Sensitive and Specific Molecular Detection of *Burkholderia pseudomallei*, the Causative Agent of Melioidosis, in the Soil of Tropical Northern Australia

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*Burkholderia pseudomallei*, the cause of the severe disease melioidosis in humans and animals, is a gram-negative saprophyte living in soil and water of areas of endemicity such as tropical northern Australia and Southeast Asia. Infection occurs mainly by contact with wet contaminated soil. The environmental distribution of *B. pseudomallei* in northern Australia is still unclear. We developed and evaluated a direct soil *B. pseudomallei* DNA detection method based on the recently published real-time PCR targeting the *B. pseudomallei* type III secretion system. The method was evaluated by inoculating different soil types with *B. pseudomallei* dilution series and by comparing *B. pseudomallei* detection rate with culture-based detection rate for 104 randomly collected soil samples from the Darwin rural area in northern Australia. We found that direct soil *B. pseudomallei* DNA detection not only was substantially faster than culture but also proved to be more sensitive with no evident false-positive results. This assay provides a new tool to detect *B. pseudomallei* in soil samples in a fast and highly sensitive and specific manner and is applicable for large-scale *B. pseudomallei* environmental screening studies or in outbreak situations. Furthermore, analysis of the 104 collected soil samples revealed a significant association between *B. pseudomallei*-positive sites and the presence of animals at these locations and also with moist, reddish brown-to-reddish gray soils.

The soil-dwelling saprophyte *Burkholderia pseudomallei* is the causative agent of melioidosis, a potentially severe tropical disease occurring in humans and animals. Melioidosis is endemic in Southeast Asia and tropical northern Australia (5, 41). Disease manifestations are protean and range from subclinical infection to localized abscess formation to pneumonia to septicemia with fulminant septic shock. In northern Australia, annual incidence rates approach 20 cases per 100,000 with case fatality rates of 19% (7, 8). Previous studies have shown that proliferation of *B. pseudomallei* is dependent on high water content of the soil and that the bacteria are mainly transmitted by contact with wet contaminated soil or surface water, by either percutaneous inoculation or inhalation (4, 10). Due to its high mortality rates, resistance to many standard antibiotics, and potential transmission by aerosols, melioidosis was classified as a category B biothreat agent (24).

Despite the detection of *B. pseudomallei* in various water and soil samples from northern Australia (3, 9, 14, 16, 23), the environmental distribution of *B. pseudomallei* is still unclear, as indeed is the global distribution of *B. pseudomallei*. The gold standard for *B. pseudomallei* detection in soil is culture, which is time-consuming and takes up to 3 weeks for final results. Molecular detection techniques such as direct soil DNA isolation and PCR have been successfully applied to detect some soil bacteria (17, 18, 32, 48), but for *B. pseudomallei* such assays often lack, or provide only sparse, specificity data (3, 15, 28) or show reduced sensitivity (35). High specificity is critical as relatives of *B. pseudomallei* such as other *Burkholderia* bacteria or *Ralstonia* sp. strains are expected to also occur in the same environment. Sensitivity is decreased by insufficient DNA extraction efficiency due to incomplete cell lysis in the complex soil mixture and also DNA adsorption to soil particles (12, 20). Moreover, humic acids derived from decomposing organic material are ubiquitous in most soils and are potent PCR inhibitors (40).

We have developed and assessed a direct soil DNA isolation assay for detection of *B. pseudomallei* in soil based on the recently published real-time PCR targeting *orf*2 of the *B. pseudomallei* type III secretion system (TTS1) (27). TTS systems have been identified in several gram-negative pathogens such as *Shigella* and *Salmonella* strains as virulence determinants injecting effector molecules into host cells (44). TTS systems in *B. pseudomallei* were found to secrete the protein BopE, which facilitates *B. pseudomallei* invasion into epithelial cells (36). Several studies have shown that a 548-bp-long TTS segment (TTS1) encompassing part of *orf*2 is ubiquitously present in *B. pseudomallei* but not in close relatives such as *Burkholderia thailandensis* or *Burkholderia mallei* (29, 34, 42, 43). It was therefore identified as an attractive target for *B. pseudomallei* detection. Novak et al. (27) developed a TTS1 real-time PCR and evaluated it with 224 *B. pseudomallei* and 136 non-*B. pseudomallei* culture isolates including isolates of *B. thailandensis* and *B. mallei* and found a sensitivity and specificity of 100%.

