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Preliminary report on the northern Australian melioidosis environmental surveillance project

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SUMMARY

An environmental surveillance programme was developed to determine whether water supplies could be a source of *Burkholderia pseudomallei* as noted during previous melioidosis outbreak investigations. Water supplies to communities in the three northern Australian jurisdictions (Western Australia, Northern Territory and Queensland) were sampled periodically during 2001 and 2002. Water and soil samples were collected from communities known to have had recent culture-positive melioidosis cases and nearby communities where no cases had been diagnosed. Clinical isolates of *B. pseudomallei* obtained from northern Australian patients during 2001 and 2002 were compared with the environmental *B. pseudomallei* isolates by ribotyping and pulsed-field gel electrophoresis. *B. pseudomallei* was isolated from 11 distinct locations, all in the Northern Territory, seven of which were associated with culture-positive melioidosis cases (>1 case at three locations). Water was implicated as a possible environmental source of melioidosis in six locations. A variety of free-living amoebae including *Acanthamoeba* and *Hartmannella* spp. that are potential hosts to *B. pseudomallei* were recovered from environmental specimens. Culturable *B. pseudomallei* was not found to be widely dispersed in the environments sampled.

INTRODUCTION

Melioidosis, a potentially fatal bacterial infection endemic in northern Australia and Southeast Asia, is thought to occur as a result of exposure to soil or water contaminated with *Burkholderia pseudomallei* [1]. Most *B. pseudomallei* infection occurs during the wet season [2]. The most likely means of transmission are direct inoculation, inhalation and ingestion [3].

A small case cluster of septicaemic melioidosis occurred in a remote northwestern Australian community just prior to the wet season in late 1997 [4]. An epidemiological, environmental and laboratory investigation implicated the community's drinking water supply as a source of *B. pseudomallei*. Environmental control measures targeted at the suspected source appeared to effectively prevent any further cases of acute, septicaemic infection. Subsequent environmental investigations identified the water treatment plant as the likely primary source of

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B. pseudomallei [5]. The unchlorinated community water supply was also implicated as a likely source of *B. pseudomallei* in another case-cluster in a remote community in the Northern Territory, which included four fatal cases [6].

A collaborative environmental surveillance project involving the three northern Australian jurisdictions Western Australia (WA), Northern Territory (NT) and Queensland (Qld) was established to follow-up these observations and determine whether drinking water supplies are a potential source of melioidosis in other parts of northern Australia.

METHODS

Clinical isolate collection

Clinical isolates were obtained by the three collaborating centres from cases of melioidosis diagnosed in their respective jurisdictions. Presumptive diagnoses (i.e. those made on clinical signs or by demonstration of seroconversion in the absence of positive cultures) were excluded from further analysis and environmental follow-up. Serological data were checked periodically to ensure that undeclared positive cultures had been referred to the collaborating centres. These centres also acted as reference laboratories for confirmation of *B. pseudomallei* identification, thus assisting the collection and collation of clinical isolates. Melioidosis was a notifiable disease in all three jurisdictions for the period under study.

Environmental sampling

Sample locations were determined by the place of residence of the clinical cases at the time of diagnosis. The communities selected for sampling had one or more dry season cases of culture-confirmed, septicaemic melioidosis. Communities where more than one acute culture-positive case had been confirmed during the wet season and adjacent communities where no cases had been documented were sampled. Multiple locations were sampled in quick succession to allow control and test samples to be compared as closely as possible, as far as geography permitted. Potable water, surface and rhizosphere soil were collected from locations suitable for human exposure by direct contact, ingestion, inoculation or inhalation.

Water samples of 250 ml to 2 litres were collected. Outlet pipes were sterilized and water run for 5 min prior to collection to reduce the risk of contamination by soil or dust. When water samples were collected from surface water, the bottle was submerged approximately 10 cm below the surface then opened, filled with the sample and then closed before being removed. Soil samples were collected at catchment area sites where water samples were collected and around the residences of melioidosis patients. The first 2–3 cm of soil was scraped away from the surface prior to collecting samples; one at a depth of 10–15 cm, and where possible another at 15–30 cm. For root soil samples, the soil was collected 60 cm from the stem of the plant.

