Melioidosis Risk in a Tropical Industrial Environment

Timothy J. J. Inglis,* Avram Levy, Adam J. Merritt, Meredith Hodge, Robert McDonald, and Donald E. Woods

Division of Microbiology and Infectious Diseases, PathWest Laboratory Medicine, Western Australia, Australia; Occupational Health, Rio Tinto Group, Australia; Department of Microbiology, University of Calgary, Canada

Abstract. An investigation into the risk of occupationally acquired melioidosis at a mine site in northern Australia found that 45 (13%) of 345 staff had serologic evidence of exposure and 14 (4%) had recent exposure to Burkholderia pseudomallei or closely related bacteria. There was only one culture-confirmed case of melioidosis in mine staff during the study period. The lack of overt infection directly attributable to work activities despite detectable B. pseudomallei on site, the absence of an association between positive serology and occupational activity on site, or duration of employment in the mining industry stand against a significant occupationally acquired infection risk on this industrial site. Workplace exposure to a dust-generating tropical environment in the melioidosis-endemic north of Australia did not appear to pose a measurable increase in infection risk. The effect of long-term climatic trends on this potential biologic threat requires further study.

INTRODUCTION

Melioidosis is a potentially fatal, endemic infection in northern Australia and Southeast Asia.¹ The small numbers of cases in rural northern Australia conceal a high incidence in the relatively sparse population. Melioidosis was previously regarded as a disease of a mainly indigenous northern Australian population with poor general health, and a high incidence of co-morbidities known to predispose to acute septicemic infection.² However, in recent years the overall melioidosis case load notified to jurisdictional health departments has included cases of travel-related and occupational infection in European Australians. Expansion of the mining industry in tropical Australia, particularly in north-western Australia, has the potential to increase the number of adults exposed to soil, surface water, and aerosols containing Burkholderia pseudomallei; the bacterial cause of this infection. Moreover, the possible disturbance to local ecology, microclimate, and hydrology by the development of existing or new mine sites are all likely to affect the risk of occupational melioidosis.

There are further plans to develop the Kimberley region of Western Australia for intensive agriculture through an expansion of the Ord River irrigation scheme. The reliability of summer rainfall in this region has prompted calls for transcontinental piping of water supplies south to Perth and southeast around of 24 to 48 hours. A preliminary visit was therefore conducted to establish the feasibility of staff blood and environmental sample collection. This visit also acted as a confirmation of cold chain arrangements for the return flight. Further visits were then made for staff blood sample and environmental sample collection at a rate of two per year for three years, to allow for bracketing of the rainy season and to maximize recruitment of staff volunteers from local and fly-in, fly-out populations.

Environmental sampling program. Multiple samples were collected from the mine site on each sampling visit. Sampling sites were selected as follows:

Potable water: Targeted sampling of potable water was performed because of previous evidence linking potable water supplies to cases of melioidosis.³ Potable water was therefore obtained from the designated mine site water sampling locations.

High-risk areas: Numerous samples were collected over areas where mineworkers were most likely to come into contact with soil, surface water, or mud, e.g., the ore processing area. These areas changed or shifted during the study period as a result of changing ore recovery and dumping locations.

Water bodies: Potential environmental sources of B. pseudomallei and its transport within the environment were of particular interest throughout the study. For this reason all accessible dams, both natural and artificial, were sampled, as were watercourses in the vicinity of the mine. Sampling included but was not limited to gauge stations where staff were likely to come into contact with water or mud.

Some sample locations were added at a later stage as a result of staff work activity questionnaires, to widen the range of environmental sample types, e.g., the underground mine shaft when it opened, gravel roads after spraying, vehicle maintenance pits after spray-cleaning, decorative flower beds after gardening, and nearby creek beds. All initial positive results were followed up by at least one additional sample from the same location. The Global Positioning System (GPS) coordinates were recorded at each sample location to assist return
to previous sample sites. However, the shifting topography of mined areas, tailings dumps, and pooled waters meant that some sample sites could not be revisited or that the type of sample from a given location changed. Soil was collected from below the most superficial 1 cm and above a depth of 20 cm. A maximum of 1 L of water was collected into sterile containers without preservative.

