Use of geostatistical tools to define strategies for environmental sampling of *Burkholderia pseudomallei* in soil

Running head: environmental sampling for *B. pseudomallei*

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Abstract

Background:

Melioidosis is a frequently fatal infectious disease caused by the soil dwelling Gram-negative bacterium *Burkholderia pseudomallei*. Environmental sampling is important to identify geographical distribution of the organism and related risk of infection to humans and livestock. The aim of this study was to use geostatistical tools to explore appropriate strategies for environmental sampling for *B. pseudomallei*.

Methods and Findings:

A fixed-interval sampling strategy was used as the basis for detection and quantitation by culture of *B. pseudomallei* in soil in two natural sites (disused land and rice field) in northeast Thailand. Spatial analysis was used to evaluate the distribution of *B. pseudomallei* and its relationship with range between sampling plots. *B. pseudomallei* was present on culture of 80/100 plot samples (80%) taken from disused land and 28/100 plot samples (28%) from the rice field. The median *B. pseudomallei* cfu/gram from positive sampling plots was 377.5 and 700, for disused land and rice field, respectively (p=0.17). “Hot-spots” of *B. pseudomallei* surrounded by areas of lower or negative bacterial count were observed in both sites. Spatial correlation in quantitative *B. pseudomallei* count was present for lag distances of 11.4 meters in disused land and 7.5 meters in rice field.

Conclusions:

We discuss the implications of the uneven distribution of *B. pseudomallei* in soil for future environmental studies, and describe a range of established geostatistical sampling approaches that would be suitable for the study of *B. pseudomallei* that take account of our findings.

Non-technical summary

Melioidosis is a severe and often fatal infection seen most often in Southeast Asia and northern Australia. The cause is an organism that exists in the soil called *Burkholderia pseudomallei*. The purpose of this study was to define the presence and amount of *B. pseudomallei* in soil in a rice paddy and an area of disused land in northeast Thailand, and to use these data to explore optimal sampling strategies. Soil sampling was performed using a fixed-interval grid of 100 sampling plots in each of the two sites, and the presence and amount of *B. pseudomallei* determined using culture. Mapping of *B. pseudomallei* counts demonstrated 'hot-spots' of variable size in both sites. Sampling plots with a high density of *B. pseudomallei* were surrounded by plots with a lower bacterial count, with a progressive step down in count moving out towards the edge of each bacterial 'island'. The proportion of sampling plots positive for *B. pseudomallei* was lower in the rice field than disused land (28% versus 80%, respectively), and the *B. pseudomallei* density moving outward from a peak count decreased more sharply in the rice field than in the disused land. These data were used as the basis for a discussion of the suitability of a range of sampling strategies in different geographical locations and for different study objectives.

Introduction

The Gram-negative bacterium *Burkholderia pseudomallei* is the cause of melioidosis and a category B select agent. This organism is present in soil and water across much of southeast Asia and in northern Australia and is increasingly being detected elsewhere, including areas of South America [1]. Melioidosis occurs as a result of exposure to environments containing *B. pseudomallei.* The route of infection is likely to be through direct skin inoculation or contamination of wounds, and more rarely by inhalation and ingestion [2]. Environmental sampling has been widely used to determine the presence of *B. pseudomallei* in an effort to identify geographical distribution of the organism and related risk of infection to humans and livestock [3–5]. *B. pseudomallei* has also been sampled from the environment to define the population genetic structure of the organism, to compare this with isolates associated with disease, and during outbreak investigations [6–8]. Environmental sampling would be a crucial component of the investigation into deliberate release of the organism associated with bioterrorist activity [9–11].

