Molecular characterization of *Burkholderia pseudomallei* and its seroprevalence in Bangladesh

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Certificate

This is to certify that the thesis entitled "Molecular characterization of *Burkholderia pseudomallei* and its seroprevalence in Bangladesh" was carried out by Md. Shariful Alam Jilani, Registration number 132, and Session: 2010 – 2011 for the fulfillment of the degree of Doctor of Philosophy from the Department of Microbiology, University of Dhaka, Bangladesh.

This original work was carried out under our supervision and the style and contents of the thesis have been approved and recommended for the award of PhD degree.

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List of Abbreviations

%	Percentage
µl	Micro liter
µm	Micro metre
^{0}N	Degree North
16S	16 Svedberg
20^{0}C	20 degree Celsius
30kDa	30 Kilo Dalton
AHL	N-acyl-homoserine lactones
AI-2	Autoinducer 2
API	Analytical profile index
APIU	Asia-Pacific International University
ASA	Ashdown's selective agar
ASB	Ashdown's selective enrichment broth
BADAS	Diabetic Association of Bangladesh
BALB/c	albino, laboratory-bred strain of the House Mouse
BIRDEM	Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders.
BSA	Burkholderia secretion apparatus
BTFC	*B. thailandensis*-like flagellum and chemotaxis
C_{10}-HSL	N-octanoyl-homoserine-lactone
CD	Cluster of differentiation
cDNA	Complementary DNA
CD	Confidence interval
cm	Centimeter
CMI	Cell mediated immunity
CR1	Complement receptor – 1
CS	Clinical Sample

DM	Diabetes mellitus
DNA	Deoxyribonucleic acid
dNTP	Deoxy nucleotide tri phosphate
EIA	Enzyme immuno assay
ELISA	Enzyme linked immunosorbent assay
EPI	Emerging pathogens institute
EPS	Exo polysaccharide
ERC	The Ethical Review Committee
Fig	Figure
G-CSF	Granulocyte-colony stimulating factor
gm	Gram
HCP	Host cell protein
HeLa	Hela cell
HIV	Human Immunodeficiency Virus
HSL	Homoserine lactone
ICDDR, B	International Centre for Diarrheal Diseases and Research, Bangladesh
IFA	Immunofluorescence assay
IgG	Immunoglobulin G
IHA	Indirect haemagglutination assay
IMC	Ibrahim Medical College
iNOS	Inducible nitric oxide synthetase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
JIMC	Jahurul Islam Medical College
Km	Kilometer
LB	Luria broth
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
M	Male
MALVA	Multilocus variable number
$MgCl_2$	Magnesium chloride

MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
mm	Millimeter
mM	Millimolar
MMC	Mymensingh medical college
MNGC	Multinuclear giant cell
N/A	Not applicable
NCBI	National Center for Biotechnology Information
NDM–1	New Delhi Metallo ß-lactamase-1
NHN	National Healthcare Network
nm	Nanometer
OD	Optical density
OPS	O-antigenic polysaccharide
orf	Open reading frame
PAMP	Pathogen associated molecular pattern
PBP	Penicillin binding protein
PBST	Phosphate Buffered Saline with Tween 20
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pH	Molar concentration of Hydrogen
PRR	Pattern recognition receptor
QS	Quorum sensing
RAPD	Random amplified polymorphic DNA
RNA	Ribonucleic acid
RND	Resistance nodulation and cell division
Rpm	Rotation per minute
rRNA	Ribosomal RNA
RTFA	Recombinant truncated flagellin antigen
RT-PCR	Real-time polymerase chain reaction
SD	Standard deviation
Sec	Second

SOMC	Sylhet Osmani Medical college
ST	Sequence type
STM	Signature –tagged mutagenesis
SWCA	Sonicated whole cell antigen
T6SS	Type six secretion systems
TLR	Toll like receptor
TMB	Tetramethylbenzidine
TMP-SMX	Trimethoprim-Sulfamethoxazole
TRIF	TIR-domain-containing adapter-inducing interferon-β
tRNA	Transfer RNA
TSB	Trypticase Soya Broth
TTS1	Type three secretion system 1
TTSS3	Type three secretion system 3
UK	United Kingdom
USA	United States of America
USM	University Sains Malaysia
UT	Urinary tract
UV	Ultra violet
VNTR	Variable number of tandem repeat
X^2	Chi square
YLF	*Yersinia*-like fimbrial

Abstract

The Gram negative bacillus, *Burkholderia pseudomallei* causes melioidosis, a rare but serious infection that can be fatal if untreated or misdiagnosed. The organism is widely distributed in the soil and water of tropical and subtropical countries. Melioidosis is naturally acquired through environmental contact with the bacterium. Although melioidosis is largely restricted to the Southeast Asia and Northern Australia, the disease has been increasingly reported from Bangladesh. However, its actual prevalence in Bangladesh is largely unknown due to the lack of systematic study and awareness of the medical community about the disease and the organism. So far, no systematic study has been done to find out the extent of exposure of *B. pseudomallei* infection among healthy individuals and to detect the source of this organism in environmental samples of Bangladesh. In order to address the issue, the first phase of the present study was designed to determine the magnitude of exposure by detecting antibodies to *B. pseudomallei* among the healthy population of selected regions of Bangladesh. In the second phase of the study, attempt was made to detect the organism in the soil by culture and molecular methods. The clinical and environmental isolates were further characterized by molecular techniques to determine the relatedness of the organisms.

In the first phase of the study, blood samples were collected from healthy population residing in rural area of four districts (Mymensingh, Sylhet, Narayangange and Kishoregange) to determine the seroprevalence of *B. pseudomallei* infection. We have used both sonicated whole cell antigen (SWCA) and recombinant truncated flagellin antigen (RTFA) of *B. pseudomallei* in an in-house indirect enzyme linked immunosorbent assay (ELISA). The cut off optical density (OD) value for SWCA and RTFA was 0.8 and 0.4 respectively. The cut off OD value was determined by mean OD of negative control + 3x SD. In the second phase of the study, we have determined the source of the organism in the soil samples from four northeastern districts of Bangladesh by culture and polymerase chain reaction (PCR). Multiple soil samples from 5–7 sampling points of 3–5 sites were collected from rural areas of four northeastern districts (Mymensingh, Sylhet, Gazipur, and Narayangange) of Bangladesh from where culture

confirmed melioidosis cases were detected earlier. Approximately 200 gm soil was taken from each point from a depth of about 20-30 cm using a shell augur disinfected with 70% alcohol in between soil collection. Collected soil was placed into a sterile plastic bag and sealed with rubber band to prevent moisture loss and was transported to the laboratory as soon as possible.

In the second phase of the study, total 179 soil samples were collected and cultured in Ashdown selective media and sub-cultured in MacConkey`s agar medium. The suspected organisms which grew on MacConkey`s agar medium at 42^0C were identified as *B. pseudomallei* by typical colony morphology, Gram staining (bipolar staining), motility, biochemical tests (including API 20NE), arabinose assimilation and resistance to colistin and aminoglycoside. Monoclonal antibody based latex agglutination test (Melioidosis Research Center, Khon Kaen, Thailand) was performed for the final identification and confirmation of the suspected colonies of *B. pseudomallei*. Phenotypically suspected colonies of *B. pseudomallei* were further confirmed by PCR using 16s rRNA specific primers and *orf2 gene* of type three secretion system1 (TTS1). In the present study, molecular analysis was performed to characterize the strains isolated from clinical specimens and environmental sources. All these isolates were analyzed by real-time PCR assay targeting TTS1, *Yersinia*-like fimbrial (YLF) gene cluster and *B. thailandensis*-like flagellum and chemotaxis (BTFC) gene cluster. Real-time PCR assay targeting TTS1 was performed to confirm phenotypically identified organisms. YLF and BTFC gene clusters were examined to demonstrate the diversity of *B. pseudomallei* from various geographical regions and sources. Multilocus sequence typing (MLST) with seven house-keeping genes was performed with all the isolates to clarify the genetic relationships between *B. pseudomallei* isolated from clinical specimens and environmental sources and also between strains isolated from Bangladesh and other countries. The primers used in the PCR amplification and sequencing of the seven housekeeping gene fragments were *ace-up* and *ace-dn, -gltB-up* and *gltB-dn, gmhD-up* and *gmhD-dn, lepA-up* and *lepA –dn, lipA-up* and *lipA –dn, narK-up* and *narK-dn, ndh-up* and *ndh-dn.*

Out of 940 blood samples, anti- *B. pseudomallei* IgG antibody against SWCA was detected in 21.5% individuals. However, ELISA using RTFA protein showed the seropositivity rate as 13.7%, which was lower than the seropositivity (21.5%) detected by ELISA using SWCA. Seropositivity rate by using SWCA was 22.6%-30.8% in three districts from where melioidosis cases were detected earlier, compared to 9.8% in a district (Kishoregange) where no melioidosis case was either detected or reported ($p<0.01$) previously. Seropositivity increased with the advancement of age from 5.3% to 30.4% among individuals aged 1–10 years and > 50 years respectively. The seropositivity rates were 26.0% and 20.6% in male and female respectively, while it was 20–27% among different occupational groups. No significant association was observed with gender ($\chi2 = 3.441$, $p = 0.064$) or any occupational group ($\chi^2 = 3.835$, $p = 0.280$). Out of 179 soil samples, *B. pseudomallei* was isolated from two (2) samples from paddy field of Gazipur district, which is located 54 km north of capital Dhaka city. Both the isolates were phenotypically identical and arabinose negative and were positive for 16s rRNA and *orf2* (TTS1) gene by conventional and real-time PCR assay respectively.

Gene cluster analysis targeting YLF and BTFC gene demonstrated that all the isolates from Bangladesh contained YLF gene cluster. None of the isolates was positive for BTFC gene cluster. YLF gene cluster is predominantly found among *B. pseudomallei* derived from Southeast Asia. Phylogenetic analysis of 24 *B. pseudomallei* isolates by MLST revealed thirteen different sequence types (STs) of which 4 STs (ST- 1352, 1124, 761 and 756) were of novel types and identified for the first time. All these isolates were from Bangladeshi patients. Strains having the above STs were isolated from patients with abscess in different organs (liver, soft tissue, lungs). It is to be noted that in the present study, ST 56 (5 cases), ST 1007 (4 cases) and ST 1005 were the most frequently isolated types. ST 56 which was present in 5 clinical isolates out of 22, was the most common variant present in Bangladesh, followed by ST 1007 revealed from 4 cases and ST 1005, found in 2 clinical and 2 environmental isolates. Presence of ST 1005 in soil of Gazipur district as well as its presence in melioidosis patients from the same district indicated soil as the source and reservoir. All the strains containing ST 56 was isolated from patients with septicemia. ST 56 was also detected earlier in 1999 from a Bangladeshi patient

living abroad. Apart from the four novel STs described above, all other STs that have been detected in the present study are also present in Thailand, Cambodia, China and Vietnam and other neighboring countries.

The present study has demonstrated that a large proportion of people residing in the rural area of four districts are exposed to the organism as determined by serology and have a potential for developing overt diseases during their lifetime. The study has also identified for the first time the presence of *B*. *pseudomallei* in the soil samples of Bangladesh and determined soil as the source of *B. pseudomallei* infection in this region. All *B. pseudomallei* isolated in Bangladesh posses YLF gene, which confirms their Asian origin. As YLF strains are more virulent than BTFC strains, so people in this region are at higher risk of severe form of infection. MLST study has revealed that a number of novel STs of *B. pseudomallei* exist in Bangladesh. Presence of unique STs of *B. pseudomallei* in our environment demands further study to understand their importance to the biology of these organisms. Presence of same ST from the soil and clinical isolates indicates soil as the source and reservoir of this organism. It is likely that human and/or animal movements between these areas played pivotal role in the dissemination of *B. pseudomallei*, however analysis of broader range of isolates from these region are required for confirmation. Further large scale study is necessary to find out the magnitude of the infection in different areas of Bangladesh and its different reservoirs in the environment along with phylogenetic distribution.

1.0 Introduction

The pioneering work of British pathologist Alfred Whitmore and his Indian colleague C.S. Krishnaswami revealed a distinct bacterial pathogen, named *Bacillus pseudomallei* as the causative agent of a newly recognized 'glanders-like illness' among Burmese morphine addicts (Whitmore, 1913; Whitmore and Krishnaswami, 1912). The organism is now known as *Burkholderia pseudomallei* and is responsible for causing the disease melioidosis. *B. pseudomallei* is a saprophyte, distributed in many different environmental niches, especially paddy field, ground and stagnant surface water and the plant rhizosphere (Cheng and Currie, 2005). The organism is an aerobic, motile, facultative intracellular Gram-negative ß-proteobacterium that afflicts both human and animals.

Melioidosis is a potentially lethal and fulminating infection manifested as community acquired pneumonia, multiple abscesses and septicemia (Wiersinga et al., 2012a)). The bacterium is intrinsically resistant to a wide range of antimicrobials and treatment with ineffective antimicrobials has case fatality rates exceeding 70% (Cheng and Currie, 2005). An estimated 165,000 human melioidosis cases occur each year worldwide, of which 89,000 (54%) die (Limmathurotsakul et al., 2016). It is an endemic disease of public health and clinical importance in many tropical and subtropical regions of the world. The ecological distribution of *B. pseudomallei* is mostly limited to 20^0 North and 20^0 South of equator, the area where the climatic condition is very favorable for the growth of this organism as it requires relatively high temperature, humidity and abundant rainfall. Humans acquire melioidosis from directly contacting, ingesting or inhaling contaminated soil, foods or dusts. Patients become infected when compromised skin surface come into contact with soil and water containing organism. Exposure through percutaneous route present a localized, suppurative cutaneous infection accompanied by regional lymphadenitis, fever, and abscess in various organ of the body (Cuadros et al., 2011). According to epidemiological evidence, pulmonary melioidosis can be acquired by inhalation after heavy rain falls and strong winds (Currie et al., 2004). Cases are reported throughout the year but peak incidences (75% of cases) are during the rainy

season (Inglis et al., 2000; Raja et al., 2005). In animal experiments, mice that inhaled aerosols contaminated with *B. pseudomallei* developed severe inflammation and neutrophil infiltration in the lungs, which mimicked the symptoms of human pulmonary melioidosis (West et al., 2012). Human can also acquire melioidosis by ingesting contaminated foods or drinks, and a melioidosis outbreak was linked to drinking water contaminated with *B. pseudomallei* (Howe et al., 1971).

Initially the bacterium seems to invade the host cells and persists for a long latent period, after which a fatal recrudescence can occur many years after infection (Currie et al., 2000). In endemic areas, different sero-epidemiologic studies have shown that infection, mostly latent, occurred at early childhood, whereas clinical melioidosis is more common in the elderly particularly in immuno-compromised host. This is due to the reactivation of primary latent infection. The organism remains quiescent intracellularly from weeks to many years (up to 62 years) and act as a potential 'time bomb' in high risk groups, like patients with diabetes mellitus, alcohol abuse, chronic lung disease, chronic renal disease (Cheng and Currie, 2005; Currie et al., 2004; Leelarasamee, 1998; White, 2003a). Diabetes mellitus is the major underlying risk factor for melioidosis, and is present in more than 50% of all melioidosis cases (Cheng and Currie, 2005). The risk of people with diabetes acquiring melioidosis is about 12 times higher than the rest of the population (Currie et al., 2004; Limmathurotsakul et al., 2010). Disease presentations are variable due to variation of host immune response, different modes of acquisition, or genomic differences among strains (Chaowagul et al., 1989; Tuanyok et al., 2007).

Melioidosis is a disease of public health importance in Southeast Asia and northern Australia where melioidosis is associated with high case-fatality rates in humans. It is the second most common cause of bacteremia and accounts for about 20% of all community-acquired septicemias in northeast Thailand where 2000 to 3000 new cases are diagnosed every year (Cheng and Currie, 2005; White, 2003b). A very high mortality rate is seen in patients with septicemic melioidosis and these patients often deteriorate quickly and death occurs within a few days after infection (Novak et al., 2006). The number of people dying in Thailand from melioidosis is now comparable to deaths from tuberculosis, and

exceeds those from malaria, diarrhoeal illness and measles combined and account for more than 1000 death in each year (Limmathurotsakul et al., 2010). Although melioidosis is largely restricted to the South East Asia and north Australia, the disease has been increasingly reported in countries including Bangladesh, India, Mauritius, South, Central and North America and several African countries, suggesting an expanding geographical distribution and/or awareness (Cheng and Currie, 2005; White, 2003a).

Melioidosis has been sporadically detected in Bangladesh over last several decades (Barai et al., 2014). The first reported case of melioidosis was published in the Lancet in 1964 in a 29 year old British sailor who was travelling through Bangladesh. His ship was carried half a mile inland near Chittagong by a cyclone, and was deposited in a paddy field. There he remained on the ship for the next 3 months and developed melioidosis later on (Maegraith and Leithead, 1964). However, the first case of melioidosis in a native Bangladeshi infant was published by International Centre for Diarrheal Diseases and Research, Bangladesh (ICDDR, B) in 1988 (Struelens et al., 1988). From 1991 to 1999, five cases were detected in United Kingdom (UK) among Bangladeshi people who migrated to UK from the Sylhet region (a northeastern district) of Bangladesh (Dance et al., 1999; Hoque et al., 1999; Kibbler et al., 1991). In 2001, the first culture confirmed suppurative melioidosis case in a 48 years old diabetic patient was detected in Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM) hospital. The patient came from Sherpur district, which is located about 140 km north of capital Dhaka (Nazimuddin et al., 2001). Later on, at least, 20 cases were reported among the diabetic patients in Bangladesh, and all of these cases were detected at BIRDEM hospital from 2001 to 2014 (Barai et al., 2014). Analyses of the reported cases strongly indicate that the disease is potentially endemic in ten districts of Bangladesh particularly in northern and northeastern parts of the country which include greater Mymensingh, Dhaka and Sylhet region.

Since the manifestations of melioidosis are highly diverse, clinical diagnosis is often difficult, so the true incidence of infection may actually be higher than suggested by current data. The true extent of the disease in Bangladesh is not known, as this disease is

not familiar to most of the physicians and microbiologists of the country. Seroepidemiological studies conducted in different countries showed that 80% of children in north-eastern Thailand were positive for antibodies against *B. pseudomallei* by the age of 4 years (Limmathurotsakul and Peacock, 2011). In Malaysia, reported seroprevalence in healthy individuals was 17-22% among rice farmers and 26% in blood donors. In north Australia 0.6 to 16% of children had evidence of infection by *B. pseudomallei* (Armstrong et al., 2005; Inglis et al., 2000; Leelarasamee, 1998). In 2012, a hospital based serological survey in Bangladesh reported 28.9% seropositive rate for *B. pseudomallei* antibody among patients attending several tertiary care hospitals for unrelated ailments. The study, however, used a very low cut off titer (1:10) of indirect haemagglutination assay (IHA) for defining seropositive cases without considering the possibility of presence of cross reactive background antibody among the local population (Maude et al., 2012). The serological tests like IHA and complement fixation tests were applied early to melioidosis diagnostics (Alexander et al., 1970). The use of IHA is problematic, especially in areas of endemicity, like Thailand, where rates of background seropositivity may be up to 30-47% in various populations (Khupulsup and Petchclai, 1986). However, IHA remains the most widely used test despite its poor sensitivity and specificity. Efforts have been made to refine the antigen targets and to develop other efficient methods to identify *B. pseudomallei*-specific antibodies. ELISA based on crude antigens, 30kDa and 200kDa proteins have been validated in a clinical context and appears to be more sensitive (74 to 82%) and specific (75 to 80%) than the IHA (Cheng and Currie, 2005). However, ELISA using recombinant truncated flagellin antigen achieved 93.8% sensitivity and 96.3% specificity and offered a more efficient candidate for sero-diagnosis of melioidosis (Chen et al., 2004). In another study, ELISA using refined recombinant 22.9 kDa type IV HCP protein (TssD-5) showed 71% sensitivity and 96% specificity (Hara et al., 2013). Serological assays using either crude or purified/refined antigen preparations for the detection of antibodies to *B. pseudomallei* demonstrated varying results in terms of sensitivities and specificities (Allwood et al., 2008; Druar et al., 2008a; Sirisinha et al., 2000). Therefore, it appears that the rate of seropositivity varies depending on the assay method and antigen used in the serological assays. In the context of above, further sero-epidemiological studies with defined

antigens and by different serological assay method should be instituted to determine the true sero-prevalence of anti-*B. pseudomallei* antibody in the Bangladeshi population.

Recently in 2013, melioidosis endemic countries of the world has been categorized into 'definite' and 'probable' country based on the presence *B. pseudomallei* in human and in environment in the respective countries (Limmathurotsakul et al., 2013). According to the above categorization, Bangladesh falls into 'probable' category of country as the presence of the organism in the environment has not yet been identified or reported even though several culture-confirmed melioidosis cases have been detected. Probability of the presence of *B. pseudomallei* in soil and water of Bangladesh is very high as the climatic condition of the country is very favorable for its growth in the environment. Therefore, isolation and identification of *B. pseudomallei* from environmental samples (e.g. soil or water) is necessary to determine the source of the organism of melioidosis cases in the country. In addition, it would help to define the status of Bangladesh as a 'definite' or 'probable' category of country for melioidosis.

Characterization of etiologic agents is central to epidemiological surveillance and public health decisions. Several molecular tests, including random amplified polymorphic DNA analysis, ribotyping and pulsed-field gel electrophoresis (PFGE) have been used to investigate the molecular characterization of *B. pseudomallei* and epidemiology of melioidosis during the last decades (Currie et al., 2001; Haase et al., 1995b). Most of these techniques suffer from significant drawbacks, including poor reproducibility within and between laboratories, and an inability to quantitate the genetic relationships between isolates (Maiden et al., 1998). To overcome these problems, a molecular typing procedure known as multilocus sequence typing (MLST), that uses nucleotide sequence data rather than DNA fragment patterns are increasingly being used. The advent of MLST provides a novel and beneficial scheme for the study of melioidosis epidemiology worldwide. This typing method is based on sequence variation within seven (7) housekeeping-gene fragments. Allele numbers are assigned to each of the seven housekeeping loci based on sequence differences and are then arranged into a string of seven integers to give the allele profile of an isolate and dendograms are constructed from the pair wise differences

in multilocus allelic profiles by cluster analysis. The *B. pseudomallei* MLST allele profile corresponds to the gene order *ace-gltB-gmhD-lepA-lipA-narK-ndh* (McCombie RL et al, 2006). The sequence type (ST) of an isolate is defined specifically by the allele profile. The relationships among the STs may then be examined by using various methods, such as eBURST which is based upon related sequence type (Godoy et al., 2003). MLST databases contain the reference allele sequences and sequence types for all *B. pseudomallei* so far isolated from different regions of the world. Phylogenetic analyses of *B. pseudomallei* isolates, performed by using MLST have led to phylogeographic associations of organisms isolated in different parts of the world (Dale et al., 2011). MLST has been used to examine the diversity of *B. pseudomallei* from various geographical regions (Cheng et al., 2008; Godoy et al., 2003; U'Ren et al., 2007; Vesaratchavest et al., 2006) and revealed patterns of geographical partitioning between Australian and Southeast Asian isolates. Also, it has been determined that Australian isolates are more likely to carry an ancestral *B. thailandensis*-like flagellum and chemotaxis (BTFC) gene cluster, whilst isolates from Asia almost exclusively carry a *Yersinia*-like fimbrial (YLF) gene cluster (Tuanyok et al., 2007). This suggests that these populations are genetically distinct due to broad scale biogeographical factors associated with establishment and persistence of the organism.

No study has yet been done to explore the STs of *B. pseudomallei* stains isolated from different human cases in Bangladesh to find out the similarities or diversities among them and also with those of other countries of the world. As has been mentioned earlier that *B. pseudomallei* found in Asian region mostly belongs to YLF gene cluster while there is no reports or study available regarding the presence or absence of the YLF gene cluster in Bangladeshi isolates. The molecular characterization involving the YLF and BTFC genes with the Bangladeshi strains would be useful to understand the dispersal of this organism in this part of the Asia. Detailed phylogenetic study of isolates from this region will advance the understanding of the biogeography of melioidosis. This would provide insight into the ecology of *B. pseudomallei* involving its evolution and dispersal.

So far, no systematic study has been done to find out the extent of exposure of *B. pseudomallei* infection among healthy individuals and to detect the source of this organism in environmental samples of Bangladesh. To serve this purpose, the present study was designed to determine the magnitude of exposure by detecting antibodies to *B. pseudomallei* among the healthy population of selected regions of Bangladesh as well as to detect *B. pseudomallei* by culture and molecular method from soil and clinical samples. The study also aimed to characterize the clinical and environmental isolates of *B. pseudomallei* by analyzing YLF, BTFC gene clusters and multi locus sequence typing (MLST).

1.1 Study purpose

Detection of melioidosis cases among Bangladeshi patients for last several decades strongly indicates that the probability of the presence of *B. pseudomallei* in our environment is very high, and Bangladeshi populations are exposed to the organism. In view of the above, the present study aimed to:

1. determine the seroprevalence of *B. pseudomallei* infection among healthy Bangladeshi population by detecting anti-*B. pseudomallei* antibodies with in-house enzyme linked immunosorbent assay (ELISA) using whole cell sonicated crude antigen
2. compare the ELISA by whole cell sonicated crude antigen with that of ELISA using recombinant truncated flagellin antigen for determination of anti- *B. pseudomallei* IgG antibodies
3. isolate and identify *B. pseudomallei* and *B. thailandensis* from environmental samples (soil) by culture
4. to identify isolated *B. pseudomallei* and *B. thailandensis* by PCR using different primer targeting 16s rRNA
5. to identify *B. pseudomallei* by real-time polymerase chain reaction (RT-PCR) targeting *orf2* gene of the *B. pseudomallei* type three secretion system 1 (TTS1) for definitive detection of the organism
6. characterize the environmental and clinical isolates of *B. pseudomallei* by real-time polymerase chain reaction (RT-PCR) targeting *orf2* gene, *B. thailandensis*-like flagellum and chemotaxis (BTFC) and *Yersinia*-like fimbrial (YLF) gene clusters
7. determine the genetic relationships among the organisms isolated from clinical and environmental sources by multi-locus sequence typing (MLST)

2.0 Review of Literatures

2.1 The Study

One of the fascinating aspects of medical microbiology is the periodic discovery of emerging and reemerging organisms. In recent years, the Human Immunodeficiency Virus (HIV), Nipah virus, Ebola virus, *Legionella* species, *Helicobacter pylori* and other new pathogens have been discovered. Although *Burkholderia pseudomallei*, the causative agent of melioidosis has been described almost a century ago and considerable progress in terms of diagnosis and treatment was achieved, *B. pseudomallei* is still "the unbeatable foe", for several reasons like under-recognition, high case-fatality rate, unacceptable relapse rate and a "time-bomb" effect for sero-positive individuals (Leelarasamee, 1998). There is a growing body of evidence that, once considered an obscurity, melioidosis is now recognised as an emerging disease of global significance. This frequently fatal infection of man and numerous other animal species is caused by the facultative intracellular Gram-negative ß-proteobacterium, *B. pseudomallei*. The world wide distribution of this organism is still unknown and our understanding of the environmental factors determining the presence of *B. pseudomallei* is rudimentary. It is believed that the organism is a ubiquitous soil and water dwelling saprophyte of tropical and subtropical regions worldwide; a relationship in the high incidence of disease among people sharing a close association with the environment. Infection can remain latent for decades before the onset of clinical signs and symptoms (Ngauy et al., 2005). Infection follows aspiration of qntaminated water, inoculation of bacteria through traumatic skin breaches or inhalation of contaminated dusts and aerosols (Chierakul et al., 2005; Wang et al., 1993). There is a substantial number of factors that have driven melioidosis research, most importantly : increasing awareness and discovery of *B. pseudomallei* in areas outside the previously recognised regions of endemicity and accelaration of bioterrorism research, the recent surge in disease associated with survivors of the December 2004 tsunami in Indonesia and the accelaration of bioterrorism research in light of the *Bacillus anthacis* mailing in United States of America which has raised public awareness of health security, and in particular the threat of bioterrorism (Chierakul et al., 2005). Whilst specific details regarding the immunopathogenesis of melioidosis are accumulating,

comparatively little is known concerning the organism`s niche and rservoir of infection. *Burkholderia* are key microbial consituents of the rhizosphere and have significant roles including the provision of nutrients to the growing plant nodule, fixation of atmospheric nitrogen, inhibition of plant psathogens including fungi, and degradatrion of complex compounds. They are versatile and inhabit ecological niches as varied as rice paddy water, and water holes and sea water. This versatility has led to use of a number of *Burkholderia* species, for biocontrol, bioremediation, and plant growth promtion. The introduction of a biocontrol strain with the planting material reesults in the coonization of the rhizosphere and the suppression of the pathogenic competing bacteria and fungi with proven economic benefits. Other *Burkholderia* strains have been widely used for the bioremediation of soils and agricultural environments due to their ability to degrade complex hydrocarbons and herbicides. Such information not only has the potential to expose new therapeutic avenue, bio-active compounds and mechanisms of bioremediation (Cain et al., 2000), but also to predict regions of hyperendemicity in which the disease is frequently misdiagnosed (Warner et al., 2007). The identification of such high risk areas may aid in advising traditional inhabitants of high risk areas in which bathing, washing and playing should be avoided.

