A CLUSTER OF MELIOIDOSIS CASES FROM AN ENDEMIC REGION IS CLONAL AND IS LINKED TO THE WATER SUPPLY USING MOLECULAR TYPING OF BURKHOLDERIA PSEUDOMALLEI ISOLATES

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Abstract. Nine cases of melioidosis with four deaths occurred over a 28-month period in members of a small remote Aboriginal community in the top end of the Northern Territory of Australia. Typing by pulsed-field gel electrophoresis showed isolates of Burkholderia pseudomallei from six of the cases to be clonal and also identical to an isolate from the community water supply, but not to soil isolates. The clonality of the isolates found in this cluster contrasts with the marked genetic diversity of human and environmental isolates found in this region which is hyperendemic for B. pseudomallei. It is possible that the clonal bacteria persisted and were propagated in biofilm in the water supply system. While the exact mode of transmission to humans and the reasons for cessation of the outbreak remain uncertain, contamination of the unchlorinated community water supply is a likely explanation.

INTRODUCTION
Melioidosis, infection with Burkholderia pseudomallei, is endemic in southeast Asia and northern Australia, but has also been reported in other tropical regions and imported cases are described in temperate countries. In northeast Thailand and the top end of the Northern Territory of Australia melioidosis is hyperendemic, being the commonest cause of fatal bacteremic pneumonia and septicaemia during the monsoonal wet season.

The organism is a soil saprophyte and can be cultured from soil and surface water in endemic regions, with postulated routes of infection being via contamination of broken skin, aspiration, inhalation of aerosolised bacteria, and possibly ingestion and sexual transmission.

Understanding of the epidemiology of melioidosis has been improved by the development of molecular typing methods for B. pseudomallei, beginning with ribotyping, followed by random amplified polymorphic DNA analysis and pulsed-field gel electrophoresis (PFGE) of restriction enzyme digested total chromosomal DNA.

Since October 1989 we have been prospectively following all cases of melioidosis in the top end of the Northern Territory. From March 1994 until June 1996 there were nine cases of melioidosis with four fatalities in people from a single remote Aboriginal community in the north of the Northern Territory of Australia, compared with only one case in the preceding 5 years. The community population at the 1996 Census was 1,286 people and over the same 28-month period there were 70 melioidosis cases with 6 fatalities amongst the 139,000 (1996 Census data) people living elsewhere in the top end. This gives a relative risk for melioidosis in community members over this period of 13.9 (95% CI 7.0–27.8). We therefore used PFGE to examine the isolates from the cases of melioidosis from the remote community together with isolates collected from an environmental study.

MATERIALS AND METHODS

Environmental isolates. Eighty-seven soil samples and 35 water samples were collected over a six-month period spanning the wet season from November 1996 to April 1997. Thirty soil sites representing domestic, congregation, and outstation locations were sampled, usually at three depths, 30 cm, 50 cm, and 70 cm. Culture of soil was in duplicate using modified Ashdown’s enrichment selective broth and Galimand and Dodin’s selective broth (both with 50 mg/L colistin added), with a method adapted from Wuthiekanun and others. Briefly, one teaspoon of soil was added to 2 mL of sterile distilled water and shaken vigorously. After settling, 0.5 mL of supernatant was added to the selective broths which were then incubated for 48 hours at 40°C, followed by plating of 10 μL of broth supernatant onto Ashdown’s selective agar, which was incubated for a further 48 hours at 40°C.

Water samples were from environmental water bodies as well as diverse pipes and tanks related to the bore water collection and distribution for the community. They were processed in the field by passing 5 L of water through a 22 micron filter and then placing the filter paper in 25 mL of Ashdown’s selective broth with 50 mg/L colistin added. The broth was incubated at 40°C for seven days, with 10 μL supernatant plated onto Ashdown’s selective agar at 2 and 7 days and incubated for 48 hours at 40°C. Suspect colonies from soil and water cultures were identified using API 20 E (API System SA, Lyon, France).

Human bacterial isolates. Isolates of B. pseudomallei were available from eight of the nine cases of melioidosis from the community, in addition to isolates from other cases for comparison. Identity was confirmed by standard biochemical tests and API 20 E.

