In Vitro Motility of a Population of Clinical *Burkholderia Pseudomallei* Isolates

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Melioidosis, a serious infection caused by Burkholderia pseudomallei, is a leading cause of community-acquired sepsis in Northeast Thailand, and the commonest cause of death from community-acquired pneumonia in the Top End of Northern Australia. The causative organism is a Gram-negative, motile bacillus that is a facultative intracellular pathogen. B. pseudomallei flagella have been proposed as a possible vaccine candidate and putative virulence determinant. Flagella expression was highly conserved for 205 clinical B. pseudomallei isolates, as defined by in vitro swim and swarm motility assays. No association was found between motility and clinical factors including bacteremia and death.

Keywords: In vitro motility, Burkholderia pseudomallei

Burkholderia pseudomallei is the cause of melioidosis and a recognized bio-threat agent mainly in South-East Asia and Northern Australia[1,2]. Infection presents with a wide spectrum of clinical manifestations, and any part of the body can be involved. Mortality rates are high in endemic areas of Thailand (50%), most likely due to a combination of severity of illness, slow response to antibiotic treatment and failure to receive intensive care unit support[2]. One fifth of disease in Thailand occurs in previously healthy children; this group has a mortality rate of around 30%. Improvement in treatment modalities and the development of preventive strategies would be informed by an understanding of the determinants of infection. These are currently poorly understood. No vaccine is available to protect those at risk of deliberate bacterial

release, and individuals in melioidosis-endemic areas where *B. pseudomallei* is present in the environment. *B. pseudomallei* is flagellate and motile, and flagellin has been proposed as a possible vaccine candidate[3,4]. Whole genome sequencing of *B. pseudomallei* K96243 has revealed five gene clusters that encode the components of a single flagella system[5]. The detection of anti-flagella antibody has also been used to determine exposure to *B. pseudomallei*[6,7]. Vaccine efficacy and serology test sensitivity will depend in part on ubiquitous flagella expression by the population of invasive *B. pseudomallei*. It is likely that the presence of genes encoding *B. pseudomallei* flagella are highly conserved; PCR detection of *B. pseudomallei* based on amplification of *fli*C has been developed to detect bacterial DNA[8-10], and combinations of PCR and RFLP or sequence analysis of the flagellin gene have been used to identify *B. pseudomallei* and differentiate this from other *Burkholderia* species[11-15]. However, it cannot be assumed from this that flagella expression

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by clinical *B. pseudomallei* isolates is ubiquitous. The first aim of the present study was to determine the proportion of invasive *B. pseudomallei* isolates that express flagella *in vitro*, as defined by phenotypic motility assays and the second was to investigate the association between motility and clinical factors of patients.

Material and Method

A total of 205 *B. pseudomallei* isolates obtained from 205 consecutive patients presenting with melioidosis to Sappasithiprasong Hospital, northeast Thailand between 2002 and 2003 were identified. These were isolated from blood (n = 99), pus (n = 55), sputum/respiratory secretions (n = 26), throat swab (n = 9), urine (n = 13), wound swab (n = 5) and other sites[4]. Motility was assessed using swim and swarm agar plate assays, as previously described[7]. Swim agar plates were composed of 1% tryptone, 0.5% NaCl, 0.3% agar. Swarm plates were composed of 0.5% Bacto Agar and 8 g of nutrient broth/liter (both from Difco, Detroit, Mich.), supplemented with 5 g dextrose/liter.

Motility assays on solid agar were performed using *B. pseudomallei* that had been cultured on Ashdown's agar at 37^0C in air for 4 days. Bacterial cells from an isolated colony was point inoculated into the center of a swim or swarm plate using a sterile toothpick. Plates were incubated at 37^0C in air for 72 hours, after which the widest colony diameter was measured and the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation

using a Vernier micrometer. *Pseudomonas aeruginosa* ATCC 27853 and *Burkholderia mallei* were used as positive and negative controls, respectively. *B. pseudomallei* strain MM35, an isogenic mutant of *B. pseudomallei* 1026b that is defective in flagella expression[16] (a gift from Professor Donald Woods, University of Calgary), was used as a negative *B. pseudomallei* control.

