A randomized soil survey of the distribution of
Burkholderia pseudomallei in rice fields in Laos

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Running Title - Burkholderia pseudomallei in Lao rice fields
Melioidosis is a major cause of morbidity and mortality in Southeast Asia where the causative organism (*Burkholderia pseudomallei*) is present in the soil. In the Lao PDR (Laos), *B. pseudomallei* is a significant cause of sepsis around the Vientiane capital and has been isolated in soil around the city adjacent to the Mekong River. We explored whether *B. pseudomallei* occurs in Lao soil distant from the Mekong River, drawing three axes across northwest, northeast and southern Laos to create 9 sampling areas in 6 provinces. Within each sampling area a random rice field site was selected composed of a grid of 100 sampling points each 5m apart. Soil was obtained from a depth of 30 cm and cultured for *B. pseudomallei*. Four of 9 sites (44%) were positive for *B. pseudomallei*, including all 3 sites in Saravane Province, southern Laos. The highest isolation frequency was in east Saravane, where 94% of soil samples were *B. pseudomallei* positive with a geometric mean (95% CI; range) concentration of 464 (372-579; range, 25-10,850) cfu/g soil. At one site in northwest Laos (Luangnamtha), only one sample (1%) was positive for *B. pseudomallei* at a concentration of 80 cfu/g soil. Therefore *B. pseudomallei* occurs in Lao soils beyond the immediate vicinity of the Mekong River, alerting physicians to the likelihood of melioidosis in these areas. Further studies are needed to investigate potential climatic, soil and biological determinants of this heterogeneity.

Keywords: Geography, soil, *Burkholderia pseudomallei*, Laos, melioidosis
Introduction

Melioidosis is an infectious disease caused by *Burkholderia pseudomallei*, a Gram-negative saprophytic soil bacterium (2, 3, 25, 30). The majority of patients are reported in southeast Asia and Northern Australia (3, 25). Risk factors include being a rice farmer and having diabetes, chronic alcoholism, renal failure, and/or thalassemia. Most patients probably contract the infection by contact with contaminated soil and water (6, 15, 22). Septicemic melioidosis, even with optimum antibiotic treatment and intensive care, has a very high mortality (19-68%) (3). In northeast Thailand, where *B. pseudomallei* is found in ~50% of soil samples, the hospital admission incidence is 137.9/100,000 inhabitants (24) and represents 18% of patients admitted with community acquired septicemia (1).

The Lao PDR (Laos) is a small land-linked country of considerable environmental and ethnic diversity situated between Burma (Myanmar), Thailand, Cambodia, Vietnam and the People's Republic of China (PRC). Most of the western border is the Mekong River. Melioidosis was first identified in 1999 (17) and subsequently (to October 2010) 409 culture positive patients have been recorded in Vientiane, the capital (unpublished data). However, there is only one laboratory with a routine accessible diagnostic service for *B. pseudomallei* in Laos and the disease is probably under-recognized outside of the capital.

In a soil survey in 1998 36% of 110 soil samples collected in rice fields around the Vientiane capital contained *B. pseudomallei* (28). The reasons for the abundance of this organism in soil in adjoining NE Thailand and around Vientiane are not understood, but...
factors such as the physical and chemical properties of soil and/or interaction with other organisms and plants may be important (5, 9, 12, 13, 15). In Australia, proximity to a stream and moist soil rich in roots were independently associated with the presence of *B. pseudomallei* in soil (11). The majority of Lao patients (86%; unpublished data) diagnosed with melioidosis in Vientiane have houses in Vientiane capital and Vientiane Province which are close to the Mekong River. However, it is uncertain whether this represents the relative lack of diagnostic facilities elsewhere or whether *B. pseudomallei* does not occur in soil in the highlands of Laos, which cover the northwest, northeast and eastern aspects of the country. We therefore explored whether *B. pseudomallei* occurs in Lao soil distant from the Mekong River.

