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THE CONTRIBUTION OF SOIL PHYSICOCHEMICAL PROPERTIES TO THE PRESENCE AND GENETIC DIVERSITY OF *BURKHOLDERIA PSEUDOMALLEI*

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Abstract. *Burkholderia pseudomallei* (Bp), the causative agent of melioidosis, is unevenly distributed in the complex soil environment. Physicochemical factors in the soil have been reported to affect microbial communities in the soil. The effect of physicochemical factors on the number and diversity of organisms in the soil has not been reported. Twenty-five each *B. pseudomallei*-positive and -negative soil samples were collected from a melioidosis-endemic area. The amount of Bp in each soil sample was measured by culture and quantitative PCR (qPCR). The following physicochemical properties from each soil sample were measured: pH, total organic carbon (TOC), total nitrogen (TN), carbon to nitrogen ratio (C:N ratio), exchangeable calcium (EC) and extractable iron (EI). All the physicochemical properties measured were significantly different between the Bp-positive and -negative soil samples. The Bp-positive soil samples had lower C:N ratios and lower EC and a higher EI ($p<0.05$) than the Bp-negative samples. The average pH was lower (3.7-5.0) in the Bp-negative samples. Among the Bp-positive soil samples, the EC was negatively correlated with the PCR copy number. The amount of bacteria detected with the qPCR method was higher than with the culture method, suggesting the presence of unculturable forms of bacteria that might re-grow when the environmental conditions was suitable. A total of 117 Bp isolates obtained from the soil samples were classified into 25 groups using BOX-PCR. The genetic diversity of Bp, did not correlate with the physicochemical factors investigated. A suitable pH range and C:N ratio may be important for the presence of Bp. The EI supports the needs and EC probably alters the growth of Bp. The genetic diversity of the bacteria was not influenced by the soil factors

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investigated in this study. This information shows the environment conducive to the growth of Bp. This gives us information about how to potentially control or decrease Bp in the soil in the future.

Keywords: *Burkholderia pseudomallei*, melioidosis, soil physicochemical factors, qPCR

INTRODUCTION

The gram-negative bacterium *Burkholderia pseudomallei* is the causative agent of melioidosis, an infectious disease in humans and other animals (White, 2003). The disease is endemic in Southeast Asia and northern Australia. Sporadic cases have been reported in travelers to endemic areas (Dance, 1991; Wilks *et al*, 1994). In Thailand, most of the cases occur in the northeastern part of the country. The average annual melioidosis incidence during 1997- 2006 in Ubon Ratchathani Province was 12.7 cases per 100,000 people per year with the highest rate being 21.3 cases per 100,000 people during 2006 (Limmathurotsakul *et al*, 2010). *B. pseudomallei* is a widely distributed environmental saprophyte found in the soil and water of many tropical countries. It is resistant to a variety of adverse environmental conditions (Cheng and Currie, 2005). Humans become infected by inoculation of contaminated soil or water through skin abrasions, by inhalation of contaminated dust and ingestion of contaminated water. Patients with recreational or occupational exposure to mud and surface water, particularly rice farmers, have a greater potential of becoming infected with this bacterium (Heng *et al*, 1998).

The amount of *B. pseudomallei* in the soil has been estimated to be 20-fold higher in northeastern Thailand than in central Thailand (Smith *et al*, 1995). This results in a 10-fold greater incidence of melioidosis in the northeastern Thailand than central Thailand (Suputtamongkol *et al*, 1994). Khon Kaen Province is endemic for melioidosis (Wuthiekanun *et al*, 1995). From our survey as previously reported, Nam Phong District has more *B. pseudomallei* in the soil than any other district in Khon Kaen. *B. pseudomallei* is more culturable in sandy soil at a pH of 5.0 to 6.0, a moisture content >10% and higher chemical oxygen demand (COD) and total nitrogen levels (Palasatien *et al*, 2008).

Soil is a complex environment and a reservoir for microbial communities (Robe *et al*, 2003). Besides the type and amount of microbes affecting the soil microbial community, soil physicochemical properties also have an effect. Besides studying microbial diversity using molecular biology techniques, the study of physicochemical properties of soil is also important (Maron *et al*, 2011). It is important to study the amount and genetic diversity of *B. pseudomallei* in association with the physicochemical properties of the soil where it is endemic. This information could improve measures for controlling and decreasing the amount of *B. pseudomallei* in the soil.

