Nutrient depleted soil is associated with the presence of *Burkholderia pseudomallei*

Running title: Nutrient depleted soil and *Burkholderia pseudomallei*

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Abstract

Burkholderia pseudomallei is a soil-dwelling bacterium and the cause of melioidosis, which kills an estimated 89,000 people per year worldwide. Agricultural workers are at high risk of infection due to repeated exposure. Little is known about soil physicochemical properties associated with presence or absence of the organism. Here, we evaluated the soil physicochemical properties and presence of *B. pseudomallei* in 6,100 soil samples collected from 61 rice fields in Thailand. The presence of *B. pseudomallei* was negatively associated with the proportion of clay, proportion of moisture, level of salinity, percentage of organic matter, presence of cadmium, and nutrient levels (phosphorous, potassium, calcium, magnesium and iron). The presence of *B. pseudomallei* was not associated with the level of soil acidity (p=0.54). In a multivariable logistic regression model, presence of *B. pseudomallei* was negatively associated with the percentage of organic matter (OR=0.06; 95%CI 0.01-0.47, p=0.007), level of salinity (OR=0.06; 95%CI 0.01-0.74, p=0.03), and percentage of soil moisture (OR=0.81; 95%CI 0.66-1.00, p=0.05). Our study suggests that in rice fields, *B. pseudomallei* thrives in those that are nutrient-depleted. Some agricultural practices result in a decline in soil nutrients, which may impact on the presence and amount of *B. pseudomallei* in affected areas.

Importance

Burkholderia pseudomallei is an environmental Gram-negative bacillus and the cause of melioidosis. Humans acquire the disease following skin inoculation, inhalation or ingestion of the bacterium in the environment. The presence of *B. pseudomallei* in soil defines geographic regions where humans and livestock are at risk of melioidosis, yet little is known about soil properties associated with presence of the organism. We evaluated the soil properties and presence of *B. pseudomallei* in 61 rice fields in East, Central and Northeast Thailand. We demonstrated that the organism was more commonly found in soils with lower levels of organic matter and nutrients including phosphorus, potassium, calcium, magnesium and iron. We also demonstrated that crop residue burning after harvest, which can reduce soil nutrients, was not uncommon. Some agricultural practices result in a decline in soil nutrients, which may impact on the presence and amount of *B. pseudomallei* in affected areas.

Introduction

Melioidosis, an infectious disease caused by the Gram-negative bacterium *Burkholderia pseudomallei*, is an important global public health threat. An estimated 165,000 cases of human melioidosis occur each year worldwide, of which 89,000 (54%) die (1). The disease is highly endemic in Southeast Asia and Northern Australia (2), and is predicted to be endemic but is grossly under-reported in many tropical and sub-tropical countries (1, 3). The crude case fatality rate for melioidosis ranges from 14% to 40% and may be as high as 70% in cases given sub-optimal antibiotic therapy (4-6). No licensed vaccine for melioidosis is currently available.

B. pseudomallei is a free-living organism found in soil and water (2), and humans acquire the disease following skin inoculation, inhalation or ingestion of the bacterium in the environment (7). In tropical developing countries, most patients are agricultural workers (typically rice farmers) with frequent contact with soil and water. Evidence-based guidelines for the prevention of melioidosis recommend that residents and visitors to melioidosis-endemic areas avoid direct contact with soil and water, and wear protective gear such as boots and gloves when in direct contact with soil and environmental water (7, 8). However, rubber boots are hot and make walking difficult in muddy rice fields, and rubber gloves are also hot and difficult to use while planting rice (9). As a result, many rice farmers continue to work in rice fields without protective gear and are at high risk of melioidosis.

