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Seasonal variation of soil environmental characteristics affect the presence of *Burkholderia pseudomallei* in Khon Kaen, Thailand

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The environmental Gram negative bacteria *Burkholderia pseudomallei*, the causative agent of melioidosis, have been found globally. *B. pseudomallei* can survive under a wide range of environmental conditions and is isolated from soil in both rainy and dry seasons. The correlation of melioidosis cases have been predominantly found present in *B. pseudomallei* during rainfall. In the present study, various physico-chemical properties of soil in rainy and dry seasons in Khon Kaen, Thailand, where melioidosis is endemic, were investigated for their correlations with the presence of *B. pseudomallei*. The results revealed that the mean soil pH of 6.05, the low percentage of water holding capacity (%WHC) and the low iron (Fe) were significantly correlated with the presence of *B. pseudomallei* ($p < 0.05$) in the rainy season, while high concentration of manganese (Mn) was correlated with the presence of this bacterium in dry season ($p < 0.05$). These data will be useful in the control of *B. pseudomallei* in the environment to reduce melioidosis cases.

Key words: Soil physico-chemical properties, *Burkholderia pseudomallei,* melioidosis.

INTRODUCTION

The incidence of melioidosis is increasing globally and is correlated with the increase of global distribution of the causative soil bacteria, *Burkholderia pseudomallei* worldwide (Currie et al., 2008; Wiersinga et al., 2012). Melioidosis patients are infected either by inhalation or percutaneous inoculation from contaminated muddy soil or stagnant water in endemic locations and have resulted in pneumonia and/or sepsis with high mortality rate (up to 40%) of treated patients (Cheng and Currie, 2005; White, 2003; Dance, 1991). In Thailand, the highest incidence of melioidosis cases has been seen in the northeast with the highest presence of *B. pseudomallei* in soil (Vuddhakul et al., 1999). A prospective cohort study in northeast Thailand during 1997 to 2006 revealed the increasing incidence of human melioidosis indicating that the disease is an emerging public health issue (Limmathurotsakul et al., 2010). The seasonal emergence of melioidosis cases in the monsoonal rainy season was reported not only in Khon Kaen, Thailand (Srisurat et al., 2008) but also in Townsville, Australia (Cheng et al., 2008), which are the most prevalent areas of melioidosis in each country. The epidemiological study revealed that, in the rainy season, the mode of exposure to *B. pseudomallei* shift from percutaneous inoculation to

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aerosol inhalation resulting to higher prevalence of severe melioidosis patients (Cheng et al., 2008; Srisurat et al., 2008).

The correlations between the presence of *B. pseudomallei* and the characteristics of environmental habitat in endemic regions have been investigated by several groups. The soil survey in endemic area in rainy and dry seasons in Thailand demonstrated the correlation of the presence of *B. pseudomallei* with low soil pH, a moisture content more than 10%, higher chemical oxygen demand and high total nitrogen (Palasatien et al., 2008). In Australia, the presence of *B. pseudomallei* was associated with grasses, livestock animals, lower soil pH and different combinations of soil texture and color (Kaestli et al., 2009).

The survival of *B. pseudomallei* is supposed to be profoundly affected by seasonal changes of the physico-chemical natures of soil, the understanding of the correlation between the presence of *B. pseudomallei* and the climatic and physico-chemical factors of soil will provide useful information for the control of the pathogens in environments. The aim of this study was to investigate the association of the presence of *B. pseudomallei* and the physico-chemical properties of soil during rainy and dry seasons in Nam Phong District, Khon Kaen, Thailand.

MATERIALS AND METHODS

Soil sampling

Forty soil samples previously reported as *B. pseudomallei* positive soil sites by Palasatien et al. (2008) were collected from rice paddy field of Nam Phong and Muang Districts, Khon Kaen, Thailand in May, 2009 (rainy season) and collection was repeated in November, 2009 (dry season) using a global positioning system (GPS) device (GPS eTrex Vista HCX Color, USA) whereas another 10 soil samples from nearby sites were also collected.