MATERIALS AND METHODS

Soil sampling

Soil samples were collected from the agricultural fields of the Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand. To determine the sampling sites, soil samples were collected from 20 randomly selected areas in the fields and the presence or absence of *B. pseudomallei* was determined by the direct culture and

the sites were designated as either positive or negative for *B. pseudomallei*. Then, each site was divided into 25, 1.5 x 1.5 m grids. Soil samples were collected from each grid. The grid locations were marked by a global position system (GPS) device (GPSMAP® 60CSx, Garmin, Lenexa, KS) using the center of each grid. Each soil sample was collected at 15 cm below the soil surface using a spade that was cleaned and disinfected prior to use. One sample was made from a pooled collection 3 sampling locations in a triangle 30 cm from the center of each grid. Soil samples were kept in a closed container at room temperature in the dark until use. Two hundred grams of soil from each sample were used for microbial culture and biological studies, while 300 g of soil were used for physicochemical property analysis.

Soil physicochemical properties

The physicochemical properties of the soil samples were determined at the Department of Land Resources and Environment, Faculty of Agriculture, Khon Kaen University, Khon Kaen, Thailand.

The soil pH was measured by mixing 20 ml sterile distilled water with 20 g soil and stirring to form a paste and then left for 60 minutes; the suspension was then re-stirred and the pH was measured immediately using a pH meter (Jones, 2001).

The total organic carbon (TOC) level of the soil was determined using the dichromate oxidation method according to Walkley and Black (1934).

The total nitrogen (TN) level of the soil, which includes both organic and inorganic forms of nitrogen,was measured using the micro-Kjeldahl digestion method with a 2 g soil sample (Bremner, 1965).

Calcium was extracted from a 5 g soil sample using 1N ammonium acetate (NH_4OAc) and the exchangeable calcium (EC) level was determined using flame photometry (Berry *et al*, 1946).

Iron was extracted from 10 g soil using diethylenetriaminepentaacetic acid solution and the extractable iron (EI) level was measured by flame atomic absorption spectrometry (Lindsay and Norvell, 1978).

Bacterial culture of the soil

B. pseudomallei in the soil was detected by direct and enrichment culture methods as described previously (Wuthiekanun *et al*, 1990). Suspected colonies are wrinkled and violet-purple in color. The bacteria were grown by direct culture; enriched culture was used if the bacteria could not be grown on direct culture. The bacteria were then identified using latex agglutination with a monoclonal antibody specific to *B. pseudomallei* (Samosornsuk *et al*, 1999). At least 5-10 colonies of *B. pseudomallei* from each grid were used for BOX-PCR analysis.

Detection of *B. pseudomallei* by semi-nested PCR

To detect *B. pseudomallei* in soil, the total DNA was extracted from soil samples using a DNA extraction kit (PowerSoil® DNA Isolation Kit; MO BIO Laboratories, Carlsbad, CA). *B. pseudomallei* DNA was detected by semi-nested PCR, using primers for the 16S-23S intergenic spacer (Inglis *et al*, 2005) with slight modification. The first-round primers were Bp1 (5′CGATGATCGTTGGCGCTT) and Bp4 (5′CGTTGTGCCGTATTCCAAT), and the second-round primers were Bp1 and Bp3 (5′ATTAGAGTCGAACAAT). For the first-round PCR, a total volume of 20 µl was used, which consisted of 0.5 units of Taq polymerase (RBC Bioscience, New Taipei City, Taiwan), 1x buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 0.2 µM each for Bp1 and Bp4 and 2 µl of DNA template. For the second-round, the Bp1

and Bp3 primers were used and 0.4 µl of the first-round product was used as the DNA template. The amplification conditions for first-round cycling were pre-denaturation at 94°C for 5 minutes, activation of the polymerase, 45 cycles at 94°C for 30 seconds for denaturation, 30 seconds at 55°C for annealing, 45 seconds at 72°C for extension and then 72°C for 7 minutes. Second-round amplification was carried out under the same conditions as the first round, except 50°C was used as the annealing temperature. The PCR products were analyzed with 1% agarose gel-electrophoresis.

DNA extraction of cultured *B. pseudomallei*

B. pseudomallei was cultured in 1.5 ml of Luria-Bertani broth and incubated at 37°C with shaking at 200 rpm for 16 hours. The DNA was extracted from the cell pellets using the SDS and Proteinase K method (Goldenberger *et al*, 1995). The DNA pellet was re-suspended in 30 µl TE buffer (2M Tris-HCl pH 8, 0.5 M EDTA pH 8) and stored at -20°C until used.