The TTS1-based soil *B. pseudomallei* DNA detection
method proved not only to be substantially faster than culture but also more sensitive. We also detected significant correlations between *B. pseudomallei*-positive sites and some environmental factors.

**MATERIALS AND METHODS**

**Soil collection and identification of soil properties.** One hundred four soil samples were collected from random locations in the Darwin rural area (within a 33-km radius of Darwin [12°S]) in the Northern Territory of Australia in the dry season from July to August 2006. The dry season lasts from May to September with virtually no rain occurring. The geographic location of all soil sampling sites was recorded using a handheld global positioning system (GPS) unit (GPS Exterra, Garmin, KS). Soil sampling locations were recorded using GPS points and satellite imagery of the Top End of Australia were imported into ArcMap Geographic Information System version 9.1 (ESRI, CA). Sampling sites were randomly chosen within the Darwin rural area by using the “Random Point Generator” extension for ArcView 3.2. If locations were not accessible, the nearest accessible point was chosen. Fifty samples were collected from 25 holes at two depths, 10 and 30 cm. The remaining 54 samples were collected at a depth of 30 cm from 54 holes. Augers and spades were cleaned with 70% ethanol between soil collections, and the soil was collected in sterile 50-ml specimen containers. Soil texture was determined by following a common soil texture flowchart (http://cse.pdx.edu/forest/soil_texture_chart.htm) and the *Australian Soil and Land Survey Field Handbook* (22). According to increasing clay content, soils were grouped into “sandy loam to loam,” “sandy clay loam to clay loam,” and “light to heavy clay.” Soil color was interpreted with the help of the Munsell Soil Color Chart, which is based on the color dimensions of hue, value, and chroma (H/V/C). Colors were grouped as “grayish brown to black” (hue 10YR: 3/2, 3/3, 4/2, 4/3, and 5/2; hue 5YR: 5/3, 2/1, 3/1, and 4/1), “reddish brown” (hue 5YR: 2/2, 3/2, 3/3, 3/4, 4/4, 5/3, 5/4, and 6/4), “reddish gray” (hue 5YR: 5/2 and 4/2), “yellowish brown” (hue 10YR: 5/4, 4/4, and 3/4) and “yellowish red” (hue 5YR: 4/8 and 4/6). Water status of soil samples was determined with the help of the *Australian Soil and Land Survey Field Handbook* (22).

**Enrichment of *B. pseudomallei* in the soil.** Soil samples of 20 g were incubated in 20 ml of selective modified Ashdown's broth (1) (containing 15 g/liter Oxoid tryptone, 5 ml/liter 0.1% crystal violet, and 50 mg/liter colistin) for 39 h with shaking at 240 rpm at 37°C. The soil was then left to settle for 30 min, and the supernatant was transferred to 1 ml of CaCO₃-saturated water. After being shaken for 30 min at 35°C, the sample was centrifuged for 20 s at 3,400 × g and the supernatant was transferred to 0.8 mg of aurintricarboxylic acid (ATA). After being spun for 45 min at 4,300 × g, the supernatant was discarded and the soil pellet (0.5 to 0.8 g) was processed for DNA extraction.

**DNA extraction from soil pellet.** The soil pellet was processed with the UltraClean soil DNA isolation kit (MoBio Laboratories) with the following modifications. After addition of the detergent-containing buffer SI, the sample was incubated at 70°C for 10 min. After bead beating, 20 μl of proteinase K (20 mg/ml) was added to the sample and incubated for 1 h at 55°C. After elution, half of the amount of DNA was further purified with the QiAamp DNA Micro Kit (QiAGEN, Hilden, Germany) and eluted in 50 μl of AE buffer. Alternatively, if the real-time PCR still showed the presence of PCR inhibitors, the second half of the eluted DNA was further purified with the PowerClean DNA Clean-Up Kit (MoBio Laboratories) and real-time PCR was repeated.

**Cloning of external inhibitor control.** In order to detect PCR inhibitors in the real-time PCR, a linearized plasmid was constructed containing a 548-bp region encompassing the real-time PCR target sequence. This 548-bp region was amplified from *B. pseudomallei* genomic DNA of culture isolate MSHR186 by using primers BPTTSF and BPTTSR as described previously (43). Isolate MSHR186 was isolated from a goat in the rural Darwin region. Molecular cloning was done following standard protocols. Briefly, PCR product was purified and ligated into pGEM T Easy vector (Promega) and transformed into XL1-Blue cells (Stratagene) by electroporation. Plasmids were purified and linearized with 5 U of Scal by cutting the vector backbone once.