The presence of *B. pseudomallei* in soil defines geographic regions where humans and livestock are at risk of melioidosis, but knowledge of environmental factors associated with the presence of the organism in the natural setting is poor and conflicting. Laboratory studies using sterile soil showed that *B. pseudomallei* grows well in soil with a high percentage of moisture (10-12), high level of iron (13), optimal acidity (pH 4-8) (11, 13), and high salinity (up to 4.2 dS/m) (13). By contrast, two cross-sectional studies in the natural environment in Northern Australia and Northeast Thailand found that the presence of *B. pseudomallei* was negatively associated with the level of iron in soil (14, 15), and a recent modelling study and an experimental field study suggested that the presence of *B. pseudomallei* was not associated with soil acidity (1, 12). Furthermore, both negative and positive correlations between the presence of *B. pseudomallei* and soil salinity have been reported (1, 12, 15). Land use can affect the biodiversity of organisms in soil (16), but there is currently no information on the association between the presence of *B. pseudomallei* and agricultural practices.

Here, we report the findings of a large cross-sectional environmental survey to determine the physicochemical characteristics of soil associated with the presence of *B. pseudomallei* in three regions in Thailand where melioidosis is considered to be highly endemic (Northeast and East) or non-endemic (Central). Our findings extend the understanding of soil properties related to environmental *B. pseudomallei*.

Materials and Methods

Study area. East, Central and Northeast Thailand consist of 7, 21 and 20 provinces that cover 34,381, 93,005 and 168,854 km^2, and have an estimated population in 2013 of 3.9, 18.7 and 23.3 million, respectively (17). Northeast Thailand is a plateau surrounded by mountain ranges, and most of the arable land consists of tropical sandy soil. East Thailand is characterized by short mountain ranges alternating with alluvial plains. Central Thailand is a large plain consisting of clay soil. Rice farming is the predominant form of agriculture in all three regions. In Thailand, for administrative purposes each province is sub-divided into districts, sub-districts, communes and villages. The majority of the population in all three regions live in rural settings and most adults are engaged in agriculture, particularly rice farming. In 2013, land used for agriculture was 57%, 48% and 60% in East, Central and Northeast Thailand, respectively (18).

To evaluate environmental factors associated with the presence of *B. pseudomallei*, we selected six, seven and seven adjacent provinces in each of East, Central and Northeast Thailand, respectively (Fig. 1). Three villages per province were randomly selected. Randomization was performed using Stata version 14.0 (StataCorp LP, College station, Texas). Soil sampling was performed in one rice field per one village. Rice fields were selected as sampling sites since rice farming is a major risk factor for melioidosis (9). The sampled fields were those that had been used for rice farming for at least 12 months prior to the sampling date. Written, informed permission was obtained from land owners prior to sampling.

The study protocol was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University (MUTM 2013-021-01) and the Oxford Tropical Research Ethics Committee, University of Oxford (OXTREC 1013-13).

Soil Sampling. Soil sampling in East, Central and Northeast Thailand was performed during the dry season (from April to June) in 2013, 2014 and 2015, respectively. We used the consensus guidelines for environmental sampling described by the Detection of Environmental *Burkholderia pseudomallei* Working Party (DEBWorP) (19). In brief, each rice field was divided into a grid system, in which 100 sampling points (10 by 10) were plotted 2.5 meters apart. At each sampling point, around 30 grams of soil was removed from the base of a 30-cm hole, placed in a zip bag, and kept at ambient temperature and protected from sunlight. We recorded the location of sampled fields using the EpiCollect application (www.epicollect.net, Imperial College, London) (20). All soil samples were processed within 48 hours of collection for the identification of *B. pseudomallei* and for soil physicochemical properties.

Identification of *B. pseudomallei*. Ten grams of soil from each sampling point was mixed with 10 ml of enrichment broth consisting of threonine-basal salt solution plus colistin (TBSS-C50 broth) and incubated at 40°C in air for 48 hours. Ten microliters of surface liquid was then sub-cultured onto Ashdown agar and incubated at 40°C in air and examined every 24 hours for 4 days for bacterial colonies suggestive of *B. pseudomallei,* which were initially identified on the basis of colony morphotype. This included the characteristic colony morphology (purple, flat, dry and wrinkled) together with six additional colony morphotypes, as described previously (21). Presumptive colonies were picked from each sample and tested immediately using a specific latex agglutination test for *B. pseudomallei*-specific CPS, as previously described (22). For positive colonies, susceptibility to amoxicillin/clavulanic acid and arabinose assimilation were determined as previously described (23). *B. pseudomallei* was defined based on the combination of colony morphology, positive latex agglutination test, susceptibility to amoxicillin/clavulanic acid and negative arabinose assimilation (23).