Despite its importance as a cause of natural disease and a bio-threat agent, there is limited evidence on which to base sampling strategies for *B. pseudomallei* that minimize the probability of error. Although the distribution of *B. pseudomallei* in soil is poorly understood, studies of other environmental organisms have demonstrated that soil structure and composition is very heterogeneous and that bacteria present in the natural environment are not uniformly distributed in soil [12–17]. These studies used a range of approaches including direct microscopy [12,13], determination of total biomass [14,15], or bacterial abundance determined by amplified fragment length polymorphism (AFLP) on whole-community DNA extracts [16,17]. Evidence of aggregation in soil bacteria comes from studies using biological soil thin-sections and bacterial visualization using microscopy, which have reported that the degree of aggregation varies depending on soil depth [12,13]. A recent study focused primarily on genotyping of environmental *B. pseudomallei* that used a fixed-interval sampling strategy reported an uneven distribution of this organism across the study site [18]. A key question, therefore, is how to limit the probability of error that arises as a result of variable distribution of *B. pseudomallei* across the sampling site. This is likely to be crucial when the presence of *B. pseudomallei*

is sparse, the organism is present in foci surrounded by areas that do not contain the organism rather than being evenly distributed, and when the distance between positive foci is large.

The variogram is a geostatistical tool for determination of the range over which measurements of soil properties are related [19]. The variogram has been used to define the range over which counts of specific environmental bacteria were related, and has been reported to range from micrometers to several metres [13,16,20]. Using AFLP DNA fingerprinting of extracted whole-community DNA, Franklin and Mills found that the spatial correlation of relative similarity of soil DNA ranged from 30 cm to more than 6 metres [16]. Using direct observation by microscopy of biological soil thin-sections, Nunan *et al* found bacterial hot spots with a range of spatial correlation of 1 mm and below [20]. However, no information has been published on the spatial correlation of *B. pseudomallei* in soil.

The aim of this study was to apply geostatistical and statistical tools to datasets from two large environmental sampling studies that defined the presence and quantitation of *B. pseudomallei* in disused land [21] and a rice field in nearby regions of northeast Thailand. Spatial correlation was defined using variograms and the data used as a basis on which to explore appropriate environmental sampling strategies for the presence of *B. pseudomallei*.

Materials and Methods

Study sites. Soil samples were collected from two locations situated in a rural rice-growing region in Ubon Ratchathani province, northeast Thailand. Sampling of an area of disused land situated to one side of road 231 in Amphoe Meung was performed in September 2005 (the rainy season), as previously described [21]. The site ran parallel to a tarmac road with a brick wall forming the distal boundary, was covered with low-lying scrub and showed no signs of cultivation. Sampling of an area of rice field situated in Amphoe Lao Sua Kok (a distance of 19 km southwest from the disused land) was performed in May 2007 (start of the rainy season). This site has been used for rice cultivation for more than 25 years and was isolated by raise earth walkways on three

sides, and by a dirt road on the fourth side. Both sites were wet but not flooded at the time of sampling. The soil type was sandy loam in both sites.

Sampling strategy. The disused land site was rectangular and its restricted size demanded a rectangular experimental grid. A grid comprising 5 x 20 plots placed 2.5 m apart on the vertical axis and 1.25 m apart on the horizontal axis (237.5 m^2) was marked out using string and wooden stakes. Plots were referenced using letters (A to E for horizontal rows viewed with back against the road, row A lying closest to the wall), and numbers (1 to 20 from left to right on the vertical axis). The rice field was several acres in size and a square experimental grid was sited in one corner of the field. A grid comprising 10 x 10 plots each measuring 2.5 m by 2.5 m (506.25 m^2) was marked out using string and wooden stakes. Sampling plots were also referenced using letters (A to I for horizontal rows as viewed with back against the earth walkway, row A lying closet to the dirt road) and numbers (1 to 10 from left to right). At each plot, a hole was dug using a clean spade to a depth of approximately 30 centimetres. A clean plastic bag was placed on weighing scales and a sample of soil (100 grams) was removed from the base of the hole and processed as previously described [21]. The spade was cleaned by alcohol before and after sampling at each point.