**Real-time PCR.** Real-time PCR targets a 115-bp stretch in orf2 of the TTS1 of *B. pseudomallei*. It was performed as described elsewhere with some minor modifications (27). Briefly, 4 μl of DNA (in 10 mM Tris HCl, 0.5 mM EDTA, pH 9.0) was amplified in duplicate in 25-μl volumes with 1 U HotStarTaq Plus DNA polymerase (QiAGEN) using final concentrations of 416 nM (each) primer, 256 nM probe labeled with 6-carboxyfluorescein and a black hole quencher (Biosearch Technologies), 6 mM MgCl₂, 0.2 mM deoxynucleoside triphosphates (including dUTP at a dUTP/dTTP ratio of 1:9), and 0.25 U uracil DNA glycosylase (Invitrogen). Nonacetylated bovine serum albumin at a final concentration of 400 ng/ml was added to bind PCR inhibitors such as humic acids (19). PCR conditions were as follows: an initial uracil DNA glycosylase incubation step at 37°C for 10 min followed by 94°C for 10 min and then 40 cycles of 94°C for 1 s and 60°C for 1 min. Nontemplate controls were added to each run and were always negative, i.e., no amplification was detected. Standard deviations of threshold cycle (*C*ₚ) values of soil sample duplicates ranged from 0.01 to 1.05 with a mean of 0.22. In order to check for PCR inhibitors, 0.3 pg of inhibitor control plasmid was amplified alone and in parallel spiked with 4 μl of sample DNA. If spiking resulted in an increase of ≤2 *C*ₚ values, which corresponds to two times the maximum standard deviation of duplicates and an approximate decrease of DNA yield of 3.7, the DNA was further purified as described above and real-time PCR was repeated. In each PCR run, the plasmid was also used as the standard positive control in a dilution series in duplicate and at final concentrations of 4.4 ng/ml, 217 pg/ml, and 11 pg/ml. *B. pseudomallei* inoculation of soil. Various soils from the Darwin area (light clay, sand, and loamy garden soil) were UV sterilized by exposing a thin layer of soil to a UV dose of more than 1,000 mW/m² in a 50-cm distance for 6 h with repeated shaking. No *B. pseudomallei* organisms were recovered from these soils by culture or by the direct soil DNA extraction method. Three questions were addressed by inoculation experiments. First, in order to establish the lowest limit of detection, these soils were inoculated in several independent experiments with serial dilutions of 0 to 4,800 CFU of *B. pseudomallei* (0 to 240 CFU/g soil) at 10 to 15 different concentrations with *B. pseudomallei* culture isolate MSHR186. Optical densities of the *B. pseudomallei* broth were measured at 600 nm, and the exact amount of *B. pseudomallei* CFU added to the soils was determined by standard plate counts in duplicate. Second, specificity was assessed by inoculating sand with 15 genetic relatives of *B. pseudomallei* at a concentration of 300 CFU/g soil. Third, in order to compare the sensitivity of soil DNA extraction with that of culture, sand was inoculated in parallel with serial dilutions of nine different concentrations from 5 to 100 CFU of *B. pseudomallei* (i.e., 0.3 to 5 CFU/g soil). Samples were blinded and processed for culture and soil DNA extraction. DNA extraction of all soils (20 g per sample) of these three sets of experiments was performed as described above, starting with 39 h of incubation at 37°C. The lowest limit of detection for sand and clay was also assessed for an enrichment of *B. pseudomallei* by culture and direct soil DNA extraction method. This method proved not only to be substantially faster than culture but also more sensitive. We also detected significant correlations between *B. pseudomallei*-positive sites and some environmental factors.

