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Colonial morphotypes and biofilm forming ability of *Burkholderia pseudomallei*

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Abstract. *Burkholderia pseudomallei* the causative agent of melioidosis, is being increasingly recognized as an important cause of morbidity and mortality in South East Asia. Biofilm formation of *B. pseudomallei* may be responsible for dormancy, latency and relapse of melioidosis. Based on the colonial morphology of the bacteria on *B. pseudomallei* selective agar medium, seven distinct morphotypes were identified. This study was conducted to assess the *in vitro* biofilm produced by *B. pseudomallei* and to investigate possible correlation between *B. pseudomallei* morphotypes with biofilm forming abilities of the isolates. Using a standard biofilm crystal violet staining assay, comparison was made between the biofilm forming ability of 76 isolates of *B. pseudomallei* and *Burkholderia thailandensis* ATCC 700388. Amongst the blood isolates, 30.2% were considered as high biofilm producers and 27.9% were low producers, 33.3% of the pus isolates were considered as high and 16% low biofilm producers. Most of the isolates were identified as morphotype group 1 which displayed a rough centre with irregular circumference on the agar medium. However, we did not find any correlation of *B. pseudomallei* morphotypes with biofilm forming abilities ($p > 0.05$). Additional studies are needed to identify internal and external factors which contribute to the high and low biofilm formation of *B. pseudomallei*.

INTRODUCTION

Burkholderia pseudomallei is an environmental saprophyte with a remarkable capacity to infect humans resulting in a potentially lethal infection known as melioidosis. The clinical spectrum of melioidosis is extremely broad ranging from an indolent local infection to septicaemia, particularly in the immunocompromised. A prolonged period of dormancy may occur between exposure to *B. pseudomallei* and clinical manifestation of infection (Puthucheary, 2009). In the laboratory, the colonial morphology of this organism varies both within and between clinical isolates which suggests that *B. pseudomallei* undergoes a process of adaptation involving altered expression of surface determinants and associated colony morphology that facilitates bacterial survival *in vivo* (Chantratita *et al.*, 2007). Such an *in vivo* adaptation of virulence "drift", either by upregulation or attenuation does occur under certain conditions such as iron availability (Ulett *et al.*, 2001). When *B. pseudomallei* was subjected to a single passage in mice (Vellasamy *et al.*, 2009), increased production of six bacterial enzymes had been demonstrated, which agrees with the hypothesis that *B. pseudomallei* undergoes *in vivo* adaptation. This gives rise to the notion that colony morphology of *B. pseudomallei* may possibly provide some indications of the virulence of a particular strain.

Bacterial biofilm infections are particularly problematic, because sessile bacteria can withstand host immune defense mechanisms and are extremely resistant to

antimicrobials. The ability to form biofilms likely contributes to the occurrence of persistent infection in the host and may also account for the greater likelihood of asymptomatic infections as in melioidosis. A major feature of melioidosis is that bacterial eradication is difficult to achieve. The clinical response to antimicrobials is slow and recurrent disease is common, despite appropriate therapy for 12 to 20 weeks (Puthucheary, 2009). In addition, the fact that levels of humoral antibodies in patients who have had melioidosis remain high and seldom come down to basal levels even years after recovery from acute infections supports the notion of persistence (Vasu *et al.*, 2003).

It is clear that *B. pseudomallei* can become adapted for survival *in vivo* (Chantratita *et al.*, 2007), but the mechanisms by which this occurs in humans have yet to be demonstrated. In this study, we have attempted (a) to differentiate *B. pseudomallei* isolates based on their colony morphologies on *B. pseudomallei* selective agar (BPSA), (b) to assess the *in vitro* biofilm production by *B. pseudomallei* and (c) to investigate possible correlation between morphotypes and ability to form biofilm.

MATERIAL AND METHODS

Bacterial strains

Seventy six clinical isolates of *B. pseudomallei* kept in our culture collection were used in this study. The isolates were obtained from several Malaysian hospitals from 1990 to 2008, the majority were from blood cultures (n=43), and pus specimens (n=12). Working cultures were maintained at 37°C aerobically on Luria Bertani (LB) agar. *Burkholderia pseudomallei* ATCC 23343, *B. pseudomallei* NCTC 13178, *Burkholderia thailandensis* ATCC 700388 were included as reference strains.

Observation of colonial morphology

A single pure colony of *B. pseudomallei* growing on LB agar was stabbed with a straight wire onto freshly made *B. pseudomallei* selective agar (BPSA) and incubated for 5 days at 37°C (Howard & Inglis, 2003). The morphological features of the colonies were recorded and the images were captured using a digital camera with macro shot mode.

