DETECTION OF *BURKHOLDERIA PSEUDOMALLEI* FROM POST-FLOOD SOIL SAMPLES IN KELANTAN, MALAYSIA

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Abstract. *Burkholderia pseudomallei* is an important causative organism of fatal community bacteremia especially in Southeast Asia and northern Australia. Outbreaks of melioidosis have been reported post-floods and -typhoons. A cross sectional study was conducted in January 2015, following a major flood in Kelantan, Malaysia to detect presence of *B. pseudomallei* from soil. A total of 89 soil samples were cultured for *B. pseudomallei* on Ashdown agar. Putative colonies underwent further staining and biochemical testing prior to confirmation by PCR. Rate of detection was 1%, although low, it nevertheless indicated a risk for melioidosis among flood victims in Kelantan. Flood affected individuals should be made aware of symptoms of melioidosis and healthcare providers must have a high index of suspicion of patients presenting with fever. Such subjects should be screened for the possibility of melioidosis and given prompt treatment to avoid preventable death.

Keywords: *Burkholderia pseudomallei*, culture method, environmental sample, melioidosis, Malaysia

INTRODUCTION

Melioidosis has been recognized as infectious disease of important public health concern as it accounts for up to approximately 20% of community acquired bacteremia and causes death in 40% of treated patients in endemic areas (White, 2003). It is caused by *Burkholderia pseudomallei,* a gram-negative bacillus that is capable of surviving in hostile environmental conditions (prolonged nutrient deficiency, dehydration, wide temperature range, acidic and alkaline environment) (Cheng and Currie, 2005). The bacteria are found in surface water and soil and transmission is usually by contact of lacerated or abraded skin with contaminated soil or water. Melioidosis may also be acquired by aspiration or ingestion of water and inhalation of dust. An increase in numbers of cases of sepsis and pneumonia due to *B. pseudomallei* have been reported during periods of monsoonal rains and floodings (Puthucheary, 2009; White, 2012; Dance, 2014).

In Malaysia melioidosis is endemic as evidenced by the many local epidemiologic studies published in the last decade

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describing patients at risk and antimicrobial of choice (How *et al*, 2005; Pagalavan, 2005; Raja, 2008; Deris *et al*, 2010; Hassan *et al*, 2010). However, there is a paucity of local data on environmental sampling using the latest technology, with two published study conducted in Pahang (Sapian *et al*, 2012) and Sarawak (Hassan *et al*, 2015), Malaysia. Earlier environmental studies were conducted by inoculating samples (intraperitoneally) in hamsters (Strauss *et al*, 1969). Availability of environmental samples could assist in determining geographical areas at risk of melioidosis. Thus, the aim of this study was to detect the presence of *B. pseudomallei* from soil from flood affected areas of 2014 Kelantan, Malaysia flood.

MATERIALS AND METHODS

Collection of soil samples

A total of 89 soil samples were collected in January 2015, about 2 weeks after the flood in Kelantan, Malaysia had receded. The sites chosen were based on areas where people resided and were most frequented before and during the flood, such as housing areas, flood relief centers, houses of worship and recreational areas. Location sites of soil sampling were recorded using a handheld global positioning system unit (GPSMAP® 76CSx) (Fig 1). Wherever possible, three soil samples were taken from each site. Samples consisted of mud (upper most layer made up of run-off soil from other areas), lower layer of soil that represents the initial surface soil prior to flooding and deeper layer (30 cm) from the initial surface soil. Samples were collected aseptically by flame sterilization method after washing the scoop with clean water. Collected samples were labelled and sent at room temperature within 48 hours of collection in sealed containers to the laboratory.