This bacterium still has many secrets left to be revealed. The purpose of this review is to present a detailed discussion of this organism, its distribtion, detection procedure and to summarise current thinking regarding its role in disease.

2.2 Discovery

The term melioidosis describes a collection of serious and often fatal disease in humans and animals, arising from infection by *Burkholderia pseudomallei*. This organism was first reported by the British pathologist Alfred Whitmore and his Indian colleague Krishnaswami in 1912 when a clinical syndrome resembling glanders, was described by them in Rangoon, Burma (Whitmore and Krishnaswami, 1912). They described cases of a newly recognized septicemic disease among Burmese morphine addicts, the sufferings they illustrate as a `hitherto undescribed glanders-like illness` (Whitmore, 1913; Whitmore and Krishnaswami, 1912). Glanders is a disease predominantly affecting

horses and other members of the Equine but can also infrequently infect humans and other mammals; a condition that without treatment is almost invariably fatal (Wilkinson, 1981). Fatal cases are characterized by extensive caseous consolidation in the lungs, ulceration of mucous membranes in the upper respiratory tract and numerous abscesses throughout body, particularly in liver, spleen, kidney, and subcutaneous tissue(Currie et al., 2010). Cultures of lung tissue, from the index case after autopsy on peptone agar and potato slopes revealed a microorganism, similar to *Bacillus mallei* (now known as *Burkholderia mallei*) the causative agent of glanders. Subsequent studies discovered that although similar, the organism differed from the etiological agent of glanders, *Burkholderia mallei* by virtue of a high motility, rapid growth and failure to invoke the Strauss reaction (severe localized peritonitis and orchitis) when inoculated into guinea pigs. It can be assumed that a new, but closely related organism had been discovered, the bacterium was named *Bacillus pseudomallei* and the sufferings expressed as 'Whitmore`s disease' or often 'pseudoglanders' (Whitmore, 1913).

Krishnaswami, for the next six years in Burma reported more than a hundred case fatalities due to Whitmore's disease. The bacterium was later identified as the aetiological agent in an outbreak of serious distemper-like illness during 1913 among laboratory animals from the Institute for Medical Research in Kuala Lumpur, Malaysia; from which it was speculated that the disease might be zoonotic. For these reasons, Stanton and Fletcher renamed the disease to 'melioidosis' in 1921, the term derived from the Greek words *'melis'* meaning distemper of asses and *'eidos'* meaning resemblance.(Stanton and Fletcher, 1925). During the mid 1950 the organism was for the first time sucessfully isolated from soil and muddy water in French Indochina and its saprophytic nature was identified (Chambon, 1955). During the Vietnam war, large number of American servicemen contracting meliodosis was thought to be due to the inhalation of dust spun up from heliopter blades (Howe et al., 1971), furher incriminate that the organism is an enviromental saprophyte.

2.3 Nomenclature and Taxonomy

B.pseudomallei has been reclassfied using a veriety of nomenclature since its discovery. It was previously known as *Bacterium whitmorii, Bacillus whitmorii*, *Malleomyces pseudomallei, Pfeifferella whitemori, Pfeifferella pseudomallei, Actinobacillus pseudomallei, Flavobacteruim pseudomallei* and more recently *Pseudomonas pseudomallei*. Similary, nurmerous pseudonyms have also been given to the diseases melioidosis. Initially it was named as Whitemore's deasease after Captain Alfred Whitmore, and also termed as pseudoglanders or morphia injector's septicemia. The disease was also known as 'Nightcliff gardener's disease' which refers to the high incidence among home gardeners. More recently the disease has termed are 'Vietnamese time bomb' due to its recrudescence among American soldiers after many years. In Southeast Asia, melioidosis has become known as 'paddy-field disease' because of its high incidence among paddy field workers (Orellana, 2004). Among clinicians, the disease is known as the 'great imitator, or great mimicker (due to diverse presentation), or 'sudden unexplained death syndrome' (Yap et al., 1991a).

The microrganism, since its first description, has been the subject of taxonomic controversy (Brindle and Cowan, 1951; Fournier, 1965). This has been fuelled, to some extent, by the complexities of bacterial taxonomy and a lack of consensus until recently on how phenotypic and genotypic taxonomic tools be used (Vandamme et al., 1996). Brindle and Cowan initially described that the bacillus belonged to the family Pseudomonadaceae group but based on the observation that the colonial pigment was not soluble in water, proposed that it could not belong to the *Pseudomonas* genus (Brindle and Cowan, 1951). Finally molecular characterization of the *pseudomonas* involving 16S rRNA sequencing, DNA: DNA homology, cellular lipid and fatty acid composition, and other phenotype characteristics resulted in creation of the genus *Burkholderia* in 1992, which encompass the species formerly grouped within the rRNA group II of the genus *Pseudomonas* (Yabuuchi et al., 1992). The genus was named after Walter Burkholder; a credit to his founding works on the plant pathogen *Burkholderia cepacia (*formerly *pseudomonas cepacia*).

Burkholderia is one of several genera belongs to the family Burkholderiaceae, in the order Burkholderiales of the Gram negative beta-proteobacterium. The classification of *Burkholderia pseudomallei* is based on rRNA/ DNA homology and common culture characteristics. The bacterium has been classified as following:

Scientific classification

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Beta Proteobacteria

Order: Burkholderiales

Family: Burkholderiaceae

Genus: *Burkholderia*

Species: *B. pseudomallei*

Approximately seventy species have been incorporated into the genus *Burkholderia*, the majority of which are soil and water dwelling saprophytes. The biochemical versatility of different species of *Burkholderia* has ensured their ubiquity throughout a broad range of environmental habitats (Poonguzhali et al., 2007) and they range from obligate plant and animal pathogens through symbiotic organisms to plant associated species that cause opportunistic infection in man. The most pathogenic species are the members of which are *B. pseudomallei, B. mallei* and in certain clinical conditions such as cystic fibrosis *B. cepacia*. The genus includes *Burkholderia thailandensis*, which co-exists with *B. pseudomallei* in the soil in Thailand but rarely causes disease and is $>10^5$ - less virulent than *B. pseudomallei* in Syrian hamsters or mice (Brett et al., 1998). *B. mallei* causes glanders in horses and is potentially highly virulent than in man, but natural disease in any host is now extremely rare. *B. pseudomallei* and its two closest relatives; *B. mallei* and *B. thailandensis* provide an interesting insight into mechanisms of environmental persistence and pathogenicity. Whilst each differ in their virulence, host range and environmental niche, all share the ability to subvert host cells to promote their intracellular replication and survival, a characteristic that are capable of causing disease in man (Martin and Mohr, 2000; Stevens et al., 2005).

The genome of *B. pseudomallei* (strain K96243 from Thailand) has been sequenced and observed that it comprises two chromosomes of 4.07 Mb and 3.17 Mb, of which the large chromosome carries many genes associated with core functions such as cell growth and metabolism, while the smaller one carries more genes encoding accessory functions that could be associated with adaptation and survival in different environments (Holden et al., 2004a). Around 6% of the genome is made up of putative genomic islands that have probably been acquired via horizontal gene transfer. These are mostly absent from the *B. thailandensis* genome (and are absent from the *B. mallei* genome), it is not clear whether these regions have a role in disease pathogenesis. The DNA microarray based on the whole genome sequence of *B. pseudomallei* K 96243 has been used to compare isolates of *B. pseudomallei*, *B. mallei* and *B. thailandensis* (Ong et al., 2004). Deleted regions in *B. mallei* had significant genomic clustering compared with those in *B. thailandensis,* which were more uniformly dispersed. This suggests that the evolutionary processes that result in divergence of the three species might have distinct mechanisms. Comparison of 16S rRNA sequences suggest that *Escherichia coli* and *Salmonella typhimurium* diverged from *B. pseudomallei* 140 million years ago, while *B. thailandensis* diverged 47 million years ago. *B. thailandensis* E264 possesses 2 chromosomes of 3.81 Mb and 2.91 Mb. The total number of genes in *B. pseudomallei* is 5854 while *B. thailandensis* has 5645. Of these, 4994 genes are conserved between the two species. Overall, the %GC content, numbers of pseudogenes, and average gene length are very similar between the two *Burkholderia* species. The whole-genome comparison between *B. pseudomallei* and *B. mallei* suggests that *B. mallei* has evolved through 'genomic down-sizing' from a single clone of *B. pseudomallei* (Godoy et al., 2003). More recently, at least two other closely related species have been described based on molecular analysis; *Burkholderia oklahomensis* and the provisionally named *Burkholderia humptydooensis*. These five species comprise a distinct lineage on the *Burkholderia* phylogenetic tree.

2.4 Microbiology of *B. pseudomallei*

Morphology

B. pseudomallei are slender Gram negative rods, 0.3-0.5 µm in width and 1-2 µm long with two to four polar flagella per cell. Microscopically the organism has rounded ends, staining is irregular and stains with a bipolar appearance (safety-pin appearance). This is due to the presence of intracellular deposits of poly-ß-hydroxybuterate, used for carbon storage and has a vaculated appearance (Sprague and Neubauer, 2004). Safety-pin appearance is particularly seen in films of pus; accumulation of poly-ß-hydroxybuterate may be demonstrated by staining with Sudan black.

Genomics of *B. psedomallei* - A highly variable and evolving genome

As mentioned earlier, the genome of *B. pseudomallei* is one of the most complex bacterial genomes sequenced to date (Wiersinga et al., 2012b). Till 2009, completely sequenced genomes of four strains are available in published databases, while another 19 have been drafted (Holden et al., 2004b). The first complete genome sequence of *B. pseudomallei* was published in 2004 which was isolated in 1996 from a 34-year-old female diabetic patient in Khon Kaen hospital in Thailand. This genome of *B. pseudomallei* strain K96243 (Figure 2.1) consists of two chromosomes of 4.07 and 3.17 megabase pairs respectively which demonstrate significant functional partitioning of genes between them (Holden et al., 2004a). The larger chromosome is involved predominantly with essential cellular functions such as metabolism, growth and replication, whilst the smaller contains genes involved in survival and adaptation to complex niches. Eighty-six percent of the prototypic *B. pseudomallei* K96243 genome is common to all strains and represent the core genome, with 14% variably present across isolates. The variable region includes multiple genomic islands containing DNA acquired from other bacteria. Genomic islands are likely to be associated with virulence and the potential for infection, although specific associations with clinical outcomes have not yet been elucidated. Genotyping of multiple *B. pseudomallei* colonies from tissue sites from four patients with acute melioidosis showed substantial genetic diversity within a single patient, indicating the capacity of the organism to evolve rapidly within the host (Price et al., 2010).

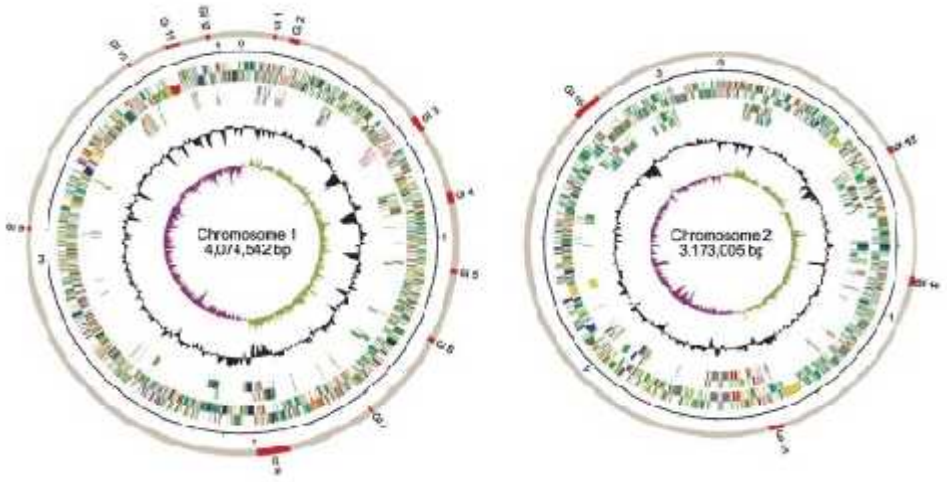


Figure 2.1 : Schematic diagrams of two chromosomes in the *B. pseudomallei* strain K96243 genome: 3,460 coding sequences are encoded by chromosome 1 whereas 2,395 coding sequences are encoded by chromosome 2. This figure is taken from Holden *et al* (2004).

Physiology

Motility and disease presentation are two major phentypic chracteristics that differentiate *B. pseudomallei* from *B. mallei*. By virtue of possessing polar flagella, *B. pseudomallei* can move by aerotaxic motility. Such aerotaxis is likely responsible for the biofilm-like pellicle formed by the organism at their air interface of broth cultures and migration of the organism toward the surface of pooled environmental water (Inglis et al., 2001; Virginio et al., 2006). Biochemically, the organism produces both catalase and oxidase and utilyses carbohydrates oxidatively. Despite the strongly aerobic nature ojf the organism, there is increasing evidence that it can utilise nitrate or arginine under anaerobic conditions, thus may have the capacity of anaerobic phosphorylation (Wongwanich et al., 1996). All clinical isolates synthesise at least one extracellular enzyme, the majority produce a cocktail of multiple enzymetic excretions (Ashdown and Koehler, 1990). It is nutritionally non-fastidious, being able to utilse a variety of carbon and nitrogen sources and grows on most traditionally used media within a wide temperature range. Optimal incubation temperatures for prolferation of the organism are between 37^0 C and 42^0 C, although growth of several strains has been reported at temperatures as low as 4^0 C (Chen et al., 2003a; Levine et al., 1954).

Growth characteristics

B. pseudomallei grow well on simple media, including nutrient, blood, and MacConkey agars, but it does not grow on deoxycholate citrate or Salmonella-Shigella agars. After overnight incubation on nutrient agar at 37^0 C, the colonies are 1-2 mm in diameter. The growth may be smooth and mucoid or rough, with a dull, wrinkled, corrugated, or honeycombed surface and an entire edge, have a metallic appearance; although mucoid in consistency, they are easily emulsified (Figure 2.2, 2.3).

Figure 2.2: Colony morphology of *B. pseudomallei* USM strain after 72hrs at 42ºC (Smooth surface, dry, dark purple colony with umbonate elevation).

Figure 2.3: Colony morphology of *B. pseudomallei* USM strain after 72hrs at 37ºC (Rough surface, irregular outer edge, deep purple, mucoid colony)

After several days they become opeque, yellowish-brown, uneven and often umbonate. The growth has a characteristic earthy or musty odour after several days incubation in air, although sniffing of agar cultures is discouraged on safety ground. α-hemolysis is observed on sheep blood agar; older cultures produce ß-hemolysis.

Isolation of *B. pseudomallei* is predominently carried out on Ashdown's selective and differential agar as colonial morphology is best observed in this medium. This is a simple agar containing crystal violet, glycerol, and gentamicin; colonies concentrate the dye from the medium and grows rugose or smooth (Ashdown, 1979). The high glycerol content of Ashdown's agar induces distinctive wrinkling on the medium. This medium was later modified Howard and Inglis in 2003 and contained less glycerol than the original; crystal violet, which apparently inhibitory for some mucoid isolates, was replaced by Nile blue (Howard and Inglis, 2003). Many strains grow poorly below 25^0 C but all grow at 41^0 C.

Chantratita et al in 2007 demonstrated various types of colony morphology and the association of morphotype and phenotype changes (Chantratita et al., 2007). Seven unique colony morphotypes have been identified according to its surface texture of the colony center, outer edge of colony, srface roughness in outer half of colony, colony diameter and colony color (Figure 2.4). The colonies are shown here as defined after 4 days incubation at 37 C° in air on Ashdown's selective and differential agar (Chantratita et al., 2007). Type II and V are distinguished by size since type V does not always have a central crater. Types III and VI are also distinguished by size colour differentiation is not a consistently reliable indicator. Typical wrinkled, mauve colonies of *B. pseudomallei* are seen growing on Ashdown`s selecetive and differential agar after five days incubation at 37^oC.

Figure 2.4: The seven major morphotypes of *B. pseudomallei*.

Dance et al. (1989) recommended a simple screening procedure for the confirmation of identity of *B. pseudomallei* for an oxidase positive, gram negative rod showing bipolar or irregular staining (Dance et al., 1989a). Organisms are tested for resistence to disks containing colidtin 10 μg and gentamicin 10μg and subcultured in Ashdown's medium and incubated for 48-72 hours. The API 20NE kit proved to be highly efficient for the confirmation of the identity of *B. pseudomallei*, but false identification of some other Psedomonads as *B. pseudomallei* some times occurs. Wuthiekanum et al in 1990 evaluated Ashdown's medium with and without pre-enrichment in selective broth (containing colistin and crystal violet) for the identification of *B. pseudomallei* from specimen and concluded that enrichment improved the chances of recovery of the organismfrom site with an extensive normal flora (Wuthiekanun et al., 1990). The inclusion of colistin (50 mg/l) to Ashdown's enrichment broth and also to Gallimand's medium (basal salt medium containing L-threonine) marked increase the yield of organism from both clinical and environmental specimen (Wuthiekanun et al., 1995).

Susceptibility to Physical and chemical agents

B. pseudomallei is able to survive in different harsh environmental conditions including low moisture content soil, acidic environments, widely fluctuating temperature, the presence of detergents and even a lack of nutrients (Cheng and Currie, 2005; Wuthiekanun et al., 1995). It has the ability to grow on 0.1% cetrimide agar and is resistant to various dyes. It survives for upto 30 months in moist clay soil, but a much shorter timein dry sandy soil (Thomas et al., 1979). Wuthiekanum and his colleagues describe the organism's survival in triple distilled water without any additional nutrient for more than 3 years (Wuthiekanun et al., 1995), and can suvive in distilled water for at least 16 years (Pumpuang et al., 2011), a character which presumably enables it to survive in its relatively nutrient-poor saprophytic environment. A study of *B. pseudomallei* survival in water showed that a membrane protein, BPSL0721, which was up regulated, may be involved in the adaptation for surviving in the aqueous environment. However, this gene is not essential for survival as the BPSL0721 deficient mutant was still able to survive in water (Moore et al., 2008). *B. pseudomallei* can survive in various conditions of high water content, pH chnges, osmotic pressure and

chemical stress, but is less tolerant to ultra-violet light exposure. Another factor that influences disease outbreaks is the weather conditions, with many cases of melioidosis occurring in the rainy season (Wiersinga et al., 2006). As *B. pseudomallei* is susceptible to ultraviolet light, this may explain the relatively low incidence of melioidosis during the dry season when natural sunlight levels are very high (Sagripanti et al., 2009).

2.5 Epidemiology

The tip of the iceberg metaphor has been commonly used to describe the evidence for global melioidosis distribution (Dance, 1991; John et al., 1996). It has been observed that, so far a few case report and series regarding melioidosis had been published as culture facilities are not available in most of the rural tropics where the infection is likely to be prevalent. The detection of melioidosis is often linked with the strengthening of medical and laboratory services in developing countries (Chaowagul et al., 1989). Since the manifestations of melioidosis are highly diverse, clinical diagnosis is often difficult, so the true incidence of infection may actually be higher than suggested by current data.

Geographic distribution (Figure 2.5)

The extent of melioidosis endemic region is reported to be within latitudes 20^0 north (Tropic of Cancer) and 20^0 south (Tropic of Capricorn) from the equator (Redfearn et al., 1966). The region includes most of the South East Asia (particularly North Eastern Thailand), Northern Australia, and parts of equatorial Africa and the Americas. Reports of animal melioidosis, in particular, have extended this region to 40^0 latitude. Importation of melioidosis and subsequent maintenance of the reservoir of infection in temperate regions seems possible (Currie et al., 1994). It is to be noted that, since the initial reports of melioidosis by Whitmore and Krishnaswami, only a handful of cases have since been reported from Myanmar (Whitmore, 1913; Whitmore and Krishnaswami, 1912). It seems unlikely that the index cases reported from Rangoon resulting from the work of Whitmore and Krishnaswami represents an isolated outbreak that does not reflect national endemicity. Since the withdrawal of its colonial administration many countries may have struggled to maintain the facilities that were established by expatriate

colonization and therefore different causes of febrile disease that may have provided scientific curiosity to expatriates, have since been overlooked.

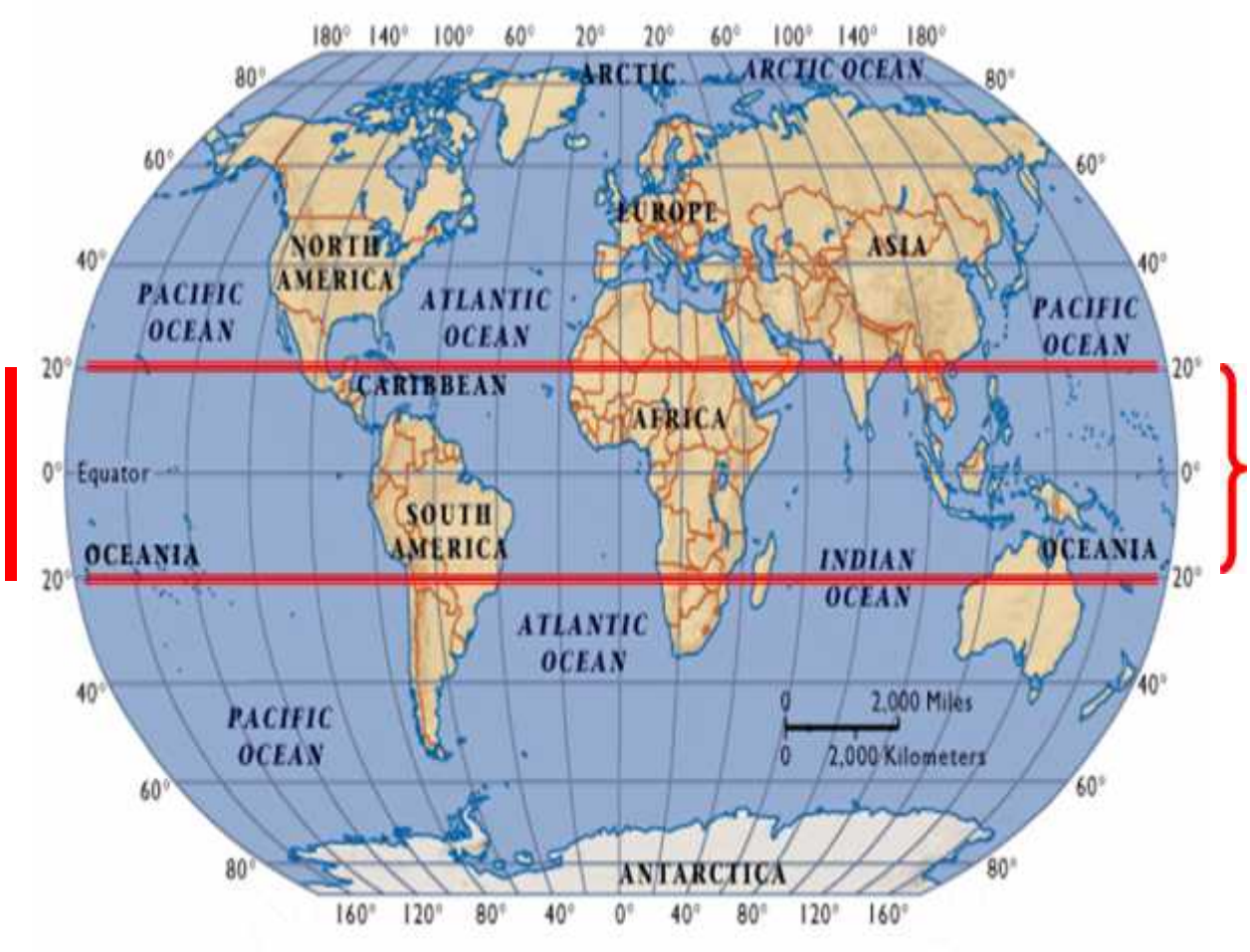


Figure 2.5: Global map showing the distribution of *B. pseudomallei*.

Global distribution and burden of *B. pseudomallei*

Recently in 2013, melioidosis endemic countries of the world has been categorized into 'definite' 'probable' and 'possible' country based on the presence *B. pseudomallei* in human and in environment in the respective countries (Limmathurotsakul et al., 2013; Prakash et al., 2014). There was 'definite' evidence for the presence of environmental *B. pseudomallei* in 18 countries (Table 2.1). Nine were either in southeast Asia (Cambodia, India, Lao PDR, Malaysia, Singapore, Thailand and Vietnam) or Oceania (Australia and

Papua New Guinea), with the remainder (n=9) being Brazil, Burkina Faso, China, France, Iran, Madagascar, Niger, Sri Lanka and Taiwan (Limmathurotsakul et al., 2013; Prakash et al., 2014). In France, soil culture positive for B. pseudomallei were initially reported in the 'Jardin des Plantes' in Paris where soil cultures positive for *B. pseudomallei* were initially reported after an outbreak of melioidosis, which was thought to have originated from a panda imported from China, but organism was subsequently reported to have been detected in soil throughout the country (Limmathurotsakul et al., 2013).

A further 33 countries were assigned to the 'probable' category based on clinical evidence of indigenous melioidosis but lack of environmental studies. Two studies described the molecular identification or genotyping of environmental *B. pseudomallei* isolates from Ecuador, Kenya and Venezuela but no environmental sampling studies positive for *B. pseudomallei* were identified for these countries in the published literature (Godoy et al., 2003; Tomaso et al., 2005). A total of 4 countries assigned to the 'possible' category (Table 2.1) based on inadequate bacterial confirmation of putative environmental *B. pseudomallei* combined with a lack of evidence for indigenous melioidosis (Limmathurotsakul et al., 2013).The global distribution of *B. pseudomallei* and the burden of melioidosis is poorly understood. Very recently Limmathurotsakul D and et al in 1916 documented human and animal cases and the presence of environmental *B. pseudomallei* and combine this in a formal modeling framework to estimate the global burden of melioidosis. They estimated there to be 165,000 human melioidosis cases per year worldwide, from which 89,000 people die (Limmathurotsakul et al., 2016). This study also suggested that melioidosis is severely ender-reported in the 45 countries in which it is known to be endemic and that melioidosis is probably endemic in a further 34 countries that have never reported the disease. The large numbers of estimated cases and fatalities emphasize that the disease warrants renewed attention from public health official and policy makers.

Table 2.1: Global distribution of *B. pseudomallei*

Level of evidence	Definition	Countries
Definite	a. Organism isolated from soil or water with adequate identification by culture or a *B. pseudomallei*-specific PCR, and b. Evidence for melioidosis having been acquired in that country.	Asia: Cambodia, China, India, Iran, Lao PDR, Malaysia, Singapore, Sri Lanka, Taiwan Thailand and Vietnam. Oceania: Australia, and Papua New Guinea. Africa: Burkina Faso, Madagascar, Niger. Europe: France. South America: Brazil
Probable	a. No report identified of *B. pseudomallei* isolation from soil or water, and b. Evidence for melioidosis having been acquired in that country.	Asia: Bangladesh, Brunei, Egypt, India, Indonesia, Myanmar, Pakistan, Philippines and Saudi Arabia Oceania: Fiji Africa: Chad, Gambia, Kenya, Nigeria, Sierra Leone, South Africa and Uganda Central America: Costa Rica, El Salvador, Honduras, Mexico and Panama South America: Colombia, Ecuador, Puerto Rico and Venezuela Europe: Turkey Others: Aruba, Guadeloupe, Guam, Mauritius, Martinique, New Caledonia, Puerto Rico
Possible	a. Organism isolated from soil or water that was considered to *be B. pseudomallei*, but identification process not sufficient to exclude other non-pathogenic environmental *Burkholderia* spp. such as *B. thailandensis*, and b. No evidence for melioidosis having been occurred in that country	Coˆ te d'Ivoire, Haiti, Italy and Peru.