Results

P. aeruginosa ATCC 27853 and *B. mallei* EY100 which were isolated from an infected horse (a gift from Dr. Sumalee Tangpradubkul, Mahidol University, Thailand) were examined in triplicate in three independent assays on swim and swarm plates following incubation at 37^0C in air for 24, 48, and 72 h. This confirmed the ability of the assay to define the presence and absence of bacterial motility (Fig. 1). *B. pseudomallei* strain MM35 was used to develop cut-off values for swim and swarm assays, below which isolates were considered negative. The MM35 was inoculated onto 30 swim and 30 swarm plates, and the mean colony diameter and standard deviation calculated for each assay after incubation at 37^0C for 72 h. The mean colony diameter of the MM35 on swim plates was 8.5 mm $\pm$ 1.3 mm; any *B. pseudomallei* isolate with a zone size within or below this range was defined as negative in this assay. The mean colony diameter of the MM35 on swarm plates was 22.2 mm $\pm$ 1.2 mm; any *B. pseudomallei* isolates with a zone size within or below this range was defined as negative in this assay. Clinical isolates were
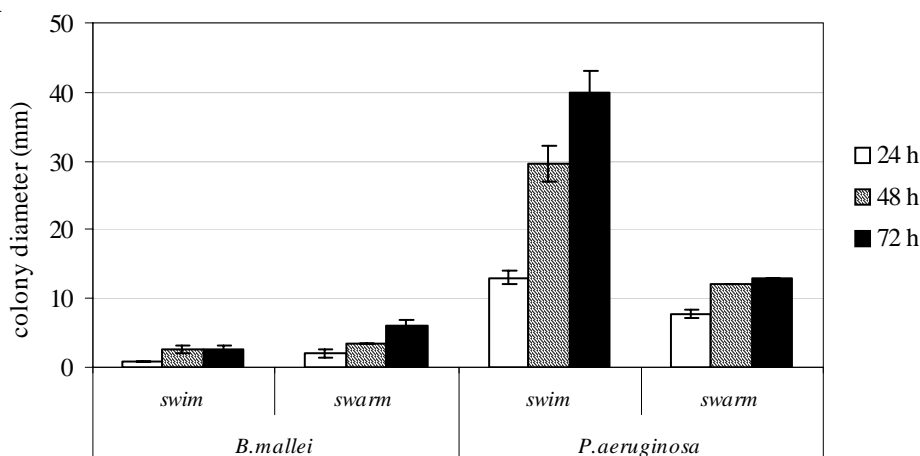

Fig. 1 Colony diameter of *P. aeruginosa* ATCC 27853 (positive control) and *B. mallei* EY100 (negative control) on swim and swarm motility agar plates following incubation at 37^0C in air for 24, 48, 72 h. Values shown are mean (SD) for triplicate plates in each of three independent experiments

initially assessed using a primary screen, in which each isolate was tested once. Isolates with a colony diameter below the relevant cut-off were re-tested more intensively, in which each strain was tested on three plates in triplicate independent experiments (9 plates).

The frequency distribution of colony diameter for 205 invasive *B. pseudomallei* isolates on swim plates after incubation at 37^0C in air for 72 h is shown in Fig. 2A. Colony diameter ranged from 11 mm to 88 mm (median 53 mm, interquartile range (IQR) 47 to 61 mm). Thus, all isolates in this population demonstrated swim motility. The frequency distribution of colony diameter for these isolates on swarm plates after incubation at 37^0C in air for 72 h is shown in Fig. 2B. Colony diameter ranged from 7.7 mm to 88 mm (median 52.5 mm, IQR 26 to 88mm). A total of 18 isolates were initially defined in the primary screen as putative non-swarmers (diameter range 11 to 23 mm, median 19 mm). On re-testing in triplicate the mean value for 15 of 18 isolates fell above the cut-off, the diameter ranging from 27 mm to 88 mm. Three isolates remained below the cut-off (mean diameter for each strain 11.5 mm, 18.5 mm, and 21 mm). It is possible this represents the lower biological range of motility on swarm plates, rather than non-swarming. The second aim of the present study was to examine the relationship between motility (*in vitro* swim and swarm) and clinical factors of patients from whom the isolates were derived. Clinical information was available for 192 of the 205 patients. The host factors considered were death (88/192, 46%), presence of bacteremia during admission (113/192, 59%), fever clearance time, and type of clinical specimen positive for *B.*

pseudomallei. Statistical analysis was performed using Intercooled STATA, version 8.0 (College Station, Tex.), using non-parametric tests (Spearman's Rho or Kruskal-Wallis test, as appropriate). No association was found between the results for swim or swarm motility assay and any of the clinical factors ($p > 0.05$ in all cases).