**TABLE 1. Detection rate of *B. pseudomallei* by culture versus soil DNA extraction and TTS1 real-time PCR method.*

<table>
<thead>
<tr>
<th>Soil type and depth (cm) at which collected</th>
<th>Total collected</th>
<th>No. of real-time PCR/culture result</th>
<th>Detecion rate (%) for method:</th>
<th>Culture</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sandy loam to loam</td>
<td>26</td>
<td>21</td>
<td>2</td>
<td>3</td>
<td>19.2</td>
</tr>
<tr>
<td>10</td>
<td>9</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>11.1</td>
</tr>
<tr>
<td>30</td>
<td>17</td>
<td>13</td>
<td>1</td>
<td>3</td>
<td>23.5</td>
</tr>
<tr>
<td>Sandy clay loam to clay loam</td>
<td>53</td>
<td>43</td>
<td>2</td>
<td>8</td>
<td>18.9</td>
</tr>
<tr>
<td>10</td>
<td>16</td>
<td>14</td>
<td>1</td>
<td>1</td>
<td>12.5</td>
</tr>
<tr>
<td>30</td>
<td>37</td>
<td>29</td>
<td>1</td>
<td>7</td>
<td>21.6</td>
</tr>
<tr>
<td>Light to heavy clay</td>
<td>25</td>
<td>20</td>
<td>3</td>
<td>2</td>
<td>20.0</td>
</tr>
<tr>
<td>10</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>30</td>
<td>25</td>
<td>20</td>
<td>3</td>
<td>2</td>
<td>20.0</td>
</tr>
<tr>
<td>Overall (all types)</td>
<td>104</td>
<td>84</td>
<td>7</td>
<td>13</td>
<td>19.2</td>
</tr>
<tr>
<td>10</td>
<td>25</td>
<td>22</td>
<td>1</td>
<td>1</td>
<td>12.0</td>
</tr>
<tr>
<td>30</td>
<td>79</td>
<td>62</td>
<td>5</td>
<td>12</td>
<td>21.5</td>
</tr>
</tbody>
</table>

* One hundred four soil samples were collected at a depth of 10 cm and 30 cm in the Darwin rural area. ND, not done.
the 104 soil samples were analyzed for associations between the occurrence of B. pseudomallei and different environmental factors. For 25 sites, a sample was collected at depths of both 10 and 30 cm. For comparison of broad vegetation class and animal occurrence, only one sample (from 30 cm) per site (hole) was included in order to avoid bias due to the inclusion of more than one sample for some holes. Broad vegetation class was categorized as either open terrain (grass, crops, or shrubs) or single trees and forest. Animal occurrence was further distinguished between domestic animals (dogs, pigs, horses, and chickens) and large native animals (mainly wallabies [family macropodidae]). The latter variable was defined as positive if resting wallabies were seen close by but mainly if droppings were found <1 m from the sampling site. Furthermore, all 104 soil samples were analyzed for soil factors such as water status, pH, soil color, and texture. Associations between soil pH and occurrence of B. pseudomallei or animal resting places were analyzed using a two-sample t-test with unequal variances. Logistic regression analyses and likelihood ratio tests were performed to calculate the odds ratio (OR) for occurrence of B. pseudomallei. The ORs were calculated and compared unadjusted or in multivariate logistic regression analyses, with adjustment for various factors such as soil water status, animals, soil pH, or soil color. All tests were two-tailed and considered significant if P values were smaller than 0.05.

RESULTS

Sensitivity and linear dynamic range of TTS1 real-time PCR. The detection limit of the TTS1 real-time PCR assay was found to be 15 fg of genomic DNA of B. pseudomallei isolate MSHR186 corresponding to two B. pseudomallei genome equivalents (GE) based on a B. pseudomallei genome size of 7.2 Mb and a GC content of 68% (26). Serial dilutions of B. pseudomallei genomic DNA over 6 orders of magnitude showed a linear dynamic range of 40 GE to 1 × 10^6 GE with a coefficient of determination (R^2) of 0.998 and an amplification efficiency of 92%. C_T values were in the linear range between 16 and 33.

Inoculation of different types of soil with B. pseudomallei. In order to establish the lowest limit of detection of the DNA extraction method, soil samples (20 g) representing different soil types in Darwin (i.e., light clay, sand, and loamy garden soil) were UV sterilized and inoculated with dilution series of 0 to 240 B. pseudomallei CFU/g soil. No B. pseudomallei organisms were detected (by culture and DNA extraction) in noninoculated, UV-sterilized soils. In sand and clay, the detection limit was 1 CFU of B. pseudomallei/g soil. Intermittently, samples with only 3 CFU B. pseudomallei added in total were positive. C_T values were in the range of 26 to 35. Loamy garden soil rich in decomposing organic material and humic acids showed a lower sensitivity of 1.5 CFU B. pseudomallei/g soil. Depending on the amount of inoculated B. pseudomallei, about 2 to 7 µg DNA was recovered from the approximately 0.5-g soil pellet after the soil supernatant was spun. Clay inoculation series showed a slightly higher yield of recovered B. pseudomallei DNA than did those of sand with an enrichment of 39 h (Fig. 1). The opposite was evident with an enrichment of 1 h. In order to compare growth rates of B. pseudomallei in clay and sand during the initial enrichment step of 39 h, sand and clay were inoculated with 3 CFU of B. pseudomallei/g soil. Inoculated soils were incubated in 25 ml of Ashdown's broth over 40 h, and at five time points 3 ml of supernatant was retrieved for culturing. Samples were blinded. Clay-derived samples were not only B. pseudomallei culture positive in a shorter period of time, indicating an increased amount of B. pseudomallei in these samples, but some sand samples did not grow B. pseudomallei at all (data not shown). This indicates that B. pseudomallei shows an increased growth rate in clay compared to that in sand in the initial enrichment step whereas sand shows a higher soil DNA extraction yield. The lowest limit of detection with 1 h of enrichment was 10 CFU/g soil.