Pulsed-field gel electrophoresis. Block preparation was carried out using a modification of the method of Haase and others. Pure colonies were grown in Tryptone-soya broth (Oxoid, Heidelberg West, Victoria Australia) for 3 days at 37°C. Cells were pelleted, washed twice in normal saline and adjusted to 240–300 × 10^6 cells/ml in PET IV (10 mM Tris, 1M NaCl). This suspension was mixed with an equal volume of 2% low melting point (FMC) agarose and pipetted into block moulds (0.8 × 0.4 × 0.2 cm). Solidified blocks were incubated in lysis buffer (100 mM EDTA, 0.5% Brij 58, 0.2% Na deoxycholate, 0.5% N-lauroylsarcosine) containing 20 mg/ml lysozyme and 10 μg/μl RNAsase at 37°C overnight. Blocks were then transferred to proteinase K buffer (0.5 M EDTA, 1% N-lauroylsarcosine) containing 1mg/ml proteinase K and incubated at 55°C overnight. Blocks were washed twice in proteinase K buffer and stored at 4°C until use. Half-blocks were dialysed overnight in low EDTA TE buffer (10 mM Tris, 0.1 mM EDTA). Blocks
were pre-incubated in 200 μl of digestion buffer for 30 minutes, then digested with 10 units of Spe I (Pharmacia) in fresh buffer at 37°C for 4 hours. Digests were run on 1% agarose gels in 0.5× TBE on a BioRad Chef DR-III system, with a 10–60 second pulse ramp at 6V/cm for 24 hours with yeast chromosomal marker. Gels were stained with ethidium bromide, visualised with UV light, and analysed with the BioRad molecular analyst package (BioRad Laboratories, Hercules, CA).

The study was approved by the community involved in the outbreak and the Joint Institutional Ethics Committee of the Royal Darwin Hospital and the Menzies School of Health Research, which has an Aboriginal Sub-Committee with power of veto.

RESULTS

Six of the 30 soil sites and one of the 35 water samples grew B. pseudomallei. Of the seven soil isolates, four were from samples collected at 70 cm, one from 50 cm, and two from 30 cm, with one site positive at both 30 and 70 cm. Five soil isolates grew from Galimand and Dodin’s broth and two from Ashdown’s broth, with no sample being positive from both broths, although the two isolates from different levels of the same site were one from each broth. The single water isolate was from water collected from one of the two storage tanks for the community water supply, which is piped from a bore 1–2 km outside the community.

PFGE showed six of the eight community case isolates (Lanes B, C, G, H, I, and J; Figure 1) and the water isolate (Lane K) to be identical. The other two case isolates were different (lanes D, E). An isolate from a community member who lived in Darwin and died from septicemic melioidosis during the same period was different on PFGE (lane F). An isolate from the only other case in the community between October, when melioidosis is unusual. We have isolated the clonal strain from the community water supply, which is not chlorinated. A similar clonal cluster of melioidosis cases has recently been reported from northern Western Australia, also implicating contamination of the domestic water supply.10 Previously we used ribotyping to show clonality in a cluster of isolates from farm animals, a farmer, and soil in the temperate southwest of Australia.11 That cluster was presumed to have been introduced to the non-endemic region by importation of an infected animal, but the current case series occurred within the hyperendemic region of tropical northern Australia, where there is large genetic diversity amongst human, animal, and environmental B. pseudomallei isolates, as shown by the soil isolates and previous studies.6

The mode of transmission of B. pseudomallei in this outbreak remains speculative, but appears likely to have originated from persisting contamination of the community water supply, possibly due to biofilm protection and propagation of bacterial colonies within the water supply system.12 The limited number of environmental samples in our study is insufficient to fully characterise the distribution of B. pseudomallei in local soil and water. We have isolated B. pseudomallei from bore water in other locations in the top end of the Northern Territory (unpublished data), and studies in Thailand have noted persistence of B. pseudomallei near to bores during the dry season.8 In one of the clonal cases an inoculating forehead lesion was culture-positive. Percutaneous inoculation, inhalation of aerosolised

that PFGE is excellent for differentiating strains of B. pseudomallei. To confirm the PFGE findings using Spe I digestion, the chromosomal DNA from each isolate was digested with Pac I. Again the fragment patterns of the six clinical isolates and the water isolate were identical (data not shown).