BOX-PCR genomic typing

Repetitive sequence-based PCR using the BOXA1R primer (5′CTACG-GCAAGGCGACGCTGACG) (de Versalovic *et al*, 1998) was used for BOX-PCR typing. The 25 µl PCR reaction mixture consisted of 2 units of *Taq* polymerase (Roche, Indianapolis, IN), 1xbuffer, 50 ng DNA templates, 600 µM dNTPs, 1.3 µl of primer (300 ng/µl), 0.2 µl BSA (20 mg/ml) and 10% v/v of dimethyl sulfoxide. Pre-denaturation of template DNA was done at 96°C for 10 minutes and then *Taq* polymerase was subsequently added. The PCR reaction was conducted for 30 rounds using denaturation at 92°C for 30 seconds, primer annealing at 50°C for 1 minute, elongation at 65°C for 8 minutes and then 65°C for 8 minutes. The PCR products were then analyzed with 2% agarose gel-electrophoresis using TAE buffer (Mirza *et al*, 2009).

Quantitative PCR (qPCR)

The quantitative PCR was carried out using the iTaqTMFast SYBR Green Supermix with a ROX kit (Bio-Rad, Hercules, CA). The 20 µl reaction mixture consisted of 10 µl iTaqTM Fast SYBR Green Super mix with ROX, 10 ng of DNA sample and 100 nM of each 16S-23S spacer primer. The expected size of the first round PCR product was 302 bp and the second round was 285 bp. Amplification was performed using the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The PCR amplification conditions were 95°C for 5 minutes, 40 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and then extension at 72°C for 30 seconds. Specificity of the qPCR products was confirmed using melting curve analysis. Standard curves using 10^1-10^7 copies per reaction of 16S-23S intergenic spacer were generated with plasmid DNA containing a fragment of the 16S-23S spacer. The qPCR efficiency (E) was calculated using the equation E=10[-1/slope]. The real-time efficiency of the 16S-23S spacer was 1.79 ± 0.01 and the R^2 of the standard curve was 0.99.

Statistical analysis

The program SPSS, version 16.0 (IBM, Armonk, NY) was used to analyze the physicochemical properties of *B. pseudomallei*-positive and -negative soil. The independent samples *t*-test was used when two separate sets of independent data were normally distributed. The non-parametric test, Mann-Whitney *U* test, was used when two separate sets of independent data were not normally distributed. The generalized linear model (GLM) method was used to study the

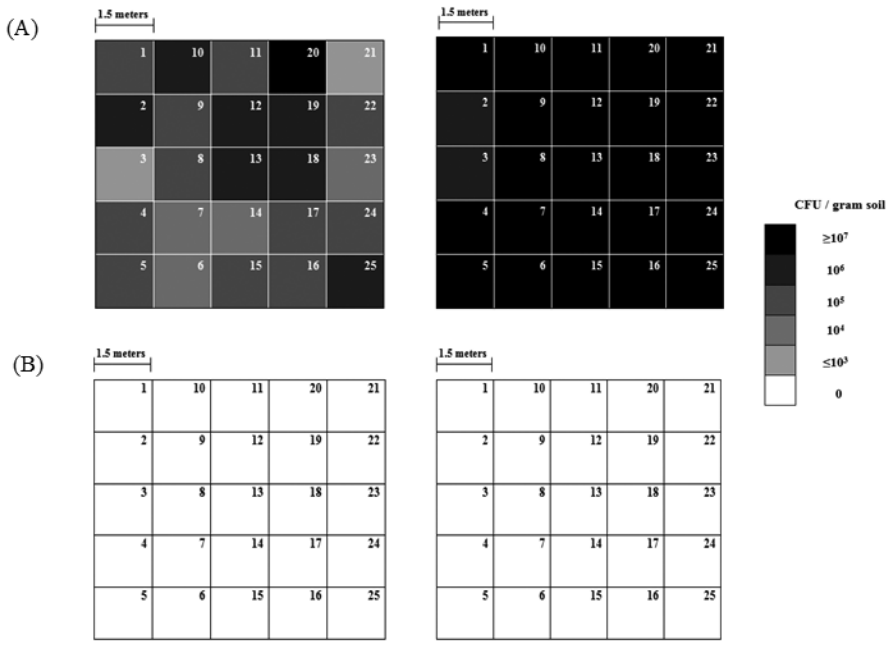

Fig 1–Culture of *B. pseudomallei* from the soil. (A) Colony count obtained from positive soil by direct culture (left) and enriched culture (right). (B) Results of all negative soil direct culture (left) and enriched culture (right).

correlation between soil physicochemical factors and the numbers of *B. pseudomallei* in the positive soil samples.

