THE PRESENCE OF FLAGELLA AND MOTILITY OF CLINICAL *Burkholderia pseudomallei* ISOLATES FROM NORTHEAST THAILAND

KHAEMAPORN BOONBUMRUNG

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Miss Khaemaporn Boonbumrung
Candidate

Assoc.Prof.Sirirat Rengpipat
Ph.D. (Food Science)
Major-Advisor

Assoc.Prof.Virapong Prachayasittikul
Ph.D. (Molecular Biology)
Co-Advisor

Dr. Sharon Peacock
Ph.D.
Co-Advisor

Asst. Prof. Salee Kiewkarnka, Ph.D
Acting Dean
Faculty of Graduate Studies

Assoc.Prof. Virapong Prachayasittikul
Ph.D. (Molecular Biology)
Chair
Doctor of Philosophy Programme
in Medical Technology
Faculty of Medical Technology

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Khaemaporn Boonbumrung
.....
Miss Khaemaporn Boonbumrung
Candidate

Sirirat Rengpipat
.....
Assoc.Prof.Sirirat Rengpipat
Ph.D. (Food Science)
chair

Virapong
.....
Assoc.Prof.Virapong Prachayasittikul
Ph.D. (Molecular Biology)
Member

Sharon Peacock
.....
Dr. Sharon Peacock
Ph.D.
Member

Byaporn na Nagara
.....
Asst.Byaporn Na Nagara
Ph.D.
Member

Salee Kiewkarnka
.....
Asst. Prof. Salee Kiewkarnka, Ph.D
Acting Dean
Faculty of Graduate Studies
Mahidol University

Chatchai
.....
Assoc.Prof. Chatchai Sornchai
M.D.
Dean
Faculty of Medical Technology
Mahidol University

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Khaemaporn Boonbumrung

THE PRESENCE OF FLAGELLA AND MOTILITY OF CLINICAL *Burkholderia pseudomallei* ISOLATES FROM NORTHEAST THAILAND

KHAEMAPORN BOONBUMRUNG 4336815 MTMT/D

Ph.D.(MEDICAL TECHNOLOGY)

THESIS ADVISORS: SIRIRAT RENGPIPAT, Ph.D.(FOOD SCIENCE), VIRAPONG PRACHAYASITTIKUL, Ph.D.(MOLECULAR BIOLOGY), SHARON PEACOCK, Ph.D.

ABSTRACT

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. Expression of flagella has been defined as a virulence determinant for *B. pseudomallei* in an animal model, but detailed study of flagella and motility *in vitro* has not been reported. The aim of this study was to undertake an in-depth examination of flagella expression and motility *in vitro* for a large population of clinical *B. pseudomallei* isolates. The principle methodologies used were motility agar assays, flagella staining followed by microscopic observation and counting, and delineation of motility using Real Time Microscopy. These were related to factors such as colony morphology on Ashdown's and swarm agar. *B. pseudomallei* demonstrated swarm and swim motility but was negative for twitching motility. Swim motility was ubiquitous for the clinical isolate collection, although swarming was apparently absent in a small minority of isolates. Flagella expression was defined over a growth curve, and was found to be a highly regulated process in which expression was lowest in lag and very early exponential growth phase, peaking at 8-10 hours. Flagella expression was strongly influenced by position within a colony on swarm agar; expression was significantly higher in bacterial cells from the edge compared with the colony centre. Marked inter-and intra-strain variability in swarm motility was observed. This was related in part to the morphology of the colony which was highly variable, indicating variable states of flagella expression even within small regions of the colony. There was no evidence of hyper-flagellation or change in bacterial microscopic morphology in bacteria from the edge of a swarm colony, as previously described for *Proteus* or *Serratia spp.* For flagella-positive cells, the majority expressed a single polar flagellum (around 75%). *B. pseudomallei* can undergo a reversible switch in colony morphology in response to environmental stress which is likely to be related to changes in phenotype. Flagella expression was examined for two isogenic colony variants (termed Types III and VI) of two clinical *B. pseudomallei* isolates (strains 153 and 164, parental morphotype Type I). Differences were seen between parental and isogenic variant morphotypes in terms of the number of flagella expressed. However, the changes seen were not consistent between assays testing change during growth in broth and those in a swarm colony. This could indicate that the presence of flagella is phenotypically plastic, and may become either up- or down-regulated in response to environmental signals.

KEY WORDS : FLAGELLA / MOTILITY / *Burkholderia pseudomallei* / MELIOIDOSIS

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แฟลกเจลลาและ การเคลื่อนที่ของเชื้อ *Burkholderia pseudomallei* แยกได้จากผู้ป่วยโรคเมลิออยโดสิสจากภาคตะวันออกเฉียงเหนือของประเทศไทย (THE PRESENCE OF FLAGELLA AND MOTILITY OF CLINICAL *Burkholderia pseudomallei* ISOLATES FROM NORTHEAST THAILAND)

เขมาภรณ์ บุญบำรุง 4336815 MTMT/D

ปร.ด. (เทคนิคการแพทย์)

คณะกรรมการควบคุมวิทยานิพนธ์ : ศิริรัตน์ เร่งพิพัฒน์, Ph.D(FOOD SCIENCE), วีระพงศ์ ปรัชญญาสิทธิกุล , Ph.D(MOLECULE BIOLOGY), SHARON PEACOCK, Ph.D

บทคัดย่อ

เมลิออยโดสิสเป็นโรคที่เกิดจากการติดเชื้อแบคทีเรียแกรมลบทรงแท่ง*Burkholderia pseudomallei* พบมากในแถบเอเชียตะวันออกเฉียงใต้และบริเวณภาคเหนือของประเทศออสเตรเลีย เชื้อนี้มีชีวิตอยู่ได้ภายในเซลล์ สามารถสร้างแฟลกเจลลาและ เคลื่อนไหวได้ และพบว่าแฟลเจลลาเป็นปัจจัยหนึ่งของความรุนแรงของโรค ปัจจุบันยังไม่มีการศึกษาความสัมพันธ์กับแฟลกเจลลาและรูปแบบการเคลื่อนที่ งานวิจัยนี้จึงได้มีการศึกษาการสร้างแฟลกเจลลาและการเคลื่อนที่ในหลอดทดลองจากกลุ่มตัวอย่างเชื้อ*B. pseudomallei* ที่แยกได้จากผู้ป่วยและหาความสัมพันธ์ระหว่างลักษณะโคโลนีบนอาหารเลี้ยงเชื้อ Ashdown's agar กับการสร้างแฟลกเจลลา ผลการตรวจสอบพบว่าเชื้อ *B. pseudomallei* สามารถเคลื่อนในรูปแบบ swim และ swarm ด้วยแฟลกเจลลา แต่ไม่พบการเคลื่อนที่แบบ twitching ในการเคลื่อนที่แบบ swarm จะพบว่ามีความแตกต่างทั้งในสายพันธุ์และระหว่างสายพันธุ์ ซึ่งมีความสัมพันธ์กับความหลากหลายของเชื้อบนอาหารเลี้ยงเชื้อ ส่วนการสร้างแฟลกเจลลาพบสูงสุดในช่วงปลายของการเจริญแบบทวีคูณที่เวลา 8-10 ชั่วโมงของการเจริญ และยังพบว่าเซลล์ที่ขอบของโคโลนีจะสามารถสร้างแฟลกเจลลาได้มากกว่าเซลล์จากตรงกลางโคโลนีของบริเวณswarm ในทุกสายพันธุ์ โดยจำนวนของแฟลกเจลลาที่สร้างและขนาดของเชื้อ *B. pseudomallei* แตกต่างจากที่พบในเชื้อ *Proteus* หรือ *Serratia* spp. แต่คล้ายคลึงกับ *Pseudomonas aeruginosa* ความหลากหลายของลักษณะโคโลนีบนอาหารเลี้ยงเชื้อ Ashdown's agar เนื่องจากการตอบสนองต่อสภาวะแวดล้อมที่แตกต่างไปนั้น ได้มีการศึกษาถึงการสร้างแฟลกเจลลาระหว่างสายพันธุ์ดั้งเดิมและเปรียบเทียบกับ isogenic strains เมื่อศึกษาจากเซลล์ที่ขอบของโคโลนีจาก swarm agar พบว่าใน isogenic strains สามารถสร้างแฟลกเจลลาได้มากกว่าสายพันธุ์ดั้งเดิม ซึ่งอาจจะเกิดจากกลไกที่ตอบสนองต่อสภาพแวดล้อม

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LIST OF ABBREVIATIONS

^{0}C	degree celsius
40X	40 magnificent
ASA	Ashdown's Selective Agar
B	Bulkholderia
BA	5% sheep blood agar
BALB/c	The BALB/c is popular as a general purpose inbred strain for many different research disciplines. It exhibits good breeding performance and long reproductive life span and is often used for the production of monoclonal antibodies using hybridomas of BALB/c origin.
bp	base pairs
BSL	Biosafety laboratory
C	center
C (genes)	cytosine
CCW	Counter clockwise
Cfu/ml	colony forming unit per millitre
cm	centimetre(s)
CW	Clockwise
Da	Dalton
DNA	Deoxyribonucleic acid
E	edge
E.M.	Electron microscopy
et al.	et alli
fla genes	genes involved in the synthesis of flagella
fliC	flagellin gene
G	guanine
g	gram
HIV	human immunodeficiency virus
IQR	Inter Quartile Range

LPS	lipopolysaccharide
Mac	MacConkey agar
ml	millilitre
mm	millimetre
mol	mole
Muc1	Mucine 1
nm	nanometre
OD	Optical density
pH	Potenz power + H (symbol for hydrogen)
rpm	revolutions per minute
RTM™	The Richardson Real Time Microscope™
RTM-3	Real Time Microscope-3
SSR	single repeat sequence
Swarm 1’	First swarm plate
Swarm 2’	Second swarm plate
TSB	Tryptic Soy Broth
µl	microlitre (s)
µm	micrometre(s)
w/v	weight by volume

CHAPTER 1

INTRODUCTION

Melioidosis, an infection caused by the bacterial pathogen *Burkholderia pseudomallei*, is an infectious disease seen mainly in South-East Asia and northern Australia (Dance, 1990). 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. 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. Predisposing factors for melioidosis in adults are diabetes, renal failure and other diseases that impair the immune system. Contact with soil (usually during occupational exposure) is a key risk factor. The role of bacterial factors in determining disease, and during disease pathogenesis once infection has occurred, is poorly defined.

The expression of flagella has been shown to be a virulence determinant in an animal model. Outcome was reduced in animals inoculated with wild type compared with the same strain defective in flagella expression (Chua *et al*., 2003). It is possible that the bacterium relies on motility to disseminate from one body site to another (for example from the respiratory tract or a wound to other parts of the body such as liver, spleen or blood). Detailed study of *B. pseudomallei* flagella and motility in the laboratory has not been undertaken to date. This study set out to define motility and to examine the relationship between flagella expression (number and type) versus motility.

CHAPTER 2

LITERATURE REVIEW

2.1 General background

Melioidosis is caused by *B. pseudomallei*, a Gram-negative soil saprophyte. The disease was first described by Whitmore and Krishmaswami in 1912 (Whitmore and Krishnaswami, 1912) in 38 cases of fatal pneumonia amongst the destitute and morphine addicts in Rangoon, Burma. Stanton and Fletcher coined the term 'melioidosis' nine years later. This is derived from the Greek words meaning glanders-like condition, thus used because melioidosis clinically and patho-physiologically resembled glanders (Stanton and Fletcher, 1921). Many cases occurred in US servicemen several years after their return from Vietnam, leading to use of the term "Vietnamese Time Bomb"(Clanton *et al.*, 1973).

2.1.1 The genus Burkholderia

The genus was named after W.H. Burkholder, an American microbiologist who discovered the etiological agent of diseased onions. On the basis of rRNA-DNA homology, Yabuuchi et.al (Yabuuchi *et al.*, 1992b) proposed that the seven species in homology group II were sufficiently different from each other to assign them to a new genus, *Burkholderia*. Seven species, all formally *Pseudomonas spp*., were validated in Validation List No. 45, 1993 (Ashdown, 1981). These are: *B. cepacia, B. mallei, B. pseudomallei, B. ickettii, B. gladioli, B. caryophylli* and *B. solanacearum*. The DNA molar percentage of guanine plus cytosine (G+C) of the genus is 64-68.5 mol%. All species are aerobic, oxidase-positive, non-spore forming, Gram-negative rods 1-5 μm long and 0.5-1.0 μm wide. DNA hybridization studies of *Burkholderia* have shown

that *B. mallei* and *B. pseudomallei* are the most closely related species, with a DNA homology of about 90% (Yabuuchi *et al.*, 1992a).

The *B. pseudomallei* genome is 7,247,547 bp in size with a G+C content of 68.1%, and consists of two chromosomes of 4,074,542 bp and 3,173,005 bp, respectively (www.sanger.ac.uk). The genome has many markers of genomic plasticity. These include: (i) variable presence of genomic islands in a population of *B. pseudomallei* isolates from cases of invasive disease and from soil; (ii) presence of multiple copies of insertion sequences, and (iii) presence of single sequence repeats (SSR) in the coding or promoter region of many hundred of genes. SSR are regions of increased mutability and can result in the variable expression (phase variation) of putative virulence determinants. Variable phenotypic expression can also occur through gene regulation, a process that is usually dependent on the production of small regulatory molecules that alter gene expression via interactions with the gene promoter region (Holden *et al.*, 2004).

B. pseudomallei usually exhibit bipolar staining on Gram stain of positive clinical specimens and pure laboratory culture. This gives an appearance of a safety pin. Cells may appear as long parallel bundles of "filaments" which represent closely associated strings of rods (Miller and Pennel, 1948). *B. pseudomallei* grows well on peptone medium and in chemically defined media (Levine et al., 1954). For selective isolation from contaminated specimens, Ashdown's selective medium is helpful (Ashdown, 1979b). This contains crystal violet (the colony often takes up the dye to give a purple colony), the antibiotic gentamicin (the majority of strains are resistant), and glycerol (enhances the formation of wrinkled colonies) (Ashdown, 1979a). Up to four colonial variants may be seen for one strain, giving the impression of a mixed culture. The commonest colonial types are often described as "rough" and "smooth" colonies. Rough colonies usually predominate in fresh clinical isolates. A yellow pigment is produced by a minority of strains; this is best observed on clear agar such as Columbia agar. Most strains grow well at 42 ^{0}C, and are also able to grow at pH 5.6 (Wetmore and Gochenour, 1956), and to tolerate 5^{0}C for 100-190 days (Arakawa *et al.*, 1993). Colonies on heart infusion agar or blood agar are usually small and shiny after 20 hours incubation at 35^{0}C. Beta-haemolysis is usually seen around areas of confluent growth, but not around individual colonies. On MacConkey agar colonies

are usually pink (Dance, 1990). After 72-96 hours incubation on Ashdown's agar colonies are 7-10 mm in diameter, dull and wrinkled and have a strong musty, earthy odor (Salisbury and Likos, 1970). A thick pellicle often develops on the surface of liquid medium.

Activities of catalase and indophenoloxidase are strongly positive. The energy for growth is acquired by respiration but not by fermentation *(Bokman et al.*, 1957). Glucose and galactose are oxidized to gluconic acid and galactonic acid, respectively (Dowling and Levine, 1956). However, the organism grows anaerobically when nitrate or arginine is present. *B. pseudomallei* can be easily distinguished from other related species by the property of resistance to the polymyxin group antibiotic (polymyxin B and colistin) (Arakawa *et al.*, 1993). Commercial kits enable any laboratory to identify sporadically encountered isolates. The API 20NE system correctly identified 97.5% of isolates in one study (Chaowagul *et al.*, 1989).

Comparison of biochemical characteristics between isolates that were once considered to be 'invasive' and 'non-virulent environmental' types of *B. pseudomallei* by Wuthiekanun and colleagues indicated that the two were different in their ability to assimilate L-arabinose (Wuthiekanun *et al.*, 1996). Further work by Brett *et al.* entailing a comprehensive 16S and 23S phylogenetic analysis supported the existence of a new *Burkholderia* species named *Burkholderia thailandensis* (Brett *et al.*, 1998).

2.1.2 *B. pseudomallei* as an environmental saprophyte

B. pseudomallei is an environmental saphrophyte that exists in the soil and surface water within melioidosis-endemic areas. These are tropical and subtropical climates, although it is widely assumed that factors other than climate must be important in determining the distribution of *B. pseudomallei*. Tong and colleagues demonstrated the optimal temperature and pH for survival to be 24-32^{0}C and pH 5-8, respectively (Tong *et al.*, 1996). *B. pseudomallei* survive during both the dry and wet seasons in the clay layer of the soil 25-30 cm below the surface in Australia, and 12-20 cm in Malaysia. The organism can be readily isolated from rice paddies, still or stagnant water and moist soil. Soil and surface water are highly complex ecosystems in which a vast range of physical, chemical and biological factors interact

(Puthucheary *et al.*, 2001). Extracellular polysaccharide may be important for survival in this environmental (Kanai and Kondo, 1994).

2.1.3 Relationship between presences of *B. pseudomallei* in soil and disease

Human acquisition of *B. pseudomallei* occurs through skin inoculation, or more rarely by inhalation of contaminated debris. In most cases, inoculation is thought to occur following contact with soil. This may be one of the reasons for the seasonal variation in disease in Thailand, with most cases occurring during the rainy season when rice farmers are planting and harvesting. The infection rate in patients attending government hospitals in the northeast (137.9 per 100,000 in patients) was significantly higher than other parts of Thailand; *B. pseudomallei* was cultured from 50% of sites sampled in North-east Thailand. It is suggested that melioidosis, which is endemic in Thailand, is associated with the presence of *B. pseudomallei* in soil (Vuddhakul *et al.*, 1999). The potential for long latent periods before disease occurs may lead to failure to remember an exposure event (Dance, 2000).

2.1.4 *B. pseudomallei* infection in man

2.1.4.1 Epidemiology

2,000 – 3,000 cases of clinical melioidosis are estimated to occur each year in Thailand with a population of 60,000,000. The incidence rate in highly endemic areas has been calculated to be 3.6 – 5.5 cases per 100,000 population. Disease is seasonal and occurs mainly during the rainy season (Suputtamongkol *et al.*, 1994). Melioidosis may present at any age but peak incidence is in the forth and fifth decades of life, coinciding with the development of underlying predisposing illness (White, 2003). Of the predisposing conditions, diabetes mellitus is the most frequent (Suputtamongkol *et al.*, 1999). Serological studies in Thailand show that exposure occurs as the infant makes contact with soil and water; about 25% of children seroconvert every year for the first 4 years of life (Kanaphun *et al.*, 1993).

2.1.4.2 Risk factors

Melioidosis is acquired by contact with infectious soil and water through penetrating wounds or existing skin abrasions, ulcers, burns or by inhalation of dust particles, by aspiration of contaminated water during near-drowning episodes, iatrogenic inoculation and by laboratory accidents. Aerosols containing the organism are infectious to humans, although the infective dose is unknown (Egan and Gordon, 1996). Exposure can occur occupationally or recreationally. Outbreaks have also occurred in relation to drinking water, although these appear to be uncommon. Human-to-human transmission is very rare, with one possible case of male-to-female sexual transmission involved a man with chronic prostatic *B. pseudomallei* infection (McCormick *et al.*, 1975). Two cases of maternal-to-child transmission of *B. pseudomallei* were reported from Australia's tropical north (Ralph *et al.*, 2004). One infant died of overwhelming sepsis. Both lactating mothers had mastitis. In another case, *B. pseudomallei* was isolated from breast milk which was identical on pulsed-field gel electrophoresis with that of isolates from infant blood and cerebrospinal fluid (Abbink *et al.*, 2001). There is one report from Thailand of transmission to a sister with diabetes who cared for her brother who had chronic melioidosis (Kunakorn *et al.*, 1991).

2.1.4.3 Clinical manifestations

In recent years, it has been recognized that fulminating cases of melioidosis in humans represent the tip of the iceberg and that mild or even sub-clinical infections are not unusual (Guard *et al.*, 1984). The spectrum of melioidosis in humans varies from sub-clinical to overwhelming sepsis, and includes chronic infection that resembles tuberculosis. As a result, melioidosis has been termed “the great imitator” of every infectious disease, as virtually every organ can be affected (Leelarasamee and Bovornkitti, 1989). Melioidosis is usually perceived as an acute pulmonary illness characterized by prostration and marked toxicity that are often out of proportion to objective physical findings or chest radiographic findings. However, melioidosis has been recognized to give rise to transient bacteraemia, asymptomatic pulmonary infiltration, acute localized suppurative lesions, acute pulmonary infection,

disseminated septicaemic infection, non-disseminated septicaemic infection or chronic suppurative infection. Since melioidosis is a multi-system disease and the signs and symptoms are non-specific, the clinical classification of melioidosis has been controversial. The subdivision into acute, sub-acute and chronic melioidosis is somewhat unsatisfactory since clinical disease is on a continuum, and the less acute or localized forms of disease may rapidly progress to the septicaemic form, especially if there is concomitant debilitating illness. The same is true of a classification by organ involvement. Perhaps the most useful clinical classification for melioidosis is septicaemic versus non-septicaemic melioidosis. The overall mortality of non-septicaemic melioidosis is 15-20 %, which is very much lower than for septicaemic melioidosis. Septicaemic melioidosis may present in many forms, from a simple bacteraemia with no obvious focus of infection to the most severe form of disseminated bacteraemia with fulminant shock and multi-organ involvement. Histopathologic study has shown the formation of abscesses in the acute stage and granuloma in the chronic form (Piggott and Hochholzer, 1970).

2.1.4.4 Outcome

Relapse is not uncommon in melioidosis. This can occur despite appropriate and prolonged antimicrobial therapy. Relapse is defined as the reappearance of clinical signs and laboratory findings of infection together with positive culture, after initial clinical response (Puthucheary *et al.*, 2001). In Thailand, the median time to relapse is 21 weeks. Risk of relapse is related to adherence to treatment and the initial extent of disease, but not to other underlying conditions (Chaowagul *et al.*, 1993).

Melioidosis has a high mortality of 30-70% (table 1). Overall mortality in adults in Thailand is about 50 %.

Table 1: Classification of melioidosis by clinical assessment (Leelarasamee, 2004).

	No. organs involved [a]	Blood culture	Severity of illness	Mortality rate [b] (%)
Associated with septic shock	Any	Positive	Fulminating sepsis/septic shock	80 – 95
Disseminated septicemic	>1	Positive (mostly)	Sepsis to severe sepsis	40 – 50
Septicemic	1	Positive	Sepsis to severe sepsis	10 – 40
Localized	1	Negative	Fever to sepsis	0 – 10
Bacteremic	0	Positive	Nil to fever	0
Asymptomatic [c]	0	Negative	Nil or healthy	0

[a] classified by clinical assessment
[b] cases treated with ceftazidime
[c] cases with positive serological test for melioidosis

2.2 Determinants of melioidosis

2.2.1 Host factors

Infection in the first year of life is more frequent than between the ages of 10 and 29 years, perhaps reflecting greater environmental exposure during play or outdoor activities (Suputtamongkol et al., 1994). Most people in melioidosis-endemic areas develop a detectable antibody response to *B. pseudomallei* by the age of four years, indicating widespread exposure from an early age. However, these antibodies have not been show to be protective. The fact that melioidosis occurs predominantly in people with one or more underlying predisposing conditions suggests that host susceptibility is an important factor. The commonest of these conditions is diabetes mellitus. It has also been postulated that insulin deficiency may contribute directly to the association between melioidosis and diabetes mellitus (Sexton *et al.*, 1993). Renal failure, renal calculi, malignancy, steroid therapy, alcoholism, occupational exposure, trauma and intravenous drug abuse are also predisposing factors. Severe impairment of specific cellular immunity, such as with advanced HIV infection, does not appear to be risk factor for infection (Cheng and Currie, 2005).

2.2.2 Bacterial factors

Though *B. pseudomallei* is an aerobic microorganism, it has the ability for anaerobic respiration (Wongwanich *et al.*, 1996). This implies that it can multiply in an oxygen rich environment as well as survive under oxygen-limited conditions depending on the energy generated by anaerobic respiration. Thus this organism can persist for long periods in the natural environment where oxygen tension may fall. The organism produces an array of secreted and cell associated antigens that may contribute to the progression of disease. The specific role of these putative virulence factors in pathogenesis has not been well defined, primarily due to the lack of suitable techniques for the manipulation of this organism at the molecular level. A number of pathogenic mechanisms and virulence factors have been identified that protect *B. pseudomallei* against host defenses and enable them to multiply. These include lipopolysaccharide, fimbriae, extracellular polysaccharide and flagella (Cheng and

Currie, 2005). A requirement for bacterial pathogenicity is attachment to host cells, but it is not known whether this is due to fimbriae or surface proteins (Ahmed *et al.*, 1999). *B. pseudomallei* is highly motile on account of its flagella. The flagellin of *B. pseudomallei* has been isolated and, by differential centrifugation its molecular weight was found to be 43,400 Da (Brett *et al.*, 1994). The bacteria from a single isolation exist in three electron microscopically distinguishable forms. These are a macro-encapsulated form with a medium density fibrogranular layer of 0.1-0.25 um, a micro-encapsulated form with a more compact granular layer of 0.086 um, and a non-encapsulated form (Puthucheary *et al.*, 1996). The variants with the electron-dense particles, which probably represents glycocalyx, suggest that adaptation by *B. pseudomallei* and *B. mallei* could account for latency and relapse in the disease they cause.

Two alpha-haemolytic activities may be produced (Liu *et al.*, 2002). Almost all isolates have a heat-stable, weakly haemolytic activity that is neutralized by human sera. It is visible around confluent growth, but not around individual colonies. A much stronger heat-labile haemolytic activity visible round individual colonies is inhibited by sterols, especially cholesterol, and is most active at about pH 5.5, but it is not affected by reducing agents of SH-modifying agents (Ashdown and Koehler, 1990). An acid tyrosine and serine phosphatase has been characterized in *B, pseudomallei* (Kondo *et al.*, 1991b). It is stable at 60^0C and markedly inhibited by 0.1% $ZnCl_2$. Tyrosine phosphatases are recognized as virulence factors in a number of organisms and have a role in cell growth and differentiation (Saito and Strenli, 1991). An association between phosphatase acitivity and *B. pseudomallei* pathogenicity has been proposed, but its role in virulence remains unclear (Ismail *et al.*, 1994). Exotoxins have been identified and partly purified from culture filtrates of *B. pseudomallei*. On the basis of their different pH sensitivities, two heat-labile toxins have been separated from culture filtrates; one produces necrotic lesions and the other is lethal to mice (Heckly and Nigg, 1958). An iron-dependent metallo-protease of 36 kDa, with an activity optimum at pH 8 and 60^0C has been isolated and characterized (Sexton *et al.*, 1994). Activity was lost above 60^0C but it was stable for 2 months at 4^0C. Iron could not be removed from the active site of the protease, and it was thought to be tightly bound, but enzyme activity was inhibited by chelating agents. The

enzyme degrades a variety of substrates, including immunoglobulins, haemoglobulin and transferin. It may digest substances involved in host defense and assist invasion of host tissues by digesting collagen VIII and elastin. It may also be involved in iron acquisition.

Biofilm provides a protective niche against changes in oxygen tension and changes in degree of moisture (Kondo *et al.*, 1991a). Bacterial biofilms also protect against bacterial factors such as antibiotics, antibodies and bacteriophages(Costerton *et al.*, 1987). Bacteria persist in biofilms even in the presence of an intact host immune system. At the same time their ability to cause acute inflammatory disease is compromised, because they release fewer toxins, with the result that they cause chronic and indolent infection with disseminated inflammatory episodes(Vorachit *et al.*, 1995). In the chronic phase of disease, the organisms present in biofilms are especially refractory to antibiotics treatment.

B. pseudomallei appears to be completely resistant to host defense mediators, such as defensins (cationic peptides) and protamine, an indicator of sensitivity to biological defense substances (Kaufmann, 1993). Complement is very rapidly activated by *B. pseudomallei* and complement components are deposited on the bacterial surface. Opsonisation greatly increases ingestion by polymorphonuclear leucocytes and oxidative burst occurs, but the reason for resistance to lysis is not clear (Egan and Gordon, 1996).

2.2.3 Determinants of *B. pseudomallei* motility

Bacterial motility occurs through the action of flagella. This is widespread in the microbial world, and more than 80% of known bacterial species possess these organelles (Moens and Vanderleyden, 1996). Bacteria use flagella for chemotaxis, which is the motion of bacteria toward chemical attractants or away from chemical repellents. A cell can move toward regions that are more favorable by measuring changes in the concentrations of certain chemicals in its environment (mostly nutrients), and modulating the direction of rotation of its flagella.

Motility by means of flagella is very expensive for cellular economy in terms of the number of genes and the energy required for flagella biosynthesis and functioning (Macnab, 2003). Consequently, it is not surprisingly that the synthesis of flagella is

highly regulated by external factors (including the interaction of bacteria with their hosts). Some bacterial species have lost the capacity to synthesize flagella, for example, *Yersinia enterocolitica* and *Escherichia coli*, but possess the flagella genes (Kapatral *et al.*, 1996). The absence of motility in these species may reflect differences in pathogenesis or life cycles and/or the existence of particular adaptations in response to similar conditions that necessitate motility in related stains.

Motility is not only subject to having one or more flagella, but depends also on other factors. Surface motility is generally dependent on moist conditions, the amount of movement under laboratory conditions being associated with agar concentrations. For example *Pseudomonas aeruginosa* demonstrates swarming (the movement of a group of bacteria) optimally on 0.5%-0.7% agar and generally does not swarm at concentrations above 1% (Kohler *et al.*, 2000). Matsuyama et al. (1992) demonstrated that exolipids such as surfactants promote swarming motility by lowering the surface tension and improving surface wetness (Matsuyama *et al.*, 1992). A second factor in motility is the availability of nutrients. Some species prefer nutrient-rich conditions, while others favor nutrient-poor conditions. Temperature is also important, with some temperatures being inhibitory for swarming. For example, swarming and other kinds of motility are inhibited for *S. marcescens* above 32^0C (Alberti and Harshey, 1990). One hypothesis is that inhibition of motility relates to the absence of surfactant synthesis that occurs at higher temperatures.

Motility can also be mediated through other organelles such as pili. Pili or fimbriae are thin protein tubes originating from the cytoplasmic membrane and are found in virtually all gram-negative bacteria but not in many gram-positive bacteria. There are two types of pili; (i) short attachment pili that are usually quite numerous; and (ii) long conjugation pili that are very few in number. Short attachment pili are organelles of adhesion that allow bacteria to colonize environmental surfaces or cells and resist flushing, while long conjugation pili are involved in genetic recombination (Meta, 1997). Henrichsen suggested the possible involvement of polar pili in a bacterial movement termed twitching (Henrichsen, 1975). Bradley proposed that retraction of pili was the driving force for certain bacterial movement (Bradley, 1980).

2.3 *B. pseudomallei* flagella

2.3.1 Description of *B. pseudomallei* flagella

The flagellum is composed of three parts as figure 1 (Howard, 1999) shows:

(i) A long helical filament, the propeller

(ii) A short curved structure called the hook, which is a flexible coupling or universal joint

(iii) A basal body consisting of a central rod and several rings. The basal body is embedded in the cell surface, beginning within the cytoplasm and ending at the outer membrane, whereas the hook and filament are external to the cell.

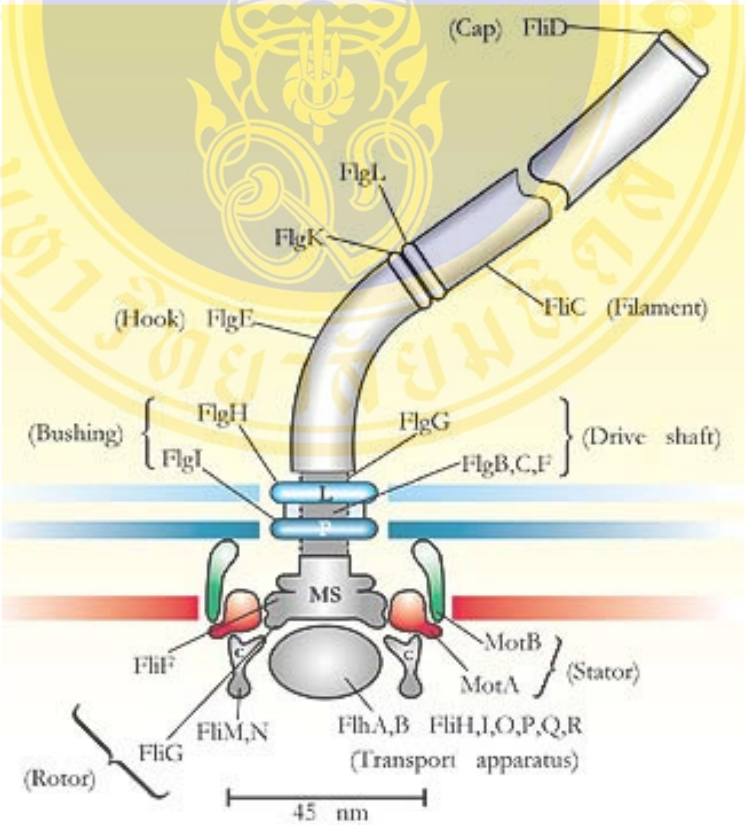


Figure 1: Bacterial motor and flagellum (Howard, 1999).

The flagella filament is a left-handed helix of variable length (5 to 10 μm). Motor rotation is possible in two directions, clockwise (as seen by an observer on the outside of the cell looking down at the hook) and counterclockwise. Switching of direction is possible. The motor is driven by the flow of protons from the outside to the inside of the cell. The source of energy is a transmembrane electrical potential or pH gradient (or both), generated by respiration for cells grown aerobically (Howard, 1999).

The specific structure and arrangement of flagella on the cell differ between species and both seem to be related to the specific environments in which the cells reside (Joys, 1988). Flagella can be arranged on the cell body in a variety of configurations, including single polar, multiple polar and many peritrichous (or lateral) configurations. Mixed flagellation is also possible, when two structurally unrelated flagella types are on the same cell.

2.3.2 Genes encoding flagella

Genes encoding flagella proteins are termed the *fla* genes. A second set of *mot* genes are involved in motility or generation of torque. There are also a variety of genes that encode specific chemoreceptors. The proteins MotA and MotB in *E. coli* are thought to constitute the elements of the stator (the stationary component of the motor within which the rotor turns), which is composed of two integral membrane proteins, MotA and MotB, which form a complex with a ratio of four MotA to two MotB proteins (Kojima and Blair, 2004). The proteins that make up the flagellum are present in multiple copies. For example, there are about 5000 molecules of FliC (flagelline) per helical turn of the filament, which can have as many as six turns. The filament grows at the distal end, with molecules of FliC being added under a distal cap made of protein FliD. The growth process is subject to exquisite genetic control. FliC, for example, is not made until the assembly of the basal body is completed (Macnab, 2003).

2.3.3 Regulation of gene expression

The flagella synthesis genes form a cascade. The genes are grouped in several clusters on the bacterial chromosome. Gene expression occurs in an ordered cascade (1 to 2 to 3 to 4 etc.), creating a flagellar hierarchy. At the top of this hierarchy there is a 'master operon'. Environmental signals and global regulatory proteins control the expression of this operon. The first level of this hierarchy might constitute an important target for motility regulation by external factors. Multiple transcriptional start sites were identified by primer extension analysis in a natural isolate of *Pseudomonas aeruginosa* (Arora *et al.*, 1997), including two major *fleQ* transcription initiation sites. Such a complex promoter structure makes possible multiple regulations of these genes in response to specific environmental conditions. The transcription of master regulator genes located at the top of the hierarchy is growth phase dependent in *Pseudomonas* strains. The complex regulatory cascade of peritrichous and lateral flagella is shown in figure 2 (Olga and Philippe, 2003).

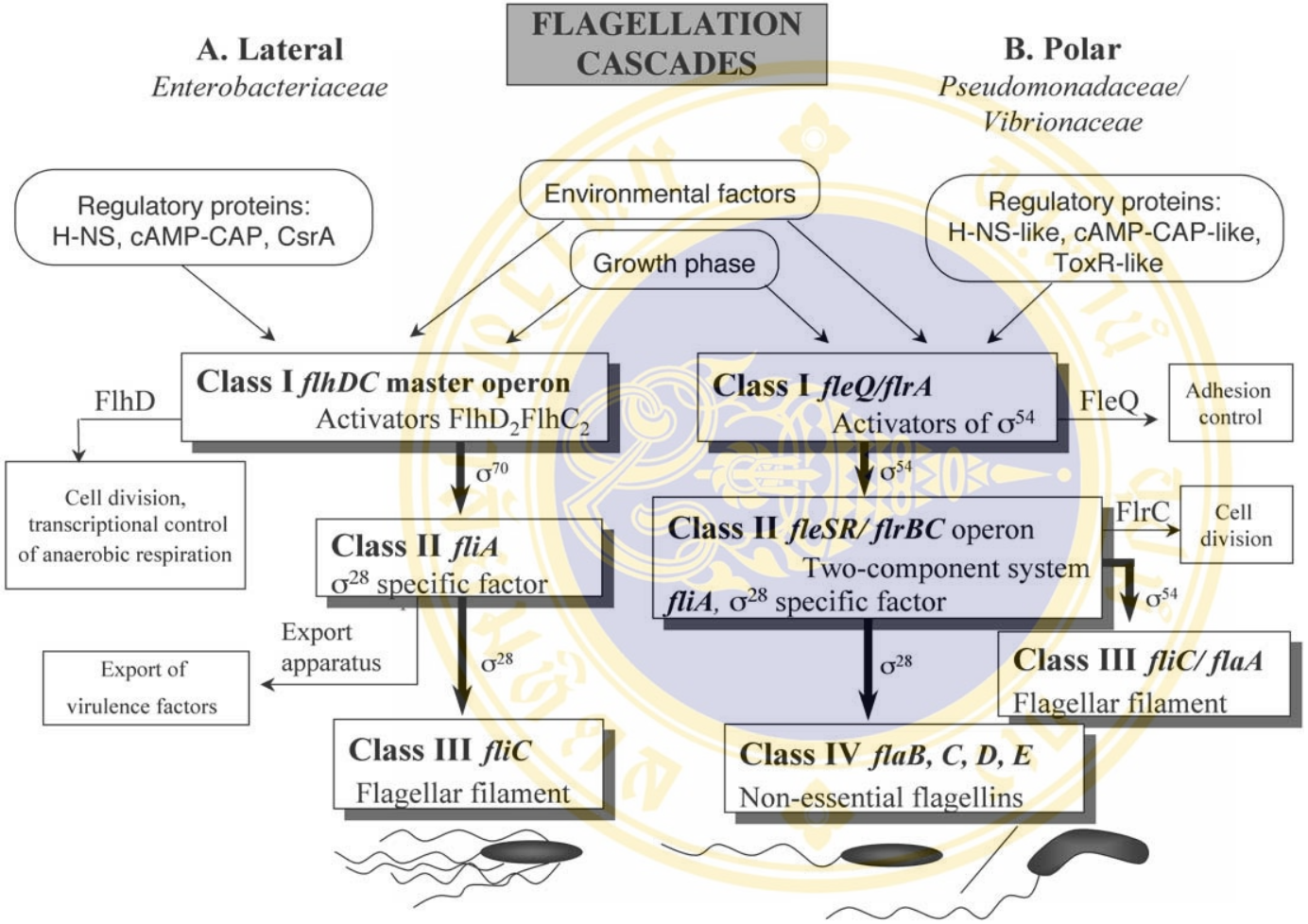


Figure 2: Flagellation cascades of Lateral flagella by Enterobacteriaceae family and polar flagella by Pseudomonadaceae and Vibrionaceae families (Olga and Philippe, 2003).

Flagellum biosynthesis is usually co-regulated together with other virulence factors within the same network. The large number of genes involved in both the biosynthesis and functioning of flagella, the numerous regulators and environmental factors implicated in motility control and the multiple interactions with other cellular processes highlight the extreme complexity of this regulatory network.

2.3.4 Interactions between flagella and environment

Flagellated microorganisms can survive under a wide variety of environmental conditions through the action of bacterial motility and chemotaxis. This allows bacteria to move towards favorable conditions or to avoid detrimental environments, and allows flagellated bacteria to successfully compete with other microorganisms. This is a very complex process and is controlled by gene expression in response to external stimuli (Macnab, 1996). Bacterial flagella also play a crucial role in adhesion, biofilm formation and colonization of microorganisms (Fehlner-Gardiner *et al.*, 2002).

2.3.5 Factors influencing expression of flagella

The *flhDC* flagellar master operon of *Escherichia coli* is controlled by numerous environmental signals, e.g. temperature, osmolarity and pH and by global regulatory proteins, such as H-NS and the cAMP-CAP (catabolite gene activator protein) complex (Olga and Philippe, 2003). Flagella appear to function indefinitely as long as their structure remain intact. However, a break within the basal body does not result in re-grow; rather, new flagella are synthesized (Okino *et al.*, 1989). Optimal flagellar expression occurs when bacteria are in exponential phase of growth (Kodaka *et al.*, 1982). This is well demonstrated by *Helicobacter pylori* for which flagella expression is strongly influenced by age of bacterial culture (Mulugeta *et al.*, 1999).

2.3.6 Interactions between flagella and host immunity

Little is known about putative virulence factors of *B. pseudomallei*. The organism is difficult to kill, like many other soil bacteria (Wuthiekanun *et al.*, 1995). This is partly due to the production of hydrolytic enzymes that are secreted into the extracellular milieu including protease, lipase and lecithinase (Woods *et al.*, 1999). *B. pseudomallei* is also resistant to the action of complement. Bacteria can also survive within human macrophages and in several eukaryotic cell lines (Egan and Gordon, 1996).

B. pseudomallei produce a highly hydrated glycocalyx polysaccharide capsule (Steinmetz *et al.*, 1995). The capsule facilitates formation of micro colonies in which the organism is both protected from antibiotic penetration and phenotypically altered, resulting in reduced susceptibility to antibiotics. LPS (lipopolysaccharide) is present on the bacterial cell wall; endotoxin (part of the molecule) leads to septic shock during human disease. Charuchaimontri *et al.* (Charuchaimontri *et al.*, 1999) reported that high concentrations of antibodies to LPS were associated with improved survival in melioidosis.