Melioidosis in Asian countries

Melioidosis were first described in Rangoon, Myanmar in 1911 (Whitmore and Krishnaswami, 1912) and was soon reported in other parts of Asia including Singapore, Vietnam, Thailand and Malaysia (Stanton and Fletcher, 1925). In Singapore, melioidosis has been a notifiable disease since 1989; an annual rate of 1.7 cases of melioidosis was documented between 1989 and 1996 with majority (89%; 337 cases) culture-confirmed cases (Heng et al., 1998). In Malaysia, reported seroprevalence in healthy individuals was 17-22% among rice farmers and 26% in blood donors. It was not until the later Vietnam conflict that considerable interest in the disease was generated due to the large number of US servicemen contracting the disease (Brundage et al., 1968; Weber et al., 1969). Most melioidosis cases from Asia have been reported from Thailand where it has been extensively studied and reviewed (White, 2003a). Features include regional clustering with a hyperendemic focus in the north east province with only sporadic cases being reported from Bangkok and elsewhere (Chaowagul et al., 1989). Seroepidemiological studies conducted in different countries showed that 80% of children in north-eastern Thailand were positive for antibodies against *B. pseudomallei* by the age of 4 years (Limmathurotsakul and Peacock, 2011). Despite its proximity to Vietnam and Thailand, melioidosis was not recognized in Laos until prospective efforts were taken to diagnose it in 1999 (Phetsouvanh et al., 2001). It is now becoming apparent that the country represents an important endemic region with soil prevalence of *B. pseudomallei* is comparable to that found in Thailand (Rattanavong et al., 2011). Similarly melioidosis was only formally documented from Cambodia in 2008, however 58 cases were documented in the ensuing three years (Overtoom et al., 2008). On mainland China, *B. pseudomallei* has been isolated from 4.2% of soil and water specimens in Hainan Island and adjoining coastal provinces as north as 25^{o}N, confirmed by Human cases and seroprevalence (IHA>1:40) of up to 34% in farmers in the region (Yang, 2000; Yang et al., 1998). In the 7 isolates tested from culture-positive cases, a high rate (57%) of ceftazidime resistance was observed (Yang et al., 1998).

Melioidosis in Australia

An outbreak of melioidosis in sheep on a Winton property (central Queensland) during 1949 was the first time the disease was recognized n Australia (Cottew, 1950; Cottew et al., 1952). The following year, the first human case in Australia was recorded when a 32 year old diabetic man from Townsville, north Queensland succumbed to septicemic melioidosis (Rimington, 1962). Shortly after, an outbreak of melioidosis among sheep in the Townsville district was reported, and the organism was also isolated from a goat and a pig in the same region (Lewis and Olds, 1952). The first indication that melioidosis was endemic in the Torres Strait was published in 1967 with two cases reported from Brisbane, one of the patients having recently been on Thursday island (Magee et al., 1967). Despite now being recognized as having a higher incidence of melioidosis than Queensland, melioidosis was not described from the North Territory until 1963 (Crotty et al., 1963). In the Northern Territory, melioidosis is the most common cause of fatal community-acquired pneumonia seen at the Royal Darwin hospital. Disease incidence is estimated to be 19.6 cases per 100,000 on average but has peaked at 50.2 cases per 1200,000 in 2009/2010 during a period of higher than average rainfall (Currie et al., 2010). In north Australia 0.6 to 16% of children had evidence of infection by *B. pseudomallei* (Armstrong et al., 2005; Chen et al., 2004; Inglis et al., 2000; Leelarasamee, 1998). Melioidosis has only recently been classified as a notifiable disease in parts of Australia, so prevalence of annual incidence is open to underestimation. In Townsville, 15.25% cases per year can be expected with a fatality rate of 25% (Cheng et al., 2003).

Other regions

Outside of Southeast Asia and Australia the distribution of melioidosis remains to be clarified. Melioidosis has been reported in numerous countries spanning tropical regions of the globe. The Americas have isolated reports from Mexico (Barnes et al., 1986), Puerto Rico whilst Brazil in particular is emerging as an endemic focus (Miralles et al., 2004). Reports of melioidosis from Africa have emerged from South Africa(Van der Lugt and Henton, 1995) but more extensively in Western Africa including Sierra Leone (Wall et al., 1985), Gambia (Cuadros et al., 2011), Burkina Faso (Ferry et al., 1973) and Nigeria

(Salam et al., 2011). It is likely, although remains to be clarified, that the extent of melioidosis in Western Africa is far greater than that reported. In Eastern Africa, melioidosis has been reported from Kenya (Bremmelgaard et al., 1982) and the island of Madagascar (Borgherini et al., 2006).

Melioidosis in Bangladesh

Melioidosis has been sporadically detected in Bangladesh over last several decades (Barai et al., 2014). The first reported case of melioidosis was published in the Lancet in 1964 in a 29 year old British sailor who was travelling through Bangladesh. His ship was carried half a mile inland near Chittagong by a cyclone, and was deposited in a paddy field. There he remained on the ship for the next 3 months and developed melioidosis later on (Maegraith and Leithead, 1964). However, the first case of melioidosis in a native Bangladeshi infant was published by International Centre for Diarrheal Diseases and Research, Bangladesh (ICDDR, B) in 1988 (Struelens et al., 1988). From 1991 to 1999, five cases were detected in United Kingdom (UK) among Bangladeshi people who migrated to UK from the Sylhet region (a northeastern district) of Bangladesh (Dance, 1991; Hoque et al., 1999; Kibbler et al., 1991). In 2001, the first culture confirmed suppurative melioidosis case in a 48 years old diabetic patient was detected in Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM) hospital. The patient came from Sherpur district, which is located about 140 km north of capital Dhaka (Nazimuddin et al., 2001). Later on, at least, 20 cases were reported among the diabetic patients in Bangladesh, and all of these cases were detected at BIRDEM hospital from 2001 to 2014 (Barai et al., 2014). Analyses of the reported cases strongly indicate that the disease is potentially endemic in ten districts of Bangladesh particularly in northern and northeastern parts of the country which include greater Mymensingh, Dhaka and Sylhet region.

Molecular epidemiology

The epidemiology of melioidosis is complicated due to the environmental persistence of the organism and is subject to distinct differences in the organism`s distribution on soil, disease-presentation, and incidence rates among different areas of endemicity. Molecular

typing method has been employed in an attempt to reveal quantifiable association between environmental and clinical clones so to provide conclusive evidence for a reservoir of infection and a mode of transmission. A variety of molecular tools have been used to infer genetic relatedness between isolates of *B. pseudomallei*. These have demonstrated that environmental isolates can be identical to epidemiologically-related human or animal strains, that recurrent infection is usually due to relapse with the same strain rather than reinfection with a different strain and that outbreaks of infection may be clonal (Currie et al., 1994; Currie et al., 2000; Currie et al., 2001; Desmarchelier et al., 1993; Haase et al., 1995a; Haase et al., 1995b).

Phylogenetics

Molecular tests that have been employed to segregate B. pseudomallei isolates into genetic sub-clusters include: pulsed field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD), multilocus sequence typing (MLST) and ribotyping (Haase et al., 1995a; Inglis et al., 2002; Maiden et al., 1998). Ribotyping was first reported for use in melioidosis epidemiology by Lew and Desmarchelier using *Bam*HI digests of chromosomal DNA (Lew and Desmarchelier, 1993). Fragments were separated by agarose gel electrophoresis and transferred to a nylon membrane. The membrane was subjected to hybridization with a cDNA copy of *Escherichia coli* tRNA probes labeled with horseradish peroxydase, based on studies by Stull (Stull et al., 1988). Twenty two rDNA patterns resulted which demonstrated greater diversity than phenotypic typing schemes such as serology and therefore provided the basis for an epidemiological tool. Haase et al showed that ribotyping could be further discriminated by using RAPD PCR, where short arbitrary primers under low-stringency conditions bind and produce products displayed as a pattern when separated by agarose gel electrophoresis (Haase et al., 1995a). This technique proved to be faster and able to discriminate the unrelated isolates within a single ribotype. However, these techniques suffer from significant drawbacks, including poor reproducibility within and between laboratories, limited availability of reagents and an inability to quantitate the genetic relationships between isolates (Maiden et al., 1998). To overcome these problems, a molecular typing procedure known as MLST, that uses nucleotide sequence data rather than DNA fragment patterns are

increasingly being used and emerged as the preferred choice, not only as it provides superior resolution of microbial diversity allowing discrimination of vastly more genotypic groups, but because the data can be easily stored on a stored on a central database allowing comparison of isolates between laboratories (Godoy et al., 2003).

Multilocus sequence typing (MLST)

Godoy et al optimized the MLST scheme for melioidosis epidemiology and phylogenetic analysis that may provide information at least as sensitive as PFGE macro-restriction pattern. The MLST typing scheme for *B. pseudomallei* targets seven of the most highly conserved genes which are involved in essential metabolic processes (Godoy et al., 2003). The genes were chosen based on their proximity to other housekeeping genes and their relationship to nearby genes that could be considered to be under diversifying selection. This typing scheme utilizes electronic portable nucleotide sequence data for the characterization of genome that can be easily shared and compared between laboratories worldwide via the internet (Maiden et al., 1998). The advent of MLST provides a beneficial methodology for the study of melioidosis epidemiology worldwide. This typing method is based on sequence variation within seven housekeeping-gene fragment. Allele numbers are assigned to each of the seven housekeeping loci based on sequence differences and are then arranged into a string of seven integers to give the allele profile of an isolate and dendograms are constructed from the pair wise differences in multilocus allelic profiles by cluster analysis. The *B. pseudomallei* MLST allele profile corresponds to the gene order *ace-gltB*- profile. The relationships among the STs may then be examined by using various methods, such *gmhD-lepA-lipA-narK-ndh* (McCombie et al., 2006). The sequence type (ST) of an isolate is defined specifically by the allele as eBURST which is based upon related sequence type (Godoy et al., 2003). MLST databases contain the reference allele sequences and sequence types for all *B. pseudomallei* so far isolated, and also isolate epidemiological data. The websites contain interrogation and analysis of software which allow users to query their allele sequences and sequence types.

Multiple-locus variable number of tandem repeat (VNTR) analysis (MALVA)

MALVA of *B. pseudomallei* is useful for detecting fine-scale genetic diversity due to the highly mutable nature of the targeted VNTR regions (U'Ren J et al., 2007; U'Ren et al., 2007). The ability of the MALVA to resolve fine-scale genetic diversity has been applied to soil isolates from Northeast Thailand and demonstrated much higher diversity than MLST on the same isolates. Furthermore, the technique has allowed successful resolution of within-host evolution of the organism during acute infection (Price et al., 2010). However, the application of 32-locus VNTR to multiple isolates is time consuming and is only suitable for comparisons between closely related organisms. For that reason MLST is still required to determine the relatedness of isolates to others on a global scale.

***B. pseudomallei* diversity**

Whilst narrow genetic diversity of environmental B. pseudomallei isolates has been reported in Thailand and Papua New Guinea, other studies have found high diversity within small geographic regions (Chantratita et al., 2007; Pearson et al., 2007; U'Ren et al., 2007; Warner et al., 2007). Phylogenetic analyses of *B. pseudomallei* isolates, performed by using MLST have led to phylogeographic associations that can be used to track melioidosis epidemics, examine the diversity of *B. pseudomallei* from various geographical regions, and reveal patterns of geographical partitioning between Australian and Southeast Asian isolates(Cheng et al., 2008; Dale et al., 2011; Godoy et al., 2003; U'Ren et al., 2007). Also, it has been determined that Australian isolates are more likely to carry an ancestral *B. thailandensis*-like flagellum and chemotaxis gene cluster (BTFC), whilst isolates carry a *Yersinia*-like fimbrial gene cluster (YLF) almost exclusively from Asia (Tuanyok et al., 2007). This suggests that these populations are genetically distinct due to broad scale biogeographical factors associated with establishment and persistence of the organism. More recently, whole genome sequencing has resolved that Asian isolates of *B. pseudomallei* share an Australian ancestral root (Pearson et al., 2009).

Environmental microbiology, transmission and epidemiology

B. pseudomallei is a resilient organism capable of surviving in hostile environmental conditions, including prolonged nutrient deficiency (up to 10 years), antiseptic and detergent solutions, acidic environments (pH 4.5 for up to 70 days) and a wide natural temperature range (24^0C to 32^0C), dehydration (soil water content <10% for up to 70 day) but not exposure to ultraviolet light (Gal et al., 2004; Tong et al., 1996; Wuthiekanun et al., 1995). It is likely that harsh environmental conditions may confer a selective advantage for the growth of *B. pseudomallei*.

The saprophytic nature of *B. pseudomallei* was first recognized in 1955. Some early studies implicated the aerosolization of dry dusts as a route of acquisition for American servicemen in Vietnam based on the high incidence in helicopter crews (Howe et al., 1971). However, further studies have demonstrated highest yields of bacteria occurred from moist soils and pooled surface water (Strauss et al., 1969; Wuthiekanun et al., 1995). The association between surface water and melioidosis is supported by the strong relationship with monsoonal rains and with occupational and recreational exposure to surface water and mud, particularly with flooding of rice fields and planting at the commencement of the monsoonal season (Chaowagul et al., 1989; Currie et al., 2000; Currie and Jacups, 2003; Leelarasamee and Bovornkitti, 1989). The finding that higher rainfall is significantly associated with sepsis and pneumonia and may suggest that environmental conditions during the monsoonal season may be associated with inhalation rather than inoculation as the primary mode of acquisition (Currie and Jacups, 2003). In particular, moist clay soils seem to be favored by the organism and populations residing on these soil types in Darwin have a higher rate of disease (Thomas et al., 1979). Sampling studies in Australia have suggested that bacterial counts increase to a death of 60-90 cm, but the finding that dry, shallower soils may be culture negative yet PCR positive has led to the suggestion that the organism may persist in a 'viable but not-culturable state' (Brook et al., 1997). Factors that may influence the distribution of *B. pseudomallei* in the environment may include physical factors such as rainfall, humidity, ultraviolet radiation and temperature, and chemical factors such as soil composition, other vegetation and the use of fertilizers and recent soil disturbance such as excavation

and ploughing (Inglis et al., 2000). The implications of global climate change for the epidemiology of melioidosis is as yet unknown (Currie et al., 2010).

2.6 Pathogenicity

From environmental saprophyte to opportunistic pathogen

Burkholderia pseudomallei is a resilient organism capable of surviving in a variety of hostile conditions, including nutrient deficiency, acid and alkaline pH, in disinfectant and antiseptic solutions like detergent and chlorine, exposure to many antibiotics and at extremes of temperature. It is likely that harsh environmental conditions may confer a selective advantage for the growth of *B. pseudomallei.* This oranism is a terrestrial bacterium that has adapted to survive in the environment primarily in rice fields, stgnant waters, and moist tropical soil and may persist in the environment as a viable but non-culturable state(Brook et al., 1997). However, the virulence of environmental isolates is not significantly different from clinical isolates. People with occupational or recreational exposure to stgnant water have higher incidence of disease and during the rainy season when bacteria are leached from the soil there are more cases of melioidosis (Chaowagul et al., 1989; Currie et al., 2000). Therefore, the bacterium is able to quickly adapt from a terrestrial lifestyle to the eukaryotic environment. *B. pseudomallei* obtains nutrition from rotting organic matter and opportunistically from invasion to protozoa. Initial adhesion of *B. pseudomallei* to the free-living protozoan *Acanthamoeaba astronyxis* involves polar attachment via flagella (Inglis, 2003). Following engulfment by pseudopodia, the viable bacteria are observed both within vacuoles and free in the cytoplasm (Inglis, 2000). It is predicted that mechanisms similar to those used for invasion and survival within this environmental niche are also used during infection of human macrophages.

Molecular typing methods have shown that there is a significant diversity within both environmental and clinical isolates of *B. pseudomallei*, however, individual isolates from either grouping can be identical (Cheng and Currie, 2005). The closely related species *Burkholderia thailandensis*, which was initially identified as an avirulent environmental *B. pseudomallei* isolate, has a similar environmental niche, but is unable to cause disease.

The reason for the attenuation of *B. thailandensis*, in comparison with *B. pseudomallei*, has been associated with the presence of a functional arabinose biosynthesis operon in *B. thailandensis*, which is largely deleted in *B. pseudomallei*. Introduction of the complete *B. thailandensis* arabinose biosynthesis operon into *B. pseudomallei* resulted in the down regulaton of a number of type III secretion genes and the strain displayed reduced virulence in Syrian hamsters (Moore et al., 2004). *Burkholderia thailandensis*, which shares many similarities with *B. pseudomallei*, including intracellular invasion, has been used as a model organism in which to study *B. pseudomallei* virulence. Significant genetic differnces have been reported between *B. pseudomallei* strains that differ in virulence potential, but the level of virulence of *B. pseudomallei* strains isolated from the environment is not significantly different from that of the clinical strains (Vesaratchavest et al., 2006). Furthermore, no clear difference in virulence was obseved between strains isolated from fatal and nonfatal melioidosis cases (Ulett et al., 2001). Thus, while *B. pseudomallei* strains differ in their individual ability to cause disease, the outcome also clearly depends on the immune status and response of the infected host.

Molecular and cellular mechanism of *B. pseudomallei* virulence

A number virulence factors have been proposed to be involved in the pathogenesis of *B. pseudomallei*. However, it is important to note that these virulence determining factors are mechanisms developed (in evolutionary terms) by the organisms to survive in its new ecological niches. The bacterium also avoids host immune responses by utilizing these virulence factors. However, infection of these hosts by *B. pseudomallei* is accidental and is not likely to provide an evolutionary advantage for this environmental organism. This fact is reflected in the poor characterization of bacterial products as being truly virulent in animal studies and its primary affinity for hosts with impaired immunity. They have low disease-causing potential in healthy hosts despite its ubiquity in the environment. This stands in contrast to other parasitic organisms, like *Staphylococcus aureus* whose ecological niche is in humans and animals, can affect immunocompetent individuals and where virulent factors such as the Panton-Valentine leukocidin correlate with severe disseminated disease.

Virulence determinants of *B. pseudomallei*

The spectrum of diseases in immunocompromised host caused by *B. pseudomallei* suggests that the organism uses a variety of virulent determinants for survival during infection in mammals. These factors have been proposed to be involved in the pathogenesis of *B. pseudomallei*. It is well adapted to its many hosts by producing secreted proteins like, proteases, lipases, lecithinase, catalase, peroxydase, superoxide dismutase, haemolysins, a cytotoxic exolipid, and a siderophore. The organism is resistant to complement, lysosomal defensin and cationic peptidases and can survive within many eukaryotic cell lines including professional phagocytes such as neutrophils and macrophages (White, 2003a).

A number of virulence factors have been proposed to be involved in the pathogenesis of *B. pseudomallei*. Some of these virulent factors are located on the surface of the bacteria as cell associated antigens while others are secreted by the bacteria as secretory proteins. Role of the virulent factors like, capsule, lipopolysaccharide (LPS), the type three secretion system3 (TTSS3), flagella, morphotype switching and certain quorum-sensing (QS) molecules have been documented. The effect on virulence of a number of other factors including pili, the type 6 secretion system (T6SS), secreted factors and regulatory genes has also been studied and current data indicating that each plays a moderate to minor role in virulence (Table 2.2)

Table 2.2 Identified virulence factors of *B. pseudomallei*

Virulence factor	Role in virulence	Reference
Capsule	Epithelial attachment, resistance to complement mediated lysis	(Ahmed et al., 1999)
LPS	Resistance to complement and defensin	(Burtnick and Woods, 1999; DeShazer et al., 1998)
Flagella	Motility	(DeShazer et al., 1998)
Pili	Epithelial attachment; micro-colony formation	(Brown et al., 2002; Essex-Lopresti et al., 2005)
Quorum sensing	Stationary phase gene regulation, including secreted enzymes and oxidative stress protein	(Lumjiaktase et al., 2006; Song et al., 2005)
TTSS3	Invasion and vacuolar escape	(Stevens et al., 2003; Stevens et al., 2002)
Morphotype switching	Alteration of surface determinants for in vivo phenotypic changes	(Chantratita et al., 2007)

Capsular polysaccharides

Many bacterial pathogens produce extracellular capsular polysaccharide which has been proposed to serve a variety of functions including attachment to cell surface, evasion of host defenses by preventing complement mediated lysis, opsonization, and finally phagocytosis. Electron microscopy analysis has demonstrated several morphologically distinct capsule variants of the organism. One group of bacteria is surrounded by a very thick macrocapsule of approximately 0.1-0.25 μm in thickness, while another group had microcapsules of approximately 0.086 μm thick, and finally the other group of bacteria appeared to lack any capsule (Puthucheary et al., 1996). Whether this differences was simply due to the amount of capsule produced or whether these two capsule-producing

strains were producing different variants of capsule has not been determined. However, the macrocapsule, observed to encase several cells at once in a micro-colony, may in fact constitute the initial stages of the glycocalyx biofilm formation (Vorachit et al., 1995).

B. pseudomallei has been shown to produce four polysaccharide structures, but only three capsule biosynthesis loci have so far been identified on the K96243 genome (Kawahara et al., 1998; Knirel et al., 1992; Masoud et al., 1997; Nimtz et al., 1997; Perry et al., 1995). The organism been shown to produce a polysaccharide molecule designated as type 1 O-antigenic polysaccharide (type 1 O-PS), which is an unbranched high-molecular-weight polymer of 1, 3-linked 2-O-acetyle-6-deoxy-ß-D-manno-heptopyranose (Knirel et al., 1992; Perry et al., 1995). Although initially characterized as one of the O-PS of lipopolysaccharide, this polysaccharide was independently reclassified as a capsular polysaccharide by an identification of the biosynthetic locus (Reckseidler et al., 2001; Reckseidler-Zenteno et al., 2005) and the absence of lipid A (Isshiki et al., 2001). Studies using signature-tagged mutagenesis (STM) identified a number of genes located within the type 1 O-PS biosynthetic locus including *wcbN, wcbC, wzm2, wcbQ* and *wcbB*. Inactivation of any of these genes in the biosynthetic locus of the type 1 capsule led to reduced survival in mice (Atkins et al., 2002; Cuccui et al., 2007; Lazar Adler et al., 2009) and Syrian hamsters(Reckseidler et al., 2001). Moreover, the *wcbB* mutants were also shown to be 1000 times more sensitive to the bactericidal effects of human serum, which could be reversed by the addition of purified capsule. Western blot analysis revealed more C3b complement deposition on the surface of capsule deficient mutants than the wild-type strain, which agrees with the sensitivity of the mutant strain to human serum. The capsule may act as a barrier, blocking access of the complement receptor – 1 (CR1) on phagocytes to the C3b deposited on the bacterial surface. Additionally, type 1 O-PS capsule has been shown to be important in the context of macrophage infections following opsonization with serum components as the *B. pseudomallei* capsule mutant was internalized in greater numbers than the wild-type following incubation with human serum (Reckseidler-Zenteno et al., 2005). These data indicate that expression of type 1 O-PS capsule *in vivo* helps *B. pseudomallei* to resist phagocytosis. The absence of type 1 OPS capsule from *B. thailandensis*, is likely to account for the observation that *B.*

thailandensis is avirulent in Syrian hamsters (Brett et al., 1997; Brown et al., 2000; Reckseidler et al., 2001). Whether lack of a capsule is the sole factor associated with the avirulence of *B. thailandensis* is unknown.

A second capsular polysaccharide (Type II) was determined to be a linear unbranched polymer of the tetra-saccharide: -3)-2-O-Ac-ß-D-Galp-(1-4)-α-D-Galp-(1-3)-ß-D-Galp-(1-5)-ß-D-KDOp-(2-. This capsule was expressed by 12 strains from various geographic regions and reacted strongly with antibodies in patient sera (Masoud et al., 1997; Nimtz et al., 1997; Steinmetz et al., 1995). The third capsular polysaccharide (Type III) isolated from B. pseudomallei has been to be a 1-4 linked glucan. *B. pseudomallei* strains lacking expression of the 1-4 linked glucan showed a delayed time of death in mice of 7.8 days compared to wild-type (3 days). An acidic fourth polymer (Type IV) composed of galactose, rhamnose, mannose, glucose and uronic acid (3:1:0.3:1:1 ratio). Mutants of these sugars also showed a delayed time of death (11.6) in mice (Kawahara et al., 1998; Sarkar-Tyson et al., 2007).

Biofilm formation

Multicellular communities of surface-attached bacteria are known as biofilm. *B. pseudomallei* sometimes produce confluent biofilm which comprises bacteria encased in a carbohydrate-based fibrous matrix. Biofilms are commonly associated with the pathogenesis of bacterial infections, especially nosocomial and chronic infections, as antibiotics and host immune responses cannot thoroughly penetrate the matrix surrounding bacterial aggregates (Costerton et al., 1999; Fux et al., 2005; Reisner et al., 2005). Biofilm has been observed in electron micrographs of *B. pseudomallei*-infected lung tissue from guinea pigs and a human patient. As mentioned above, the bacteria producing very thick capsules (0.1-0.25 μm) were sometimes shown to aggregate into a microcolony completely surrounded by a polysaccharide structure, which may represent the beginning of biofilm production (Vorachit et al., 1995). *B. pseudomallei* produces biofilm that increase resistance to ceftazidime and co-trimoxazole (Vorachit et al., 1993). Biofilm production of *B. pseudomallei* is regulated by *oxyR*, the alternative sigma factor RpoE, and quorum sensing (Korbsrisate et al., 2005; Loprasert et al., 2002). It has been

shown that the *rpoE* mutants were demonstrating a 50% reduction in biofilm formation. Electron microscopy indicated that the *rpoE* mutants were found in chains rather than aggregated clusters as observed for the wild-type *B. pseudomallei* (Korbsrisate et al., 2005). Overall, there are significant differences in the amount of biofilm produced by *B. pseudomallei* strains. A study of 50 *B. pseudomallei* strains confirmed significant variability in the amount of biofilm produced by *B. pseudomallei* strains. It has been observed that there is no correlation between biofilm production and virulence in the BALB/c mouse melioidosis model. Even strains lacking biofilm production were still as virulent as wild-type bacteria. Furthermore, biofilm mutants were recognized by capsule specific antibodies, indicating that biofilm formation by *B. pseudomallei* does not involve capsular polysaccharide (Taweechaisupapong et al., 2005). Biofilm production is dispensable during early stages of infection; however, its production during latent infection has not been studied. Therefore, biofilm formation does not appear to be essential for virulence, although it is likely to have an important role in persistence in harsh environments, thus allowing survival for later infection.

Quorum sensing

Quorum sensing (QS) is a system of stimuli and response utilized by many species of bacteria and also some insects that correlate to population density. Bacteria use quorum sensing to co-ordinates the expression of many genes according to the density of their local population and to coordinate certain behaviors such as biofilm formation, virulence, and antibiotic resistance. Some social insects, like ants, honey bee also use this system in a similar fashion to determine where to nest (Fuqua et al., 1994; Miller and Bassler, 2001). Quorum sensing can occur within a single bacterial species or between diverse species, and can regulate a host of different processes, in essence, serving as a simple indicator of population density or the diffusion rate of the cell`s immediate environment.