As there were differences in sample processing methods between the three centres, a proportion of environmental samples from NT and Qld were collected in duplicate for analysis by the WA centre in order to assess consistency of results.

Water testing

At each sample site the temperature, pH and residual and free chlorine levels of the water were measured. Water samples were prepared for culture by membrane filtration. The membranes were added to selective broths and incubated at 37 °C. The cultures were spread on Ashdown's medium [7] and BPSA plates [8]. The plates were examined after 48 h incubation at 37 °C and after a further 5 days at room temperature (Table 1).

Water samples were analysed for free-living amoebae by centrifugation using a standard method. In brief, 250 ml of the water sample was centrifuged with live AWQC *Escherichia coli*. The pellet was resuspended in 2 ml of the supernatant and spread on NNA plates. The plates were examined for areas of lysis after 72 h incubation at 42 °C. A light microscope was used to identify the genus of the amoebae detected.

Soil testing

The pH of each soil sample was measured. Soil samples were prepared for culture by shaking the soil in demineralized water and allowing it to sediment. The supernatant was added to selective broths and incubated at 37 °C. The cultures were spread on Ashdown's medium and BPSA plates. The plates were examined after 48 h incubation at 37 °C and after a further 5 days at room temperature (Table 1).

Identification of *B. pseudomallei*

Colonies showing morphology typical of *B. pseudomallei* were tested for oxidase production and

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Table 1. *Laboratory methods*

	Collaborating centre		
	WA	NT	Qld
Water testing			
Volume filtered	250 ml	1–2 l	250 ml
Membrane pore size	0·22 μm	0·45 μm	0·22 μm
Selective broths	TSB*	TSB* and Ashdown's broth†	TSB*
Broths monitored	7 d	14 d	2 d
Soil testing			
Quantity tested	5 g	20 g	10 g
Shaking	4 h	Overnight	4 h
Selective broth step	(1) TSB* (2) Ashdown's broth†	(1) TSB* and Ashdown's broth†	(1) TSB* (2) Ashdown's broth†
Broths monitored	7 d	14 d	2 d
Identification of *B. pseudomallei*	(1) Oxidase test (2) Gram stain (3) Gentamicin resistance (4) API20NE (5) Nested PCR [10–12]	(1) Oxidase test (2) Gram stain (3) In-house agglutination [9] (3) Microscan® Walkaway 96 (4) PCR [13]	(1) Oxidase test (2) Biomérieux Vitek

* Contains 10 μg/ml gentamicin.
† Contains 50 mg/l colistin.

Gram-stained (Table 1). Gentamicin resistance was established by disc diffusion testing using 10 μg/ml gentamicin discs on blood agar. Definitive *B. pseudomallei* identification was determined by substrate utilization [API20NE strips, Microscan® Walkaway 96 (Dade Diagnostics, West Sacramento, CA, USA) and Biomérieux Vitek (Baulkham Hills, NSW, Australia)], in-house agglutination [9] and polymerase chain reaction [10–13].

Molecular typing of *B. pseudomallei*

Molecular typing was performed on isolates identified as *B. pseudomallei* using DNA macrorestriction and ribotyping as described previously [14]. In brief, pulsed-field gel electrophoresis (PFGE) was performed using *Xba*I and loaded gels were electrophoresed with a pulse time and ramp of 5·5–52 s for 20·2 h at 200 V. An automated *Eco*RI protocol (RiboPrinter™, Dupont-Qualicon, Wilmington, DE, USA) was used for ribotyping. Gel images and ribotype patterns were collated and analysed using a molecular typing analytical software package (Bionumerics Version 1.0, Applied Maths, Kortrijk, Belgium).

RESULTS

There were 41 human cases of culture-confirmed melioidosis in northern Australia during 2001 and 43 in 2002, 62% of which were from the NT, 30% from Qld and 8% from WA. These cases are less in number than in 2000 and coincide with drier years for the three participating jurisdictions. In 2001 the difference in rainfall was most notable in Qld where several areas within the state had a total rainfall well below average, based on Bureau of Meteorology data collected from 1961 to 1990. In 2002, areas in all three jurisdictions had low total rainfall readings.

