Epidemiological study of *Burkholderia pseudomallei* isolates from animals and soil in an endemic area of Ratchaburi by

**Pulsed-Field Gel Electrophoresis**

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**ABSTRACT**

*Burkholderia pseudomallei* is an environmental saprophytic bacterium found in water and soil in tropical and subtropical regions. It is the causative organism of melioidosis in humans and animals. This study analyzed genetic diversity of *B. pseudomallei* isolates from animals and soil in an endemic area in Ratchaburi Province. A total of 36 isolated originated from 22 clinical animal cases occurred during 2006-2010 and 14 soil samples that were collected during January-March 2011 in the goat farm where melioidosis cases have been identified. The chromosomal DNA of all isolates were digested with enzyme SpeI and run by pulsed-field gel electrophoresis (PFGE) to analyze for DNA fingerprint patterns. PFGE patterns of the 36 isolates appeared in 7 different genotypes. The dominant PFGE pattern belonged to 28 isolates which consisted of 14 isolates from animals and 14 isolates from soil. This study indicated the possible role of *B. pseudomallei* contaminated soil as a source of *B. pseudomallei* infection in animals. Melioidosis outbreaks in animals in Ratchaburi province conformed to the presence of *

**Keywords:** *Burkholderia pseudomallei*, Animals, Soil, Pulsed Field Gel Electrophoresis
INTRODUCTION

Melioidosis is a potential severe tropical disease occurring in humans and animals caused by *Burkholderia pseudomallei* (Koonpaew et al., 2000; Sprague and Neubauer, 2004; Kaestli et al., 2007). The bacterial infection is occurred as a result of exposure to contaminated soil or water (Dance, 2000; Inglis et al., 2004; Koonpaew et al., 2000). It is isolated frequently from soil in regions where the disease occurs. The most likely of transmission are direct percutaneous abrasion, inhalation and ingestion (Currie et al., 2000). The epidemiology of melioidosis is complicated due to the environmental persistence of the organism and is subject to distinct differences in the organism’s distribution in soil, disease presentation and incidence rates among different areas of endemicity (Robert et al., 2006). In Thailand, the endemic of melioidosis has been labeled as the main area (Dance, 1991). The greatest number has reported with an estimated 2,000-3,000 cases each year (Leerarasamee, 2000). The risk of the disease varies according to the region of the country because of unevenly distributed of organism, with most cases being found in the northeastern part of Thailand (Chaowagul et al., 1989). The epidemiological study about the distribution of virulent strains and the source of melioidosis infection are available, 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(Smith et al., 1995; Wuthiekanun et al., 1995; Brook et al., 1997; Vuddhakul et al., 1999). The genotyping of isolates from the environment and cases of disease is an essential component of outbreak investigations to link isolates to a common contaminated source (Currie et al., 2001). Understanding the epidemiology of melioidosis can be undertaken by molecular typing which offers a very discriminatory tool for differentiating strains, several studies have used pulsed field gel electrophoresis (PFGE) to study clonal relatedness because of its high discriminative power. (Currie et al., 2001; Azura et al., 2011; Chua et al., 2011).

In this study, we effort to find distribution of *B. pseudomallei* which contaminated in soil surrounding the goats farm from the disease outbreak in Ratchaburi province and compare the genetic relatedness between isolated from infectious animals strain and soil isolates by using PFGE.
MATERIALS AND METHODS

Soil and Infectious Animals isolates

A total of 459 soil sampling was collected from 51 soil sites, 153 holes in Ratchaburi province, within 300x500 m² surrounding goats farm where the melioidosis occurs during the dry season (January to March 2011). Approximately 500 g of each soil sample was removed from each hole at a depth of 15, 30 and 45 cm and transported immediately to the laboratory. Soil was taken to homogeneity and cultured for the presence of *B. pseudomallei*, using 5 g of each soil sampling put into a sterile tube, mixing well with 40 ml of sterile distilled water, and shaken vigorously for 15 min, allowed to settle for sedimentation 15 min, 2 ml of the soil supernatant were transferred into 10 ml of Ashdown’s selective enrichment broth and incubate at 37°C for 48 h. A further 1 ml of supernatant was serial 10-fold diluted to concentrations of 10⁻² and 10⁻³ and 100 μl of each dilution was spread onto modified MacConkey agar which 5 mg/liter of gentamicin contained, incubated aerobically at 37°C for 48-72 hr. The culture plates were observed daily for the morphological appearance of *B. pseudomallei* colonies.