**Laboratory processing.** Processing of soil and water samples and phenotypic characterization of isolates was performed according to previously published methods. Isolates were confirmed by a combination of *lpxO* polymerase chain reaction (PCR) and *recA* PCR followed by sequencing of the amplified product.

**In situ processing.** Before field deployment of the *B. pseudomallei* PCR method, the procedure was validated as follows: *B. pseudomallei* was cultured in 10 mL of tryptone soya broth (Oxoid, Basingstoke, Hampshire) for 24 hours to give a suspension of ~1 × 10^8 colony forming units (CFU)/mL. This was then washed by centrifugation and resuspended in 1 mL of sterile distilled water. The suspension was then diluted in sterile distilled water in 10-fold serial steps to 10^−6. Culture-negative soil from the Kimberley region was sterilized by autoclaving at 121°C for 15 minutes. After cooling, 100-mg samples were each spiked with the *B. pseudomallei* dilution series. The mixture was thoroughly mixed by vortexing and allowed to stand at room temperature for 30 minutes. The mixture was then extracted as described below. The extraction process was also performed on an unsterilized sample of the same soil to demonstrate efficacy on “real-world” samples. Soil samples not spiked with *B. pseudomallei* were processed as negative controls. Real-time PCR was performed in duplicate as described previously, to demonstrate that *B. pseudomallei* DNA had been successfully extracted from the spiked samples. In this instance *lpxO* real-time PCR detected *B. pseudomallei* DNA in the 10^−7 spiked sample (~1−2 CFU of *B. pseudomallei*). During the final series of site visits the investigators brought a portable molecular biology laboratory to the mine site to provide preliminary detection data on environmental samples and thus guide further sample collection. Samples of ~100 mg of soil or 100 µL of liquid were added to 1-mL TSB amended with 10 µg/mL each of gentamicin sulphate and vancomycin. These were then enriched for 4 hours with shaking in a dark, shaded outdoor location (26−36°C). Samples were centrifuged at full speed (~14,000 rpm) in a microfuge (Hettich Mikroliter, Tuttlingen, Germany) for 5 minutes, then the supernatant was aspirated and pellet extracted using SoilMaster DNA extraction kit (Epicentre, Madison, WI) as per the manufacturer’s instructions. The resulting DNA extract was used as the template for another *lpxO* PCR on a conventional thermal cycler (Applied BioSystems 2720, Singapore). The amplified product was then resolved in a microfluidic laboratory chip on a Bioanalyzer (Agilent Bioanalyzer 2100, Waldbronn, Germany). Positive and negative controls were included with each chip run. Preliminary results allowed the researchers to conduct a second more targeted sample collection the next day before the conclusion of the fieldwork phase and return to the central laboratory for definitive analysis.

**Notifiable disease data.** Melioidosis has been a notifiable disease in Western Australia since 2000. A Burkholderia culture collection has been maintained by PathWest Laboratory Medicine WA and one of its predecessor organizations, PathCentre since then. *Burkholderia pseudomallei* isolates from culture-positive cases were obtained from hospital laboratories whenever they had not already been sent to our center for definitive confirmation or addition to the Burkholderia Culture Collection.

**Molecular epidemiology.** Environmental isolates from the mine site, the single clinical isolate from a staff member who had culture-confirmed soft tissue melioidosis, and clinical isolates from epidemiologically unrelated cases in other parts of the state were compared by DNA macrorestriction analysis using a technique we described previously. Band patterns were compared using the unweighted pair group method with arithmetic mean (UPGMA) and Dice coefficient to produce a dendrogram (BioNumerics, Applied Maths, Kortrijk, Belgium).

**Occupational exposure questionnaire.** All staff volunteering to participate in the serologic survey were asked to complete an occupational activities questionnaire whose entries were to be archived alongside the serology results. The survey included questions on the type of work performed on site, potential exposure to soil, water, aerosols and dust, place of residence, time in the industry, and time on the specific site. No questions specific to gender or ethnicity were included.