Quorum sensing bacteria produce and release a variety of different chemical signal molecules called autoinducers or pheromones that increase in concentration as a function of cell density. The detection of a minimal threshold stimulatory concentration of an inducer leads to an alteration in gene expression. A variety of different autoinducer molecules are secreted which influences the behavior of many bacterial populations

through the production and extracellular secretion of N-acyl-homoserine lactones (AHL) in case of Gram negative bacteria, oligopeptides in Gram positive bacteria and autoinducer – 2 (AI-2) in both Gram positive and Gram negative bacteria(Miller and Bassler, 2001). These organisms also possess a receptor that can specifically detect the signaling molecule (AHL). When AHL binds the receptor, it activates transcription of certain genes, including those for inducer synthesis. There is a low likelihood of a bacterium detecting its own secreted inducer. Thus, in order for gene transcription to be activated, bacteria must encounter signaling molecules secreted by other cells in its environment. When only few bacteria are present in the vicinity, diffusion reduces the concentration of the autoinducer (AHL) in the surrounding medium to almost zero, so the bacteria produce little inducer. However, as the population grows, the concentration of the inducer passes a threshold, causing more inducer to be synthesized. This forms a positive feedback loop, and the receptor becomes fully activated. Activation of the receptor induces the up-regulation of other specific genes, causing all of the bacteria to begin transcription at approximately the same time. This coordinated behavior of bacterial cells can be useful in a variety of situation, like the bioluminescent luciferase, produced by *Vibrio fischeri* would not be visible if it were produced by a single cell. By using QS to limit the production of luciferase to situation when cell populations are large, *V. fischeri* cells are able to avoid wasting energy on the production of useless product. Furthermore, there is mounting data suggesting that bacterial autoinducers elicit specific responses from host organisms. Although the nature of the chemical signals, the signal relay mechanisms, and the target genes controlled by bacterial QS systems differ, in every case the ability to communicate with one another allows bacteria to coordinate the gene expression, and therefore the behavior, of the entire community. Presumably, this process bestows upon bacteria some of the qualities of higher organisms. The evolution of QS systems in bacteria could, therefore, have been one of the early steps in the development of multicellularity (Miller and Bassler, 2001).

B. pseudomallei constitutively produce and secret AHL signaling molecules. The K96243 genome of *B. pseudomallei* expresses *LuxIl* homologues, which encodes the AHL synthase proteins. As the concentrations of bacteria increase, AHLs promote of the

transcriptional regulator, *LuxR* (Lazdunski et al., 2004; Ulrich et al., 2004). The *B. pseudomallei* genome is reported to contain 3 (three) *LuxI* and 5 (five) *LuxR* quorum sensing homolog. The regulators become activated upon binding their cognate AHL, and subsequently mediate transcription of QS-regulated bacteria. Disruption of these 8 genes, encoding *LuxI* and *LuxR* QS homolog lead to a significant increase in LD_{50} in Syrian hamsters after intraperitoneal challenge, and increased the time to death and reduced organ colonization in aerosolized BALB/c mice (Ulrich et al., 2004). Mass spectrometry analysis of *B. pseudomallei* culture supernatants has demonstrated the presence of numerous signaling molecules. These include different types AHLs and so far seven (7) AHLs have been detected in *B. pseudomallei* supernatant from various strains. These are N-octanoyl-homoserine-lactone (C_8HSL), N-(3-oxyoctanoyl)-L-homoserine-lactone (3-oxo-C_8-HSL), N-(3-hydroxyoctanoyl)-L-homoserine-lactone (3-hydroxy-C_8-HSL), N-decanoyl-homoserine-lactone(C_{10}-HSL), N-(3-hydroxydecanoyl)-L-homoserine-lactone (3-hydroxy-C_{10}-HSL), N-(3-hydroxydodecanoyl)-L-homoserine-lactone (3-hydroxy-C_{12}-HSL) and N-(3-oxotetradecanoyl)-L- homoserine-lactone (3-oxo-C_{14}-HSL). A LuxI-LuxR homolog, which directs the synthesis of C_{10}-HSL and is involved in regulation of a metalloprotease, is essential for full virulence in a mouse model (Chan and Chua, 2005; Lumjiaktase et al., 2006; Song et al., 2005; Ulrich et al., 2004; Valade et al., 2004). A homolog termed BpsI-BpsR is also required for optimal expression of virulence and secretion of exoproducts (Song et al., 2005). Quorum-sensing-controlled virulence factors such as siderophore and phospholipase C and biofilm formation are probably partially depended on BpeAB-OprB, a multidrug efflux pump of B. pseudomallei also known to be responsible for conferring antimicrobial resistance to aminoglycosides and Macrolides (Chan and Chua, 2005). BpeAB mutants showed attenuated cell invasion and cytotoxicity towards human lung epithelial cells (A549) and human macrophage (THP-1) cells (Chan and Chua, 2005). This suggests that possibility of attenuating *B. pseudomallei* virulence through the use of inhibitors of the BpeAB-OprB efflux pump.

Secretion Systems

Eukaryote and prokaryote organisms have evolved mechanisms that allow them to best survive in their preferred environment. As interactions with other cells or environmental

factors generally occur first through surface receptors, it is essential to have some systems in place that allow the translocation of necessary proteins to the surface of these cells. In Gram-negative bacteria, like *B. pseudomallei*, this process is made more complex by the double membrane structures of the organism. Proteins bound for the surface or which are secreted must cross the inner membrane, pass through the periplasm and peptidoglycan layer, and finally cross the outer membrane before reaching their final destination. Translocation of these molecules to the surface is performed by several export or secretion systems and these are diverse in their composition and function. Bacteria use secretion systems to express virulence factors on the surface of the bacterium or to direct virulence proteins onto or into host cells. A number of different proteins involved in the secretion machinery have been identified and currently, there are six different secretion systems described in Gram-negative bacteria which include type I to type VI. In *B. pseudomallei*, some of these systems like type II, type III and type VI secretion device have been well defined while others have yet to be characterized. Among these devices, type III secretion system (TTSS) is a major virulence factor for *B. pseudomallei* (Winstanley et al., 1999).

Type III Secretion System (TTSS)

Type III Secretion System (TTSS), also known as injectosome, has received special attention for its apparent ability to `inject` effector proteins into host cells, thereby enabling the bacterium to manipulate certain cellular processes (Figure 2.6). The system possesses a needle-like structure used as a sensory probe to detect the presence of eukaryotic organisms and secret proteins that help the bacteria infect them. The secreted effector proteins are secreted directly from the bacterial cell into the eukaryotic cell, where they exert a number of effects that help the pathogen to survive and to escape an immune response. The TTSS of *B. pseudomallei* belongs to the *inv-mxi-spa* gene clusters, which is known as a key virulence determinant in *Salmonella enterica* and *Shigella flexneri* (Attree and Attree, 2001; Rainbow et al., 2002; Stevens et al., 2002). In *B. pseudomallei*, TTSS is encoded by the *Burkholderia* secretion apparatus (*bsa*) gene cluster. The secreted proteins encoded by this gene cluster are required for invasion,

escape from endocytic vacuoles, intracellular spread and pathogenesis (Stevens et al., 2002).

Following internalization, *B. pseudomallei* escapes from endocytic vacuoles into the cytoplasm of infected cells. Induction of actin polymerization at one pole leads to the formation of membrane protrusions and cell to cell spread. *B. pseudomallei* mutants lacking components of the *bsa* secretion and translocation apparatus have reduced replication in murine macrophage-like cells, an inability to escape endocytic vacuoles and cannot form membrane protrusions and actin tails (Stevens et al., 2002). Inactivation of BopE, a TTSS protein encoded adjacent to the *B. pseudomallei bsa* locus that is homologous to S. enterica SopE/SopE2, a guanine nucleotide-exchange factor, leads to impaired bacterial entry into HeLa cells, indicating that BopE facilitates invasion (Stevens et al., 2003). *B. pseudomallei bipD* mutants lacking a component of the translocation apparatus are attenuated following intraperitoneal or intranasal challenge of BALB/c mice, and have impaired bacterial replication in liver and spleen (Stevens et al., 2004). *B. pseudomallei bipB* has been shown to mediate multinucleated giant cell formation, cell-to-cell spreading of bacteria and apoptosis of infected host cells (Suparak et al., 2005). *B. pseudomallei bipB* mutants are also associated with attenuated following intranasal challenge of BALB/c mice (Suparak et al., 2005).

The TTSS1 gene cluster was first described in 1999 (Winstanley et al., 1999). This is homologous to a TTSS of the plant pathogen *Ralstonia solanacearum* but is absent from *B. mallei and B. thailandensis* (Holden et al., 2004b; Rainbow et al., 2002; Thibault et al., 2004b). TTSS2 is present in *Ralstonia solanacearum, B. pseudomallei, B. mallei and B. thailandensis.* The role of these two systems in pathogenesis is not known. As TTSS1 is absent from other Burkholderia, so this highly conserved gene is used for molecular detection of *B. pseudomallei.* TTSS3 has been shown to be required for full in a hamster model of infection (Warawa and Woods, 2005).

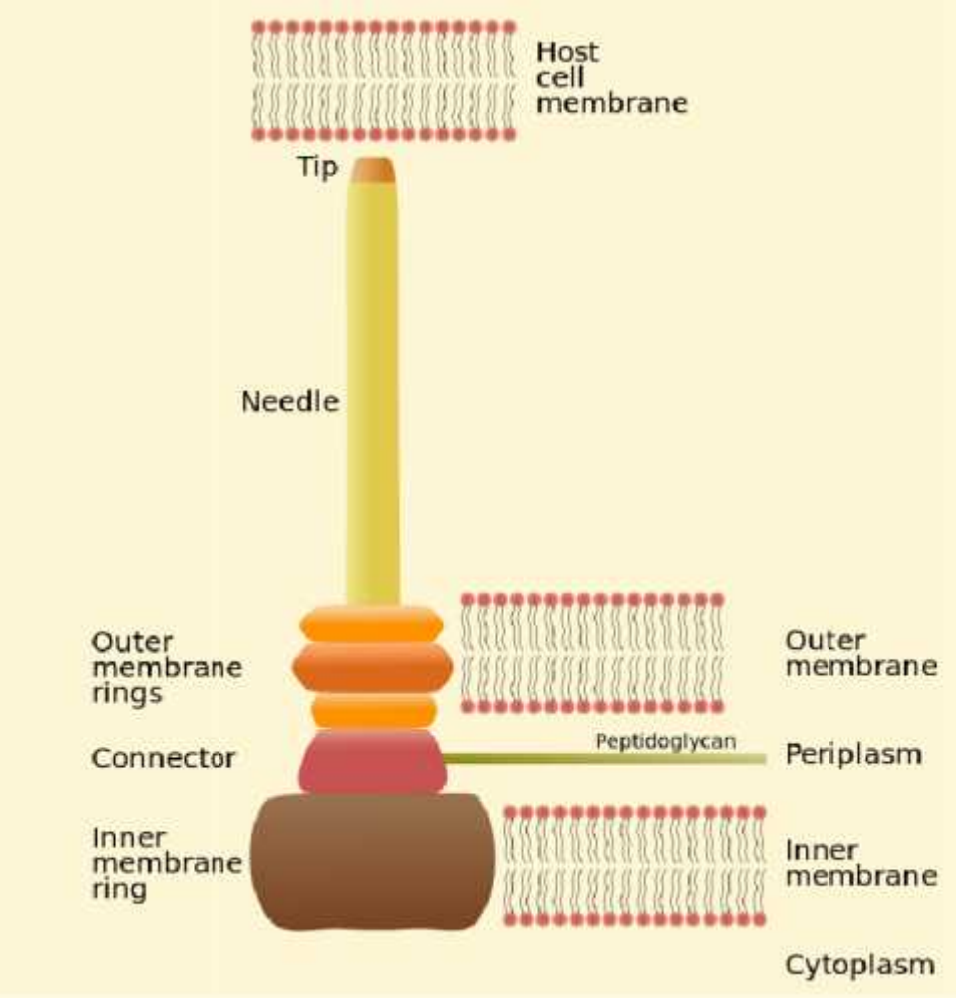


Figure 2.6: The TTSS needle complex

Lipopolysaccharide (LPS)

B. pseudomallei LPS (formally termed type II O-antigenic polysaccharide) appears to differ in several respects from the LPS of other Gram-negative organisms. *B. pseudomallei* LPS exhibits weaker pyrogenic activity in rodents compared enterobacterial LPS, but stronger mitogenic activity in murine splenocytes (Matsuura et al., 1996). LPS-mediated activation of a mouse macrophage cell line in vitro is slower for LPS from *B. pseudomallei* compared with LPS from *Escherichia coli* (Utaisincharoen et al., 2000). The genes responsible for LPS production have been studied and it has been shown that mutants in some of these genes resulted in *B. pseudomallei* strains that had increased sensitivity to polymixin-B (Burtnick and Woods, 1999), increased sensitivity to the bactericidal effects of human serum, increased uptake by phagocytes, and attenuated virulence in animal models (DeShazer et al., 1998). Therefore, LPS can be considered as an important virulence determinant in *B. pseudomallei.*

Flagella and pili

B. pseudomallei is a motile organism and electron microscopy studies have demonstrated the presence of flagella and the variable expression of pili on *B. pseudomallei* (Vorachit et al., 1995). Flagella are structures that are composed of many proteins homologous to molecules involved in the formation of type three secretion systems(Lee and Schneewind, 2001). Flagella consist of a central shaft, anchored in both membranes by two ring structures connected to the flagellar filament by a hook. Rotation of the filament allows the propulsion of the bacteria (Lee and Schneewind, 2001). A polar tuft of two to four flagella confers motility on *B. pseudomallei.* Flagella synthesis requires the *fliC* gene, which encodes a 39.1 –kDa flagellin protein (DeShazer et al., 1997). Polyclonal antiserum against *fliC* was able to inhibit motility in all but one of the 65 *B. pseudomallei* strain tested (Brett et al., 1994).

There are conflicting data on the importance of flagella in virulence; a *fliC B. pseudomallei* 1026b transposon mutant was not attenuated via the intraperitoneal route in the diabetic rat or Syrian hamster melioidosis models (DeShazer et al., 1997). However, unlike wild type *B. pseudomallei*, the *fliC* mutants were unable to adhere to cells of the

free living amoeba, *Acanthamoeba astronyxis*, a critical step for efficient invasion of this organism (Inglis et al., 2003). These data indicate that flagella have an important role in virulence, but it is possible that this role can be overcome or subverted in more acute infection in models, such as diabetic rats or hamsters.

Pili are essential for adherence and colonization of many species of bacteria. Genes involved in type 1 pilus expression namely *fimA, fimC* and *fimD* from *B. pseudomallei* 1026B have been cloned and sequenced. The *B. pseudomallei* K96243 genome contains multiple type IV pilin-associated loci, of which the major subunit is *pilA* (Essex-Lopresti et al., 2005). A *pilA* deletion mutant has reduced adherence to human epithelial cells and is less virulent in the nematode model of virulence and the murine model of melioidosis, suggesting a role of type IV pili in *B. pseudomallei* virulence. In addition, type IV pilus producing gene with strong homology to *pilB, pilC* and *pilD* gene of other Gram negative bacteria have been identified. The role of these genes and their products in adherence and pathogenesis is still unknown.

Morphotype switching

The virulence of an individual strain is further complicated by the ability of *B. pseudomallei* to alter surface determinants and change its observed colony morphology. As mentioned earlier, seven distinct morphotypes have been described, although the characteristic wrinkled type 1 morphotypes predominates (Chantratita et al., 2007). Morphotype switching can be induced by a range of *in vitro* stresses, including starvation, heat shock, iron limitation and sub-inhibitory antibiotic concentration. Phenotypic differences, including changes in Biofilm formation, secreted enzymes and motility, were observed between morphotypes. These differences affected intracellular survival in macrophage and epithelial cells. Furthermore, differential lethality and persistence were seen in BALB/c mice, suggesting that morphotype switching *in vivo* provides a strong survival advantage for *B. pseudomallei* (Chantratita et al., 2007).

2.7 Pathogenesis

A key element of the pathogenesis of *B. pseudomallei* is its ability to invade, survive and replicate within both phagocytic and non-phagocytic cells of the host (Allwood et al., 2011). Adhesion to the external surface of host cells is considered the first step in the pathogenesis of *B. pseudomallei* prior to an intracellular invasion. The initial attachment of *B. pseudomallei* to host cells is mediated by a bacterial capsule (Galyov et al., 2010). Subsequently, an intracellular invasion of the organism occurs either in macrophages or epithelial cell lines. It is likely that the TTSS plays an important role in the bacterial invasion as well as escaping from endocytic vesicles (Stevens and Galyov, 2004). Once inside the host cell cytoplasm, the ability to survive intracellularly is a key factor for developing the infection while avoiding host immune surveillance. Strategies of evasion of the host immune response by *B. pseudomallei* include escaping from the endosome to the cytosol, inducing macrophage cell death and cell-to-cell spreading through actin polymerization (Gan, 2005). *B. pseudomallei* can form actin based membrane protrusions by continuous nucleation of actin at one pole of the bacterial cell, allowing intracellular motility. The bacterial movement to adjacent cells leads to cell fusion and the formation of multinuclear giant cells (MNGC), which indicates the progression of infection (Lazar Adler et al., 2009).

Host infection with environmental saprophyte

B. pseudomallei is a terrestrial bacterium that has adapted to survive in the environment primarily in rice fields, stagnant surface waters, moist tropical soils and roots of plants (Brett and Woods, 2000; Holden et al., 2004b). Its persistence is demonstrated in that it is able to survive for prolonged periods in low nutrient conditions and had been cultured from distilled water 10 years after inoculation (Wuthiekanun et al., 1995). The organism may persist in the environment as a viable but non-culturable state (Brook et al., 1997). However, *B. pseudomallei* grows best in soil with a water content of 15%, and most infections occur during the rainy season when bacteria are leached from the soil (Brett and Woods, 2000; Leelarasamee, 2004). Studies with Vietnam helicopter crews suggested that inhalation was a major route of infection and infection following inhalation of aerosolized organisms is also observed during the monsoon seasons when

the total number cases of disease increased dramatically (Currie and Jacups, 2003; Howe et al., 1971). Alternatively, inoculation of *B. pseudomallei* into skin lesions is also a significant route of infection for this disease. During periods of harvesting, rice farmers spend long hours in the wet soils where the organism is found in significant quantities, and commonly contract minor wounds to the feet and legs (Chaowagul et al., 1989). Inoculation in skin breaks can be problematic, as presentations may progress rapidly in a manner similar to necrotizing fasciitis (Wang et al., 2003). Certainly, both inoculation into open wounds in the skin and inhalation of aerosolized bacteria are major routes of infection. Other modes of infection include ingestion and person-to-person transmission, but the significance of these routes of infection is believed to be very low (Holland et al., 2002; Howe et al., 1971). Molecular typing methods have shown that there is a significant diversity within both environmental and clinical isolates of *B. pseudomallei*; however, individual isolates from either grouping can be identical (Cheng and Currie, 2005). Furthermore, no clear difference in virulence was observed between strains isolated from fatal and non-fatal melioidosis cases (Ulett et al., 2001). The closely related species *B. thailandensis*, which was initially identified as an avirulent environmental *B. pseudomallei* isolate, has a similar environmental niche, but is unable to cause disease. The reason for the attenuation of *B. thailandensis*, in comparison with *B. pseudomallei*, has been associated with the presence of a functional arabinose biosynthesis operon in *B. thailandensis*, which is deleted in *B. pseudomallei*. Introduction of the complete *B. thailandensis* arabinose biosynthesis operon into *B. pseudomallei* resulted in the down-regulation of a number of TTSS gene and the strain displayed reduced virulence in Syrian hamsters (Moore et al., 2004).

Attachment to epithelial cells

The major routs of infection by *B. pseudomallei* appear to be from inoculation in the skin and inhalation. During infection, it is certain that *B. pseudomallei* encounter epithelial barriers in the skin and/or in the lungs. To date, three structures, namely capsule, flagellum, and pilus, and two individual proteins, namely BoaA and BoaB, have been shown to mediate attachment of the bacterium to epithelial cells. This highly pathogenic bacterium binds these cells at relatively low. The bacterial capsule adheres to pharyngeal

cells, though it is not known what component of the capsule is responsible for this attachment levels (Ahmed et al., 1999). Another macromolecular structure implicated in binding to epithelial cells is the type IV pilus (Essex-Lopresti et al., 2005), though this process also remains poorly defined. The role of the flagellum in adherence was shown in an amoeba model, which revealed the wild-type *B. pseudomallei* 1026b was attached to the surface of the amoeba by the flagella while the flagella mutant strain lacked this adherence (Inglis et al., 2003). Studies on the regulation of the structural pilus component, pilA, show that adherence mechanisms appear to vary between strains (Boddey et al., 2006). In addition to large surface structures displaying attachment to epithelial cells, several single molecule adherence factors have recently been characterized including the auto-transporters BoaA and BoaB (Balder et al., 2010). As with all auto-transporters BoaA and BoaB possess C-terminal ß-barrels that insert into the outer membrane of the bacterium and are necessary for the secretion of the passenger domains. The BoaA and BoaB proteins in *B. pseudomallei* were shown to be important for binding to the respiratory epithelial cells lines, A549 (type II pneumocyte) and Hep2 (laryngeal). The BoaA and BoaB mutant strains were also found to have lower levels of adherence to a primary culture of normal human bronchial epithelium (Balder et al., 2010).

Pathogen recognition by host

On first encounter with *B. pseudomallei*, cells of the innate immune system recognize conserved surface motifs termed "pathogen-associated-molecular-patterns" or PAMPs via host cell "pattern recognition receptor" or PRR. The family of commonly known PRRs is the toll-like receptors (TLRs), which are type1 integral membrane glycoproteins. Extracellular domains of TLRs contain varying numbers of leucine-rich-repeat (LRR) motifs. They are important members of this surveillance system which initiate the innate immune response, and form a key link between innate and adaptive immunity (Takeda et al., 2003). *B. pseudomallei* express several PAMPs for which the corresponding TLR is known. For example, LPS activates the cells of the immune system via a receptor complex that consists of a ligand-binding molecule (CD14) and TLR4 as the signal-

transducing element. Other candidate *B. pseudomallei* TLR ligand includes peptidoglycan (TLR2), flagellin (TLR5), and bacterial DNA (TLR9).

Invasion of cells

Following adherence to epithelial cells, *B. pseudomallei* is able to invade, survive, and replicate. One molecule shown to be involved in the invasion of cells by the organism is BopE. This protein was shown to be secreted by the Bsa Type three secretion system (TTSS) in *B. pseudomallei* and expression of BopE in eukaryotic cells led to dramatic rearrangements in the actin cytoskeleton and membrane ruffling. *B. pseudomallei* BopE mutants were unable to invade epithelial cells at wild type levels (Stevens et al., 2003) Additionally, a *B. pseudomallei* bipD mutant, which lacks expression of the functional TTSS, has an even greater defect in invasion than the BopE mutant alone, suggesting that more TTSS effectors may also be involved in this process (Stevens et al., 2003). Following invasion of cell lines, *B. pseudomallei* is able to escape from the endocytic vacuoles into the cytoplasm (Harley et al., 1998). Subsequent studies in cell lines and in animal models have shown that once in the cytoplasm, the organism is capable of manipulating cellular process to survive. Specifically, the organism is able to bind and polymerize actin to move intercellularly while protected from extracellular immune recognition (Gauthier et al., 2001). *B. pseudomallei* express three functional TTSSs. The TTSS3 is not only important for invasion of epithelial cells but also shown to be important for the intracellular life style of *B. pseudomallei*. Mutants that were unable to form a functional TTSS3 demonstrated defects in the ability to escape from the vacuole, survive, and replicate within these cells (Stevens et al., 2002).

Intracellular survival of *B. pseudomallei*

After entry into human, the initial host defense against *B. pseudomallei* includes natural antibiotic peptides, the complement system and phagocytic cells. Antimicrobial peptides called defensin are naturally produced to kill bacteria attacking host cells. However, *B. pseudomallei* is resistant to this natural immunity (Jones et al., 1996). In addition, *B. pseudomallei* can produce an extracellular polysaccharide capsule that prevents

complement mediated lysis. Moreover, this capsule reduces the deposition of complement factor C3b, resulting in anti-phagocytosis (Reckseidler-Zenteno et al., 2005). *B. pseudomallei* is able to survive and proliferate within a variety of phagocytic cells such as macrophages and polymorphonuclear leukocytes and also within non-phagocytic cell lines and *Acanthamoeba* (Inglis et al., 2000; Pruksachartvuthi et al., 1990; Stevens and Galyov, 2004). Data from *in vitro* studies indicate that *B. pseudomallei* are equipped for intracellular survival and replication within macrophages and neutrophils; and employ multiple mechanisms to escape phagocytic killing and evade host defense (Egan and Gordon, 1996; Jones et al., 1996; Kespichayawattana et al., 2000). Ultra-structural studies suggest that *B. pseudomallei* reside within membrane-bound compartments (Pruksachartvuthi et al., 1990), particularly phagolysosomes, making use of its ability to survive and grow in acidic environments (Dejsirilert et al., 1991; Egan and Gordon, 1996; Pruksachartvuthi et al., 1990). Moreover, the majority of internalized bacteria are able to avoid normal phagosome/lysosome fusion in macrophages by escaping into the cytoplasm, where the organisms multiply to high numbers and eventually rupture the cells (Nathan and Puthucheary, 2005). Following phagocytosis by mouse peritoneal macrophages, *B. pseudomallei* has been observed in the cytoplasm following destruction of the phagosome. This indicates that, *B. pseudomallei* is able to lyse the plasma membrane-derived phagocytic vacuole and proliferate in the cytoplasm (Harley et al., 1998). Furthermore, *B. pseudomallei* is able to invade mouse macrophages without activating inducible nitric oxide synthetase (iNOS), an enzyme that is required for the generation of reactive nitrogen intermediates and important for intracellular bacterial killing (Utaisincharoen et al., 2001). *B. pseudomallei* can escape from endocytic vesicles into the cytoplasm by lysing the endosome membranes as early as 15 minutes after internalization by phagocytic cells (Harley et al., 1998; Stevens et al., 2002). *B. pseudomallei* also induces apoptosis of macrophages in a caspase-1-dependent manner, a process requiring bacterial entry and pore formation in the cell membrane. The Inv/Mxi-Spa-like type three secretion system (*bsa*/TTSS3) was essential for intracellular replication and escape from endocytic vesicles (Stevens et al., 2002), and for *B. pseudomallei*-induced apoptosis of macrophages. The ability of *B. pseudomallei* to

survive intracellularly could account for the latency, recrudescence, difficulties in treatment, granuloma associated with melioidosis.

Actin-based motility

Following escape from endocytic vesicles into the cytoplasm, *B. pseudomallei* is able to polymerase actin at one pole, forming actin tails within and protruding from infected cells (Kespichayawattana et al., 2000). This process involves the *bsa*/TTSS3 locus including the effector protein BipB, the auto-secreted protein BimA (Stevens et al., 2005; Stevens et al., 2002; Suparak et al., 2005).

Cell fusion and multinucleated giant cell (MNGC) formation

After entry into both phagocytic and non-phagocytic cells, *B. pseudomallei* can induce cell fusion resulting in the formation of MNGCs (Harley et al., 1998; Kespichayawattana et al., 2000). MNGCs lead to formation of granuloma which are associated with chronic melioidosis, and "giant" cells containing many intracellular bacteria have been recovered from human melioidosis patients during post-mortem and from biopsies from melioidosis patients with bone damage, indicating that MNGC formation occurs *in vivo*; however, the relevance of MNGCs to virulence and pathogenesis is currently unclear (Sirikulchayanonta and Subhadrabandhu, 1994).

Interactions with human epithelial cells *in vitro*

B. pseudomallei adheres to cultured human epithelial cell lines derived from alveolar, bronchial, laryngeal, oral, conjunctival and cervical tissues (Brown et al., 2002). Adherence of *B. pseudomallei* to cell lines *in vitro* was enhanced when incubated at a temperature of 30^0C compared with 37^0C (Brown et al., 2002). *B. pseudomallei* is more efficient in invasion, adherence and induction of cellular damage of respiratory epithelial cells compared to *B. thailandensis* (Kespichayawattana et al., 2004).

Latency and recrudescence

Perhaps most concerning is the ability of *B. pseudomallei* to remain quiescent in the host for decades after initial exposure (Morrison et al., 1988). The longest recorded period was

about 62 years in a World War II prisoner of war who served in Southeast Asia (Ngauy et al., 2005). This aspect of the disease has lead to the moniker 'Vietnam time-bomb', reflecting the estimated 225,000 Vietnam veterans who were serologically positive for melioidosis at the end of the wars (Howe et al., 1971). Mechanisms by which *B. pseudomallei* evade the host immune system for prolonged periods, prior to the onset or recrudescence of disease is presumably a consequence of its facultative intracellular nature. The organism can invade and persist within numerous cell types (Jones et al., 1996), where it is effectively hidden from the host immune response. Compounding disease severity, recrudescence or delayed onset melioidosis is linked to the host's status; compromised cellular immunity such as that observed in late onset diabetes, chronic renal disease, patients with organ transplants and those with chemotherapy are permissive to the development of melioidosis. It is likely that these immunodeficient hosts are responsible for the higher morbidity and mortality observed in recrudescent or late onset melioidosis.