Soil culture and *B. pseudomallei* identification and quantitation. Soil was cultured for the presence of *B. pseudomallei* as previously described [21]. Colonies of *B. pseudomallei* were initially identified on the basis of colony morphotypes [22]. Colonies suspected to be *B. pseudomallei* were tested using the oxidase test, and positive colonies confirmed as *B. pseudomallei* using a highly specific latex agglutination test (positive for *B. pseudomallei* but negative for *B. thailandensis)* [23]. Following confirmation of bacterial identity, colonies with an identical morphotype on a given agar plate were considered to represent *B. pseudomallei*, and a colony count performed to allow calculation of the number of *B. pseudomallei* colonies per gram of soil at each sampling point. The lower and upper limit of detection of the methodology were 1 to $\geq$10,000 CFU/gm soil, respectively. Proportions were compared by the Chi-square test and bacterial counts were compared using the Wilcoxon-Mann-Whitney test.

Analysis of spatial correlation. Spatial correlation was analysed to quantify the relationship between *B. pseudomallei* count and lag distance between sampling plots. *B. pseudomallei* count was log transformed to reduce the effect of skew. Variance of log CFU count was calculated between each pair of sampling plots and graphed versus lag distance to produce a variogram. The formula used for the variogram was one half of the squared difference of all pairs of observations that are the distance (*h*) apart, as follows:

$$\gamma(\mathrm{h}) = \frac{1}{2 \mid \mathrm{N(h)} \mid} \sum_{N(h)} \mid Z(u_i) - Z(u_j) \mid^2$$

Figure 1 demonstrates a theoretical variogram. The line of the variogram generally rises from the origin to an upper asymptote called the *sill*. The sill indicates that all of the variation at this level of investigation has been encompassed. The distance at which this occurs is called the *range* of spatial correlation or the limit of spatial dependence. The variogram may have a positive value at the same point (lag distance equals zero), which is known as *nugget* variance; this indicates the spatial correlation over distances less than the smallest sampling interval, any measurement error, or purely random variation. The nugget/sill ratio was used to determine nugget effect on overall variability. The variogram was modelled using the Gaussian equation, as follows:

$$\gamma(\mathrm{h}) = C_0 + C_1[1 - \exp\{-3\frac{\| h \|}{a}\}]$$

C_0 is a parameter quantifying the nugget effect, C_1 is a spatially structured component of the model, and *a* is the range. The Gaussian model was fitted with non-linear least square regressions. Variograms were also computed for the E-W and N-W directions in order to determine whether the pattern of spatial variability changed with direction in the field.

All analyses were calculated using Stata 9.0 (College Station, Texas, United States) and S-plus 6.0 with Spatial Stats module (Insightful Corp, Seattle, United States).

Results

Presence and quantity of *B. pseudomallei* in two sampling sites. A total of 80 (80%) sampling plots in the disused land site were culture positive for *B. pseudomallei,* compared with 28 (28%) positive plots in the rice field site ($p<0.001$, Chi-square test). The median *B. pseudomallei* count for disused land was 377.5 cfu/gram soil (range 1 to >10,000, interquartile range (IQR) 55 to 1119), while the median count for the rice field was 700 cfu/gram soil (range 10 to >10,000, IQR 50 to 2810). There was no difference in *B. pseudomallei* count in the positive plots of the two sites ($p=0.17$, Mann-Whitney test). *B. thailandensis* was not detected in either field.

Mapping of *B. pseudomallei* distribution Mapping of log *B. pseudomallei* counts demonstrated 'hot-spots' of variable spatial size in both sites (Figure 2). Sampling plots with a high density of *B. pseudomallei* were surrounded by plots with a lower bacterial count, with a progressive step down in count moving out towards the edge of each bacterial 'island'. The highest density of *B. pseudomallei* (>10,000 CFU/gram) was similar in both fields, but the bacterial count demonstrated a more rapid decrease from the plot with the highest count in the rice field compared with disused land. The size of the bacterial 'island' was also smaller in the rice field. The degree of asymmetry in bacterial distribution (skew) was higher in the rice field than in disused land.