Soil properties. One kilogram of soil from each sampling field was made by aggregating 100 soil samples (10 g per each sampling point) and evaluated for four main properties, as follows. (1) Physical properties: texture (proportion of sand, silt and clay) and moisture (%w/w). (2) Acidity and salinity: pH, lime requirement (to adjust soil acidity; kg/100sqm) and electrical conductivity (dS/m). (3) Chemical properties: total nitrogen (mg/kg), available phosphorous (mg/kg), exchangeable potassium (mg/kg), exchangeable calcium (mg/kg), available magnesium (mg/kg), extractable sulphur (mg/kg), total iron (g/kg), total cadmium (mg/kg), exchangeable sodium (mg/kg) and cation exchange capacity (cmol/mg). (4) Biological related factors: organic matter (%w/w) and carbon to nitrogen ratio (C:N ratio) (see Table S5 in the supplemental material). All soil properties were evaluated by iLab Asia (Kanchanaburi, Thailand) except for total iron and total cadmium which were evaluated by Central Laboratory (Bangkok, Thailand). Both laboratories were registered with the Ministry of Agriculture Thailand as standardized national soil testing laboratories.

Agricultural practices. A closed-end interviewee-based questionnaire was used to collect the information about agricultural practices. For illiterate participants, the questionnaire was read to the participant and completed by trained research staff in accordance with their responses.

Questions included fertilizer used, rice field management (before planting and after harvest) in the 12 months before the sampling date.

Sample size calculation. To determine the optimal sample size, we performed a pilot study of soil sampling in four rice fields in Chachoengsao province, East Thailand. Three of four rice fields (75%) were culture positive for *B. pseudomallei*. We calculated that 60 rice fields (3 rice fields per province) were needed to determine environmental factors associated with *B. pseudomallei* with a power of 80% at an alpha error of 5%.

Statistical analysis. The outcomes of interest were positivity of *B. pseudomallei* in rice fields and its association with soil properties. Binary and continuous variables were compared by using the Fisher's exact test and Mann-Whitney test, respectively. Soil properties associated with the presence of *B. pseudomallei* were evaluated using univariable and multivariable logistic regression. The final multivariable logistic regression models were developed using a purposeful selection method (24). Sensitivity analysis was conducted using region-stratified analysis. We also used ordered logistic regression to evaluate the association between soil properties and quantity of *B. pseudomallei*. The number of positive sampling points for *B. pseudomallei* within a rice field was used to represent the quantity of *B. pseudomallei* distribution in the field. The Spearman correlation coefficient was used to evaluate the correlation between soil properties. All statistical tests were performed using Stata version 14.0 (StataCorp LP, College station, Texas). The final database with the data dictionary are publicly available online (https://figshare.com/s/b44c335a9b321ab19325).

Results

Distribution of *B. pseudomallei* in Northeast, East and Central Thailand. Of 6,100 soil samples collected from 61 rice fields (100 soil samples per rice field), 1,046 were culture positive for *B. pseudomallei* (Fig. 1). A total of 30 of 61 rice fields (49%) had at least one sampling point that was culture positive for the organism. Percentages of rice fields culture-positive for *B. pseudomallei* were 57% (12 of 21 rice fields), 68% (13 of 19 rice fields) and 24% (5 of 21 rice fields) in Northeast, East and Central Thailand, respectively. The percentage of rice fields culture-positive for *B. pseudomallei* in the Northeast and East were higher than that in Central Thailand (57% vs. 24%, p=0.06 and 68% vs. 24%, p=0.01), while the percentage was not significantly different between the Northeast and East (57% vs. 68%, p=0.53).

For the rice fields that were culture-positive for *B. pseudomallei*, the median number of positive sampling points were 53 (range 2 to 98), 16 (range 1 to 81) and 1 (range 1 to 63) in Northeast, East and Central Thailand, respectively (see Table S1 in the supplemental material). The median number of positive sampling points in the Northeast and East were both higher than that in Central Thailand (p=0.01 and p=0.002), while the number was not significantly different between the East and Northeast (p=0.61).

