the disease is endemic in southeast Asia, possibly reflecting Australia's geographic isolation. For local epidemiological investigations, PFGE typing and MLST provide similar results.

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TABLE 2. Isolates epidemiologically linked but polyclonal on PFGE: comparison with MLST^a

Date	Isolate no.	PFGE clone	ST	Assigned allele no. for each gene:						
				<i>ace</i>	<i>gltB</i>	<i>gmhD</i>	<i>lepA</i>	<i>lipA</i>	<i>narK</i>	<i>ndh</i>
February 1995	362	3	141	4	16	3	4	1	9	6
March 1994	287	4	125	1	14	20	1	15	9	1
October 1994	343	4	125	1	14	20	1	15	9	1
January 1995	356	5	149	1	2	14	2	1	6	1

^a See Table 1, footnote a.

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