In several bacterial pathogens, virulence has been associated with flagella and motility (Carsiotis *et al.*, 1984). It is not clear how flagella function as virulence factors, but the motility phenotype imparted by these organelles often correlates with the ability of an organism to cause disease. For example, Chua et *al.* (Chua *et al.*, 2003) mutated a gene involved in motility, *fliC*, which encodes flagellin (DeShazer *et al.*, 1997). This mutant failed to produce flagella. The virulence of this mutant was compared with that for the wild type parental strain in an animal model. Bacteria were inoculated into the nasal cavity. All of the mice infected with the mutant survived bacterial inoculation, whilst all of those infected with the parent strain died. The motility of the bacterium is markedly reduced when mixed with antibodies raised against the *B. pseudomallei* flagellin protein. This has been shown to provide passive protection against *B. pseudomallei* infection in animal models (Brett *et al.*, 1994).

Flagella also function as an adhesin. Adhesion is a fundamental requirement for many bacteria in order to colonize the host and cause disease. Lillehoj and colleagues demonstrated that *P. aeruginosa* flagella are responsible for bacterial binding to Muc1, a mucin secreted by epithelial cells *(Lillehoj et al.,* 2002). *B. pseudomallei* has been

shown by Smith *et al.* (Smith *et al.*, 1987) to adhere to buccal epithelial cells, and Chua et al. (Chua et al., 2003) concluded from their animal results that attenuated virulence of the *fliC* mutant could have been due to a loss of adhesion. Many pathogenic bacteria also mediate adhesion via pili.

Swarming involves differentiation of vegetative cells into hyper-flagellated swarm cells that undergo rapid and coordinated population migration across solid surfaces (Fraser *et al.*, 2002). Hyper-flagellation (flagella expression) is coupled to the ability to enter host cells and to up-regulation of virulence-associated proteins such as hydrolytic enzymes.

2.4 Evaluating the role of flagella in disease

2.4.1 Experimental animal models

Published data on the role of flagella in *B. pseudomallei* virulence is conflicting. DeShazer *et al.* reported that there was no significant difference in virulence between wild type and a Tn*5*-OT182 disrupted *fliC* mutant when the pathogens were injected intra-peritoneally into young diabetic rats and Syrian hamsters (DeShazer *et al.*, 1997). In 2003, Chua *et al.* used a virulent isolate of ***B.*** *pseudomallei*, KHW, to construct an isogenic deletion mutant with a mutation in the flagellin gene (*fliC*). This was aflagellate and non-motile in semisolid agar (Chua *et al.*, 2003). The mutant was equally virulent in slow-killing assays involving *Caenorhabditis elegans*, but was avirulent during intranasal infection of BALB/c mice. The difference in the findings could have been due to the use of different animal models since disease in diabetic rats and Syrian hamsters is more acute than that in mice. Investigation into the interaction between *B. pseudomallei* strain MM35, a flagellum-defective *fliC* knockout derivative of *B. pseudomallei* strain 1026b, and *Acanthamoeba astronyxis* trophozoites, free-living amoebae, indicates a role for the bacterial flagellum during the early stages of cellular invasion (Inglis *et al.*, 2003).

2.4.2 In vitro models

Several bacterial pathogens appear to require motility in the initial phases of the infection rather than once the infection has been established. This includes *Salmonella spp*, *Escherichia coli* (intestinal infections), *Bordetella bronchiseptica* and *B. pertussis*. Flagella can serve as adhesive appendages in the initial phases of colonization (Josenhans and Suerbaum, 2002). Both the virulent *B. pseudomallei* and avirulent *B. thailandensis* are taken up by phagocytic and non-phagocytic cells (Jones *et al.*, 1996). *B. pseudomallei* is more efficient than *B. thailandensis* in the process of invasion of non-phagocytic epithelial cells, and this is associated with an ability to induce cellular damage as represented by plaque formation in the same A549 cell monolayers. It is possible that flagella play a role in this process. The differences between the two *Burkholderia* species may be related to several other potential factors including the type III protein secretion system (Kespichayawattana *et al.*, 2004).

AIM AND OBJECTIVES

There is evidence to suggest that expression of flagella is a putative virulence determinant of *B. pseudomallei*. The broad aim of this project is to examine flagella expression and motility in clinical isolates of *B. pseudomallei*. Specific aims are to:

1. Develop and/or modify assays to determine flagella expression and motility of *B. pseudomallei*.
2. Define flagella expression and motility *in vitro* of a large collection of *B. pseudomallei* isolates from patients with melioidosis in northeast Thailand.
3. Examine in more detail the expression of flagella and motility during a range of conditions *in vitro*, including expression
    a. Over a growth curve
    b. In static or shaken culture
    c. From the centre or edge of colonies on swarm agar
    d. From the pellicle of broth culture
4. Define the relationship between flagella expression and motility, and colony morphology. This will be defined using isogenic strains of *B. pseudomallei* with three different colony morphology types.

CHAPTER 3
MATERIALS AND METHODS

3.1 Chemicals and reagents.

All chemicals and reagents were obtained from Sigma-Aldrich Company Ltd. or Merck Ltd. Bacterial culture media was obtained from Oxoid. Sterile plastic ware was obtained from Falcon, Becton Dickinson Ltd. and Terumo.

3.2 Bacterial culture and storage conditions.

(a) *Liquid media*: *B. pseudomallei* was grown in Trypticase soy broth (TSB) either static or under constant rotation at 37^0C in air. Cultures were routinely grown in 10 ml media contained in 35 ml universal containers. M9 medium (Difco) was used to grow bacteria used in chemotaxis assays; this was incubated at 200 rpm at 37^0C in air.

(b) *Solid media*: All agar plates used in this study were prepared at the Wellcome Unit, Bangkok as recommended by the manufacturer (Appendix I). *B. pseudomallei* was routinely cultured on Ashdown's Selective Agar (ASA) at 37^0C in air for 96 hours. Swarm, swim and twitching plates were used for motility tests. These were cultured at 37^0C in air for 48, 72, 80 and 96 hours.

(c) *Bacterial storage*: *B. pseudomallei, B. mallei* and *P. aeruginosa* were suspended in TSB containing glycerol (15%, v/v). All isolates were stored at -80^0C.

3.3 Microscopic techniques

Real Time microscope (RTM-3) slides (pre-cleaned, Swiss glass 75 x 25 mm) were obtained from J. Melvin Freed Co. (Canada). Glass coverslips (22 x 22 mm no.15 thickness), were obtained from Richardson Technologies Inc. Canada. Immersion oil type DF non-drying for fluorescence and general microscopy was obtained from Cargille Laboratories Inc., U.S.A. All accessories for real time microscopy were as recommended by Richardson Technologies Inc.

3.4 Laboratory facilities

B. pseudomallei is classified as a hazard group 3 organism (Advisory Committee on Dangerous Pathogens, 2004). All experimental work involving viable cells of *B. pseudomallei* was performed in a Category 3 containment laboratory (Figure 3). Biosafety level (BSL) 2 practices, equipment, and containment have been recommended for laboratory work with *B. pseudomallei* cultures, or clinical samples that could contain the organism (Jonathan and McKinney, 1999). Consistent with these recommendations, aerosol-generating procedures were performed in a Class II safety cabinet. Sniff testing of opened culture plates was strictly prohibited (Jonathan and McKinney, 1999).

A

B

Figure 3: (A) Category 3 containment laboratory, (B) Biosafety level (BSL) 2 cabinet

3.5 Bacterial isolates

3.5.1 *B. pseudomallei*

3.5.1.1 Bacterial identification

The *B. pseudomallei* isolate collection used during this work was maintained in locked –80^{0}C freezers at the Wellcome Unit, Faculty of Tropical Medicine, Mahidol University. These were isolated from samples from patients presenting to Sapprasithiprasong Hospital, Ubon Ratchathani (northeast Thailand) with melioidosis between 2002 and 2003. All isolates were identified in the clinical diagnostic laboratory in Ubon Ratchathani as *B. pseudomallei* using the following criteria:

(i) Growth on Ashdown's agar with typical colony morphology after 3 or 4 days of incubation at 37^{0}C in air (see Figure 4).

(ii) Oxidase test positive

(iii) Resistance to Gentamicin (disc strength 10μg) and Colistin (disc strength 10μg) after overnight incubation at 37^{0}C in air on Columbia agar (National Committee for Clinical Laboratory Standards, 2000).

(iv) Bacterial colonies on agar plates were confirmed as *B. pseudomallei* using an in-house latex agglutination reaction (Kanaphun et al., 1993). In brief, a small amount of colony is emulsified using a sterile toothpick into a solution containing latex particles coated with a mixture of three monoclonal antibodies specific for a 200-KDa surface antigen of *B. pseudomallei*. The reaction is read within 1-2 min. The presence of agglutination is taken as a positive reaction. *E. coli* is used as a negative control (Figure 5).

(v) API 20NE biochemical strips were used as recommended by the manufacturer (bio-Merieux. Inc.), if any uncertainly remained about the bacterial species (Figure 6). Typical API profiles are 1156577, 1556577 or 1156576 by APILAB Plus software; this gives an automated interpretation on a computer workstation (bioMerieux, 2005).

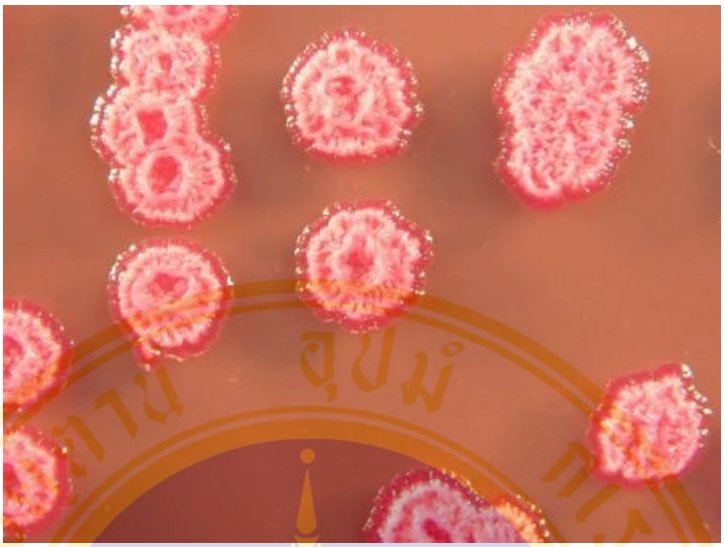


Figure 4: Typical colonies of *B. pseudomallei* on Ashdown's agar after incubation for 4 days at 37^0C in air

Figure 5: Latex agglutination reaction with *B. pseudomallei*

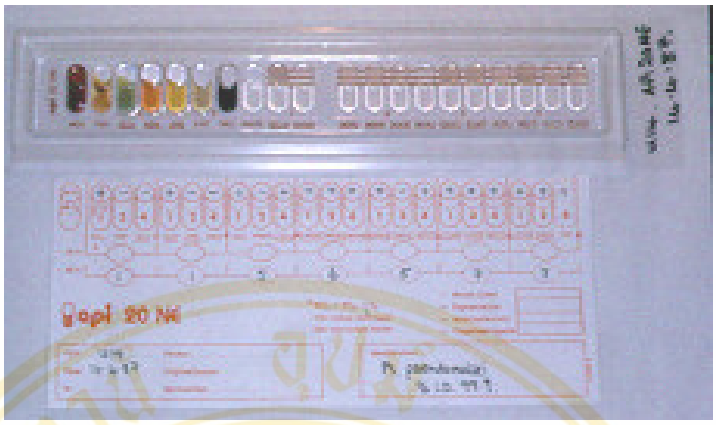


Figure 6: API 20 NE biochemical reaction test and result datasheet

To exclude the possibility that some of the isolates were *B. thailandensis*, all isolates were tested for their inability to assimilate arabinose. This was performed in batches at the end of the rainy season of each year in the Wellcome Unit, Bangkok. *B. pseudomallei* is negative and *B. thailandensis* is positive (Wuthiekanun et al., 2002). Isolates were point inoculated onto agar plates containing arabinose as the only source of nutrition. *B. pseudomallei* fails to grow on this medium after 48 hours incubation at 37^0C in air, while *B. thailandesis* shows growth (Figure 7).

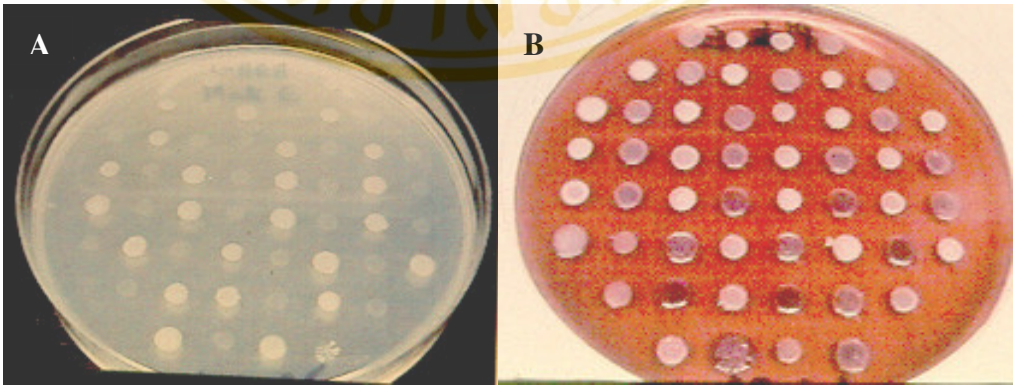


Figure 7: Arabinose assimilation test differentiates between *B. pseudomallei* and *B. thailandensis*. Plate A, arabinose plate; plate B, growth of the same organisms on Ashdown's agar.

3.5.1.2 Clinical sample types positive for *B. pseudomallei* isolates

The clinical sample types yielding the isolates used in this study are shown in summary form in Table 2 below. A full list of isolates and clinical sample types is provided in Appendix II.

Table 2: Site of isolation for 211 of *B. pseudomallei* isolates used in this study

Specimen site	Number of samples	%
Blood	99	46.9%
Pus from normally sterile sites*	55	26.1%
Respiratory secretions (Sputum or tracheal aspirates)	26	12.3%
Urine	13	6.2%
Throat swab	9	4.3%
Wound swab	5	2.3%
Other (Pleural fluid 3, PD fluid 1)	4	1.9%
Total	211	100.0%

* Known sites are as follows: spleen (1), parotid gland (9), liver (7), joint (8), brain (1), soft tissue abscess (3), lymph node (1) and bone (1). Sites of other pus samples not recorded.

3.5.1.3 Choice of *B. pseudomallei* isolates used

The initial, large isolate collection was assembled to represent unselected, consecutive clinical isolates. This was done to avoid bias that could be introduced by using one or more selection criteria. Pilot studies of motility that were performed to establish standardized methodology at the start of the project used 10 isolates selected on the basis of morphology. The rationale for this was to test a range

of phenotypic bacterial types. (These are described later, and isolates are listed in Appendix II). Population based studies of motility (swimming, swarming and twitching) were then performed on the complete bacterial collection. A single isolate (termed strain 49) originally isolated from splenic pus was used for study of flagella expression over time. This isolate had the typical colony morphology on Ashdown's agar (pale purple, round, irregular and rough edge, and dry rugose surface texture). More detailed studies of motility and associated bacterial factors were performed using *B. pseudomallei* strains 153 and 164, together with isogenic strains with difference phenotype that were generated during bacterial starvation (described in 3.11 below).

3.5.2 Control isolates

Burkholderia mallei strain EY 100 was used as a negative control for motility assays. This species is non-motile; the genome of *B. mallei* contains numerous flagellar and chemotaxis genes, most of which appear to be intact. However, one methyl-accepting chemotaxis gene has a frameshift mutation and may account for lack of flagella expression (Upadhyay et al., 2004). *Pseudomonas aeruginosa* ATCC 27853 was used as the positive control in motility assays. This is known to be motile and to be positive on assays of swimming, swarming and twitching (Eric et al., 2001).

3.6 Methodology used to examine motility on solid agar

All 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. This period of incubation was chosen since colony morphology is clearly identifiable at this stage. This was important since colony morphology proved central to motility during the period of this project. This is described in more detail in later sections.

3.6.1 Swimming motility

Swimming motility was assessed as previously described (Eric et al., 2001). In brief, Tryptone swim plates (1% tryptone, 0.5% NaCl, 0.3% agar) were inoculated with a small amount bacterial colony using a sterile toothpick. Plates were incubated at 37^0C in air, and observed at 24, 48, 72, 80 and 96 hours. Motility was assessed qualitatively by examining the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation (Eric et al., 2001). Positive and negative controls were prepared on each day of the experiment. Typical colony appearance after 72 hours incubation is shown in Figure 8.

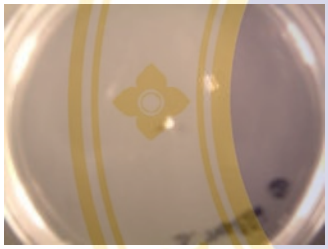
		
B. mallei EY100 (negative control)	*P. aeruginosa* ATCC 27853 (positive control)	*B. pseudomallei*

Figure 8: Swimming motility of *B. pseudomallei* at 37^0C in air, at 72 hours

3.6.2 Swarming motility

Swarming motility was assessed as previously described (Eric et al., 2001). In brief, 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 of dextrose/liter. These were dried overnight at room temperature. Bacterial cells were point inoculated with a sterile toothpick. Plates were incubated at 37^0C in air, and observed at 24, 48, 72, 80 and 96 hours (Eric et al., 2001). Evidence of bacterial swarming was taken as the presence of bacterial growth around the point of inoculation. The widest diameter of this zone was measured also done for swim. Positive and negative controls were prepared on each day of the experiment. Typical colony appearance after 72 hours incubation is shown in Figure 9.

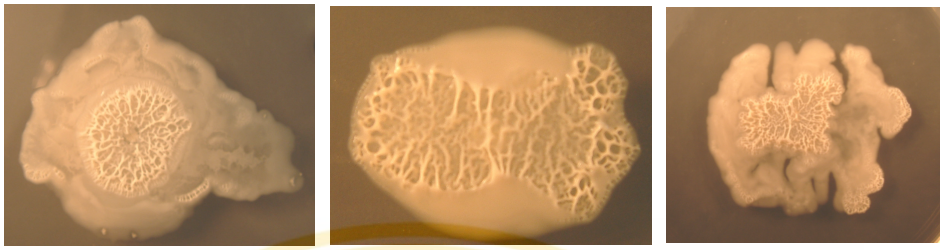


Figure 9: Swarm plates for three isolates of *B. pseudomallei* on swarm plates at 37^0C in air, at 72 hours

3.6.3 Twitching motility

The top of an isolated bacterial colony was touched with a sterile toothpick and stab-inoculated through to the bottom of twitching agar plates. These were incubated at 37^0C in air, and observed at 24, 48 and 72 hours. A hazy zone of growth at the interface between the agar and the polystyrene surface was observed. The ability of bacteria to strongly adhere and form a biofilm on the polystyrene surface was then examined by removed the agar with a disposable loop. Contaminated agar was placed into 1.0% w/v virkon disinfectant. The Petri dish was carefully rinsed with tap water to remove unattached bacterial cells. The remaining attached bacteria were fixed with 99 % methanol for 7 min at room temperature, after which the plate was left to dry. The plate was then stained for 30 min with 150 µl of 1 % crystal violet. Excess stain was rinsed off by placing the plate under running tap water (Eric et al., 2001). Evidence of twitching was taken as the presence of bacterial attached to the Petri dish. *Pseudomonas aeruginosa* ATCC 27853 (positive control) and *B. mallei* EY 100 (negative control) were used in each plate. Typical results after 72 hours incubation are shown in Figure 10.

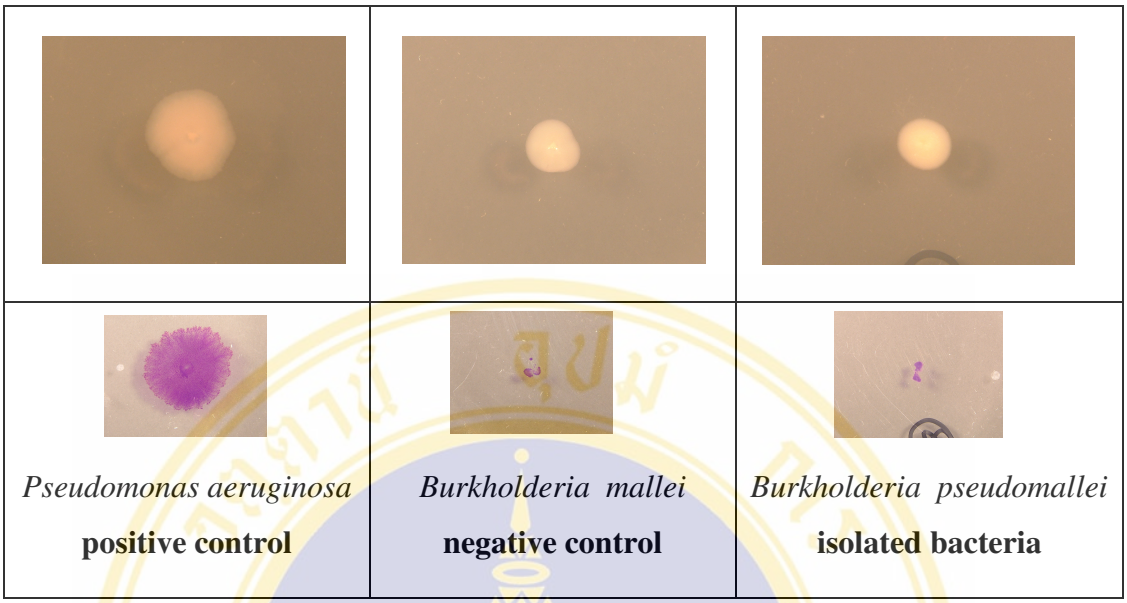


Figure 10: Twitching motility at 37^0C in air, at 72 hours

Top pictures show bacterial colony on top surface of agar plate. Bottom pictures represent stained bacteria adherent to the bottom of the Petri dish after removal of the agar.

3.7 Real Time Microscopy (Richardson's RTM-3)

3.7.1 Description of RTM-3 microscope and its capabilities

The RTM™ is a highly specialized microscope that has been designed and developed by Richardson Technologies Inc (RTI), Ontario Canada, 2004 (Figure 11). The RTM™ was designed to provide fast and easy access to images of living cells and organisms, with maximum resolution, contrast and colour fidelity. It causes minimum impact on the sample and avoids the need for the normal techniques of fixation, staining or dehydration.

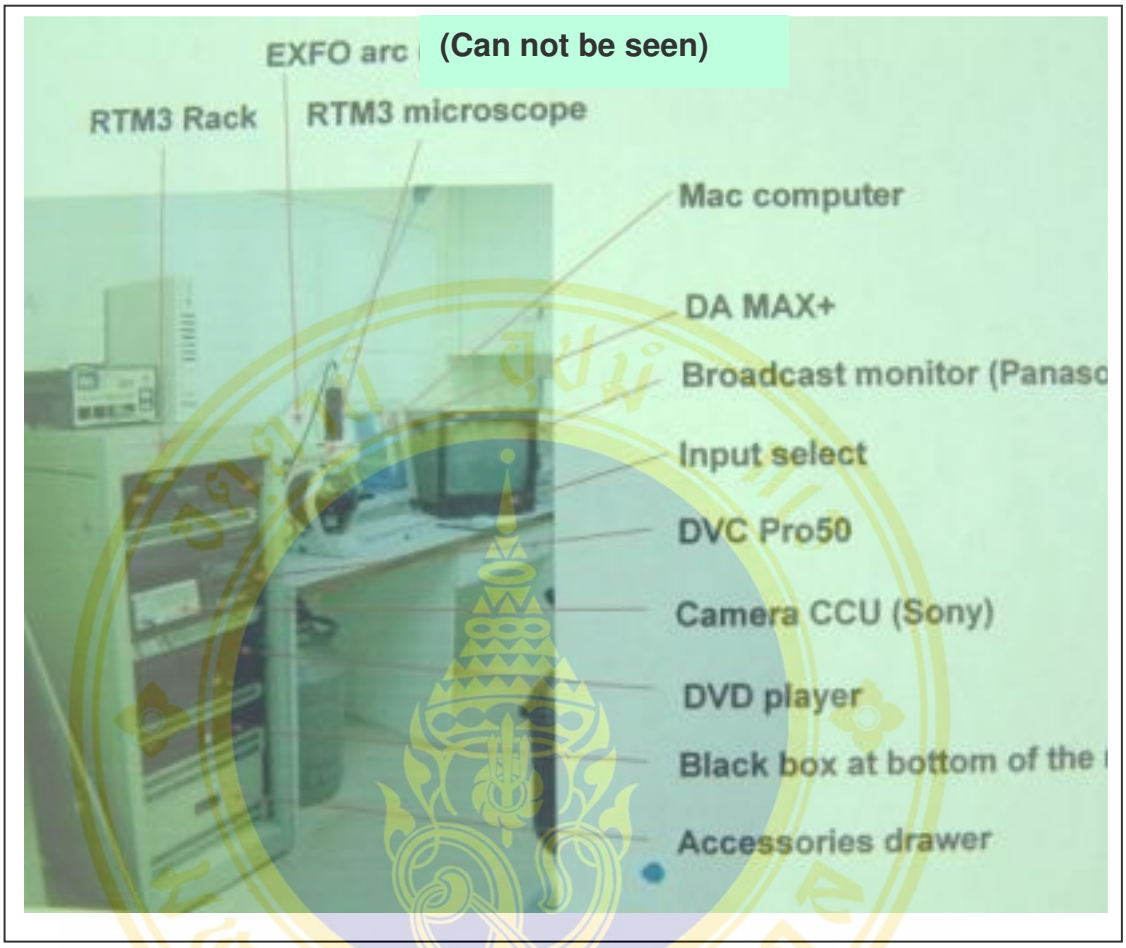


Figure 11: Diagram of RTM-3

The principle behind the RTM™ is inverted dark-field contrast in an ultra-stable, ultra clean microscope system with full colour imaging capability. Compared with conventional techniques, it provides higher contrast, higher resolution images of living cells, in surroundings more similar to their natural environments. The RTM™ was used during this project to examine flagella expression by dead, stained bacteria, and to analyze motility of living bacteria. This provided high quality views, and allowed:

(i) enumeration of flagella by taking high resolution pictures that were stored by the RTM™ software and examined at a later date as still frames, and

(ii) definition of motility by observation of video footage.

3.8 Analysis of flagella expression

3.8.1 Preparation of the slide prior to staining

Isolates were grown on agar or in broth culture, depending on the experiment. Bacteria were prepared for microscopy using either live or formalin-fixed bacteria. Live bacteria were used for motility experiments, while bacteria were fixed with formalin prior to evaluation of the number and position of flagella.

To examine live bacteria from a colony on agar, a colony was lightly touched with a bacteriological loop containing sterile water, and the resulting bacterial cells carefully spread onto a microscope slide. Manipulation was kept to a minimum to avoid shearing of flagella. For live bacteria from broth culture, 5ul of neat broth culture was placed directly onto a clean glass slide followed by a coverslip. To examine fixed bacteria, 500ul bacterial suspension (either neat broth of cells from colony suspended in fresh TSB) was added to 50ul formalin (37% w/v). A loopful of suspension was placed onto a microscope slide followed by a coverslip.

3.8.2 Staining of flagella by Modified Ryu's stain

Approximately 5-10 minutes after preparation of the slide, two drops of filtered Ryu's stain (~10 ul) was pipetted along the edge of the coverslip utilizing the effect of capillary action (Heimbrook et al., 1989). Details of Ryu's flagella stain are given in Appendix I. The slide was left at room temperature for between 5-15 minutes to allow staining to occur, after which they were examined at 40X. In the case of fixed slides, nail polish was applied to the edges of the coverslip to form a seal and preventing drying for around one month, in case slides required re-examination.

3.8.3 Terminology used to describe flagella number and position

Stained *B. pseudomallei* were classified as having no flagella, or one flagellum, two flagella, three flagella, four flagella, five flagella or six flagella. The position of flagella was defined as unipolar (single flagellum at one pole), bipolar (flagella at both poles), lateral (on the side of the bacterium) or peritrichous (a

combination of at least one polar and one lateral flagellum). This is depicted in Figure 12 below.

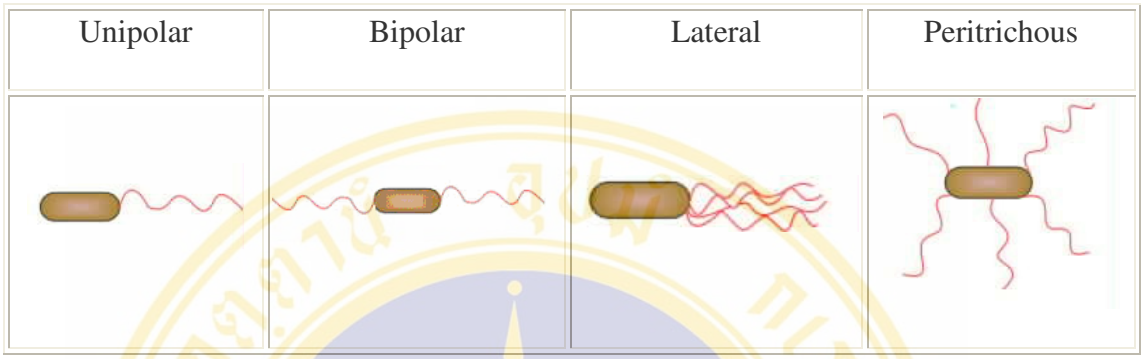


Figure 12: Position of flagella

3.8.4 Methodology for counting number of flagella

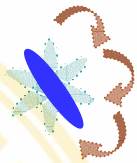
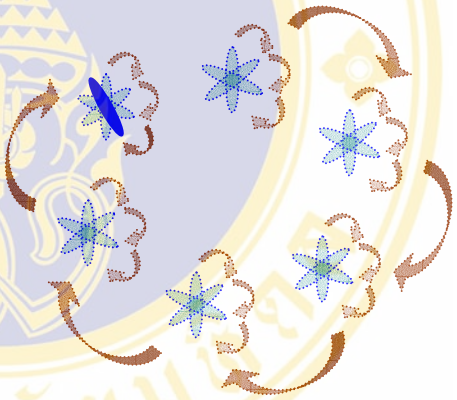
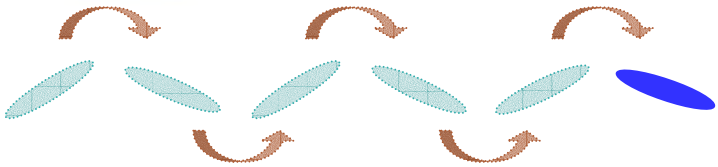
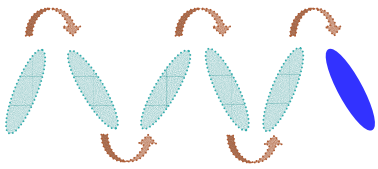
Slides were observed using the RTM-3 microscope under 40X magnification. Images of bacteria were taken and stored for each isolate using the microscope software. Flagella expression was assessed for 100 bacterial cells chosen at random per slide for each isolate. The exception to this was the observation of *B. pseudomallei* strain 153 and 164 together with their isogenic colony variants; 500 bacterial cells were used during several experiments.

3.9 Analysis of motility

3.9.1 Terminology to describe bacterial movement as observed by RTM-3

Terminology for bacterial movement was developed using RTM-3 microscope video footage. Slides were prepared as described above. Prior to reading, slides were maintained in a moisture chamber to prevent drying. One minute of video footage was recorded for each of 10 fields per slide. A pilot study was undertaken using 15 *B. pseudomallei* isolates in which each isolate was examined using a single slide (isolates will be described in detail in results). Five types of movement were defined (Table 3).

Table 3: Terminology to describe bacterial motility.

Term	**Description**
Vibration	The cell is moving (vibrating). There is no displacement. 
Rotation	The cell is rotating around one pole (CW, CCW). There is no displacement 
Rotating displacement	The cell is rotating around one pole (CW, CCW). There is displacement, mostly in one direction. 
Waving	The cell is moving in an undulating pattern (zigzag). There is displacement mostly in one direction. 
Tumbling	The cell is alternating tumbling around its two poles. There is displacement mostly in one direction. 
Non movement	There is no movement at all. 

3.9.2 Scoring for the presence of bacterial movement type

Each isolate was examined using a single slide. Ten fields per slide were chosen at random and recorded for 1 minute. The five types of movement were scored as present (+) if one or more bacteria were observed with the specific movement during the 5 minute observation period. Movements were scored as absent (-) when they did not occur in any of the fields.

3.9.3 Scoring for dominant bacterial movement type

Each isolate was examined using a single slide. One hundred bacterial cells were chosen at random per bacterial isolate and the specific movements recorded on videotape. A movement was defined as DOMINANT when more than 50% of the cells counted had the same specific movement.

3.10 Expression of flagella during growth

A single colony of *B. pseudomallei* taken from Ashdown's agar after incubation at 37^0C in air for 4 days was suspended in 20 ml of TSB. This was further incubated overnight at 37^0C in air at 200 rpm, and then diluted in fresh TSB to reach a starting OD of 0.025-0.03 at wavelength 600 nm (~10^6 cell/ml). This was further diluted 1 in 100 in TSB in a 500 ml Duran bottle to give a final concentration at time 0 hour of approximately 10^4 CFU/ml. The bottle was incubated at 37^0C in air both static and shaken at 60 rpm and sampled in triplicate every 2 hours. One aliquot was used to define colony count as determined by serial dilution and spread plating onto Ashdown's agar in duplicate. The second aliquot was used to measure optical density at a wavelength of 600 nm. The third aliquot was used to examine flagella expression, flagella length and bacterial cell length. Bacterial suspension was mixed with 37% formalin in a 1:10 ratio prior to flagella staining. Flagella expression was defined as described above. To determine bacterial cell and flagella length, 100 cells were chosen at random and the bacterial length recorded using the RTM-3. A measuring ruler function (RTM-3 software) was applied to still images to measure bacterial cell and flagella length in micrometers.

3.11 Observation of bacterial colony morphology

A single investigator defined colony morphotype throughout the study. Spread plates were prepared directly from the freezer vial. Frozen bacterial colony was scrapped from the top of the vial, suspended in sterile saline, serially diluted and spread plated onto Ashdown's agar to give approximately 100 single colonies per plate. The first 50 isolates were examined in a pilot study to determine the optimum day of colony observation. Plates were incubated at 37^0C in air and observed every day for 7 days using a combination of a hand held magnifier and colony photography. The following features of colony morphology were recorded: colony size (measured in mm by ruler), color (red, purple, pink), translucency (opaque or translucent), degree of color (pale, bright and dark), wetness (wet or dry), outer edge of colony (smooth or irregular), surface texture of center of colony, presence of roughness in outer half of colony, and overall surface shape (convex, crater, lobulated, radial, radio-umbilicated, radio-umbonated, peaked, rugose, segemented-rugose, segmented-umbilicated, umblicated, umbilicated with irregular edge, umbilicated with heaped up irregular edge, umbonated). From this it was determined that day 4 gave the maximum observable colony variation between strains. The remaining samples were then examined. A total of seven morphotypes were defined, hereafter termed Type I-VII (Appendix III). An algorithm was developed to include discriminatory features using data mining Clementine 7.2 software (detailed in Appendix III).

3.12 Isolation of isogenic strains with variable colony morphology

Type I *B. pseudomallei* strains 153 and 164 were randomly selected from clinical isolates identified in 2002-3 from those observed to have more than one morphotype present in a single sample. A single colony of clearly defined morphology was harvested from an Ashdown's plate after incubation at 37^0C in air for 4 days and placed into 3 ml of TSB. This was maintained static at 37^0C in air for 28 days after which a spread plate was prepared on Ashdown's agar. Colonies with Type I, III and VI morphology (see Appendix III) were inoculated into a freezer vial containing TSB with 20% glycerol which were maintained at -80^0C. Isogenic and parental strains were directly inoculated onto agar or broth from these vials for all further assays.

3.13 Statistical analysis

Statistical analysis was performed using the statistical program Intercooled STATA, version 8.0 (College Station, TX). Motility for the bacterial in population was analyzed using mean and standard deviation. Data for swarm colony size were not normally distributed; those are presented using median, 95% confidence interval and inter-quartile range, and analyzed using the Wilcoxon signed-rank test Fisher's exact test was used to test proportions.

CHAPTER 4

RESULTS

4.1 Motility assays for control strains *P. aeruginosa* and *B. mallei*

4.1.1 Swim assay

The positive and negative control strains (*P. aeruginosa* ATCC 27853 and *B. mallei* EY100, respectively) were examined in triplicate in a given assay. The results of the colony diameter after 24, 48 and 72 h incubation at 37^0C in air on swim agar for these isolates is shown in table 4. The colony appearance is shown on Figure 13. The mean colony diameter on swim plates for these control organisms is compared in Figure 16.

Table 4: Colony diameter (mm) for *P. aeruginosa* ATCC 27853 and *B. mallei* EY100 on swim agar plate after 24, 48 and 72 h incubation at 37^0C in air.

Strains	24 hrs.			48 hrs.			72 hrs.		
	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3
B. mallei EY100	1	1	1	3	2	3	3	2	3
P. aeruginosa ATCC 27853	14	12	13	30	27	32	40	37	43

The results were as predicted, the swim zone diameters for *B. mallei* and *P. aeruginosa* being consistent with the absence or presence of swim motility, respectively.

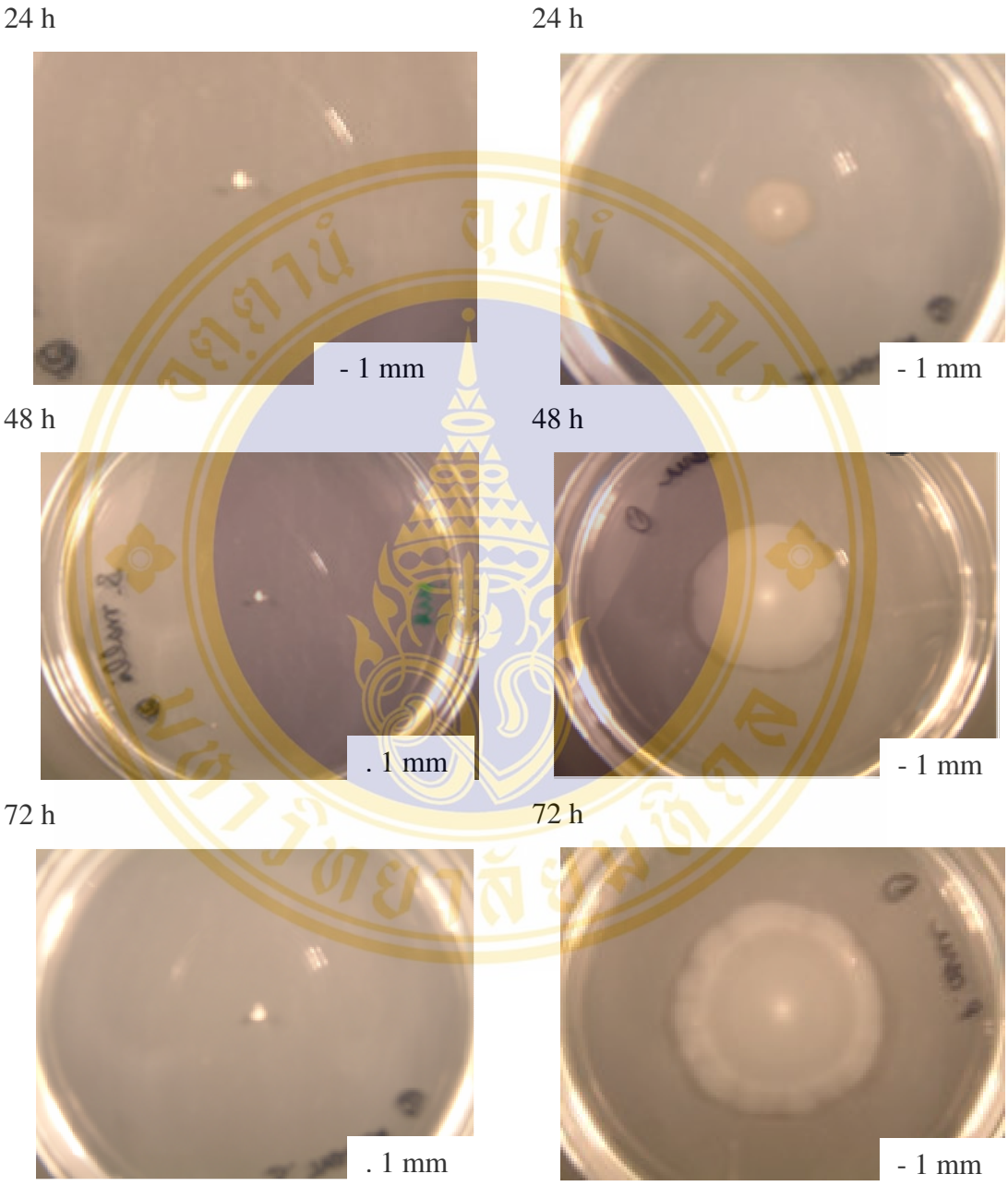


Figure 13: Colony appearance of *P. aeruginosa* ATCC 27853 and *B. mallei* EY100 on swim agar after incubation for 24, 48 and 72h at 37^0C in air.

4.1.2. Swarm assay

The positive and negative control strains (*P. aeruginosa* ATCC 27853 and *B. mallei* EY100, respectively) were examined in triplicate in a given assay. The results of the colony diameter after 24, 48 and 72 h incubation at 37^0C in air on swarm agar for these isolates is shown in table 5. The colony appearance is shown on Figure 14. The mean colony diameter on swim plates for these organisms is compared in Figure 16.

Table 5: Colony diameter (mm) for *P. aeruginosa* ATCC 27853 and *B. mallei* EY100 on swarm agar plate after 24, 48 and 72 h incubation at 37^0C in air.

Strains	24 hrs.			48 hrs.			72 hrs.		
	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3
B. mallei EY100	3	2	2	4	4	4	6	7	5
P. aeruginosa ATCC 27853	8	8	7	12	12	12	13	13	13

The results were as predicted, the swarm zone diameters for *B. mallei* and *P. aeruginosa* being consistent with the absence or presence of swarm motility, respectively.