Characteristics of soil and agricultural practices. Overall comparison of soil properties among three regions showed that soil from Central Thailand had the highest median percentage of clay (53%), followed by the Northeast (45%) and East (32%). Soil acidity (pH) varied considerably, ranging from very acid (pH=4.9) to carbonate-rich soil (pH=8.1), but was not significantly different between the three regions (p=0.68). Soil salinity, as determined by electrical conductivity and expressed in dS/m, was very low in all fields sampled (<2.0 dS/m).

Farmers were interviewed about land management before and after rice planting (including the fertilizer used, and crop residue burning before and after harvest) in the 12 months before the sampling date. Of 61 rice fields evaluated, 54 (89%) were treated with chemical fertilizer, 17 (28%) with organic fertilizer made from plant material, 22 (36%) with organic fertilizer made from animal dung, and 39 (64%) with biological fertilizer such as effective microorganisms. Owners of 24 (39%) rice fields burned their fields between rice planting seasons. The median percentage of organic matter in fields with a history of burning was not significantly lower than that of others (0.81 vs. 0.84 %w/w, p=0.82).

Association between soil physicochemical properties and *B. pseudomallei.* We found that the presence of *B. pseudomallei* was associated with nutrient-depleted soil (Fig. 2; see also Table S2 in the supplemental material). Presence of the organism was negatively associated with the percentage of soil moisture (p<0.001), the level of soil salinity (p=0.001), presence of cadmium (p<0.001) and levels of multiple nutrients including available phosphorous (p=0.03), exchangeable potassium (p<0.001), exchangeable calcium (p=0.001), available magnesium (p=0.002) and total iron (p=0.002). Levels of overall nutrients and total nutrient fixing capacity of soil determined by organic matter and cation exchange capacity, respectively, were also negatively associated with the presence of *B. pseudomallei* (both p values<0.001). The carbon to nitrogen ratio, which is used to determine how easily bacteria can decompose organic material in soil, was also negatively associated with the presence of *B. pseudomallei* (p=0.01). Presence of the organism was positively associated with the proportion of sand (p=0.02), negatively associated with the proportion of clay (p=0.002), and not associated with the proportion of silt (p=0.68). Presence of *B. pseudomallei* was not associated with soil acidity (p=0.54), or agricultural practices. Many soil physicochemical properties were strongly correlated (see Table S3 in the supplemental material).

We used multivariable logistic regression analysis and found that the presence of *B. pseudomallei* was negatively associated with the percentage of organic matter (OR=0.06; 95%CI 0.01-0.47, p=0.007), level of salinity (OR=0.06; 95%CI 0.01-0.74, p=0.03), and level of soil moisture (OR=0.81; 95%CI 0.66-1.00, p=0.05) (Table 1). A sensitivity analysis was conducted by including region as a stratification variable, which gave comparable results.

In addition, we also used ordered logistic regression to further evaluate the association between the quantity of *B. pseudomallei* distribution in rice fields and soil physicochemical factors. We observed that the number of sampling points culture positive for *B. pseudomallei* was also negatively associated with the percentage of organic matter (OR=0.06; 95%CI 0.01-0.32, p=0.001), level of soil moisture (OR=0.78; 95%CI 0.66-0.91, p=0.002) and level of salinity (OR=0.07; 95%CI 0.01-0.53, p=0.01) (see Table S4 in the supplemental material).