Spatial correlation. Spatial correlation of log *B. pseudomallei* counts in disused land and the rice field was present for up to 11.4 meters and 7.5 meters of lag distance, respectively, as demonstrated by the variogram shown in Figure 3. Directional variogram analysis showed that the patterns of spatial variability were not significantly different with direction in either field (data not shown). Nugget/sill ratios were calculated. A value approaching 1.0 indicate that a large degree of the variability is associated with the within sample measurements, and that relatedness between spatially separated measurements is limited. A value close to zero indicates that the relatedness of spatially separated measurements within the range is strong. The nugget/sill ratios were 0.50 and 0.52 for disused land and rice field, respectively, indicating a moderate degree of spatial dependence at both sites.

Sampling Strategy. Findings from our geostatistical analysis were combined with a statistical approach to develop a sampling strategy for the detection of *B. pseudomallei* in soil. The presence of *B. pseudomallei* in the soil is hazardous and of the potential errors associated with soil sampling, we considered a false negative result as the one of greatest significance. In light of this, we considered the optimal sampling number based on the reliability of a negative culture result, as defined by the exact 95% binomial confidence interval (CI). For example, if only 10 samples are randomly taken in one area and all are negative, the exact 95% binomial CI ranges from 0 to 30.8%; thus, the probability of a false negative is high. If 100 samples are taken, the exact 95% binomial CI is 0 to 3.6%, and if 1000 samples are taken the CI is 0 to 0.4%, and so on. The number of samples actually taken can then be based upon the balance between feasibility of sample size and the acceptable degree of inaccuracy (false negativity). This approach is applicable in regions where the positivity rate is likely to be high (such as northeast Thailand), and low (for example, Southern Thailand and Northern Australia). The quantitative count data defined in this study allowed us to determine the optimal distance between sampling plots. This is based on an established guideline that the optimal distance between sampling points should be half the range of the correlation observed in the variogram [24]. For northeast Thailand, we propose that the distance between sampling points during future studies should be 5 meters (range 4 to 6 meters).

Discussion

This study has demonstrated that *B. pseudomallei* is unequally distributed in the soil and forms bacterial hot-spots, in which an area of high bacterial density is surrounded by regions of decreasing bacterial density with a step down in count towards the edge of the bacterial 'island'. These hot-spots were commonly surrounded by areas that were culture negative for *B. pseudomallei*. This finding is consistent with previous studies of other microorganisms present in soil [12,16]. Several explanations have been proposed for this phenomenon. Bacterial communities may be affected by an uneven distribution of organic matter from which soluble compounds are diffusing, with bacterial density greatest close to the organic matter [25]. Another possibility is that the spatial

pattern may reflect an effect of regulation within the bacterial community itself, in which release of specific bacterial factors involved in bacterial gene regulation influences growth within the community. This is supported by a growing body of evidence that bacterial quorum-sensing occurs in soil [26–28].

This study also demonstrated a marked difference in the proportion of samples positive for *B. pseudomallei* in the disused land site versus the rice field site, despite the observation that the *B. pseudomallei* count per gram of soil at positive spots were equivalent in the two sites. This is reflected in the shorter range of spatial correlation for the rice field compared with disused land (7.5 meters versus 11.4 meters). Rice fields undergo repeated flooding, ploughing, planting, rice stubble burning and the application of chemical fertilizers and pesticides. *B. pseudomallei* may also be influenced by the presence of rice. A difference in bacterial communities present in rice field and unused land has been described previously in regions where *B. pseudomallei* was not present, and agricultural practices have been reported to lead to reversible changes in the community structure of environmental *Burkholderia* species other than *B. pseudomallei* [29,30]. In addition, the proportion of *Burkholderia* strains present in soil with antagonistic activity against *Rhizoctonia solania* AG-3, a plant pathogenic fungus, has been shown to decrease in association with agricultural management [30].