A total of 745 environmental samples were collected across northern Australia during the study period (Table 2), 52% of which were water samples and 48% soil samples. All samples collected in Qld, and 45% of the samples collected in the NT were analysed in duplicate. Duplicate analysis revealed a statistically significant agreement in the results obtained by each jurisdiction (Fisher's exact test, $P = 0{\cdot}0005$), however, samples were more often found to be positive by one centre than by both (Table 3).

Eleven water samples and 11 soil samples from the NT were found to be culture-positive for *B. pseudomallei* (Table 4). Both of the two positive water samples from 2001 (NTE003 and NTE265) were collected during the wet season from bores on NT properties where animal infection occurred (pigs and a dog respectively). Two positive soil samples were collected from another NT property where goats had melioidosis. Two soil samples collected for control purposes at different depths below the surface of a construction

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Table 2. *Environmental sample collection*

	WA	NT	Qld	Total
Sample runs performed	24 (30%)	40 (50%)	16 (20%)	80
Water samples collected	182 (47%)	130 (34%)	73 (19%)	385
(no. processed in duplicate)	(0)	(103)	(73)	(176)
Soil samples collected	44 (12%)	154 (43%)	162 (45%)	360
(no. processed in duplicate)	(0)	(24)	(162)	(186)
Total samples collected	226 (30%)	284 (38%)	235 (32%)	745

site associated with no clinical cases contained *B. pseudomallei* (NTE065 and NTE066). During the 2001 dry season soil samples were collected around the residences of two NT human melioidosis cases. Both properties were grassed and watered regularly at the sites of four positive soil samples (NTE086, NTE090, NTE098 and NTE100). The fifth positive soil sample was from an area exposed to regular vehicle traffic (NTE102).

The first positive water sample for 2002 was collected after the wet season ended, from a shaded area at the edge of a running creek in the NT (NTE270). Six of the positive water samples collected in the 2002 dry season were from bores at two NT residences. One of the residences belonged to a human melioidosis case and had *B. pseudomallei* in water from the bore-water holding tank (NTE280) and in water samples taken from an irrigation pipe at the start and end of a 2-month period (NTE273 and NTE282). The water from a bore filter system used to filter the bore water for household use was also positive for *B. pseudomallei* (NTE279 and NTE114). The other residence was not connected with a case of melioidosis but had *B. pseudomallei*-positive water collected from the bore-water holding tank (NTE289) and the bore-head tap (NTE290). At the beginning of the 2002 wet season a water sample collected by the NT local water authority from a bore-head tap at a different location was also positive for *B. pseudomallei* (NTE326).

Other *Burkholderia* species with phenotypic similarities to *B. pseudomallei* were isolated from 4 WA water samples, 2 WA soil samples, 10 NT water samples, 47 NT soil samples and 22 Qld soil samples. These were excluded on the basis of PCR results.

Of the 315 water samples tested for free-living amoebae, 22, 44 and 4% of the samples collected from WA, NT and Qld respectively contained amoebae. *Hartmannella* was the most common genus found, and was detected in 60% of the positive water samples. *Acanthamoeba* was the next most common and was detected in 39% of the positive water samples.

Table 3. *Comparison of duplicate specimen results also tested in WA*

	B. pseudomallei result		
WA results	+*	−†	Total
+	4	11	15
−	7	340	347
Total	11	351	362

* *B. pseudomallei* isolated from NT or Qld sample.
† *B. pseudomallei* not isolated from NT or Qld sample.

Molecular typing results

PFGE and ribotype patterns of 24 environmental *B. pseudomallei* isolates (12 from 11 water samples and 12 from 11 soil samples) were compared with 9 related clinical isolates and 26 unrelated clinical isolates for 2001 (6 from WA, 13 from the NT and 7 from Qld). The Bionumerics molecular typing analytical software generated a composite dendrogram demonstrating the degree of relatedness between the 59 *B. pseudomallei* isolates (Fig.).