Culture method

A 100 ml aliquot of distilled water was mixed with 100 g of soil sample and left overnight at room temperature. The upper layer from each sample (50 ml) was transferred into sterile tube and homogenized before spreading an aliquot onto selective Ashdown media (ISOLAB, Shah Alam, Malaysia) following method described by Sapian *et al* (2012) with some modification. Plates were incubated at 37ºC for 2-3 days and then left at room temperature for 4-5 days. Plates were inspected daily for growth. Putative *B. pseudomallei* colonies were tested by Gram staining and if were gram-negative bacilli, colonies were tested further by oxidase, motility, indole and triple sugar iron (TSI) tests. If found to be oxidase positive, motile and indole negative, and if TSI test showed no changes of butt and slant agar with no gas and hydrogen sulfide production, colonies was sub-cultured on blood agar and subjected to PCR assay for *B. pseudomallei.*

PCR protocol

PCR method was conducted as described by Brook *et al* (1997). In brief, a single colony was picked from an overnight culture on blood agar plate and suspended in 20 μl of sterile distilled water. The suspension was heated in a water bath at 100°C for 20 minutes and then centrifuged at 10,000*g* for 10 minutes. Two microliters of the supernatant were used in each 25-μl PCR reaction solution. Forward primer BP1 (5′ AATCATTCTGGCTAATACCCG 3′) and reverse primer BP2 (5′ CGGTTCTCTTTCGAGCTCG 3′) were used to amplify 16 rDNA region of *B. pseudomallei*. Amplicons (550 bp) were analysed by 1.2% agarose gel-electrophoresis at 130 V for 1 hour.

Fig 1–Soil sampling sites in 8 districts of Kelantan, Malaysia.

RESULTS

Out of the 89 samples cultured on Ashdown media, 64 (72%) samples showed bacterial growth, with 42 samples having colony morphology of *B. pseudomallei.* After subculturing on blood agar and biochemical tests, only one putative sample (detection rate of 1%) was confirmed as *B. pseudomallei* by PCR. The positive sample had the typical wrinkled, deep pink to purple colonies on selective Ashdown media with characteristic 'safety pin' appearance upon Gram staining (Fig 2). *B. pseudomallei* was isolated from a deep soil sample.

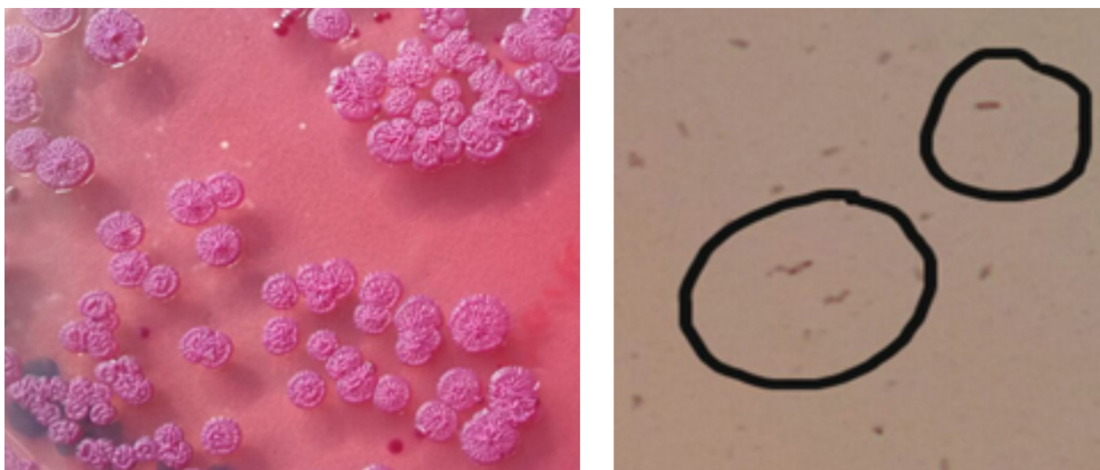

Fig 2–Typical appearance of *Burkholderia* spp on Ashdown media (left panel) and upon Gram staining (right panel).