Variable degrees of correlation were found in these inoculation dilution series with only sand showing a coefficient of determination (R^2) of >0.9 for 39 h and 1 h of enrichment (Fig. 1). In clay-containing samples, large precipitates of fine sediments were often observed during DNA extraction, reducing DNA solute volume and yield. In loamy soil, the presence of PCR inhibitors in some samples reduced the R^2. In order to check for the presence of PCR inhibitors, the linearized plasmid containing the real-time PCR target sequence was included in each run alone and in parallel spiked with soil DNA samples. Three percent of sand, 8% of clay, and 26% of garden loamy soil samples contained a detectable amount of PCR inhibitors as shown by a C_T value increase of >2 after the positive control was spiked. Sand showed a significantly smaller C_T value increase in spiked controls (median increase of 0.04) compared to clay with 0.27 and 0.40 for loamy soil (Mann-Whitney test between sand and clay, P = 0.005; sand and loam, P = 0.002). Samples with a C_T value increase of >2 in spiked positive controls were further purified as described above and in the following real-time PCR, fewer PCR inhibitors or none were detected in all samples, with C_T value increases of <2.

By comparing TTS1 copy numbers of B. pseudomallei-posi-
tive soil samples with the standard curves of the same broad soil type, a rough quantification is possible. Due to low R² values for clay- and loam-containing samples, this was done only for positive sandy samples (n = 4) and a median of 2.6 B. pseudomallei CFU/g soil was found (range, 0.3 to 411 CFU/g soil) in these soils.

Enrichment with Trypticase soy broth containing 10 mg/ml gentamicin was found to be only slightly inferior with a one- to threefold decrease of B. pseudomallei DNA yield compared to enrichment with Ashdown’s broth, whereas enrichment with distilled water was found to be considerably less sensitive with more-than-20-fold-less B. pseudomallei DNA.

Inoculation of soil with close genetic relatives of B. pseudomallei. When testing DNA of 136 non-B. pseudomallei culture isolates on the TTS1 real-time PCR, Novak et al. (27) found a specificity of 100%. We wanted to confirm that this high specificity is also applicable to DNA extracted from soil. Sandy soils inoculated with 6,000 CFU of 15 genetic relatives of B. pseudomallei were all negative by soil DNA extraction and real-time PCR. No PCR inhibitors were detected in these samples. These 15 culture isolates consisted of one Burkholderia thailandensis, three Burkholderia cepacia complex, three Burkholderia spp. cluster A, two Burkholderia spp. cluster B, two Cupriavidus spp., two Ralstonia taiwanensis, and two other Betaproteobacteria culture isolates.

Comparison of sensitivities of culture and DNA extraction method. All nine sandy soil samples which were inoculated with serial dilutions of 0.3 to 5 CFU of B. pseudomallei/g soil were positive when processed with the direct soil B. pseudomallei DNA detection protocol. However, culturing did not detect the lowest concentrated sample, which was inoculated with a total of 5 CFU of B. pseudomallei.

B. pseudomallei was detected in 13 of the 104 randomly collected soil samples from rural Darwin by both culture and soil DNA extraction methods. However, a further seven samples were positive by DNA extraction but not by culture. Six of these seven samples were also positive when they were screened with a B. pseudomallei-specific real-time PCR targeting the gene wcbG (30), which is involved in B. pseudomallei capsule production. The one sample which was not amplified with the latter assay was collected from the same hole as a B. pseudomallei culture-confirmed sample. A further three of the seven samples which were positive only by the soil DNA extraction method were also collected from the same holes as B. pseudomallei culture-positive samples. Two of these were shallower (10 cm) than the culture-confirmed B. pseudomallei-positive samples (30 cm). The other four soil DNA extraction-positive and culture-negative samples were collected within a radius of 100 m of culture-confirmed B. pseudomallei-positive sites. Around 5% of all samples contained a detectable amount of PCR inhibitors as shown by a Cₚ value increase of >2 after spiking of the positive control. These samples were further purified as described above, and in the following real-time PCR, fewer PCR inhibitors or none were detected with Cₚ value increase of <2. The soil texture of all samples containing traces of PCR inhibitors was “sandy clay loam” (50 to 80% sand, 20 to 35% clay, and up to 30% silt) (25). No samples were B. pseudomallei culture positive but real-time PCR negative.