Discussion

The role of flagella as virulence factors are uncertain; no difference in virulence was detected between an isogenic aflagellate mutant and wild-type *B. pseudomallei* in diabetic rat and Syrian hamster infection models[16]. This contrasts to a second study in which the mutant was less virulent than wild type following intraperitoneal infection of BALB/c mice, and bacterial numbers of the mutant were markedly reduced in the lung and spleen of BALB/c mice following intranasal infection[17]. This assumes that quantitative motility *in vitro* reflects flagella function during human disease; the authors accept that this may not be the case. The authors conclude that flagella expression is highly preserved for a large population of invasive clinical isolates under laboratory conditions. This provides further evidence for its utility as a putative vaccine candidate and target for serological testing, although the presented data does not extend to the study of flagella expression in vivo during human disease.
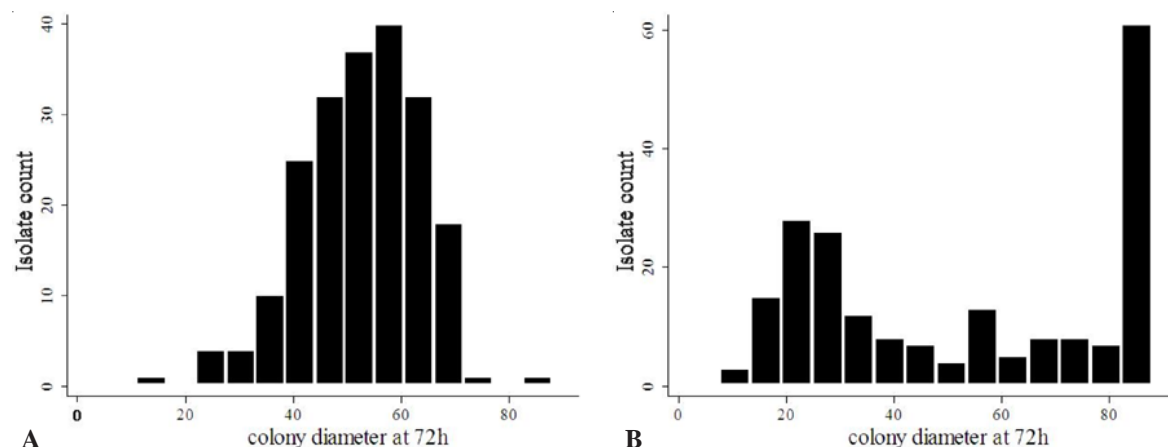
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Fig. 2 Colony diameter on swim (A), and swarm (B) motility agar plates of 205 clinical *B. pseudomallei* isolates following incubation at 37^0C in air for 72 h

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การเคลื่อนที่ของเชื้อ Burkholderia pseudomallei ในกลุ่มผู้ป่วยโรคเมลิออยโดสิส

เขมาภรณ์ บุญบำรุง, วรรณพร วุฒิเอกอนันต์, ศิริรัตน์ เร่งพิพัฒน์, Nicholas PJ Day, Sharon J Peacock

เมลิออยโดสิสเป็นโรคที่เกิดจากการติดเชื้อแบคทีเรียแกรมลบทรงแท่ง Burkholderia pseudomallei พบมากในแถบเอเชียตะวันออกเฉียงเหนือของประเทศไทย และบริเวณภาคเหนือของประเทศออสเตรเลีย เชื้อนี้สามารถอาศัยอยู่ภายในเซลล์และก่อโรค สามารถสร้างแฟลกเจลลาและเคลื่อนไหวได้ โดยพบว่าแฟลเจลลาเป็นปัจจัยหนึ่งในความรุนแรงของโรค การศึกษานี้มีวัตถุประสงค์เพื่อหาความสัมพันธ์ของแฟลกเจลลาและการเคลื่อนที่ของเชื้อ B. pseudomallei ในหลอดทดลองกับลักษณะอาการทางคลินิกของผู้ป่วยโรคเมลิออยโดสิส โดยทดสอบเชื้อ B. pseudomallei จากผู้ป่วยโรคเมลิออยโดสิส 205 สายพันธุ์ และพบว่าการเคลื่อนที่ในรูปแบบ swim และ swarm ด้วยแฟลเจลลานั้นไม่มีความสัมพันธ์กับลักษณะอาการที่แสดงออกทางคลินิกในกลุ่มผู้ป่วยโรคเมลิออยโดสิส