RESULTS

B. pseudomallei culture and DNA extraction from the soil

The 25 *B. pseudomallei* direct culture positive samples had concentrations of 1×10^3-6.6×10^6 colonies/g of soil. The concentration of *B. pseudomallei* with the enriched culture was $\geq10^7$ colonies/g soils (Fig 1A). The 25 soil sites that positive by culture were all positive by nested-PCR. For the negative site, *B. pseudomallei* was confirmed to be negative by both direct and enriched methods in all 25 sites (Fig 1B). When these 25 negative soil samples were confirmed by nested-PCR, 2 of them were positive for *B. pseudomallei* at the second round of amplification. Therefore, these 2 samples were excluded from physicochemical factors analysis of the negative site.

Physicochemical properties of soil

The pH, TOC, TN, EC, and EI from *B. pseudomallei*-positive and -negative soil sites were compared. The average values for all the physicochemical properties tested were significantly different between the positive and negative sites ($p<0.05$) (Table 1, Fig 2).

The pH for all the soil samples was below 7, indicating generally acidic soil in the study area. The pH values in the *B. pseudomallei*-positive soil site ranged from 4.13 to 5.05 and the average pH value of 4.6 in the *B. pseudomallei*-positive soil was higher than the 4.4 in the *B. pseudomallei*-negative soil (range 3.66 to 4.42).

Table 1
Physicochemical properties of *B. pseudomallei*-positive and -negative soil samples.

Factor	Positive site (n=25)	Negative site (n=23)	p-value
pH	4.578±0.2238	4.149±0.2244	<0.001[b]
Total organic carbon (%)	0.385±0.067	0.559±0.065	<0.001[b]
Total nitrogen (%)	0.027±0.005	0.023±0.004	0.012[a]
Carbon to nitrogen ratios	14.65±1.937	24.69±5.248	<0.001[b]
Exchangeable calcium (mg/kg)	43.60±27.82	67.20±39.85	0.016[a]
Extractable iron (ppm)	45.36±18.44	17.88±7.219	<0.001[b]

Each value represents the mean ± standard deviation.
[a]Significant at p<0.05; [b]Significant at p<0.001.

Table 2
Correlation between real time PCR results and soil physicochemical properties predicted by a generalized linear model.

Variable	Estimate	Std error	t-value	p-value
Intercept	-903375.0	830807.3	-1.087	0.290
pH	333338.4	208090.1	1.602	0.126
Total organic carbon	-482759.4	819030.0	-0.589	0.563
Total nitrogen	-978734.7	8453178.7	-0.116	0.909
Exchangeable calcium	-3432.2	1492.4	-2.300	0.033*
Extractable iron	472.1	2772.4	0.170	0.867

* Statistically significant (p<0.05).

The TOC in the *B. pseudomallei*-positive soil ranged from 0.26% to 0.52%, and the average in the *B. pseudomallei*-positive soil (0.39%) was lower than the 0.56% in the *B. pseudomallei*-negative soil (ranged from 0.30 to 0.63%). The average TN in the *B. pseudomallei*-positive soil was 0.027% (ranged from 0.02% to 0.04%), which was higher than the average of 0.023% (ranged from 0.02% to 0.03%) in the *B. pseudomallei*-negative soil. The C:N ratio in the *B. pseudomallei*-positive soil ranged from 12.23 to 21.00 and the average value is 14.7, which is lower than the average 24.7 in *B. pseudomallei*-negative soil (ranged from 15.20 to 36.69).

The EC in the *B. pseudomallei*-positive soil ranged from 10 to 100 mg/kg and the average value 44 mg/kg was lower than 67 mg/kg in the *B. pseudomallei*-negative soil (ranged from 20 to 190 mg/kg). The EI in the *B. pseudomallei*-positive soil ranged from 17 to 90 ppm and the average of 45.4 ppm was significantly higher than 17.9 in the *B. pseudomallei*-negative soil (ranged from 8 to 35 ppm).