Determination of biofilm forming ability

Biofilm formation of *B. pseudomallei* was determined using a modified protocol (Taweechaisupapong *et al.*, 2005). Briefly, overnight cultures of *B. pseudomallei* were inoculated into brain heart infusion broth (BHI) and incubated aerobically overnight in a shaking incubator at 37°C. Prior to the test, the bacterial density was standardized using a spectrophotometer (OD_{600} =1). For each isolate, 200 µl bacterial suspensions was added to 8 wells of a 96-well flat bottomed plastic tissue culture plate (Nunc, Denmark) and incubated at 37°C for 48 hours. Uninoculated BHI broth was used as control. Following incubation, the wells were washed 3 times with phosphate buffered saline (PBS) to remove non-adherent bacteria, and fixed with 99% (v/v) methanol for 15 minutes and air-dried at room temperature. The wells were then stained with 200 µl filtered 2% crystal violet for 5 minutes. Excess stain was washed with water and the wells were air dried. The crystal violet bound bacterial cells were solubilized with 200 µl of 95% (v/v) ethanol and the released stain was measured using a microplate reader at 595nm. Eight replicates were performed for each isolate and the average OD reading was taken.

Statistical analysis

Biofilm forming ability of *B. pseudomallei* was determined by comparing the OD readings of the eluted crystal violet from each isolate with that of *B. thailandensis* ATCC 700388. MINITAB 14 was used to calculate descriptive statistics and box plot analysis was used to determine high (X>Q3), medium ($Q1 \leq X \leq Q3$) and low (X<Q1) biofilm producers, as well as outliers, where X was the relative fold difference of biofilms formed as compared with that of *B. thailandensis*. The association of the morphotypes with biofilm formation was determined using ANOVA statistical analysis. A P value of >0.05 was considered as significant.

RESULTS AND DISCUSSION

Variations in colonial morphology of *B. pseudomallei* often pose difficulties to the untrained eye in the clinical diagnostic laboratory. Mixed *B. pseudomallei* morphologies such as large and small colony variants have been observed when cultured from individual clinical samples especially from the blood samples (Puthucheary, 2009). Three selective media, i.e. Ashdown's medium, *Burkholderia cepacia* medium and *B. pseudomallei* selective agar (BPSA) (Chantratita *et al.*, 2007; Chen *et al.*, 2009) had been used to compare the efficacy of growth of *B. pseudomallei* and all 3 media demonstrated equivalent sensitivity (Peacock *et al.*, 2005).

The 76 *B. pseudomallei* isolates cultured on the BPSA agar, showed smooth, moist and dome shaped colonies with entire borders as well as wrinkled purple colonies centrally umbonated with radiating ridges to the periphery as well as irregular borders. Seven different groups or colonial morphotypes of *B. pseudomallei* on BPSA agar were recorded (Figure 1). The colonial appearances could be differentiated into 2 major groups: one where the surface texture had a mixed appearance and another where the surface texture was uniform. The mixed surface textured group could be divided into 4 subgroups, i.e. (i) central rough surface with radiating wrinkling up to the edge, (ii) mixture of rough, wrinkled and smooth surface with irregular edges, (iii) central rough surface with smooth circumference and (iv) wrinkled central area with smooth circumference. The uniform textured group consisted of 3 subgroups, i.e. (i) convex, mucoid, with smooth colony surface, (ii) rough texture of entire colony with irregular edges, and (iii) wrinkled surface of entire colony. The most predominant morphotype was group 1 (40.8%), next was group 2 (18.4%) and close to it was group 3 (15.8%) (Figure 1).

Biofilm forming abilities of *B. pseudomallei* and *B. thailandensis* ATCC 700388 were compared and ranked based on the relative fold differences compared to that of *B. thailandensis*. Box-plot analysis identified 20 *B. pseudomallei* isolates as high ($X>11.01$), 37 as medium ($3.45\leq X\leq 11.01$) and 19 as low biofilm ($X<3.45$) producers. The high biofilm producing isolates demonstrated as high as 45.23 fold differences while the low biofilm producers showed at least 1.25 fold difference when compared to *B. thailandensis* ATCC 700388 strain (Figure 2). In addition to the positive skewness (2.08) and a Kurtosis value of (4.4) ($k>0$) demonstrating a sharper peak than the bell of a normal distribution in the box plot analysis, a small difference was found between the mean and median of the relative fold difference of biofilm for each morphotype group (Figure 2), indicating that the majority of *B. pseudomallei* were low biofilm producers. Amongst the blood isolates, 30.2% were considered high biofilm producers and 27.9% as low producers, 33.3% of the pus isolates were considered high and 16% as low biofilm producers, respectively. A total of 9.5 and 28.5% of isolates from unknown clinical sources were considered as high and low biofilm producers, respectively.