Our study was based on a fixed-interval sampling strategy which was used for its simplicity, and because this is a necessary strategy for the generation of variograms [31]. Alternative sampling strategies have been described, including random sampling, stratified sampling, adaptive sampling and multistage sampling [32]. The most appropriate sampling strategy will depend on the objectives of the study, and whether any information is already available for the geographical area to be sampled. In stratified sampling, the experimental area is divided into zones or strata and unequal numbers of samples are taken from each stratum. Stratified sampling may be used where sampling areas differ greatly or prior information indicates that suggests the *B. pseudomallei* prevalence varies across the study area. Adaptive sampling is a suitable approach for the detection of *B. pseudomallei* in an area where the presence and/or distribution of *B. pseudomallei* is unknown, and is based on a stepped approach to sampling. For example, a pilot could be performed in a defined experimental area in which 20 random plots are

sampled and tested for the presence of *B. pseudomallei*. If any sampling plot is positive for *B. pseudomallei*, this confirms the presence of the organism and therefore an area of risk to humans and livestock. If no sampling plots are positive for *B. pseudomallei*, a second round of sampling is done in which a larger number of random plots are sampled in the same area, the number taken being a function of the level of statistical confidence required. Based on our data, we recommend that a minimum of 100 sampling points be taken during stage 2 in the event that the first round of sampling is negative.

Random sampling with ad hoc strategies has been used previously to define the presence of *B. pseudomallei* throughout southeast Asia and northern Australia [3–5,33,34]. The methodology for sampling including calculations or justification of sample size was not usually specified, and the number and position of sampling plots were probably selected on an ad hoc basis. This has provided important information on geographical areas of positivity and has shown that *B. pseudomallei* load is higher in areas of south Asia including northeast Thailand and Laos than in northern Australia. However, the sampling strategies such as those used previously in Thailand in which a small number of sampling plots were tested per field and/or distances between the plots were short could potentially underestimate the geographical distribution of positive sites in an area of low prevalence. We propose that multistage sampling would be suitable to determine geographical distribution of *B. pseudomallei* within a region such as the province of Ubon Ratchathani. This sampling method involves primary sample units and subsamples. Defined areas of land (the primary sample unit) are selected from the entire region using a sample size calculation. Each experimental area is then sampled using an adequate number of plots (for example, n=100) using a sampling grid size (in northeast Thailand) of 5 meters. Sampling strategies could be based on random sampling, fixed-interval sampling, stratified sampling or adaptive sampling, as described above. This extensive dataset would give a broad insight into the distribution of *B. pseudomallei* across this region.

The optimal grid size is calculated from half of the range of spatial correlation and this calculation is based on the theory of information [24]. Variability in *B. pseudomallei* count in soil within and between different countries is well described [3–5], and the proposed sampling distance may not hold true in areas where the predicted *B.*

pseudomallei count in soil is markedly different from that found in this study, since the range of correlation is likely to differ [16]. To evaluate the range of spatial correlation in different regions in order to determine the optimal sampling grid size, fixed-interval sampling of at least 100 sampling points is required [31].

This study has focused on the sampling design (the strategy of placement and the number of samples), but of equal importance is the sampling technique. We detected *B. pseudomallei* by a culture method with a detection range of 1 CFU/gm to >10,000 CFU/gm of soil. The methodology involved the addition of water to the soil sample followed by vigorous manual mixing and overnight sedimentation, after which the supernatant was removed for culture. This may underestimate the true number of founder organisms, since bacteria in macro-aggregates may remain in the soil fraction, and bacteria may sediment back into the soil fraction. It is also possible that *B. pseudomallei* may either replicate or die during the overnight sample preparation, and some strains may not grow well in standard laboratory culture media. Further studies are required to optimize the culture strategy for *B. pseudomallei*, and to define the role of molecular detection techniques such as PCR. DNA extraction from soil followed by detection of *B. pseudomallei* by real-time PCR has been reported to be more sensitive than culture [35], and further studies are required in which PCR is evaluated in a range of geographical settings. The possibility that soil from multiple sampling points in a field could be bulked and DNA extracted and tested as a single sample would allow for more rapid coverage of a potentially large sample size [30].