B. mallei EY100 *P. aeruginosa* ATCC 27853

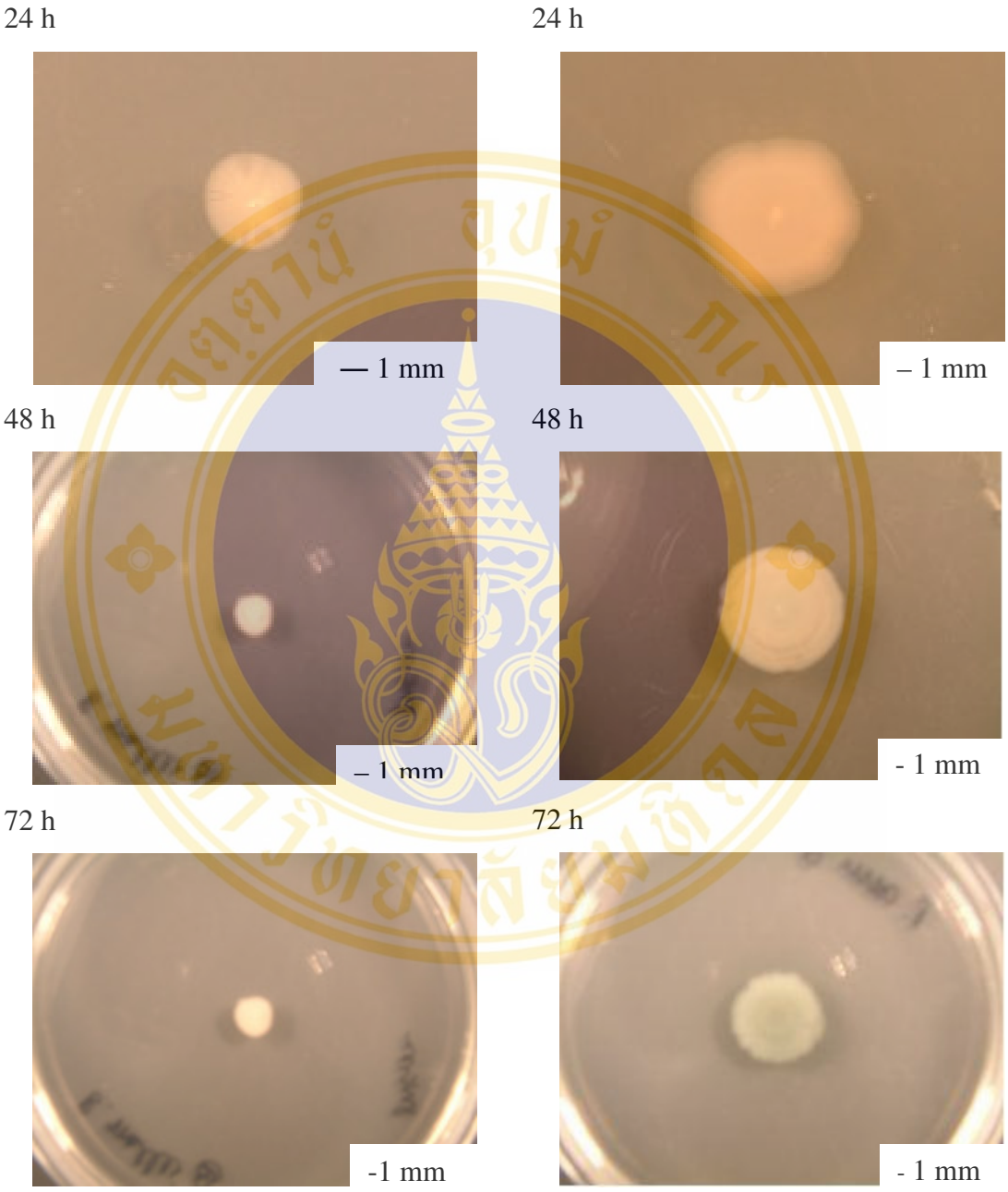


Figure 14: Colony appearance of *P. aeruginosa* ATCC 27853 and *B. mallei* EY100 on swarm agar after incubation for 24, 48 and 72 h at 37^0C in air.

4.1.3 Twitching motility assay

The positive and negative control strains (*P. aeruginosa* ATCC 27853 and *B. mallei* EY100, respectively) were examined in triplicate in a given assay. The result of the stained zone present on the petri dish after removal of the agar and staining by crystal violet after incubation for 24, 48 and 72 h at 37^0C in air on twitching agar is shown in table 6.

Table 6: Twitch diameter zone of *P. aeruginosa* ATCC 27853 and *B. mallei* EY100 at 24, 48 and 72 h at 37^0C in air.

Strains	24 hrs.			48 hrs.			72 hrs.		
	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3
B.mallei EY100	0	0	0	1	1	1	1	1	1
P.aeruginosa ATCC 27853	7	7	7	11	12	13	17	17	17

These were as predicted, the zone diameters for *B. mallei* and *P. aeruginosa* being consistent with the absence or presence of twitching motility, respectively. The mean diameter on stained zones for these organisms is compared in Figure 16.

These positive and negative controls were used in every motility assay hereafter.

B. mallei EY100 *P. aeruginosa* ATCC 27853

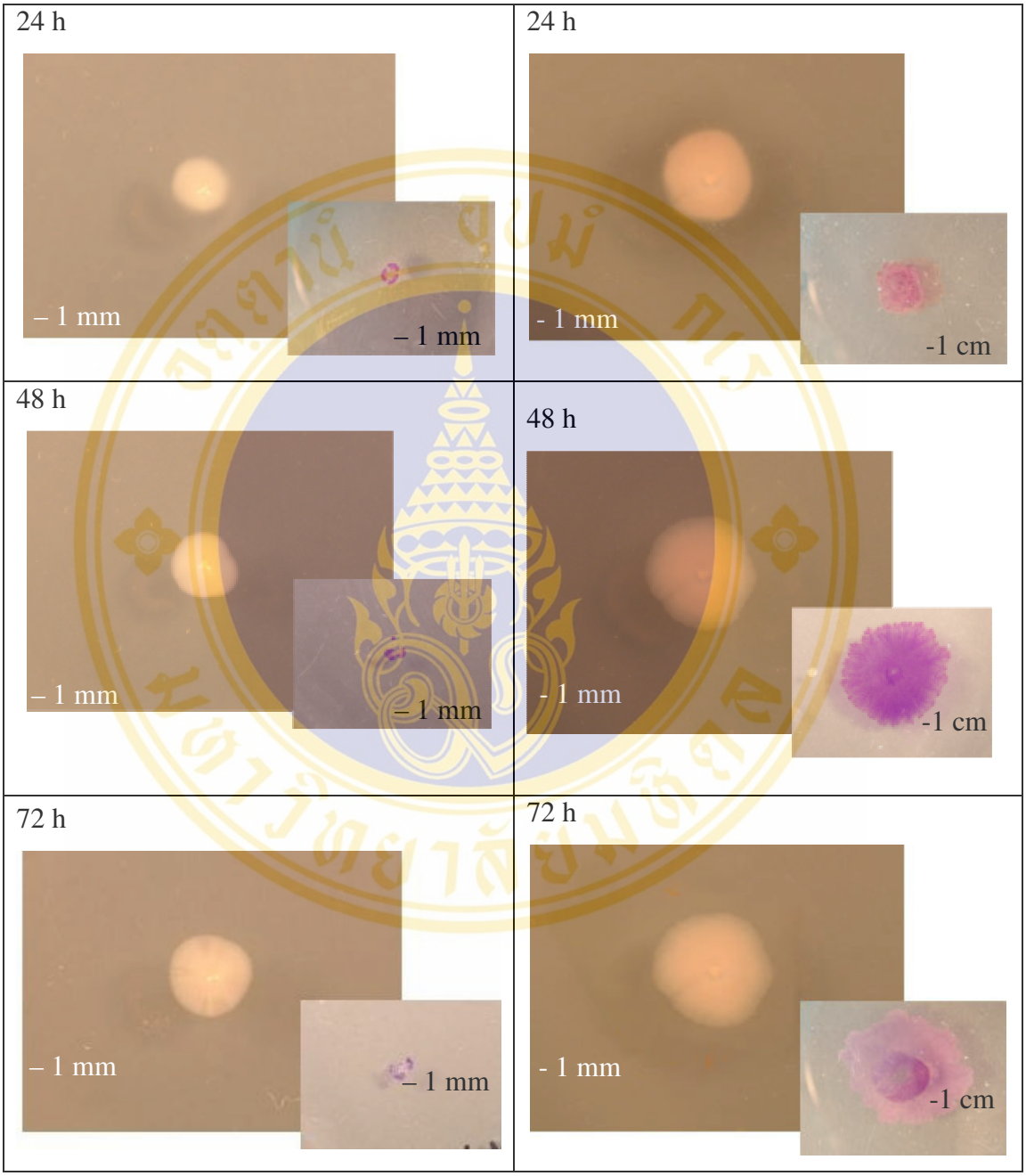


Figure 15: Colony appearance of *P. aeruginosa* ATCC 27853 and *B. mallei* EY100 on twitch motility agar after incubation for 24, 48 and 72 h at 37^0C in air.

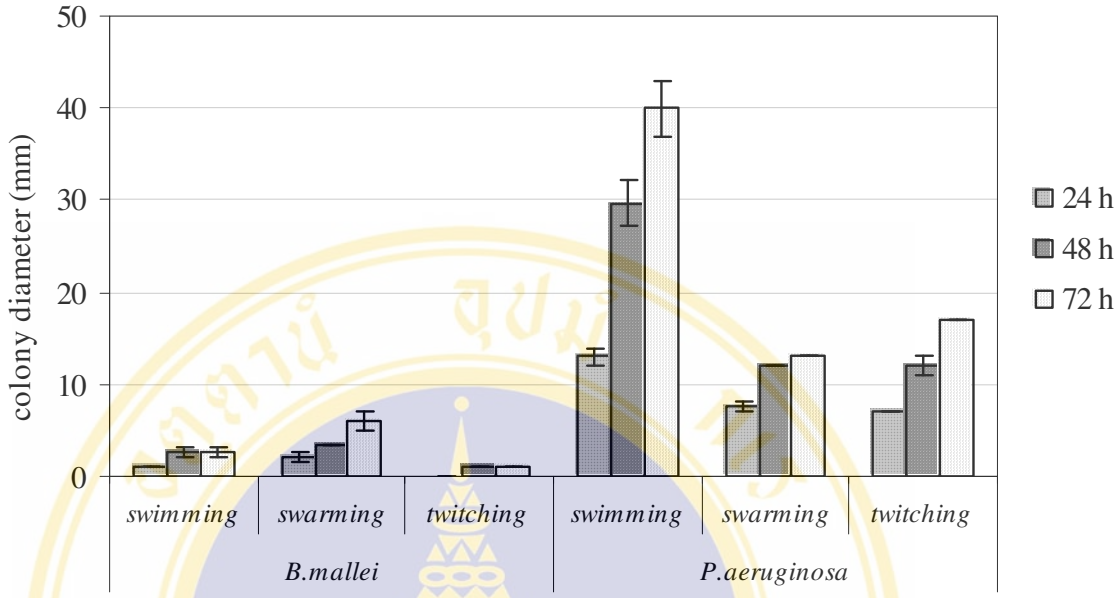


Figure 16: Colony diameters of positive and negative control strains in three motility assays. The graph shows the mean diameter (and standard deviation) of triplicate plates performed three times, measured after 24, 48h and 72 h of incubation at 37^0C in air.

4.1.4 Summary measurement of motility

A summary measurement of motility has previously been described in which motility was assumed to be linear over time and described as distance traveled per hour (mm per hour) (Semmler et al., 1999). This was applied to the results for the control strains in the three assays after 48 and 72 h incubation at 37^0C in air, as *B. mallei* does not swarm or swim. The colony diameter of *B. mallei* was approximately the same in all three assays, suggesting that this reflected colony growth rather than motility (rate less than 0.1 mm per hour).

Table 7: Rate of colony diameter expansion (mm per hour) of *B. mallei*, and *P. aeruginosa* in 2 types of motility assay following 48 and 72 h incubation at 37^0C in air.

Condition		Swimming motility	Swarming motility
B. mallei at	48 h	0.056	0.073
	72 h	0.037	0.083
P.aeruginosa at	48 h	0.618	0.264
	72 h	0.556	0.176

4.2 Motility of *B. pseudomallei* isolates

Motility was initially tested in a pilot study, in which 10 strains from 5 *B. pseudomallei* isolates were randomly selected from the stock of clinical *B. pseudomallei* isolates (some isolates had more than one colony morphotype after 4 days incubation on Ashdown's agar, and each was tested). The colony appearance of these strains is shown in Figure 17.

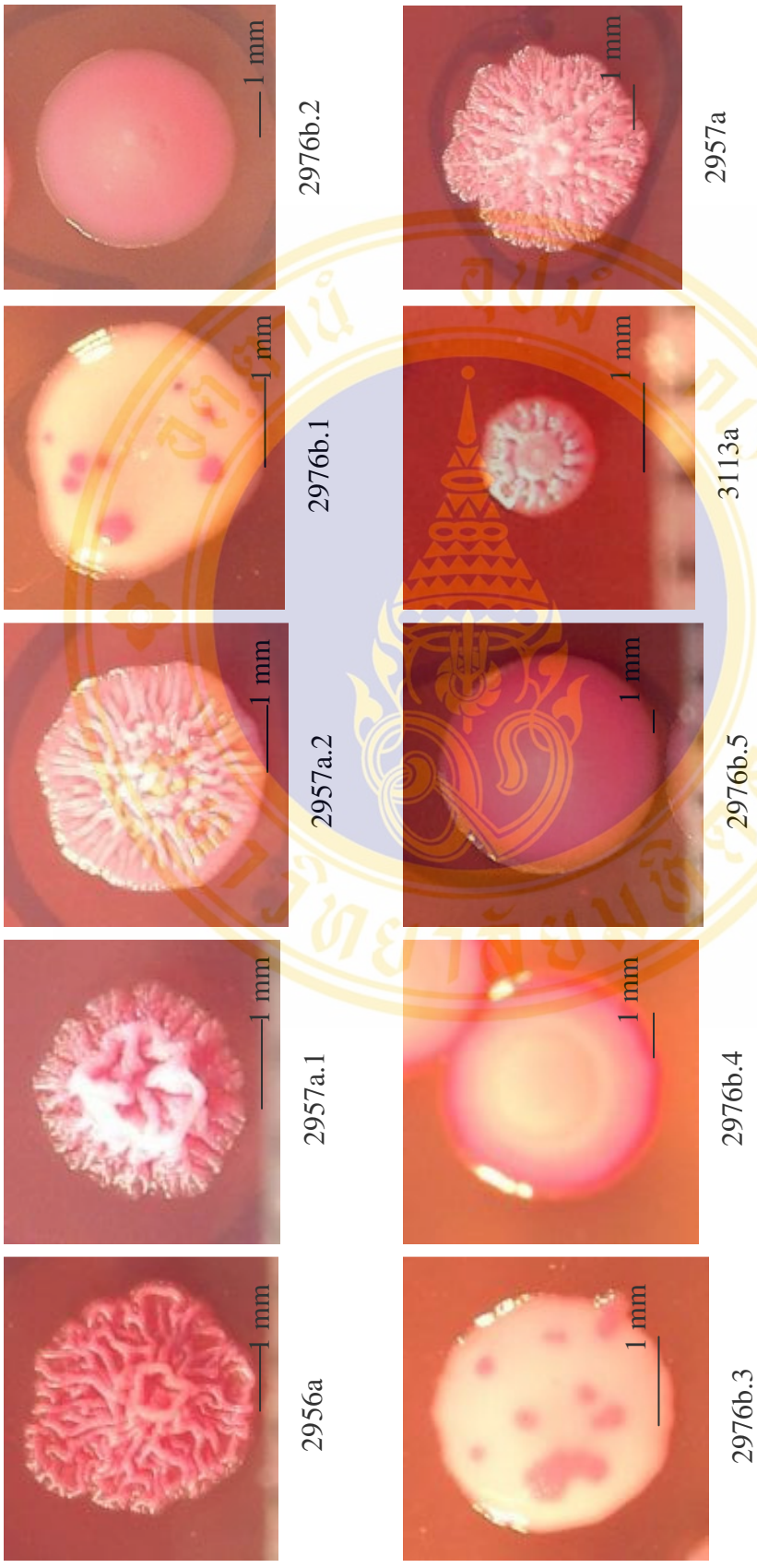


Figure 17: Clinical strains of *B. pseudomallei* tested in pilot study of motility. Colony appearance is on Ashdown's agar after 4 days incubation at 37^0C in air. Some isolates had more than one colony morphotype, and are denoted by the suffix (1, 2 and so on). In this section, isolates are termed 'strains' to denote the fact that some are from the same isogenic background.

4.2.1 Swim assay

Colony diameter of *B. pseudomallei* after incubation on swim agar for 24h, 48h and 72 h at 37^0C in air is shown in Table 8. The mean colony diameter for the 10 strains was 18.3, 37.2 and 46.6 mm at each of the three time points, respectively (Figure 18). The colony appearance on swim agar is shown in figure 19. These results indicate the presence of swim motility for *B. pseudomallei.*

Table 8: Colony size of 10 strains of *B. pseudomallei* after incubation on swim agar for 24, 48 and 72 h at 37^0C in air.

B. pseudomallei isolates	Incubated 24 hrs.			Incubated 48 hrs.			Incubated 72 hrs.		
	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3
2956a	18	19	19	38	41	44	52	55	58
2957a.1	13	14	15	30	31	31	41	40	42
2957a.2	15	15	16	31	34	34	38	45	45
2976b.1	18	18	21	35	38	44	45	45	56
2976b.2	21	22	28	37	39	42	43	47	47
2976b.3	15	17	20	33	37	40	42	46	48
2976b.4	16	21	22	36	37	43	43	42	55
2976b.5	13	22	23	28	42	45	34	45	53
3113a	14	17	17	33	34	36	43	48	45
2957a	18	21	21	42	43	38	53	54	47

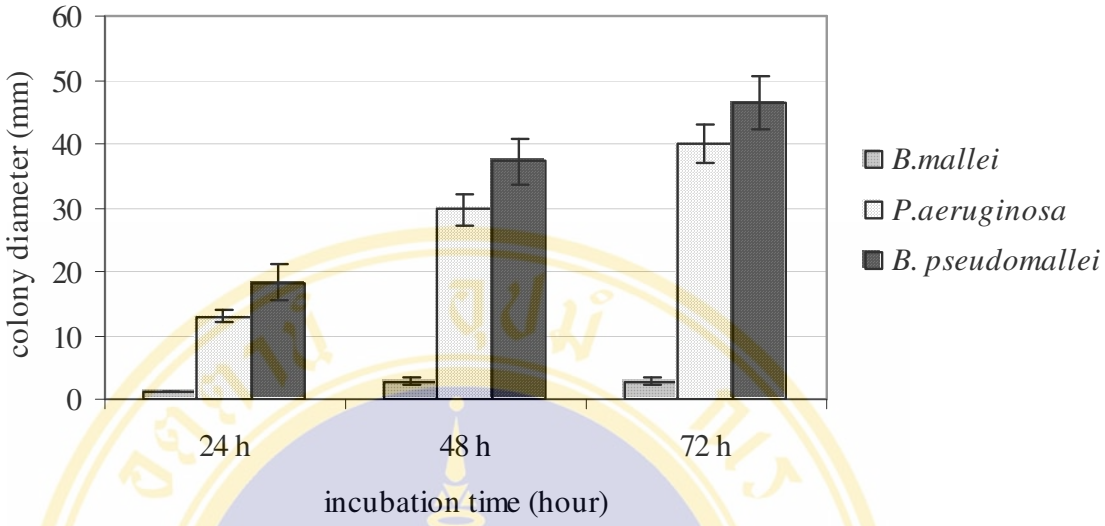


Figure 18: Colony diameter on swim agar for 10 *B. pseudomallei* strains after incubation for 24, 48 and 72 h at 37^0C in air. Results shown are the mean and standard deviation for the 10 strains performed in triplicate.

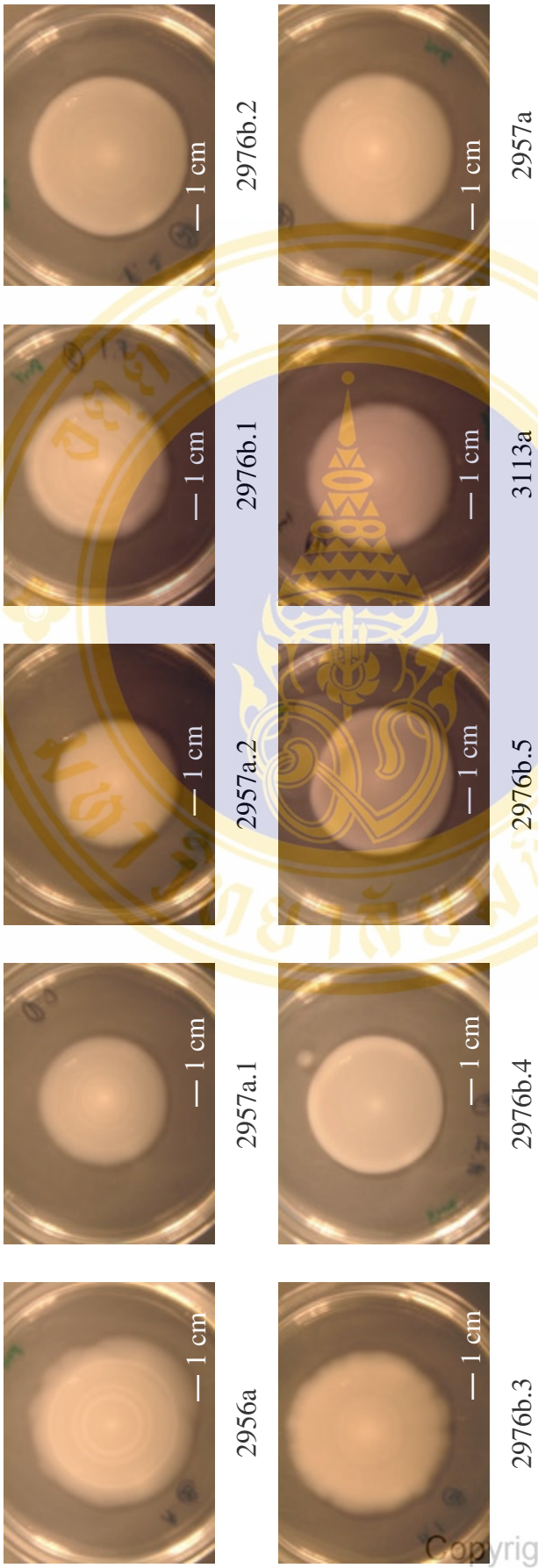


Figure 19: Colony appearances of 10 *B. pseudomallei* strains after incubation on swim agar for 24, 48 and 72 h at 37^0C in air.

4.2.2 Swarm assay

Colony diameter of *B. pseudomallei* after incubation on swarm agar for 24, 48 and 72 h at 37^0C in air is shown in Table 9. The colony appearance is shown in figure 21. The mean colony diameter for the 10 strains was 5.8, 16.8 and 34.5 mm at each of the three time points, respectively (Figure 20).

Table 9: Colony size of 10 strains of *B. pseudomallei* after incubation on swarm agar for 24, 48 and 72 h at 37^0C in air.

B.pseudomallei isolates	Incubated 24 hrs.			Incubated 48 hrs.			Incubated 72 hrs.		
	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3	No. 1	No. 2	No. 3
2956a	3	6	7	17	24	27	39	40	41
2957a.1	2	2	3	5	5	7	8	10	13
2957a.2	12	14	16	26	30	29	38	33	43
2976b.1	6	6	6	14	16	17	24	36	28
2976b.2	3	4	4	13	14	16	23	29	19
2976b.3	5	7	7	16	18	18	30	33	34
2976b.4	2	2	3	9	14	13	29	29	22
2976b.5	3	3	5	16	18	23	28	34	32
3113a	8	8	9	16	18	15	80	61	61
2957a	7	8	8	13	19	20	33	55	50

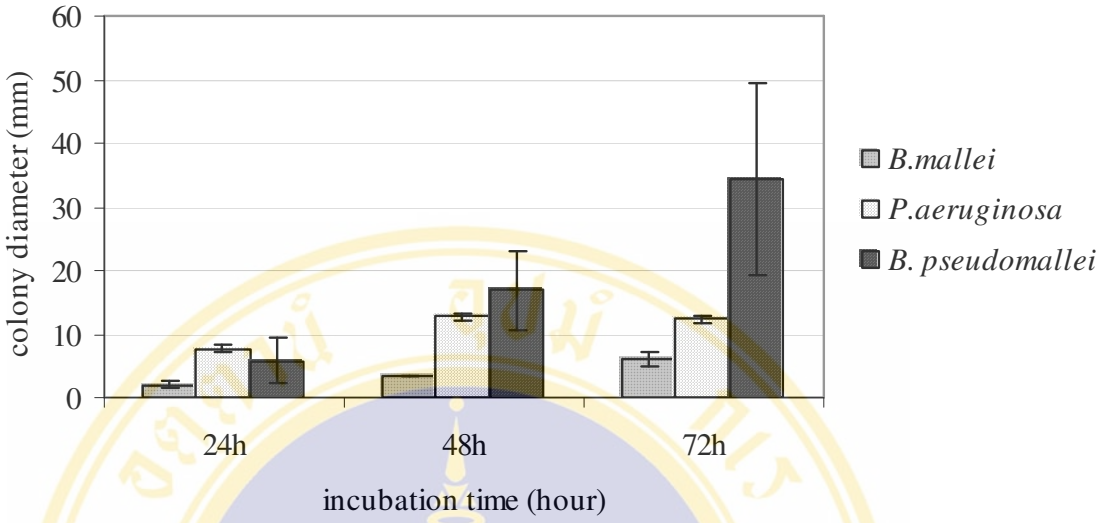


Figure 20: Colony diameter on swarm medium for 10 *B. pseudomallei* strains after incubation for 24, 48 and 72 h at 37^0C in air. Results shown are the mean and standard deviation for the 10 strains performed in triplicate.

These results indicate the presence of swarm motility for *B. pseudomallei*. The large error bars in Figure 20 for colony diameter after 72 h incubation suggests either intra- or inter-strain variability. This was explored further, as described in section 4.3.3, below.

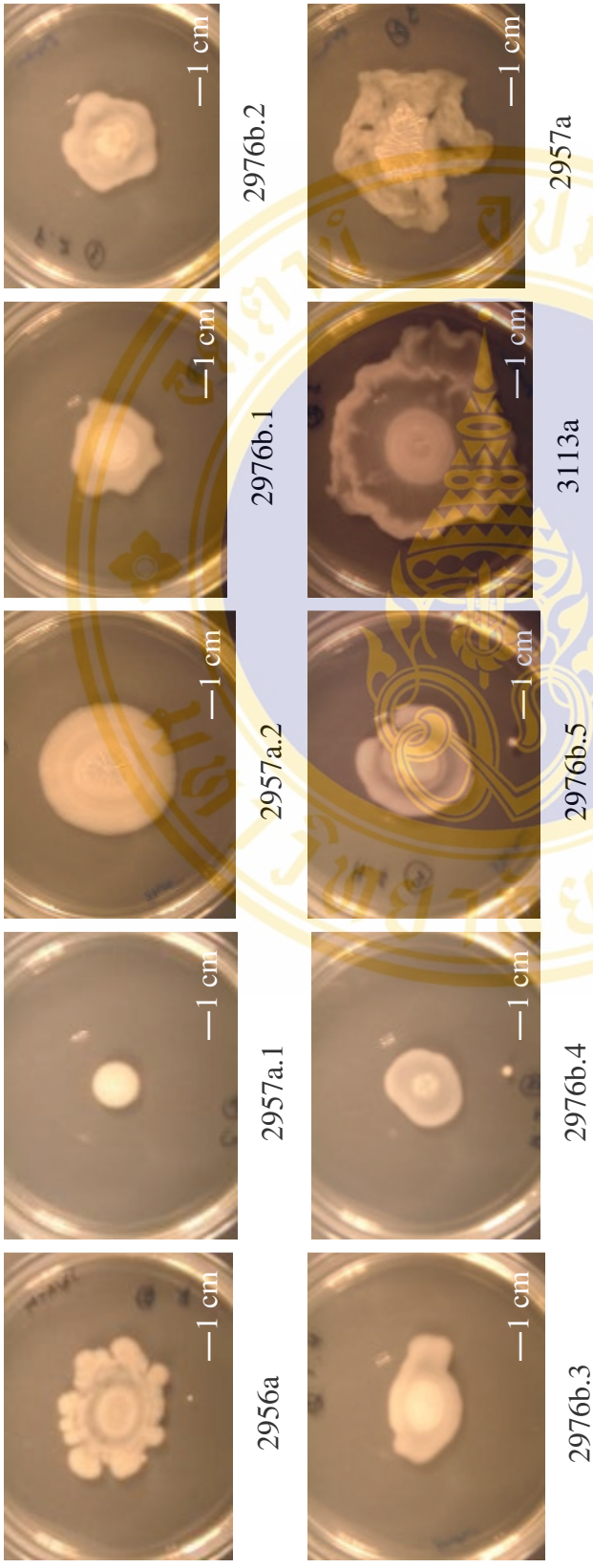


Figure 21: Colony appearance of 10 *B. pseudomallei* strains after incubation on swarm agar for 72 h at 37^0C in air. Colony morphotype showed irregular branching which began after 48 h.

4.2.3 Twitching motility assay

The appearance of the stained petri dish of twitching motility assays of 10 *B. pseudomallei* strains after incubation at 37^0C in air for 24, 48 and 72 h is shown in figure 22. There was no stained zone visible. These results indicate the absence of twitching motility as defined by this assay for *B. pseudomallei*.

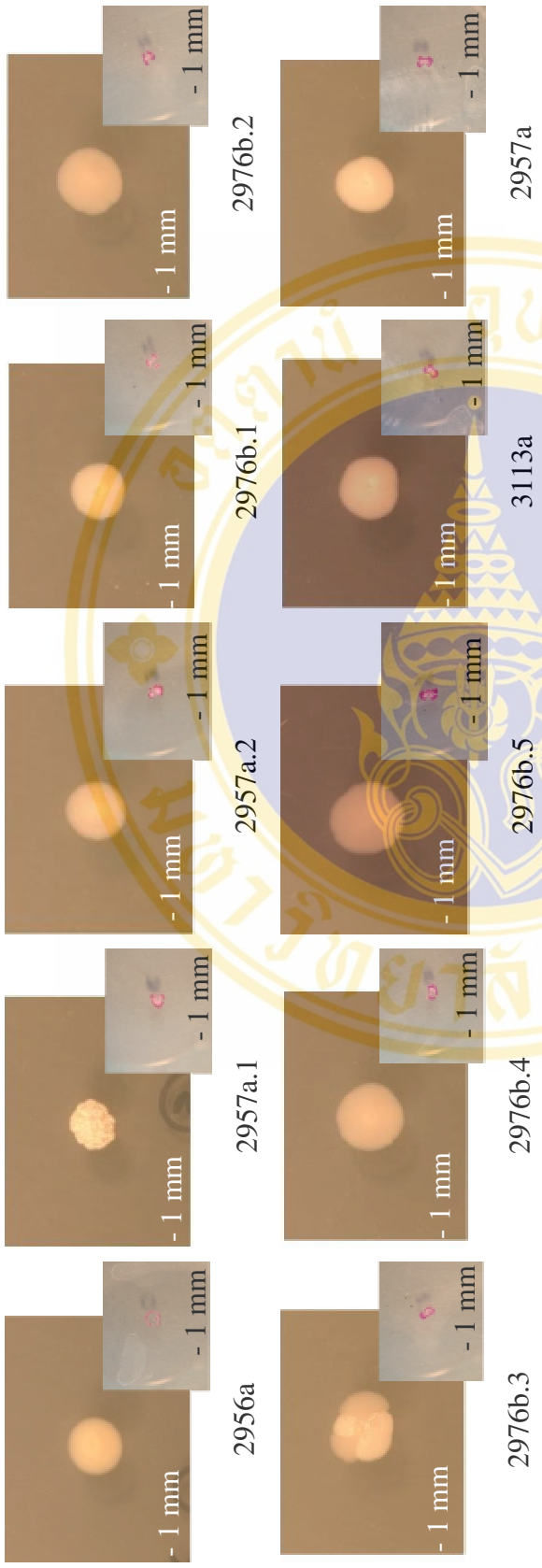


Figure 22: Twitch agar plates stained with crystal violet after incubation for 72 h at 37^0C in air.

4.3 Further defining the optimal incubation time for swim and swarm plates

In order to further characterize the experimental conditions for swimming and swarming, colony zone sizes were examined over a wider time course than that previously described in the literature. Later experiments were planned that aimed to look at both qualitative (presence or absence) and quantitative measurements of motility for a population of *B. pseudomallei* isolates. Demonstrating a difference in zone sizes between isolates depended on the colony being in exponential phase of growth, and not having reached maximal growth (that is, the edge of the agar plate).

4.3.1 Motility of *B. pseudomallei* on swim plates over a time course

Five isolates were selected at random from the clinical strain collection for use in pilot studies; these were: U2670, U2632, U2667, E0237 and U2762. Swim plates were inoculated in triplicate as described in section 3.6.1 above, and examined daily for 8 days during incubation at 37^0C in air. Figure 23 shows the colony diameters for each plate over time.

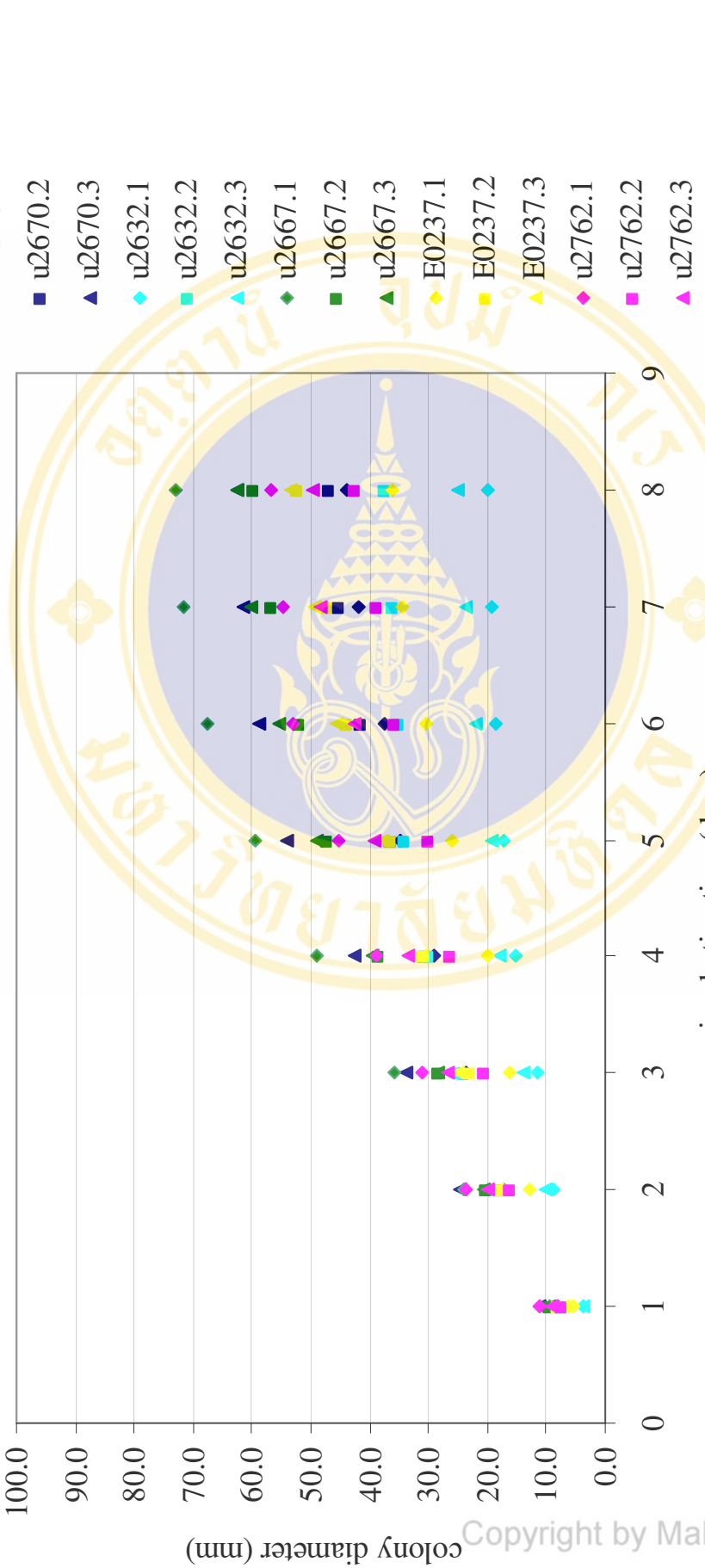


Figure 23: Colony diameter of 5 *B. pseudomallei* isolates tested in triplicate during growth over 8 days on swim agar incubated at 37^0C in air.

These results indicate a linear increase in colony diameter from days 2 to day 4 for most isolates, with a leveling off in colony growth thereafter. The exception was strain U2632, which had the smallest colony diameter. There was increasing divergence in swim colony results between plates from day 2 onwards. It was concluded that day 3 or 4 represented both practical and scientifically sound days for this assay.

4.3.2 Motility of *B. pseudomallei* on swarm plates over a time course

The same five isolates as used above (U2670, U2632, U2667, E0237 and U2762) were examined for swarm motility over time. Swarm plates were inoculated in triplicate as described in section 3.6.2 above, and examined daily for 8 days during incubation at 37^0C in air. Figure 24 shows the colony diameters for each plate over time.

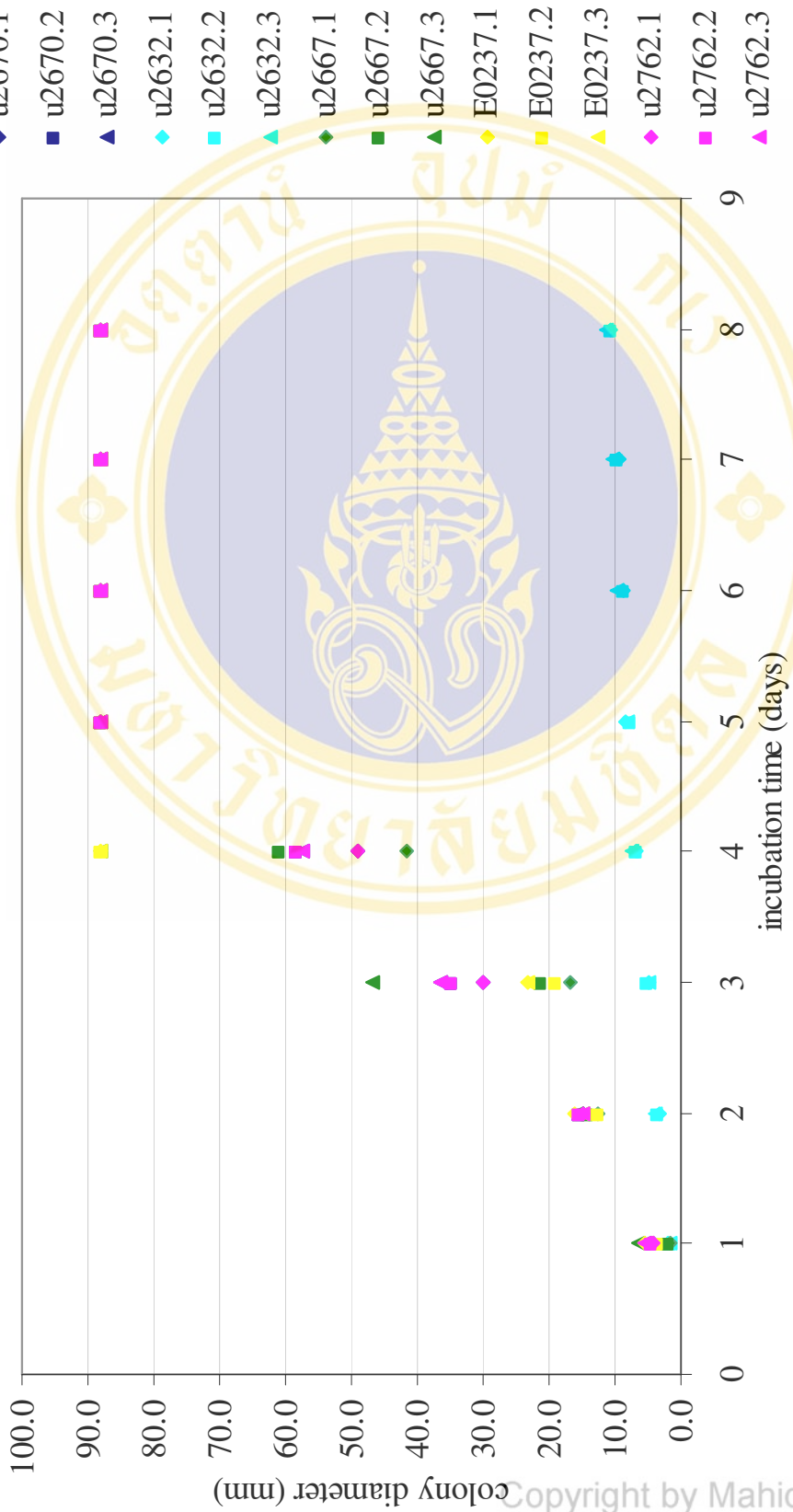


Figure 24: Colony diameter of 5 *B. pseudomallei* isolates tested in triplicate during growth over 8 days on swarm agar incubated at 37^{0}C in air.

These results indicate an initial exponential increase in colony diameter, with cessation of colony expansion from day 5 that was caused by the colony reaching the edge of the agar plate. The exception was strain U2632, which was considered negative for swarm motility. Most variation between isolates was observed after incubation for 3 or 4 days. It was concluded that day 3 or 4 represented both practical and scientifically sound days for this assay.