**Prospective serologic survey.** Mining staff were invited to volunteer participation in a prospective serologic survey. Informed consent was obtained for all participants prior to venesection, in accordance with the ethics requirements of the board of the Mineral and Energy Resources Institute of Western Australia (a State Government body). Ten milliliters venous blood was drawn on the first occasion and on each subsequent occasion they were present and an investigator was visiting the mine site. Because a high proportion of staff were fly-in, fly-out shift workers resident in urban Western Australia, a follow-up sample request form was provided for use in six months time or sooner if a confirmatory sample was needed. Positive and borderline results triggered a request for a confirmatory sample, as explained at the time of consent. Staff contact details were recorded on recruitment. Volunteers were advised that follow-up samples at six months should be provided even if they ceased to work for the mining company. No test costs were borne by the staff. No identifiable results were passed to the mining company, health authority, or any other third party except at the express instruction of the staff member (e.g., to their family doctor in the event of a rising titer). Melioidosis serology was determined by an indirect haemagglutination test using a standard tri-valent test antigen derived from one reference strain (*B. pseudomallei* NCTC 10276), one local outbreak strain (*B. pseudomallei* NCTC 13177), and a local phenotypic variant (*B. pseudomallei* DM98). The sensitivity of this method had been previously confirmed by exchange of sera between several Australian diagnostic laboratories. Results with a titer of <40 were categorized as negative, borderline on a titer of 40, and positive if the titer was >40. These interpretive standards were used only as a guide for comparative analysis of sera collected in the course of the present study, and were not used for diagnostic purposes. When possible, repeat sera were obtained to confirm borderline or positive results, or a potential seroconversion. Specificity of these results was evaluated by Western blot of 100 sera, including all positives, and a range of borderline and clear negative sera in an independent laboratory.
Meteorologic data. Rainfall, wind speed, and wind direction data were obtained from the weather monitoring station near to the mine site for the two decades up to and including the 3.5-year study period.

Statistical methods. Statistical analysis was conducted with a combination of standard methods, including the $\chi^2$ test, frequency distribution, linear regression, and Spearman’s correlation coefficient with a biomedical software program (GraphPad Prism version 4.0, San Diego, CA).

RESULTS

The first environmental culture of *B. pseudomallei* obtained from the mine site was isolated from roadside surface water (Figure 1: Site A, Table 1). This location was resampled later in the study at which time *B. pseudomallei* was again isolated. *Burkholderia pseudomallei* was also isolated from a transient roadside pool of water near a large reservoir used to supplement mine water, from water at a gauge station on a water course, from the edge of a tailings runoff dam, and from a creek fed by runoff from the same tailings but at a different location (Figure 1: Sites B–E). Of these five culture-positive sites, all but Site B were PCR-positive when direct detection was performed. Site B did not contain pooled roadside water in subsequent visits and was not resampled. Three sites, runoff from another dam, a further gauge station, and the opposite side of the dam from Site D were repeatedly PCR positive, but no *B. pseudomallei* was isolated (Figure 1: Sites F, G, and I). During the last sampling run an even more distant gauge station was sampled, this also returned a PCR positive result for *B. pseudomallei* but was negative by culture (Figure 1: Site H). The designated mining area, underground waters, maintenance, and accommodation areas were all culture negative, and where tested by PCR-based protocol in 2006, were also PCR-negative for *B. pseudomallei* (Figure 1). Other *Burkholderia* spp. were also isolated: *B. cenocepacia* (dam water), *B. cepacia* (pooled fountain water at staff quarters, stagnant roadside water), *B. thailandensis* (pooled water in open-cut pit), and *B. ubonensis* (sediment from gauge station). *Ralstonia* spp. were recovered from a range of samples obtained from mine processing, mine tailings, and potable water. Isolates of genus *Bordetella*, *Chromobacterium*, *Comamonas*, *Enterobacter*, *Klebsiella*, and *Pandorea* were also identified in the course of sample analysis.

The one clinical isolate of *B. pseudomallei* from a mine worker (BCC220) was distinct from all environmental *B. pseudomallei* isolates from the mine site and its surrounds (Figure 2), and was also distinct from previously encountered *B. pseudomallei* clinical and environmental isolates from other parts of WA (data not shown). The DNA macrorestriction analysis did not distinguish A0601, A0602, and A0603 (Figure 2),
which were all obtained from the same water sample collected from around the tailings dam (Site D), thus indicating these were likely to represent the same strain of *B. pseudomallei*. Isolates A0501 and A0503 from Site B and isolate A0702 from Site A were dissimilar to each other and to any other isolates from the study area. These isolates were also dissimilar to any clinical isolates from Western Australia tested to date. The group of environmental isolates from 2006 (A06D01–Site E, A0704–Site A, A01401 and A01402–Site C) produced an indistinguishable DNA macrorestriction pattern to a cluster of isolates previously encountered in both a clinical and environmental setting. One of these was the WA outbreak strain NCTC 13177 (BCC 6), originally seen 500 km to the West in late 1997.3,10 No negative controls set up during processing of soil samples were culture positive for *B. pseudomallei*, and strain NCTC 13177 was not used as a positive control for this process.