This is the first published study to apply geostatistical tools to the study of environmental *B. pseudomallei*. Our data are likely to be specific to northeast Thailand rather than the rest of the world, and our focus was primarily the issue of false negatives. As such, it does not attempt to determine optimal strategies necessary for all applications, such as the strategy necessary to obtain an unbiased snapshot of bacterial population genetic structure. However, our findings have major implications for future environmental studies of *B. pseudomallei*, and highlight both the critical importance of study design and methodological approach and the need for further studies in this area.

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Competing Interests

The authors have declared that no competing interests exist.

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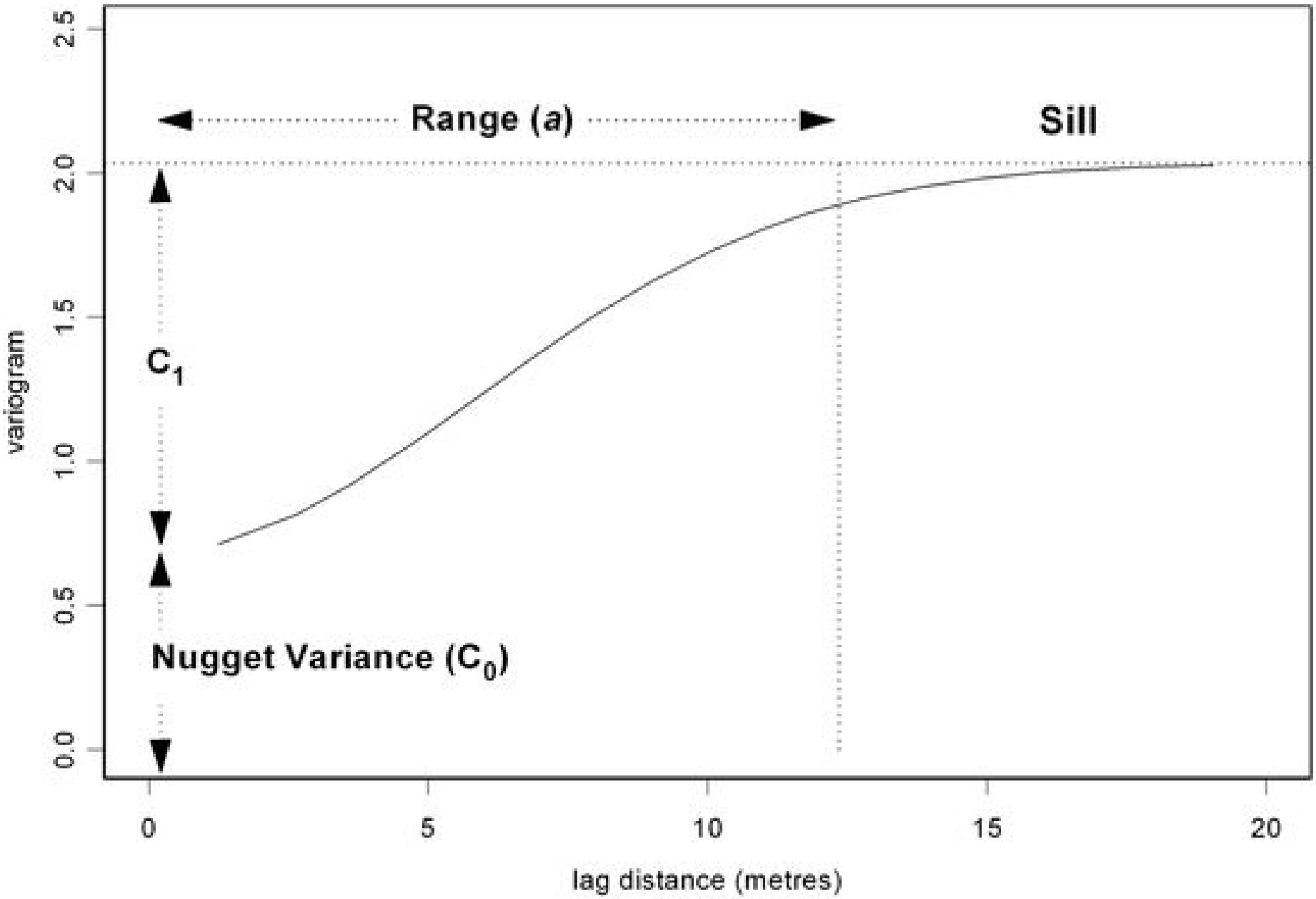
Figure legends