4.3.3 Experimental reproducibility of swarming motility

During the pilot study and the assessment of colony growth over a time course above, it was noted that there appeared to be considerable variability in colony size between triplicate plates for the same isolate. This was assessed further by selecting the isolate with the most rapid in vitro swarm motility (E0237), plating this onto 10 separate plates that were incubated at 37^0C in air and examined daily over a six-day period. The results are shown in Figure 25.

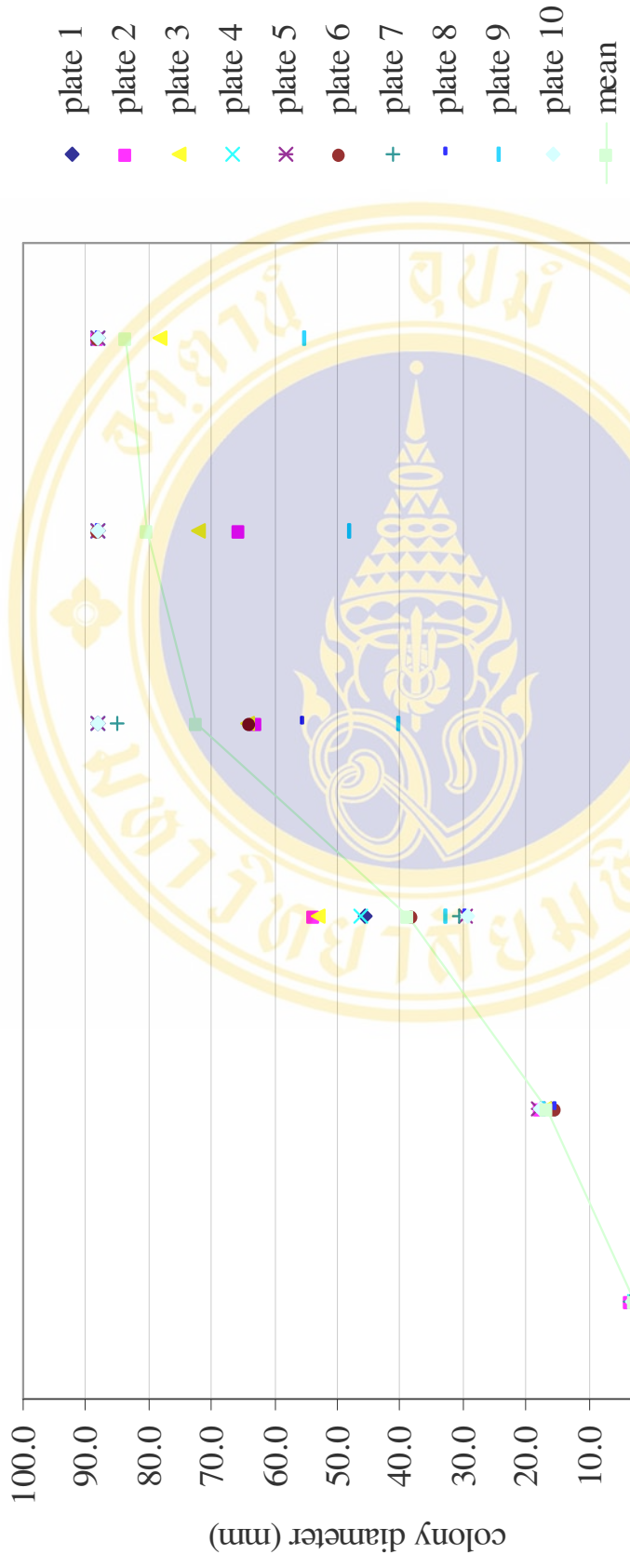
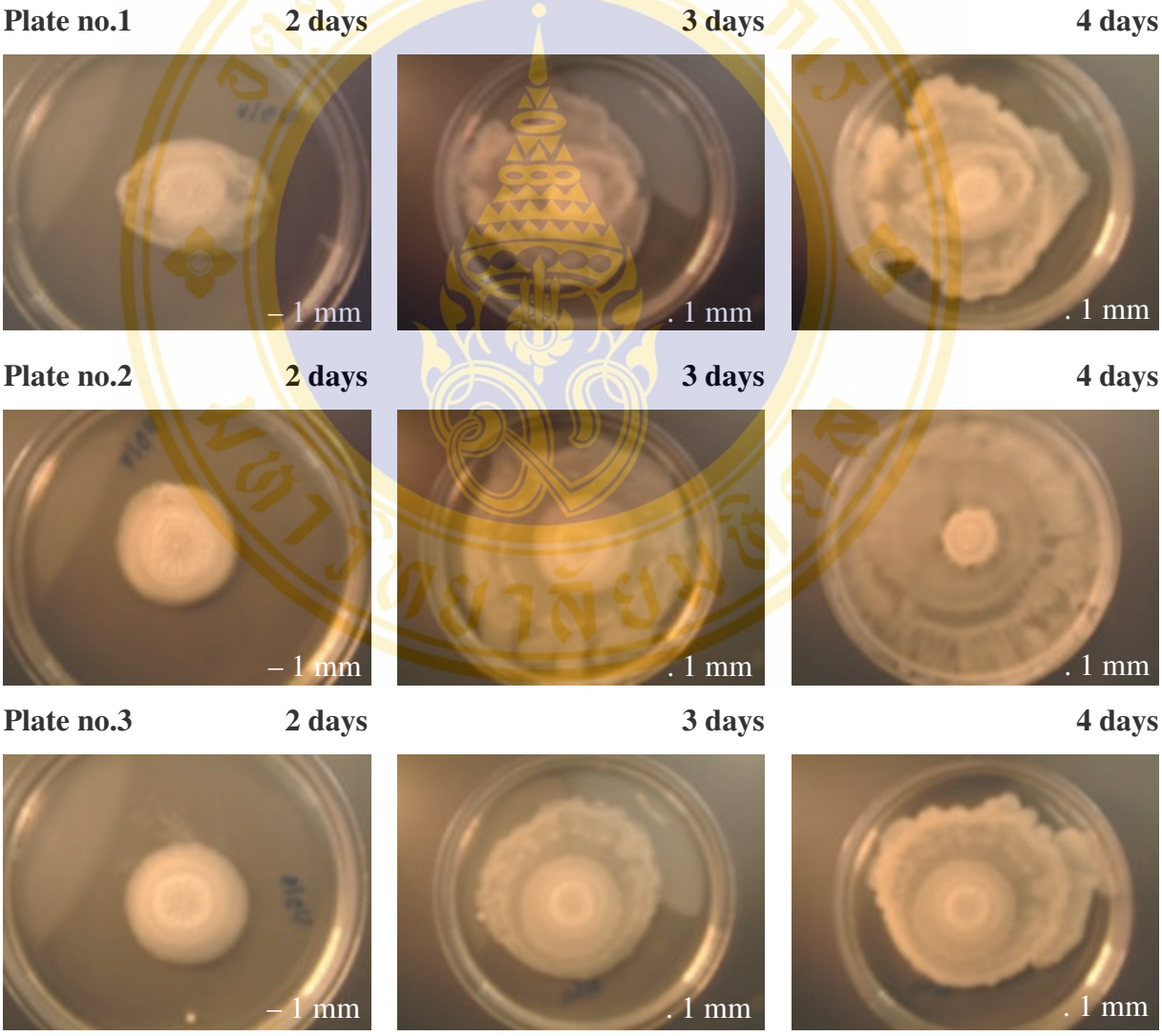


Figure 25: Colony diameter on swarm plates of 10 replicates of *B. pseudomallei* E0237 during incubation for 6 days at 37^0C in air.

Variability in swarm colony size for this single isolate become apparent by day 3, and continued during days 4 to 6, being most marked on day 4. Swarm colony appearance was carefully examined to determine the reason for this variability. This is shown in figure 26.



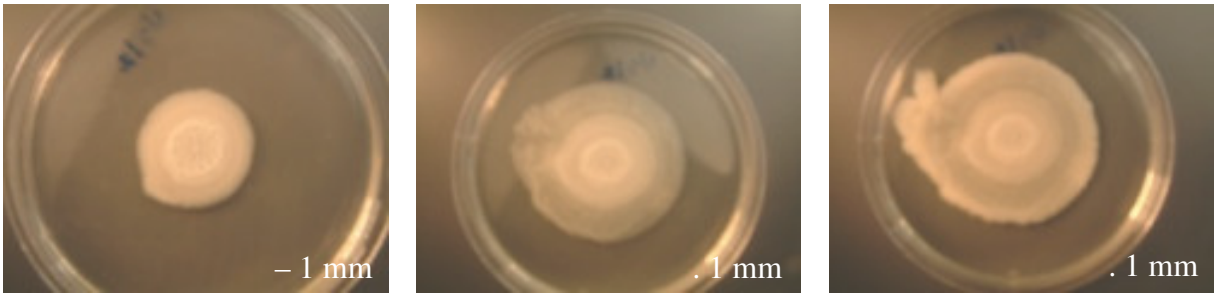
continue

Plate no.4 **2 days** **3 days** **4 days**

Plate no.5 **2 days** **3 days** **4 days**

Plate no.6 **2 days** **3 days** **4 days**

Plate no.7 **2 days** **3 days** **4 days**



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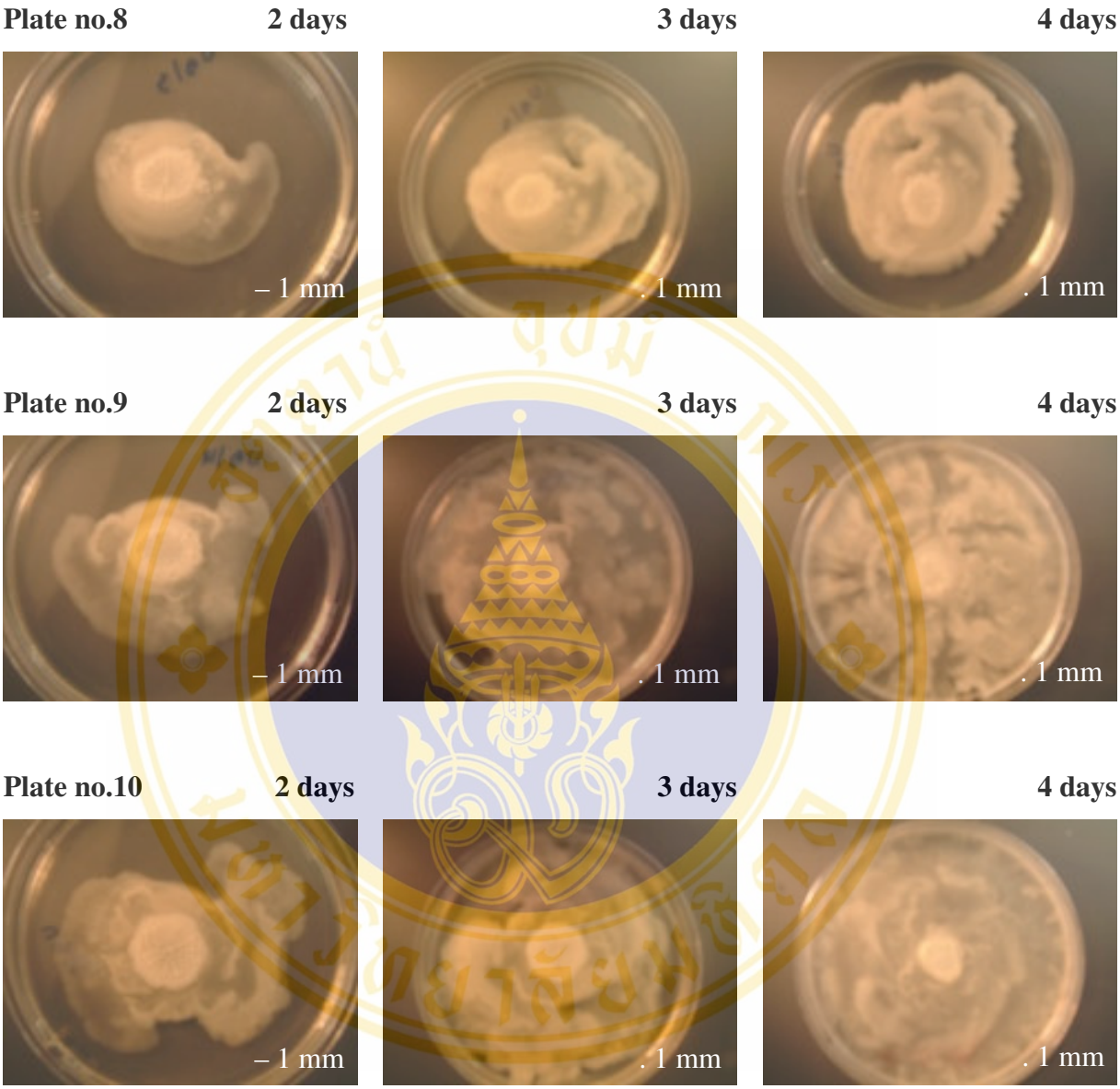


Figure 26: Colony appearance on swarm agar plates of 10 replicates of *B. pseudomallei* E0237.

The variability in colony size appeared to be related to several observations. Some colonies were slower to undergo a transition from a tight colony to a more amorphous swarming colony (for example, as seen on comparison of plate 7 and 10 at 72 hours). It was not uncommon for swarming to initially occur in an irregular fashion

from the central colony (see plates 6, 9 and 10 at 48 hours). After incubated for 4 days, 5 plates were completely swarming over the agar.

4.4 Motility in population of *B.pseudomallei*

4.4.1 Swim motility of a population of *B. pseudomallei*

A population of 266 clinical *B. pseudomallei* isolates was examined for swim motility *in vitro*. The isolates studied were as described in methods section 3.5.1.2. The primary aim was to determine whether swimming motility was present or absent. This was performed using a 'primary screen' in which isolates were tested once. Isolates that appeared to lack the ability to swim *in vitro* were then further evaluated to determine reproducibility of this result. An estimate of rate of motility of the population was determined using the single measurement.

Swim plates were prepared as before and examined after incubation at 37^0C in air for 72 and 96 h. Qualitative ability to swim was decided on the basis of a cut-off below which an isolate was defined as negative. This was determined by performing swim experiments for a laboratory strain of *B. pseudomallei* strain MM35 that was genetically manipulated so as to be defective in flagella expression (a gift from Professor Don Woods, University of Calgary). The colony diameter of this strain was examined over a time course for 30 separate swim plates. The mean was calculated together with the standard deviation. The results after 72 h and 96 h incubation were 8.5 mm $\pm$ 1.3 mm and 10.6 mm $\pm$ 1.6 mm respectively, giving a range from 7.2 to 9.8 mm at 72 h, and 9.0 to 12.2 mm at 96 h. A clinical isolate was defined as being negative for swim motility on the primary screen if the colony diameter fell within or below this range at a specified time point. Such isolates were then re-tested on triplicate plates in three independent experiments. A range was also determined for the parental strain *B. pseudomallei* strain 1026b; the mean (SD) diameter at 72 and 96 h incubation time was 70 mm $\pm$ 3.9 mm and 77.7 mm $\pm$ 2.0 mm, giving a range from 66.1 to 73.9 at 72 h, and 75.7 to 79.7 mm at 96 h.

The colony diameter of a single isolate (strain 3020a) failed to increase in size over time of incubation; (colony diameter 9 mm at 48 h, 11 mm at 72 h and 12 mm at 96 h), and fell within the parameters set for 'no swim'. Swim motility was re-tested in

triplicate; the mean of 9 measurements for each time point was: 49.4 mm at 48 h, 70 mm at 72 h, and 77.7 mm at 96 h. This shows that strain 3020a was able to swim *in vitro* on repeated testing. Thus, all isolates in this population demonstrated swim motility.

4.4.2 Swarm motility for a population of *B. pseudomallei*

A population of 266 clinical *B. pseudomallei* isolates was examined for swarm motility *in vitro*. The isolates studied were as described in methods section 3.5.1.2. The primary aim was to determine whether swarming motility was present or absent. Each isolate was initially tested on one occasion ('the primary screen'); those that were negative were then repeated to determine reproducibility. An estimate of rate of motility of the population was determined using the single measurement.

Plates were examined after incubation at 37^0C in air for 72 and 96 h. A cut-off was required in order to determine the inability to swarm. This was determined by performing swarm experiments for a laboratory strain of *B. pseudomallei* that was genetically manipulated so as to be defective in flagella expression (strain MM35). The colony diameter of this strain was examined over a time course for 30 separate swarm plates. The mean was calculated together with the standard deviation. The result at 72 h and 96 h was 22.2 mm $\pm$ 1.2 mm and 28 mm $\pm$ 2 mm, giving a range from 21 to 23.4 mm and 26 to 30 mm. A clinical isolate was defined as being potentially negative for swarm motility on the primary screen if the colony diameter fell within this range. Such isolates were then re-tested in three independent experiments in which three plates were used per experiment. *B. pseudomallei* strain 1026b was also tested; the mean diameter at 72 h and 96 h was 38.2 mm $\pm$ 20.8 mm and 73.4 mm $\pm$ 10.4 mm, giving a range from 17.4 to 59 mm and 63 to 83.8 mm, respectively.

The frequency distribution of the colony diameters for the population at 72 h and 96 h is shown in Figure 27. At 72 h the colony diameter ranged from 11 mm to 88 mm (mean and 2SD 53.1 $\pm$28.8 mm). At 96 h the colony diameter ranged from 12 mm to 88 mm (mean and 2 SD 68.7 $\pm$23.2 mm). There was a biphasic result at 72 hours, with 78 strains having reached 88 mm and the remainder (n=36) forming a second smaller peak around 20 mm. In view of this finding, swarm was examined at 48 hours,

the results of which are shown in Figure 28. This demonstrated that the majority of isolates were centered around the 15-20 mm point, with a long right hand tail of isolates that had higher swarming ability.

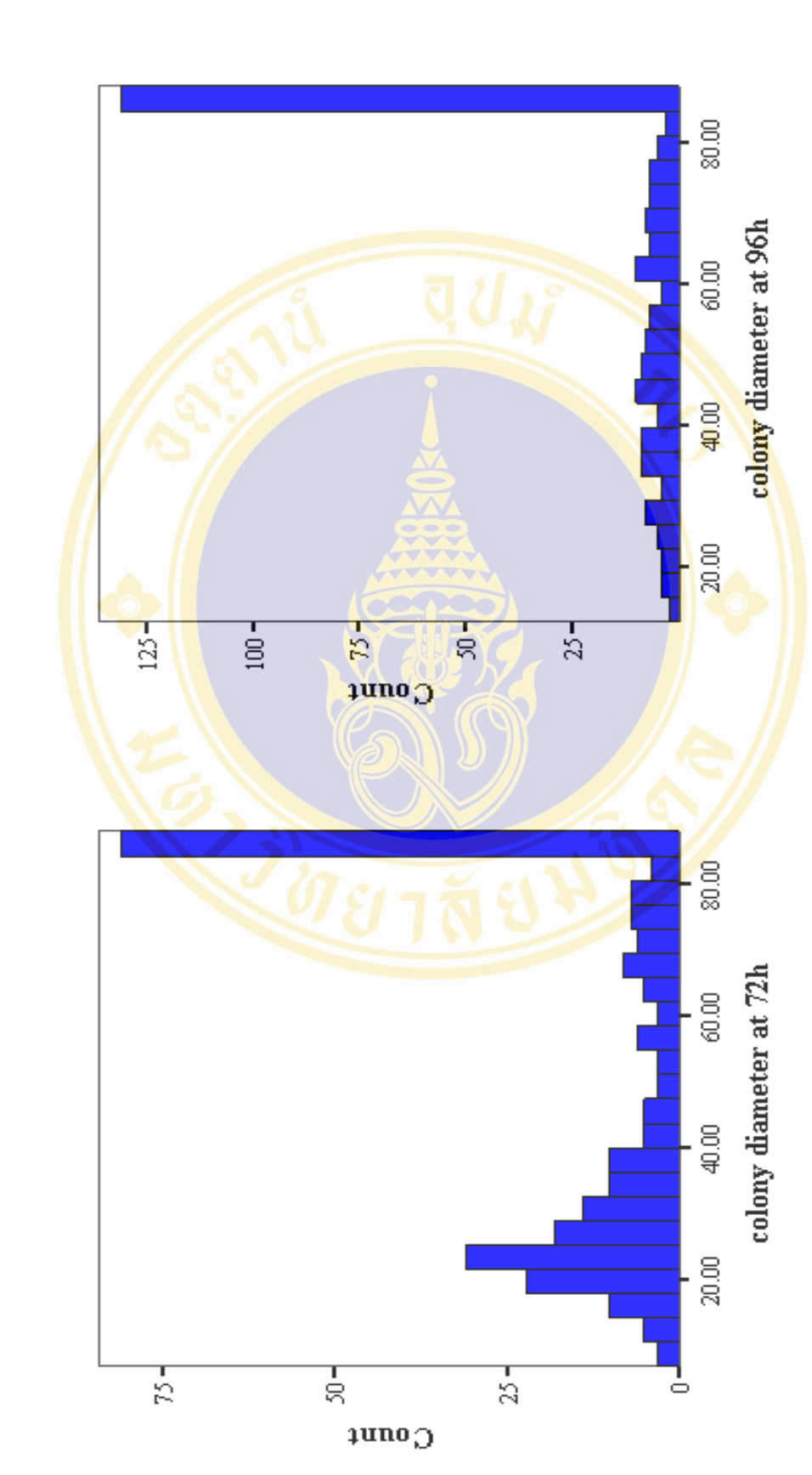


Figure 27: Histogram of colony diameter of 266 isolates of *B. pseudomallei* on swarm plates after incubation at 37^0C in air for 72 and 96 hours.

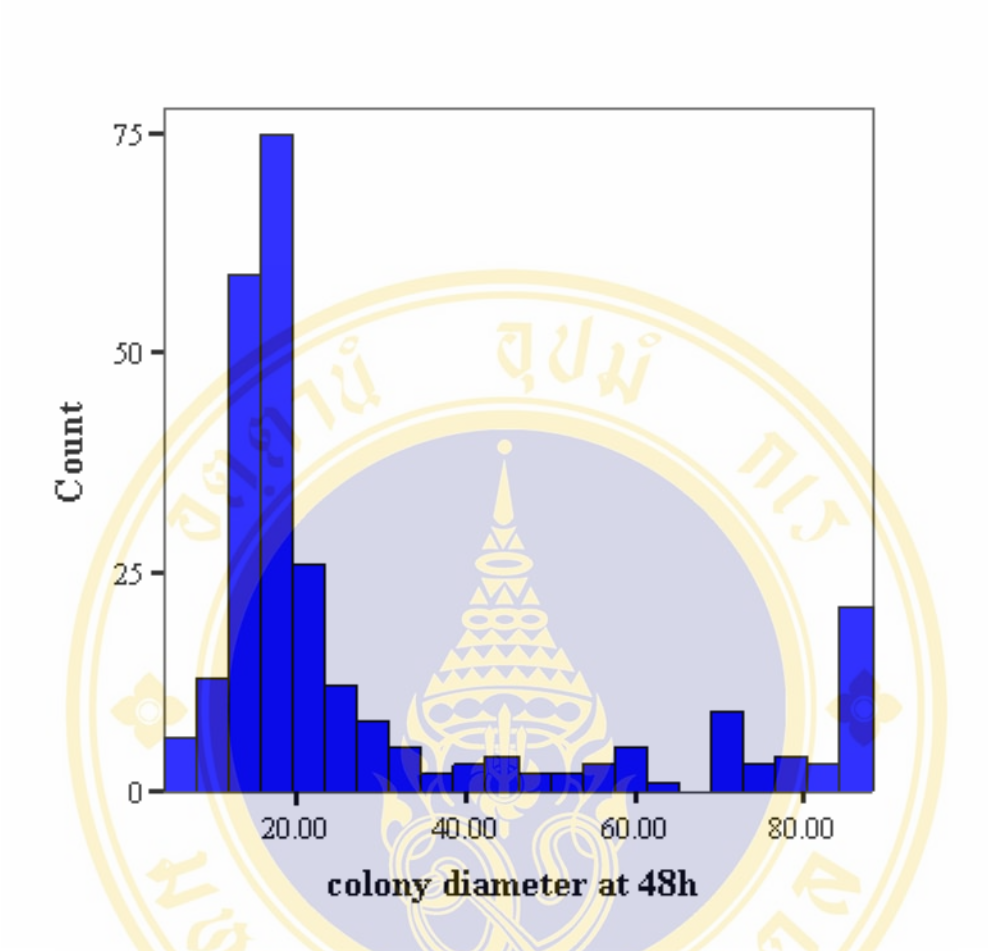


Figure 28: Histogram of colony diameter of 266 isolates of *B. pseudomallei* on swarm plates after incubation at 37^0C in air for 48 hours.

A total of 25 isolates were initially defined in the primary screen as being possibly unable to swarm. These are shown in table 10, together with colony diameter over a time course.

Table 10: Colony diameter (mm) of isolates defined as putative non-swarmers during primary screen of bacterial population over a time course.

Strain	48 h	72 h	96 h
U3062	10	11	12
U3119a	12	13	14
U3164b	12	14	16
U3090	8	15	17
U3119b	15	16	17
U3139a	7	11	17
U3083	16	18	20
U3109	16	17	21
U3082b	18	19	21
U3132	16	19	21
U3034	19	19	24
U3069a	20	22	24
U3179a	19	21	24
U3157	21	22	25
U3239	11	12	25
U3069b	18	21	26
U3082a	22	22	26
U3164a	21	23	26
U3224	18	19	27
U3205a	24	24	28
U3172	20	22	28
U3168	20	22	29
OH	27	28	29
U3202	20	20	30
U3076	23	28	30

These isolates were re-tested in triplicate; 20 of 25 isolates demonstrated swarming (data not shown) and five isolates were persistent non-swarmers (shown in Table 11).

Table 11: Colony diameter (mm) range on swarm agar of persistent non-swarmers

Strain	48 h	72 h	96 h
U3090	7.6 to 9	10.5 to 12	13.5 to 15
U3119a	12 to 15	15 to 18.4	18 to21
U3119b	15.4 to 17	19 to 22	24 to 27
OH	20 to 21	22 to 24	25 to 26
U3179a	15 to 16	19 to 24	27 to 31

4.4.3 The colony dimension of single strain

The frequency distribution of the colony diameters for the population at 72 h and 96 h is shown Figure 29. At 72 h the colony diameter ranged from 11 mm to 88 mm (mean and 2SD 52.3 $\pm$11.7 mm). At 96 h the colony diameter ranged from 12.4 mm to 88 mm (mean and 2 SD 60.7 $\pm$12.6 mm). The results for 72 h appeared normally distributed, while those for 96 h show an overall shift to the right and a longer left tail, consistent with a proportion but not all isolates approaching the maximum zone size of 88 mm.

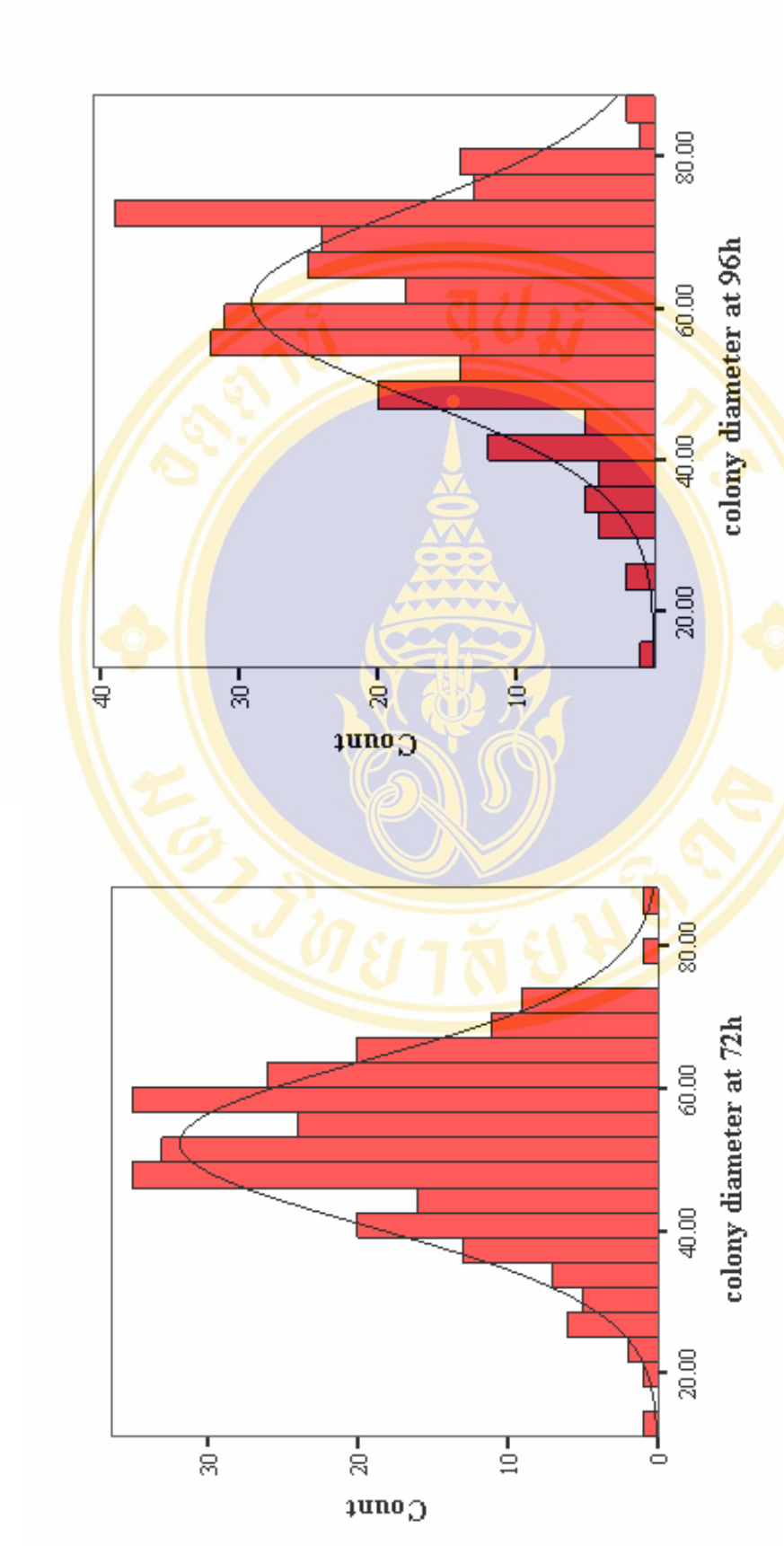


Figure 29: Histogram of colony diameter for 266 isolates of *B. pseudomallei* on swim plates after incubation at 37^{0}C in air for 72 and 96 hours.

The mean rate of colony expansion obtained in the primary screen was calculated for the bacterial population at each time point. This is shown in Figure 30a. The highest rate was found at 48 h, with the rate falling over time. It is possible that the fall off in expansion is related to the population approaching maximum colony diameter size. The analysis was repeated for each time point after removing isolates that had reached 88 mm for a given time point (maximum colony diameter). The rate of colony expansion was recalculated, as shown in Figure 30b. The reduction in colony expansion over time remained, indicating the presence of alternative factors, the most obvious of which is limitation of nutrients.

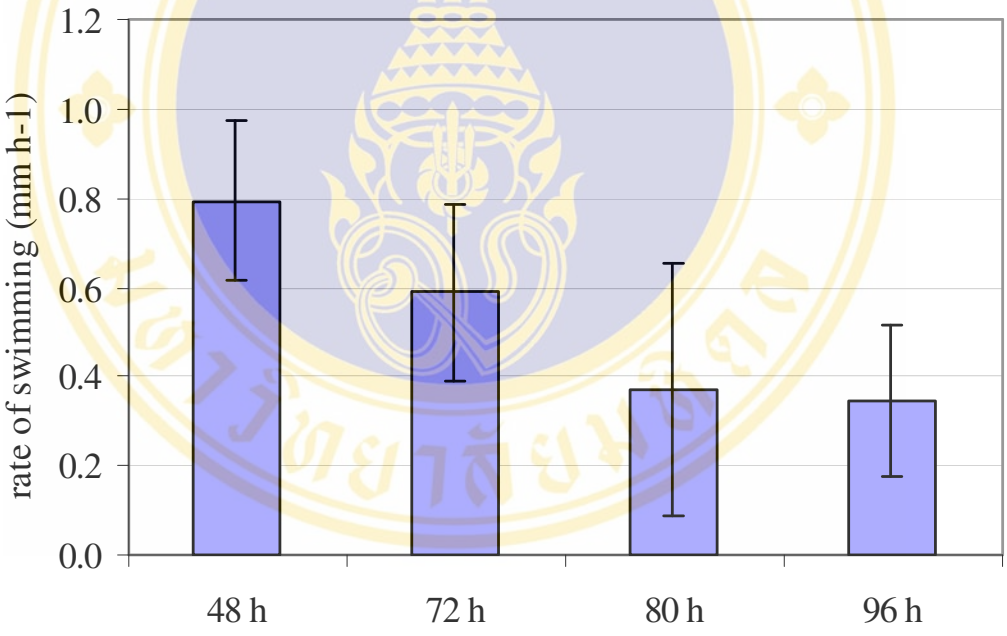


Figure 30a: Mean rate of colony expansion (mm per hour) for the bacterial population at each incubation time point. The error bar represents 2 standard deviations.

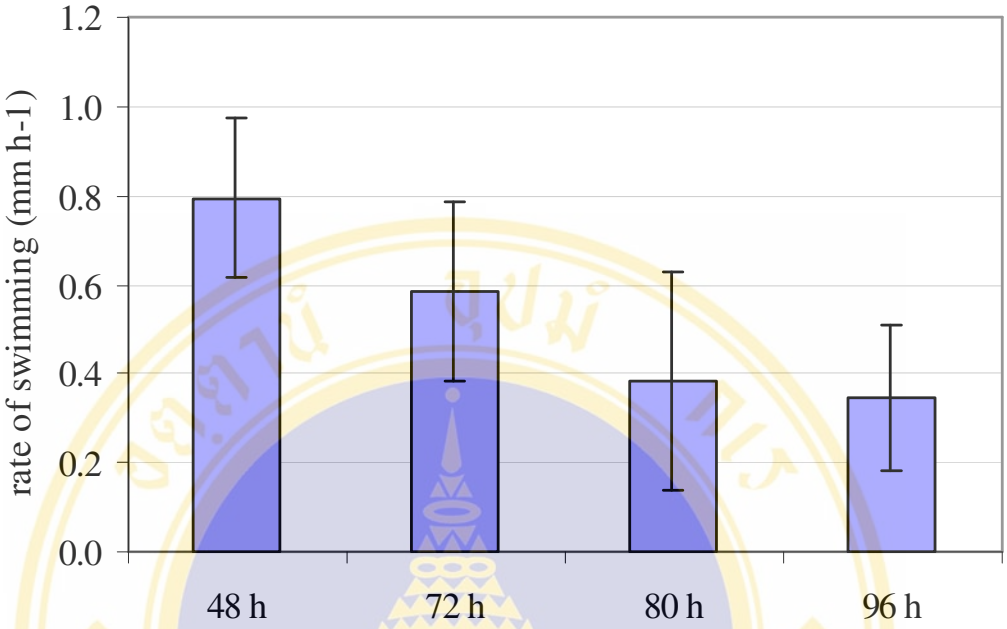


Figure 30b: Mean rate of colony expansion (mm per hour) after censoring isolates that had reached 88 mm. The error bar represents 2 standard deviations.

4.5 Colony appearance of *B. pseudomallei* on swarm plates

4.5.1 Colony appearance of *B. pseudomallei* 1026b and MM35, defective in flagella expression, on swarm plate

B. pseudomallei strain MM35 is a mutant of strain 1026b that is defective in flagella expression. Swarm assays were performed for wild type and mutant over a time course using 30 plates for each strain. The colony appearance of wild type and mutant are shown below (figure 31).

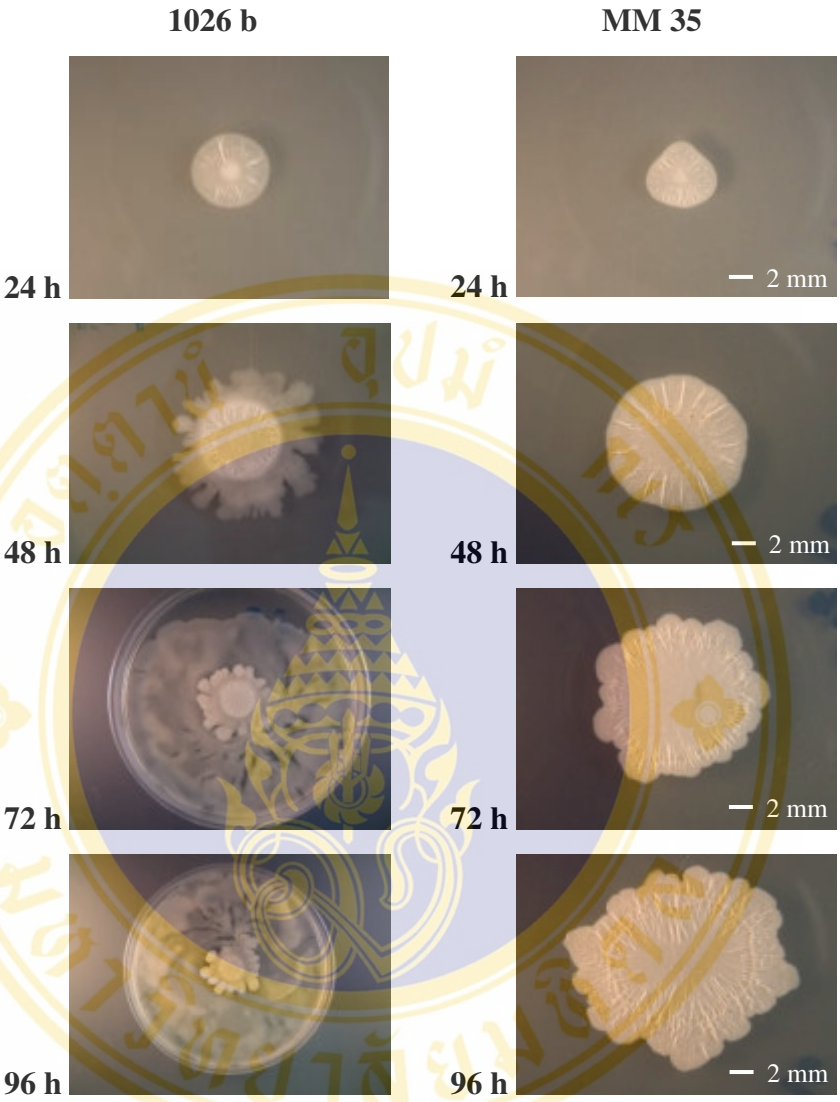


Figure 31: Colony appearance of *B. pseudoamllei* strain MM35, defective in flagella expression, and parental strain 1026b cultured at 37^0C in air for 24, 48, 72 and 96 h.

4.5.2 Colony appearance of clinical isolate *B. pseudomallei* on swarm agar

On observation of *B. pseudomallei* colonies growing on swarm plates, it was clear that colony morphology demonstrated marked variability between strains. It was hypothesized that colony morphology was related to quantitative ability to swarm. This was tested on the first 60 unselected isolates, as follows.

Three characteristics that were known from pilot studies to vary were edge of colony, surface texture of centre of colony, and transparency of colony. The regions of the colony to which these refer are shown in Figure 32.

A = surface texture of centre of colony
B = transparency of colony
C = edge of colony

Figure 32: Three areas of *B. pseudomallei* swarm colonies after incubation at 37^0C in air for 72 h

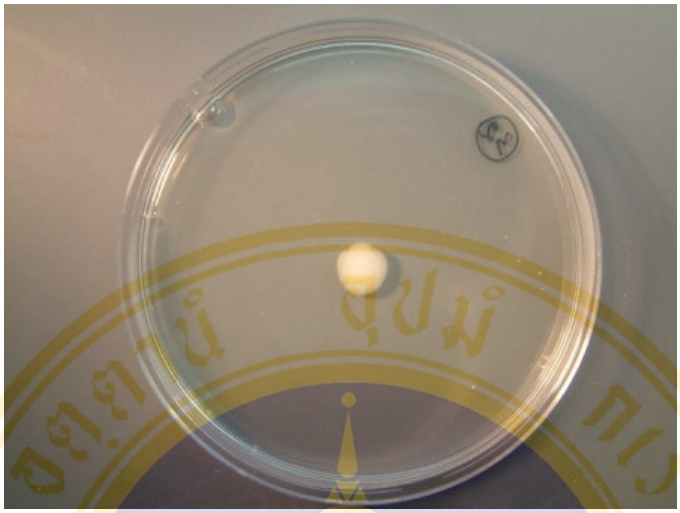
These features were defined for colonies on swarm agar after incubation at 37^0C in air for 72 h, and the combinations designated as Type A to E as shown in Table 12. Figure 36 shows the appearances of each Type. A single plate was prepared was prepared for each strain.

Table 12: Typing scheme for characterization of *B. pseudomallei* colony morphology after incubation on swarm agar at 37^0C in air for 72 h.