Soil samples from each site were collected in a depth of 30 cm for 4 holes at the corners of a 1 m^2 area. One kilogram soil from each hole were pooled and separated using the quartering method. One kilogram of pooled soil from each site was packed in a plastic zip bag and kept in a cool plastic box to protect from sunlight and transferred for analysis within the same day.

Isolation and identification of *B. pseudomallei* from soil samples

Soil samples (100 g each) were mixed with 100 ml sterile distilled water, stand still overnight at room temperature to allow soil particles to settle down (Wuthiekanun et al., 2009), and then 10,100 and 500 µl of the supernatant was plated onto modified Ashdown's agar (Peacock et al., 2005; Wuthiekanun et al., 1990). The agar plates were incubated at 37°C and visually inspected daily for 4 to 7 days for colonies of *B. pseudomallei* as direct plate count. Another 500 µl of the soil supernatant was transferred into 3 ml of selective enrichment broth (threonine basal salt solution with 20 mg/l colistin) (Wuthiekanun et al., 1995). After incubation at 42°C for 48 h with agitation, all samples were enumerated by 10-fold serial dilution and plating of 100 µl aliquots in triplicate onto modified Ashdown's agar. The *B. pseudomallei* suspected colonies were confirmed by the following biochemical testing; Triple sugar iron (TSI), glucose and arabinose assimilation by using a minimal salts solution with 10% glucose and 10% L-arabinose (*B. pseudomallei* could assimilate glucose but not arabinose), antibiotics (augmentin and colistin) susceptibility test (*B. pseudomallei* is resistant to colistin but sensitive to augmentin). Finally, monoclonal antibody based latex agglutination test was performed for confirmation (Anuntagool et al., 2000; Wuthiekanun et al., 2002).

Physico-chemical properties of soil

For physico-chemical analysis, soil samples were air-dried and sieved to <2 mm. The following parameters were analyzed; soil pH was determined in soil paste (1:1) using pH meter (Eutech, EcoScan pH5, Singapore). Electrical conductivity (EC_e) was determined in 1:5 soil-water extracts using Bench-Top EC meter (Mettler Toledo, CH-8603, Switzerland) (Jones, 2001). Soil moisture content (MC) was determined by the percentage of weight loss before and after drying at 105°C in hot air oven for 24 h.

Soil water holding capacity (WHC) was measured gravitationally using 47 mm Buchner funnels connected to rubber tube and clamp. Sieved soil (100 g) was placed on the funnel and soaked in water overnight and allowed to drain until it stopped dropping (about 6 h). Dry weight of the soil was determined after drying at 105°C in hot air oven for 24 h. The WHC was calculated as a percentage of the weight loss when compared with the dried weight of the sample.

Soil organic matter (SOM) or readily oxidizable carbon was measured by Walkley-Black method (Walkley and Black, 1934). The oxidizable carbon was oxidized by $K_2Cr_2O_7$ under acidic condition. The remaining $K_2Cr_2O_7$ was back-titrated with $FeSO_4$ (ferrous -sulfate). Soil organic carbon (SOC) was converted from SOM by dividing by 1.724 (Nelson and Sommers, 1996). Loss on ignition (LOI) for organic matter determination was also measured at 500 ± 2°C for 3 h. The weight loss after cooling in desiccator was weighted and calculated as the percentage of dried weight sample.

Soil microbial biomass carbon

Soil microbial biomass carbon was measured by chloroform fumigation-extraction (FE) method (Ladd and Amato, 1989). After chloroform fumigation in a desiccator overnight (16 h), oxidizable carbon flux of non-fumigated soil and chloroform-fumigated soil was determined. Soil samples were extracted by 0.5 M K_2SO_4 and the dissolved organic carbons were analyzed by acidic dichromate method and converted to biomass. Soil biomass carbon was calculated using K_{ec} of 0.35 as the constant (Sparling et al., 1990).