The two isolates from positive water samples collected in 2001 from NT bores associated with animal infections (NTE003 and NTE265) grouped together in a distinct cluster (Cluster A) featuring the associated porcine and canine clinical isolates (NTC011, NTC012, NTC014, NTC015, NTC016 and NTC017). The two *B. pseudomallei* isolates from soil samples collected around a NT goat farm (NTE055 and NTE057) and the associated caprine clinical isolate (NTC009), a soil connected with a primate case of melioidosis (NTE076), two representative clinical isolates from this jurisdiction (NTU028 and NTU034) and an isolate from one of five water samples

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Table 4. *Sites positive for* B. pseudomallei

Jurisdiction sampled	Sample site	Type of case	Sample ID	Date of collection	Type of sample	Species of *Burkholderia*	Clinical isolate
NT	Piggery	Porcine	NT[a]E[b]003	23.ii.01	Water	*B. pseudomallei*	NTC[c]011, NTC012, NTC014, NTC015, NTC016
NT	Goat farm	Caprine	NTE055	30.iii.01	Soil	*B. pseudomallei*, *B. multivorans*	NTC009
			NTE057	30.iii.01	Soil	*B. pseudomallei*	
NT	Construction site	No cases	NTE065	2.v.01	Soil	*B. pseudomallei*	
			NTE066	2.v.01	Soil	*B. pseudomallei*	
NT	Animal park	Primate	NTE068	8.v.01	Soil	*B. pseudomallei*	n.a.
			NTE076	31.v.01	Soil	*B. pseudomallei*	
NT	Residence	Human	NTE086	4.vii.01	Soil	*B. pseudomallei*, *B. cepacia*	n.a.
NT	Residence	Human	NTE090	11.vii.01	Soil	*B. pseudomallei*, *B. cepacia*	NTC008
			NTE098	11.vii.01	Soil	*B. pseudomallei*	
			NTE100	11.vii.01	Soil	*B. pseudomallei*	
			NTE102	11.vii.01	Soil	*B. pseudomallei*	
NT	Residence	Canine	NTE265	13.xii.01	Water	*B. pseudomallei*	NTC017
NT	Running creek	No cases	NTE270	26.iv.02	Water	*B. pseudomallei*	
NT	Residence	Human	NTE273	28.v.02	Water	*B. pseudomallei*	NTC83
			NTE279	8.viii.02	Water	*B. pseudomallei*	
			NTE280	8.viii.02	Water	*B. pseudomallei*	
			NTE282	8.viii.02	Water	*B. pseudomallei*	
			NTE114	8.viii.02	Water	*B. pseudomallei*	
NT	Residence	No cases	NTE289	23.viii.02	Water	*B. pseudomallei*	
			NTE290	23.viii.02	Water	*B. pseudomallei*	
NT	Water authority sample point	No cases	NTE326	27.xi.02	Water	*B. pseudomallei*	

[a] Isolated from the Northern Territory, as indicated by 'NT' in prefix.
[b] Isolated from an environmental sample, as indicated by 'E' in prefix.
[c] Isolated from a clinical sample, as indicated by 'C' in prefix.
n.a., Not available.

collected from the residence of a NT human case (NTE273) were all grouped in this cluster. The isolates from the other four water samples from this site, but collected at a later date (NTE279, NTE280, NT282 and NT114), did not group with this cluster and had a similarity of at least 85% to each other. All were distinct from the geographically related human clinical isolate (NTC83).

A second cluster of isolates was evident in the dendrogram (Cluster B). This consisted of two of the four *B. pseudomallei* isolates found in soil samples collected around the residence of a human case (NTE090 and NTE102), the respective clinical isolate (NTC008), a soil isolate from the residence of a second human case (NTE086), and three unrelated clinical isolates from the same jurisdiction (NTU033, NTU035 and NTU036).

Though the unrelated clinical isolates often grouped with other clinical isolates from the same jurisdiction, these isolates were genetically diverse. Moreover, some similarities between isolates from the three jurisdictions were evident.

DISCUSSION

The previous finding that potable water supplies contained *B. pseudomallei* that were indistinguishable from clinical isolates in two separate melioidosis outbreaks highlighted the potable water supply as a potential source of the infection. The present surveillance project was established to follow-up the findings of those two outbreak investigations in a structured, prospective study.