Association of B. pseudomallei occurrence and environmental factors. The collected soil samples (n = 104) were analyzed for association between the occurrence of B. pseudomallei and different environmental factors such as broad vegetation class, occurrence of animals, or soil characteristics. For the analysis of the former two variables, only one sample per site (at a depth of 30 cm) was included, resulting in 17 B. pseudomallei-positive and 62 negative sites. Despite low sample numbers, we found a significant majority of B. pseudomallei-positive sites in open terrain (grass, crops, or shrubs) (88%) compared to B. pseudomallei-negative sites with 50% being open terrain (Fisher’s exact test, P = 0.005). Furthermore, a significant correlation between B. pseudomallei-positive sites and the occurrence of animals was detected. Ninety-four percent of B. pseudomallei-positive sites had either domestic or native animals in close proximity as opposed to 52% of negative sites (P = 0.001). If animals were further distinguished between domestic animals (dogs, pigs, or horses) and large native animals (mainly wallabies), we found a significant association of B. pseudomallei-positive sites with the occurrence of native animals (P = 0.004) but not with domestic animals (P = 0.41). We tested whether animal droppings and urine changed the pH of the soil and found a significantly lower pH at native animal resting places (median pH of 5; bootstrap estimate of standard error [SE], 0.14) compared with sites with no native animals (median pH of 5.5, SE of 0.08) (two-sample t test with unequal variances, P = 0.032). While pH was lower at B. pseudomallei-positive sites (median pH of 5, SE of 0.27) than at sites negative for the organism (pH of 5.5, SE of 0.06), the difference was nonsignificant statistically.

When comparing soil water status, texture, and color, all collected soil samples (20 positive and 84 negative sites) were included in the analysis, as soil characteristics often changed with increasing depth. This resulted in an additional 25 samples collected at a depth of 10 cm from holes from which a sample was also collected at 30 cm. A higher B. pseudomallei detection rate at the deeper level of 30 cm was evident but not significant. All three samples positive at 10 cm were also positive at 30 cm. The majority of B. pseudomallei-positive samples were retrieved from moist to wet soil (P = 0) (Fig. 2). Only 5% (n = 4) of B. pseudomallei-positive soil samples were dry in contrast to 71% of B. pseudomallei-negative samples. Three of these four B. pseudomallei-positive dry soil samples were collected at a depth of 30 cm and one was collected at 10 cm. Three of these sites were waterlogged in the wet season, and one was normally irrigated. The soil color “reddish gray” was significantly associated with the occurrence of B. pseudomallei (P = 0.012), as was the combination of “reddish gray” and “reddish brown” (P = 0.024) (Fig. 2). “Reddish gray” was also significantly associated with moist to wet soil (P = 0.003). Despite the association with soil color, we did not find a significant association of B. pseudomallei-positive sites with soil texture type when differentiating according to increasing clay content between “sandy loam to loam,” “sandy clay loam to clay loam,” and “light to heavy clay.”

Multivariate logistic analyses were performed for all environmental factors adjusting for moist to wet soil as confounder (Table 2). ORs for animals and wallabies were also adjusted for vegetation and soil pH while vegetation was also adjusted for the presence of animals. On multivariate analyses, the
occurrence of \( B. \) pseudomallei was still significantly associated with animals but no longer with open terrain. When results were adjusted for moist to wet soil, the soil color combination of “reddish gray” and “reddish brown” was still significantly associated with \( B. \) pseudomallei (Table 2).