There was no evidence to indicate a source of the one culture-positive soft tissue infection on the mine site, and no evidence to suggest that the environmental isolates from the area surrounding the mine had caused any of the culture-confirmed infections in the mine site region. This infection occurred in an otherwise healthy worker who was resident in tropical northern Australia, and who required hospital admission for antibiotic treatment of a soft tissue infection.

The majority of serologic tests for melioidosis in mine site workers were negative (Figure 3). Those who had repeat tests after an initial negative remained seronegative in the majority of cases even when their tests bracketed one or more wet seasons. However, there was a significantly higher proportion (\(\chi^2 = 29.38, df = 4, P < 0.0001\)) of each of positive and borderline results immediately after a severe weather event in May 2005 (MAY-05; 60 negative, 1 borderline, and 2 positive, compared with JUN-05; 6 negative, 5 borderline, and 4 positive; DEC-05; 121 negative, 11 borderline, and 10 positive). These results were confirmed by Western Blot, although the isolation of *B. thailandensis* on site meant that neither indirect haemagglutination test nor Western Blot results could discriminate between exposure to *B. pseudomallei* and *B. thailandensis*. No occupational or home location variable was associated with a higher likelihood of positive serology (Table 2). The increased median time in the mining industry increased only from 7.00 to 9.00 years (negative to borderline to positive serology) and was not statistically significant (\(P < 0.05\), Mann-Whitney U test). Overall, there was no significant association between

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**Table 1** Summary of environmental samples and bacteriological results

<table>
<thead>
<tr>
<th>Samples</th>
<th>B. pseudomallei detected by culture</th>
<th>B. pseudomallei detected by PCR</th>
<th>Other <em>Burkholderia</em> species isolated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtered*</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Unfiltered†</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Mine and processing‡</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Surrounding area§</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Clear waters only filtered.
† Turbid waters, soils, and sediments.
‡ Unvegetated areas.
§ Vegetated areas.

PCR = polymerase chain reaction; Bc = *B. cepacia*; Be = *B. cenocepacia*; Bt = *B. thailandensis*; Bu = *B. ubonensis*.

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**Figure 2.** *Burkholderia pseudomallei* XbaI macrorestriction analysis and unweighted pair group method with arithmetic (UPGMA) dendrogram. Reference numbers A0501, A0503, and A0702 represent *B. pseudomallei* isolated from environmental waters. The cluster, including isolates A0601-3, were all isolated from a tailings dam water sample. The cluster from A06D01–1402 was indistinguishable from NCTC 13177, the WA outbreak strain. The BCC series isolates are from the State *Burkholderia* culture collection and come from patients with culture-confirmed melioidosis in Western Australia. They include an isolate from a worker on another mine site who had septicaemic infection (BCC 234).
The daily, monthly, and annual rainfall trends in the vicinity of the mine are depicted in Figure 4. The wet season corresponds to the months of December through to March. Dry season rainfall was at or close to zero from May to September. Annual rainfall around the mine area has doubled in two decades, with a cyclical pattern of peaks and troughs every 5–7 years. Daily rainfall reached its highest recorded total (200 mm; the normal monthly total for that time of year) during the study period shortly before the June 2005 seroepidemiology sample collection. When daily 9 am wind speed and total rainfall were plotted against each other, the highest combination was also recorded on that day. This was one of only two occasions in over two decades when in excess of 150-mm daily rainfall coincided with strong winds. The April 2005 weather event was therefore the most extreme of its kind in more than two decades.