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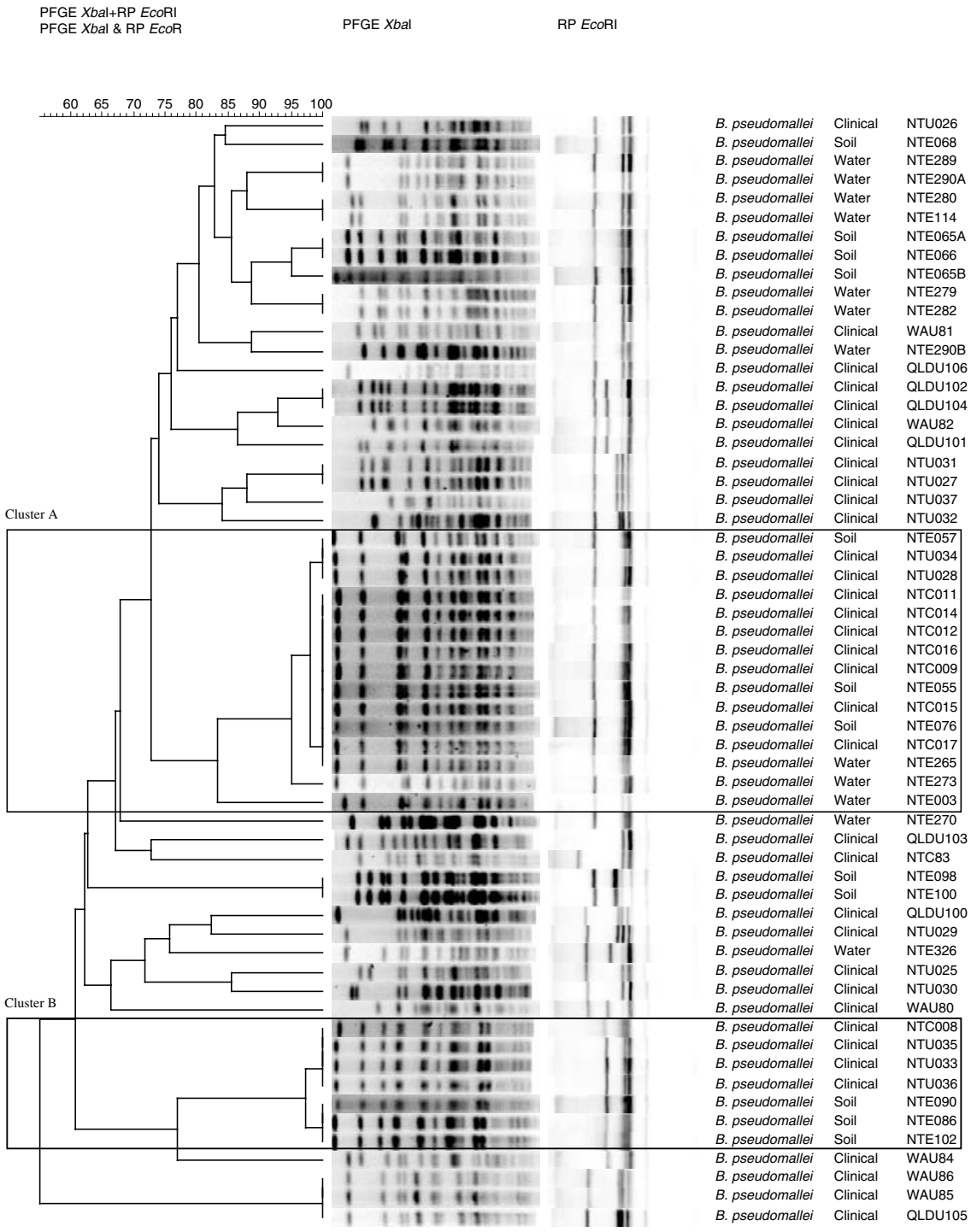

Fig. Composite dendrogram of PFGE and ribotype patterns for 59 *B. pseudomallei* isolates. Isolate names represent the jurisdiction where the isolate was obtained (NT, Northern Territory; WA, Western Australia; QLD, Queensland) and the nature of the sample from which it was isolated (E, environmental sample; C, clinical sample related to an environmental site sampled; U, clinical sample unrelated to the environmental sites sampled).