**DISCUSSION**

In order to screen for \( B. \) pseudomallei in soil, a direct soil DNA extraction method was developed to detect \( B. \) pseudomallei DNA using a modified protocol from a recently published real-time PCR that targets the \( B. \) pseudomallei TTS1. By inoculating different types of soil with dilutions of \( B. \) pseudomallei, we determined a lowest limit of detection of 1 to 1.5 CFU of \( B. \) pseudomallei/g soil depending on the soil type. In studies conducted in Laos and Thailand, environmental \( B. \) pseudomallei load was assessed by using quantitative culture of soil samples. A concentration range of 10 to 1,200 CFU/g soil with a geometric mean of 39 CFU/g soil was found for the Laotian samples (45) and a median of 10 and 230 CFU/g soil for central and northeast Thailand, respectively (33). This is well within our detection range. In a comparison of the sensitivity of the DNA extraction method with that of culture in 104 randomly collected soil samples from the Darwin rural area, \( B. \) pseudomallei DNA was detected in 19.2% of DNA extraction samples compared with 12.5% of samples from culture. Inoculating soil with close genetic relatives of \( B. \) pseudomallei species produced no false-positive real-time PCR results.

This DNA extraction method aims to provide a fast, sensitive, and specific result for the presence or absence of \( B. \) pseudomallei in the soil. However, accurate quantification is

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**FIG. 2.** Comparison of soil profile (water status and soil color) of \( B. \) pseudomallei-positive and -negative sites. Percentages and labels in bold indicate statistically significant shifts of this factor between \( B. \) pseudomallei-negative and -positive sites \((P < 0.05, \text{two-tailed Fisher’s exact test})\). Significant shifts were found for water statuses “dry” \((P = 0)\) and “moderately moist” \((P = 0.001)\) and for soil colors “reddish gray” \((P = 0.012)\) and the combination of “reddish gray” and “reddish brown” \((P = 0.024)\).

**TABLE 2. ORs for occurrence of \( B. \) pseudomallei and different environmental factors**

<table>
<thead>
<tr>
<th>Factor associated with occurrence of ( B. ) pseudomallei</th>
<th>Adjustment status</th>
<th>OR</th>
<th>95% confidence interval</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open terrain</td>
<td>Unadjusted</td>
<td>7.5</td>
<td>1.58–35.59</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>Adjusted for moist to wet soil and animals</td>
<td>2.4</td>
<td>0.39–15.03</td>
<td>0.333</td>
</tr>
<tr>
<td>Presence of animals</td>
<td>Unadjusted</td>
<td>15.0</td>
<td>1.87–120.16</td>
<td>0.011</td>
</tr>
<tr>
<td></td>
<td>Adjusted for moist to wet soil, open terrain, and soil pH</td>
<td>9.3</td>
<td>1.08–79.93</td>
<td>0.012</td>
</tr>
<tr>
<td>Presence of wallabies</td>
<td>Unadjusted</td>
<td>6.0</td>
<td>1.88–18.86</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>Adjusted for moist to wet soil, open terrain, and soil pH</td>
<td>2.9</td>
<td>0.74–11.80</td>
<td>0.124</td>
</tr>
<tr>
<td>Soil water status: moist to wet soil</td>
<td>Unadjusted</td>
<td>9.4</td>
<td>2.87–31.07</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Adjusted for soil color “reddish brown to reddish gray”</td>
<td>10.5</td>
<td>3.05–36.03</td>
<td>0.000</td>
</tr>
<tr>
<td>Soil color: reddish brown to reddish gray</td>
<td>Unadjusted</td>
<td>3.8</td>
<td>1.18–12.37</td>
<td>0.026</td>
</tr>
<tr>
<td></td>
<td>Adjusted for moist to wet soil</td>
<td>4.5</td>
<td>1.27–16.12</td>
<td>0.012</td>
</tr>
</tbody>
</table>

* ORs are either unadjusted or adjusted in multivariate logistic regression analysis for moist to wet soil and, depending on factor, for animals, open terrain, soil pH, or soil color “reddish brown to reddish gray.” Values in bold indicate significant \( P \) values of <0.05.
difficult with this approach. Accuracy is impaired not only by the initial enrichment step with nonlinear bacterial growth and different growth rates in different soils but also by different DNA extraction efficiencies with different types of soils and possible PCR inhibitors. Spiking of soil samples with an internal control (17) can adjust for different DNA extraction efficiencies and PCR inhibitors. When different soil types were inoculated with serial dilutions of *B. pseudomallei*, only sand was found to have a satisfying coefficient of determination (without enrichment). Sand also showed a higher DNA extraction efficiency. Accurate quantification was often hampered by the presence of PCR inhibitors in loamy soil, whereas in clay-rich soils, large precipitates of fine sediments were often observed during soil DNA extraction, reducing DNA solute volume and yield. For *B. pseudomallei*-positive sandy soil samples (*n* = 4), we performed a rough quantitative analysis by comparing TTS1 copy numbers of these samples with the standard curve of inoculation series in sand. A median of <5 CFU/g soil was found in positive sandy soils. These soil samples were collected during the dry season when the incidence of melioidosis is drastically reduced. Sampling during the wet season will show whether and by how much *B. pseudomallei* load in soil changes with the seasons. Of 63 dry soil samples, only four were *B. pseudomallei* positive and all of these sites were either waterlogged in the wet season or normally irrigated. Hence, all *B. pseudomallei*-positive sites of this study were waterlogged, waterlogged during the wet season, or irrigated. A study in northeast Thailand with culturing of *B. pseudomallei* from soil during the dry season also found a strong association between *B. pseudomallei* occurrence and irrigated sites (46).