Type	Surface texture of centre of colony (A)	Surface colony surround the centre (B)	Edge of colony (C)
A	Smooth	Opaque*	Regular
B	Rough	Opaque	Regular
C	Rough	Transparent**	Regular
D	Rough	Opaque	Irregular
E	Rough	Transparent	Irregular

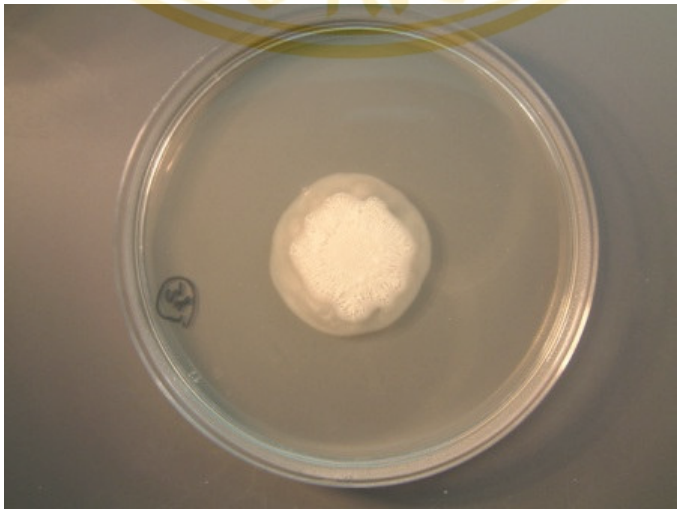
* The majority of the colony surrounding the centre is opaque

** The majority of the colony surrounding the centre is transparent



Type A

Type B



Type C

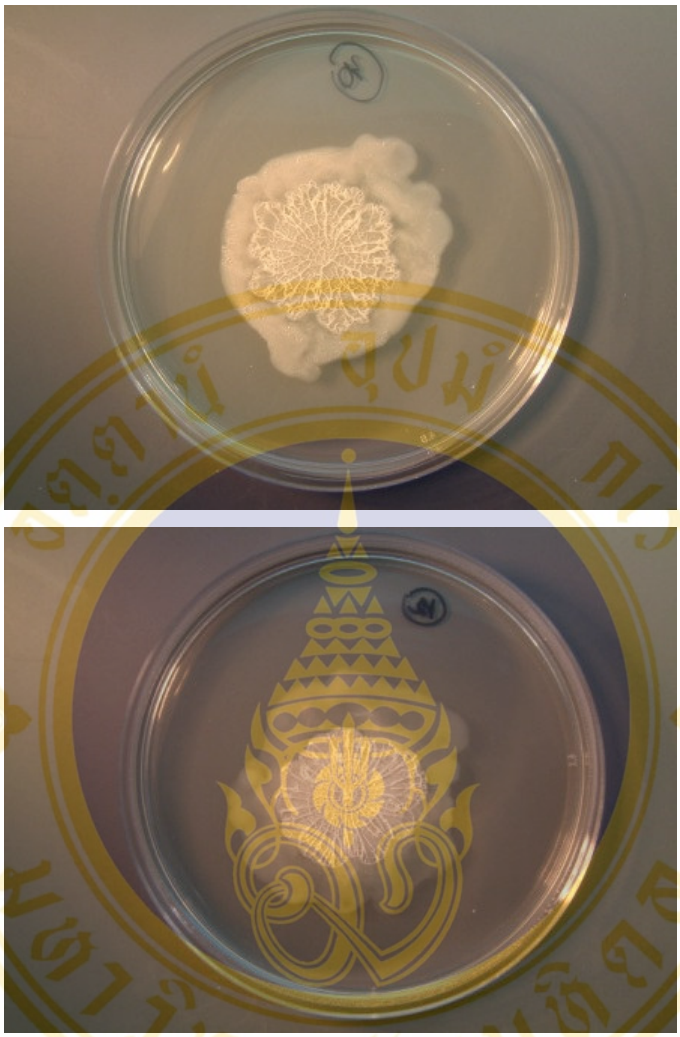


Figure 32: *B. pseudomallei* colony types (A to E) on swarm agar after incubation at 37^0C in air for 72 h

Table 13 gives results for the proportion of each colony type for the first 60 isolates, together with the mean colony diameter. Three isolates could not be typed since the colony spread across the agar plate and the edge of the colony could not be determined.

Table 13: Swarm colony type and diameter for 57 *B. pseudomallei* isolates

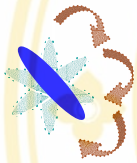
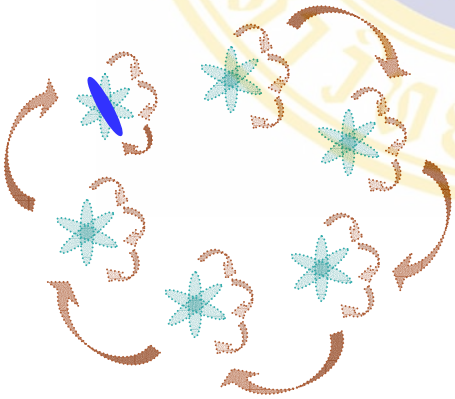
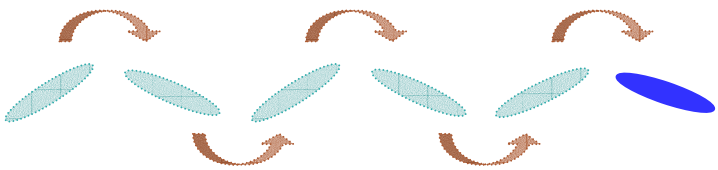
Type	Count	%	Mean colony diameter	Standard deviation	Range
A	1	1.8%	9.5	-	-
B	9	15.8%	34.5	4.9	24.5 – 40.7
C	2	3.5%	34.7	5.5	30.8 – 38.6
D	16	28.1%	46.4	18.7	28.5 – 88
E	29	50.9%	49.0	18.5	26.6 – 88

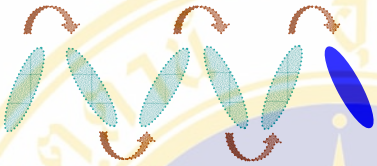
4.6 Motility of *B. pseudomallei* on Real Time Microscopy

4.6.1 Development of terminology to describe bacterial movement under RTM-3

Terminology to describe bacterial movement was developed in a pilot study using 15 clinical isolates of *B. pseudomallei* that were chosen at random from the larger population (listed below). Five types of movement were discriminated using RTM-3 together with a category for movement.

Table 14: *B. pseudomallei* isolates used to in pilot study of motility and shows each type of movement as defined by serial still frames of RTM-3 images.

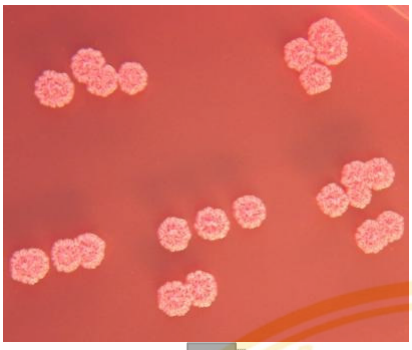
Type of movement: description	*B. pseudomallei* strains	Morphotype on Ashdown's agar
Vibration: The cell is moving (vibrating). There is no displacement. 	U3060 U3170 U3215	Type 4 Type 1 Type 1
Rotation: The cell is rotating around one pole (CW, CCW). There is no displacement 	U3100 U3148	Type 1 Type 1
Rotating displacement: The cell is rotating around one pole (CW, CCW). There is displacement, mostly in one direction. 	U3141 U3241	Type 4 Type 1
Waving: The cell is moving in an undulating pattern (zigzag). There is displacement mostly in one direction. 	U3109 U3162 U3240	Type 3 Type 1 Type 6

Type of movement: description	Bacterial isolate of *B. pseudomallei*	Morphotype on Ashdown's agar
Tumbling: The cell is alternating tumbling around its two poles. There is displacement mostly in one direction. 	U3102 U3155	Type 1 Type 1
Non movement: There is no movement at all. 	U3037 U3130 U3233	Type 1 Type 1 Type 1

4.7 Variability in colony size and flagella expression in bacterial colony on solid agar

4.7.1 Bacterial colony on swarm plates

To examine swarm motility on solid agar, one colony of *B. pseudomallei* was sub-cultured onto a fresh Ashdown's plate, incubated at 37^0C for four days. On the fourth day, a colony was picked using a sterile toothpick and inoculated onto a swarm plate (plate I). This was incubated at 37^0C in air for 72 hours. Bacteria from the colony edge and colony center of swarm plate I were sampled for motility and flagella expression, and were used to subculture two independent swarm plates (plate II center and plate II edge). These were again incubated at 37^0C in air for 72 hours. This process is summarized in Figure 33. The colony size (diameter) of all swarm plates was measured.

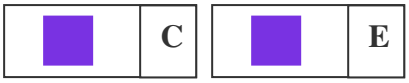


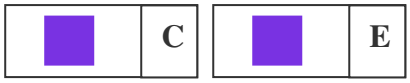
Single colonies on Ashdown's agar after 4 days at 37^0C in air. A small amount of bacterial cells from one colony was inoculated onto swarm plate I which was incubated for 72 hours at 37^0C in air.

Bacterial cells from the edge and center of the colony were used to determine motility and flagella expression, and were inoculated onto further swarm plates which was incubated for 72 hours at 37^0C in air II.

2' swarm plate from center cell

2' swarm plate from cell





Further slides were prepared using bacteria from the center and edge of each of the two secondary plates

Figure 33: Study protocol for assessment of flagella expression and motility of clinical isolates of *B. pseudomallei*

Table 15 gives the results of bacterial colony size (diameter) for 40 *B. pseudomallei* isolates after 72 hours incubation for swarm plates I, IIC and IIE. Inclusion of naturally variable morphotypes ensures that the widest range of bacterial phenotypes were included in the study. Summary statistics are shown in Table 16.

Table 15: Colony size on swarm plates

Strain number	Colonial Morphotype	Plate I (mm)	Plate IIC (mm)	Plate IIE (mm)
U3037	1	71	48	67
U3038	1	51	39	63
U3039	1	64	65	60
U3043	4	49	55	63
U3044	1	71	41	88
U3044	1	41	29	37
U3053	4	73	61	88
U3058	7	33	27	25
U3060	3	44	22	31
U3063	1	20	24	22
U3069	5	37	38	32
U3071	6	34	20	28
U3082	1	27	19	22
U3084	2	33	17	19
U3085	6	42	39	32
U3091	1	88	88	68
U3096	3	46	27	24
U3100	1	43	57	67
U3102	5	31	21	30
U3102	6	38	21	20
U3109	8	10	15	17

continue

Strain number	Colonial Morphotype	Plate I (mm)	Plate IIC (mm)	Plate IIE (mm)
U3130	1	81	37	47
U3139	1	19	30	31
U3139	1	61	75	88
U3141	4	10	43	72
U3141	3	44	75	88
U3148	1	30	20	80
U3155	1	88	88	88
U3162	1	47	32	64
U3164	2	17	12	13
U3169	1	30	41	88
U3170	1	50	48	88
U3204	5	25	38	24
U3215	1	59	67	88
U3233	1	36	40	77
U3239	5	74	34	55
U3240	5	63	53	50
U3241	1	88	88	88
U3241	1	80	74	88
U3252	1	23	40	34

Table 16: Summary statistics for swarm plate assays

Swarm plate	Mean diameter (mm)	Median diameter (mm)	95% CI*	IQR**
I	46.8	43.5	39.7-53.9	30.5-63.5
IIC	42.7	39.0	35.8-49.6	25.5-56.0
IIE	53.9	57.5	45.3-62.4	29.0-84.0

*CI denotes confidence interval; ** IQR denotes inter-quartile range

The data for swarm colony size were not normally distributed for all three groups. This is because the results for 2e were found to be bimodal. In view of this, a non-parametric test was used (Wilcoxon signed-rank test) to compare swarm I versus swarm IIC, swarm I versus IIE, and swarm IIC versus IIE. The results are shown below. These indicate that the colony size for the IIE group was significantly greater than that for IIC.

Comparison	P value*
Swarm I – Swarm IIC	0.12
Swarm I – Swarm IIE	0.19
Swarm IIC – Swarm IIE	0.0006

* Using Wilcoxon signed-rank test

The maximum possible size of swarm colony was 88mm. The data were re-analyzed for IIC versus IIE after removing results of 88mm, to determine whether colony size was larger in the IIE group for the remaining isolates. The p value for this was 0.0132. A paired t-test was also performed for IIE and IIC. This reflected the result of the non-parametric test results, with a p value of 0.0003 and a mean difference of 11.2 mm (95% CI 16.8-5.5). Colony size was relatively consistent for the three plates for any given strain. This indicates that swarming is a function of the gene complement and expression for a given strain.

4.7.2 Flagella expression on swarm plates

To examine further the swarm colony, and in particular to explore the reasons for the variation in swarm colony appearance, flagella expression was compared between bacteria from different parts of the colony after incubation for 72 h at 37^0C in air on swarm agar. A clinical isolate of *B. pseudomallei* (strain 2965a), was chosen at random from the population for this experiment. The colony sampled is shown below in Figure 34. The areas selected were:

1. Central colony
2. The mid point between the centre and the edge of the colony
3. The edge of the colony in an area where the colony was translucent
4. The edge of the colony in an area where the colony was opaque.

Presence and number of flagella per cell was counted as described in section 3.8. Each sample area was examined in triplicate. Each specimen was measured by counting 100 bacterial cells chosen at random.

Figure 34: Swarm colony appearance and the areas of the colony examined

The number of bacteria that expressed one or more flagella is shown in Figure 35 (where it is expressed as a proportion). The centre of swarm colony had the lowest proportion of positive cells. This rose across the colony from center to edge. The number of flagella present on flagella-positive bacterial cells was enumerated for each sampling site (Figure 36). The number of bacteria with 2 flagella rose between samples from center to colony edge.

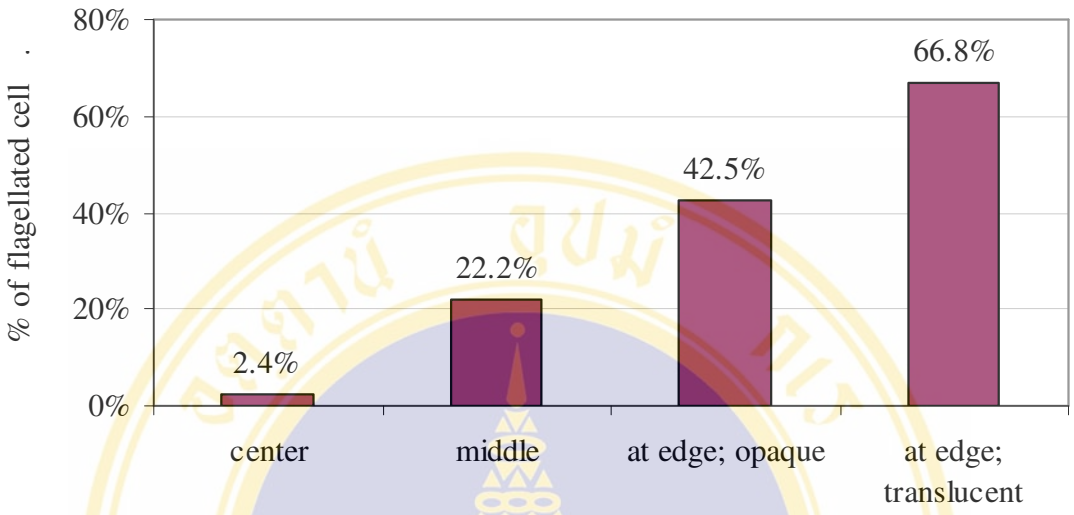


Figure 35: % of flagellated cell in difference positions within a swarm colony

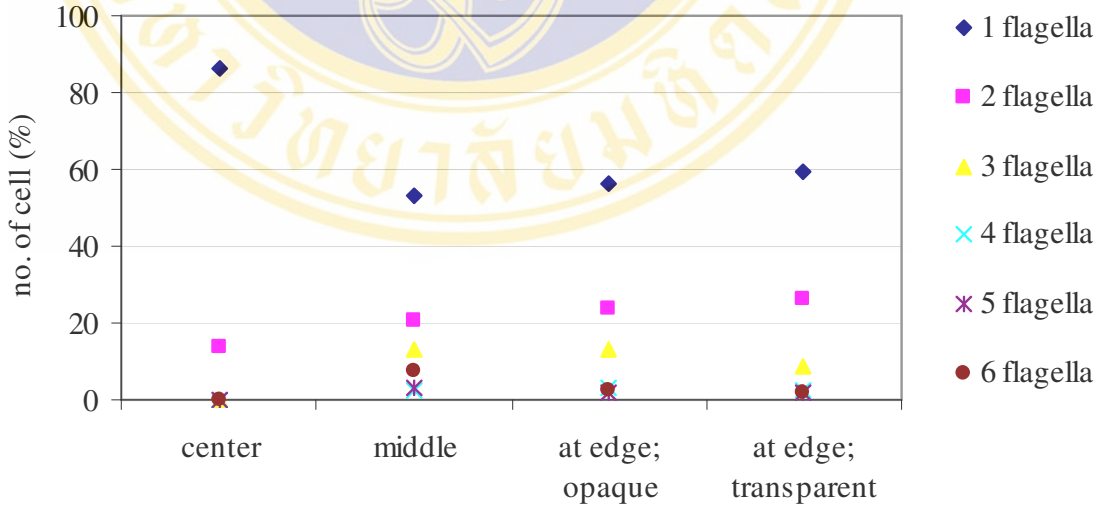


Figure 36: The number of flagella per bacterial cell for bacteria that were flagella positive

4.7.2.1 Flagella expression by bacteria sampled from primary plate

Figure 37 shows flagella expression for bacteria sampled from the center and edge of the first swarm plate. The data shown is a summary for 40 strains (100 bacterial cells per site sampled), (in total, 4,000 observations for center, and 4,000 for edge). Results are shown as the proportion of bacterial cells positive for flagella.

Bacteria from the center of the colony (black bars) were usually non-flagellate. Of the 15% with one or more flagella, 10% had one flagellum, 3% two flagella, 1% three flagella, less than 1 % had four or five flagella and none of the cells had six flagella. Bacteria from the edge of the colony were more often flagellate (60% positive). Of these, 32% had one flagellum, 13% two flagella, 9% three flagella, 4% four flagella, 1% five flagella and 1% had six flagella. Comparing center and edge cells, it is clear that center cells expressed less flagella compared with edge cells, and that the number of flagella were lower overall in the flagella-expressing group.

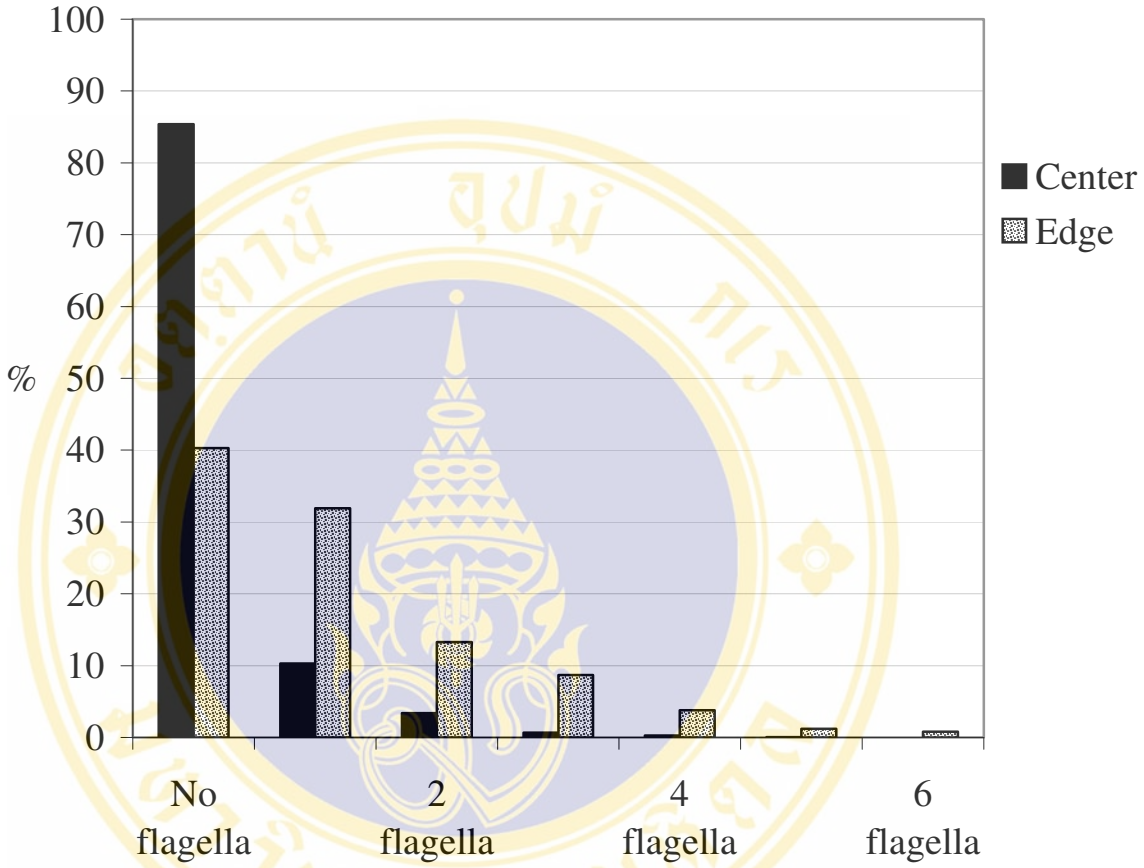


Figure 37: Results of flagella expression for 40 isolates of *B. pseudomallei* after sub-culture on swarm plate for 72 hours at 37^0C in air. A total of 100 bacteria were examined per strain. 'Edge' refers to bacteria taken from the edge of the swarm colony; 'center' to the centre of the swarm colony

4.7.2.2 Expression by bacteria from secondary swarm plate

Figure 38 shows flagella expression for bacteria sampled from the centre and edge cells of the two secondary swarm plates IIE and IIC. Data shown represents results for 100 bacterial cells per site sampled, chosen at random per slide. The results for Center-Center (bacteria originated from center of first swarm plate colony and were then sampled from colony of second plate) and Edge-Center (bacteria

originated from edge of first swarm plate colony and were sampled from the center of the second swarm colony), were not statistically different. On average (for the two combined), 83 % expressed no flagella, 13 % had one flagella, 4 % two flagella, 1% three flagella and none of the cells expressed more than three flagella.

There was no significant difference between Centre-Edge and Edge-Edge cells. The average for the expression of flagella was: 32 % no flagella expression, 29 % one flagella, 19 % two flagella, 12 % three flagella, 6 % four flagella, 2 % five flagella and 1 % expressed six flagella. Overall, center cells produced more flagella than edge cells.

For statistical analysis of flagella expression see Appendix IV.

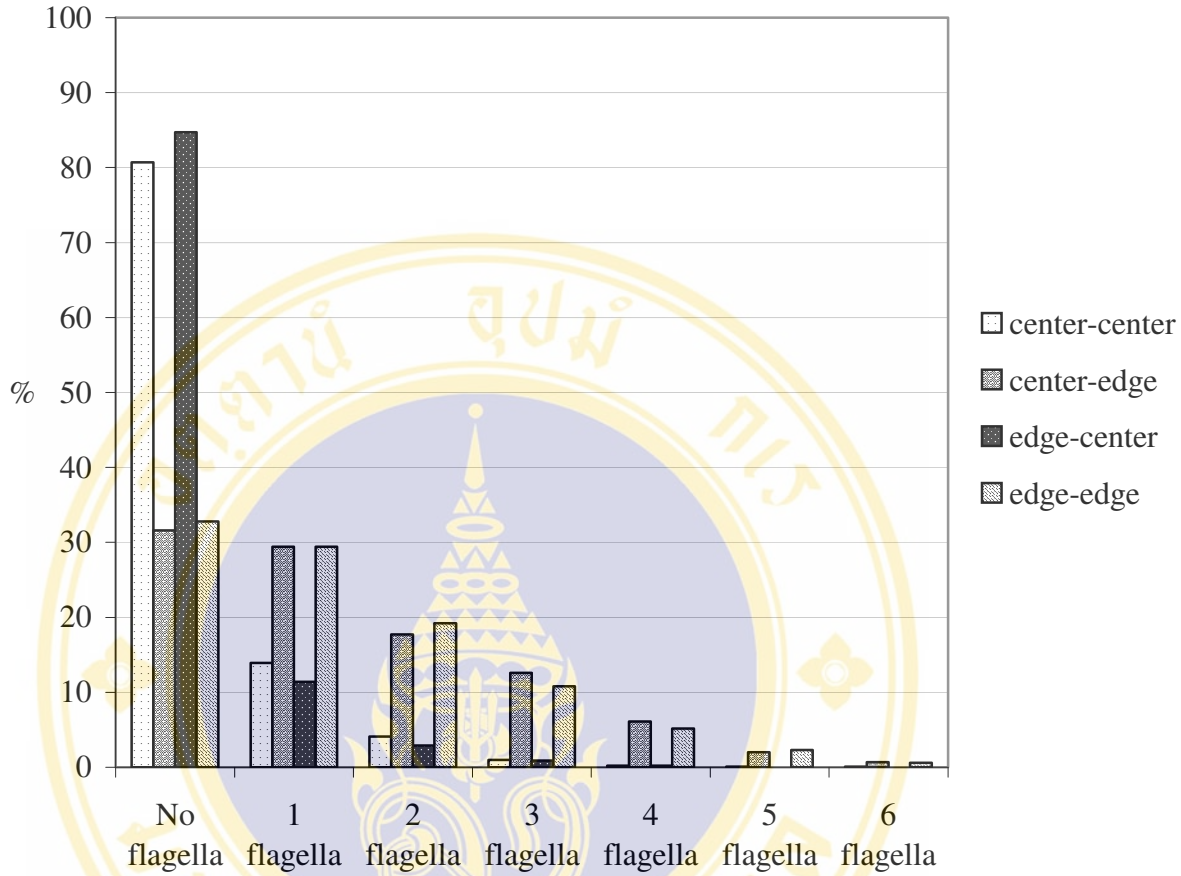


Figure 38: **F**lagella expressed by *B. pseudomallei* after sub-culture from one swarming plate to another. After 72 hours, bacteria were picked from the center and edge of the primary swarm plate onto two secondary swarm plates. A total of 100 bacteria were examined per strain. 'Center-center' means bacteria in the center of the primary swarm plate were sub-cultured onto a secondary swarm plate, and those colonies were picked from the center of this for examination; and so on**.**

4.7.2.3 Position of flagella; center and edge cells

An evaluation of the position of flagella versus position in swarm colony was performed, results for which are shown in summary form in Table 17.

For the bacterial cells sampled from the edge of a swarm colony, when one flagellum was expressed then this was most commonly polar. When more than one

were expressed, the commonest positions were all at one pole. Expression at both poles, lateral or peritrichous positions were less common.

Overall, bacterial cells sampled from the center of a swarm colony expressed fewer flagella, and most cells failed to express flagella. When flagella were expressed, a single flagellum was most often present at one pole. When more than one flagella were expressed, this was most commonly seen at one pole. There were few cells with flagella expressed at both of the poles, in a lateral or peritrichous position (figure 39).

Table 18 and Figure 39 show the results of the flagella positions for combined data for bacteria from the center and edge of the colony.

Table 17: Number of observations and row proportions (95% confidence intervals for row proportions)

Experiment	No flagella	Unipolar	Bipolar	Lateral	Peritrichous	Total
Centre						
Number	3415	443	29	83	30	4000
%	85.4%	11.1%	0.7%	2.1%	0.7%	(100%)
95%CI	(80.6-89.1)	(7.9-15.4)	(0.42-1.3)	(1.5-2.9)	(0.5-1.1)	
Centre-centre						
Number	3226	508	51	176	39	3900
%	80.6%	12.7%	1.3%	4.4%	1.0%	(100%)
95%CI	(73.4-86.3)	(9.2-17.3)	(0.8-2.1)	(2.9-6.7)	(0.5-1.8)	
Edge-centre						
Number	3387	419	21	140	33	4000
%	84.7%	10.5%	0.5%	3.5%	0.8%	(100%)
95%CI	(78.4-89.4)	(7.1-15.2)	(0.3-0.8)	(2.5-4.8)	(0.5-1.4)	
Edge						
Number	1572	1516	97	393	322	3900
%	40.3%	38.9%	2.5%	10.1%	8.3%	(100%)
95%CI	(32.5-48.6)	(33.4-44.7)	(1.7-3.6)	(7.9-12.8)	(5.7-11.8)	
Edge-Edge						
Number	1310	1556	125	522	487	4000
%	32.7%	38.9%	3.1%	13.1%	12.2%	(100%)
95%CI	(24.3-42.5)	(33.6-44.5)	(2.3-4.2)	(11.0-15.4)	(9.2-16.0)	
Centre-Edge						
Number	1262	1648	129	455	596	4000
%	31.6%	41.2%	3.2%	11.4%	12.6%	(100%)
95%CI	(22.7-42.0)	(35.4-47.2)	(2.2-4.8)	(9.6-13.4)	(8.9-17.7)	

Table 18: Flagella positions, combined data for center and edge cells. Number of observations and row proportions (95% confidence intervals for row proportions).

Experiment	No flagella	Unipolar	Bipolar	Lateral	Peritrichous	Total
Centre (combined)						
Number	10,028	1,370	101	399	102	12,000
%	83.6%	11.4%	0.8%	3.3%	0.8%	100%
95% CI	(80.2-86.5)	(9.3-13.9)	(0.6-1.2)	(2.6-4.2)	(0.6-1.2)	
Edge (combined)						
Number	4,144	4,720	251	1370	1405	11,890
%	34.8%	39.7%	2.9%	11.5%	11.0%	100%
95% CI	(29.8-40.2)	(36.4-43.0)	(2.4-3.6)	(10.3-12.8)	(9.1-13.3)	

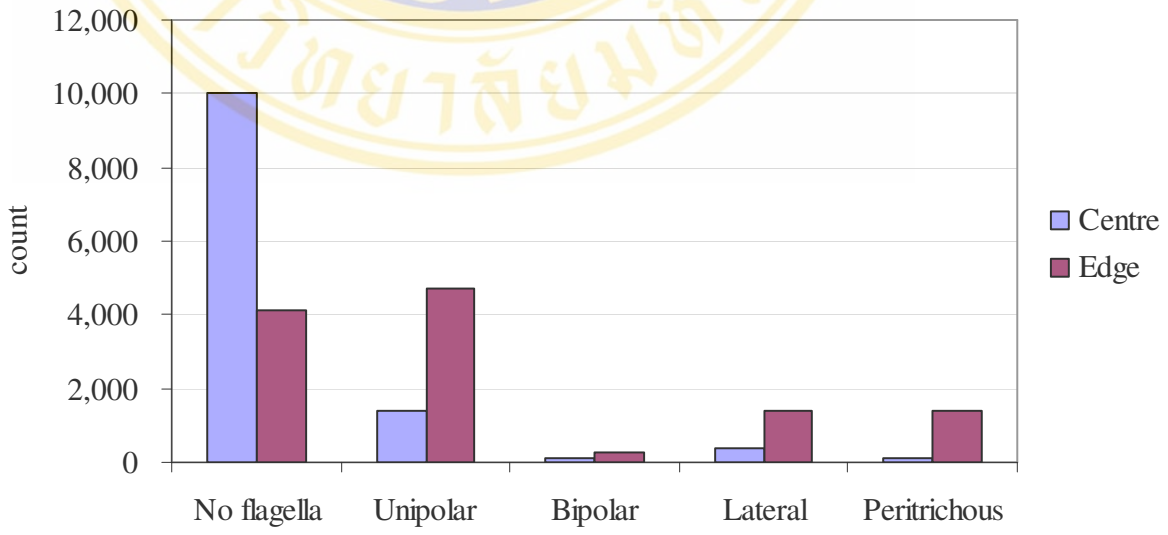


Figure 39: Position of flagella from center and edge cells versus final position on swarm plate.

A comparison was made between the number of flagella, and the position from which there were expressed. This has been displayed in Figure 40 in the form of a pie chart for each of one, two, three, four, five and six flagella. Figures are based on every cell counted, thus all the cells that were producing one or more flagella were included. It can be observed that the percentage of peritrichous flagella increases with the number of flagella expressed, and at the same time the percentage of one pole position falls.

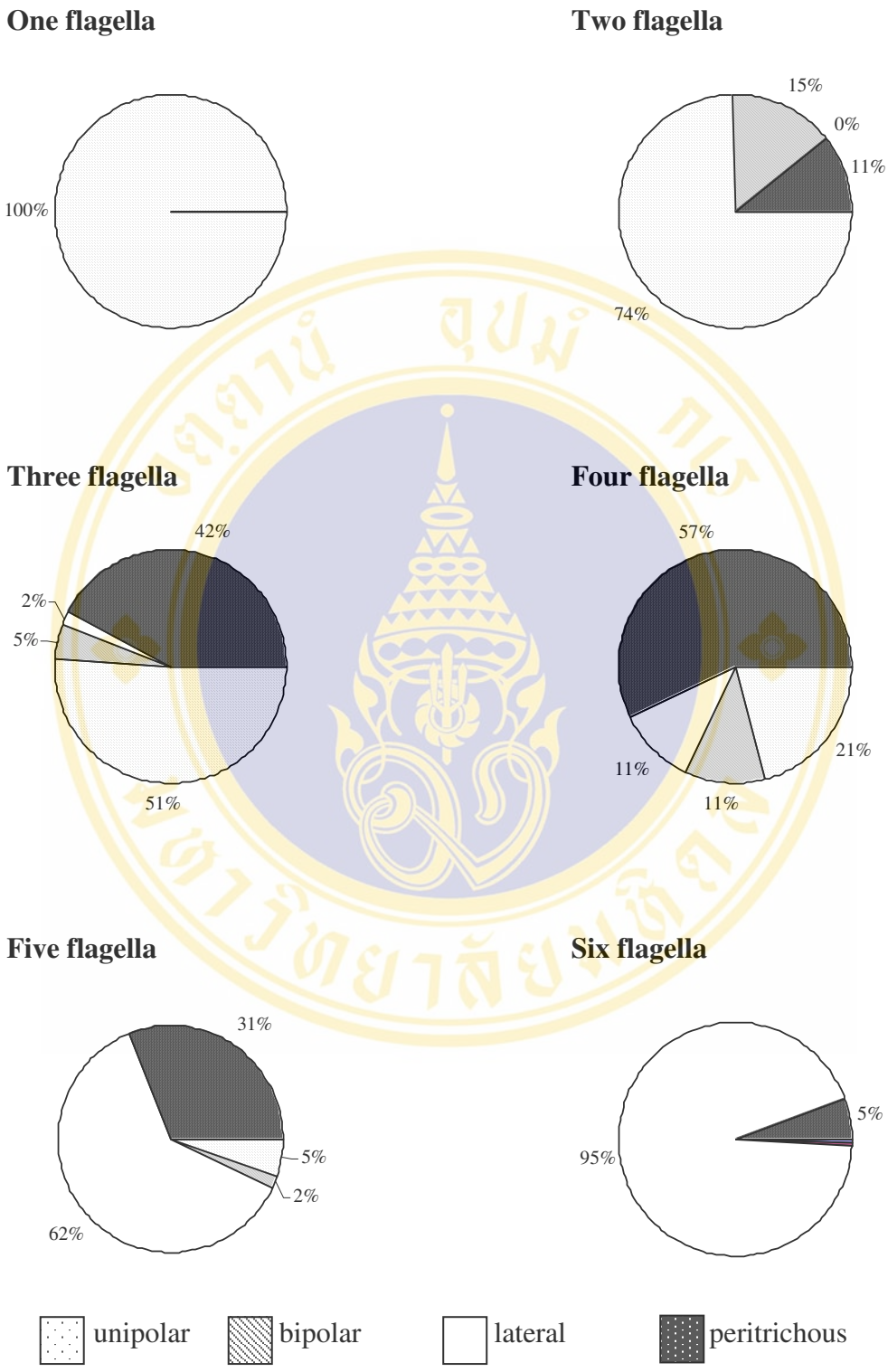


Figure 40: Results for the position of one to six flagella expressed by *B.pseudomallei*

4.8 Motility by RTM-3 from swarm plates

The dominant movements of bacterial cells were defined for each of the swarm plates. The dominant movement for the first swarm plate was tumbling for the center cells and waving for the edge cells. The dominant movements from the second swarm plate were the same for center (center-center and edge-center) and edge cells (center-edge and edge-edge). Thus tumbling and waving were the dominant movements respectively, for the center and the edge cells. For a complete overview of the data on motility, see Appendix V.

4.9 Relationship between flagella expression and motility on RTM-3

Table 19: Dominant movement versus number of flagella

Dominant movement	**Number of flagella**					
	0	**1**	**2**	**3**	**4**	**Total**
Vibration	24	11	4	1	0	**40**
Rotational displacement	1	1	1	0	0	**3**
Waving	42	35	24	6	1	**108**
Tumbling	76	2	0	0	0	**78**
Non-movement	2	3	0	0	0	**5**
Total	**146**	**51**	**29**	**7**	**1**	**234**

It appears from this comparison that vibration and tumbling motility as defined by RTM-3 may not be dependent on the presence of flagella. These types of movement may relate to other bacterial factors that were not visualized by RTM-3, or to the appearance of Brownian motion which is not dependent at all on motility factors. There was a strong relationship between waving (forward progressive) motion

and the presence of flagella. Over half of bacteria in this group had one or more flagella. However, 42 of 108 (39%) bacterial strains for whom waving was the dominant movement did not have a visible flagellum. For these, either an alternative motility factor was propelling the bacterium, or the flagella were lost from bacteria during processing. This is possible, since it is recognized that flagella are shed during growth, and are also liable to shearing through physical stress. The association between the presence of flagella but lack of motility could be due to the presence of dead bacteria, or relate to defects in expression of genes encoding factors involved in flagella movement.

4.10 Relationship between *B. pseudomallei* growth curve and spectrophotometric measurements

The aim of this experiment was to define the relationship between optical density (OD) and colony forming units per ml of broth culture over a time course so that OD could be used as a surrogate for colony forming units in later experiments. One colony of *B. pseudomallei* strain 49 was inoculated from Ashdown's medium into one of two 100ml of TSB. Both samples were incubated at 37^0C in air for 24 h (one sample static, and the other at 60 rpm). Two aliquots were taken from each sample at 2 h intervals, one of which was used to measure the OD_{600}, and the other to quantify CFU/ml by serial dilution on Ashdown's agar. OD and cfu were compared over time (Figure 41). The turbidity of shaken broth was higher than statically incubated broth but the colony count in the two conditions were very similar. The most likely explanation for this is that static broth was not sufficiently suspended prior to removal of the aliquot for OD assessment.

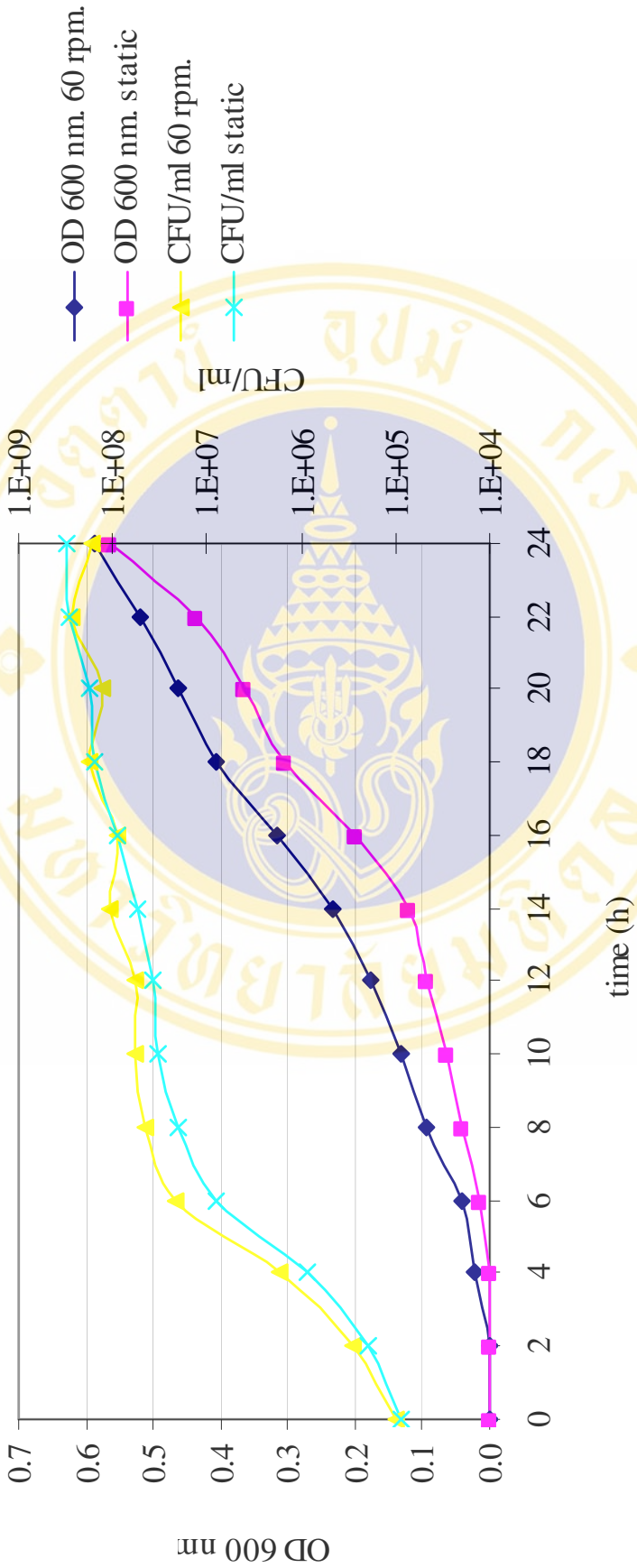


Figure 41: Colony forming units versus optical density of TSB containing *B. pseudomallei* strain 49 over an incubation time course

4.10.1 Expression, number and position of flagella by *B. pseudomallei* during growth

The aim of this experiment was to define the expression of flagella during a growth curve *in vitro*. This was enumerated using a flagella stain followed by microscopy, as described previously. Bacterial incubation was maintained throughout at 37^0C in air, but the effect of shaking was defined. This was considered important for two reasons. First, shaken culture is more oxygenated, and this can alter factors such as expression and regulation of bacterial factors. Second, it was considered possible that flagella could be sheared off during the process of shaking. Thus, flagella were simultaneously examined from static culture, and from culture that was shaken at 60rpm.

A single colony of *B. pseudomallei* strain 49 was taken from Ashdown's agar and inoculated into one of two 10 ml TSB in 50 ml Falcon plastic tubes that were incubated static or shaken at 60 rpm overnight at 37^0C in air. These were used to inoculate two 100 ml samples of TSB contained in 500 ml Duran bottles. These were again incubated static or shaken at 60 rpm at 37^0C in air, and were sampled every 2 hours for measurement of OD_{600} and flagella counting. Figure 42 shows the results of flagella expression for shaken (42A) and static culture (42B).