A total of 22 isolates of *B. pseudomallei* were available from melioidosis cases of 13 goats, 4 deer, 4 swine and 1 porcupine in Ratchaburi province during 2006-2011. Identity was confirmed by conventional method and preserved in cryobank (Mask Diagnostics, Germany) at -80°C. Working culture were maintained in 5% sheep blood agar, incubated at 37°C for 48 h.

Identification of isolates

All isolates were identified by biochemical tests, which were positive to oxidase, motility, nitrate reduction, oxidative fermentative to glucose, maltose and lactose. The positive colonies confirmed as *B. pseudomallei* using polymerase chain reaction for determined the presence of the specific amplicons of the 16S rRNA gene (411 bp), used primer BS4R (5’-CAC TCC GGG TAT TAG CCA G-3’) and U33 (5’-AAG TCG AAC GGC AGC ACG G-3’), followed by Dharakul et al. (1996). PCR amplification was performed in a total volume of 50 μl containing PCR buffer (contain 1.5 mM MgCl₂), 1.25 unit HotStarTaq DNA Polymerase(QIAGEN, Germany), 200 μM of each dNTP mix, 2 mM MgCl₂, distilled water and 0.5 μM each of primer BS4R/U33, used reference strain DMTC 27191 for positive control and distilled water for negative control. The reaction mix was activated initially 95°C for 15 min, followed by 35 cycles of denaturation 95°C for 30 sec, annealing 62°C for 30 sec and extention 72°C for 30 sec, and a final extention 72°C for 10 min. PCR products were separated on 1.5% agarose gel.
Pulsed-field gel electrophoresis

Agarose block preparation

DNA and block were prepared by modification of the method of Thong et al. (1994). Pure colonies were grown in 5% sheep blood agar at 37°C for 24 h. Cells were suspended and adjusted to optical density at 600 nm of 1.2 in 10 mM Tris-HCl (pH 7.5) and 1 M NaCl, washed once and mixed with an equal volume of 2% low melting agarose, then loaded into block moulds, allowed to solidify in 4°C for 10 min and incubated overnight at 37°C in lysozyme solution (6 mM Tris-HCl, 1 mM NaCl, 100 mM EDTA, 0.5% Brij, 0.22% deoxycholate, 0.5% Sarkosyl and 2 mg/ml lysozyme). Agarose block were then transferred to proteinase K buffer containing 1 mg/ml proteinase K, 0.5 M EDTA, 0.5% Sarkosyl and incubated at 50°C for 48 h. The cell debris and proteinase K were then removed by five wash in TE buffer for 20 min at room temperature and stored at 4°C until use.

Digestion and Electrophoresis

DNA plugs were equilibrated in RE buffer at 37°C for 30 min, then digested with 10 units of SpeI in 100 μl of RE buffer at 37°C overnight. The digests were washed once in TE buffer and run on 1% agarose gel on a Bio-Rad CHEF DRIII (Biorad, USA), with a 3-50 sec pulses ramp at 6v/cm for 30 h. Gels were stained with ethidium bromide, visualized with ultraviolet light in GelDoc.It (UVP, USA), and analyzed with the molecular analyst program on Bio1D++ (Vilber Lourmat, Germany).