**Materials and Methods**

We defined sampling sites across Laos based on three transects (Fig 1). To facilitate this Vientiane and Saravane Provinces were divided into 2 (western and eastern) and 3 (western, central and eastern), respectively, areas of equal width (Fig 1). The transects were from western Vientiane Province to Oudomxay Province to Luangnamtha Province in the northwest (towards the Chinese border), from eastern Vientiane Province to Xiengkuang Province to Huaphanh Province in the northeast (towards the Vietnamese border), and from the Mekong River close to the Thai border in southern Laos across Saravane Province, towards the Vietnamese border, giving a total of 9 sampling areas. All areas, except that in Huaphanh, are in the watershed of the Mekong River.
Within each sampling area, one site was selected randomly according to all of the following criteria: (i) within a rice field of area $\geq 1$ hectare; (ii) $\leq 100$ m from a perennial river; (iii) $\geq 50$ m from a known area of unexploded ordnance (UXO); and (iv) $\geq 50$ m from buildings. The distribution of rice fields was determined from land use data (2001-2003; Forest Inventory and Planning Division (FIPD), Government of the Lao PDR) and photographs taken by satellite (Landsat 7 ETM. http://glovis.usgs.gov/) from 2008 to 2009. National Geographic Department (NGD, Government of Lao PDR) land use data were also used to finalize the choice to identify rice fields located $\leq 100$ m from river edges. Data from the Ministry of Communication, Transport and Post were used to confirm the absence of buildings in a proposed sampling site. Laos is the most heavily bombed country in the world (23) and UXO remains a major public health and economic problem. Therefore the digging of holes in eastern Laos poses important safety risks and to reduce the risk of detonating buried munitions, data from the Lao National Unexploded Ordnance programme were checked to ensure that the sampling site was $\geq 50$ m of a known bombing area. In addition, the local UXO clearance authorities were consulted during the field survey and the grid area checked by them if they considered this necessary. Soil data were provided by the National Agriculture and Forestry Research Institute (NAFRI), Government of Laos, from the GIS coordinates. ArcGIS (Environmental Systems Research Institute, Inc., Redlands, CA, www.esri.com.) was used to map these variables and perform the randomization with three points selected for each site, in case the 1st and 2nd were not accessible or appropriate. Each random point defined the location of the SE corner of a grid of 45x45m in which 100 soil samples were collected from holes dug 5 m apart. If the predetermined grid was not included in one rice field, or if the grid included habitats other than rice fields, the nearest point in the nearest rice field able to include the 45x45
A grid map was taken. The random sampling points were found using a GPS (Global Positioning System GARMIN GPS Map 60CSx). A tape measure was used to mark out the grid of 100 holes separated from each other by 5 m and 100 grams of soil removed from a depth of 30 cm (30). Oral consent for soil samples was obtained from the farmers concerned and they were compensated for any crop losses. Samples were maintained at ambient temperature during transport and processed within 48 hours. B. pseudomallei was cultured, identified by colonial morphology, latex agglutination test and antibiotic resistance to colistin and susceptibility to co-amoxiclav and quantitated for each soil sample as previously described (30). The 200-kDa exopolysaccharide latex agglutination test used is positive for B. pseudomallei but not for B. thailandensis (27).

Lao place name spellings follow Sisouphanthong & Taillard (20).

Results

Sampling was performed in June 2009 at the start of the rainy season. All sampling sites had to be moved to the nearest point in the nearest rice field able to include the 45x45 m grid, as the randomly selected rice field was too small (4 sites) or other habitats, such as a cemetery, forests or pools, were within the site (5 sites). B. pseudomallei was isolated from 4 of the 9 (44%) sites, including Luangnamtha Province in the northwest and all 3 sites in Saravane Province in the south (Figure 1, Table 1). The most easterly sampling point in Saravane Province, farthest (99km) from the Mekong River, had the highest number of positive soil samples (94/100), followed in frequency by the two other sampling sites in this province. By contrast, the site from which B. pseudomallei was isolated in Luangnamtha Province was positive for only 1/100 samples. The frequency of positive samples was mirrored to some extent by the quantitative count of B. pseudomallei, in that the sampling site in Saravane Province with the highest number of
positive samples had the highest geometric mean (95% CI; range) count of 464 (372-579; 25-10,850) cfu/g soil, exceeding those of western Saravane where 61% of holes were positive with geometric mean (95% CI; range) concentration of 82 (47-141; 2-22,250) cfu/g soil and central Saravane where 42% of holes were positive with a geometric mean (95% CI; range) concentration of 122 (65-227; 2-4,700) cfu/g soil. The mean concentration of *B. pseudomallei* at the 3 sites in Saravane were significantly different (ANOVA; p<0.001).