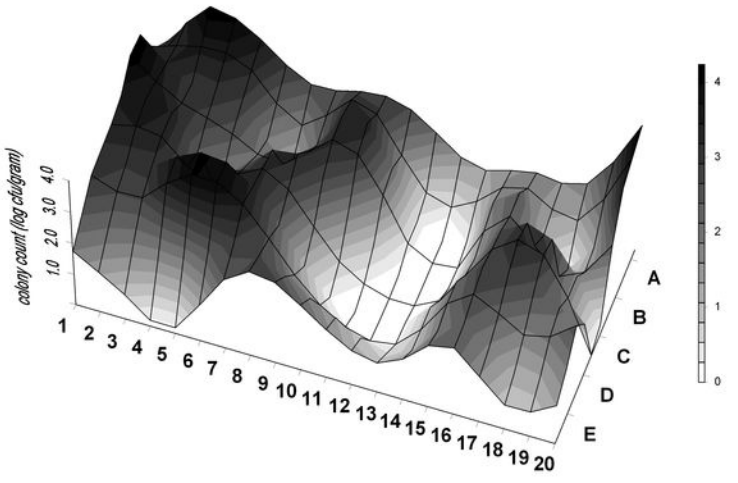
Figure 1. Theoretical variogram showing an increase in variance as lag distance increases within *range (a)* (spatial correlation), and upper asymptote (*sill*) as lag distance exceeds the range (no spatial correlation). The *nugget* variance (C_0) exhibits a positive variance at lag distance 0. The variogram has range of correlation at 12 meters, nugget variance 0.7, and sill 1.8.

Figure 2. Presence and count (log cfu/gm soil) of *B. pseudomallei* in 100 spaced sampling plots within an area of 237.5 m^2 of disused land (2A) and 506.25 m^2 of a rice field (2B). The gray scale represents the amount of *B. pseudomallei* in soil, with pure white indicating a point negative for *B. pseudomallei*.

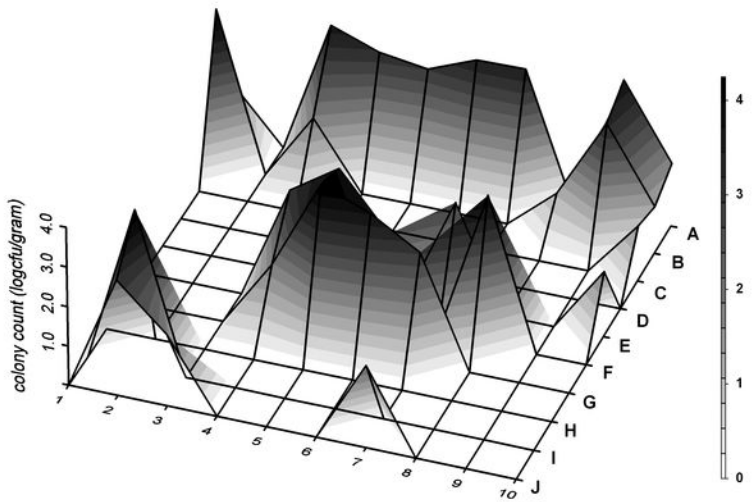
Figure 3. Variograms for quantitation of *B. pseudomallei* (log cfu/gm soil) over the lag distance (in meters). The solid line represents the fitted Gaussian model. In the disused land (3A), *range* of correlation is up to 11.4 meters, *nugget* variance is 0.96, and sill is 1.91. In the rice field (3B), *range* of correlation is up to 7.5 meters, *nugget* variance is 1.05, and sill is 2.04.