DISCUSSION

Detection rates of *B. pseudomallei* vary among studies. The rate in this study was higher compared to a study conducted in Hong Kong but was much lower than a study in Thailand. The study in Hong Kong involved 1,420 soil samples taken from an oceanarium with a detection rate of 0.6% (Lau *et al*, 2014), whilst the study in Thailand consisted of 200 samples taken from two rice fields with a detection rate of 74.5% (Limmathurotsakul *et al*, 2012). Although the study in Thailand had a much higher detection rate, no further confirmatory test either by PCR or biochemical testing was carried out as was in our study and the study in Hong Kong. A study conducted in East Malaysia reported a PCR confirmed detection rate of 3% (9/304 samples) for *B. pseudomallei* from soil and water samples taken from various areas with risk of exposure including timber logging areas, hydro electric project, school compound, Iban and Malay villages and a nearby river bank (Hassan *et al*, 2015). Another study carried out in response to an outbreak during a search and rescue operation in Pahang, Malaysia revealed a detection rate of 14.3% (Sapian *et al*, 2012). However, colonies were identified by biochemical testing and no confirmation by PCR was performed. Our study had 47% putative *B. pseudomallei* colonies but only one sample was confirmed by PCR. Thus, it is important to conduct confirmatory test as part of testing of environmental samples for *B. pseudomallei* so as to reduce the number of false positive results. This was one of the recommendations by an international working party (Detection of Environmental *B. pseudomallei* Working Party, DEBWorP) formed during the VIth World Melioidosis Congress in 2010 (Limmathurotsakul *et al*, 2013).

In this study, the traditional extraction of bacteria from soil by mixing with distilled water was used. Detection rate of *B. pseudomallei* in this study may have been higher if extraction of the bacteria was conducted by mixing the soil samples with enriched broth or other solutions. Trung *et al* (2011a,b) reported higher recovery of viable *B. pseudomallei* from soil samples using polyethylene glycol and sodium deoxycholate detergents. They also reported higher recovery when enriched broth was used for extraction. In order to improve detection rate of *B. pseudomallei* from soil samples, DEBWorP proposed using L-threonine-buffered salt solution (TBSS or Galimand and Dodin broth) or Ashdown broth in bacteria extraction (Limmathurotsakul *et al*, 2013).

Although one of the transmission modes of melioidosis is through inhalation of dust, studies have shown that the detection rate of *B. pseudomallei* in soil samples are higher for samples taken at a depth of 20-40 cm from the surface (Kaestli *et al*, 2007; Rolim *et al*, 2009). In our study, the sample that was culture positive for *B. pseudomallei* was from soil taken at a depth of 30 cm. This depth was also recommended in the DEBWorP guidelines (Limmathurotsakul *et al*, 2013).

Data on environmental isolates are useful in risk assessment of melioidosis as well as in investigation of outbreaks and other epidemiological investigations. It could also be used for disease prediction and targeted measures to control infections in endemic areas, especially during extreme weather events (floods, typhoons and tsunamis).

Culture method has been the gold standard for detection of *B. pseudomallei* in environmental samples as it has

100% specificity (Limmathurotsakul *et al*, 2012). However, it is labor intensive and may take up to 3 weeks before obtaining the final result. Alternatively, direct *B. pseudomallei* detection from soil samples by PCR technique has been developed and assessed (Kaestli *et al*, 2007; Trung *et al*, 2011a; Lau *et al*, 2014). To date, PCR technique has a higher positivity rate compared to culture method, with a detection limit reported from as low as 1-1.5 colony forming unit of *B. pseudomallei*/g of soil (Kaestli *et al*, 2007). PCR also has the advantage of shorter processing time and receiving timely results are important especially in outbreaks.

In conclusion, this study was successful in detecting isolates of *B. pseudomallei* from soil samples in Kelantan, Malaysia. Although the detection rate was low, nevertheless it indicated the presence of risk for melioidosis among flood victims in Kelantan. Further research should be conducted to map the distribution of *B. pseudomallei* especially in flood prone areas. This could assist in informing flood affected victims of the risk of melioidosis and alert healthcare providers to have a high index of suspicion for melioidosis among those seeking treatment as the disease has diverse clinical manifestations. Heightened awareness and prompt appropriate medical treatment could prevent death due to this potentially infectious disease.

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