The elevation of sampling sites ranged from 140-581 m above mean sea level. The three sites with *B. pseudomallei* at high density were at relatively low elevation (140-191m) but the site at Luangnamtha with *B. pseudomallei* detected was at relatively high elevation (557m), unlike the other sites of intermediate elevation where *B. pseudomallei* was not detected (Fig 2, Table 1). Rainfall at the provincial capitals was of similar seasonality but highest in the south at Salavane, with high *B. pseudomallei* density, than in the north at Luangnamtha and Huaphanh, with low or zero *B. pseudomallei* density (Table 1). The gross soil type (8) at the three Saravane sites, with high *B. pseudomallei* soil density, were similar ferric soils to those in Vientiane Province where *B. pseudomallei* was not detected. The other 4 sites had markedly different soil types (Table 1).

**Discussion**

The results from this pilot study, the first randomized survey of *B. pseudomallei* distribution in soil in Asia (10), demonstrates that the organism is common in southern Lao soil and is not confined to soil in the immediate valley of the Mekong River. Saravane East, 99 km east of the Mekong River (Fig 2), had the highest geometric mean
B. pseudomallei soil density described in any survey to date. Interpretation of past soil surveys are hard to interpret if B. pseudomallei was not distinguished from B. thailandensis (18, 21, 24). B. pseudomallei soil concentration around the Lao capital Vientiane, in central Laos was relatively low with a mean (range) B. pseudomallei concentration of 90 (10-1,200) cfu/g (28). Notably, unlike the previous study (28) we did not find B. pseudomallei in soil at either site in Vientiane Province but, as recently discussed (12), the distribution of B. pseudomallei is very heterogeneous and the sample size is small. There is a tendency for the frequency of B. pseudomallei isolation in rice fields to be lower at higher latitudes (Fig 3). This suggestion could be confounded by different sampling and culture techniques and seasons of sampling in different studies and confusion with B. thailandensis. However, the northerly boundary of B. pseudomallei soil rice field frequency >10% is at approximately 20° N, which has been also suggested as the northern boundary for a high incidence of melioidosis (3). This pilot study has important limitations, including the fact that only 9 sites were sampled (of note the processing of 900 soil samples took 772 person-hours) and that the physical, biological and chemical characteristics of the soil samples and farming techniques were not investigated. However, these data suggest that health workers in NE and NW Laos may be able to put melioidosis lower on their differential diagnosis of sepsis than in southern Laos. There is no accessible local blood culture service in Saravane Province but these data strongly suggest that melioidosis will be an important public health problem. This has important economic implications given the high relative cost of empirical ceftazidime therapy (17). The factors determining the geographical distribution of B. pseudomallei in soil are not understood. The gross soil type was not related to the distribution of B. pseudomallei in soil in Laos. However, there is relatively little published information on the comparative chemistry and ecology of Lao soil (7,
19). In Thailand there is evidence that acidic rice field pH may be associated with the presence of *B. pseudomallei* in soil (14, 15). Further work investigating the distribution of this organism in relation to climatic, physical, chemical, botanical features are farming practices in diverse parts of Laos are needed and would inform public health decisions about this severe disease.

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Table 1: Geographical and climatic features and distribution of *B. pseudomallei* at 9 sampling sites in Laos. Soil description follows (8).

*Rainfall data, courtesy of the Department of Meteorology and Hydrology, Lao PDR, is from the provincial capital and hence only listed once for each province sampled.*