2.8 Host immune response

While healthy individuals can contract melioidosis, most patients have an underlying predisposition, suggesting that the immunological status of the patient affects the disease initiation and progression. In particular, diabetes mellitus and renal disease are common underlying conditions in melioidosis patients; other factors which result in immune suppression, such as alcohol abuse, have also been identified as risk factors (White, 2003a). Melioidosis has several disease outcomes (asymptomatic, acute, chronic or latent), which are believed to determined by host immune response (Gan, 2005). The murine melioidosis models of acute and chronic infections mimic the acute and chronic disease in humans (Leakey et al., 1998).

Innate immune response

Alternative complement pathway becomes activated when *B. pseudomallei* enter into the host, but the membrane attack complex is deposited on an external polysaccharide and hence is not bactericidal (Egan and Gordon, 1996). Opsonization with a complement enhances, but is not necessarily result in intracellular killing of the bacteria (Harley et al.,

1998). Resistance of *B. pseudomallei* to lysosomal defensins and cationic peptides has been demonstrated (Gan, 2005). This resistance mechanism, attributed to the presence of the capsule and lipopolysaccharide, allow *B. pseudomallei* to survive within phagocytes and in human serum.

Following interaction with *B. pseudomallei*, mouse tissue shows a rapid influx and activation of neutrophils (Barnes et al., 2001). When C57BL/6 mice are depleted of neutrophils, an acute *B. pseudomallei* infection is established (Easton et al., 2007), indicating the importance of neutrophils in innate immunity. However, macrophages are also essential for the control of *B. pseudomallei i*nfection. Depletion of macrophages from BAL/c or C57BL/6 mice significantly increases mortality rates (Barnes et al., 2008). Macrophages from melioidosis patients demonstrate a reduced level of lysosomal fusion compared with healthy individuals, resulting in higher bacterial numbers (Puthucheary and Nathan, 2006). These data suggest that acute melioidosis result from an ineffective cellular innate immune response.

Toll-like receptors (TLRs) recognize conserved pathogen associated molecular pattern (PAMP) and mediate an inflammatory immune response. Activation of TLRs occurs via various signaling adaptor proteins, including MyD88 and TRIF. MyD88 knockout mice demonstrated increased susceptibility to *B. pseudomallei* infection as a result of reduced neutrophil recruitment and activation (Wiersinga et al., 2007). Melioidosis patients suffering septic shock have increased expression of TLR1, TLR2 and TLR4 and its co-receptor CD14, the expression of TLR2, TLR4, and CD14 was decreased upon recovery (Wiersinga et al., 2007).

Humoral immune response

Antibody to *B. pseudomallei* is usually present in people living within melioidosis endemic region, although the percentages of seropositive individuals vary significantly between regions and subpopulations (Barnes et al., 2004; Bryan et al., 1994). This variability may be due to *B. pseudomallei* antigens cross-reacting with related, avirulent *Burkholderiaceae* species (Cheng and Currie, 2005; Gilmore et al., 2007). The role of

antibodies in protection from infection is equivocal. Notably, recurrent infections can occur in the presence of high antibody levels.

Cellular immune response

Once intracellular invasion by *B. pseudomallei* has occurred, a cell-mediated immune (CMI) response, in which T cells play an important role, is required for bacterial clearance. However, melioidosis patients demonstrate reduced T cell counts following stimulation with *B. pseudomallei* lysates (Ramsay et al., 2002). T cells from patients with subclinical melioidosis demonstrated higher proliferation levels as well as higher Gamma-Interferon production than those from patients with clinical melioidosis. These data suggest that a strong CMI response is essential for protection against the progression of *B. pseudomallei* infection. Furthermore, optimal bactericidal activity against *B. pseudomallei* was observed only when both lymphocytes and macrophages were present (Barnes et al., 2004).

2.9 Clinical features

Despite the discovery of various antibiotics and immunisation regimes, many infectious diseases retain their ability kill and thus undrstanding their pathogenicity has important implications for human health. Melioidosis is one such infectious disease and is endemic in Southeast Asia and northern Australia, where it accounts for the majority of fatal community acquired fatal pneumonia and septicemia (Currie and Brewster, 2001). Compounding the severity of disease are delays or incorrect diagnosis due to lack of clinical awareness and poorly resourced laboratory services; situations which are common in many regional communities where the disease is endemic. Correct and earliear diagnosis is essential as the organism is innately resistant to many antibiotics used treat common acute bacteraemic infections (Moore et al., 1999). Furthermore, recovery from infection does not confer immunity to the disease, which sheds doubt on the efficacy of future vaccine development (Maharjan et al., 2005). For these reasons and the ease at which infection can occur through inhalation wnich have lead to the classification of *B. pseudomallei* as a caegory B select agent by the Centres for Disease Control and Prevention (Rotz et al., 2002).

Risk factors

Melioidosis primarily affects persons who are in regular contact with stagnant surface water and soil. Infection usually occurs from percutaneous inoculation by means of a penetrating injury or open wound, inhalation during severe weather or ingestion through contaminated food or water. Melioidosis can be considered as a seasonal disease 75 to 81% of cases occur during the rainy season (Cheng and Currie, 2007). Incidence peaks between 40 and 60 years of age, but melioidosis is well recognized in children and the disease has been transmitted to infants through breast milk from mothers with mastitis (Cheng and Currie, 2007). Since up to 80% of patients with melioidosis have one or more risk factors for the disease, it has been suggested that melioidosis should be considered an opportunistic infection. It is unlikely to have a fatal outcome in a previously healthy person, provided that the infection is diagnosed early and appropriate agents and intensive care resources are available (Currie et al., 2010). Risk factors for melioidosis include diabetes present in 23 to 60% of patients, heavy alcohol use in 12 to 39%, chronic pulmonary disease in 12 to 27%, thalassemia in 7%, glucocorticoid therapy in <5% and cancer in<5% (Currie et al., 2010; Limmathurotsakul et al., 2006; Limmathurotsakul et al., 2010). Patients with diabetes mellitus have a high incidence of melioidosis with up to 60% of patients with pre-existing or newly diagnosed type 2 diabetes (Currie et al., 2000; Suputtamongkol et al., 1999; Suputtamongkol et al., 1994). Studies have examined risk factors in patients with melioidosis were compared with septic and non-septic to estimate a relative risk. In a Thai study, diabetes, Thalassemia, renal disease and occupational exposure to surface water were all associated with an increased risk of melioidosis (Suputtamongkol et al., 1994). A population-based study in Australia defined adjusted relative risks of 4.0 (3.2 to 5.1) for those aged >45 years, 2.4 (1.9 to 3.0) for males, 3.0 (2.3 to 4.0) for aboriginal Australians, 13.1 (9.4 to 18.1) for diabetics, 2.1 (1.6 to 2.6) for those with excess alcohol consumption, 4.3 (3.4 to 5.5) for chronic lung disease and 3.2 (2.2 to 4.8) for chronic renal disease. The reason for these specific risk factors is not clear, but many have implicated the effect of these co-morbidities on neutrophil function, known to be important in the pathogenesis of melioidosis (Currie et al., 2000; Jones et al., 1996; Suputtamongkol et al., 1994).

Incubation period

The incubation period for melioidosis has been evaluated in a single published study in which 25% of patients who recalled a specific event such as an injury had clinical manifestations 1 to 21 days (mean, 9 days) later (Currie et al., 2000). The inoculating dose, strain virulence, mode of infection, and risk factors in the host are all likely contributors to the incubation period, clinical presentation, and outcome. An incubation period of a day or less was documented after aspiration of *B. pseudomallei* in a near-drowning event, whereas the longest recorded apparent incubation period was 62 years (Chierakul et al., 2005; Ngauy et al., 2005).

Clinical manifestations

B. pseudomallei infection has protean clinical manifestations, and severity varies from an acute fulminant septic illness to a chronic infection. Chronic infection manifest as the presence of symptoms for >2 months, accounting for 11% of all cases that may mimic cancer or tuberculosis (White, 2003a). Sub-acute disease involves prolong febrile illness involving multiple abscesses and *B. pseudomallei* can be detected in bodily tissues and secretions, including blood, urine, and pus (Leelarasamee and Bovornkitti, 1989). In a descriptive study involving 540 patients in tropical Australia over a 20-year period, the primary presenting feature was pneumonia in 51% of patients, followed by genitourinary infection in 14%, skin infection in 13%, bacteremia without evident focus in 11%, septic arthritis or Osteomyelitis in 4%, and neurologic involvement in 3% (Currie et al., 2010). The remaining 4% of patients had no evident focus of infection. Over half of patients have bacteremia on presentation, and septic shock develops in approximately one fifth (Currie et al., 2010). Internal-organ abscesses and secondary foci in the lungs, joints, or both are common.

A notable difference in presentation between patients in tropical Australia and those in South-east Asia is suppurative Parotitis, which accounts for up to 40% of cases of melioidosis in children in Thailand and Cambodia but extremely rare in Australia (Harris et al., 2011). In Australia, prostatic melioidosis is present in approximately 20% of male patients, and neurologic melioidosis is manifested as brain-stem encephalitis, often with cranial-nerve palsies (especially cranial nerve VII), or as myelitis with peripheral motor

weakness (Currie et al., 2010). Recurrent melioidosis occurs in approximately 1 in 16 patients, often in the first year after the initial presentation (Currie et al., 2010; Limmathurotsakul et al., 2006). Roughly a quarter of recurrences are due to reinfection, with the reminder due to relapse from a persistent focus of infection (Limmathurotsakul et al., 2006). Mortality rates for melioidosis are approximately 40% in northeast Thailand (35% in children) and 14% in Australia (Currie et al., 2010; Limmathurotsakul et al., 2006).

2.10 Diagnosis and management of melioidosis

The high fatality rate of melioidosis, as well as the potential use of *B. pseudomallei* in biological warfare necessitates unequivocal and rapid detection and identification. The capacity for accurate identification of aetiologic agents is central to epidemiological surveillance and public health decisions. A delay in diagnosis can be fatal, since empirical antibiotic regimens used for suspected bacterial sepsis often do not provide adequate coverage for *B. pseudomallei*. Guidelines for empirical treatment of community-acquired pneumonia in endemic regions recommend the administration of antibiotic agents with activity against *B. pseudomallei* in patients with risk factors for melioidosis.

Laboratory diagnosis: Phenotypic characteristics - Culture based methods

A culture of *B. pseudomallei* from any clinical sample is the sine qua non for the diagnosis of melioidosis. Therefore the capacity of the laboratory personnel to isolate and identify *B. pseudomallei* and the extent to which the laboratory services are available in developing countries is a crucial factor in determining the significance of melioidosis (White, 2003a). Laboratory procedures for maximizing the culture and identification of *B. pseudomallei* have been developed, but a delay in the identification of *B. pseudomallei* or a misidentification as another species is not uncommon in laboratories that are unfamiliar with the organism (Peacock et al., 2008). Selective media have long been used for the isolation of B. pseudomallei from non-sterile fluids and environmental samples, utilizing the broad antibiotic resistance of the organism. Ashdown tested his eponymous medium, with tryptase soy agar with glycerol, crystal violet, neutral red and gentamycin

(4mg/L), in 8,000 clinical specimens in Townsville in 1979. *B. pseudomallei* was isolated in 8 specimens, with *Klebsiella* spp (n=73), *Pseudomonas aeruginosa* (n=57), *Enterococcus faecalis* (n=23), *B. cepacia* (n=17), and *Serratia marcescens* (n=14) the most common contaminants (Ashdown, 1979). A modified Ashdown`s broth with colistin, is now used, particularly in cultures from non-sterile sites such as, throat, rectal and wound swabs (Dance et al., 1989b; Wuthiekanun et al., 1990).

Time to blood culture positivity, reflecting the density of bacteremia, correlated with mortality in Thailand; 73.7% of patients died if blood cultures became positive with 24 hours, compared to 40.9% of those with a time to detection of >24 hours (Tiangpitayakorn et al., 1997). Alternative blood culture methods could decrease the time to obtain a positive culture, but at the cost of reduced sensitivity. Compared with conventional broth-based blood culture (median time to positivity 61.8 hours; n=42), the Isolator lysis centrifugation had 81% sensitivity with time to positivity 39.3 hours, and the pour plate method had 61% sensitivity with median time to positivity 45.5 hours (Simpson et al., 1999).

Substrate utilization tests

As *B. pseudomallei* can utilize a wide arrange of substrates. It has an unusually broad utilization in commercial phenotypic systems for a member of the *Pseudomonadaceae*. Traditionally it has been identified as being motile, oxidase and catalase positive, indole negative, citrate positive, gelatin positive, producing acid from glucose and maltose with no gas. It is lactose negative, arginine positive and ornithine is negative as is lysine (Lipuma et al., 2007). Urease is not produced. It can be differentiated from the *Pseudomonas aeruginosa* group by the lack of typical pigment; *Alcaligenes* spp by the rapid oxidation of glucose, *B. thailandensis* by assimilation of arabinose, and from *B. cepacia* by the rapid reduction of nitrate to nitrogen with the production of amylase (Smith et al., 1975).

Commercial phenotypic identification systems

Since the introduction of commercial phenotypic identification systems, the API 20E and API 20NE (bioMerieux, Baulkham hills, NSW) have been traditionally used, although the lack of diversity of isolates in the analytical index, has provided uncertainty in the past (Dance et al., 1989b). There are conflicting opinions as to the reliability of the API 20NE test panel; two studies have reported good results with this manual system as with the API 20E system (Dance et al., 1989b; Lowe et al., 2002). However another study found that 6 of 50 *B. pseudomallei* strains at a West Australian laboratory were misidentified, most commonly as *Chromobacterium violaceum,* and a further 4 strains gave indeterminate results (Inglis et al., 1998). However, Ashdown found the API 20E, to be useful with a combination of other simple test (Ashdown, 1979).

So, it is evident that the new generation of automated phenotypic identification systems has demonstrated diverse results. The Vitek automated system (bioMerieux, Baulkham Hills, NSW) is widely used; the Vitek 1 system, but not the Vitek 2 system appeared to identify *B. pseudomallei* reliably. The Vitek 2 system was only able to correctly identify 19% of isolates that the Vitek 1 and API systems agreed upon (Lowe et al., 2002). Commercial phenotypic identification systems are as only as accurate as the data base allows, and a system that does not include *B. pseudomallei* in the analytical profile would misidentify the isolate as the closest available (Koh et al., 2003). More importantly, the microbiologist should be aware of the limitations of systems as a "black box".

However, it is reasonably clear that most systems which include *B. pseudomallei* in the profile database all are useful and mostly accurate in the identification of *B. pseudomallei* particularly when reactions are read at 48 hour (Dance et al., 1989b). The systems may legitimately struggle with the identification of bacteria from environment. The ecology of *B. pseudomallei* is so uncertain that there may be any number of closely related species newly recovered with characteristics which are similar but are not represented in commercial databases. The history of *B. thailandensis*, the organism originally identified as biotypes of *B. pseudomallei* but differentiated by a limited number of substrate utilization tests, including the ability to utilize arabinose, and its relative avirulence but

similar serology, highlights this well (Brett et al., 1998; Dharakul et al., 1999; Smith et al., 1997; Wuthiekanun et al., 1996).

B. pseudomallei-specific antigen detection methods using monoclonal antibodies such as the latex agglutination assay (Melioidosis Research Center, Khon Kaen, Thailand) and direct Immunofluorescence appear to be useful for rapid identification of *B. pseudomallei*. Latex agglutination methods are routinely used in Thailand and northern Australia and have shown good, but these tests are not commercially available (Price et al., 2012).

Minimum identification criteria for developing countries

Regions where melioidosis is endemic are often resource poor and unable to support commercial bacterial identification systems. A simple set of criteria can be used in laboratories where melioidosis is endemic (Dance et al., 1989b). These criteria include: colonial morphology on Ashdown agar (mostly rugose, mauve colonies with metallic sheen and distinctive earthy, sweet odor – particularly after 48 hour incubation at 42^0C), Gram stain (small gram negative rods with bipolar staining), Oxidase (positive), gentamycin and colistin susceptibility (both resistant). The additional criterion for augmentin susceptibility (sensitive) will aid in the discrimination of B. cepacia which is resistant to augmentin (Dance et al., 1989b; Lipuma et al., 2007). In laboratories outside traditional zones of endemicity, a small oxidase positive, Gram-negative rod which exhibits resistance to gentamycin and sensitive to augmentin, doxycycline and cotrimoxazole, should be enough to garner suspicion.

Serology

An ideal serological test for melioidosis should be one that is sensitive, specific, rapid, simple and cost-effective. The test should be able to provide results for initial clinical decision making, for monitoring the progression of disease and for epidemiological assessment of the impact of disease. Serological results without culture have been used to provide evidence of infection when accompanied with a high level of clinical suspicion particularly associated with an outbreak (Currie, 1993; Johnson, 1967). Serology has

been reported to provide evidence of human to human transmission (McCormick et al., 1975). However, the use of serology is complicated by the high background titers apparent in areas of high endemicity (Kanaphun et al., 1993), particularly in areas of Thailand where samples from up to 80% children at four years of age when analyzed demonstrate significant sero-reactivity. This is in contrast to Australia where the highest background antibody titers, in endemic regions is not over 13% (Currie et al., 2000).

Antibody and antigen detection

Serological tests that have been developed to date for rapid and presumptive results include an indirect haemagglutination (IHA) test, immunofluorescent assay (IFA) and Enzyme linked immunosorbent assay (ELISA), all using a variety of antigens(Anuntagool et al., 1993; Ashdown, 1981; Ashdown et al., 1989; Leelarasamee, 1985). These tests are not sufficiently sensitive or specific in detecting the infection during the early stage. The IHA test was applied early to melioidosis diagnostics and remains the most widely used test despite its poor sensitivity and specificity (Alexander et al., 1970). It was first described in 1965 and has been used extensively in sero-surveys (Ashdown, 1987; Ileri, 1965; Yap et al., 1991b). The use of the IHA in sero-epidemiological study is problematic in endemic areas, particularly where rates of background seropositivity may be high presumably due to subclinical exposure to organisms related to *B. pseudomallei* (Khupulsup and Petchclai, 1986). Therefore, IHA cut off titer for defining seropositive case should be optimized for specific geographical area as has been done previously for studies involving Australian and Thai population. The lower cut-off titer of 1:40 was used in Australia compared to that of 1:160 in Thailand (Currie et al., 2000). Efforts have been made to refine the antigen targets including refinement of a 30kD exotoxin and 19.5kD, 40kD and 200kD proteins. ELISA based on these proteins has been validated in a clinical context; IgG, but not IgM appears be more sensitive (74-82%) and specific (75-80) than the IHA, but still lack the performance necessary for clinical use (Sirisinha et al., 2000). To minimize the problem, more specific and purified recombinant truncated flagellin antigen (RTFA) was used in different studies. ELISA using recombinant truncated flagellin had achieved sensitivity of

93.8% and a specificity of 96.3% and offered a more efficient sero-diagnosis of melioidosis (Chen WC et al, 2005).

A rapid immunochromogenic test (PanBio Ltd, Australia) for IgM and IgG appeared to perform well in a series of 121 patients. However the high sensitivity (IgG 100%,IgM 93%) and specificity (both assays 95%) reported were compared against IHA, rather than culture, as a gold standard (Cuzzubbo et al., 2000). Although a variety of antigen detection methods have been studied, none are yet commercially available. Antigen tests have been developed for use on direct specimens or in blood culture supernatant; of this latex agglutination (Melioidosis Research Center, Khon Kaen, Thailand) for culture identification and IFA from direct specimens (such as sputum, urine or pus) have been used in research laboratory in Thailand (Price et al., 2012).

Molecular diagnostics

Diagnostic laboratories that regularly encounter *B. pseudomallei* have found phenotypic identification methods such as substrate utilization panel unreliable for confirmation species identity (Glass and Popovic, 2005; Inglis et al., 1998). The advent of new molecular techniques has enabled the development of improved methods for more accurate species identification (Godoy et al., 2003). Moreover, the development of molecular diagnostics has lead to rapid and specific assays, which have aided in decreasing the time required to confirm diagnosis for infectious diseases. Targets for the development of specific primers to rapidly identify *B. pseudomallei* have initially concentrated on 16S and 23S rRNA sequences (Tyler et al., 1995). However, the complete sequence of the *B. pseudomallei* genome reveals several gene targets for the identification of this species (e.g., 16S rRNA genes, *fliC, rpsU*, and TTS1 gene cluster (Holden et al., 2004a; Moore et al., 2004; Tomaso et al., 2005). This approach to polymerase chain reaction (PCR) target identification and primer and probe design and improved methods for fluorescence labeling of DNA allow for the in silico design and evaluation of real-time PCR assays specific for a given species (Mackay, 2004). The advent of real-time PCR for identification of *B. pseudomallei* isolates potentially offers a faster and more reliable diagnosis of disease in regions endemic for melioidosis.

Although PCR has been used to detect the genome of the organism from the environment with high sensitivity, the presence of avirulent biotypes or yet uncategorized species may limit the relevance of this technique as a standard in determining soil prevalence of *B. pseudomallei* (Brook et al., 1997). Dharakul et al developed a multiplex PCR that is able to discriminate between *B. pseudomallei* and *B. thailandensis* (Dharakul et al., 1999). This assay is useful in confirmation of *B. thailandensis* over other species which may share the phenotypic characteristic of arabinose assimilation.

The TTS1 gene cluster encompassing part of open reading frame 2 (orf2) was found to be present in *B. pseudomallei* and not in other *Burkholderia* species (Rainbow et al., 2002; Winstanley et al., 1999). The detection of TTS1 gene cluster and its association with virulence provides the basis for targets of PCR primer sets may have clinical and epidemiological application (Winstanley et al., 1999). So it is evident that the real-time PCR targeting *orf2* of the *B. pseudomallei* TTS1 is a powerful tool for the rapid identification of *B. pseudomallei* and can potentially complement current confirmatory diagnostic procedures for melioidosis. Further studies to evaluate the TTS1 real-time assay on a larger panel of environmental samples are needed.

Antibiotic sensitivity and resistance

B. pseudomallei is known to be one of the most antibiotic resistant bacteria. It exhibits resistance to diverse groups of antibiotics that limit therapeutic options for the treatment of melioidosis. The limited arsenal of antibacterial agents available for meliodosis therapy is mostly due to the inherent resistance of *B. pseudomallei* to many antibiotics. A susceptibility of *B. pseudomallei* to 35 antimicrobial agents in terms of minimum inhibitory concentration (MIC), determined by agar dilution in Mueller-Hinton medium have shown that the organism is intrinsically resistant to numerous antibiotics, including penicillin, ampicillin, first and second generation cephalosporins, macrolides, fluoroquinolone, fosfomycin, clindamycin, colistin and aminoglycosides, but is usually susceptible to amoxicillin-clavulanate, chloramphenicol, doxycycline, trimethoprim-sulfamethoxazole, ureidopenicillins, ceftazidime and carbapenems (Cheng and Currie, 2005; Thibault et al., 2004a; White, 2003a). Bacteria possess an impressive antimicrobial

resistance armamentarium. Various mechanisms of acquired antibiotic resistance have been identified, including exclusion of the drugs from the cell due to permeability issues bestowed by constituents of the bacterial cell envelope, efflux from the cell, enzymetic inactivation, alterations in the antibiotic target site, and amino acid changes in *pen*A, the gene encoding the highly conserved class A ß-lactamases (Rholl et al., 2011; Schweizer, 2012; Trunck et al., 2009). Antibiotic penetration into bacterial cells are limited mainly by three mechanisms of action. Firstly, bacterial lipopolysaccharide (LPS) restricts the binding of cationic antibiotics, including aminoglycosides and polymyxins. It also renders the bacteria resistant to cationic peptides of the human innate immune system. Secondly, low cell membrane porin protein content may affect the permeability of water-soluble antibiotics into the bacterial cell. Thirdly, transport proteins, namely efflux pumps, are involved in antibiotic resistance. Antibiotics can be removed from the bacterial cell by the specific efflux pumps before being delivered to their cellular targets. Moreover, biofilm formation may cause the bacteria to resist an antimicrobial agent. So attention has been focused on the role of biofilms in protecting bacteria against antibiotics. In one study, *B. pseudomallei* was incubated on a silastic surface for 16 hours. Electron microscopy demonstrated that cells in biofilms were still viable after 24 hours of exposure with 800 µg/ml ceftazidime and 8000 µg/ml, which is 200 times the MIC of the planktonic cells (Vorachit et al., 1993). Antibiotic combinations active against *B. pseudomallei* in bifilm shown to be ciprofloxacin/clarythromycin, ciprofloxacin/ azithromycin, and imipenem/azithromycin (Vorachit et al., 2000). Possible resistant mechanisms in the biofilm include reduced drug diffusion through the biofilm, drug inactvation within the biofilm, the presence of less susceptible stationary-phase organisms, and the up-regulation of biofilm associated antibiotic resistance genes (Lipuma et al., 2007).

In many instances, bacterial antibiotic resistance is mediated by mobile elements, such as plasmids, transposons or integrin, but all resistance documented to date in *B. pseudomallei* is mediated by chromosomally encoded genes (Schweizer, 2012). Sequencing of the genome of strain K96243 has identified upto seven Ambler class A, C, and D ß-lactamases (including a cephalosporinase and oxacillinase) genes but not a class

B (metallo ß-lactamases gene), upto ten multidrug efflux systems of the resistance nodulation and cell division (RND) family and a putative aminoglycoside acetyl transferase (Holden et al., 2004a). Most of these resistance mechanisms are putative and, to date, have been found in neither resistant or clinical isolates (Schweizer, 2012). Functionally, out of seven Ambler class A, C, and D ß-lactamases, the most important of these is the Bush-Jacoby-Medeiros 2e ß-lactamases BPS-1, encoded by the gene *Bla*A (or PenA; Ambler class A) that hydrolyses most cephalosporins but is readily inhibited by clavulanate (Cheung et al., 2002; Livermore et al., 1987). Acquired resistance to ß-lactam antibiotics while on treatment with a ß-lactam/ ß-lactamases inhibitor combination or ceftazidime resulted from three distinct phenotypic changes; de-repression of the chromosomal enzyme, an insensitivity to inhibition by ß-lactamase inhibitors and a ß-lactamase specific for ceftazidime (Godfrey et al., 1991). Although a class C ß-lactamase was initially identified from the genome sequence, it is likely that this represents a class metallo-ß lactamase; the clinical significance of this metallo-ß lactamase is not yet known as resistance to carbapenems remains uncommon (Niumsup and Wuthiekanun, 2002; Smith et al., 1996).

The mechanism of resistance to aminoglycosides and macrolides can be explained by the presence of a multidrug efflux system in *B. pseudomallei*. The BpeAB-OprB pump in *B. pseudomallei* was accountable for the efflux of the aminoglycosides gentamicin and streptomycin, the macrolide erythromycin and the Dye acriflavine (Chan et al., 2004; Van Bambeke et al., 2000). In addition, further antibiotics that *B. pseudomallei* is moderately resistant to include fluoroquinolons, co-trimoxazole and rifampicin. These antimicrobial agents are involved in the disruption of DNA replication and RNA synthesis. On the other hand, carbapenems, the third generation cephalosporins (ceftazidime and cefotaxime), other ß-lactams (piperacillin and piparacillin/tazobactam and co-amoxiclav) are efficient for killing this bacteria. However, resistance to ceftazidime, which is currently used as the first line treatment for melioidosis, has been reported during antimicrobial therapy (Chantratita et al., 2011). This observation indicate that searching new therapeutic agents for melioidosis treatments is very much essential.

Melioidosis therapy

Melioidosis has a notoriously protracted course; cure is difficult without a prolonged course of appropriate antibiotic. Owing to rapid disease progression and the predilection of this bacteria to establish latent infection, melioidosis therapy is biphasic and lengthy (Wuthiekanun et al., 2006). An initial intensive phase involving intravenous administration of antibioticsis followed by an maintenance or eradication phase to minimize the risk of relapse.