Discussion

The results of our large environmental study demonstrated an association between the presence of *B. pseudomallei* and nutrient-depleted soil in rice fields in Thailand. Negative associations between the presence of *B. pseudomallei* and nutrient levels in the soil were observed for each of the nutrients evaluated (with the exception of total nitrogen, exchangeable sodium and extractable sulphur) and for organic matter and cation exchange capacity, which represent levels of overall nutrients and total nutrient fixing capacity of soil, respectively. This is also supported by the negative association between the presence of *B. pseudomallei* and the level of salinity, which could also represent the level of soil nutrients in the environment (12). Our findings are important because nutrients in the soil are effected by agricultural practices, and crop residue burning after harvest is not uncommon in Thailand and many other tropical countries. There is strong evidence to show that burning can reduce soil nutrients by eliminating crop residues and soil organisms present on the soil surface (25). Poor agricultural practices could impact on the presence and amount of *B. pseudomallei*. This suggests that changes in agricultural practice and improvement of soil nutrient content might also be essential to reduce the distribution of *B. pseudomallei* and incidence of melioidosis.

Our study also highlights the difference between findings from experimental soil inoculated with *B. pseudomallei,* environmental studies in small areas where melioidosis is endemic, and this large environmental study. For example, soil moisture was positively associated with presence of *B. pseudomallei* in experimental soil studies (10-12), and environmental studies of small areas where melioidosis is endemic (26, 27). It has been postulated that *B. pseudomallei* can move from deeper soil layers to the surface during the rainy season and rising water table where it may then multiply (28). Our study shows that soil in Northeast Thailand (where *B. pseudomallei* is abundant in soil) is mostly sandy soil with a low level of organic matter and moisture, while soil in Central Thailand (where *B. pseudomallei* is less abundant), is mostly clay soil with high level of organic matter and moisture. This is also supported by a recent finding of the presence of *B. pseudomallei* in a desert region outside the wet tropics in Northern Australia (29).

Organic matter in soil contains vital nutrients and influences the diversity and biological activity of soil organisms (25). The negative association between soil organic matter and the presence of *B. pseudomallei* is consistent with two previous environmental studies in Northern Australia (15) and Northeast Thailand (30), which showed that the level of organic carbon was negatively associated with presence of *B. pseudomallei*. The level of organic carbon is a measure of the carbon contained within the soil organic matter. It is possible that soils with high organic matter have high biotic stress because abundant soil microorganisms are competing for substrates, water or growth factors (31), which may inhibit the survival or growth of *B. pseudomallei*. This is supported by an environmental study showing that low microbial density in soil is associated with the presence of *B. pseudomallei* (27, 32) and that *Bacillus amyloliquefaciens* extracted from soil samples can inhibit the growth of *B. pseudomallei* (32). It is also possible that depletion of individual nutrients such as iron supports the growth of *B. pseudomallei,* which has a range of mechanisms to persist in low iron environments (33). An additional possibility is that environmental stress selects for persister cells of *B. pseudomallei*, as has been recently shown for *Pseudomonas aeruginosa* in nutrient-limited conditions and in biofilm (34). *B. pseudomallei* are taken up by amoebae, which in vitro are associated with survival in the presence of disinfecting agents and antimicrobial drugs (35, 36), and may represent an additional survival advantage for *B. pseudomallei* in nutrient-depleted soil.

Our findings suggest that extremely low levels of salinity (such as <0.1 dS/m) may be an indirect measure of nutrient depletion in rice fields. This is because soil salinity as estimated by measuring electrical conductivity represents soluble salts of soil nutrients, including sodium, chloride, magnesium, calcium, potassium and nitrate. Our finding is consistent with an experimental study in Northern Australia (12), which showed that *B. pseudomallei* grew well in soil with low electrical conductivity (0.1 dS/m) but could not survive in commercial soil, which has a high level of organic based compost and high electrical conductivity (0.7 dS/m). Although a recent modelling study proposed a positive association between salinity level and presence of *B. pseudomallei*, this estimation was based on soil salinity for all land (undisturbed land, agricultural land, sports fields, etc) with an electrical conductivity ranging from 0 to >20 dS/m (1). It is also possible that the effect of salinity in rice fields may be different from non-rice fields. For example, rice fields may be intentionally flooded and drained repeatedly to reduce salinity to a very low level (<2.0 dS/m) (37), and this could lead to the loss of water-soluble nutrients from the soil (38-40).

B. pseudomallei can survive well in soil under laboratory condition with pH ranging from 4 to 8 (13), and our study supports the lack of association between presence of *B. pseudomallei* and pH.