A

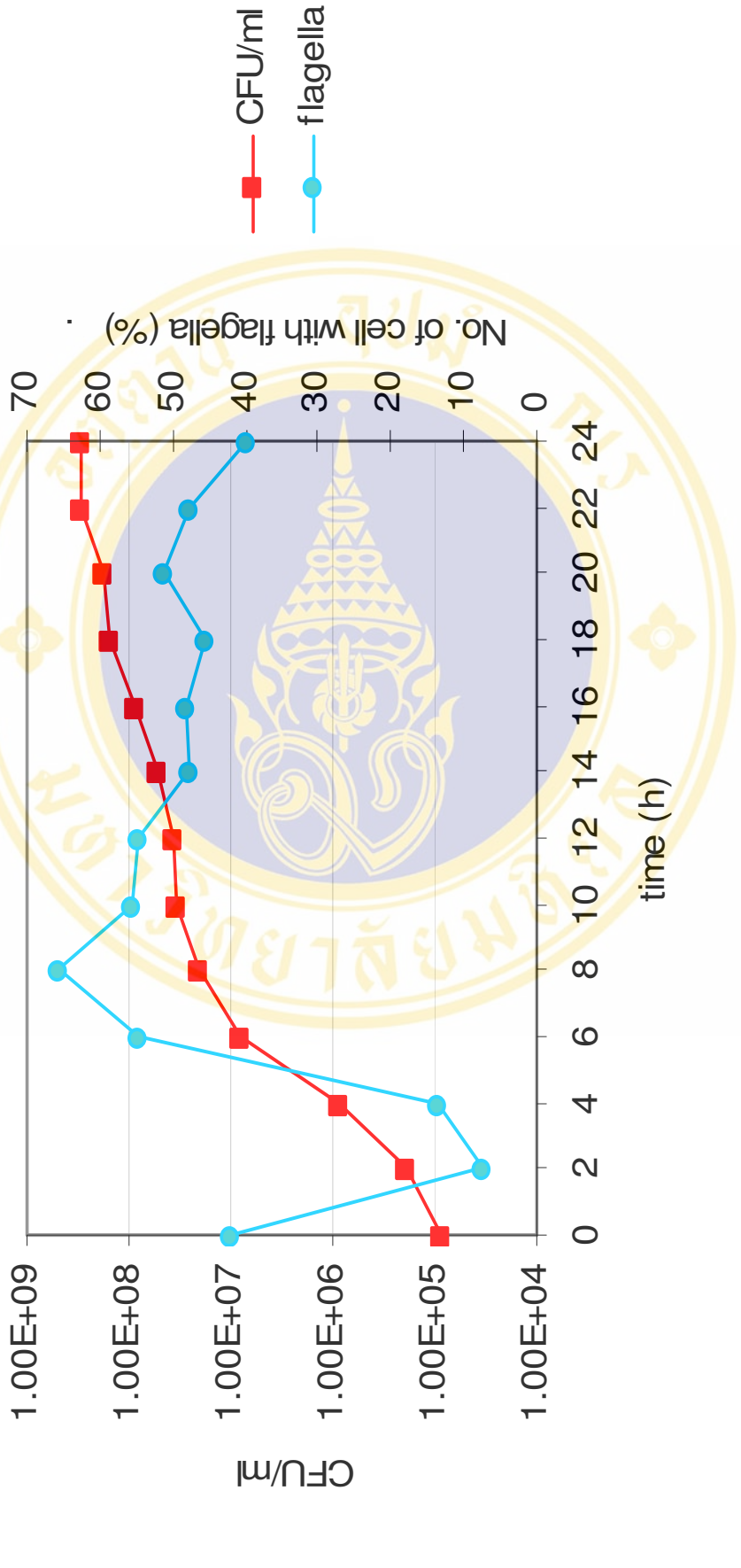


Figure 42A: Proportion of *B. pseudomallei* strain 49 expressing flagella over a time course in shaken culture

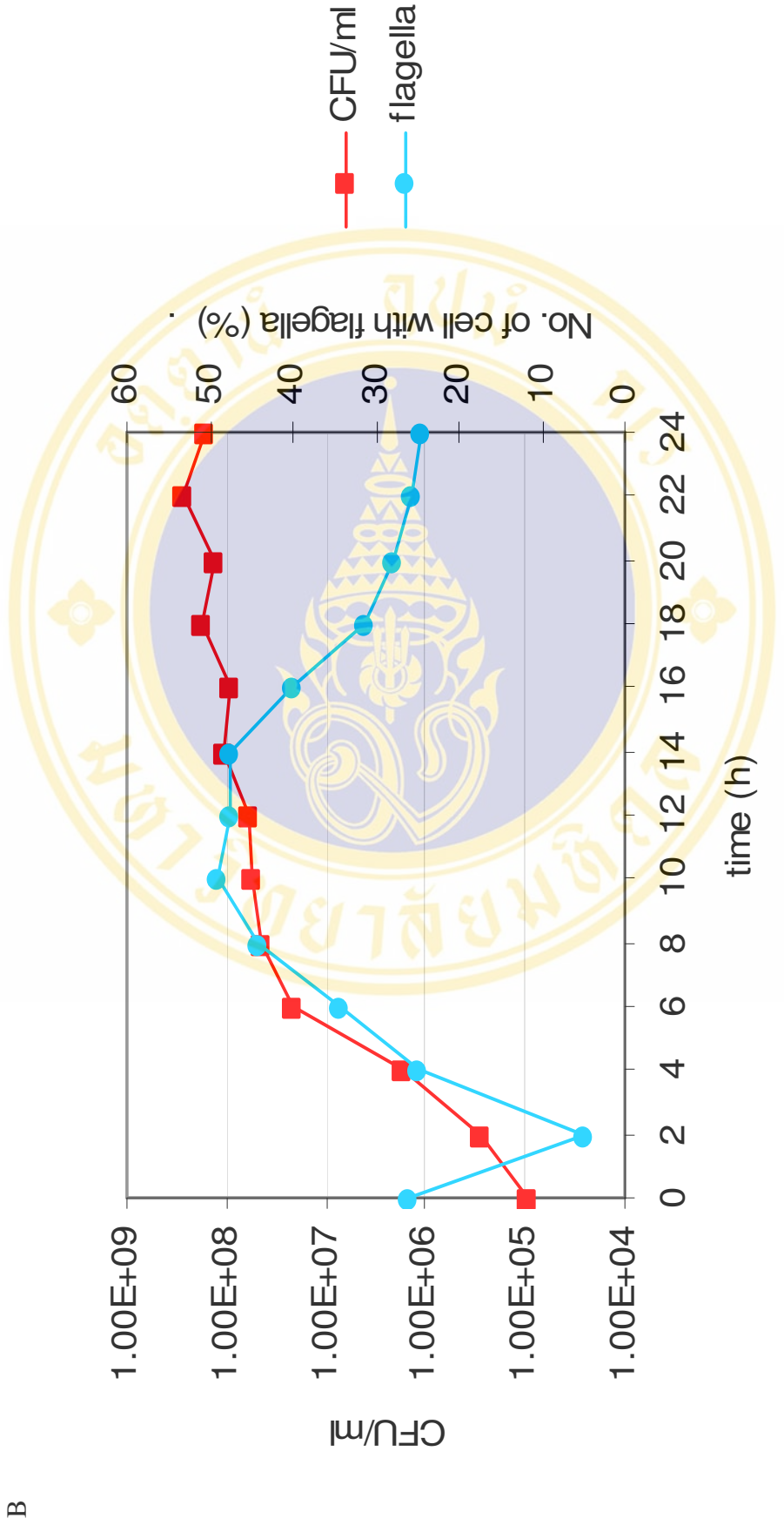


Figure 42B: Proportion of *B. pseudomallei* strain 49 expressing flagella over a time course in static culture

During the first two hours, the percentage of flagellated cells fell sharply for both conditions. For example, in the case of static culture, this fell from 42% at the start to 7.3% by two hours. A similar picture was seen for the shaken culture. The peak in flagellated cells differed between the two culture conditions. In static culture, the peak was at 8 hours, the numbers declining gradually thereafter. The peak for shaken culture was later at 10 hours.

The apparent fall in proportion of flagellate bacteria at 2 and 4 hours may relate to bacterial division prior to flagella expression during early exponential growth phase. This is consistent with the rising OD during this time period, which indicates continues bacterial growth. It is also possible that flagella present on bacteria in the stationary inoculum were shed as they entered into exponential phase and cell division. The peak of flagella expression at 8-10 hours represents late exponential/early stationary phase. It is possible that regulatory processes occur thereafter which lead to either flagella shedding, or to a process of cleavage through the action of secreted factors whose expression was low during exponential phase but became upregulated during stationary phase. The gradual loss of flagella over time in stationary phase is consistent with a process of continual flagella loss. This may be consistent with adaptive processes of the bacterium within the environment.

The difference observed between static and shaken cultures, with a more rapid peak of flagella expression by the shaken culture, may relate to differences in oxygen tension, availability of nutrients or other factors. There was no evidence that shaken cultures led to a rapid loss of flagella resulting from physical force. However, the amount of flagella in supernatant was not quantified, and it is still possible that bacteria that were incubated shaken had a greater turnover of flagella that was not manifest by this assay.

Overall, it seems reasonable to choose either assay for future flagella expression experiments, although the difference in OD between the two for the same CFU/ml should be taken account of during further experimental work.

The effect of static versus shaken culture on the number of flagella present on flagella-positive bacteria was determined over a time course (24 h) for *B. pseudomallei* strain 49. The results given in Table 20 represent a summary of all data points. Unipolar flagella expression was the most common configuration, followed by two and then 3 or more flagella per bacterium. There were no differences observed in the number of flagella between static and shaken cultures. This is consistent with the conclusion that shaking the culture does not lead to physical loss of flagella, although again, it is not possible to rule out increased turn-over.

Table 20: Proportion (SD) of flagellated cells in static versus shaken cultures over a growth curve.

Culture condition	1 flagellum	2 flagella	≥ 3 flagella
Static	77.1% ± 12.1%	15.7% ± 8.1%	7.2% ± 4.6%
Shaken at 60 rpm	74.1% ± 12.3%	18.6% ± 7.3%	7.3% ± 6.2%

4.10.2 Bacterial cell and flagella size of *Burkholderia pseudomallei*

The bacterial cell length remained constant throughout the growth cycle for *B. pseudomallei* strain 49 (Range 2.3-to 3 um, mean and SD 2.54 um $\pm$ 0.48). The flagella length varied with growth phase. During early exponential phase the length was 4.64 ± 1.65 um. The longest length was at 32 hours at 7.18 ± 3.03 um. This predictable observation is likely to relate to doubling time.

4.10.3 Variation in flagella expression within different areas of a broth culture (during incubation for 24 hours)

When *B. pseudomallei* is grown overnight in broth at 37^0C in air, a pellicle usually forms on the top of the medium. This is likely to represent a mass of bacteria embedded within an extracellular matrix or biofilm. It is not known whether flagella expression is related with presence within the pellicle.

Bacterial cells were sampled from pellicle and broth and examined for flagella. This was examined in static and shaken cultures of *B. pseudomallei* strain 49 in TSB incubated at 37^0C in air for 24 h.

Figure 43 shows the proportion of flagellate cells in each condition. The most striking feature was that the proportion of flagellate cells was significantly higher in the broth compared with the pellicle ($p=0.076$ and $p<0.0001$ for shaken and static culture, respectively). The finding that fewer bacteria expressed flagella in the pellicle of the shaken culture is likely to indicate an effect of increased oxygenation, or reduced ability to form a biofilm under conditions of movement.

An assessment was made of the number of flagella present on flagella-positive bacteria in broth and pellicle. Shaking the culture had a marked effect on the number of flagella expressed by bacteria in the pellicle. This is a clear example of how relatively minor changes in experimental methodology can lead to marked differences in results.

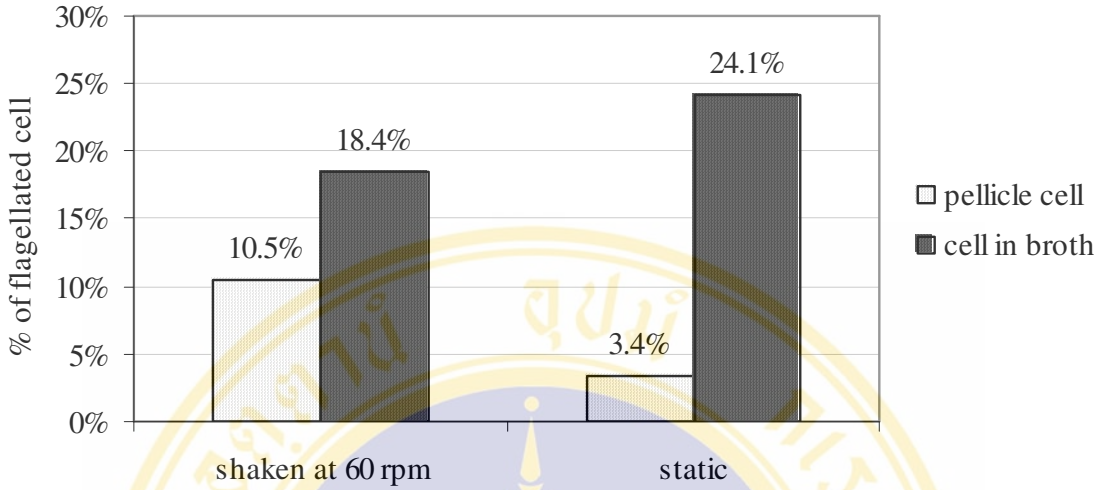


Figure 43: Proportion of flagellated cells in pellicle and broth of TSB after overnight incubation

Table 21: The number of flagellated cell (in percentage) was differentiate in number of flagella which generated in individual cell.

Culture condition	1 flagellum	2 flagella	≥ 3 flagella
Static condition			
Pellicle cell	100	-	-
Cell in broth	75.6	19.1	5.0
Shaken at 60 rpm			
Pellicle cell	66.7	24.8	8.6
Cell in broth	81	17.4	1.6

4.11 Flagella expression by isogenic colony variants of *B. pseudomallei*

The following data pertain to the question of whether isogenic colony variants vary in terms of flagella expression. Comparisons were made between isogenic colony Types I, III and VI (as described in methods) for two bacterial strains termed 153 and 164. These were randomly selected from clinical cultures; strain 153 was isolated from blood culture, and 164 from wound swab. The three morphotypes were selected on the basis that Type I appears to be the parental type, and III and VI are dominant morphotypic forms that emerge over time in starvation culture.

Two major questions are addressed here: (i) is there any difference in the number of bacteria that express flagella between isogenic colony morphotypes, and (ii) for those bacteria that are flagellate, is there any difference in the number of flagella expressed between morphotypes. This is important since the phenotypes related to each Type may represent an adaptation for a particular environmental niche. Understanding the variability in phenotype for a given morphotype may lead to an understanding of the biological advantages conferred by colony variation.

The starting point was to examine flagella expression over a growth curve, since a multitude of influences may be at play including variation in expression of secreted proteins that could cleave or in other ways modify flagella, and regulatory mechanisms that will dictate flagella expression. An important aspect of this experiment was to ensure that rates of growth were comparable since this may also affect flagella expression. Bacterial cells (%) expressing flagella were then compared at four time points between isogenic types. Further experiments were then performed using prolonged culture on swarm agar.

4.11.1 Comparative expression of flagella by isogenic Type I, III and VI *B. pseudomallei* during a growth curve

A growth curve was performed for isogenic *B. pseudomallei* Type I, III and VI strains 153 and 164. Morphotypes was identified on Ashdown's agar after incubation at 37^0C in air for 4 days. A single colony of each was suspended in PBS, and diluted in 100 ml of TSB using optical density to reach a starting concentration of approximately 10^5cfu/ml. This was incubated static at 37^0C in air and sampled

periodically for colony count. Aliquots were serially diluted and plated onto Ashdown's agar and the cfu/ml calculated. A second aliquot was used to determine flagella count, as previously described in section 3.8. Figure 44 shows the growth curve of the three Types for strains 153 and 164, and confirms that there is no difference in rate of growth between the three morphotypes.

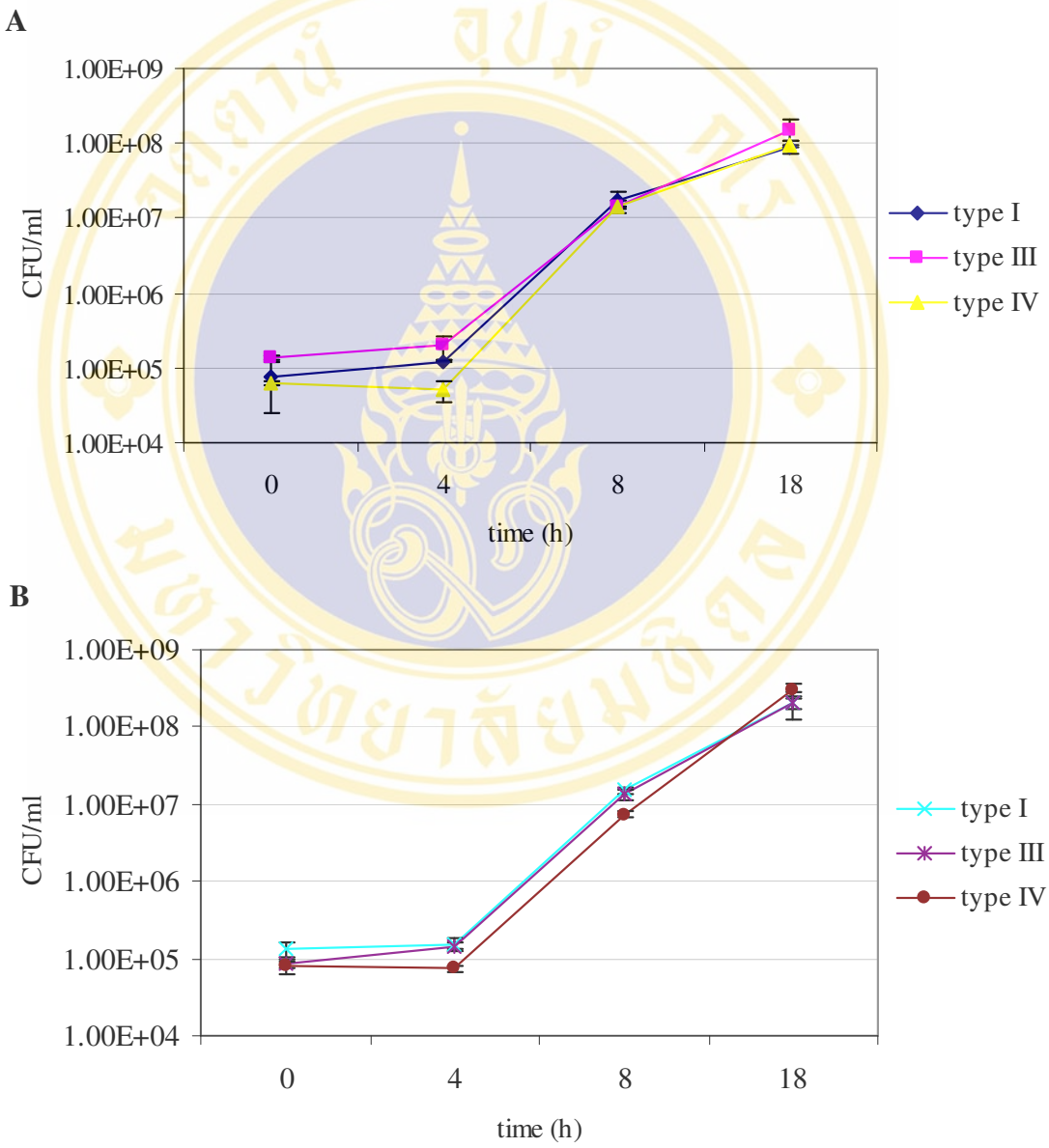


Figure 44: Growth curves for isogenic Type I, III and VI *B. pseudomallei* strains 153 (A), and 164 (B).

The number of bacterial cells that expressed one or more flagella at each time point is shown in Table 22a. At the start of culture (0 h), all three morphotypes of strain 153 were predominantly flagellate. The number positive fell dramatically by 4 hours, followed by a rise to around 50% positive by 8 hours and more than three quarters flagellate by 18 hours. There were no striking differences between Types I, III and VI. Statistical analysis in which Type I strain 153 was considered to be the reference strain against which Types III and VI strain 153 were compared is shown in Table 22b. A single significant p value for comparison of Types I and III at the zero time point is of doubtful significance after taking account of the number of comparisons made.

The overall pattern of flagella expression over time was similar for strain 164, with the exceptions that a smaller proportion were positive for flagella in the starting inoculum, and the proportion of flagellated cells was greater at 4 and 8 hours. Statistical analysis in which Type I strain 164 was considered to be the reference strain against which Types III and VI strain 164 were compared is shown in Table 22b. The striking feature is that flagella expression was markedly lower for Type VI compared with Type I during exponential growth phase, although there was no significant difference at the zero and 18-hour time points. Taking the results for the two strains together, it appears that there is inter-strain variability in biological behaviour.

Table 22a: Number of flagellated cells during a growth curve

Strain	Number positive for flagella (out of total 100 cells)			
	0 h	4 h	8 h	18 h
Strain 153; Type I	68	8	49	82
Type III	83	6	65	82
Type VI	75	14	56	83
Strain 164; Type I	36	36	83	72
Type III	45	19	89	56
Type VI	30	7	54	79

Table 22b: Statistic analysis of flagella expression during a growth curve

Strain	P value			
	0 h	4 h	8 h	18 h
Strain 153; Type I	-	-	-	-
Type III	0.02	NS	NS	NS
Type VI	NS	NS	NS	NS
Strain 164; Type I	-	-	-	-
Type III	NS	0.01	NS	0.3
Type VI	NS	<0.0001	<0.0001	NS

Paired comparisons were made using the Fisher's exact test

The next question was whether the number of flagella expressed by flagellate bacteria differed. Results are depicted below for each of the three isogenic types for strain 153 and 164. This has been divided to demonstate those bacteria with one, two or three or more flagella. The pattern of flagella expression in terms of number of flagella present per bacterium was relatively comparable both between strains, and between colony morphotypes as shown in figure 45 and 46. Bacteria with 3 or more flagella were not observed at 4 hours when single flagella predominated, but were not uncommon at the other time points. Considering the two strains together, there was no consistent difference in number of flagella expressed by Types I, III and VI.

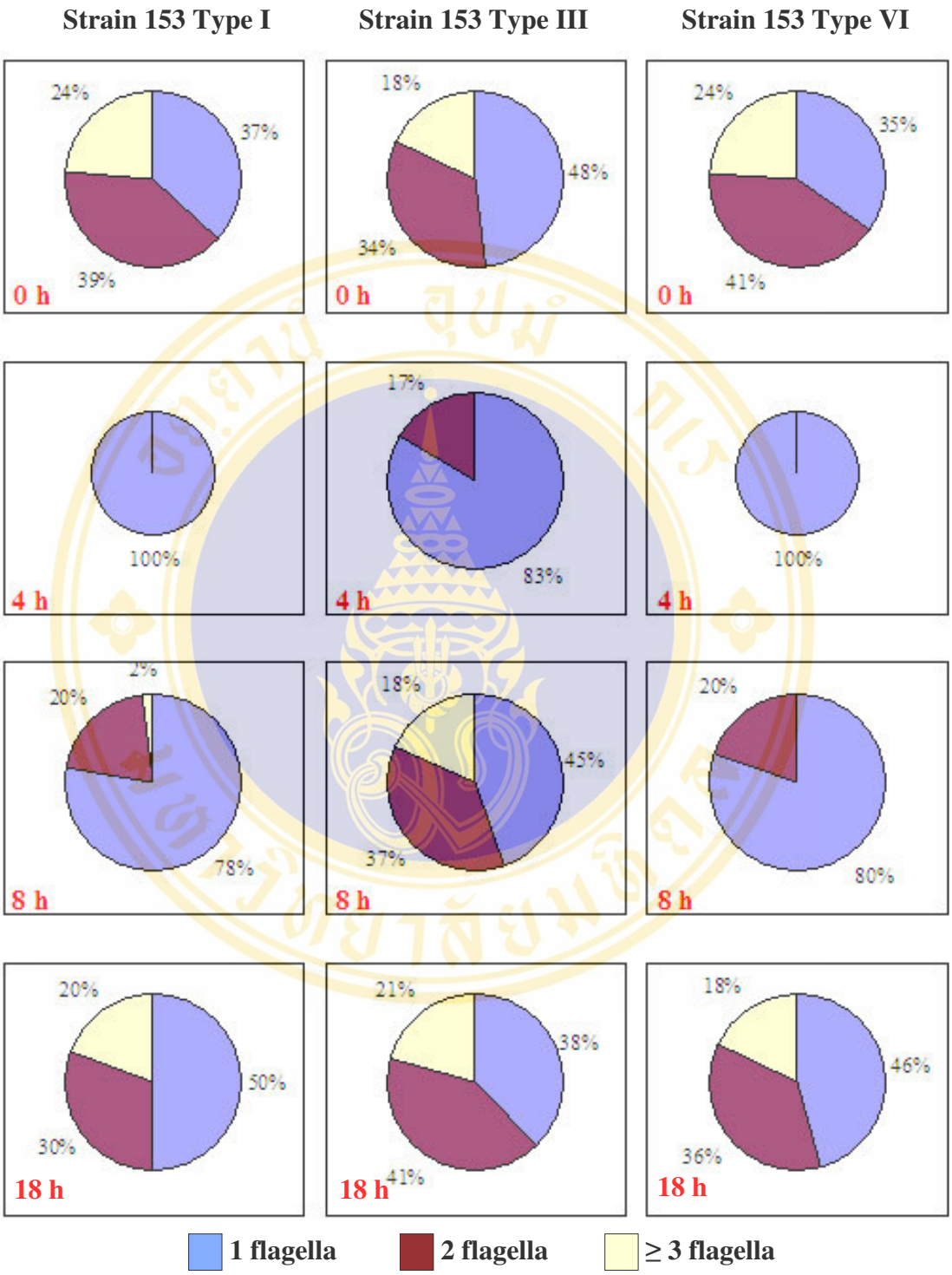


Figure 45: Number of flagella expressed by bacterial cells that had one or more flagella at each of four time points for *B. pseudomallei* strain 153 (type I, (left) III (middle) and VI (right). Each count represents the mean of 100 bacterial cells at each time point.

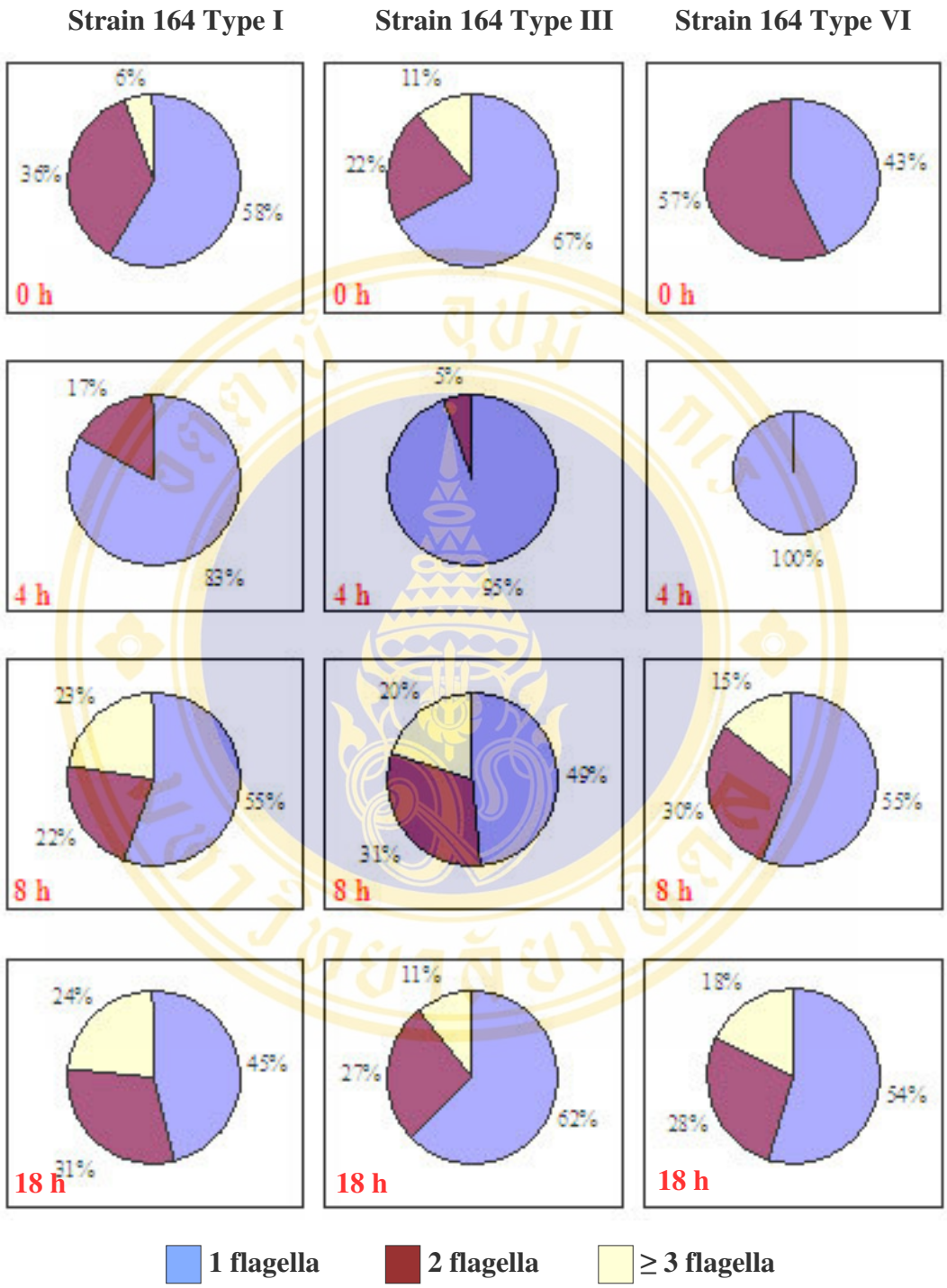


Figure 46:. Number of flagella expressed by bacterial cells that had one or more flagella at each of four time points for *B. pseudomallei* strain 164 (type I, (left) III (middle) and VI (right). Each count represents the mean of 100 bacterial cells at each time point.

4.11.2 Flagella expression by isogenic morphotypes following growth on swarm agar

A subsequent experiment was performed to define qualitative (yes/no) expression of flagella by isogenic strains over a more prolonged period of time on swarm agar. *B. pseudomallei* Type I, III or IV strains 153 and 164 were evaluated following incubation on swarm agar at 37^0C in air for 48 h and 72 h. Bacteria were sampled from the edge of the swarm colony. The proportion of bacterial cells (of 500 examined) expressing one or more flagella are shown in figure 47. Flagella expression was greater at 72 hours compared with 48 hours, although the degree to which this was seen varied between Types. Type III and VI expressed more flagella than Type I at 72 hours for both bacterial strains.

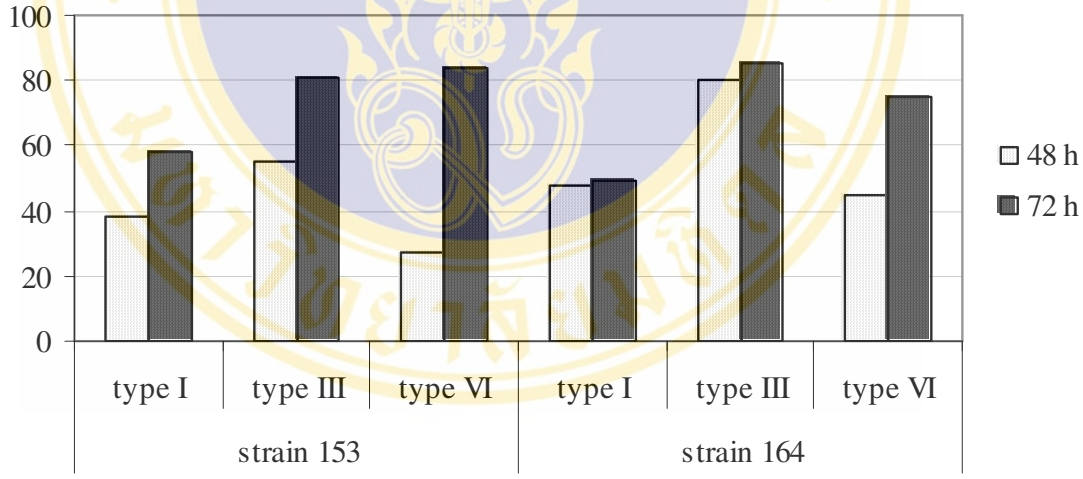


Figure 47: Proportion of bacterial cells with flagella for isogenic Type I, III and VI for strains 153 and 164 strains observed at edge of swarm colony following incubation at 37^0C in air for 48h and 72 h.

The difference in flagella expression was compared between morphotypes, in which Type I was used as the comparator for a given strain. Table 23 below demonstrates that the increase in flagella expression for Type III (48 and 72 h), and Type VI (72 h) were highly statistically significant. This is surprisingly contradictory compared with the result for flagella expression during exponential growth in broth

when expression was reduced. Thus, it appears that flagella expression of strain 164 is highly dependent on growth phase and/or *in vitro* conditions.

Table 23: Statistic analysis of flagella expression on swarm plate

Strain		P value	
		48 h	72 h
Strain 153; Type I		-	-
	Type III	<0.0001	<0.0001
	Type VI	<0.0001	<0.0001
Strain 164; Type I		-	-
	Type III	<0.0001	<0.0001
	Type VI	0.38	<0.0001

Paired comparisons were made using the Fisher's exact test

An evaluation was then performed of the number of flagella expressed by those bacteria that were flagella-positive. Figure 48 below demonstrates the proportion of bacterial cells with one, or more than one flagella for each of the three colony morphologies for strain 153 and 164. A single flagellum was more commonly expressed by Type I than the other Types for the two strains tested. The proportion of cells with one or more than one flagella was approximately the same for Type VI strains 153 and 164. The results for Type III were inconsistent between strains. This indicates that there is variability in the number of flagella expressed by different colony morphology types, and provides further evidence for inter-strain variability.

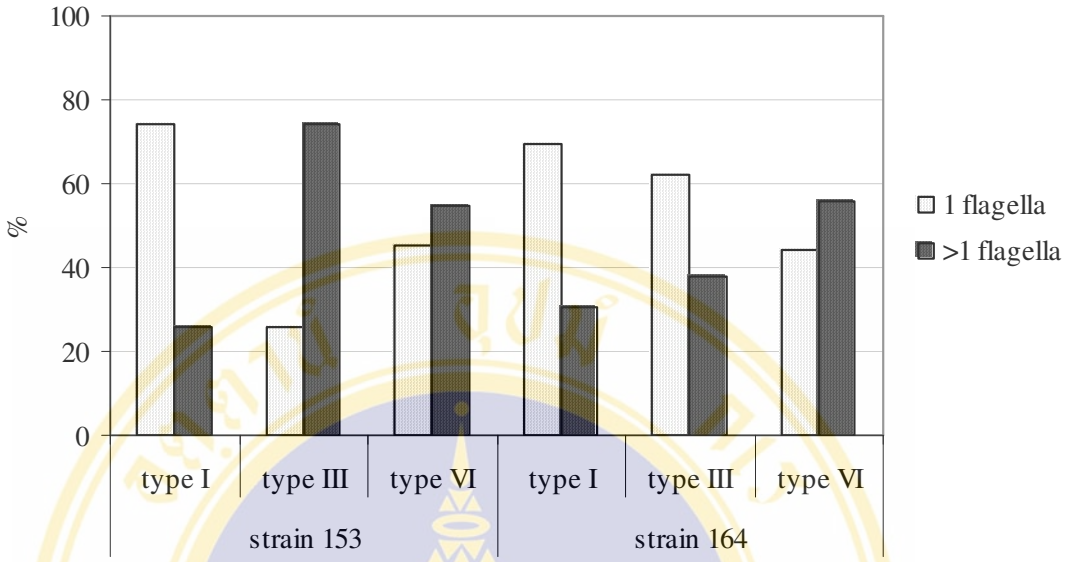


Figure 48: Number of flagella expressed by flagellate bacteria for isogenic morphotypes I, III and VI.

4.11.3 Motility by isogenic morphotypes

Presence or absence of motility was examined by RTM-3for isogenic *B. pseudomallei* Type I, III and VI strains 153 and 164 after incubation in TSB for 24 h at 37^0C in air. There were no reproducible differences between parental and isogenic morphotypes for either strain.

CHAPTER 5

DISCUSSIONS

Bacterial motility

Bacterial surface motility has been divided into six categories, as follows: swimming, swarming, gliding, twitching, sliding and darting (Henrichsen, 1975). Three types of motility were studied here (swimming, swarming and twitching). Twitching has been shown to require type IV pili (Semmler et al., 1999). Two quite different types of motility are mediated by bacterial flagella. In addition to swimming motility in liquid, flagella mediate swarming motility on solid surfaces or in viscous conditions.

Motility is important for movement in liquid, but is also involved in biofilm formation which involves three types of motility. Biofilm formation is initiated by the attachment of individual cells to a surface (pilus or "swimming" (Fla) flagella mediated), followed by their surface migration (pilus "twitching" and/or "swarming" (Laf) flagella mediated), replication to form microcolonies, and quorum-sensing regulated differentiation into the mature biofilm encased in polysaccharide by *Aeromonas* spp. (fig. 53)(Kirov, 2003).

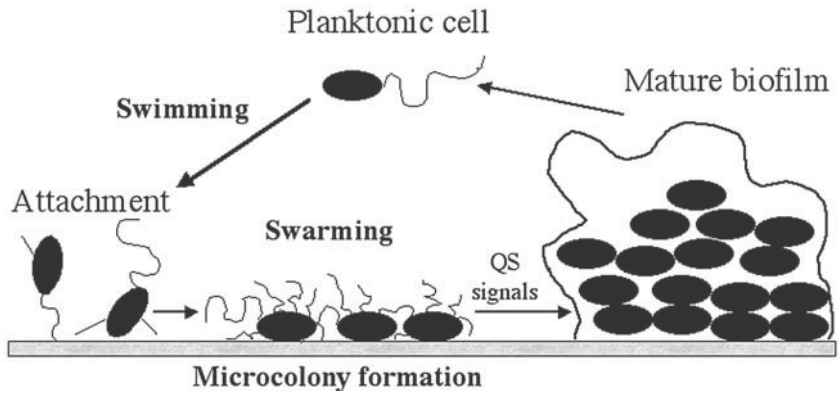


Figure 49: Model of the development of a mature biofilm from planktonic cells.

Here, a pilot study was performed to assess all three types of motility for *P. aeruginosa* and *B. mallei* were used to examine in all three types of motility prior to their use as positive and negative controls, respectively. The polar flagellum of *P. aeruginosa* is responsible for a mode of motility in aqueous environment or swimming and also mediates a mode of social motility known as swarming (Rashid and Kornberg, 2000). The polar fimbriae are presumably the principal adhesins, mediating adherence to cells or abiotic surfaces. These are responsible for the flagellum-independent mode of surface translocation called twitching motility (Beatson et al., 2002). *B. mallei* is a non-motile organism that lacks flagella yet its genome contains numerous flagellar and chemotaxis genes, most of which appear to be intact. One methyl-accepting chemotaxis gene has a frameshift mutation. In addition, a 65-kb insert flanked by IS elements disrupts the *fliP* gene (Malakooti et al., 1994), an essential gene for flagellum biogenesis, and a frameshift mutation in the flagellum motor *motB* gene eliminates its functionality. These mutations are likely to account for *B. mallei* being non-motile and non-flagellated (Upadhyay et al., 2004).

In this study, *B. pseudomallei* was observed to have swimming and swarming motility but appeared to lack twitching motility. Swimming motility rate of *B. pseudomallei* was calculated and compared with positive and negative control. These were 0.775 mm/h for *B. pseudomallei*, and 0.618 mm/h and 0.056 mm/h for *P. aeruginosa* and *B. mallei*, respectively. Rate of swimming was linear over time. A large population of clinical *B. pseudomallei* isolates were tested for their ability to swim *in vitro*. All isolates could swim on the surface of swim plates, the rate of which was highest at the 48 h time point.

The social nature of swarming indicates that extracellular and possibly cell-cell signals are central stimuli, as are intracellular physiological parameters and contact with a surface. Swarming is neither a starvation response nor an obligatory development stage. Cell density is critical to swarming as is the duration of the lag phase in *P. mirabilis* (Fraser and Hughes, 1999).

In this study, variability in swarm motility of *B. pseudomallei* was observed. This was evident between strains, and also for repeated measurements of a given strain. The methodology used was to toothpick inoculate a small amount of bacterial colony onto swarm agar, from which bacteria migrate outward and multiply. Swarm

motility of *B. pseudomallei* was greater than that for the positive control *P. aeruginosa*, with a swarm motility rate 0.351 mm/h for *B. pseudomallei*, and 0.264 mm/h and 0.073 mm/h for *P. aeruginosa* and *B. mallei*, respectively. A sub-set (9.4%) of a large population of clinical *B. pseudomallei* isolates was defined as non-swarmers based on a primary screen. On further more rigorous re-testing, non-swarmers accounted for just 1.9% of population.

Whilst considering why swarm colonies demonstrated marked variability within repeated measurements of a given strains, it was noted that colony appearance often showed marked variability. This was observed to occur between strains, and to some extend during repeated testing of a given strain. This was examined using several approaches. *B. pseudomallei* strain (1026b) was compared with an isogenic mutant defective in flagella expression (MM35). The mutant was unable to swarm, resulting in a tight colony on swarm agar. Wild type (1026b) grew as a swarm colony and reached a colony size of around 10 times the diameter of that of the mutant after 72 hours incubation.

The appearance of the swarm colony was then examined for a larger number of strains. Colony appearance took several forms, including the production of highly branched patterns or symmetrical migration. This variability was observed during repeated testing of a given strain. The colony appearance had a relationship with swarm motility. The most common swarm colony was type E (51% of 57 isolates tested), which showed an irregular colony edge, rough texture of the centre of the colony, and transparency of the colony in the outer colony area. One non-swarming isolate produced a swarm colony with a smooth texture in the colony centre that from the flagella mutant MM35. The non-swarmers identified here are likely to have one or more defects in genes that are essential for flagella expression.