Treatment consists of an intensive phase of at least 10 to 14 days of ceftazidime, meropenem, or imipenem administered intravenously, followed by oral eradication therapy, usually with trimethoprim-sulfamethoxazole (TMP-SMX) for 3 to 6 months (Chierakul et al., 2006). However, the cource of intensive therapy may be extended to four or more weeks in cases of more severe disease such as septic shock, deep sealed or organ abscesses, extensive lung disease, osteomyelitis or neurological melioidosis (Currie, 2015). In some instances, for example deep seated infections, parenteral ceftazedime can be supplemented with oral or parenteral administration of trimethoprom-sulfamethoxazole (co-trimoxazole). Resistance of *B. pseudomallei* to carbapenems has yet to be documented, and primary resistance to ceftazidime is extremely uncommon. In very rare cases, acquired resistance to ceftazidime develops during therapy, the mechanisms for acquired resistance include point mutations and gene deletion (Chantratita et al., 2011). The current recommended antibiotic therapy for melioidosis includes initial intensive therapy for minimum of 10-14 days comprising ceftazidime (50mg/kg, upto 2 g 6 hourly) or meropenem (25mg/kg, upto 1 g 6 hourly) or imipenem (25mg/kg, upto 1 g 6 hourly) and eradication therapy for Minimum of 3 months comprising TMP-SMX (6/30 mg/kg, upto 320/1600 mg 12 hourly). In cases of more severe disease (such as neurologic, cutaneous, bone, joint, and prostatic melioidosis), TMP-SMX (6/30 mg/kg, upto 320/1600 mg 12 hourly) may be added during the intensve therapy period (Currie, 2015). The mobile genetic elements, such as plasmids, seem notably absent from *B. pseudomallei* (Schweizer, 2012). However, many strains are naturally transformable, and conjugative plasmids can be introduced and, with pressure, stably maintained in *B. pseudomallei* (Choi et al., 2008; Thongdee et al., 2008). Some

conjugative multi-resistance determinants, especially those containing carbapenemeases, including New Delhi Metallo ß-lactamase (NDM–1) are disseminating rapidly worldwide and challenge the treatment of Gram-negative infections (Nordmann et al., 2011; Yong et al., 2014). Since many the regions where carbapenemases and other resistance determinants are emerging overlap with those that are endemic for , it will be wise to monitor drug-resistant for resistance determinants known to be associated with mobile elements. As resistance to carbapenems has not yet been observed and, apart from clinical manifestation that warrant their use, carbapenems should therefore remain drugs of last resort. To preserve the utility of carbapenems, meropenem therapy is only recommended when conditions during ceftazidime therapy worsen or repeat blood culture remain positive (Schweizer, 2012). Carbapenems have lower minimum inhibitory concentrations and superior results in invitro time-kill studies than ceftazidime, but a randomized comparative study in Thailand did not show a survival advantage of imipenem over ceftazidime (Simpson et al., 1999).

Understanding resistance mechanisms provides tangible benefits, such as the development of PCR-based asseys for rapid detection of known resistance alleles. For example, a SYBR Green-based mismatch amplification mutation assay was developed for the detection of single nucleotide polymorphism in *B. pseudomallei penA* that result in ceftazidime resistance (Sarovich et al., 2012). Ceftazidime-resistant isolates carrying the PBP3 deletion do not grow on common laboratory media unless they are supplemented with glycerol (Chantratita et al., 2011). The ceftazidime-resistant Thai isolates were detected because they were fortuitously plated on Ashdown's agar, which contains glycerol (Ashdown, 1979). However this is not common practice and one of the lessons learned from this study is that, following ceftazidime therapy, patient isolates should be routinely plated on to Ashdown's agar so that, growth deficient variants can be detected early.

The agent of choice for the at least three month eradication phase is oral co-trimoxazole when dealing with susceptible *B. pseudomallei* and no documented allergy in patient for this drug. In cases where the organism is resistant or patients are intolerant to co-

trimoxazole, the second line of choice is amoxycillin-clavulanic acid (co-amoxiclav). The oral antibiotic TMP-SMX, with or without doxycycline and chloramphenicol, is used for the prolonged eradication phase. They have been demonstrated to be of little activity in the acute intensive phase and are bactriostatic *in vitro* (Dance et al., 1989a; White et al., 1989).

Testing for TMP-SMX resistance is problematic, with disk diffusion methods probably over-estimating the extent of resistance. Methods that determine the minimum inhibitory concentration (E-tests, broth microdilution or agar dilution) are recommended and demonstrate much lower rates (3%-10%) of primary resistence. However, resistance rates, as assessed with the use of Etest, is reported to be higher (around 13%) in Thailand but much lower for Australian isolates (0 to 2.5%); the clinical signficance of which is unknown (Lumbiganon et al., 2000; Rattanavong et al., 2011). An alternative agent for eradication therapy is amoxycillin-clavulanate; although it is inferior to TMP-SMX because it is associated with a higher rate of relapse, amoxycillin-clavulanate is used in some locations in children and pregnant women (Chantratita et al., 2011).

Antibiotics with borderline MIC against *B. pseudomallei* do not appear to be clinically useful in the intensive phase; these include the tetracyclines, chloramphenicol, the quinolones and ceftriaxone (Chaowagul et al., 1989; White et al., 1989). Although the most recent recommendations no longer endorse doxycycline use in clinical melioidosis therapy, the antibiotic was previously recommended for postexposure prophylaxis and is still used as a component of eradication-phase treatment in some parts of the world (Peacock et al., 2008). In Thailand, chloramphenicol was previously included in a four-drug combination treatment, but a clinical study indicated that chloramphenicol did not provide any benefit when included in oral melioidosis therapy (Chaowagul et al., 2005). Primary resistance to doxycycline and chloramphenicol occurs infrequently in 6% cases only (Simpson et al., 1999). Acquired resistance to doxycycline has been observed when doxycycline has been used as monotherapy, and much less frequently with TMP-SXT monotherapy (Currie et al., 2000; Jenney et al., 2001). Acquired resistance to ceftazidime while on therapy is an uncommon cause of treatment failure but acquired resistance

occurs more frequently with chloramphenicol (Dance et al., 1989b). Relapse attributable to resistance may occur with either oral or intravenous agents used in treatment (Jenney et al., 2001).

The use of combination therapy in the initial intensive phase is routine in Thailand with the rationale of protection against the emergence of resistant strains during therapy and the improved intracellular penetration of TMP-SMX (Currie et al., 2000; Dance et al., 1989a; Dance et al., 1989b). However, *invitro* studies suggests antagonism; clinical evidence of this is currently lacking (Dance et al., 1989b). It is interesting to note that macrophage-permeable antibiotics were more effective than non-permeable antibiotics in the treatment of melioidosis using a mouse model of melioidosis. Furthermore, Currie and co-workers reported fatal human cases of melioidosis despite aggressive treatment with ceftazidime, meropenem, or imipenem; *B. pseudomallei* was persistantly cultured from these patients and was consistantly sensitive to these antibiotics *in vitro* (Currie et al., 2000). It seems that the intracellular nature of *B. pseudomallei* during infection is an important factor during treatment. Whether biofilm formation in vivo is related to increased antibiotic resistance, and difficulties in the effective treatment of melioidosis, requires clarification (Costerton et al., 1999; Vorachit et al., 1993).

Analogous to tuberculosis, melioidosis can relapse following therapy. Studies reported that up to 23% of melioidosis patients in thailand, and 13% of patients in Australia, relapsed despite receiving reliable eradication therapy (Currie et al., 2000; Leelarasamee, 1998). Molecular typing of isolates from patients with recurrent melioidosis showed that most relapses were from insufficient eradication rather than from re-infection (Haase et al., 1995a). This was attributed to incomplete eradication therapy, the severity of the disease, the absence of ceftazidime in initial intensive phase therapy (the use of which reduces relapse by 50%), the duration of erdication therapy, and the choice of agents for eradication therapy (Chaowagul et al., 1993; Currie et al., 2000). These observation mean that finding new drugs for developing melioidosis treatments are an urgent need.

Searching for potential new therapeutic target

It was believed that, resistance to clinically significant antibiotics in *B. pseudomallei* is relatively rare, however there is mounting evidence that resistance is more prevalent than previously thought. Not surprisingly, resistance is emerging in response to antimicrobial treatment, both during the intensive and eradication phase, and may become a more prevalent issue with more widespread use of antibiotics throughout regions with endemic melioidosis. Given the scarceness of antimicrobial agents useful for melioidosis therapy, resistance to any of the currently used key antibiotics severely undermines the ability to successfully treat the disease (Schweizer, 2012).

Several conditions including diabetes mellitus, renal failure and alcohol abuse are important risk factors for the development of melioidosis. A common link is that these are associated with impairement of neutrophil function. This has led to interest in the therapeutic role of granulocyte colony-stimulating factor (G-CSF), a cytokine that increases the circulating neutrophil count and stimulate neutrophil function. A retrospective study conducted in Australia reported a fall in mortality of melioidosis patients after the introduction of G-CSF as an adjunctive treatment of those with septic shock (Cheng et al., 2004a; Cheng et al., 2004b). A mouse model of melioidosisin which outcome was compared between mice given ceftazidime alone or in combination with G-CSF failed to show a benefit from G-CSF A randomized clinical trial of G-CSF is currently on-going in Thailand (Cheng et al., 2004a; Cheng et al., 2004b).

Many studies have been carried out using different techniques to identify proteins involved in infection and virulence mechanisms of *B. pseudomallei*, that may be targeted by novel therapies. Total 312 essential genes have been identified by a computational bioinformatics approach (Chong et al., 2006). *B. pseudomallei* protein sequences were compared with known essential genes encoding proteins from other bacterial pathogens, and also against human sequences to identify targets that did not have human homologues. The subsets of genes thus identified have been annotated as vital genes for survival, and possible potential candidates for antimicrobial drug target development. In addition, the relative levels of protein expression in *B. pseudomallei* and the non-

pathogenic *B. thailandensis* have been observed by proteomic analysis (Wongtrakoongate et al., 2007). Several proteins in *B. pseudomallei* that are up regulated compared to *B. thailandensis* have thus also been identified as potential markers of pathogenicity and virulence. They may play a key role in virulence as they were highly expressed in the virulent species but missing in the avirulent species.

A set of target proteins that stimulate the human immune response in infected patients have been identified (Su et al., 2008). The genome expression library of a clinical strain of *B. pseudomallei* D286 was constructed and immunologically screened with pooled melioidosis infected human sera. Total 109 expressed polypeptides reacted with melioidosis positive sera and the coding sequence of sero-positives were analysed for sequence identification. These identified proteins are involved in cell envelope biogenesis, cell mobility and secretion, transcription, metabolism, transportation and also include many uncharacterized proteins. Around 30% of these proteins are present in the cytoplasm. These cytoplasmic localised proteins are exposed after destruction of bacterial cells by the host immune defense. These identified immunogenic proteins may be induced and up-regulated during human infection. These immunogen could be further investigated for anti-infective target.

3.0 Materials and Methods

In the present study, blood from healthy individuals and soil from paddy fields of four north eastern districts of the country were collected to determine the magnitude of exposure to *B. pseudomallei* as well as the presence of the organism in the soil. *B. pseudomallei* was isolated in selective media, identified by biochemical tests, and finally confirmed by specific antisera. Molecular tests were performed from all environmental and clinical isolates for confirmation of phenotypic identification and to establish the genetic relationships between organisms isolated from clinical specimen and environmental sources, and also among the strains isolated from Bangladesh and other countries.

To determine the extent of exposure to *B. pseudomallei*, anti- *B. pseudomallei* IgG antibody was determined by in-house ELISA using sonicated whole cell antigen (SWCA) and recombinant truncated flagellin antigen (RTFA) of *B. pseudomallei*. Soil samples were cultured in selective media to isolate *B. pseudomallei*. Organism was identified by biochemical tests, specific antisera and PCR. Real-time PCR assay targeting *Yersinia*-like fimbrial (YLF) and *B. thailandensis*-like flagellum and chemotaxis (BTFC) gene cluster was done for revealing patterns of geographical partitioning between Australian and Southeast Asian isolates. Multilocus sequence typing (MLST) was performed to assess the phylogenetic relationship of strains isolated from clinical and soil. Details of the methods are described below.

3.1 Ethics Statement

The Ethical Review Committee (ERC) of the Diabetic Association of Bangladesh (BADAS) has approved the study. Ibrahim Medical College (IMC) is an institution under the BADAS and its ERC is the approval body for research protocols of IMC. Informed written consent was obtained from all adult participants (age 18 years and above) and from the parents/guardians of all children (age up to 17 years) prior to collection of blood samples and demographic data.

3.2 Determination of seroprevalence of *B. pseudomallei* infection

Anti- *B. pseudomallei* IgG was measured by enzyme linked immunosorbent assay (ELISA) to assess the magnitude of exposure of *B. pseudomallei* among healthy Bangladeshi individuals. Sonicated whole cell antigen (SWCA) and recombinant truncated flagellin antigen (RTFA) of *B. pseudomallei* were used in ELISA to detect antibodies.

Study population and collection of blood samples

Relatives and attendants of patients attending the rural healthcare facilities of the four districts namely Mymensingh, Sylhet, Narayangange and Kishoregange were recruited for determining the anti- *B. pseudomallei* IgG antibody. The geographical distribution of the four districts is shown in Figure 3.1. After obtaining informed consent from each person, about 5 ml of venous blood (2 ml in case of children) were collected with proper aseptic technique. A total of 940 healthy individuals residing in rural areas with no history of fever, persistent cough, wasting and suppurative lesion were included in the study. Age, sex and socio-economic conditions were recorded. In addition, sera from 51 healthy newborn babies of Dhaka city who were presumed to be not exposed to *B. pseudomallei* were enrolled as negative control and for determining the cut off optical density (OD) value of ELISA test. Serum samples from culture positive, 10 melioidosis cases admitted at BIRDEM Hospital were included in this study as positive control.

Determination of anti- *B. pseudomallei* IgG antibody by ELISA

Anti- *B. pseudomallei* IgG antibody was determined by an indirect ELISA as described by Voller A et al (Voller et al., 1978).

Preparation of sonicated whole cell antigen

The antigen was prepared from *B. pseudomallei* USM strain (obtained from University Sains Malaysia) grown on blood agar media. The strain has been isolated and identified by colonial morphology, standard biochemical and serological tests and polymerase chain reaction. The organism was propagated on nutrient agar slants and stored at 4^0C. Whole cell antigen of *B. pseudomallei* was extracted by the methods described by Chart H,

1995. To prepare sonicated antigen, 50 ml of Trypticase Soya Broth (TSB) was inoculated with 4-6 pure colonies of *B. pseudomallei* USM strain and incubated overnight at 37^0C. Organisms were harvested by centrifugation for 30 minutes at 4000 x g at 10^0C. Pellets were suspended in 3 ml of 25 mM Tris-HCL (pH 7.4) and washed three times with Tris-HCL for 30 minutes at 4000 x g at 10^0C. Deposited pellet, suspended in 5 ml of ice-cold Tris-HCL, was sonicated at 40W for 8 minutes in each pulse inside the assigned biosafety cabinet. Sonicated bacterial suspension was then centrifuged at 5000 x g at 10^0C for 30 minutes. The supernatant was separated and then stored at -20^0C in 200 µl aliquots (Chen et al., 2004).

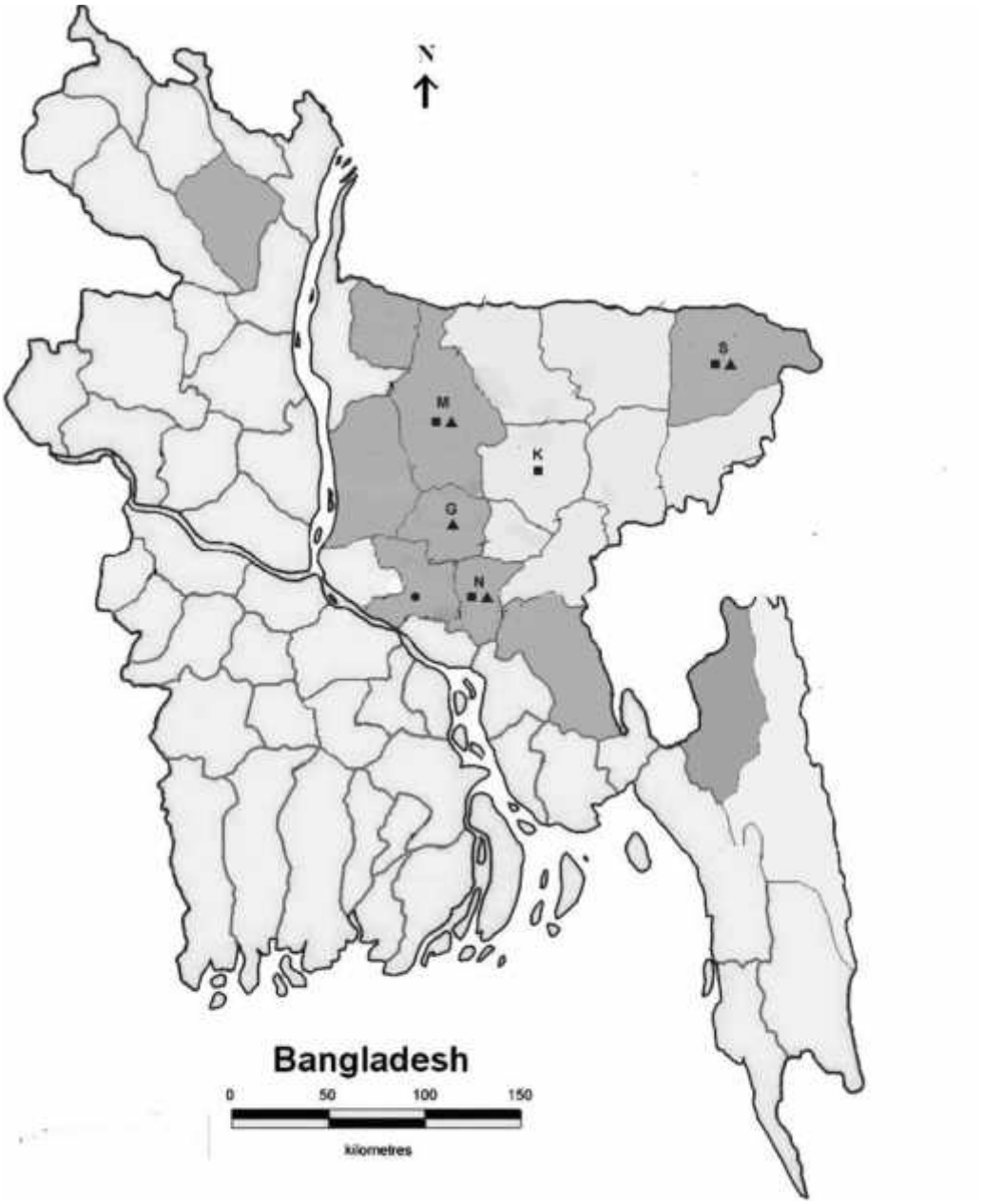


Figure 3.1: Map of Bangladesh showing collection sites (district) of soil and blood samples. The shaded districts are endemic for melioidosis. M = Mymensingh, G = Gazipur, K = Kishoregange, S = Sylhet, N = Narayangange. The distance of Mymensingh, Gazipur, Kishoregange, Sylhet and Narayangange from capital Dhaka is 115, 40, 89, 198 and 14 Km respectively. ■ = Site of soil collection; ▲= Site of blood collection; ● = Capital Dhaka

Expression and preparation of recombinant truncated flagellin antigen (RTFA)

Plasmid containing recombinant flagellin protein of *B. pseudomallei* was provided by Yao-Shen Chen, Infectious Disease Section, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan.

Flagellin protein cloned with 2-T4was overexpressed from the plasmid pGEX *flagellin* gene of *B. pseudomallei* ,For protein purification .strain 21and transformed into BL cultures and allowed (LB) ml Luria Broth 100 positive clone chosen was inoculated into -mM isopropyl 1.0 induced with ,0.6 to 0.5 reach about $_{600}$C until the OD^{o}37 to grow atβ- ml 10 Cell pellet was resuspended in .hour 4 for about (IPTG) thiogalactopyranoside-D %1 ,glycerol %10 ,mM NaCl 300 ,7.5 pH ,HCl-mM Tris 50) of ice cool lysis buffer The cell lysate was then .and subjected to sonication in the ice bath (100-Triton X 400 .C and supernatant was discarded^{o}4 minutes at 20 g for x 4,000 centrifuged atμL of h 2 C for^{o}4 was added to the supernatant and mixed at (GmbH ,Qiagen) NTA resin-$^{+2}$Ni mM 10 Bound recombinant protein was collected from the resin by adding .on a rotator The protien concentration was determined and the purified antigen .reduced glutathione was reconstituted with sterile distilled water tomake up to a c oncentratio no f1μl and /μg aliquoted fo .r further use

Determination of protein concentration of SWCA and RTFA

Protein concentration was determined by directly measuring absorbance by a spectrophotometer with Ultraviolet (UV) lamp after adjusting the wave length to 280nm. Absorbance of the protein solution was again measured after adjusting the wave length to 260nm. Protein concentration of was than estimated by using the following formula (Stoscheck, 1990):

Protein concentration (mg/ml) = [(1.55 x A280) – (0.76 x A260)] x dilution factor

Procedure for ELISA

The 96 well EIA plate (Linbro, USA) was coated with sonicated whole cell antigen. The SWCA was diluted to an optimal working dilution (10µg/ml in coating buffer (0.5 M carbonate/bicarbonate buffer, pH 9.6). The optimal concentration of antigen was determined as 10µg/ml by checkerboard titration. To each well 100 µl volume of coating buffer was added and incubated overnight at 4^0C. The plate was washed three times with phosphate buffer saline-0.05% Tween 20 (PBS-T, pH 7.4)) and blocked by incubating for 2 hours with PBS-T containing 2% bovine serum albumin (BSA) at 37^0C. The plate was then washed three times with PBS-T. A volume of 100 µl serum (1:1600 dilutions) samples was added into each well and incubated for 4 hours at 37^0C. The serum dilution of 1:1600 was predestined by checkerboard titration. After washing with PBST three times, 100 µl of horseradish peroxydase conjugated anti-human IgG antibodies (1:4000) was added and incubated at 37^0C for 2 hours. After washing three times with PBST, 50 µl of 3,3`,5,5`-Tetramethylbenzidine (TMB) substrate was added to each well and incubated at room temperature for 30 minutes in dark. Then 50 µl of 1 M sulfuric acid was added in each well. The colour developed was measured by enzyme immuno assay (EIA) plate reader (Human ELISA Reader) at 450 nm. Optimum concentration of coating RTFA and serum dilution was predetermined by checkerboard titration as 2.5µg/ml and 1:400 respectively.

Cut off OD values determination for SWCA and RTFA

A Cut off OD values for anti- *B. pseudomallei* IgG antibody with SWCA and RTFA was determined to find out the exposure rate to *B. pseudomallei* in the study population. ELISA was performed with sera from 51 healthy newborn babies of Dhaka city, who were presumed to be not exposed to *B. pseudomallei*. The mean OD + 3xSD of these sera were taken as cut-off OD value to determine the exposure rate. The mean OD±SD of 51 healthy newborn babies were 0.2±0.2 and 0.2±0.06 for SWCA and RTFA respectively. Therefore, the calculated cut-off OD value was 0.8 (0.2+3x0.2) and 0.4 (0.2+3x0.06) respectively for SWCA and RTFA (Table 3.1). Any sample showing OD above this cut-off value was considered positive and referred to as exposed to *B. pseudomallei* infection.

It may be mentioned that the mean OD value of ten culture-positive cases (positive control) was 2.26±0.2.

Table 3.1: Calculated cut off OD value for ELISA using SWCA and RTFA

Antigen	Mean OD value	±SD	Cut-off value (Mean+3xSD)
SWCA	0.2	0.2	0.8
RTFA	0.2	0.06	0.4

Note: SWCA= sonicated whole cell antigen
RTFA= recombinant truncated flagellin antigen

Specificity of ELISA against SWCA

To determine the specificity of anti- *B. pseudomallei* IgG by ELISA using SWCA, a sub-set of 24 known positive serum samples were adsorbed with whole cell killed *Pseudomonas aeruginosa* (*P. aeruginosa*) and *B. pseudomallei* USM strain (1x 10^8 organisms/ml) by incubating overnight at 4^0C. The adsorbed serum samples were centrifuged at 10,000 x g for 5 minutes to remove the bacteria. Anti- *B. pseudomallei* IgG antibody was then determined in adsorbed serum by ELISA as described above. Decline of antibody concentration in terms of OD values after adsorption with *B. pseudomallei* indicated presence of specific antibody to *B. pseudomallei* in serum samples while decline with *P. aeruginosa* indicated antibodies cross reacting to pseudomonas antigens. The adsorption assay showed that mean antibody level of the positive sera reduced significantly, after adsorption with *B. pseudomallei* compared to pre-adsorbed value from OD 1.11 to 0.65 (Fig 3.2). The mean OD value decreased below the cut off OD of 0.819 following absorption. But, the OD value decreased insignificantly from 1.11 to 0.91 following adsorption with *P. aeruginosa*.

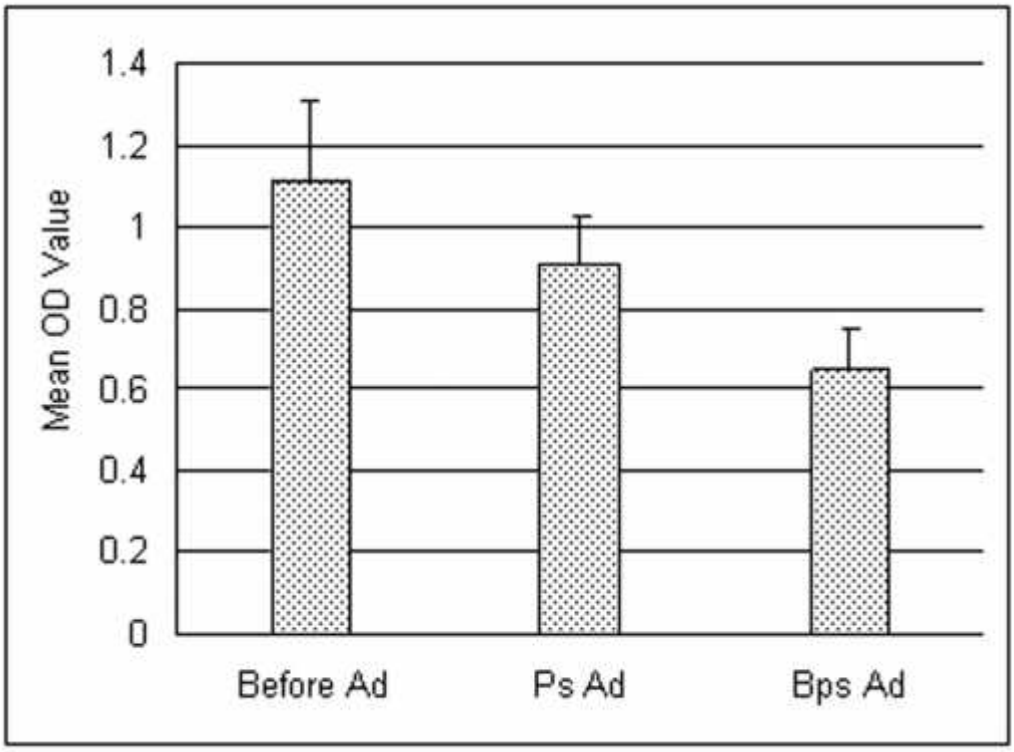


Figure 3.2: The effect of adsorption with whole cell *P. aeruginosa* and *B. pseudomallei* on the anti- *B. pseudomallei* IgG antibodies. Before Ad= before adsorption; Ps Ad= Adsorption with *P. aeruginosa*; Bps Ad= Adsorption with *B. pseudomallei*. n=24 seropositive sera in each group.

3.3 Isolation and characterization of *B. pseudomallei* from soil

In order to determine the source of the organism in the environment and its characterization, soil samples from north eastern districts of Bangladesh were collected and tested for the presence of *B. pseudomallei*.