Correlation between the concentration of bacteria in the soil and the soil physicochemical properties

A qPCR with standard curve was used to quantify the amount of *B. pseudomallei*

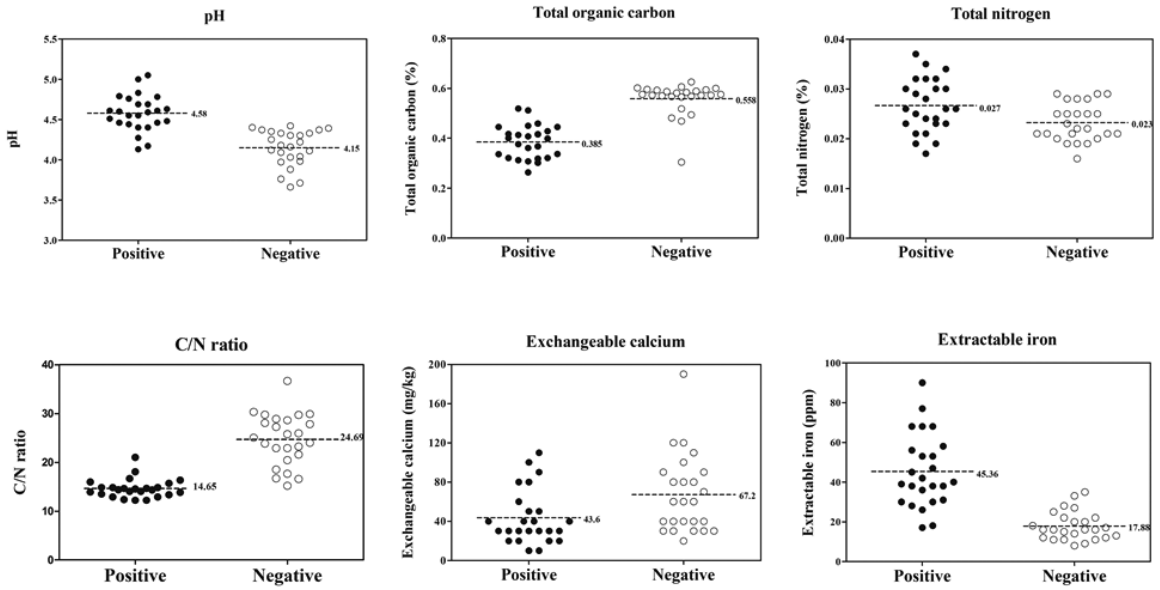

Fig 2–Scatter dot plot of the physicochemical factors investigated in positive and negative soil sites. The pH, total organic carbon, extractable iron and C:N ratio were significantly different between negative and positive soil sites.

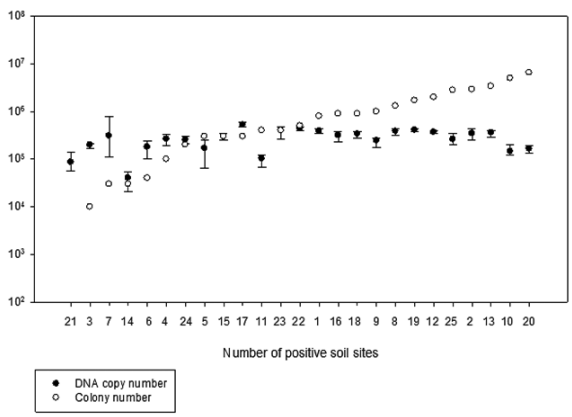

Fig 3–Scatter plots for DNA copy numbers by qPCR and direct soil culture.

in soil. There were 6 sites that DNA copy was higher than colony count, whereas 13 sites showed higher colony count than DNA copy number and 6 sites equivalent (Fig 3). The average of *B. pseudomallei* copy number was 2.82×10^5. The generalized linear model (GLM) method was used to study the correlation between soil physicochemical properties in *B. pseudomallei*-positive soil and DNA copy number of *B. pseudomallei*. The GLM showed only a negative correlation between EC and the number of DNA copies of *B. pseudomallei* ($p<0.05$) (Table 2).