Julkowska *et. at.* has reported that swarm appearance that produced by *Bacillus subtilis* is variable. This forms amorphous, largely confluent growth zones through relatively simple radial branching, to highly complex colonies that are generated by successive waves of moving cells (Julkowska et al., 2004). There are many factors that influence this, including moisture, nutrients, slime, temperature and signal transduction (Harshey, 2003). Genetic analysis has revealed that swarmer cells need to up-regulate the number of flagella in order to move. For example, in the case of

E. coli and *S. typhimurium*, the flagellar genes are expressed in a hierarchical manner (Macnab, 2003). Expression of the *flhDC* operon is influenced by environmental signals such as glucose availability, osmolarity, heat shock, acetyl phosphate, as well as by cell cycle regulation (Harshey, 2003). The front edge of the swarm zone is preceded by a transparent zone devoid of bacteria (Julkowska *et. al.).* This transparent zone may contain the wetting agent surfactin, secreted by the bacteria to aid their translocation across the surface. The biosurfactant required for swarming of *P. aeruginosa* is rhamnolipid (Kohler et al., 2000). Haussler *et. al.* has examined the effect by *B. pseudomallei* rhamnolipid on bacterial behaviour *in vitro* (Haussler et al., 2003). It is possible that rhamnolipid is produced during swarm motility of *B. pseudomallei*, based on the presence of a transparent zone that is present in many other bacteria such as *Serratia liquefaciens* and *S. marcescens* (Eberl et al., 1999).

Cell of difference area on swarm colony was measured that centre cell was smallest and edge cell was greatest by around 1.5 times of centre cells. From this result it related with former study that most active swarming occurs near the periphery of colony, where the longest and most flagellated cells are found such as *Proteus* sp. (Fraser and Hughes, 1999) and *Serratia liquefaciens MG1* (Eberl et al., 1996). Cells in the interior of the colony move less and appear to de-differentiate to a morphology more typical of broth-grown vegetative or shorter with fewer flagella (Harshey, 1994). Toutain *et. al*. studied the difference in cell length between cells at the edge and the center of the swarm for *P. aeruginosa* which found similar to that observed for cells grown planktonically in M8 medium when measured in stationary phase versus exponential phase, suggesting that the smaller cells at the center of the swarm were nutrient limited (Toutain et al., 2005). Similar results regarding the size of the cells were also observed by TEM for *P. aeruginosa* PA14 (Kohler et al., 2000), as reported previously for strain *P. aeruginosa* PA01 (Rashid and Kornberg, 2000).

Unlike swarming and swimming, *B. pseudomallei* did not demonstrate twitching motility. Twitching motility is influenced by cell density, cell contact-dependent intercellular signals, as well as nutritional status for example in *N. gonorrhoeae* (McBride, 2001). A *pilA* mutant of *P. aeruginosa* has been constructed that lacked twitching (Rashid and Kornberg, 2000). Similar the loss of gene function in *B. mallei*, it is possible that gene loss in *B. pseudomallei* has led to loss of twitching motility.

In an investigation of a *P. aeruginosa* mutant defective in twitching motility, colonies were observed to be smooth and domed on solid agar compared with wild-type colonies that were flat, spreading and rough (Semmler et al., 1999). The colony appearance of this mutant is very similar to that for most strains of *B. pseudomallei*. A screen of *P. aeruginosa* mutants to detect those with impaired virulence in Drosophila showed that all strains that were strongly impaired in fly killing also lacked twitching motility. The majority of mutations occurring in the *pilGHIJKL-chpABCDE* chemosensory gene cluster, although this appeared to be a consequence of *chp* control of other virulence factors as other non-twitching variants had normal virulence (David et al., 2001). A further study has defined *PilT*-deficient mutants of *N. gonorrhoeae* that retain type IV pilus expression but which lack the ability to take up DNA (Wolfgang, 1998).

Flagella expression

The number of flagella expressed by *B. pseudomallei* was found to be variable during this study. The distribution of the number of flagella present on flagella-positive cells appears to be constant over a growth cycle, suggesting that the number, as well as the length, of filaments per cell is coupled with the cell cycle (Iino 1974). During bacterial growth, the proportion of cells that were flagellated fell sharply in first 2 hours. This is similar to findings for *S. typhimurium*, for which the first cell division occurs after 70 min, and the second at about 120 min after inoculation (Aizawa and Kubori, 1998). A new *S. typhimurium* flagellum as observed by electron microscopy occurs in the third bacterial generation (around 135 min), but these were not observed until the fourth generation by dark-field microscopy (Aizawa and Kubori, 1998). A flagellum that becomes broken within the basal body fails to re-grow. In this case, new flagella are synthesized (Okino et al., 1989).

In the study here, the highest number of flagellated *B. pseudomallei* cells during growth was observed at 8 or 10 hours for static or shaken culture, respectively. In *E. coli*, the level of *flhDC* expression is highest during the exponential growth phase (Pruss and Matsumura, 1997). However, the expression of the level 2 genes peaks in late exponential phase, and swimming speed of cells is highest in the post-exponential phase, indicating a delay in flagella construction (Amsler et al., 1993). Cell division

and the regulation of flagella gene expression are interdependent. That is, cell division affects the expression of the flagellar regulons, and flagellar gene expression in turn could affect cell division (Aizawa and Kubori, 1998). The commonest configuration for flagella in *B. pseudomallei* was unipolar, representing around 75% of flagellated cells. Unipolar flagella are required for swimming motility in liquid conditions in other bacterial species.

Swarming involves differentiation of vegetative cells into hyper flagellated swarm cells that undergo rapid and coordinated population migration across solid surfaces. Vegetative cells at the colony margin differentiate into elongated, hyper flagellated swarm cells that assemble into multi-cellular rafts and migrate away from the colony (Fraser and Hughes, 1999). In contrast to other swarming organisms, there is no evidence that *P. aeruginosa* changes cell morphology and produces lateral flagella when swarming (Rashid and Kornberg, 2000). The findings of the swarming studies for *B. pseudomallei* here were similar to that for *P. aeruginosa* in that cell morphology did not change. However, the edge cells expressed higher numbers of flagella than the center cells of a colony on a swarm agar plate. It is likely that a co-ordinated process of flagella expression in occurring during this process.

One reason why colony size and flagella expression was examined after plating from the center or the edge of a primary swarm colony was to determine whether bacteria carry across with them a high (edge cells) or low (center cells) motility phenotype. There was no evidence for this. Rather, bacteria on the edge behave the same regardless of whether they have been derived from the edge or center of a colony. The same was true for bacteria sampled from the center of a colony. Thus, regulatory changes that result in high or low rates of flagella expression are short lived. Bacteria sampled from the center of the swarm colony produced almost no flagella and when present these are usually a single unipolar flagellum. Bacteria from the edge of the colony frequently expressed unipolar flagellum, but these were also expressed in lateral and peritrichous positions.

Flagella are commonly recognized as important virulence determinants expressed by bacterial pathogens since the motility phenotypic imparted by these organelles often correlates with the ability of an organism to cause disease (Penn and Luke, 1992). Previous studies have demonstrated that a significant degree of size and

antigenic homogeneity amongst flagellin proteins expressed by *B. pseudomallei* isolates. Furthermore, Brett *et. al.* have shown that flagellin specific antiserum is capable of passively protecting diabetic infant rats against a *B. pseudomallei* challenge (Brett *et al.*, 1994). DeShazer *et al.* reported that there was no significant difference between the virulence capacities associated with a wild-type strain of *B. pseudomallei* and non-motile mutants in either the diabetic infant rat or Syrian hamster models of infection (DeShazer *et al*., 1997). This suggests that flagella and/or motility may not be major virulence determinant in the pathogenesis of melioidosis, although purified flagellin may still serve as a protective immunogen against *B. pseudomallei* infections (Brett and Woods, 1996). An intact flagellar apparatus assists *B. pseudomallei* entry in to *A. astronyxis*, but it is unclear how this relates to disease in humans (Inglis *et al.*, 2003). However, flagella were an important virulence determinant of *B. pseudomallei* during intranasal and intraperitoneal infection of mice (Chua *et al*., 2003). Thus, it is currently unclear whether flagella are, or are not, *B. pseudomallei* virulence determinants.

From the assays of motility as defined by RTM-3 and the comparison with data for presence and position of flagella, it was observed here that the form of progressive movement terms ‘waving’ was associated with the presence of flagella.

Flagella expression and motility by isogenic colony variants of *B. pseudomallei*

Different colony morphotypes of *B. pseudomallei* are likely to represent adaptive changes of phenotype that occur in response to environmental stimuli. This can occur by a process of switching, in which one type can change to another. Human melioidosis is very slow to respond to treatment as defined by a prolonged fever clearance time and the need for prolonged courses of antibiotics. It is also difficult to eradicate the bacterium, as defined by the rate of recurrent infection, which is around 6% in the first year in survivors. *B. pseudomallei* isolated from patients with melioidosis commonly have mixed colony morphologies, and so colony morphotype switching and variation in phenotype may have clinical significance.

Here, it was found that isogenic Type VI, which has a rugose morphotype, had a reduction in flagella expression during exponential growth phase in broth compared with parental Type I, but there was no significant difference observed during

stationary growth phase. Conversely, Types III and VI expressed more flagella than parental Type I after growth on swarm agar for 72h. This suggests that biological behaviour differs between isogenic colony morphotypes, but that this is highly influenced by external stimuli and conditions. In *Vibrio cholerae*, flagella gene expression is significant lower in the rugose phase variant compared with the smooth variant (Fitnat et al., 2004). This involved genes in class III and VI, which are involved in flagella assembly. Switching of flagella expression has obvious advantages in terms of energy conservation when the bacteria are in an environment in which motility does not confer an advantage.

CHAPTER 6

CONCLUSIONS

B. pseudomallei demonstrated swimming and swarming motility *in vitro*, but failed to demonstrate twitching motility. The presence of motility was associated with the expression of flagella. A large population of clinical *B. pseudomallei* isolates were tested for swimming and swarming. Swimming was highly conserved. The rate of swimming showed a normal distribution as defined by colony diameter on swim plate. The colony appearance on swim agar was conserved across the population. Swarm motility was not conserved in all strains. The colony appearance differed both within and between strains and could be divided into 5 types according to colony characteristics. The majority of isolates (51%) were Type E (irregular edge, rough surface of centre of colony, and transparent colony at leading edge). Thus biological behaviour of bacterial cells may vary, even within a given colony of a single isolate.

Flagella staining using a modified Ryu's stain and viewing by the RTM-3 microscope proved to be an effective method for enumeration and to determine the bacterial position of flagella. This is also possible by light microscopy, but the RTM™ provided the opportunity to store permanent images of the slides. Flagella expression during bacterial growth in broth was very low in lag and early exponential phase, peaking in late logarithmic phase of growth after which expression declined. Flagella expression differed for bacterial cells taken from the center of a colony growing on swarm agar compared with cells from the colony edge. Overall, 84 % of center cells expressed no flagella, 12 % had one flagellum, and 5 % expressed >1 flagella. Overall, 36 % of edge cells expressed no flagella, 31 % one flagella and 34 % expressed >1 one flagella. There was an increase in the frequency of peritrichous flagella as the number of flagella per bacterium increased.

The dominant movement for bacterial cells taken from the center of a swarm colony was tumbling. The dominant movement for bacterial cells taken from the edge of a swarm colony was waving (forward progressive motion). There was an association between flagella expression and waving motility, and it seems likely that the two are directly related. Tumbling motility may represent Brownian movement since most bacterial cells from the center of a swarm colony are aflagellate.

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APPENDIX

APPENDIX A

Appendix A-1: Composition and preparation for flagella stain

Modified Ryu's flagella Stain

Composition

Staining solution I (mordant):

5% carbolic acid (phenol) solution	10 ml
Tannic acid	2 g
Aluminum potassium sulfate, 12 hydrate saturated aqueous solution	10 ml

Staining solution II (stain):

saturated solution of crystal violet in ethanol	12 g per100 ml

Preparation

Final stain:

Solution I	10 parts
Solution II	1 part

Store at ambient temperature, dispense from a syringe fitted with a 0.22 um porous membrane filter before use.

Formalin for fixative bacterial cells

50 ul of formalin added to 500 ul of sample

Appendix A-2: Composition and preparation of bacteria medium

Ashdown's Agar (in liter)

Composition

Trypticase soy broth	10 g
Agar	15 g
Glycerol	40 ml
0.1% (w/v) Crystal violet	5 mg
1% Neutral red	5 ml
DW.	950 ml

Preparation

Autoclave at 121^{0}C for 15 mins. Cool to 50^{0}C and add gentamicin to a final concentration of 5 mg/l. The new stock of 0.1% crystal violet solution needs to be incubated at 37^{0}C for at least 7 days before use.

Swim plates (in liter)

Composition

Tryptone	10 g
NaCl	5 g
Bacto agar	3 g
DW.	1000 ml

Preparation

Autoclave at 121^{0}C for 15 mins. 15 ml of sterile swim medium at 50^{0}C is poured into a sterile Petri dish should not be stored upside down.

Swarm plates (in litre)

Composition

Nutrient broth	8 g
Dextrose	5 g
Bacto agar	5 g
DW.	1000 ml

Preparation

Autoclave at 121^{0}C for 15 mins. 15 ml of sterile swarm medium at 50^{0}C is poured into a sterile Petri dish. Swarming plates are allowed to dry at room temperature overnight before being used.

Twitch plates (in litre)

Composition

Bacto agar	15 g
LB medium	20 g
DW.	1000 ml

Preparation

Autoclave at 121^{0}C for 15 mins. 18 ml of sterile swarm medium at 50^{0}C is poured into a sterile Petri dish, at ~3 mm agar thickness.

APPENDIX B

Appendix B: Database of population studies in *Burkholderia pseudomallei* 212 isolates

NO.	Ubon	Strain	Specimen	Date
1	U3032	2918a	Sputum	14-Sep-02
2	U3034	2919a	Right ankle pus	17-Sep-02
3		2919e*	Wound swab	5-Oct-02
4	U3035	2920a	Blood culture	17-Sep-02
5	OH	2921a	Pus	16-Sep-02
6	U3036	2922a	Pus from knee	18-Sep-02
7	U3037	2923a	Sputum	19-Sep-02
8	U3038	2924a	Tracheal suction	22-Sep-02
9	U3039	2925a	Blood culture	21-Sep-02
10	U3040	2926a	Knee pus	25-Sep-02
11	U3041	2927a	Blood culture	24-Sep-02
12	U3042	2928a	Pus	23-Sep-02
13	U3043	2929a	Tracheal suction	24-Sep-02
14	U3044	2930a	Sputum	26-Sep-02
15	U3045	2931a	Blood culture	20-Sep-02
16	OH	2932a	Blood culture	25-Sep-02
17	U3046	2933a	Pus from back	27-Sep-02
18	U3047	2934a	Liver pus	2-Oct-02
19	U3048	2935a	Wound swab	30-Sep-02
20	U3049	2936a	Throat swab	1-Oct-02
21	U3050	2937a	Blood culture	2-Oct-02

NO.	Ubon	Strain	Specimen	Date
22	U3051	2938a	Urine	3-Oct-02
23	U3053	2939a	Blood culture	4-Oct-02
24	U3052	2940a	Blood culture	5-Sep-02
25	U3054	2941a	Blood culture	7-Oct-02
26	U3056	2943a	Blood culture	12-Oct-02
27	U3058	2944a	Throat swab	15-Oct-02
28	U3059	2945a	Right knee aspirate	18-Oct-02
29	U3060	2946a	Thigh aspirate	21-Oct-02
30	U3061	2947a	Blood culture	18-Oct-02
31	U3062	2948a	Urine	22-Oct-02
32		2948b	Tracheal suction	23-Oct-02
33	U3063	2949a	Blood culture	28-Oct-02
34	U3064	2950a	Blood culture	29-Oct-02
35	U3065	2951a	Brain pus	1-Nov-02
36	U3066	2952a	Thigh pus	25-Oct-02
37	U3067	2953a	Tracheal suction	2-Nov-02
38	U3068	2954a	Throat swab	3-Nov-02
39	U3069	2955a	Sputum	5-Nov-02
40	U3070	2956a	Blood culture	7-Nov-02
41	U3071	2957a	Blood culture	18-Nov-02
42	U3072	2958a	Urine	23-Nov-02
43	U3073	2959a	Blood culture	26-Nov-02
44	U3074	2960a	Blood culture	28-Nov-02
45	U3075	2961a	Pus	29-Nov-02
46	U3076	2962a	Blood culture	29-Nov-02
47	U3077	2963a	Blood culture	28-Nov-02

NO.	Ubon	Strain	Specimen	Date
48	U3078	2964a	Pus	27-Nov-02
49	U3079	2965a	Splenic pus	29-Nov-02
50	U3080	2966a	Blood culture	1-Dec-02
51	U3081	2967a	Blood culture	5-Dec-02
52	U3082	3068a	Blood culture	14-Dec-02
53	U3083	2969a	Blood culture	24-Dec-02
54	U3084	2970a	Pus	16-Dec-02
55	U3085	2971a	Urine	19/12/002
56	U3086	2972a	Sputum	22-Dec-02
57		2972b	Blood culture	22-Dec-02
58	U3087	2973a	Blood culture	24-Dec-02
59	U3088	2974a	Blood culture	27-Dec-02
60	U3089	2975a	Liver pus	19-Dec-02
61	U3090	2976a	Blood culture	4-Jan-03
62	U3091	2977a	Blood culture	4-Jan-03
63	U3092	2978a	Blood culture	6-Jan-03
64	U3093	2979a	Pus	9-Jan-03
65	U3094	2980a	Blood culture	10-Jan-03
66	U3095	2981a	Liver pus	18-Jan-03
67	U3096	2982a	Blood culture	14-Jan-03
68	U3097	2983a	Blood culture	3-Jan-03
69	U3098	2984a	Pleural fluid	29-Jan-03
70	U3099	2985a	Blood culture	15-Nov-02
71	U3100	2986a	Blood culture	5-Feb-03
72	U3101	2987a	Pleural fluid	24-Jan-03
73	U3102	2988a	Blood culture	8-Feb-03

NO.	Ubon	Strain	Specimen	Date
74	U3103	2989a	Blood culture	14-Feb-03
75	U3104	2990a	Sputum	3-Mar-03
76	U3130	2991a	Pus	7-Mar-03
77	U3105	2992a	Sputum	11-Mar-03
78	U3106	2993a	Blood culture	12-Mar-03
79	U3107	2994a	Blood culture	18-Mar-03
80	U3108	2995a	Pus	22-Mar-03
81	U3109	2996a	Blood culture	29-Mar-03
82	U3110	2997a	Blood culture	1-Apr-03
83	U3111	2998a	Blood culture	8-Apr-03
84	U3112	2999a	Pleural fluid	16-Apr-03
85	U3113	3000a	Pus	15-Apr-03
86	U3114	3001a	Blood culture	28-Apr-03
87	U3115	3002a	Pus	10-May-03
88	U3116	3003a	Pus	9-May-03
89	U3117	3004a	Blood culture	6-May-03
90	U3118	3005a	Blood culture	10-May-03
91	U3119	3006a	Blood culture	8-May-03
92	U3120	3007a	Blood culture	10-May-03
93	U3121	3008a	Blood culture	11-May-03
94	U3122	3009a	Blood culture	14-May-03
95	U3125	3010a	Pus	22-May-03
96	U3126	3011a	Blood culture	26-May-03
97	U3127	3012a	Pus	27-May-03
98	U3128	3013a	Sputum	27-May-03
99	U3130	3014a	Left hip pus	27-May-03

NO.	Ubon	Strain	Specimen	Date
100	U3131	3015a	Ankle tap	1-Jun-03
101	U3132	3016a	Blood culture	3-Jun-03
102	U3133	3017a	Blood culture	5-Jun-03
103	U3134	3018a	Blood culture	6-Jun-03
104	U3135	3019a	Right parotid pus	7-Jun-03
105	U3136	3020a	Right parotid pus	8-Jun-03
106	U3139	3021a	Pus	10-Jun-03
107	U3140	3021b	Parotid pus	14-Jun-03
108	U2958	3022a	Blood culture	10-Jun-03
109	U3137	3023a	Blood culture	9-Jun-03
110	U3138	3024a	Blood culture	11-Jun-03
111	U3140	3025a	Pus	15-Jun-03
112	U3140	3025b	Blood culture	15-Jun-03
113	U3141	3026a	Blood culture	15-Jun-03
114	U3142	3027a	Blood culture	17-Jun-03
115	U3143	3028a	Pus	17-Jun-03
116	U3145	3029a	Pus	18-Jun-03
117	U3146	3030a	Urine	9-Jun-03
118	U3147	3031a	Right parotid pus	20-Jun-03
119	U3148	3032a	Blood culture	19-Jun-03
120	U3149	3033a	Blood culture	20-Jun-03
121	U3150	3034a	Blood culture	22-Jun-03
122	U3151	3035a	Blood culture	22-Jun-03
123	U3152	3036a	Pus	22-Jun-03
124	U3153	3037a	Urine	25-Jun-03
125	U3154	3038a	Tracheal suction	27-Jun-03

NO.	Ubon	Strain	Specimen	Date
126	U3155	3039a	Blood culture	28-Jun-03
127	U3156	3040a	Blood culture	29-Jun-03
128	U3157	3041a	Sputum	2-Jul-03
129	U3159	3042a	Left frontal pus	4-Jul-03
130	U3160	3043a	Throat swab	3-Jul-03
131	U3161	3044a	Blood culture	2-Jul-03
132	U3162	3045a	Blood culture	3-Jul-03
133	U3163	3046a	Left parotid pus	2-Jul-03
134	U3164	3047a	Tibia aspirate	7-Jul-03
135	U3165	3048a	Tracheal suction	8-Jul-03
136	U3166	3049a	Urine	6-Jul-03
137	U3167	3050a	Right parotid pus	7-Jul-03
138	U3169	3051a	Blood culture	10-Jul-03
139	U3170	3052a	Blood culture	10-Jul-03
140	U3171	3053a	Blood culture	10-Jul-03
141	U3172	3054a	Sputum	12-Jul-03
142	U3173	3055a	Blood culture	10-Jul-03
143	U3168	3056a	Tracheal suction	9-Jul-03
144	U3174	3057a	Blood culture	11-Jul-03
145		3057b	Blood culture	12-Jul-03
146	U3176	3058a	Urine	15-Jul-03
147	U3177	3059a	Throat swab	14-Jul-03
148	U3178	3060a	Sputum	16-Jul-03
149	U3179	3061a	Blood culture	15-Jul-03
150	U3180	3262a	Blood culture	18-Jul-03
151	U3181	3063a	Tracheal suction	17-Jul-03

NO.	Ubon	Strain	Specimen	Date
152	U3182	3064a	Sputum	17-Jul-03
153	U3183	3065a	Blood culture	11-Jul-03
154	U3184	3066a	Blood culture	17-Jul-03
155	U3185	3067a	Throat swab	18-Jul-03
156	OH	3068a	Pus	18-Jul-03
157	U3186	3069a	Blood culture	21-Jul-03
158	U3187	3070a	Blood culture	22-Jul-03
159	U3188	3071a	Blood culture	24-Jul-03
160	U3189	3072a	Blood culture	24-Jul-03
161	U3191	3073a	Sputum	24-Jul-03
162	U3192	3074a	Right groin pus	24-Jul-03
163	U3193	3075a	Blood culture	21-Jul-03
164	U3194	3076a	Blood culture	21-Jul-03
165	U3195	3077a	Right frontal pus	25-Jul-03
166	U3196	3088a	Urine	27-Jul-03
167	U3197	3079a	Liver pus	25-Jul-03
168	U3198	3080a	Right foot wound swab	25-Jul-03
169	U3199	3081a	Parotid pus	29-Jul-03
170	U3200	3082a	Sputum	29-Jul-03
171	U3201	3083a	Blood culture	28-Jul-03
172	U3202	3084a	Parotid pus	1-Aug-03
173	U3204	3085a	Throat swab	1-Aug-03
174	U3215	3086a	Right knee aspirate	31-Jul-03
175	U3205	3087a	Blood culture	1-Aug-03
176	U3206	3088a	Blood culture	11-Aug-03
177	U3206	3088b	Blood culture	3-Aug-03

NO.	Ubon	Strain	Specimen	Date
178	U3208	3089a	Blood culture	3-Aug-03
179	U3209	3090a	Blood culture	4-Aug-03
180	U3212	3091a	Blood culture	5-Aug-03
181	OH	3092a	Pus	6-Aug-03
182	U3214	3093a	Right cheek wound swab	11-Aug-31
183	U3216	3094a	Blood culture	9-Aug-03
184	OH	3095a	Sputum	11-Aug-03
185	U3225	3096a	Lymph node pus	19-Aug-03
186	U3223	3097a	Sputum	19-Aug-03
187	U3224	3098a	Blood culture	19-Aug-03
188	U3227	3099a	Urine	20-Aug-03
189	U3226	3100a	Sputum	20-Aug-03
190	U3228	3101a	Elbow pus	20-Aug-03
191	U3229	3102a	Liver pus	26-Aug-03
192	U3230	3103a	Liver pus	26-Aug-03
193	OH	3104a	Blood culture	26-Aug-03
194	U3231	3105a	Blood culture	26-Aug-03
195		3105b	Urine	29-Aug-03
196	U3202	3106a	Blood culture	24-Aug-03
197		3106b	Throat swab	29-Aug-03
198	U3233	3107a	Right parotid pus	26-Aug-03
199	OH	3108a	Pus	26-Aug-03
200	U3235	3109a	Blood culture	28-Aug-03
201	U3236	3110a	Sputum	29-Aug-03
202	U3237	3111a	Liver pus	2-Sep-03
203	U3238	3112a	Blood culture	31-Aug-03

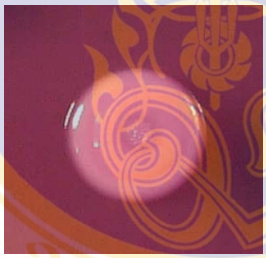
NO.	Ubon	Strain	Specimen	Date
204	U3239	3113a	Pus	1-Sep-03
205	U3240	3114a	Throat swab	2-Sep-03
206	U3241	3115a	Urine	4-Sep-03
207	U3241	3115b	Wound swab	4-Sep-03
208	U3242	3116a	Blood culture	3-Sep-03
209	U3243	3117a	Urine	3-Sep-03
210	U3245	3119a	Peritoneal fluid	3-Sep-03
211	U3246	3120a	Blood culture	4-Sep-03
212	U3252	3127a	Blood culture	10-Sep-03

APPENDIX C

Appendix C-1: Seven unique *B. pseudomallei* colony morphotypes on Ashdown's agar identified from 212 unselected cultures from patients with melioidosis. Figures represent proportion of each morphotype within this population.

Type I (75.1%)

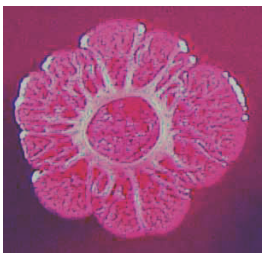
Type II (5.8%)



Type III (5.4%)

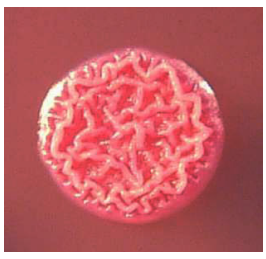
Type IV (5.0%)

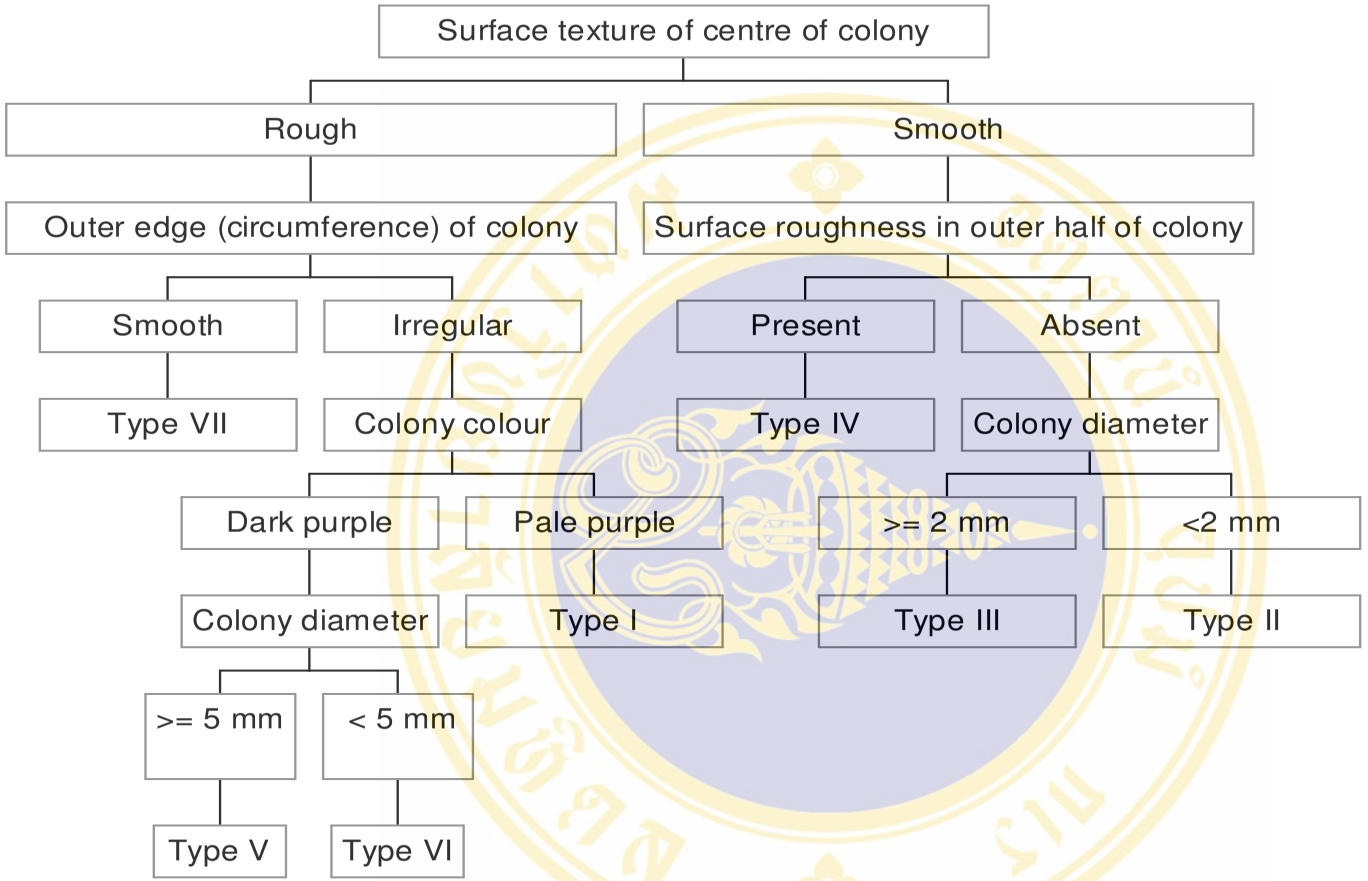
Type V (3.3%)



Type VI (3.7%)

Type VII (1.7%)



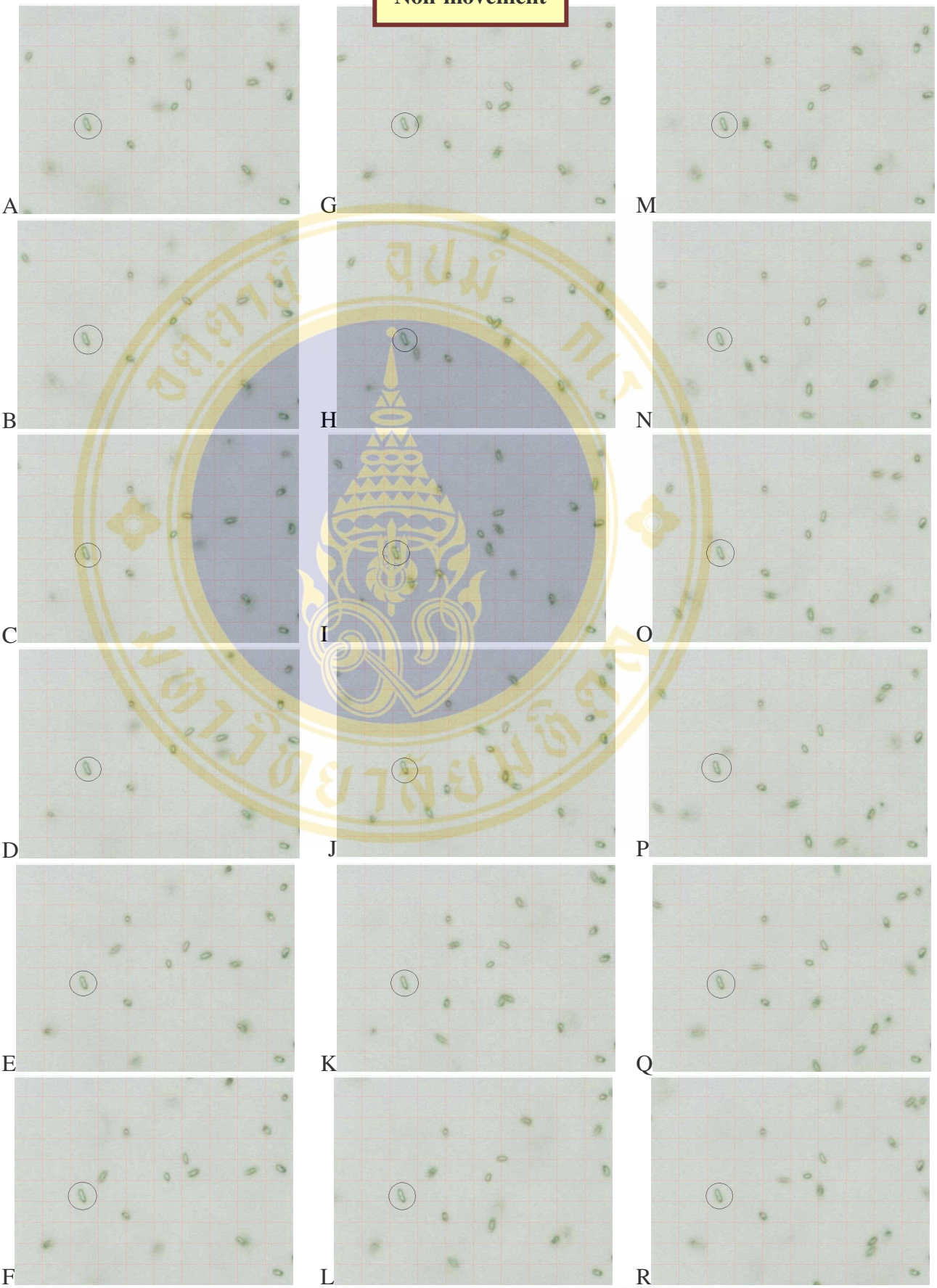


Appendix C-2: The regions (center and outer half) of the colony described above can best be thought of as a Mexican hat. Type V and VI are distinguished by size since Type V does not always have a central crater. Types II and III are distinguished from each other by size since the color of type III colony is not consistent.

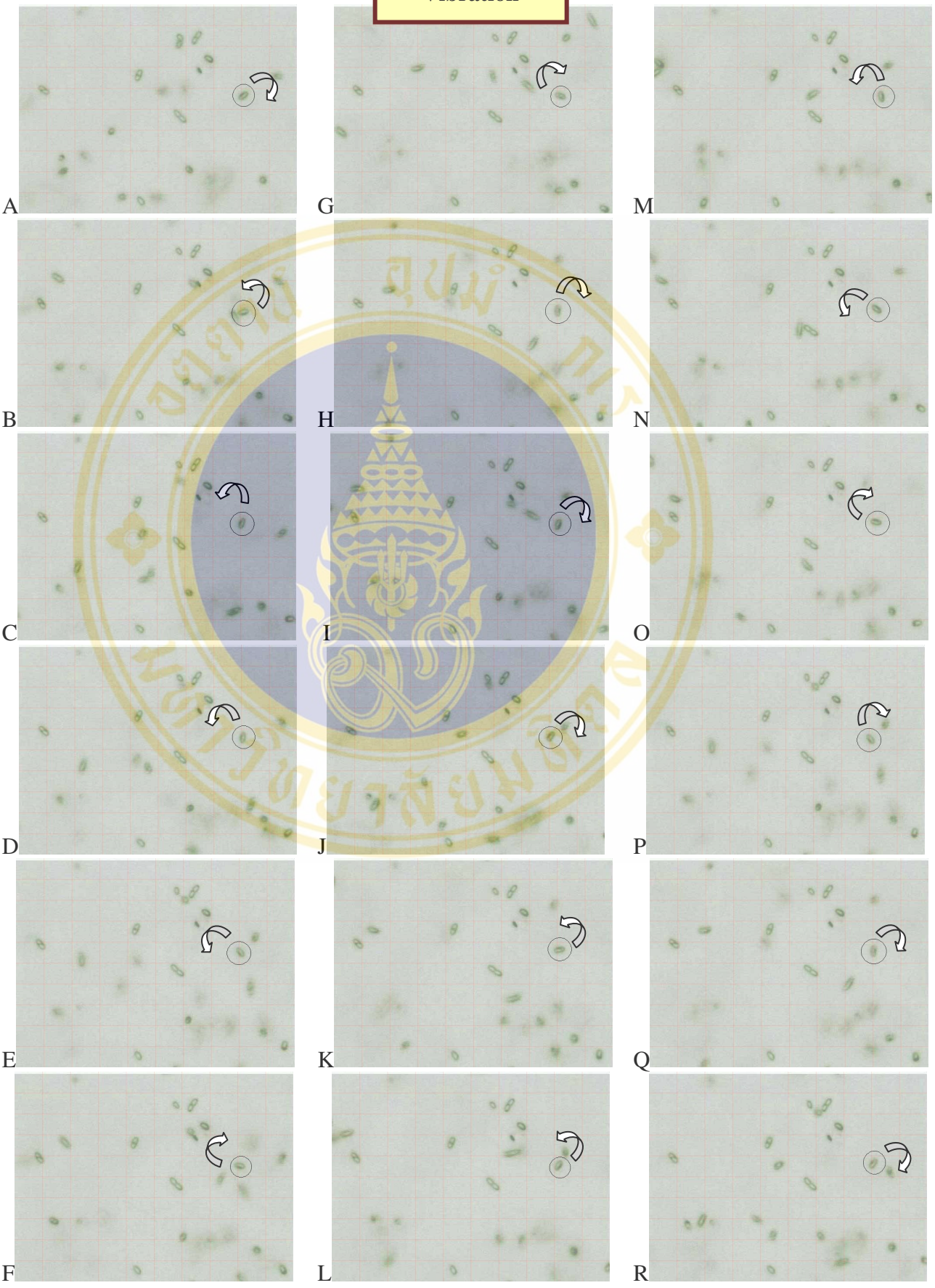
APPENDIX D

Appendix D: Bacterial movement pictures using RTM-3 time-lapse video microscopy. Frame A to R are at 1 second intervals. 5 types of bacterial movement were defined as: vibration, rotation, rotational displacement, tumbling and waving. Bacteria cells in circles are being followed; the arrow shows the direction of movement.