Selection of sites and collection of soil samples

Soil samples were collected from rural areas of four known melioidosis endemic districts of Bangladesh (Barai et al., 2014). Three districts namely Mymensingh, Sylhet and Gazipur are situated in the north and northeast of capital Dhaka city while one district (Narayangange) is located south of Dhaka city. The locations of soil sampling district and their distance from capital Dhaka is shown in Figure 3.1. In each district, 3-5 sites were selected for collection of soil samples. Each sampling site was about 5 km apart from the next sampling sites. At each site three to five sampling points were identified which were about 30 meters apart from each other. Three to five soil samples were collected from each sampling point. The preferred collection site was a moist area within a rice field. Approximately 200 gm soil was taken from each point from a depth of about 20-30 cm using a shell augur disinfected with 70% alcohol in between soil collection. Collected soil was placed into a sterile plastic bag and sealed with rubber band to prevent moisture loss and was transported to the laboratory as soon as possible.

Isolation and identification of *B. pseudomallei* from soil samples by culture

Soil samples were processed for culture as described by Brook et al (Brook et al., 1997). Twenty gram of soil was mixed with 40 ml sterile distilled water and the suspension was shaken vigorously for one minute and allowed to settle for 5-10 minutes. The supernatant fluid was collected. For enrichment, one ml of supernatant fluid was inoculated into nine ml of modified Ashdown's selective enrichment broth (ASB) and incubated at 37^0C for 48 hours (Wuthiekanun et al., 1990). After enrichment, 10 µl of broth was streaked onto modified Ashdown`s selective agar (ASA) medium. The plates were incubated for 48-72 hours to allow typical colonies to grow. Purple colored dry, wrinkled and oxidase positive colonies were then sub cultured on MacConkey`s agar medium and incubated at 42^0C. The organisms which grew on MacConkey`s agar medium at 42^0C were identified

as *B. pseudomallei* by typical colony morphology, Gram staining (bipolar staining), motility, biochemical tests (including API 20NE), arabinose assimilation and resistance to colistin and aminoglycoside (Lipuma et al., 2007). Monoclonal antibody based latex agglutination test (Melioidosis Research Center, Khon Kaen, Thailand) was performed for the final identification and confirmation of the suspected colonies of *B. pseudomallei*.

3.4 Molecular Characterization of *B. pseudomallei*

In the present study, molecular analysis was performed from the strains isolated from both clinical specimen and environmental source from different areas of Bangladesh using molecular methods, like conventional polymerase chain reaction (PCR) targeting conserved genes of *B. pseudomallei*, Real-time PCR assay targeting *orf*2 gene of Type III secretion system (TTSI) for confirmation of phenotypic identification. Detection of *Yersinia*-like fimbrial gene cluster (YLF) and *B. thailandensis*-like flagellum and chemotaxis (BTFC) gene cluster were used to examine the diversity of *B. pseudomallei* from various geographical regions. Multilocus sequence typing (MLST) was used for epidemiological studies and to clarify the genetic relationships between *B. pseudomallei* isolated from clinical specimen and environmental source, and also among strains isolated from Bangladesh and other countries.

PCR assay and primer selection

In this study, we have selected two sets of oligonucleotide primers from previously published papers (Brook et al., 1997; Dharakul et al., 1999). Phenotypically confirmed *B. pseudomallei* isolates from soil samples and clinical cases were further confirmed by PCR using specific primers constructed from 16s rRNA region of *B. pseudomallei* (Brook et al., 1997; Dharakul et al., 1999). Primer set 1 (BPS16-42L and BPS16s-266R) was used to detect both *B. pseudomallei* and *B. thailandensis*, Primer set 2 (PPM3 and PPM4) designed from 16s rRNA region of *B. pseudomallei* to amplify a fragment of 550 bp in length specific for *B. pseudomallei.* PCR using all these primers was performed with 87 suspected soil isolates and 22 phenotypically confirmed clinical isolates. The USM strain was considered as positive control and different phenotypically confirmed organisms (*Escherichia coli, Pseudomonas aeruginosa, Burkholderia cepacia,*

Comamonas testosterone, Chrysobacterium indologenes, Pseudomonas alcaligenes, Pseudomonas putida etc) were used as negative control. Primers used in this study are shown in the Table-3.2.

Table 3.2 Primers used for specific PCR for identification of *B. pseudomallei*

Target gene for	**Primer**	**Sequence**	**PCR product bp**	**References**
Primer set 1: *B. pseudomallei* & *B. thailandensis*	Bps16S-42L	F-5` CGGCAGCRCGGGCTTCGG 3`	243	(Dharakul et al., 1999)
	Bps16S-266R	R-5` TGTGGCTGGTCGTCCTCTC 3`		
Primer set 2: *B. pseudomallei*	PPM3	F-5`AATCATTCTGGCTAATACCCG 3`	550	(Brook et al., 1997)
	PPM4	R-5` CGGTTCTCTTTCGAGCTCG 3`		

Preparation of DNA

Total genomic DNA was prepared by RealLine DNA Extraction Sample Kit" (BIORON Diagnostics GmbH, Germany). Briefly, 500 µl preheated (at 56^0C) lysis reagent with sorbent was added to 100 µl bacterial suspension. The tube was vortexed for 10 seconds followed by incubation in thermo shaker for 10 minutes at 1300 rpm. DNA/RNA solution was added to the tube and again vortexed and centrifuged at 13000 x g for 5 minutes. Supernatant was discarded and Wash Solution Number 1 was added to the tube, vortexed and centrifuged at 13000 x g for 5 minutes. Supernatant was again removed and 300 µl Wash Solution Number 2 was added to the tube, vortexed and centrifuged at 13000 x g for 5 minutes. Supernatant was discarded and the pellet was dried by opening the cap for 2-3 minutes at room temperature. Two hundred µl specimen diluents was added to the air dried tube and vortexed vigorously for 10 seconds followed by

incubation at thermo shaker for 10 minutes at 56^0C at 1300 rpm and then centrifuged at 13000 x g for 1 minute. Supernatant containing DNA was finally collected and stored at -20^0C until used.

PCR assay

PCR amplification was carried out in a 25 µl final volume containing 2.0 µl DNA, 2.5 µl 1 x PCR buffer, 1.5 mM $MgCl_2$, 25 µM of each dNTP, 10 pM of each primer, and 1.25 unit of Taq DNA polymerase enzyme. Samples were subjected to initial denaturation at 94^0C for 2 minutes followed by denaturation at 94^0C for 60 sec, primer annealing at 55^0C for 60 sec and extension at 72^0C for 90 sec. Final extension was for 10 minutes at 72^0C. Amplification was performed in Master Cycler (Eppendorf) programmed for 35 cycles.
Amplified PCR product was analyzed by electrophoresis in 1.5% agarose gel containing ethidium bromide (0.5 µg/ml) in TBE buffer (0.04 M Tris acetate, 0.001 M EDTA, pH 8.6) and photographed under UV illumination. The bands were compared to the band obtained with a positive *B. pseudomallei* DNA control. In all assays, DNA from known *B. pseudomallei* was included as positive control. A tube without DNA served as no template DNA control.

Real Time PCR for molecular characterization

Molecular analysis of the strains isolated from both clinical specimen and environmental sources was performed. The epidemiological data of the clinical and environmental isolates are given in Table–3.3. Real-time PCR assay was performed targeting *orf*2 gene of Type III secretion system (TTSI) for confirming identification of *B. pseudomallei*. Yersinia-like fimbrial gene cluster (YLF) and *B. thailandensis*-like flagellum and chemotaxis gene cluster (BTFC) were assayed for determining whether the isolates were Asian or Australian origin. Multilocus sequence typing (MLST) was done for epidemiological studies to determine the genetic relationships between *B. pseudomallei* isolated from clinical specimen and environmental source and also between strains isolated from Bangladesh and other countries. All suspected soil and clinical *B. pseudomallei* isolates were sent to the Emerging Pathogens Institute (EPI),

University of Florida, USA for species identification TTS1, YLF, BTFC by real-time PCR assay. MLST was also performed at the EPI.

A multiplex real-time PCR assay using SYBR green as a fluorescent dye targeting YLF and BTFC gene cluster was developed by Tuanyok A et al in 2007 (Tuanyok et al., 2007). Primers used for detecting TTS1, YLF and BTFC genes in real-time PCR are listed in the Table-3.4

Table 3.3 - Epidemiological data of *B. pseudomallei*

Strain Id	**Year of isolation**	**Geographic Location**	**Source /Specimen**	**Organ involved**	**Underlining Medical Condition**
K23	June, 2012	Gazipur	Soil	N/A	N/A
K35	June, 2012	Gazipur	Soil	N/A	N/A
CSK1	Feb,2013	Gazipur	Sputum	Lung	DM
CSK2	Feb,2010	Gazipur	Urine	Prostate, Knee Joint	DM
CS27	Oct,2013	Narayangange	Blood	Blood	DM
CS6318	Sep,2013	Gazipur	Blood	Blood	DM
CS88	Nov,2009	Mymensingh	Liver Pus	Liver	DM
CS90	Oct,2014	Mymensingh	Synovial fluid	Knee Joint	DM
PM55	Aug,2014	Chittagong H.T	Pus	Soft Tissue	DM
SP14	Feb,2010	Savar, Dhaka	Sputum	Lung	DM
CS2317	Nov,2009	Tangail	Urine , Pus	Prostate	DM
CS35	Oct,2013	Gazipur	Pus	Supra-clavicular abscess	DM
CS 6260	2015	Mymensingh	Synovial fluid, Blood	Septicemia, Arthritis	DM
CS 5414	2015	Tangail	Blood	Pleural effusion, Septicemia	DM
CS 6301	2015	Tangail	Blood	Septicemia	DM
CS 6887	2015	Tangail	Blood	Septicemia	DM
CS89	2015	Tangail	Blood	Septicemia	DM

Table 3.3 - Epidemiological data of *B. pseudomallei*…..Contd

Strain Id	**Year of isolation**	**Geographic Location**	**Source /Specimen**	**Organ involved**	**Underlining Medical Condition**
CS60	2015	Gazipur	PUS	Abscess in Scalp	DM
CS31	2015	Dhaka	Urine	UTI, Fever	DM
CS3623	2015	Munsiganj	Pus	Abscess	DM
CS4987	2015	Comilla	Sputum	Lung abscess,	DM
CS6410	2015	Narayangange	Blood, Urine	Septicemia, UTI	DM
CS6437	2015	Mymensingh	Synovial Fluid	Arthritis	DM
CS3504	2015	Work at Brunei	Sputum	Lung Abscess, Fever	DM
USM	2008	Malaysia	Not known	Not known	Not known

DM = Diabetes mellitus, UT= Urinary Tract, N/A= Not Applicable, M=Male, F=Female. CS K1 – Jahan, CS K2 – N. Badde, PM 55 - Pus (55)

Table-3.4: Primers used for TTS1, YLF and BTFC genes in real-time PCR

Target gene	**Primer Sequence**	**References**
TTS1	F 5`-CGTCTCTATACTGTCGAGCAATCG-3` R 5` -CGTGCACACCGGTCAGTACTC-3`	(Novak et al., 2006)
YLF BPSS0120 BPSS0120	F 5`TGACCCATTCAGGCAAGGGATTCT-3` R 5`TCCGTCCTGTTCGGTGATTTCGAT-3`	(Tuanyok et al., 2007)
BTFC btfc-orf18 btfc-orf18	F 5`GTCGATTTCGGCTGCGAAACAACA-3` R 5`ATGCCGTCGCAACCATTGATGATG-3`	(Tuanyok et al., 2007)

Multi-locus sequence typing

MLST was performed according to the methods of Godoy et al (Godoy et al., 2003). The primers used in the PCR amplification and sequencing of the seven housekeeping gene fragments are listed in Table 3.5. The following prime pairs were used in the PCR amplification of seven housekeeping gene fragments from all B. pseudomallei strains analyzed: *ace-up* and *ace-dn, -gltB-up* and *gltB-dn, gmhD-up* and *gmhD-dn, lepA-up* and *lep –dn, lipA-up* and *lipA –dn, narK-up* and *narK-dn, ndh-up* and *ndh-dn.* Properties of the loci used in the *B. pseudomallei* MLST scheme is shown in table 3.6

Table 3.5 Primers used in amplification and sequencing of seven housekeeping loci for MLST (McCombie et al., 2006)

Locus	Primer name	Primer sequence (5` to 3`)
ace	ace-up	GCT CGG CGC TTC TCA AAA CG
	ace-dn	CAT GTC CGT GCC GAT GTA GC
gltB	gltB-up	GGC GGC AAG TCG AAC ACG G
	gltB-dn	GCA GGC GGT TCA GCA CGA G
gmhD	gmhD-up	CTC GCG CAG GGC ACG CAG T
	gmhD-dn	GTC AGG AAC GGC GCG TCG TA
	gmhD-dn(outer)	GGC TGC CGA CCG TGA GAC C
lepA	lepA-up	CGC TTG ATC GGC ACT GAA TGG
	lepA-dn	CGA ACC ACG AAT CGA TGA TGA G
lipA	lipA-up	CAT ACG GTG TGC GAG GAA GC
	lipA-dn	CAG GAT CTC GTC GGT CGT CT
narK	narK-up(outer)	GCC GCG CAC GAC CAG CGC
	narK-up	CGG ATT CGA TCA TGT CCA CTT C
	narK-dn	CGG CAC CCA CAC GAA GCC C
ndh	ndh-up	GCA GTT CGT CGC GGA CTA TC
	Ndh-dn	GGC GCG GCA TGA AGC TCC A

Note: dn= down

Table 3.6 Properties of the loci used in *B. pseudomallei* MLST scheme (Godoy et al., 2003)

Locus	Gene function	No. of alleles	No. of variable sites	Genome location (bp)
ace	Acetyl coenzyme A reductase	4	7	1780
gltB	Glutamate synthetase	7	12	3761
gmhD	ADP glycerol-mannoheptose epimerase	15	19	3,023
lepA	GTP-binding elongation factor	6	10	2,938
lipA	Lipoic acid synthase	7	12	448
narK	Nitric extrusion protein	14	18	2,784
ndh	NADH dehydrogenase	7	12	1,400

Data analysis

For each housekeeping locus, the different sequences obtained from the *B. pseudomallei* isolates were assigned as distinct alleles by using the Macintosh Program Sequence Output (available from www.mlst.net). Each isolates was defined by a string of seven integers (the allelic profile), which correspond to the allele numbers at the seven loci, in the order of *ace-gltB-gmhD-lepA-lipA-narK-ndh*. Each unique allelic profile is considered a clone and is assigned a sequence type (ST), which also provides a convenient descriptor for the clone (Godoy et al., 2003; Maiden et al., 1998; Spratt, 1999). An MLST database containing the sequences of all alleles, the allelic profiles, and information about the *Burkholderia* isolates, together with analysis tools, is maintained at Imperial College (London, United Kingdom) and can be found on the *Burkholderia* pages mlst website (www.mlst.net).

The nucleotide sequences obtained in this study are available at the National Center for Biotechnology Information (NCBI). Nucleotide sequences of the 7 housekeeping genes (*ace-gltB-gmhD-lepA-lipA-narK-ndh*) are included in the *B. pseudomallei* database (http://bpseudomallei.mlst.net/).

4.0 Results

Blood samples from 940 healthy individuals and soil from four districts were collected. *B. pseudomallei* was isolated in selective media, identified by biochemical tests, and finally confirmed by specific antisera. Real-time PCR assay targeting Type III secretion system (TTSI), *Yersinia*-like fimbrial (YLF) and *B. thailandensis*-like flagellum and chemotaxis (BTFC) gene cluster was done. Phylogenetic analysis of *B. pseudomallei* isolates using multi-locus sequence typing (MLST) were performed with isolated organisms.

4.1 Anti- *B. pseudomallei* IgG antibody in study population

A total of 940 samples were collected from apparently healthy individuals residing in four northeastern districts of Bangladesh, namely Mymensingh (MMC), Sylhet (SOMC), kishoregange (JIMC) and Narayangange (NHN). Presence of anti-IgG antibody were determined by an indirect ELISA using both sonicated whole cell antigen (SWCA) and recombinant truncated flagellin antigen (RTFA) of *B. pseudomallei*. In addition, sera from 51 healthy newborn babies of Dhaka city who were presumed to be not exposed to *B. pseudomallei* were included as negative control and 10 sera from culture proven, melioidosis cases were included as positive control.

Cut-off value determination

A cut off value of ELISA for anti- *B. pseudomallei* IgG was determined by performing ELISA with sera from 51 healthy newborn babies. The mean OD+3xSD of these sera were taken as cut-off OD value to determine the exposure rate. Therefore, an OD value of 0.8 (0.2+3x0.2) was taken as the cut off value for SWCA. Any sample from any individual giving OD above this cut off value (0.8) was considered positive and referred to as exposed to *B. pseudomallei* infection. The cut off OD values for anti- *B. pseudomallei* IgG antibody against recombinant flagellin protein was determined as 0.4 (0.2+3x0.06). Any sample showing OD value above this cut-off value of 0.3 was considered positive and referred to as exposed to *B. pseudomallei* infection. Table-4.1 shows the cut off OD values for SWC and RTF antigens used in ELISA assay.

Table-4.1: Cut off OD values for SWC and RTF antigens used in ELISA assay

Antigen	Mean OD value	SD±	Cut-off value (Mean+3xSD)
Sonicated whole cell sonicated	0.2	0.2	0.8
Recombinant truncated flagellin	0.2	0.06	0.4

Anti- *B. pseudomallei* IgG with SWCA

Out of 940 healthy subjects, anti- *B. pseudomallei* IgG antibody against sonicated antigen higher than the cut-off value (>0.8) were detected in 203 (21.5%) individuals. Mean OD value of positive samples (1.2) were found significantly higher than the mean OD of negative samples (0.34). The OD values of all culture proven melioidosis cases were much higher (mean OD - 2.5) than the cut-off OD value, whereas the OD value was lower (mean OD - 0.2) in all newborn healthy controls (Table 4.2).

Table 4.2: Mean OD values of the study population

Study population	Total No	Anti- IgG Positive N (%)	Anti- IgG Negative N (%)	Mean (±SE) OD of	
				Positive cases	Negative cases
Healthy	940	203 (21.5)	737 (78.5)	1.2	0.3
Positive control	10	10 (100)	0	2.5	-
Negative control	51	0	51	-	0.2

Note: Positive control=Culture positive cases; Negative control = Newborn babies;
*Cut-off value =.8

District wise sero-positivity rate

Highest sero-positive result was obtained from Mymensingh district (30.8%) from where most of the melioidosis cases reported to BIRDEM Hospital. The rate was only 9.8% in Kishoregange district from where no melioidosis case has yet been reported (Lovely, 2014). Melioidosis cases have been sporadically detected from Narayangange and Sylhet districts earlier and the seropositivity among healthy individuals in these two districts were also found considerably higher (22.6% and 27.5% respectively). The detail district wise rate of sero-positivity is shown in Table 4.3.

Table 4.3: Seroprevalence of anti- *B. pseudomallei* IgG antibodies among the study population of four districts and mean OD values of positive and negative cases

Place	Total No. of Sample	Positive for anti- *B. pseudomallei* IgG		Mean (±SE) OD of	
		Number	% (95% CI)	Positive cases	Negative cases
Kishoregange	294	27	9.2 (5.8-12.5)	1.1 (0.04)	0.3 (0.01)
Mymensingh	221	68	30.8 (24.7-36.8)	1.2 (0.05)	0.3 (0.02)
Narayangange	214	58	22.6 (17.0-28.2)	1.4 (0.05)	0.4 (0.02)
Sylhet	211	50	23.7 (18.4-29.9)	1.1 (0.03)	0.4 (0.02)
Total	940	203	21.5 (18.9-24.9)	1.2 (0.03)	0.3 (0.01)

Cut off OD value for positive cases= 0.8; CI= Confidence interval;
OD= Optical density

Socio-demographic sero-prevalence

Anti-*B. pseudomallei* IgG antibody revealed that seropositivity increased with increase of age. The maximum number (30.4%) of positive cases belonged to over 50 years of age. The seropositivity rate of 41-50 and >50 years age groups was significantly higher ($p<0.05$) than that of lower age groups (1- 20, 21-30 and 31-40 years age groups; Table 4.4)

Gender breakdown of seroprevalence rate of anti- *B. pseudomallei* IgG antibody showed that males were exposed more frequently (25.9%) to *B. pseudomallei* than the females (20.6%) population (Table 4.5). However, no significant association of seropositivity rate between male and female were observed ($\chi 2= 3.44$, $p=0.064$). Positive rate of anti - *B. pseudomallei* IgG antibody ranged between 20 to 27% among different occupational groups of the study population. Although, the seropositivity rate is higher among businessmen (27.7%) than the house-wives (20.5%) of rural community, the association is not statistically significant ($p=0.280$).

Table 4.4: Age specific seropositivity rate of anti- *B. pseudomallei* IgG antibodies of study population

Age group (years)	Total No of samples	Positive for anti-*B. pseudomallei* IgG		95% CI
		No	%	
1-20	147	23	15.7	10.6-22.5
21-30	153	22	14.4	8.9-19.8
31-40	174	25	14.4	9.2-19.6
41-50	214	56	26.2	20.3-32.0
>50	253	77	30.4	24.8-36.1
Total	940	203	21.5	18.8-24.1

Cut off OD value for positive cases=0.8; CI= Confidence interval. Age specific trend of seropositivity is shown graphically in the inset.

Table 4.5 Distribution of occupation and sex of *B. pseudomallei* seropositive cases

Characteristics	Total No of sample	Positive for anti- *B. pseudomallei* IgG		95% CI
		No	%	
Male	366	95	25.9	20.1-28.6
Female	523	108	20.7	16.3-23.0
House wife	473	97	20.5	16.8-24.2
Farmer	102	27	26.5	17.9-35.0
Service holder	137	33	24.1	16.9-31.2
Business	112	31	27.7	19.4-35.9
Others	115	15	23.4	15.7-31.2

No association with gender ($\chi 2$= 3.44, p=0.064) and with any occupational group ($\chi 2$= 3.835, p=0.280); CI= Confidence interval

Seropositivity by RTFA and SWCA

Anti- *B. pseudomallei* antibody with RTFA by ELISA Seroprevalence of anti- *B. pseudomallei* IgG antibodies was determined with RTFA and the result was compared with the seroprevalence rate obtained with SWCA. Anti- *B. pseudomallei* IgG antibody with RTFA was determined with previously collected blood samples of 940 healthy individuals from 4 northeastern districts. Compared to 21.5% positivity rate with SWCA, the seroprevalence rate was 13.7% with RTFA. The rate of seropositivity ranged from 8.8% to 16.6% in individuals residing in four different districts. The detail comparative rate of seroprevalence is given in Table -4.6.

Table 4.6: Comparative seroprevalence of anti- *B. pseudomallei* IgG antibodies of study population with SWC and RTF antigens

Place	Total No. of Sample	Positive for anti- Bps IgG with SWCA		Positive for anti- Bps IgG with RTFA	
		Number	% (95% CI)	Number	% (95% CI)
Kishoregange	294	27	9.2	26	8.8
Mymensingh	221	68	30.8	34	15.4
Narayangange	214	58	22.6	34	15.9
Sylhet	211	50	23.6	35	16.6
Total	940	203	21.5	129	13.7

4.2 Isolation and identification of *B. pseudomallei* from soil samples

In order to determine the source of the organism in the environment, total 179 soil samples from four melioidosis endemic districts were tested for the presence of *B. pseudomallei*. Out of 179 soil samples, 87 yielded growth of oxidase positive non-fermenting, aminoglycoside and colistin resistant suspected colonies on the Ashdown selective media after enrichment at 42ºC. Out of 87 suspected isolates, only two isolates (K23 and K35) were identified as *B. pseudomallei* by specific monoclonal anti-sera based latex agglutination test and by PCR (Table 4.7; Figure 4.1 and Figure 4.2). The remaining 85 soil isolates were negative by PCR. Both the isolates were negative for *B. thailandensis* by PCR and were also arabinose negative. The two soil isolates were also positive for TTS1 by real-time PCR. Two soil samples that yielded growth of *B. pseudomallei* were collected from paddy field of Gazipur district. In addition to *B. pseudomallei*, other suspected soil isolates yielded 12 other different types of Gram negative, aerobic saprophytic bacteria (Table 4.8).

Table 4.7 Results of culture of soil samples for the detection of *B. pseudomallei* from four different melioidosis endemic districts of Bangladesh

Place (District)	No. of Site	No. of Sampling point	Location of sampling point	No of Sample	No. of suspected isolates	No. positive for *Bps*
Gazipur	4	7	4 paddy field 1 cattle shed 1 poultry shed 1 riverbank	41	15	02
Mymensingh	3	5	All paddy field	26	04	0
Narayangange	5	5	All paddy field	31	08	0
Sylhet	5	5	All paddy field	81	60	0
Total	19	22	-	179	87	02

Bps=B. pseudomallei

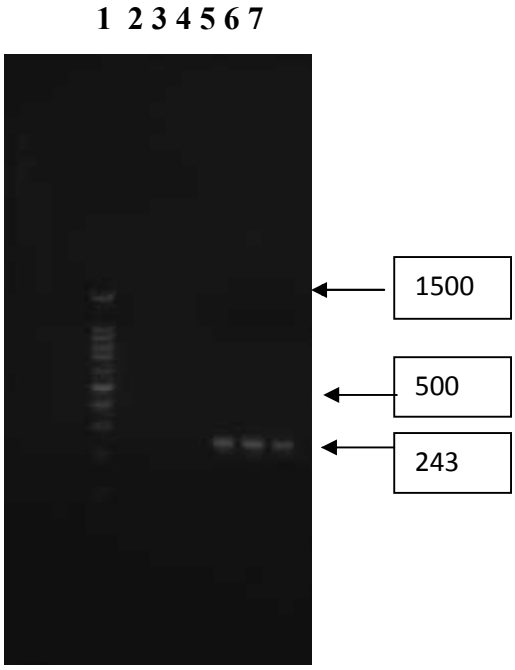


Figure 4.1a

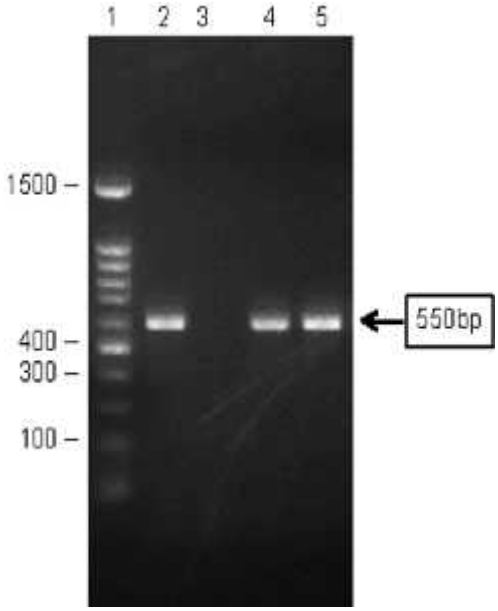


Figure 4.1b

Figure 4.1: PCR analysis of *B. pseudomallei* isolates from Gazipur district.

a. Using primer set 1: Lane 1- 100bp Marker, Lane 2 to lane 4 - Negative control, Lane 5 - Positive control (USM strain of *B. pseudomallei*, Lane 6- K23 (Gazipur soil isolate) and Lane 7- K35 (Gazipur soil isolate);

b. Using primer set 2: Lane 1- 100bp Marker, Lane 2- Positive control (USM strain of *B. pseudomallei*, Lane 3- Negative control (distilled water), Lane 4- K23 (Gazipur soil isolate) and Lane 5- K35 (Gazipur soil isolate**)**

Table 4.8 Name of other organisms isolated from the soil samples

Sl. No	Organisms
1.	*Burkholderia cepacia*
2.	*Chrysobacterium meningosepticum*
3.	*Comamonas testosteroni*
4.	*Pseudomonas alcaligenes*
5.	*Photobacterium damselae*
6.	*Chromobacterium violaceum*
7.	*Pseudomonas putida*
8.	*Pseudomonas fluorescens*
9.	*Achromobacter xylosoxidans*
10.	*Aeromonas salmonicida*
11.	*Chrysobacterium indologenes*
12.	*Pseudomonas aeruginosa*

4.3 Molecular characterization of *B. pseudomallei*

Molecular analysis of the strains isolated from both clinical and environmental sources from different areas of Bangladesh was performed by real-time PCR assay targeting *orf*2 gene of Type III secretion system (TTSI), Yersinia-like fimbrial gene cluster (YLF) and *B. thailandensis*-like flagellum and chemotaxis gene cluster (BTFC) for determining whether they are Asian or Australian origin. In the current study, attempt was made to demonstrate genetic relationships among the *B. pseudomallei* strains isolated from clinical and environmental sources by MLST. Genetic relationship was also analyzed among the strains isolated from Bangladesh and other countries.