The diversity of *B. pseudomallei*

One hundred seventeen *B. pseudomallei* isolates were collected. Their genotypes were analyzed using a BOX-PCR. Amplification of the *B. pseudomallei* DNA using BOXA1R primers generated variety of numbers and sizes of amplicons. The fingerprint patterns generated by the BOX-PCR consisted of 14-24 DNA fragments with a range of 0.28-10.0 kb. The band patterns from gel-electrophoresis were analyzed and classified into 25 distinct patterns (Fig 4A). Each isolate was classified with the BOX-PCR correlated

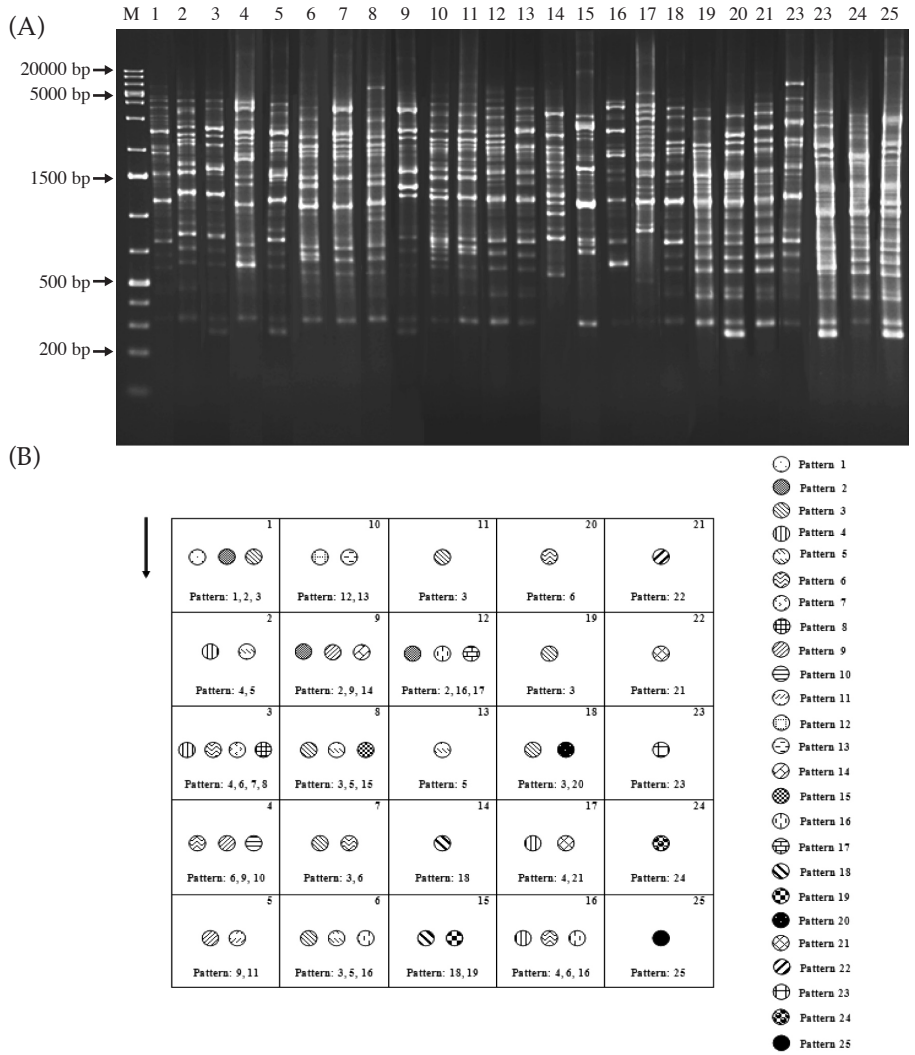

Fig 4–Type and diversity of *B. pseudomallei* in soil. Twenty-five BOX-PCR patterns seen on agarose gel (A) and diversity of *B. pseudomallei* as seen by spots of BOX-PCR obtained from each grid (B).

with its grid (Fig 4B). The more patterns per grid indicated the greater the diversity of *B. pseudomallei.*

The correlation between soil physicochemical properties and the genetic diversity of *B. pseudomallei* at each site was examined by plotting each physicochemical property against the number of genotypes of *B. pseudomallei* in each grid, plotted from the lowest to the highest number. The genetic diversity of *B. pseudomallei* tended to increase with increases in TOC, TN and EI values, but this trend was not significant (Fig 5).