Unusually dry weather was associated with lower than average totals of culture-confirmed melioidosis cases during 2001 and 2002. Of those occurring in locations that were accessible to the project team (48), six clinical isolates were closely related by molecular typing to *B. pseudomallei* recovered from associated

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water specimens. This equated with two of the four NT locations where clinical isolates of *B. pseudomallei* were linked by molecular typing to associated environmental isolates. In both instances, the clinical isolates were from infections in animals.

Great care was taken to avoid making spurious links between environmental and clinical isolates. Genotypic confirmation of all suspect *Burkholderia* spp. was performed using PCR-based protocols. Molecular typing of *B. pseudomallei* was then performed using two distinct methods, automated ribotyping and PFGE. While the stringency of this approach may have reduced the numbers of water supplies implicated as possible *B. pseudomallei* sources, it allowed a greater confidence in the links that were established. Moreover, the collection and collation of *B. pseudomallei* genotyping data from across northern Australia has led to the creation of a library of typing data that will assist the molecular epidemiological assessment of clinical or environmental isolates at a later stage.

The differences in methods used to isolate *B. pseudomallei* from environmental specimens reflected the practical difficulties of conveying samples from remote communities to collaborating centres. However, less than 2% of samples analysed in duplicate were found to be positive by the NT or Qld laboratories and negative by the WA laboratory after samples were transported there. This decrease in analytical sensitivity could be attributed to transport, difference in sample volumes and subtle differences in methods used by each centre.

While *B. pseudomallei* was recovered from several locations in the NT, we were unable to find evidence to support the unattributed but often repeated assertion that *B. pseudomallei* is widely distributed in the environment. Given the measures taken to recover *B. pseudomallei* from the environment, including potential intracellular locations, the extent of environmental contamination is remarkably limited in locations associated with clinical cases. If our preliminary findings are repeated in the later stages of this study, it would appear that a patchy environmental distribution with pockets of culturable bacteria is more likely. The alternative explanation that has yet to be disproved is that the species is more widely distributed in either a viable but non-culturable form or is present in a sequestered (e.g. deep rhizosphere or even intracellular) habitat. Viable *B. pseudomallei* was not recovered from any amoebic trophozoite lysate preparations during this stage of the project, nor was the species isolated from any root lysate preparations.

In human cases of melioidosis it is often possible to surmise a likely route of initial disease transmission. Even with this information it was not possible to do more than speculate on a likely mode of final transmission in the previously documented water-related outbreaks. In the present study, the available information does not allow us to attempt to distinguish soil from water exposure in the cases linked to positive environmental cultures. Nor is it possible to establish whether soil or water was contaminated first, or if both occurred through simultaneous contamination from an external source. Nevertheless, none of the positive water supplies were chlorinated and would thus not have been protected against downstream dissemination of *B. pseudomallei*. These results add to previous observations of water-supply contamination by *B. pseudomallei*. In none of those was chlorination continuously and properly maintained. In the majority of the reported cases, the water had not been treated with chlorine at all.

The number of water-related melioidosis cases were too small to allow useful analysis of water quality, hydrological and geological data. It is expected that the additional cases expected during the remainder of this surveillance project will allow such an analysis. From the available data it is clear that the detection of *B. pseudomallei* in a potable water specimen is uncommon and has potential public health significance. It remains to be seen whether potable water supplies in northern Australia need periodic testing for *B. pseudomallei*.

As a result of the first stage of the northern Australian melioidosis surveillance project, cases of melioidosis have been linked to potential environmental sources of infection by systematic environmental sampling, careful bacteriological and molecular typing methods. In two instances of sporadic infection, water has been identified as a potential source and in both of these the source was not chlorinated. Priorities for the remainder of this investigation are the identification of other *B. pseudomallei*-contaminated water supplies, possibly by further improvement of environmental recovery methods, and determination of the role that free-living amoebae such as *Acanthamoeba* and *Hartmannella* spp. may play at these locations. Measures are being taken in the later stages of this study to ensure that conventional water-quality data and local geographical factors such as hydrological and geological features can be used to

understand the ecology of *B. pseudomallei* distribution in the northern Australian environment.

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