A limitation of our study is that soil sampling was only performed during the dry season over a period of three years. We chose to sample during the dry season to control for variation in the presence of *B. pseudomallei* and soil physicochemical properties associated with seasonal changes. Recent environmental studies showed that soil properties were not different between the dry and wet season (14), and that changes in the presence of *B. pseudomallei* in the soil with very low salinity level (<2.0 dS/m) measured over three years were minimal (12). It is possible that the presence of *B. pseudomallei* in rice fields would have been generally higher if the study was conducted during the rainy season. Although the difference in percentage of organic matter between fields with and without a history of burning was not observed in our study, this could be because of the cross-sectional study design or other confounding factors. For example, some fields were burned more than 12 months before the study was conducted.

In summary, our large cross-sectional environmental survey has shown that the presence of the important human pathogen *B. pseudomallei* is associated with nutrient-depleted rice fields. Further investigations are required to evaluate whether changes in agricultural practices could effectively enhance soil nutrients, and whether these could reduce the distribution of *B. pseudomallei* in rice fields.

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Figure legends

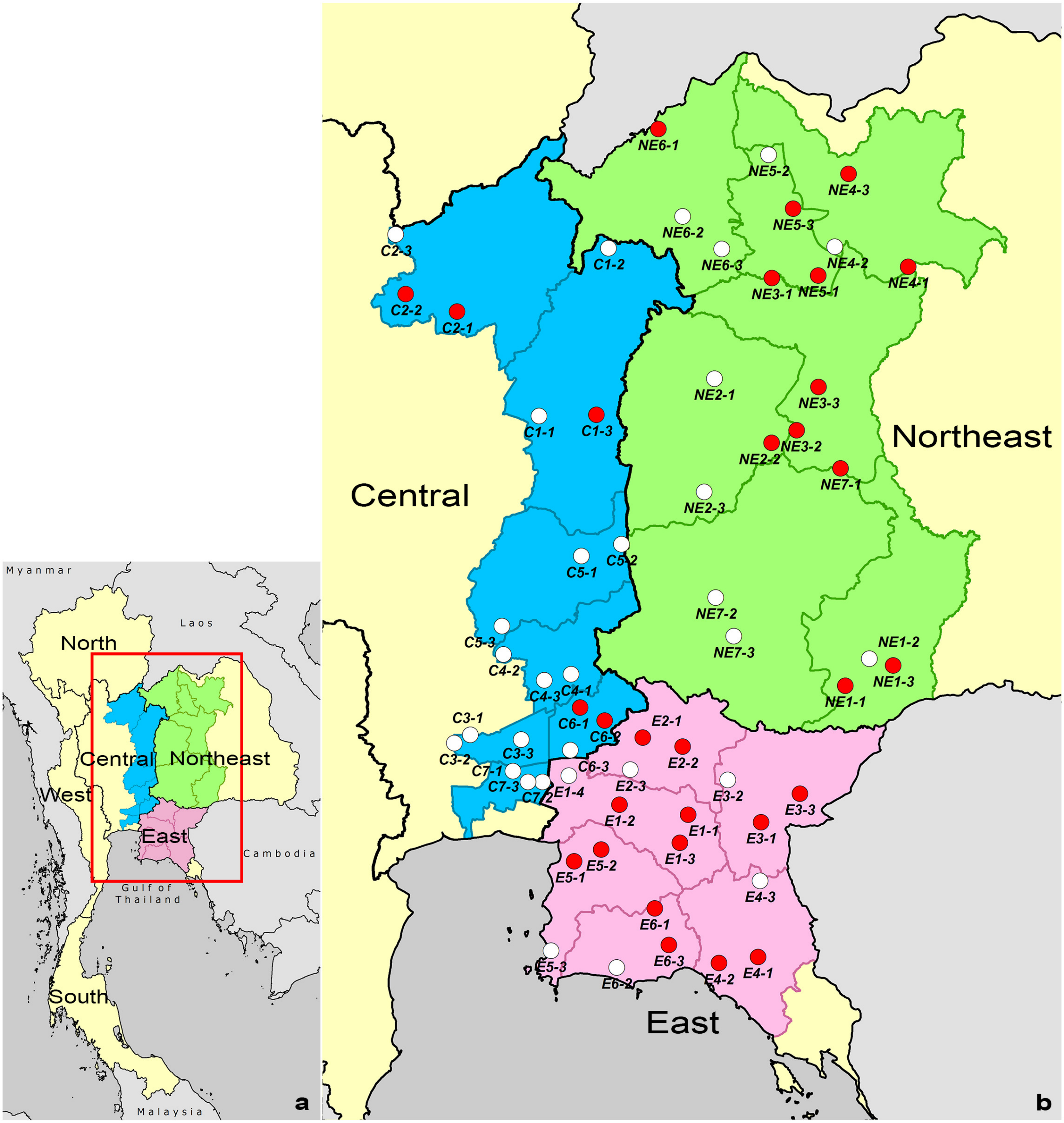
FIG 1 Distribution of *B. pseudomallei* in Central, East and Northeast Thailand. (a) Map of Thailand. (b) Location of the 61 rice fields evaluated. Red and white circles, culture positive and negative for *B. pseudomallei,* respectively. Province codes represent Phetchabun (C1), Phitsanulok (C2), Pathum Thani (C3), Saraburi (C4), Lopburi (C5), Nakhon Nayok (C6) and Bangkok (C7) in Central Thailand, Chachoengsao (E1), Prachinburi (E2), Sa Kaeo (E3), Chanthaburi (E4), Chonburi (E5) and Rayong (E6) in the East, and Burirum (NE1), Chaiyaphum (NE2), Khon Kaen (NE3), Udon Thani (NE4), Nong Bua Lam Phu (NE5), Loei (NE6) and Nakhon Ratchasima (NE7) in the Northeast.