DISCUSSION

B. pseudomallei can be generally found in Thailand, particularly in the northeast where 50.1% of soil samples are positive (Vuddhakul *et al*, 1999). Northeastern Thailand is also known as the endemic area of melioidosis (Suputtamongkol *et al*,

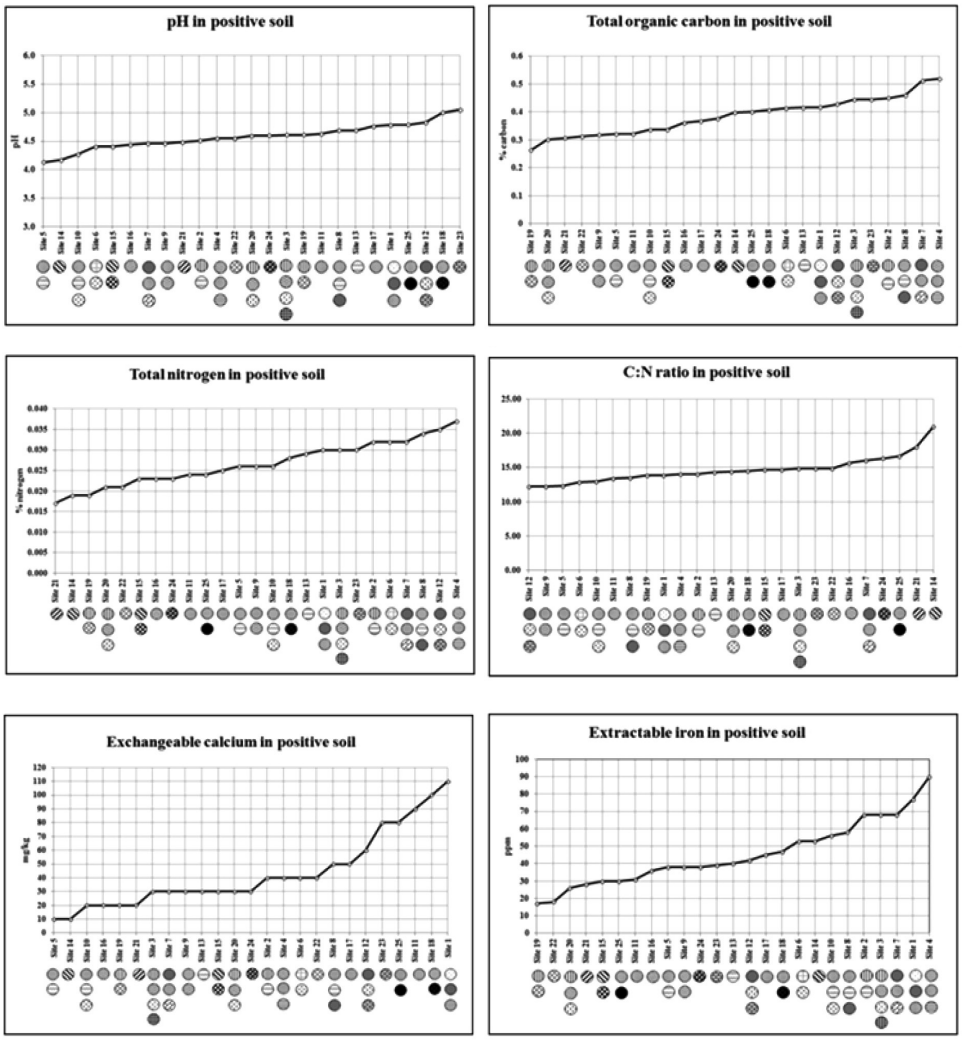

Fig 5–Plot of the diversity of *B. pseudomallei* for each positive site and the values for each physicochemical factor. BOX-PCR banding patterns found at each site (Y axis) and values for the physicochemical factors (X axis).

1999). A soil pH<6, moisture content >10% and higher carbon and nitrogen levels are all important for the growth of *B. pseudomallei* (Palasatien *et al*, 2008).

In our study, *B. pseudomallei*-positive soil sites had significantly different values for pH, TOC, TN, C:N ratio, EC and EI from *B. pseudomallei* -negative soil. The overall pH in the studied area was quite acidic. In this and previous study (Palasatien *et al*, 2008), the *B. pseudomallei*-positive soil samples have a pH less than 6. In the present study, the average pH in the *B. pseudomallei*-negative soil was 4.15 suggesting the pH critical range for *B. pseudomallei* is about 4-6. This finding is in agreement with a previous study that found *B. pseudomallei* cannot survive at pH< 4 (Wang-Ngarm *et al*, 2014). The optimal pH range for most plants and soil bacteria is 5.5-7.1. Soil in the northeastern Thailand is almost acidic (pH < 7)

(Boonsompopphan *et al*, 2008). Nearly half of the soil in northeastern Thailand contains laterite, which is rich in iron and aluminium. Those metal elements have a potential to generate hydrogen ions (H^+) which make the soil acidic (Cawte and Boyd, 2010). Iron is a soil micronutrient in and cofactor for microbial metabolism (Paul, 2006). In this study, distribution of iron rich soil correlated well with *B. pseudomallei*-positive soil. The low pH of the soil helps keep the iron in an available form for microbes.