RESULTS

A total of 459 soil samples was collected from 153 hole in 51 sites surrounding goats farm in Ratchaburi province. The isolation rate of *B. pseudomallei* at a depth of 15 down to 45 cm were observed 3.05% (14/459). The isolates of sample collected at a depth of 15 cm showed 4.58% (7/153) more frequently than the isolates at a depth of 30 and 45 cm which were found 2.61% (4/153) and 1.96% (3/153). (Table 1)

The PFGE of macrorestricted chromosomal DNA from a total of 14 soil samples and 22 infectious animal isolates revealed 7 pattern types different. The majority isolates, 28 out of 36 which consisted of 11 out of 12 goats (within soil sampling sites), 2 out of 4 deer (2 km nearby), 1 porcupine (35 km distant) and 14 soil isolates were exhibited predominant type A. The other distinct patterns observed 1 out of 12 goats isolates which located within soil sampling sites and another one 75 km distant were found type B and type C. Whereas 2 out of 4 deer were showed
type D and type E, these were deer raising within the same farm which was found type A. Interestingly, 4 swine isolates which located 3 km nearby showed 2 pattern types different, by 3 isolates were found as type F and another one was type G. (Table 2)

A dendrogram depicting the estimated phylogenetic relationship constructed based on pairwise comparison of PFGE banding. Overall, 28 predominant isolates namely type A revealed 100% genetically identical. The isolates from infection animals were found to be genetically diverse, more than 75% displayed genetic similarity between type A and type B, these were goats raised within the same farm. Whereas isolates among type C from goat which farther located distance 75 km and type F from 3 out of 4 swine showed genetic relatedness more than 85%. Moreover, approximately 75% was observed genetic similarity between 1 out of 4 swine and 1 out of 4 deer as type E and type G, while there was one deer namely type D showed 55% genetic similarity amongst all isolates. (Figure 1)

Table 1  Detection of *B. pseudomallei* in soil samples collected from an endemic area in 300x500 m² surrounding goats farm in Ratchaburi province

<table>
<thead>
<tr>
<th>Depth of soil sampled (cm)</th>
<th>Number of detected samples / number of collected samples (Percentage of isolates)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>7/153(4.58%)</td>
</tr>
<tr>
<td>30</td>
<td>4/153(2.61%)</td>
</tr>
<tr>
<td>45</td>
<td>3/153(1.96%)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>14/459(3.05%)</strong></td>
</tr>
</tbody>
</table>
Table 2  Number of isolates and distribution of B. pseudomallei SpeI-pulsotypes

<table>
<thead>
<tr>
<th>Sources</th>
<th>Distance far from soil sampling site (km.)</th>
<th>Number of isolates</th>
<th>Total of pattern types</th>
<th>Number of isolates and PFGE pattern types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goats</td>
<td>within sampling sites</td>
<td>12</td>
<td>2</td>
<td>11</td>
</tr>
<tr>
<td>Goat</td>
<td>75</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Deer</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Porcupine</td>
<td>35</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Swine</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Soil sampling sites*</td>
<td>14</td>
<td>14</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>36</td>
<td>7</td>
<td>28</td>
</tr>
</tbody>
</table>

* sampling sites: 300x500 m² surrounding the goat houses

Discussion

This study investigated the distribution of B. pseudomallei in soil during the dry season over a period of 3 months (January-March, 2011). Soil sampling site was conducted in 300x500 m² surrounding goats houses in Ratchaburi province, where the melioidosis in animals occurred.

The isolation rate of B. pseudomallei from the soil samples collected at a depth of 15 down to 45 cm were different, 4.58% isolates appeared at a depth of 15 cm, whereas 2.61% and 1.96% were observed at a depth of 30 and 45 cm. A total of 3.05% positive isolates from 14 out of 459
samples collected was found less than that previous studies, in northeast Thailand at different time periods during the rainy season by Vuddhakul et al. (1999), Chantratita et al. (2008) and Wuthiekanun et al. (2009), which were reported the higher appearance of *B. pseudomallei* 20.4%, 80% and 28%, respectively at a depth of approximately 30 cm in soil. This is not surprised anything because in northeast Thailand was remarked as hyperendemic area, the soil in this region harbors *B. pseudomallei* more frequently than the soil in the other regions (Vuddhakul et al., 1999). In addition, the prevalence of isolates in the wet season were more possibly found than dry season because surviving bacterial in underground were carried by the rising water through the upper soil layer and multiply under more favourable conditions. However, a seasonal variation is not a major determinant of *B. pseudomallei*, previous studies of Rolim et al. (2009) during dry season in northeastern Brazil was present the positive culture at 58.8% of soil samples collected at a depths between 20-40 cm, whereas the studies of Wuthiekanun et al. (1995) and Brook et al. (1997) in northeast Thailand and Northern Territory, Australia, also recovered the positive isolates from soil during the dry season. However, the increase numbers and survival of the organisms in the soil layers were correlated with the soil type and high rainfall (Strauss et al., 1969), heavy clay soils are much better at supporting bacterial persistence than well-drained, light and sandy soils (Inglis and Sagripanti, 2006). Soil with a water content of less than 10% led to the death of *B. pseudomallei* within 70 days, while soil with a water content of more than 40% maintained bacterial life for 726 days (Tong et al., 1996). The difference of physical, biological and chemical soil features appear to influence the survival of isolates (Dance, 2000; Inglis and Sagripanti, 2006). Additionally, the discrepancy could be due to the differences in specimen collection, isolation and identification techniques, or to ecologic factors influencing the environmental presence of *B. pseudomallei* (Tong et al., 1996).