Total Kjeldahl nitrogen (TKN) was performed using micro Kjeldahl digestion tube (Buchi, Digestion Unit K-435 with Scrubber B-414, Switzerland) and the ammonium salt was distilled by Ammonia distillation unit (Buchi, Distillation Unit K-350, Switzerland) (Jackson, 1958). In brief, 2 g of prepared samples were digested with H_2SO_4 and 1.1 g catalyst of K_2SO_4:$CuSO_4$:Se (100:10:1) at 360°C for 3 to 5 h until no more sulfuric fume was seen. The cool mixture was then added with 40% NaOH and distilled under water steam. The condensate was collected using indicator H_3BO_3 and titrated with 0.005 NH_2SO_4 standard.

Available phosphorus (P_{avai})

Available phosphorus was analyzed using Bray II extraction (Bray and Kurtz, 1945) and the extracted phosphorus was measured using molybdenum blue method (Murphy and Riley, 1962). Five grams of prepared soil samples were extracted using Bray II at the dilution of 1:10. The mixture was filtered followed by mixing with 2 ml of Riley's reagent. One milliliter of 2.5% ascorbic acid was used

Table 1. Summary of *B. pseudomallei* positive soil sites by culture method in rainy and dry seasons.

Seasons	*Bp.* positive sites	*Bp.* negative sites
Rainy	11/50 (22%)	39/50 (78%)
Dry	3/50 (6%)	47/50 (94%)

as reducing agent. The blue color of solution after left for 30 min was determined at 882 nm by UV-visible spectrophotometer (Shimadzu, UV-160 A, Japan).

Total iron

0.1 g of prepared soil was mixed with 4 ml of HNO_3:$HClO_4$ (3:1) in a 250 beaker. The mixture was digested at 200°C on a hot plate until dried before simmered with 15 ml of 6 N HCl at 70°C for 15 min. The mixture was then filtered using Whatman filter paper (No. 542) before dilution to 100 ml in a volumetric flask. Total iron in the solution was analyzed by flame atomic absorption spectrophotometer (Perkin Elmer, AA300, USA).

Total manganese

0.5 g of prepared soil sample was mixed with 10 ml concentrated HCl and 5.0 g NH_4F in a 250 ml beaker and warming to 90°C until almost dry to remove organic matter. After cooling to room temperature, 5 ml of 60% $HClO_4$ was added and the temperature was gradually increased to 200°C on a hot plate until dried before simmered with 5 ml of 6 N HCl at 70°C for 15 min; the solution was replenished with water to 100 ml using volumetric flask. Total manganese was determined by flame atomic absorption spectrophotometer (Perkin Elmer, AA300, USA).

Total aluminum

0.1 g of prepared soil was mixed with 20 ml concentrated HNO_3 in a 250 ml beaker to remove soil organic matter. The beaker was then covered by a watch glass and simmered at 70°C for 1 h. After cooling down to room temperature, 10 ml of 60% $HClO_4$ was added and digested at 200°C on a hot plate in a fume cabinet until no more white fume was seen. Digested mixture was filtered and adjusted to 100 ml using volumetric flask. The mixture was then analyzed for aluminum concentration using flame atomic absorption spectrophotometer (Perkin Elmer, AA300, USA). All physico-chemical parameters of soil in this study were measured three times independently.

Statistical analysis

Statistical analyses were performed using SPSS software. The significance of differences of the presence of *B. pseudomallei* and the physico-chemical properties of soil within rainy and dry seasons were addressed by a non-parametric Mann Whitney test. A p value < 0.05 was considered statistically significant.

RESULTS

Using a direct culture method, *B. pseudomallei* was isolated from rice paddy, cassava and horticulture fields with the prevalence of 11/50 sites (22%) in rainy season and only 3/50 sites (6%) in dry season (Table 1). By colony morphology, all *B. pseudomallei* isolates were classified as the Type I (Chantratita et al., 2008). The number of total bacteria in soil samples collected during dry season was significantly lower than that collected during the rainy season ($p < 0.05$) (data not shown).