Of the six patients with identical isolates, three had fatal septicaemic pneumonia, one had non-fatal sputum culture-positive pneumonia, and two had non-fatal neurological illnesses. Of the latter, one had a B. pseudomallei culture-positive (presumed inoculating) ulcer on the left forehead, with brainstem encephalitis with left lower motor neurone facial nerve palsy, left abducens nerve palsy, and peripheral motor weakness. Two of the six patients presented in the dry season (June and October), when melioidosis is unusual.

The cases were evenly distributed throughout the community, with no evident common soil exposure from congregation or hunting. The only epidemiological link between the cases, apart from residence in the same small community with a common water supply, was that five of the six cases with clonal isolates were known to ingest kava, which is commonly used in this and other Aboriginal communities in the region. The kava is prepared by placing commercially available powder of the root of the plant Piper methysticum in a cloth, which is then placed into a container of cold water. After infusion, the mixture is drunk, usually from a common cup. Three samples of prepared kava from the community were culture-negative for B. pseudomallei.

DISCUSSION

Using PFGE we have shown that at least six of a cluster of nine cases of melioidosis occurring over a 28-month period in a small remote tropical community are from a clonal strain of B. pseudomallei. We also isolated the clonal strain from the community water supply, which is not chlorinated. A similar clonal cluster of melioidosis cases has recently been reported from northern Western Australia, also implicating contamination of the domestic water supply.10 Previously we used ribotyping to show clonality in a cluster of isolates from farm animals, a farmer, and soil in the temperate southwest of Australia.11 That cluster was presumed to have been introduced to the non-endemic region by importation of an infected animal, but the current case series occurred within the hyperendemic region of tropical northern Australia, where there is large genetic diversity amongst human, animal, and environmental B. pseudomallei isolates, as shown by the soil isolates and previous studies.6
bacteria, aspiration, and ingestion all remain possible from contaminated water. Following community concerns about the deaths from melioidosis an extensive education program was implemented by community educators in conjunction with the health staff. General health and hygiene issues; specific details about melioidosis, including the known risk factors such as diabetes, alcohol excess, and renal disease; the risk of cutaneous inoculation from exposure to wet-season soils; and the concerns of excess kava consumption were all discussed. Whether the association seen in the Northern Territory between kava consumption and melioidosis is causal, possibly being related to metabolic or immunological defects from kava excess or even ingestion of contaminated prepared kava, remains speculative and is currently being studied. Any changes in behaviour subsequent to the education program were not quantified, although the community moved to ban kava. A number of public works were also carried out, including scouring of the water storage tanks and installation of some new piping. No further isolates of \( B. pseudomallei \) have been recovered from the water supply, although it remains unchlorinated. There was no further melioidosis in the community for almost three years, until a death in July 1999, where the \( B. pseudomallei \) isolate was different on PFGE than the clone (data not shown).

Of interest is the occurrence of two cases of neurological melioidosis from a \( B. pseudomallei \) clone which also caused four cases of pneumonia. This would support neurological melioidosis being caused in these cases by direct bacterial invasion of the nervous system rather than it being the result of toxin-induced damage from \( B. pseudomallei \) strains producing a neurotropic exotoxin.\(^{11}\)

None of the eight environmental isolates utilized arabinose on API 20 NE testing. Arabinose assimilation differentiates between pathogenic strains of \( B. pseudomallei \), which do not assimilate arabinose (Ara-), and nonvirulent \( B. pseudomallei \)-like organisms, which assimilate arabinose (Ara+).\(^{12}\) All clinical melioidosis isolates are Ara-; in hyperendemic northeast Thailand 75% of soil isolates are Ara-, while in central Thailand where melioidosis is rare soil isolates have all been Ara+.\(^{13}\) All 43 environmental isolates of \( B. pseudomallei \) from the top end of the Northern Territory tested by us have been Ara- (unpublished data), consistent with the region being hyperendemic for melioidosis and suggesting that the nonvirulent \( B. pseudomallei \)-like organisms found in Thailand are absent or uncommon in the Northern Territory.

In conclusion, in this small community at least six of the cluster of melioidosis cases were from a clonal strain of \( B. pseudomallei \) which was also found in the water supply. While the exact nature of transmission of the bacteria and the reasons for cessation of the outbreak remain uncertain, contamination of the community water supply is a possible explanation. Further studies are required in melioidosis endemic regions to quantitate the presence of \( B. pseudomallei \) in potable water and to establish guidelines for management of community water supplies where \( B. pseudomallei \) contamination is considered a potential risk.

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