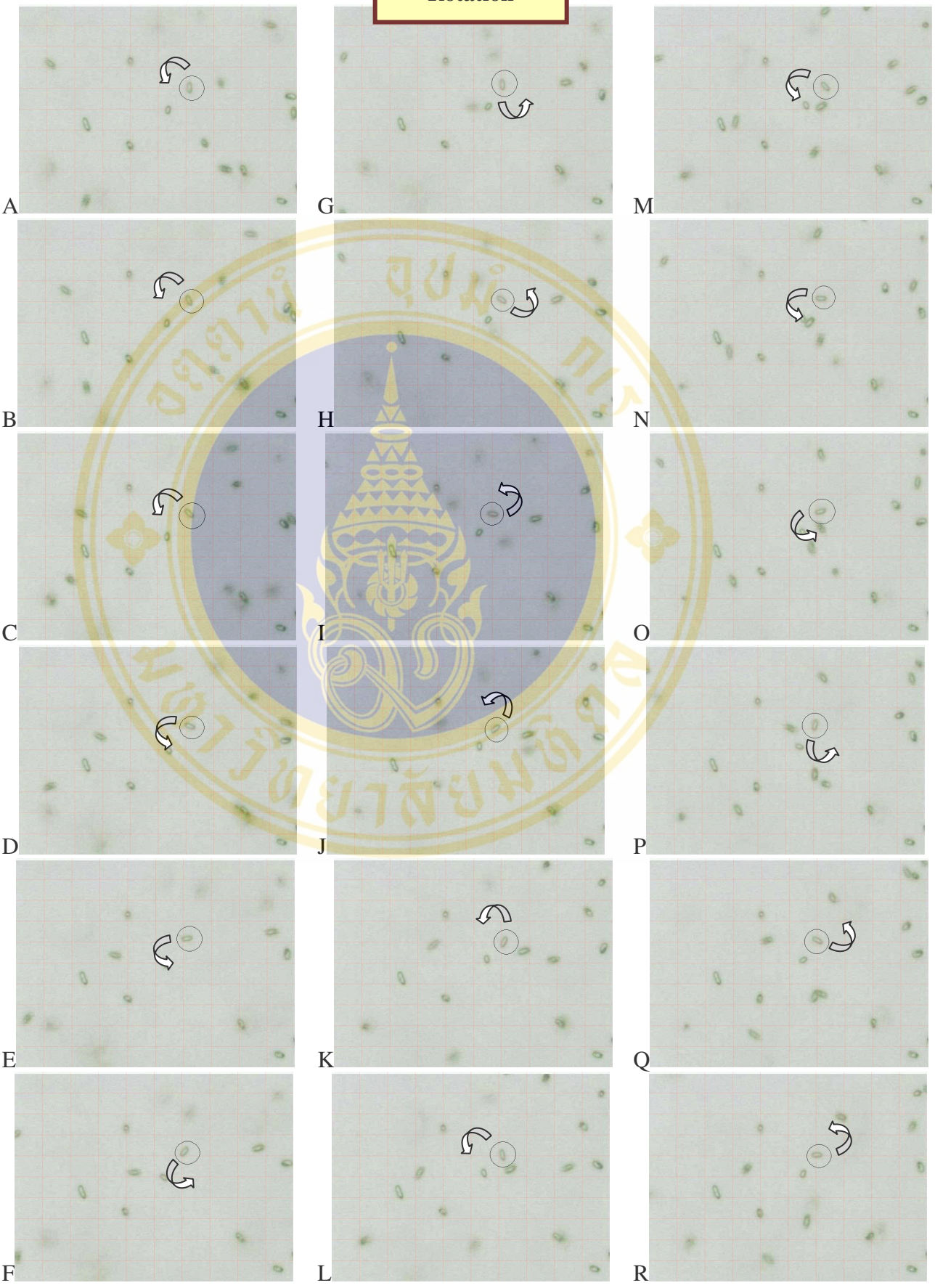
Non-movement



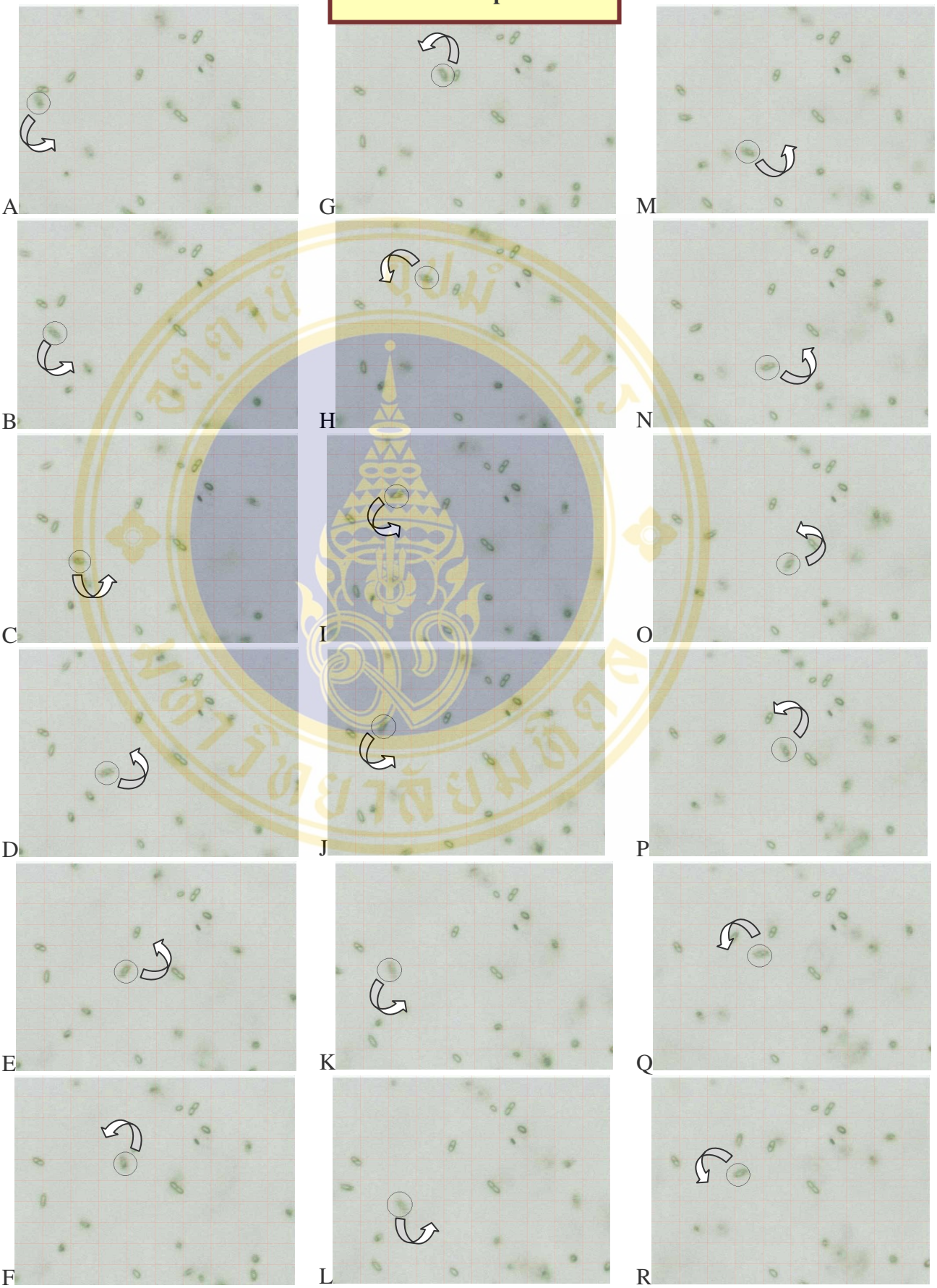
Vibration

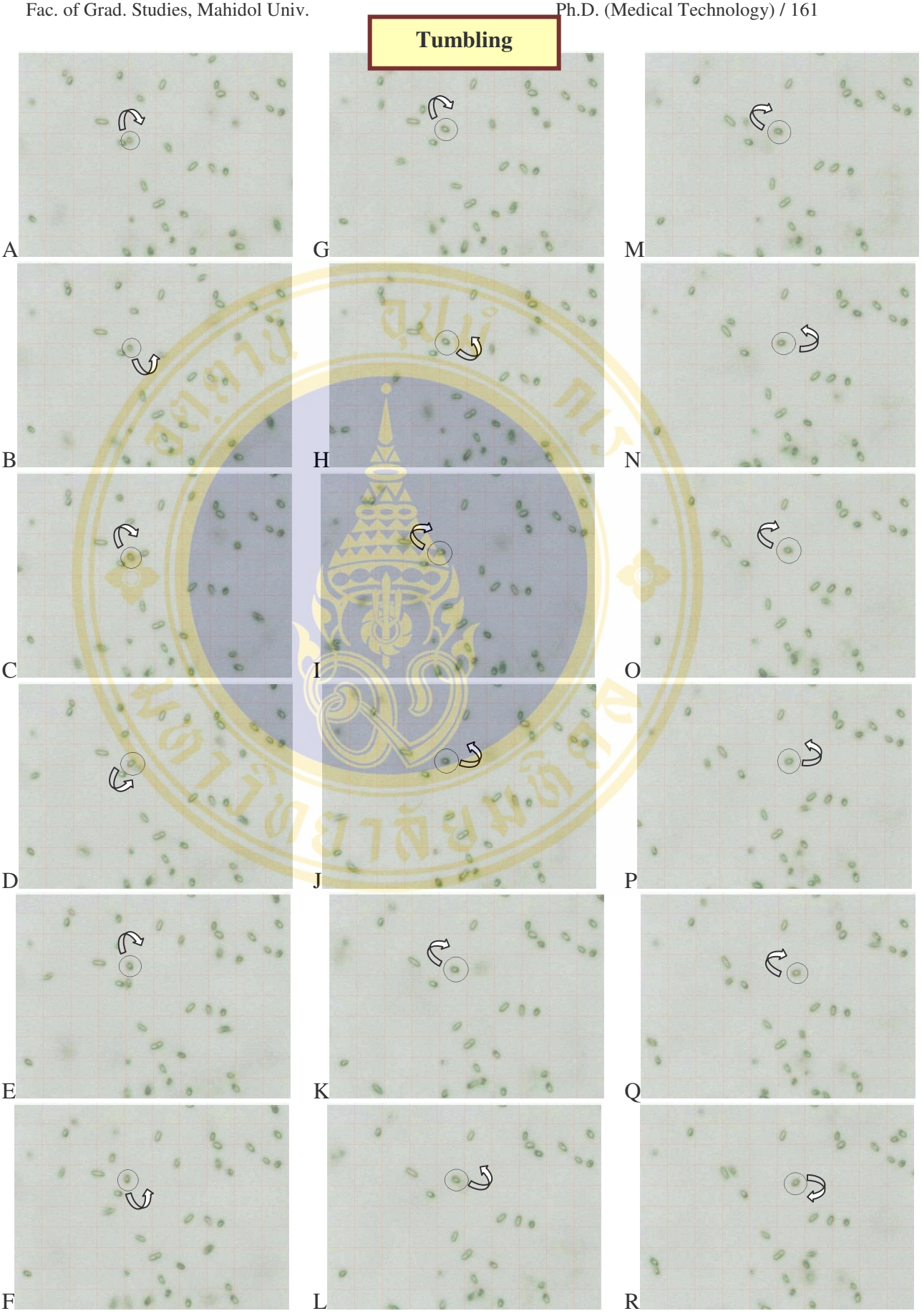


Rotation

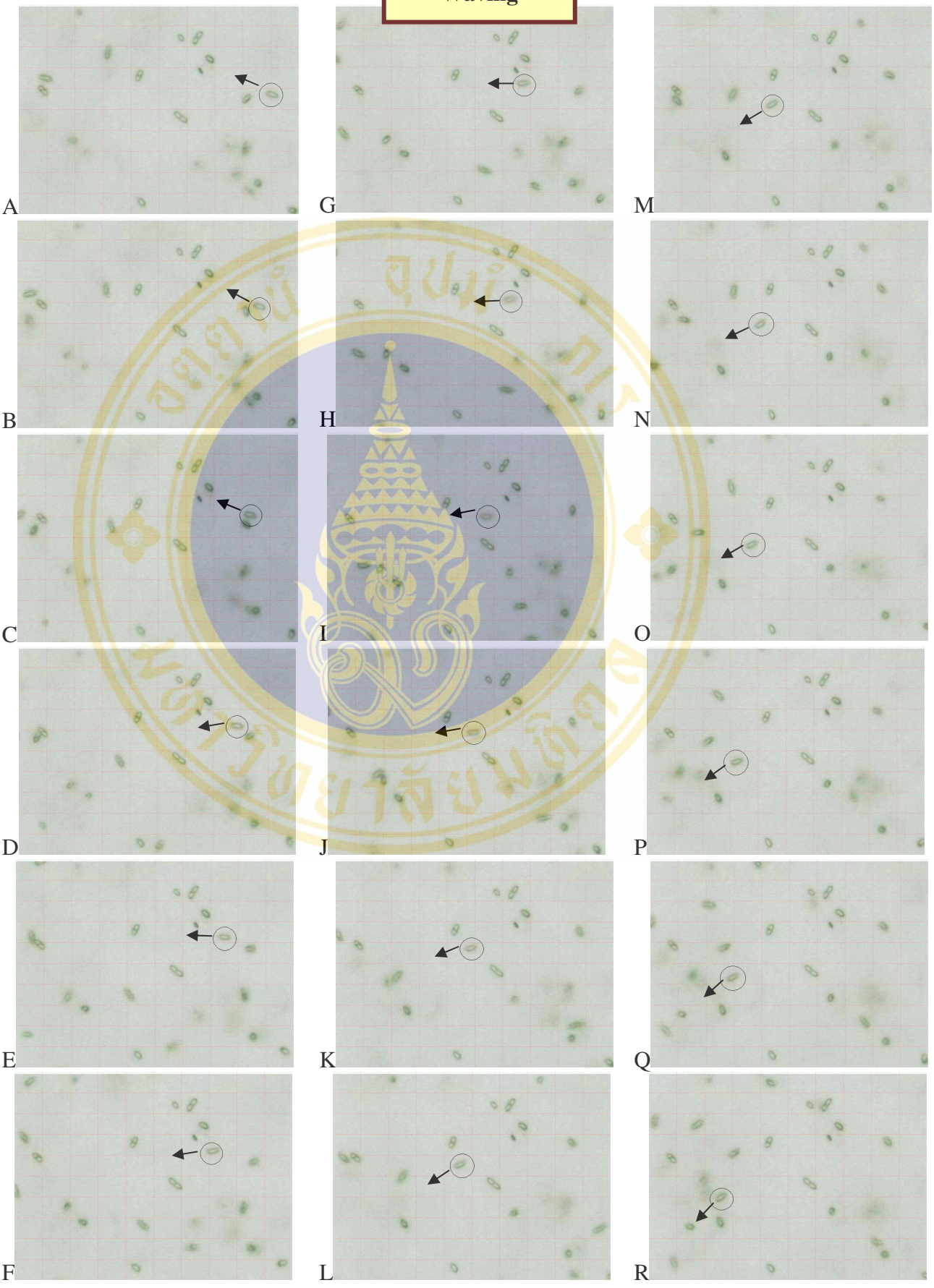


Rotational displacement





Waving



APPENDIX E

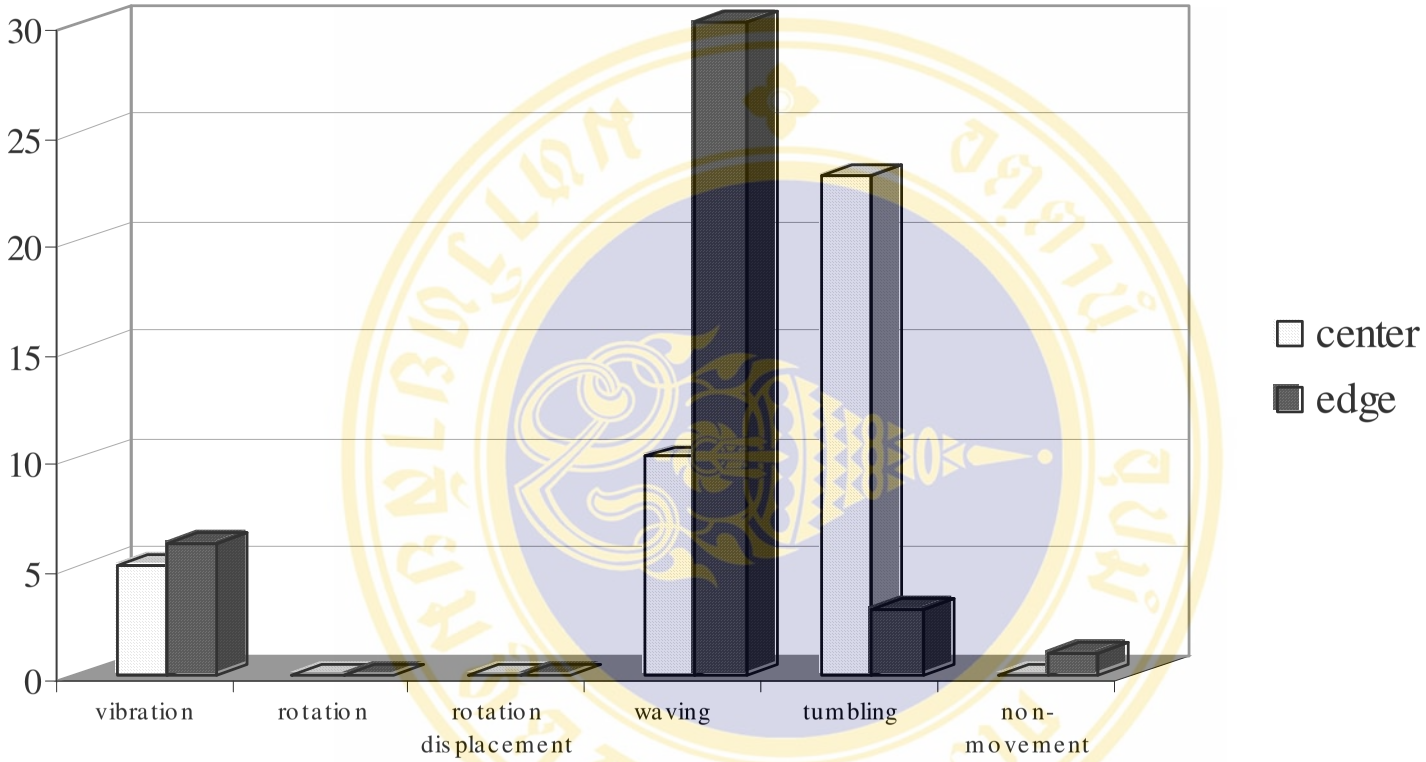
Appendix E: Number of observations and row proportions (95% confidence intervals for row proportions)

Experiment	No flagella	1 flagellum	2 flagella	3 flagella	4 flagella	5 flagella	6 flagella
Centre	3415	410	138	26	10	1	0
%	85.4%	10.2%	3.4%	0.65%	0.25%	0.02%	
95% CI	(80.6-89.1)	(7.8-13.3)	(2.2-5.4)	(0.4-1.1)	(0.1-0.5)	(0.003-0.2)	
Centre-centre	3226	555	163	41	8	5	2
%	80.6%	13.9%	4.1%	1.0%	0.2%	0.1%	0.05%
95% CI	(73.4-86.3)	(10.5-18.1)	(2.5-6.7)	(0.6-1.8)	(0.008-0.5)	(0.02-0.6)	(0.01-0.2)
Edge-centre	3387	457	115	34	6	0	1
%	84.7%	11.4%	2.9%	0.8%	0.1%		0.02%
95% CI	(78.4-89.4)	(8.2-15.7)	(1.8-4.6)	(0.4-1.8)	(0.04-0.5)		(0.03-0.2)
Edge	1572	1245	519	339	148	46	31
%	40.3%	31.9%	13.3%	8.7%	3.8%	1.2%	0.8%
95% CI	(32.5-48.6)	(27.5-36.7)	(10.9-16.1)	(6.5-11.6)	(2.4-5.8)	(0.6-2.3)	(0.4-1.5)
Edge-edge	1310	457	115	34	6	0	25
%	32.7%	29.3%	19.1%	10.8%	5.1%	2.2%	0.6
95% CI	(24.3-42.5)	(25.8-33.1)	(15.3-23.6)	(8.6-13.5)	(3.6-7.2)	(1.4-3.6)	(0.3-1.2)
Centre-edge	1262	1177	708	502	242	81	28
%	31.6%	29.4%	17.7%	12.6%	6.1%	2.0%	0.7%
95% CI	(22.7-42.0)	(25.3-33.9)	(14.3-21.8)	(9.7-16.1)	(4.2-8.7)	(1.2-3.3)	(0.3-1.4)

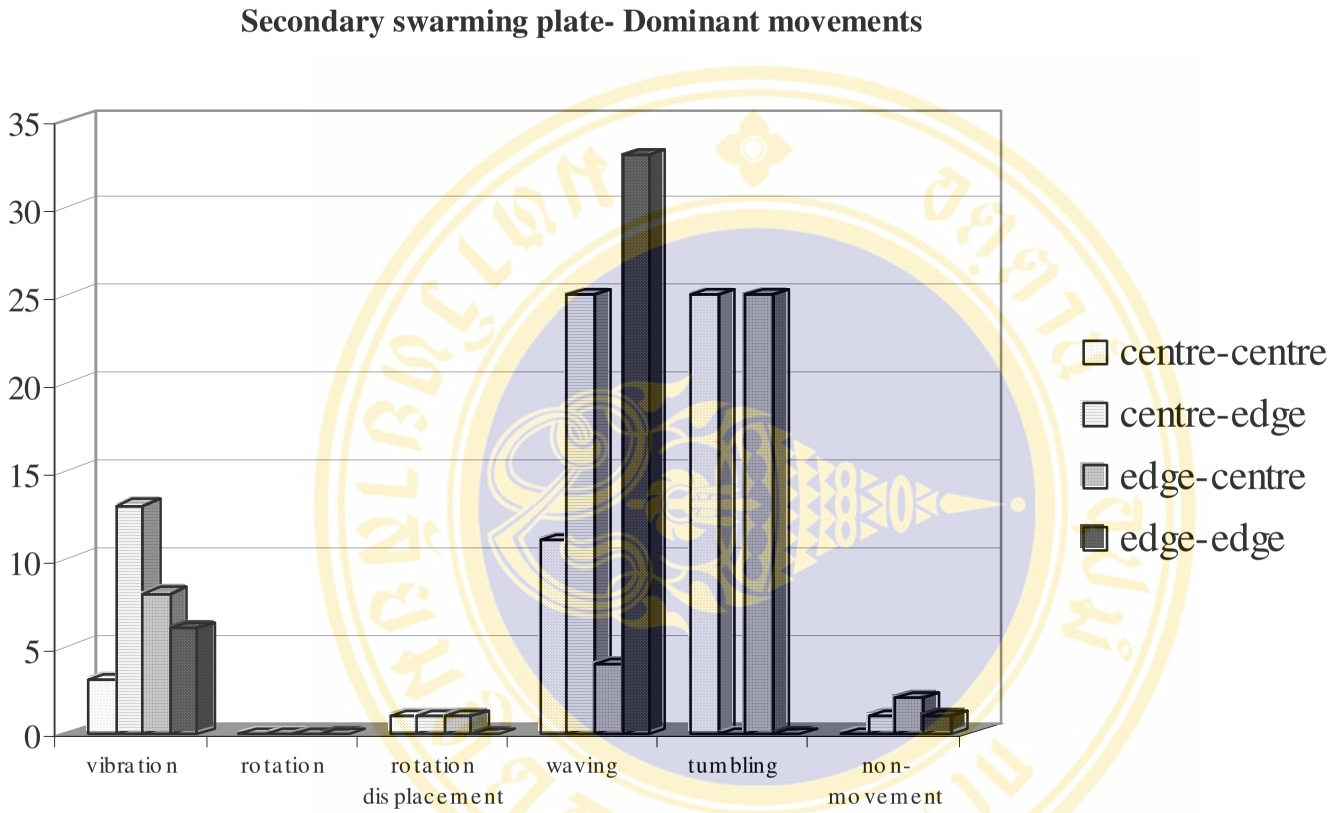
Experiment	No flagella	1 flagellum	2 flagella	3 flagella	4 flagella	5 flagella	6 flagella
Centre (combined)							
Number	10,028	1422	416	101	24	6	4
%	83.6%	11.8%	3.5%	0.8%	0.2%	0.05%	0.02%
95% CI	(80.2-86.5)	(10.0-14.0)	(2.6-4.6)	(0.6-1.2)	(0.1-0.3)	(0.03-0.2)	(0.008-0.08)
Edge (combined)							
Number	4,144	2595	1992	1272	596	217	84
%	34.8%	20.2%	16.7%	10.7%	5.0%	1.8%	0.7%
95% CI	(29.8-40.2)	(27.8-32.7)	(14.8-18.9)	(9.2-13.4)	(4.0-6.2)	(1.3-2.5)	(0.5-1.0)

APPENDIX F

Appendix F: Results of motility for 40 isolates of *Burkholderia pseudomallei*

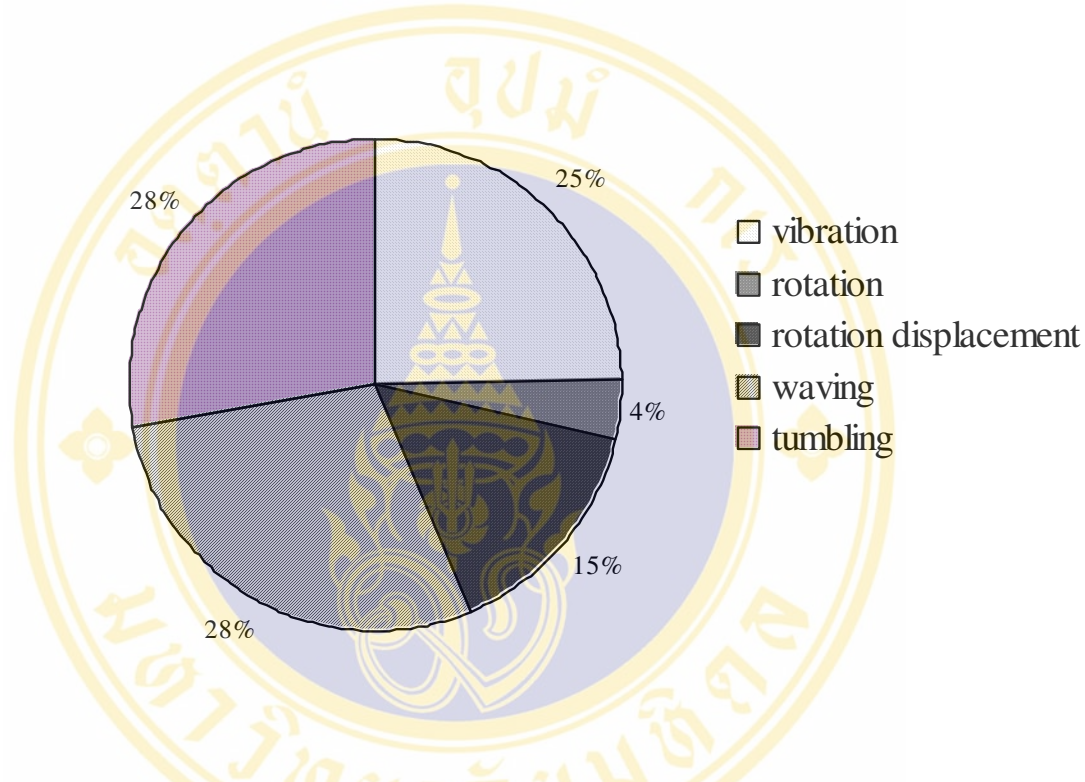


Appendix F-1: Results of the dominant movements of *B. pseudomallei* after sub-culture on a swarm plate for 72 hours at 37°C in air. A total of 100 cells were examined per strain. The type of movement is shown. The dominant movement for center cells is tumbling, and the dominant movement for edge cells is waving. In both type of cells there was no rotation or rotating displacement. For all the center cells, 1% of the edge cells had no movement, thus 99 % had one type of movement.



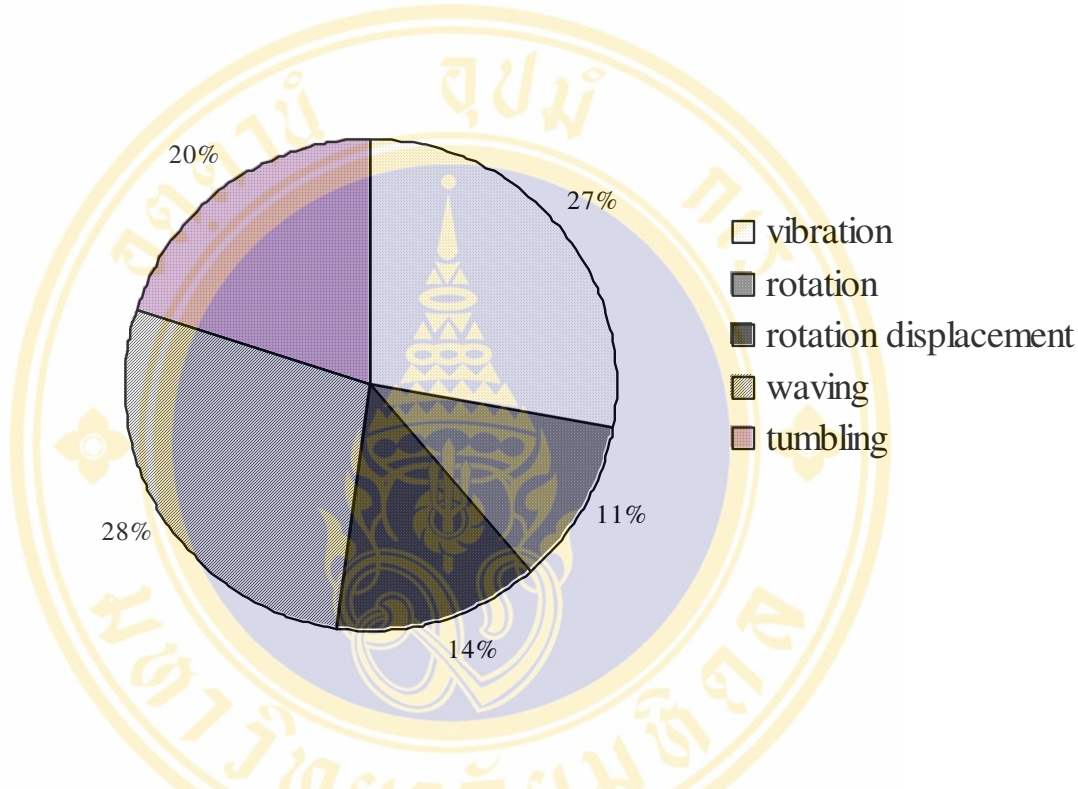
Appendix F-2: Results for the dominant movements of 40 isolates of *B. pseudomallei* after sub-culture on secondary swarm plates for 72 hours at 37^0C in air. A total of 100 cells were examined per strain. After 72 hours on the first swarm, bacteria were picked onto two secondary swarm plates and these were incubated at 37^0C in air for a further 72 hours. The dominant movement for center cells is tumbling and the dominant movement for edge cells is waving. In both type of cells there was no rotation.

Type of movement: First swarming plate- Centre



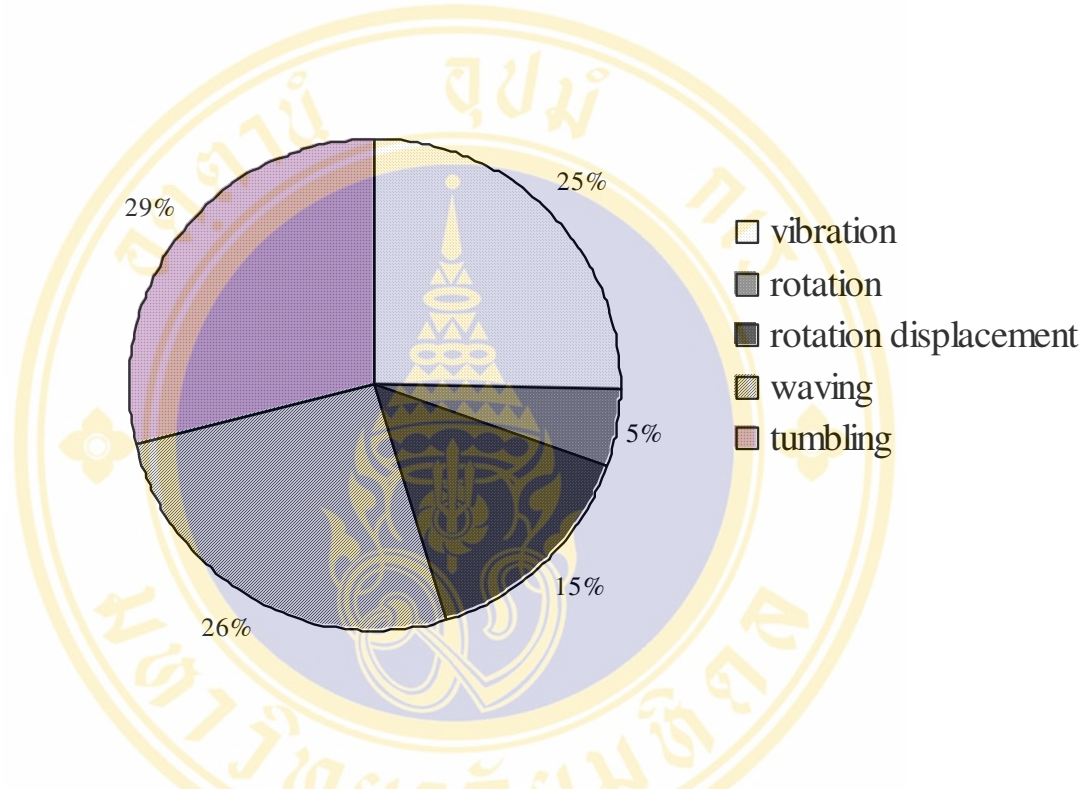
Appendix F-3: Results for the dominant movements of 40 isolates of *B. pseudomallei* after sub-culture on secondary swarm plates for 72 hours at 37^0C in air. A total of 100 cells were examined per strain. Most of the cells were waving or tumbling.

Type of movement: First swarming plate- Edge



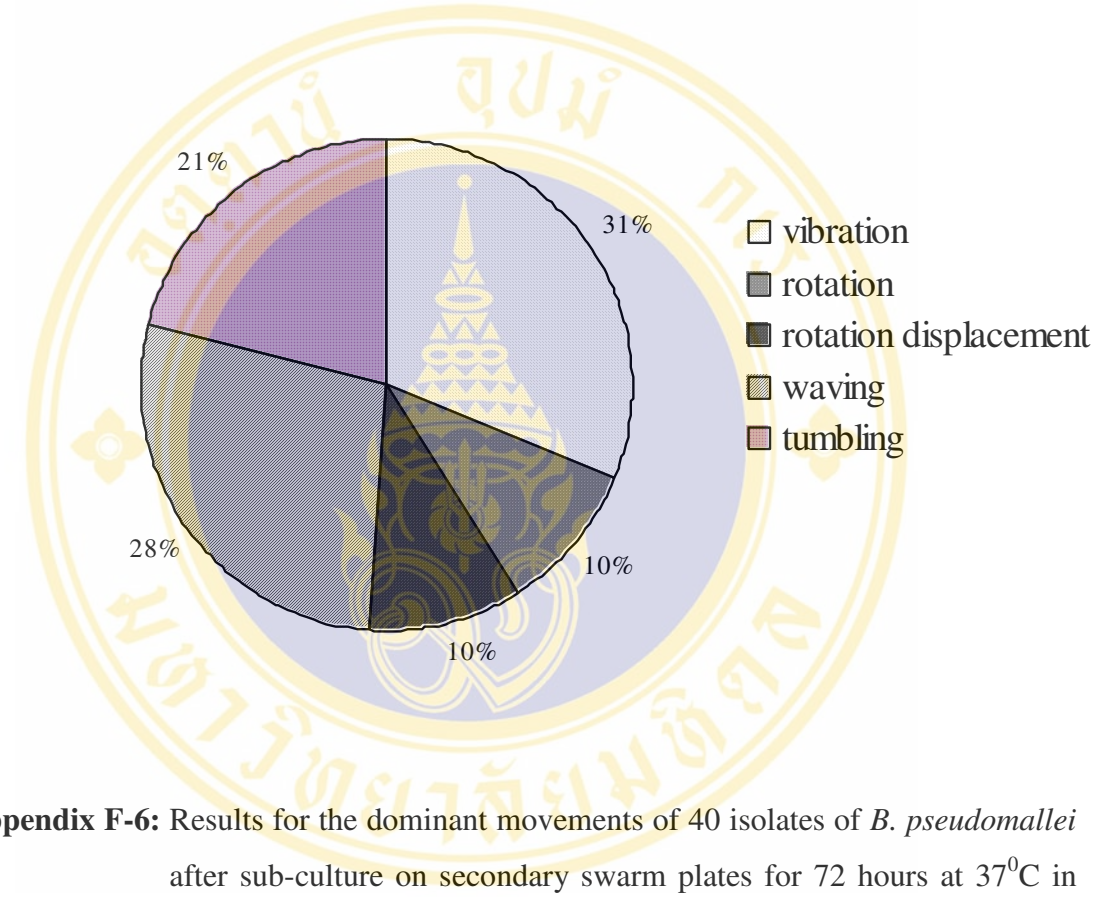
Appendix F-4: Results for the dominant movements of 40 isolates of *B. pseudomallei* after sub-culture on secondary swarm plates for 72 hours at 37^0C in air. A total of 100 cells were examined per strain. Most of the cells were waving or vibrating.

Type of movement: Second swarming plate- Centre-Centre



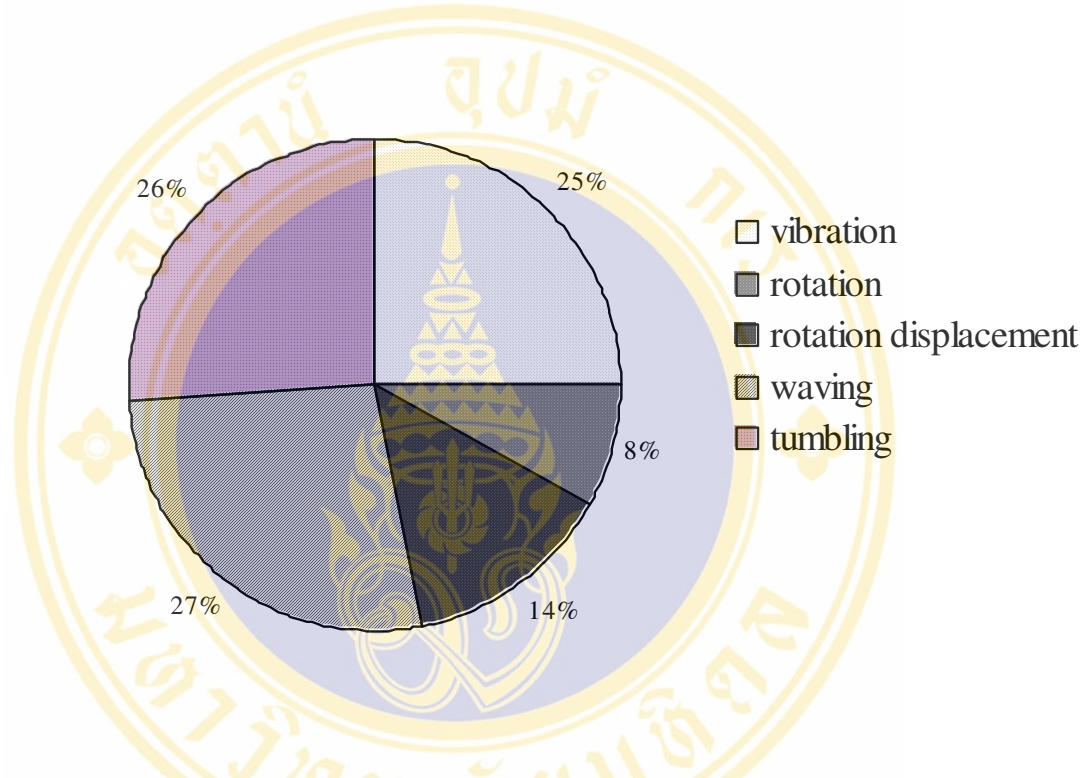
Appendix F-5: Results for the dominant movements of 40 isolates of *B. pseudomallei* after sub-culture on secondary swarm plates for 72 hours at 37^0C in air. A total of 100 cells were examined per strain. Most of the cells were tumbling, waving or vibrating.

Type of movement: Second swarming plate- Centre-Edge



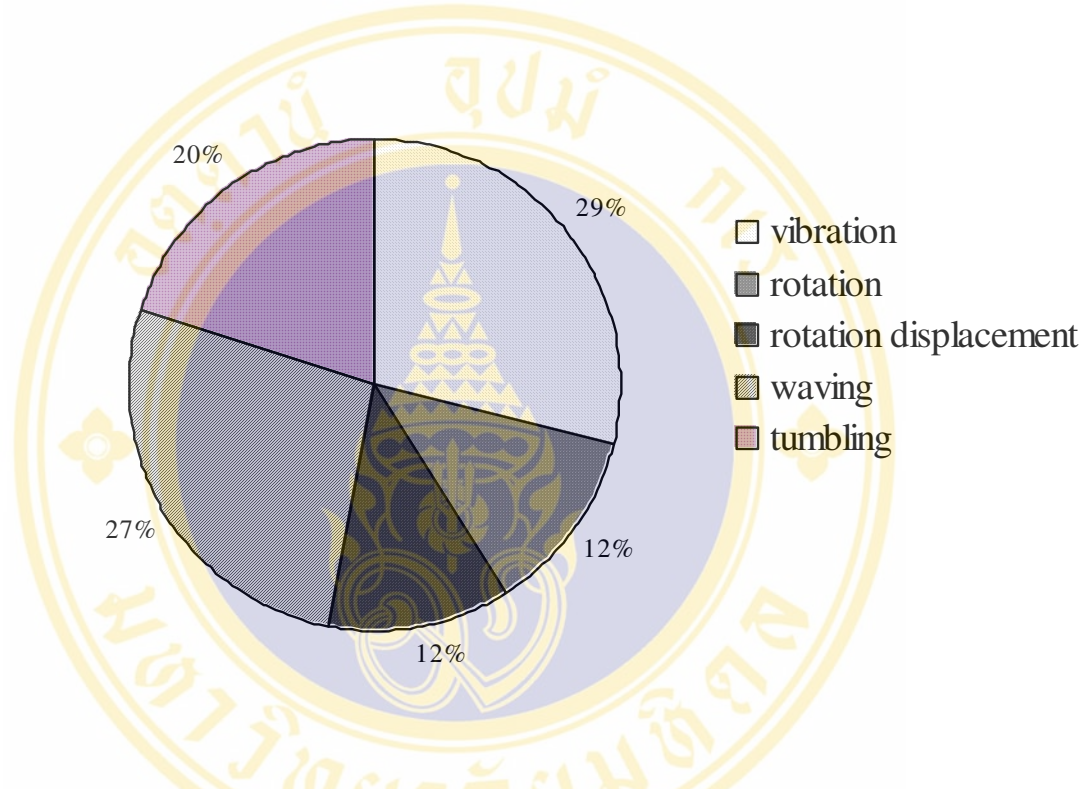
Appendix F-6: Results for the dominant movements of 40 isolates of *B. pseudomallei* after sub-culture on secondary swarm plates for 72 hours at 37^0C in air. A total of 100 cells were examined per strain. Most of the cells were waving or vibrating.

Type of movement: Second swarming plate- Edge-Centre



Appendix F-7: Results for the dominant movements of 40 isolates of *B. pseudomallei* after sub-culture on secondary swarm plates for 72 hours at 37^0C in air. A total of 100 cells were examined per strain. Most of the cells were tumbling, waving or vibrating.

Type of movement: Second swarming plate- Edge-Edge



Appendix F-8: Results for the dominant movements of 40 isolates of *B. pseudomallei* after sub-culture on secondary swarm plates for 72 hours at 37^0C in air. A total of 100 cells were examined per strain. Most of the cells were vibrating or waving.

BIOGRAPHY

NAME Miss Khaemaporn Boonbumrung

DATE OF BIRTH 7 January 1975

PLACE OF BIRTH Bangkok, Thailand

INSTITUTIONS ATTENDED Chulalongkorn University, 1995 :
Bachelor of Medical Technology
Chulalongkorn University, 1999 :
Master of Biotechnology

POSITION&OFFICE Faculty of Allied Health Science, Chulalongkorn University, Bangkok
Position: scientist
Tel. 0-2218-9832
E-mail: khaemaporn.b@chula.ac.th

HOME ADDRESS 100/112 Chonlada Village Soi 22, Bangkrau-sai-noi Road, Bang-baue-thong, Nonthaburi 11110, Thailand
Tel. 0-2924-0034