In the rainy season, 3 out of 13 physico-chemical properties of soil were significantly different between *B. pseudomallei*-positive and –negative soil samples. As shown in Table 2 and Figure 1, the mean pH of the soil of *B. pseudomallei*-positive sites was 6.05 (range from 5.05 to 7.53), whereas that of negative site was about 5.51 with the range of 4.40 to 7.65. The percentage water holding capacity (%WHC) of the soil samples at *B. pseudomallei*-positive sites was 31.92% with the range of 17.75 to 53.31%, which was lower than that of the negative sites (mean of 40.17% with the range of 20.8-63.56%). Iron concentration of the soil at *B. pseudomallei*-positive sites was 16.33 mg/kg with the range of 6.17 to 288.48 mg/kg, while that of the negative sites was 109.86 mg/kg with the range of 6.38 to 351.32 mg/kg. All other parameters were not significantly different between *B. pseudomallei*-positive and negative sites.

In the dry season, soil samples collected at *B. pseudomallei*-positive sites contained higher manganese (mean of 138.46 mg/kg with the range of 126.12 to 146.50 mg/kg) than that collected at *B. pseudomallei*-negative sites (mean 46.88 mg/kg with the range of 5.90 to 197.29 mg/kg) (Table 2 and Figure 2).

DISCUSSION

To the best of our knowledge, this is the first study to compare physico-chemical properties of soil in rainy and dry seasons affecting presence of *B. pseudomallei* in Thailand. The prevalence of *B. pseudomallei*-positive soil in rainy season (22%) was higher than that of dry season (6%). This seasonal difference coincided well with the high emergence of melioidosis cases in rainy season in Khon Kaen, Thailand (Srisurat et al., 2008). The *B. pseudomallei*-positive sites were frequently found in orchards with livestock. In such a place, the fruit trees may provide a shadow to protect from sunlight and increase organic matters that facilitate the survival of *B. pseudomallei*. *B. pseudomallei* have been revealed to be associated with rhizosphere or root zone (Inglis et al., 2000; Levy et al., 2003). Recently, Kaestli et al. (2011) revealed the localization of *B. pseudomallei* in exotic grasses in Australia which could provide hydrophilic environments to facilitate the spreading of the pathogens in soil in endemic area. These observations suggest that environmental factors apart from physico-chemical factors of soil are also involved in the survival of *B. pseudomallei*.

Table 2. The soil physico-chemical factors at *B. pseudomallei*-positive and-negative sites in rainy and dry seasons.

Soil physico-chemicals (median)	Rainy season (n = 50)		Dry season (n = 50)	
	***B. pseudomallei* positive sites (n = 11)**	***B. pseudomallei* negative sites (n = 39)**	***B. pseudomallei* positive sites (n = 3)**	***B. pseudomallei* negative sites (n = 47)**
pH	6.05* (5.05-7.53)	5.51 (4.40-7.65)	5.76 (5.44-5.78)	5.75 (5.01-6.82)
% MC	17.08 (8.26-40.82)	22.51 (6.48-46.00)	17.29 (9.93-30.51)	13.03 (2.08-46.80)
EC (dS m^{-1})	0.05 (0.03-0.93)	0.06 (0.03-0.17)	0.07 (0.05-0.08)	0.05 (0.01-0.45)
% SOM	1.28 (0.25-3.69)	1.61 (0.29-3.55)	1.81 (1.07-2.10)	1.28 (0.14-3.46)
% WHC	31.92* (17.75-53.31)	40.17 (20.80-63.56)	45.23 (27.44-45.94)	38.39 (19.54-64.42)
% C	0.74 (0.15-2.14)	0.94 (0.17-2.06)	1.05 (0.62-1.22)	0.74 (0.08-2.01)
% N	0.0125 (0.0074-0.027)	0.013 (0.008-0.082)	0.0093 (0.0058-0.0098)	0.0069 (0.0019-0.0158)
C/N ratio	55.50 (20.27-95.31)	67.91 (14.93-106.36)	112.90 (106.9-124.49)	105.48 (22.86-189.66)
P_{avai} (mg/kg)	4.79 (0.03-124.39)	6.00 (0.02-122.03)	36.90 (4.88-45.80)	10.91 (1.80-133.89)
Soil microbial biomass (µg g^{-1}soil)	161.57 (22.19-656.58)	164.13 (22.07-403.25)	86.73 (20.84-311.84)	251.96 (20.13-579.68)
Total Fe (mg/kg)	16.33* (6.17-288.48)	109.86 (6.38-351.32)	189.04 (111.03-275.88)	97.54 (9.67-278.01)
Total Mn (mg/kg)	31.16 (1.89-112.77)	56.19 (0.39-255.21)	138.46* (126.12-146.50)	46.88 (5.90-197.29)
Total Al (g/kg)	3.18 (0.83-18.74)	7.38 (0.96-25.32)	9.44 (3.70-12.21)	7.25 (0.71-27.69)

*Statistically significant difference ($p < 0.05$) between positive and negative within season using Mann Whitney test.