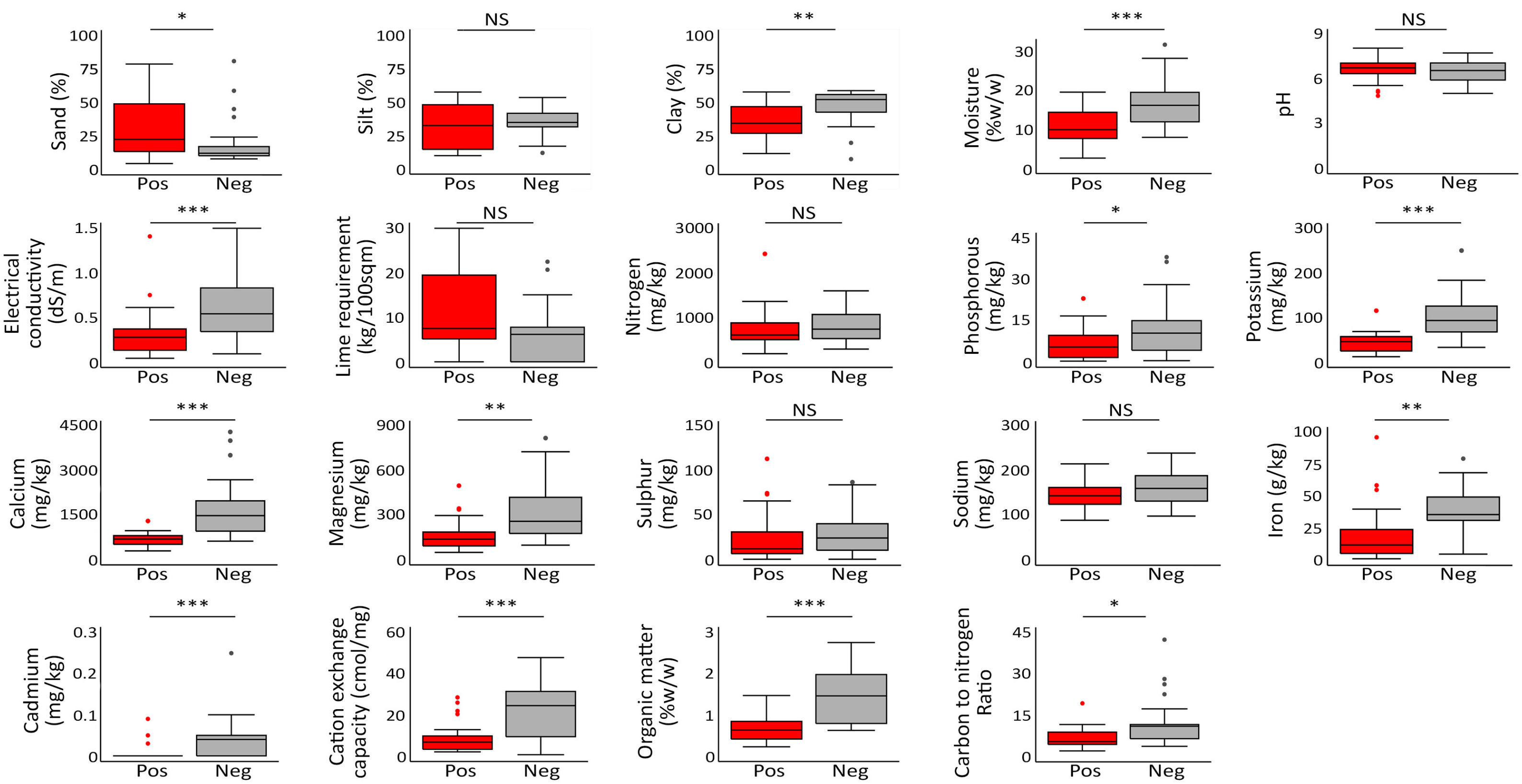
FIG 2 Soil physicochemical properties associated with the presence of *B. pseudomallei* Box–whisker plots indicate median, interquartile range and distribution of the data. Dots indicate the outliers (data located outside 1.5 times of interquartile range) (41). Red and grey boxes represent rice fields culture positive (Pos) and negative (Neg) for *B. pseudomallei*, respectively. *P≤0.05, **P≤0.01, ***P≤0.001 and NS=Not Significant.