DISCUSSION

No previous investigation of occupational melioidosis risk has been undertaken in the mining industry, or any other industrial workforce group, whether prospective, retrospective, or point prevalence. This study therefore addresses a significant knowledge gap for occupational health practitioners working in tropical Australia. Melioidosis is an uncommon occupational disease in Australia. The number of suspected occupational infections is low, even when possible under-reporting is taken into account. However, a low prevalence of melioidosis detracts from a relatively high incidence in the northern Australian population, which has seen rapid recent growth because of an influx of workers to meet the workforce demands of an expanding mineral resource industry. Even if the industrial build-up does not directly affect dissemination of the disease, the expected increase in the regional adult population is expected to result in additional cases of melioidosis.

The low prevalence of melioidosis in north Western Australia makes conventional case finding and descriptive epidemiology unsuitable for predictive risk analysis. In the present research only one culture-positive case was recorded among the mining workforce on this site during the entire 3.5-year period. The B. pseudomallei isolate from the patient’s soft tissue lesion belonged to a distinct genotype that has not been seen before or since in either clinical or environmental isolates. Importantly, that isolate did not resemble environmental B. pseudomallei isolates recovered from the mine site. Therefore, it is not possible to attribute this infection to occupational exposure, nor can this interpretation be completely excluded. The balance of probability is that the one culture-positive case of melioidosis during the study period was the result of an environmental encounter elsewhere in the region or in another state. Most of the environmental isolates recovered were genotypically distinct from clinical isolates from Western Australia during the previous decade. The exception was the 2006 isolate cluster, which was indistinguishable from the WA outbreak strain, NCTC 13177. The WA outbreak occurred 500 km to the West around eight years before the June 2005 seroepidemiology sample collection. When daily 9 am wind speed and total rainfall were plotted against each other, the highest combination was also recorded on that day. This was one of only two occasions in over two decades when in excess of 150-mm daily rainfall coincided with strong winds. The April 2005 weather event was therefore the most extreme of its kind in more than two decades.

Table 2: Mine site staff descriptors and serology results

<table>
<thead>
<tr>
<th>Group</th>
<th>Negative</th>
<th>Borderline</th>
<th>Positive</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plant operators</td>
<td>89</td>
<td>9</td>
<td>6</td>
<td>NS</td>
</tr>
<tr>
<td>Office staff</td>
<td>49</td>
<td>2</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>On site &lt;12 months</td>
<td>84</td>
<td>6</td>
<td>7</td>
<td>NS</td>
</tr>
<tr>
<td>On site 1–3 years</td>
<td>55</td>
<td>3</td>
<td>5</td>
<td>NS</td>
</tr>
<tr>
<td>On site &gt;3 years</td>
<td>87</td>
<td>4</td>
<td>7</td>
<td>NS</td>
</tr>
<tr>
<td>Mean time in industry</td>
<td>9.8 years</td>
<td>10.4 years</td>
<td>11.2 years</td>
<td>NS</td>
</tr>
<tr>
<td>Resident interstate</td>
<td>13</td>
<td>2</td>
<td>4</td>
<td>NS</td>
</tr>
<tr>
<td>Resident in Kimberley</td>
<td>96</td>
<td>7</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>Resident in south WA</td>
<td>165</td>
<td>13</td>
<td>12</td>
<td>NS</td>
</tr>
</tbody>
</table>

* NS = not significant. No variable differed significantly by occupational activity, industry experience, or place of residence \( (P < 0.05, \chi^2 \text{ test, with Yates' correction for small numbers}).
favor a genuine environmental event, but the implications of this interpretation dictate a higher standard of supporting evidence before this reading of the data can be accepted with certainty. Resampling, definitive genotyping of all similar isolates, and possibly even whole genome sequencing may resolve this issue.

The locations where *B. pseudomallei* were isolated from soil samples were all outside the principal mine areas: ore recovery, processing, maintenance, administrative or living areas. The ore recovery and processing areas were very dusty but had no topsoil and very little organic material. *Burkholderia pseudomallei* was not detected or isolated from these areas despite repeated and extensive sampling. The majority of *B. pseudomallei* culture-positive locations were in transitional habitats, where native vegetation was present, but had been subject to disturbance, either from human habitation and development in the area, or specifically from mining activity. The sole exception was a *B. pseudomallei* isolate obtained from an active tailings dam site devoid of any vegetation. There is evidence for changes in vegetation affecting bacterial biodiversity in other tropical mine sites.12 Sites in which *B. pseudomallei* was detected, either by PCR or by culture, were not from areas frequented by mine staff.