In this study, in the rainy season, the near neutral pH soil favored the presence of *B. pseudomallei* as compared to the acidic pH soil in the *B. pseudomallei*-negative sites. Contrary to our results, Palasatien et al. (2008) reported that, in dry season, both *B. pseudomallei*-positive and negative soils were slightly acidic. In the laboratory condition, *B. pseudomallei* can survive and grow in a wide pH range from 5 to 8 (Tong et al., 1996; Chen et al., 2003). Soil pH might alter other environmental factors that determine the presence of *B. pseudomallei*.

The percentage of %WHC of the *B. pseudomallei*-positive soil in rainy season was lower than that of the negative soil. This lower %WHC might support the survival of bacteria. WHC is controlled by both soil texture and the organic matter contents. Soil with larger sand particles have larger pores to lower the %WHC, whereas soils with smaller particles such as silt and clay have small pore and allow soil to hold more water. The lower %WHC of the *B. pseudomallei*-positive soil in the rainy season agrees with the earlier finding on the presence of *B. pseudomallei* in sandy soil type (Palasatien et al., 2008). However, there was no correlation between the presence of *B. pseudomallei* and soil texture type in northern Australia (Kaestli et al., 2007).

In the present study, the iron concentration was lower in the *B. pseudomallei*-positive soil than in the negative samples. In contrast, in Australia, most of *B. pseudomallei* were found in soil with high iron content (Kaestli et al., 2007). Iron is an essential element for bacterial metabolism but excessive concentration is toxic to bacteria (Weinberg 1989, 1990). The presence of low iron in soil samples in this study may be suitable for *B. pseudomallei* survival in rainy season.

In the present study, high manganese concentration was related to the presence of *B. pseudomallei* in the dry season. Manganese is required as a terminal electron transport acceptor under anaerobic condition and also plays an important role in promoting plant growth and influence the growth of the rhizosphere microflora and soil bacteria (Lovley, 1991). Since soil condition is tough for *B. pseudomallei* survival and growth during dry season, high manganese soil may promote plant growth to provide better environment for the rhizosphere microflora and soil bacteria. Whether the manganese concentration directly affect *B. pseudomallei* survival or indirectly affect it through plant growth