Tables

TABLE 1 Soil physicochemical properties associated with the presence of *B. pseudomallei* in a multivariable logistic regression model

Soil physicochemical characteristics	Adjusted odds ratio (95% CI)	p value
Organic matter (%w/w)	0.06 (0.01-0.47)	0.007
Electrical conductivity (dS/m)	0.06 (0.01-0.74)	0.03
Moisture (%)	0.81 (0.66-1.00)	0.05


Myanmar
Laos
North
Central
Northeast
West
East
Cambodia
Gulf of Thailand
South
Malaysia
a
NE6-1
NE5-2
NE4-3
NE5-3
NE6-2
C2-3
C1-2
NE6-3
NE4-2
NE4-1
NE3-1
NE5-1
C2-2
C2-1
NE2-1
NE3-3
C1-1
C1-3
Northeast
NE3-2
NE2-2
NE7-1
Central
NE2-3
C5-2
C5-1
NE7-2
C5-3
NE7-3
NE1-2
C4-2
NE1-3
C4-3
C4-1
NE1-1
C3-1
C6-1
C6-2
E2-1
C3-2
C3-3
E2-2
C7-1
C6-3
E2-3
C7-3
C7-2
E1-4
E3-2
E3-3
E1-2
E1-1
E3-1
E1-3
E5-1
E5-2
E4-3
E6-1
E6-3
E5-3
E4-2
E4-1
E6-2
East
b



Sand (%)
Silt (%)
Clay (%)
Moisture (%w/w)
pH
Electrical conductivity (dS/m)
Lime requirement (kg/100sqm)
Nitrogen (mg/kg)
Phosphorous (mg/kg)
Potassium (mg/kg)
Calcium (mg/kg)
Magnesium (mg/kg)
Sulphur (mg/kg)
Sodium (mg/kg)
Iron (g/kg)
Cadmium (mg/kg)
Cation exchange capacity (cmol/mg)
Organic matter (%w/w)
Carbon to nitrogen Ratio
Pos
Neg
NS
*
**
***