Attempts to correlate *B. pseudomallei* morphotypes with virulence in mice have been described in two previous studies. Chantratita *et al.* (2007) were able to identify a *B. pseudomallei* morphotype which favored enhanced survival and persistence of the bacterium. However, in another study, no significant difference was found in the bacterial loads in the organs of mice infected by different morphotypes, although higher mortality and larger areas of abnormal liver debris were found in mice infected with two morphotypes (Chen *et al.*, 2009). Our findings show that *B. pseudomallei* isolates varied in their biofilm forming abilities (Figure 2). Individual box-plot analysis was performed to exclude outliers in order to allow a fair comparison of biofilm formation between different morphotypes. However, there was no significant difference in biofilm formation amongst the morphotypes ($p > 0.05$).

Biofilms are now acknowledged as a permanent or temporary refuge for bacteria. The structure of biofilms frequently encompasses spatial cell aggregations that are embedded in a self-produced, protective matrix of extracellular polymeric substance

that may consist of polysaccharide, proteins and nucleic acids. This interconnecting matrix links the bacterial cells together establishing an organized community, enabling microorganisms to colonize and survive unfavorable environments (Wietz *et al.*, 2009). The phenotypic plasticity of *B. pseudomallei* has important implications for treatment and vaccine development of melioidosis (Chantratita *et al.*, 2007). The development of a colony morphotyping scheme such as the one described in this

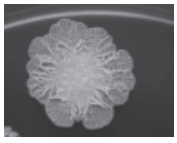
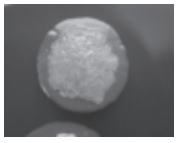
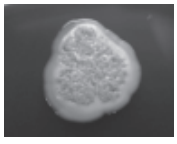
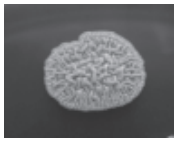
Mixed Texture		
Group	Image	Description; *No. of isolates* (%)
1		Central rough surface with radiating wrinkling up to edge; ***31*** (40.8)
2		Mixture of rough, wrinkled and smooth surface with irregular edge; ***14*** (18.4)
3		Central rough surface with smooth circumference and edge; ***12*** (15.8)
4		Wrinkled central area with smooth circumference and edge; ***5*** (6.6)
Uniform Texture		
Group	Image	Description; *No. of isolates* (%)
5		Convex, mucoid, with smooth colony surface; ***5*** (6.6)
6		Rough texture of entire colony with irregular edges; ***5*** (6.6)
7		Wrinkled surface of entire colony; ***4*** (5.2)

Figure 1. Representative pictures (Group 1–7) of the distinct 7 colonial morphotypes of *B. pseudomallei* on BPSA

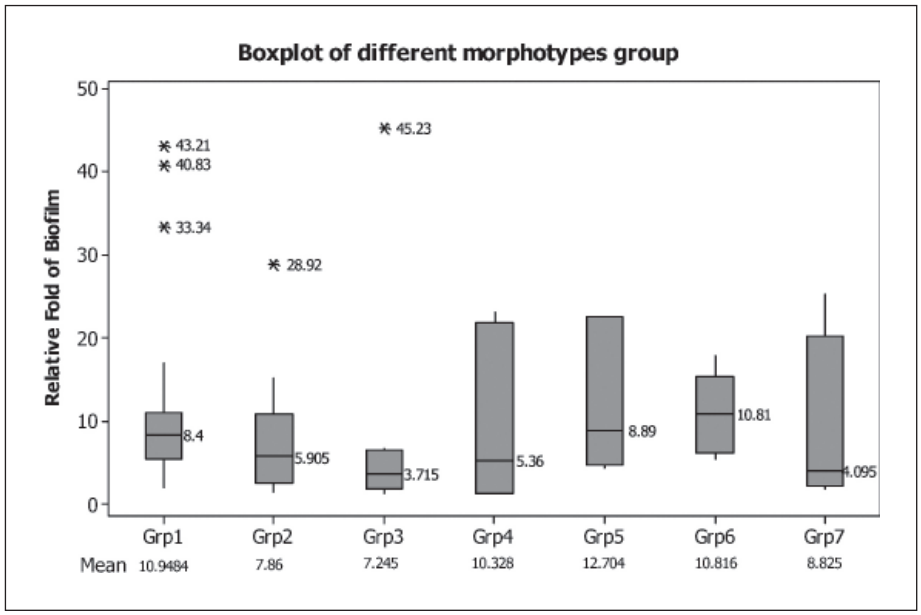


Figure 2. Boxplot analysis of biofilm formation of different morphotype groups of *B. pseudomallei* compared to that of *B. thailandensis*. The star * indicates outliers from each morphotype group. Values shown in the middle of the box plots were the median of the relative fold difference of the biofilms. The means of the relative fold difference of biofilms for each group is indicated below each plot

study maybe the first step towards understanding the phenotypic switching of *B. pseudomallei* in response to changing environmental factors. Additional studies using advanced molecular approaches are needed to identify internal and external factors which contribute to the high and low biofilm formation of *B. pseudomallei*.

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