The recovery of *B. thailandensis* and *B. ubonensis* from similar but distinct locations to *B. pseudomallei* indicates the predilection these related non-pathogenic species have for a similar habitat. Culture-based methods resulted in the isolation of both *B. thailandensis* and *B. pseudomallei*. *Burkholderia thailandensis* was isolated only once, whereas *B. pseudomallei* was isolated repeatedly. *Burkholderia thailandensis* was only recently isolated for the first time in Australia by our group from an environmental site in the Northern Territory.5 The assumption that it did not exist in Australia led to the erroneous belief that a positive non-specific serologic test for melioidosis was sufficient evidence for exposure to *B. pseudomallei*. The isolation of *B. thailandensis* on this specific mine site means that our serologic survey cannot distinguish between exposure to *B. pseudomallei* and its non-pathogenic near-neighbors. The confirmatory Western blot studies on 100 sera from our serum sample collection indicated that the main antibody component was to *Burkholderia* lipopolysaccharide, but was unable to confidently distinguish between *B. pseudomallei* and *B. thailandensis* antigen. A cautious interpretation of the serology results has therefore been taken, regarding a titer of 40 as indicative of nothing more than a borderline result. Very high single titers may be strongly suggestive of recent infection,
but only a rising titer increasing by more than 2-fold was taken as evidence of recent exposure. The discovery of *B. thailandensis* on this site means that borderline or positive antibody titers to *B. pseudomallei* may reflect exposure to *B. thailandensis*, and do not necessarily indicate *B. pseudomallei* exposure. When borderline results were excluded, the occupational activities survey showed no single association with a positive result. None of the additional questions provided even indirect evidence of occupational exposure, leading us to conclude that any occupational exposure was too infrequent on this site to be seen against a background of exposure during travel, recreational or domestic activities elsewhere in the region. The association between increasing time in the industry and stronger serologic evidence of exposure to *Burkholderia* spp. does not necessarily indicate site or occupational exposure, and was not statistically significant (Table 2). It is therefore difficult to assess the effect of additional preventive measures taken on the mine site, including dust suppression, personal protective equipment, and first aid management of minor skin trauma.

The one notable serology result was the relatively high proportion of positive and borderline results in the smaller group tested in June 2005, shortly after unusually severe weather. In 22 years, only three days recorded wind speeds of greater than 40 kph and only one of these recorded a combination of high wind speed and high rainfall (Figure 4). These extreme weather conditions would have been ideally suited to aerosolization in the area and preceded the mid-2005 serology sample collection when a significant increase in seropositives was observed (Figure 3). We also noted that annual rainfall doubled during the 22-year period and appeared to follow a 5–7 year cycle of peaks. The association between heavy rainfall and septicaemic melioidosis has been established in the Northern Territory, so it is notable that an extreme weather event on a site known to have *B. pseudomallei* present did not result in any cases of melioidosis, although the increase in seropositive serology may have been the result of this extreme weather event.

In conclusion, our research into the potential occupational health risk of melioidosis in tropical north-western Australia showed that evidence of recent exposure to the causal agent, *B. pseudomallei* or its near-neighbors, was less common than expected from an endemic area. This low level of exposure could be explained by the low frequency of positive environmental cultures, the restriction of *B. pseudomallei* to protected, regenerating sites at a distance from the main work site, and possibly by the infrequency of weather systems with conditions specifically suited to dispersal of contaminated soil in respirable particles. However, increasing summer rainfall in the region, its unpredictability, and the regional build-up of the mineral resources industry can be expected to change the melioidosis risk in the future. Periodic monitoring is required to understand the stability of environmental distribution of *B. pseudomallei* and to develop decision thresholds for environmental control measures.

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Authors’ addresses: Timothy J. J. Inglis, Avram Levy, Adam J. Merritt, and Meredith Hodge, Division of Microbiology & Infectious Diseases, PathWest Laboratory Medicine WA, Locked Bag 2009, Nedlands, Western Australia, Australia 6009. Robert McDonald, Rio Tinto, 120 Collins St, Melbourne 3000, Australia. Donald E. Woods, Department of Microbiology and Infectious Diseases, Faculty of Medicine, University of Calgary Health Sciences Centre, 3330 Hospital Drive, NW Calgary, Alberta, T2N 4N1, Canada.

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