By using an external control for PCR inhibitors, we detected PCR inhibitors especially in humic acid-rich soil such as loamy garden soil. Depending on the amount of PCR inhibitors still present after the initial DNA elution, the eluted DNA was further purified by using either the QIAamp DNA Micro Kit (QIAGEN) or the PowerClean DNA Clean-Up Kit (MoBio Laboratories). Further DNA purification made a substantial difference by turning some *B. pseudomallei*-negative results into positive results (data not shown). Furthermore, the addition of nonacetylated bovine serum albumin to real-time PCR proved to be highly valuable by binding PCR inhibitors such as humic acids.

In order to speed up the process, it is possible to incubate samples for 24 h, as this was found to be only slightly inferior (*C*ₜ increase of <2.5 with an enrichment step of 24 h) (data not shown). An enrichment step of only 1 h resulted in a detection limit of *B. pseudomallei* of 10 CFU/g soil. No increase of sensitivity for these samples was found when a nested PCR protocol was applied with a primary PCR amplifying a 267-bp fragment encompassing the real-time PCR target sequence (data not shown).

The addition of ATA to the soil proved to increase DNA yield by 6- to 11-fold. ATA is an inhibitor of nucleases and has previously been successfully used in soil DNA extractions (2, 21). Calcium carbonate is known to break up soil particles and flocculate humic acids (11). We found that the additional soil incubation step at 55°C with 1 ml of calcium carbonate-saturated water increased soil DNA recovery by five to sevenfold. Despite the small number of soil samples, we found an association between the occurrence of *B. pseudomallei* and open terrain and the presence of animals. If animals were further subdivided into native animals (wallabies) and domestic animals, there was a significant association only with the former. Wallabies have rarely been reported to contract melioidosis (6), and our finding more likely reflects the higher probability of disturbed soil and the significantly lower soil pH at these places. The latter might be due to nitrification processes of the animal urine-derived ammonium (31). *B. pseudomallei* has been shown to prefer a more acidic environment (4, 39), and we also found a lower pH at *B. pseudomallei*-positive sites, though this was not statistically significant.

In inoculation experiments, we found a higher growth rate of *B. pseudomallei* in light clay than in sand. Clay has been shown previously to be associated with *B. pseudomallei* occurrence (38). Clay is known to be rich in bacteria due to clay's small pore size withholding nutrients and water and due to possible electrostatic interactions of clay particles with bacteria (37). Furthermore, additional components in clay such as iron-containing compounds might further support growth of *B. pseudomallei* in clay (47). Yellowish and reddish soil colors indicate oxidized iron components in the soil. Yellowish brown soil was found to be associated with the occurrence of *B. pseudomallei* (38), whereas we detected most *B. pseudomallei*-positive sites in reddish brown-to-reddish gray soil. Hence, the association with red soil might reflect iron-containing soil. Gray soil on the other hand indicates anaerobic soil found in areas with a high water table, which is consistent with the known association of *B. pseudomallei* and wet soil. Despite increased growth of *B. pseudomallei* in clay compared to sand when soils were inoculated, we did not find a correlation between *B. pseudomallei* occurrence and a certain type of soil texture in the 104 randomly collected soil samples. However, it is noteworthy that all *B. pseudomallei*-positive soil samples with high sand and low clay content were derived from drainage sites or areas which are waterlogged during the wet season.

The combination of selective enrichment, soil DNA extraction, and TTS1 real-time PCR is to our knowledge the most robust methodology for detection of *B. pseudomallei* in soil to date. This method is applicable for large screening studies to assess the distribution of *B. pseudomallei* in soil, and it is not dependent on well-trained microbiologists who are able to distinguish *B. pseudomallei* colonies from close relatives in the soil. It should also be useful in outbreak situations, enabling the potential source of contamination to be rapidly identified.

**ACKNOWLEDGMENTS**

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**REFERENCES**