Real-time PCR assay targeting TTSI confirmed that all 22 clinical and 2 environmental were *B. pseudomallei* (Fig 4.2). Gene cluster analysis targeting YLF and BTFC gene was performed by multiplex real-time PCR and identified that all isolates from Bangladesh contained a *Yersinia*-like fimbrial (YLF) gene cluster which was predominantly found among *B. pseudomallei* derived from Southeast Asia (Table 4.9).

Table 4.9 Real-time PCR result targeting TTSI, YLF and BTFC gene cluster

Isolate Number	TTS1	BTFC	Ylf
Clinical Samples			
CS-90	+	-	+
CSK - 2	+	-	+
CS-27	+	-	+
CS-88	+	-	+
CS-6318	+	-	+
CSK - 1	+	-	+
PM 55	+	-	+
SP14	+	-	+
CS-35	+	-	+
CS-2327	+	-	+
CS 6260	+	-	+
CS 5414	+	-	+
CS 6301	+	-	+
CS 6887	+	-	+
CS89	+	-	+
CS60	+	-	+
CS31	+	-	+
CS3623	+	-	+
CS4987	+	-	+
CS6410	+	-	+
CS6437	+	-	+
CS3504	+	-	+
Soil samples	+	-	+
K23			
K35	+	-	+
USM (Positive control	+	-	+

Note: TTS1 - Type III secretion system1, YLF - *Yersinia*-like fimbrial,
BTFC - *B. thailandensis*-like flagellum and chemotaxis; CS indicate clinical sample

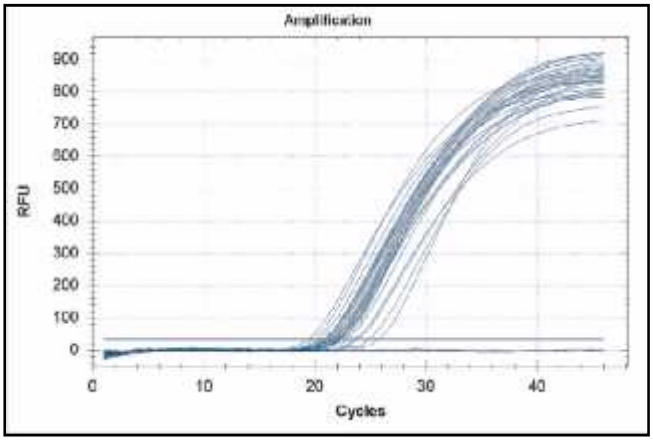


Fig 4.2: Graph showing TaqMan TTS1, real time PCR assay for confirmation of *B. pseudomallei* isolated from clinical and soil samples

Multi-locus sequence typing (MLST)

Phylogenetic analysis of *B. pseudomallei* isolates by MLST was performed with 22 clinical, 02 soil isolates and USM reference strain. Total 13 different sequence types (STs) were identified from these isolates, of which 4 STs (ST- 1352, 1124, 761, and 756) were novel and identified for the first time (Table 4.10). ST 56 (5 cases), ST 1007 (4 cases) and ST 1005 were the most frequently isolated types. These STs are also found in isolates from other countries like Thailand, Cambodia, Vietnam and China (Table 4.11). All the strains containing ST 56 were isolated from patients with septicemia (Table 4.12). Other STs were isolated from patients with abscess in different organs and body fluids (liver, soft tissue, lungs, joint fluid and pleural effusion). ST 1005 has been isolated from both clinical and soil sample and it is interesting to note that all isolates possessing ST 1005 were obtained from the same region in Bangladesh (Kapasia, Gazipur), which establishes that spatial clustering of clinical incidence is linked to environmental persistence of the organism and infection follows environmental exposure.

Table 4.10 Distribution of different sequence types and their source

Stain	Source	ST	ace	gltB	gmhD	lepA	lipA	narK	ndh
CS90	Human	1007	3	1	2	1	6	4	1
CS K2	Human	1005	3	1	4	1	6	4	1
CS27	Human	56	3	1	4	1	1	4	1
CS88	**Human**	**1352**	**3**	**1**	**2**	**1**	**6**	**36**	**1**
CS6318	Human	56	3	1	4	1	1	4	1
USM	Human	54	3	1	3	3	1	2	1
CS K1	Human	1005	3	1	4	1	6	4	1
PM55	**Human**	**1124**	**4**	**4**	**10**	**2**	**1**	**2**	**1**
SP14	**Human**	**761**	**1**	**4**	**10**	**2**	**1**	**4**	**1**
K23	Environmental	1005	3	1	4	1	6	4	1
K35	Environmental	1005	3	1	4	1	6	4	1
CS35	Human	211	3	1	3	1	1	4	1
CS2317	**Human**	**756**	**3**	**1**	**3**	**1**	**6**	**4**	**1**
CS6260	Human	56	3	1	4	1	1	4	1
CS5414	**Human**	**756**	**3**	**1**	**3**	**1**	**6**	**4**	**1**
CS6301	Human	172	1	1	3	2	6	4	1
CS6887	Human	56	3	1	4	1	1	4	1
CS89	Human	1007	3	1	2	1	6	4	1
CS60	Human	300	1	1	3	1	1	4	1
CS31	Human	1007	3	1	2	1	6	4	1
CS3623	Human	422	3	1	3	1	8	4	3
CS4987	Human	44	1	12	3	1	6	2	1
CS6410	Human	56	3	1	4	1	1	4	1
CS6437	Human	1007	3	1	2	1	6	4	1
CS3504	Human	61	1	1	2	3	8	4	1

Bold = New ST

Table 4.11 Results of MLST of *B. pseudomallei* isolated in Bangladesh and other countries

STs reported from Bangladesh				**No. of strains listed on MLST.net database**	**Bangladesh and other geographic information**
ST	**No. of isolates**	**Clinical**	**Environmental**		
1005	4	2	2	24	Thailand
56	5	5	0	14	Thailand, Cambodia, Vietnam
756	**2**	**2**	**0**	**2**	**Bangladesh**
1007	4	4	0	6	Thailand
211	1	1	0	11	Thailand, China, Vietnam
172	1	1	0	2	Thailand
300	1	1	0	6	Thailand
422	1	1	0	5	Singapore
1352	**1**	**1**	**0**	**1**	**Bangladesh**
761	**1**	**1**	**0**	**1**	**Bangladesh**
1124	**1**	**1**	**0**	**1**	**Bangladesh**
61	1	1	0	2	Australia
44	1	1	0	2	Australia

Bold = New ST

Table 4.12 Epidemiological Data of isolated *B. pseudomallei* in relation to sequence types as determined by MLST

Strain Id	**Year of isolation**	**Geographic Location**	**Sequence Type (ST)**	**Underlining Medical Condition**	**Sample/ specimen**
CS27	2013	Narayangange	56	Septicemia	Blood
CS6318	2013	Gazipur	56	Septicemia	Blood
CS 6260	2015	Mymensingh	56	Septicemia, Arthritis	Blood, synovial fluid
CS 6887	2015	Tangail	56	Septicemia	Blood
CS6410	2015	Narayangange	56	Septicemia, UTI	Blood, Urine
CS31	2015	Dhaka	1007	UTI, Fever	Urine
CS6437	2015	Mymensingh	1007	Arthritis	Synovial fluid
CS90	2014	Mymensingh	1007	Arthritis	Synovial fluid
CS89	2015	Tangail	1007	Septicemia	Blood
K23	2012	Gazipur	1005	N/A	Soil
K35	2012	Gazipur	1005	N/A	Soil
CSK1	2013	Gazipur	1005	Pneumonia	Sputum
CSK2	2010	Gazipur	1005	UTI, Arthritis	Urine, Synovial fluid
CS88	2009	Mymensingh	**1352**	Liver abscess	Pus
PM55	2014	Chittagong H.T	**1124**	Soft Tissue abscess	Pus
SP14	2010	Savar, Dhaka	**761**	Pneumonia	Sputum
CS2317	2009	Tangail	**756**	Prostate abscess	Pus
CS 5414	2015	Tangail	**756**	Pleural effusion, Septicemia	Blood, Pleural fluid

Table 4.12 Epidemiological Data of isolated *B. pseudomallei* ….Contd

Strain Id	Year of isolation	Geographic Location	Sequence Type (ST)	Underlining Medical Condition	Sample/ specimen
CS35	2013	Gazipur	211	Supra-clavicular abscess	Pus
CS 6301	2015	Tangail	172	Septicemia	Blood
CS60	2015	Gazipur	300	Abscess in Scalp	Pus
CS3623	2015	Munsiganj	422	Abscess	Pus
CS4987	2015	Comilla	44	Lung abscess	Pus
CS3504	2015	Work at Brunei	61	Lung Abscess, Fever	Pus
USM	2008	Malaysia	54	Not known	Not known

DM = Diabetes mellitus, UT= Urinary Tract, N/A= Not Applicable, M=Male, F=Female. CS K1 – Jahan, CS K2 – N. Badde, PM 55 - Pus (55), Bold & Shade = New ST

eBURST analysis

An eBURST diagram was generated to explore the reltedness of organisms isolated in the present study (24 isolates and 13 STs) to each other and those from other parts of the globe (Figure 4.3). Predicted group founders are indicated in blue and subgroup fonders are indicated in yellow. The comparative function of eBURST was used to allow for the differential identification of the 04 novel STs encountered (shown in green text) from the other existing STs already present in the *B. pseudomallei* MLST database (shown in black text).

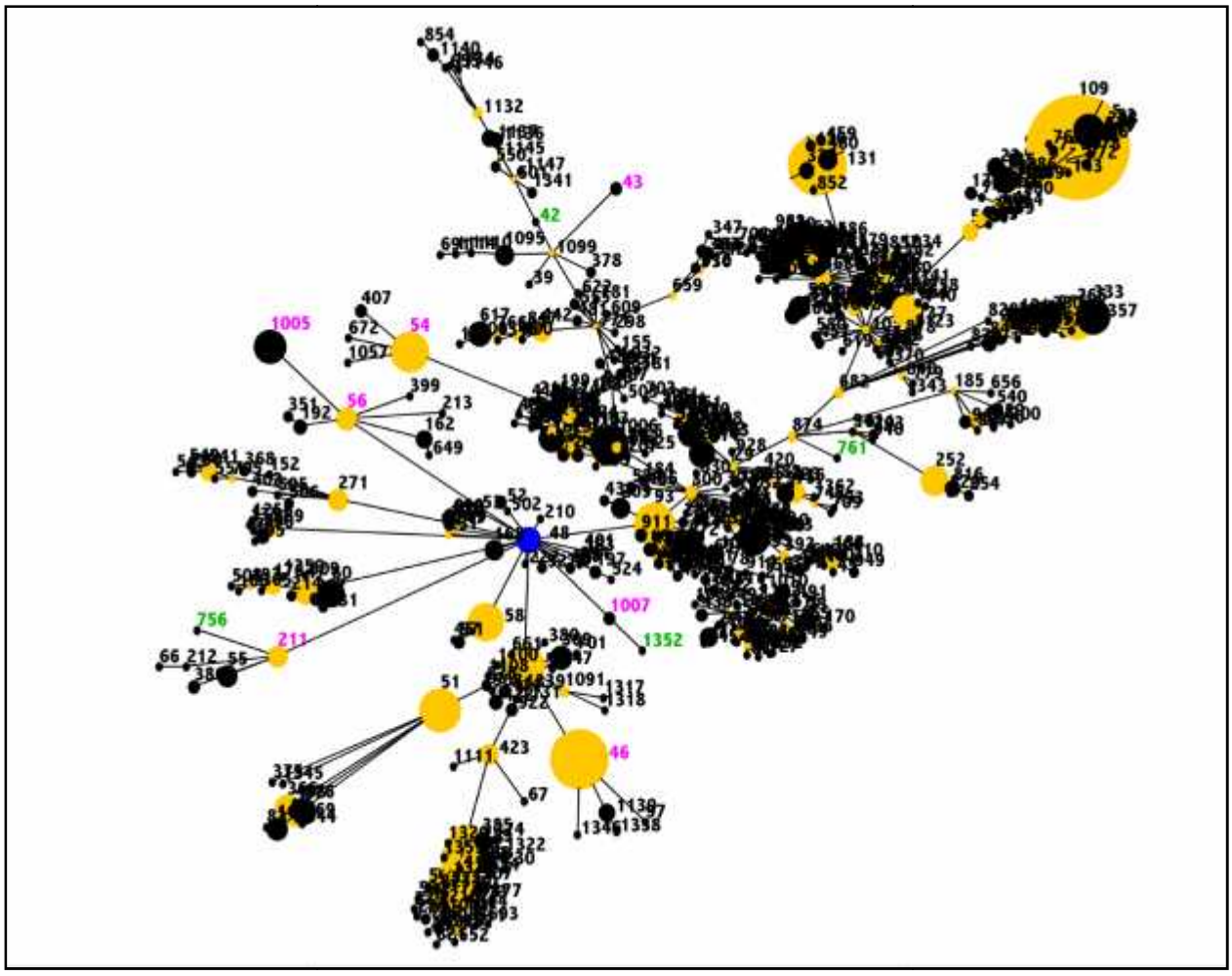
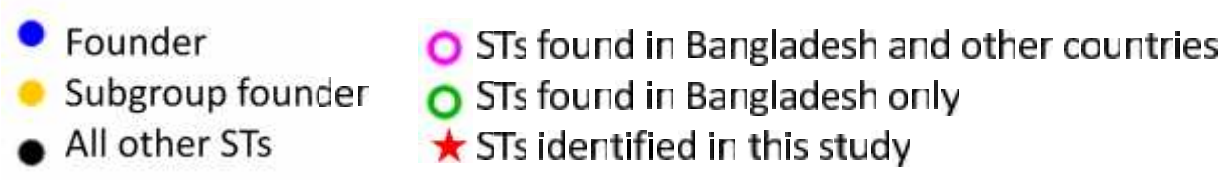


Fig 4.3 eBURST diagram displaying the relatedness of the 12 isolates and 9 sequence types of the *B. pseudomallei* strain.



5.0 Discussion

Melioidosis is largely restricted to the South East Asia and Northern Australia. However, the disease has been increasingly reported in countries outside the Asia-Pacific region including Bangladesh (Barai et al., 2014; Jilani and Haq, 2010), India (Prakash et al., 2014), South, Central and North America (Inglis et al., 2006) and West and East Africa (Cuadros et al., 2011; Salam et al., 2011). It is well established that infection follows environmental exposure, but factors contributing to the environmental persistence and dispersal of organism remain to be elucidated

Melioidosis is infrequently detected in Bangladesh for last 25 years but no systematic epidemiologic information regarding its distribution, true magnitude and source of infection is available (Barai et al., 2014). Isolation of the organism from clinical specimens in BIRDEM hospital indicates that the organism is present in our environment. However, its actual source has never been identified. The present study has been designed to determine the seroprevalence of melioidosis among apparently healthy population residing in rural areas as well as to isolate *B. pseudomallei* from the soil of four melioidosis endemic north and northeastern districts of Bangladesh. Molecular tests have been employed for confirmation of phenotypic identification and for phylogenetic studies of *B. pseudomallei* isolates.

In the first phase of the study, blood samples were collected from healthy population residing in rural area of four districts to determine the seroprevalence of *B. pseudomallei* infection. We have used both sonicated whole cell antigen (SWCA) and recombinant truncated flagellin antigen (RTFA) of *B. pseudomallei* in an in-house indirect enzyme linked immunosorbent assay (ELISA). Out of 940 blood samples, anti- *B. pseudomallei* IgG antibody against SWCA was detected in 21.5% individuals. Similar result was reported from East Timor, where the overall seroprevalence rate of 17.0% concurs with rates reported elsewhere in that region (Khupulsup and Petchclai, 1986). The seroprevalence of 21.5% in our study indicated that large proportion of people in

Bangladesh residing in the rural area is subclinically infected with the organism. These populations are potential vulnerable group for developing overt diseases during their lifetime with the alteration of their immunity as the organism may remain dormant in the body for long time like *M. tuberculosis*. The mean OD value representing the serum antibody concentration was significantly higher with SWCA in our positive sample (OD-1.2) compared to that of negative samples (OD-0.34). The findings suggest that there is a wide exposure of people to the offending organism because of its presence in the soil or other environmental sources.

However, SWCA may contain crude whole-cell preparation or bacterial lysates which may potentially lead to false-positive results. This non-specific reactivity might contribute to higher rate of seropositivity by using SWCA. In order to determine the specificity and cross-reactivity of anti- *B. pseudomallei* IgG by ELISA against SWCA, a sub-set of 24 known positive serum samples were adsorbed with whole cell killed *Pseudomonas aeruginosa* and *B. pseudomallei* USM strain. Anti- *B. pseudomallei* IgG antibody was then determined in adsorbed serum by ELISA. Decline of antibody concentration in terms of OD values after adsorption with *B. pseudomallei* indicated presence of specific antibody to *B. pseudomallei* in serum samples while decline with *P. aeruginosa* indicated antibodies cross reacting to pseudomonas antigens or related organisms. The adsorption assay showed that mean antibody level of the positive sera reduced significantly, after adsorption with *B. pseudomallei* compared to pre-adsorbed value from OD 1.11 to 0.65 (Fig 2.2). It is important to note from our adsorption study that some degree of non-specific cross reactive antibody was present in the serum which reacted with sonicated whole cell antigen used in the ELISA as was seen by reduction of OD value following adsorption with *P. aeruginosa* antigen. This may be due to the presence of some uncharacterized antigens of *B. pseudomallei* cross-reacting with other antibodies in the sera. To minimize the problem, more specific and purified recombinant truncated flagellin antigen (RTFA) was used in this study. ELISA using recombinant truncated flagellin had achieved sensitivity of 93.8% and a specificity of 96.3% and offered a more efficient serodiagnosis of melioidosis (Chen et al., 2003b). In our study, ELISA using RTFA protein showed the seropositivity rate as 13.7%, which is lower than

the seropositivity (21.5%) detected by ELISA using SWCA. However, Anuntagool N et al in a previous study showed that, ELISA with both crude and purified antigen had same sensitivity but purified antigen had higher specificity than crude antigen (Anuntagool et al., 1993). Several rapid serological diagnostic tests have been developed but the performance characteristics of many of these have not been adequately evaluated. ELISA studies using either crude or purified antigen preparations for the detection of antibodies to *B. pseudomallei* demonstrated varying results in terms of sensitivities and specificities (Allwood et al., 2008; Druar et al., 2008b; Sirisinha et al., 2000). In addition to crude and recombinant flagellin antigen, other antigen preparations such as purified exopolysaccharide (EPS), lipopolysaccharide (LPS) and Bip proteins have been reported as potential diagnostic antigens in an ELISA format, however, sensitive and reliable antigens are yet to be identified (Hara et al., 2013).

The seroprevalence of melioidosis usually reflects geography. In our study, seropositivity rate was 22.6%-30.8% in three districts from where melioidosis cases were detected earlier, compared to 9.8% in a district (Kishoregange) where no melioidosis case was either detected or reported previously. It reflects that significantly higher proportions ($p<0.01$) of people residing in the endemic districts were subclinically exposed to the organism compared to those in non-endemic areas. Similar situation prevails in northeast Thailand, which is defined as a hyperendemic area where 80% of children have specific antibodies against *B. pseudomallei,* compared with 10-29% of healthy people in other parts of Thailand (Limmathurotsakul et al., 2011). In Malaysia, reported seroprevalence in healthy individuals was 17-22% among rice farmers and 26% in blood donors. In north Australia 0.6 to 16% of children had evidence of infection by *B. pseudomallei* (Armstrong et al., 2005; Chen et al., 2004; Inglis et al., 2000; Leelarasamee, 1998).

In 2012, a hospital based serological survey in Bangladesh reported 28.9% seropositive rate for *B. pseudomallei* antibody among patients attending several tertiary care hospitals for unrelated ailments and did not investigate the possible source of the organism (Maude et al., 2012). The study, however, used a very low cut off titer (1:10) of indirect haemagglutination assay (IHA) for defining seropositive cases without considering the

presence of cross reactive background antibodies among the local population. The use of the IHA in sero-epidemiological study is problematic in endemic areas, particularly where rates of background seropositivity may be high presumably due to subclinical exposure to organisms related to *B. pseudomallei* (Khupulsup and Petchclai, 1986). Therefore, IHA cut off titer for defining seropositive case should be optimized for specific geographical area as has been done previously for studies involving Australian and Thai population. The lower cut-off titer of 1:40 was used in Australia compared to that of 1:160 in Thailand (Currie et al., 2000).

In this study, seropositivity rate for *B. pseudomallei* antibody was significantly increased with advancement of age. Anti- *B. pseudomallei* antibody was highest among individuals more than 50 years of age. Lowest rate was observed in 1-10 years age group. The result suggests that the chance of exposure to *B. pseudomallei* increases with age. Most of the individuals residing in the rural settings are involved in agriculture work and frequently come in contact with soil, mud and contaminated water, as they grow older.

The rate of seroprevalence among male and female population varies in different studies (Armstrong et al., 2005; Currie et al., 2000). However, we found no significant association of seropositivity with gender or any particular occupation. Probably our adult inhabitants in rural areas irrespective of gender or occupation are equally exposed to the environment. However, the situation is reverse in some other melioidosis endemic country like Timor, where a higher seroprevalence is observed in females compared to males (Armstrong et al., 2005). In Timor, the bulk of the physical activity in rural areas rests with females, and the resulting increased contact with soil and ground-water may explain the difference in seroprevalence rates along gender lines. In view of this, it is can be delineate that susceptibility to *B. pseudomallei* infection is directly related to environmental exposure.

In the second phase of the study, we aimed to find out the presence of *B. pseudomallei* in the soil samples from the melioidosis endemic districts of Bangladesh. The strategy of selecting four endemic districts to detect *B. pseudomallei* from environmental sources has

been previously used throughout Southeast Asia and northern Australia (Vesaratchavest et al., 2006). We have collected 179 soil samples from nineteen sites of four districts of Bangladesh (Mymensingh, Gazipur, Narayangange and Sylhet). Out of these 179 soil samples, only two soil samples (K23 and K35) from paddy fields of Gazipur district yielded growth of *B. pseudomallei* in selective Ashdown medium. Both the isolates were arabinose negative suggesting that they were not *B. thailandensis*. Our failure to isolate *B. pseudomallei* from soil samples from other sites could be due to soil condition at the time of sample collection. The presence of *B. pseudomallei* is influenced by low bacterial density, rain fall, load of organic materials and oxygen contents of the soil (Trung et al., 2011a; Trung et al., 2011b).

The isolation of *B. pseudomallei* from the soil samples in one district of Bangladesh indicates for the first time that the organism is present in the local environment and is the source of infections. Probably, the organisms may also be present in others areas of Bangladesh which could be detected if more soil/water samples were tested. Possibility of the presence of *B. pseudomallei* is very high as several clinical cases of melioidosis have been reported previously from ten different districts of Bangladesh (Barai et al., 2014). So far eighteen (18) countries of the world had been designated as definitive country for melioidosis based on the presence of culture confirmed *B. pseudomallei* in clinical case as well as in the environmental samples namely soil, water, etc of the locality (Limmathurotsakul et al., 2013; Prakash et al., 2014). Our finding of *B. pseudomallei* from the soil of one of the endemic district finally confirms that Bangladesh too is a definitive country for melioidosis.

We employed molecular typing techniques to determine the phylogenetic relationships of clinical and soil isolates. All of our isolates were positive for *orf2* of the TTS1cluster. This real-time assay targeting *orf2* of the *B. pseudomallei* TTS1 accurately detect the organism and are not inhibited by human blood products or DNA (Novak et al., 2006). Two mutually exclusive gene clusters, *B. thailandensis*-like flagellum and chemotaxis (BTFC) and *Yersinia*-like fimbrial (YLF) gene cluster have been linked to geographic origin and have been suggested to differentiate distinct strains of *B. pseudomallei*

(Tuanyok et al., 2007). Real-time PCR for the fimbrial gene cluster have determined that all PCR products from Bangladesh posses the YLF type gene cluster found predominantly among isolates of Southeast Asian origin. None of the isolates was positive for BTFC gene cluster. Phylogenetic analysis of the entire *B. pseudomallei* MLST database revealed distinct clustering of Asian isolates. This suggests that these populations are genetically distinct due to broad scale biogeographical factors associated with establishment and persistence of the organism. BTFC is the ancestral state and predominantly found in strain in Australia whereas the YLF strains predominantly found outside Australia. It has been suggested that a single horizontal transfer is responsible for replacing the BTFC cluster with the YLF cluster and that this event may have taken place in Australia, with the YLF group subsequently becoming the dominant group in Southeast Asia (Tuanyok et al., 2007). It has been further observed that YLF strains are more virulent than BTFC strains. In Thailand, where group YLF is dominant, mortality rates from primary disease are higher (50%) than melioidosis endemic regions of Australia (15 to 20%) where BTFC strains are the predominant (Cheng et al., 2003; White, 2003a).

In the present study, MLST has been used to examine the diversity of *B. pseudomallei* isolated from 22 clinical and 2 soil samples from various geographical regions in Bangladesh and revealed total 13 different sequence types (STs). Phylogenetic analysis of 24 *B. pseudomallei* isolates by MLST revealed that at least 4 STs (ST- 1352, 1124, 761, and 756) were novel STs and identified for the first time. All these isolates were from Bangladeshi patients. Strains having the above STs were isolated from patients with abscess in different organs (liver, soft tissue, lungs).These novel STs found in the clinical strains need to be further studied to determine whether such ST is linked to virulence or their survival in the environment.

It is to be noted that in the present study, ST 56 (5 cases), ST 1007 (4 cases) and ST 1005 were the most frequently isolated types. The most commonly detected ST 56 which was present in 5 clinical isolates and was the most common variant present in Bangladesh, followed by ST 1007 revealed from 4 cases and ST 1005, found in 2 clinical and 2

environmental isolates. It is important to note that all these four isolates possessing ST 1005 were obtained from the same region of Bangladesh (Gazipur). Presence of ST 1005 in soil of Gazipur district as well as its presence in the isolates from melioidosis patients from the same district indicates soil as the source and reservoir. This establishes that spatial clustering of clinical incidence is linked to environmental persistence of the organism and infection following environmental exposure. This may point out that soil may function as a vehicle for the dispersal of viable *B. pseudomallei* away from a primary environmental reservoir.

It is to be mentioned that *B. pseudomallei* isolated earlier from Bangladeshi individuals living abroad reported to contain STs 42, 43, 46, 56 and 71 (Godoy et al., 2003). Although *B. pseudomallei* possessing ST 46 was first recovered in 1960 from Bangladesh and also from a monkey in Indonesia, however this ST has not been recovered in any patient in the current study. Presence of ST 46 in both humans and animals indicates that same clone of *B. pseudomallei* appear to be able to cause melioidosis in both humans and animals. Most examples of the same ST recovered from different species with disease is likely to be due to the independent acquisition of the same virulent strain from the environment, although direct transmission from infected animals to humans has been proposed (Godoy et al., 2003).

All the strains containing ST 56 was isolated from patients with septicemia. ST 56 was also detected earlier in 1999 from a Bangladeshi patient living abroad. Apart from the four novel STs described above, all other STs that have been detected in the present study are also present in Thailand, Cambodia, China and Vietnam and other neighboring countries. It is likely that human and/or animal movements between these areas played pivotal role in the dissemination of *B. pseudomallei*, however analysis of broader range of isolates from these region are required for confirmation. Further large scale study is necessary to find out the magnitude of the infection and its different reservoirs in the environment along with phylogenetic distribution.

Conclusion

The present study has demonstrated that a large proportion of people residing in the rural area of four districts are exposed to the organism as determined by serology and have a potential for developing overt diseases during their lifetime. The study has also identified for the first time the presence of *B*. *pseudomallei* in the soil samples of Bangladesh and determined soil as the source of *B. pseudomallei* infection in this region. All *B. pseudomallei* isolated in Bangladesh posses YLF gene, which confirms their Asian origin. As YLF strains are more virulent than BTFC strains, so people in this region are at higher risk of severe form of infection. MLST study has revealed that a number novel STs of *B. pseudomallei* exist in Bangladesh environment. It has been observed that, same sequence types are present in soil as well as in melioidosis patients from same geographic area. Presence of same ST from the soil isolates indicates soil as the source and reservoir of this organism.

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