The genotyping analysis of melioidosis was performed on *SpeI* macrorestriciton pattern which was found to be more discriminatory in PFGE typing for *B. pseudomallei* (Chua et al, 2011). A dendrogram depicting the estimated phylogenetic relationship based on comparison of genetic banding patterns, a total of 36 isolates were identified 7 PFGE pattern types different, by 28 dominant isolates demonstrated 100% genetically identical. The most of 14 soil isolates were observed single fingerprint pattern, contrast with previous studies of Chantratita et al. (2008) and Wuthiekanun et al. (2009) which revealed the genetic variability isolates to 12 PFGE pattern
types in soil samples from disused land in northeast Thailand and 10 PFGE pattern types isolates from rice paddy soil. These difference might be explained that there were soil isolates from a distinct geographical location and our isolates were investigated in soil taken from a small geographic area where was not to be the genetic diversity location.

The isolates obtained from infectious animals were mainly from 12 goats which raising within soil sampling sites and 4 deer located 2 km in the field nearby were observed 2 and 3 fingerprint pattern types, by 11 out of 12 goats and 2 out of 4 deer were found 100% genetic identical pattern with isolates which recovered from soil. This indicates that the soil which contaminated with *B. pseudomallei* might be the sources of animals infection in this outbreak. However, some isolates which isolated from animals nearby such as deer showed genetic similarity, this likely to be the descendant of clones of the same progeny from soil isolates, which were disseminated to different areas especially during the wet season (Strauss et al., 1969; Haase et al., 1995; Struelens, 1996). Interestingly, It was found that from 4 swine isolates which located 3 km nearby soil sampling sites showed 2 genetically pattern types distinct from the others. It is difficult to explain about the source of infection because the piggery were situated onto a concrete platform and did not expose to soil environment. It is possible that there was importation of an infected animals from the other place or the transmission modes of infection may be come from bacterial contaminated in water supply, including percutaneous abrasion, inhalation of aerosolized bacteria, and ingestion all remain possible from contaminated water (Currie et al., 2001).

The infectious animal isolates recovered from several locations were found to be genetically diverse and heterogeneous, such as a case of porcupine which located 35 km distant from soil sampled sites was found 100% genetically identical with dominant pattern isolates. Whereas the isolates of a goat which situated 75 km distant from soil sampling sites and deer located nearby appeared genetically different. This is able to support that several strain of *B. pseudomallei* is distributed in the environment. But it is difficult to speculate about the via of infection between animals and environmental contaminated, excepted, there is an environmental study such as soil or water and compare the association of strain isolated with the emerging disease. The presence of identical genetic patterns among the clinical and environmental isolates evaluated suggested a link between the pathogens present in contaminated soil and the emergence of melioidosis (Chen et al., 2010).
In conclusion, severe strain of *B. pseudomallei* is distributed in soil as an endemic organism in this goats farm. The occurrence of the disease in animals was associated with isolates in soil, this was supported by the molecular typing of soil isolates which revealed the same clonality of outbreak strain. Interestingly, it is likely to be extended of endemicity to exposure to animals in the boundaries nearby.

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