<table>
<thead>
<tr>
<th>Province</th>
<th>Sub-division</th>
<th>Nearest Village</th>
<th>Elevation (Meters)</th>
<th>Latitude</th>
<th>Longitude</th>
<th>Rainfall in 2009/mm/year*</th>
<th>Soil</th>
<th>Soil Description</th>
<th>Sample holes positive (%)</th>
<th>Geometric Mean cfu/g soil (95% CI)</th>
<th>Minimum-Maximum cfu/g soil</th>
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<tbody>
<tr>
<td>Saravane</td>
<td>West</td>
<td>NATANE</td>
<td>140</td>
<td>15° 26' 10.1&quot; N</td>
<td>105° 47' 21.0&quot; E</td>
<td>61</td>
<td>Ferric ACRISOLS</td>
<td>‘Not strongly humic; showing ferric properties within 125 cm of the surface; lacking plinthite within 125 cm of the surface; lacking gleyic properties within 100 cm of the surface.’</td>
<td>82 (47-141)</td>
<td>2-22,250</td>
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<td>Location</td>
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<td>Central</td>
<td>NADOU GNAI</td>
<td>177</td>
<td>15° 55' 33.0&quot; N 106° 14' 28.9&quot; E</td>
<td>Ferric LUVISOLS (Ferric properties within 125 cm of the surface; lacking an albic E horizon; lacking plinthite within 125 cm of the surface; lacking gleyic and stagnic properties within 100 cm of the surface)</td>
<td>42</td>
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<tr>
<td>East</td>
<td>PON TONG</td>
<td>191</td>
<td>15° 41' 31.3&quot; N 106° 29' 56.5&quot; E</td>
<td>Ferric ALISOLS (Not strongly humic; showing ferric properties within 125 cm of the surface; lacking plinthite within 125 cm of the surface; lacking gleyic and stagnic properties within 100 cm of the surface)</td>
<td>94</td>
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<td>Location</td>
<td>Town</td>
<td>Code</td>
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<td>Elevation (m)</td>
<td>Soil Type</td>
<td>Description</td>
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<tr>
<td>Vientiane</td>
<td>West XAMFON</td>
<td>385</td>
<td>19° 07′ 49.5″ N 102° 11′ 32.9″ E</td>
<td>1,483</td>
<td>Ferric LUVISOLS</td>
<td>As above</td>
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<td></td>
<td>East NAHONG</td>
<td>407</td>
<td>19° 12′ 46.3″ N 102° 14′ 20.6″ E</td>
<td>1,483</td>
<td>Ferric ALISOLS</td>
<td>As above</td>
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<tr>
<td>Luangnamtha</td>
<td>THONG CHIATAI</td>
<td>557</td>
<td>21° 00′ 13.1″ N 101° 25′ 02.2″ E</td>
<td>1,415</td>
<td>Gleyic LIxisols</td>
<td>Gleyic properties within 100 cm of surface.</td>
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<tr>
<td>Udomxay</td>
<td>NABORN</td>
<td>513</td>
<td>20° 16′ 40.3″ N 101° 36′ 00.8″ E</td>
<td>1,340</td>
<td>Calcareous CAMBISOLS</td>
<td>Ochric and are calcareous at least between 20 and 50 cm from the surface; lacking vertic properties; lacking gleyic properties within 100 cm of the surface.</td>
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<tr>
<td>Location</td>
<td>Code</td>
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<td>Latitude</td>
<td>Longitude</td>
<td>Soil Type</td>
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<tr>
<td>Xiengkhuan</td>
<td>SAN 581</td>
<td>1,318</td>
<td>19° 37' 10.7&quot; N</td>
<td>103° 33' 23.0&quot; E</td>
<td>Eutric CAMBISOLS</td>
<td>&quot;Not strong brown to red, lacking ferralic properties in the cambic; lacking gleyic properties within 100 cm of the surface&quot;</td>
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<tr>
<td>Huaphanh</td>
<td>XAMTAI 323</td>
<td>1,264</td>
<td>19° 59' 31.9&quot; N</td>
<td>104° 37' 50.5&quot; E</td>
<td>Eutric CAMBISOLS</td>
<td>As above</td>
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**Figure 1**: The distribution of *Burkholderia pseudomallei* at 9 sampling sites in Laos, on November 12, 2017.
2009. ● = positive and o = negative for B. pseudomallei. The blue line represents the course of the Mekong River.
**Figure 2:** The presence of *B. pseudomallei* in relation to elevation and distance from the Mekong: A = Northeast, B = Northwest, C = South. The vertical bar at the right end of the topographical line represents the international Lao border.

![Map of SE Asia showing the percentage of soil samples positive for *B. pseudomallei* at different sampling sites in rice fields. From Parry *et al.* (16), Wuthiekanun *et al.* (27), Wuthiekanun *et al.* (28), Wuthiekanun *et al.* (29) and this study. The horizontal dashed line is at 20°N. Only studies in which *B. pseudomallei* has been distinguished from *B. thailandensis* are included.](image-url)