Range (a)
Sill
C1
Nugget Variance (C0)
variogram
lag distance (metres)
0.0
0.5
1.0
1.5
2.0
2.5
0
5
10
15
20

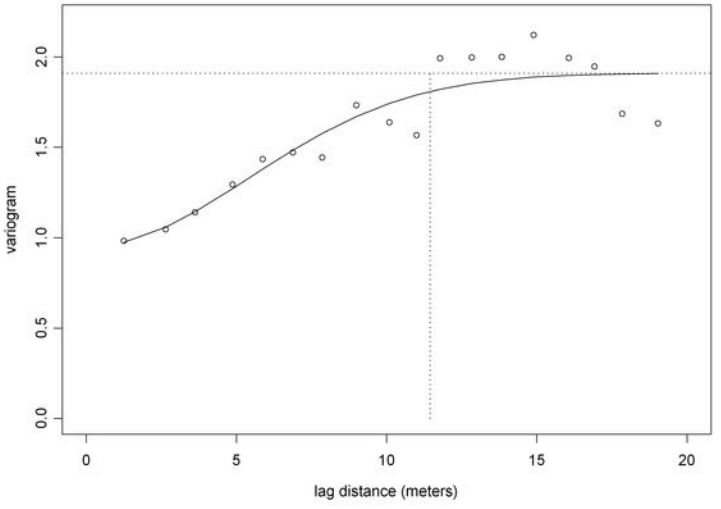
A


colony count (log cfu/gram)
1.0
2.0
3.0
4.0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
A
B
C
D
E
4
3
2
1
0

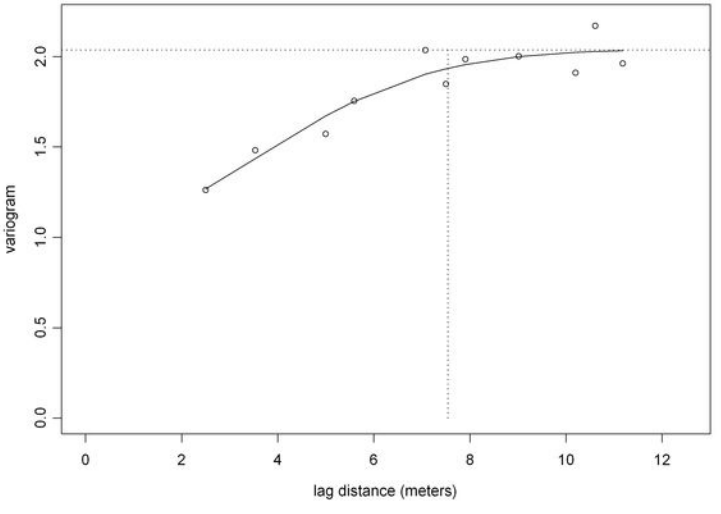
B


colony count (logcfu/gram)
1.0
2.0
3.0
4.0
1 2 3 4 5 6 7 8 9 10
A
B
C
D
E
F
G
H
I
J
4
3
2
1
0

A


variogram
0.0
0.5
1.0
1.5
2.0
0
5
10
15
20
lag distance (meters)

B


variogram
0.0
0.5
1.0
1.5
2.0
0
2
4
6
8
10
12
lag distance (meters)