The presence of and proportions of carbon and nitrogen are important for bacterial growth. TOC is used to describe all organic carbons that enter the soil through decomposition of plant and animal residues, root exudates and metabolites of living and dead microorganisms (Harris, 2007). In this study, the mean TOC level in *B. pseudomallei*-positive soil was lower than in the *B. pseudomallei*-negative soil, suggesting *B. pseudomallei* can survive under tough conditions; it has even been found to survive in water for up to 3 years (Moore *et al*, 2008). In environmental studies, the C:N ratio has been used as an indicator to express the quality of soil suitable for cultivation (Pitty, 1979). The average C:N ratio varies from country to country depending on the soil type, but values between 8 and 17 are generally found (Pitty, 1979). In this study, the mean C:N ratio in *B. pseudomallei*-positive soil (14.65) was lower than in *B. pseudomallei*-negative soil (24.69). Therefore, the pH and C:N ratio could be used for monitoring and controlling *B. pseudomallei* in the environment.

The concentration of *B. pseudomallei* in *B. pseudomallei*-positive soil was 1×10^3-6.6×10^6 CFU/g soil measured by direct culture. Interestingly, *B. pseudomallei*-negative soil was located only 100 m from *B. pseudomallei*-positive soil. The samples were both taken from a grassy field rarely used to feed cattle. In spite of their close location, the physicochemical factors were significantly different from each other.

In this study, nested qPCR was used to confirm the presence of *B. pseudomallei*. Since *B. pseudomallei* bacterium has more than one copy of the 16S-23S spacer, so the qPCR technique should be more sensitive than colony counts to quantify the number of bacteria (Supaprom *et al*, 2007). However, in our study more of the grids had lower PCR copy number than direct culture colony count numbers. Errors may occur with either method, such as errors in soil sampling, efficiency of DNA extraction and bacterial extraction from the soil. The copy numbers detected by nested qPCR was not much different among the positive sites, while colony counts varied by location. Two of the colony-negative grids were positive on the second round of the nested-qPCR, indicating low numbers of bacteria or the presence of unculturable forms of *B. pseudomallei* (Palasatien *et al*, 2008) which can persist throughout the dry environment (Inglis and Sagripanti, 2006).

As we are interested in both culturable and non-culturable forms of *B. pseudomallei* present in soil. The relationship between PCR copy numbers and the physicochemical properties of the bacteria-positive soil was examined. From this study, EC was negatively correlated with PCR copy numbers. An optimum pH of 4-6 was also important for growth of *B. pseudomallei.*

In this study, we used the BOX-PCR to type *B. pseudomallei* as it is simple and could generate high power of discrimination (Martin *et al*, 1992). The BOX-PCR has been recommended to classify *B. pseudomallei* (Currie *et al*, 2007). BOX-PCR using

the BOX-A1R primers, we found 25 patterns when evaluating *B. pseudomallei*. No statistically significant associations were seen between the various *B. pseudomallei* patterns and the various physicochemical factors examined. However, there was a non-significant association with TOC, TN and EI. An EI value of 150 to 200 mg/kg (150-200 ppm) indicates enriched soil. In our study, the average EI result was <150 mg/kg. The average EI was non-significantly higher at *B. pseudomallei*-positive sites than *B. pseudomallei*-negative sites. *B. pseudomallei* has genes coding for a two-component system that can fix iron (Lazar Adler *et al*, 2009). Interestingly, the soil sites with more diversity of *B. pseudomallei* contained higher EI than the sites with less *B. pseudomallei* diversity, suggesting EI as a possible factor affecting *B. pseudomallei* diversity, although these findings were not significant. Further study is needed to confirm this theory.

In conclusion, the studied physicochemical factors were significantly different between *B. pseudomallei*-positive and –negative soil samples. A pH range of 4-6, the C:N ratio and and EI level may be used to follow the presence of *B. pseudomallei*. The EC level may be used to control *B. pseudomallei* growth. It is unclear whether these studied factors could influence the diversity of *B. pseudomallei*.

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