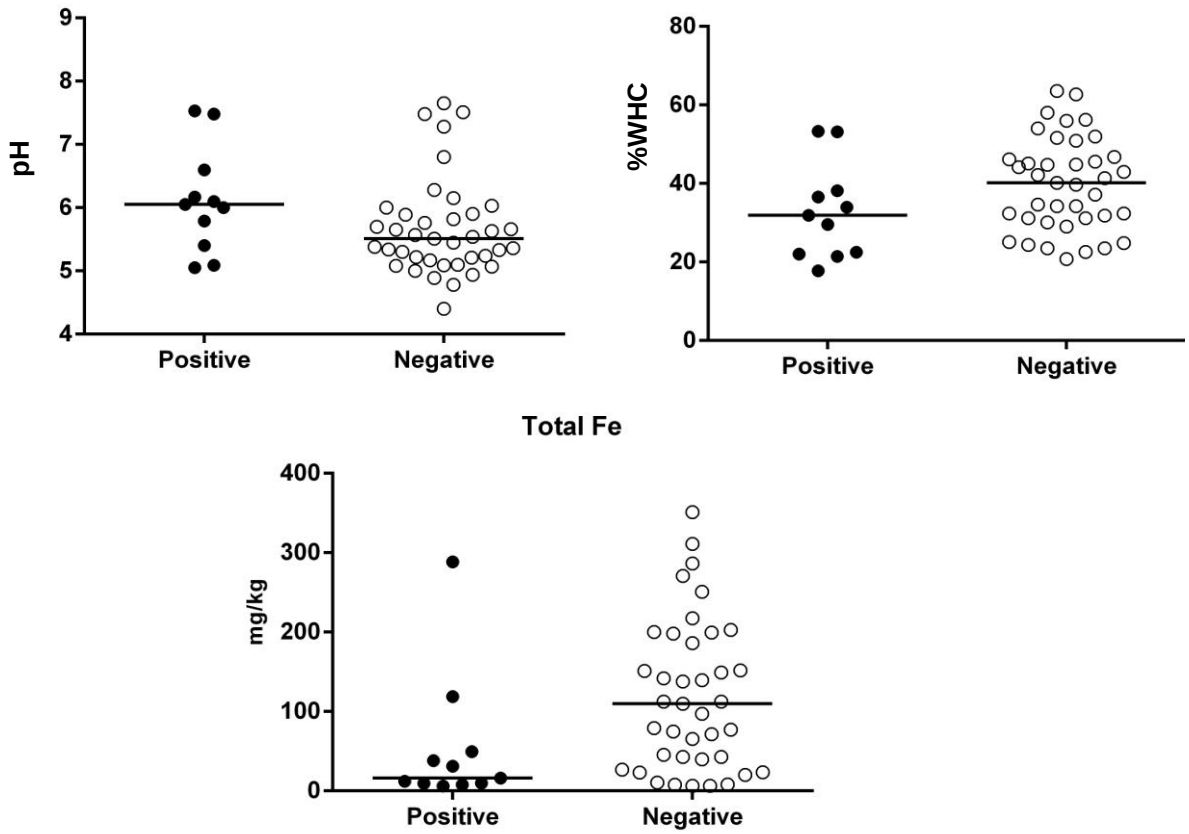

Figure 1. Scatter plot of pH, percentage water holding capacity (%WHC) and iron at *B. pseudomallei* positive and negative sites during the rainy season. The horizontal lines indicate the median of each parameter.

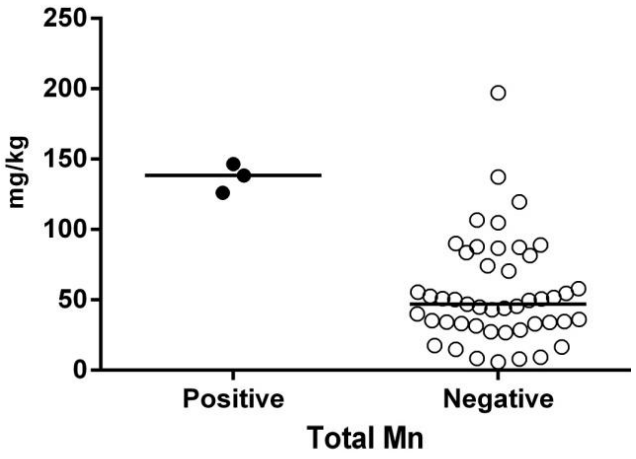

Figure 2. Scatter plot of manganese (Mn) at *B. pseudomallei*-positive and negative sites during the dry season. The horizontal lines indicate the median of the parameter.

should be investigated in the future.

The low number of *B. pseudomallei* recovered by a traditional plating technique from soil in dry season is similar to previous information even though a collection of four soil samples per site minimized the chance of missing the organism (Thomas et al., 1979). This may be because the bacteria entered a viable but non-culturable (VBNC) state in its natural environments in order to survive through the dry season (Shams et al., 2007; Inglis and Sagripanti, 2006; Chantratita et al., 2008) leading to the limitation of data analysis.

In conclusion, the results of this study demonstrate that *B. pseudomallei* is in the soil of Nam Phong and Muang districts in the Khon Kaen Province which is an endemic area of the bacteria. The information on physico-chemical factors of soil that correlated with the presence of *B. pseudomallei* in rainy and dry season may be useful to predict the presence of *B. pseudomallei* and understanding the soil factors that promote presence of bacteria through seasons which may be used to control or limit the incidence of melioidosis cases.

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