CHAPTER II
RELATED RESEARCH AND LITERATURE REVIEW

1. Background and History

Melioidosis was first described by Whitmore and Krishnaswami in 1912, as a "glanders-like" disease (Whitmore A, & Krishnaswami, 1912; Whitmore, 1913). They recognized a new organism, which could be isolated from patients in Myanmar (Whitmore, & Krishnaswami, 1912). Various reports of this bacterium were found in any names, e.g. *Bacillus whitmori*, *Pfeiffela pseudomallei*, *Actinobacillus pseudomallei* and *Malleomyces pseudomallei* (Stanton, & Fletcher, 1925). The bacterium was classified as a genus *Pseudomonas* base on its biochemical characteristics and composition of nucleic acid, called *Pseudomonas pseudomallei* (Rogul et al., 1970). In 1992, the bacterium was classified as a new genus, base on the sequences of 16S rRNA, called genus *Burkholderia,* therefore known as *Burkholderia pseudomallei* (Yabuuchi et al., 1992).

In the latter half of the 20th century, melioidosis emerged as an infectious disease of major public health importance in Southeast Asia and Northern Australia. In Ubon Ratchathani, Thailand, *B. pseudomallei* accounts for up to approximately 20% of community-acquired bacteremias (Chaowagul et al., 1989). At the Royal Darwin Hospital, Australia, it has been the most common cause of fatal community-acquired bacteremic pneumonia (Currie et al., 2000c; Douglas et al., 2004). Largely due to clinical trials in Thailand, significant improvements have been made in defining the optimal antibiotic therapy for melioidosis. However, the choice of antibiotic regimen has not been shown to have an impact on mortality within the first 48 h of admission (White et al., 1989), and severe melioidosis in Thailand is still associated with a case fatality rate of approximately 50% (White, 2003). In Australia, the mortality rate is still significant and approaches 20% among all patients with melioidosis (Currie et al., 2000b).

2. Epidemiology

Melioidosis occurs in tropical areas between latitudes 20°N and 20°S, predominantly in Southeast Asia and Northern Australia. In the past decade, reports of disease in both humans and animals have increased from countries outside the tropics (Hsueh et al., 2001). The increasing worldwide reporting of melioidosis underscores an emerging global problem. The highest number of infections are reported from Thailand (with an estimated 2,000–3,000 cases each year) (Leelarasamee, 2000), Malaysia (Puthucheary et al., 1981; Puthucheary et al., 1992; Vadivelu et al., 1997), Singapore (K. W. Chan et al., 1985; Lim et al., 1997; Tan et al., 1990; Yap et al., 1991; Yap et al., 1995) and northern Australia (Rode & Webling, 1981). The worldwide epidemiology of melioidosis has been comprehensively reviewed as shown in Figure 1 (Cheng & Currie, 2005). Reported cases are likely to represent "the tip of iceberg" (Dance, 1991; White, 2003), as confirmation of disease depends on bacterial isolation, a technique that is not available in many of the affected areas.

In Thailand, the annual incidence of 4.4 cases per 100,000 from Ubon Ratchathani province in northeast Thailand (Suputtamongkol et al., 1994). In addition, melioidosis cases are also found in Khon Kaen, Nakhon Ratchasima, Buri Ram and Udon Thani (Stone, 2007). *B. pseudomallei* is widely distributed in soil and more particularly pooled surface water such as in rice paddies (Finkelstein et al., 2000; Wuthiekanun et al., 1995). However, the rate of the closely-related but less virulent *B. thailandensis* which had previously been recognized as *B. pseudomallei* may account for the variation in disease throughout the country (Trakulsomboon et al., 1999); the ratio of *B. pseudomallei* to *B. thailandensis* found in soil, highest in the northeast, matches rates of clinical *B. pseudomallei* isolation throughout the Kingdom (Leelarasamee et al., 1997; Smith et al., 1995). These findings and the possibility of the existence of other less virulent strains of *B. pseudomallei* may also account for the much higher rates of seropositivity seen in Thailand (Kanaphun et al., 1993), compared to the endemic areas of northern Australia (Ashdown & Guard, 1984).

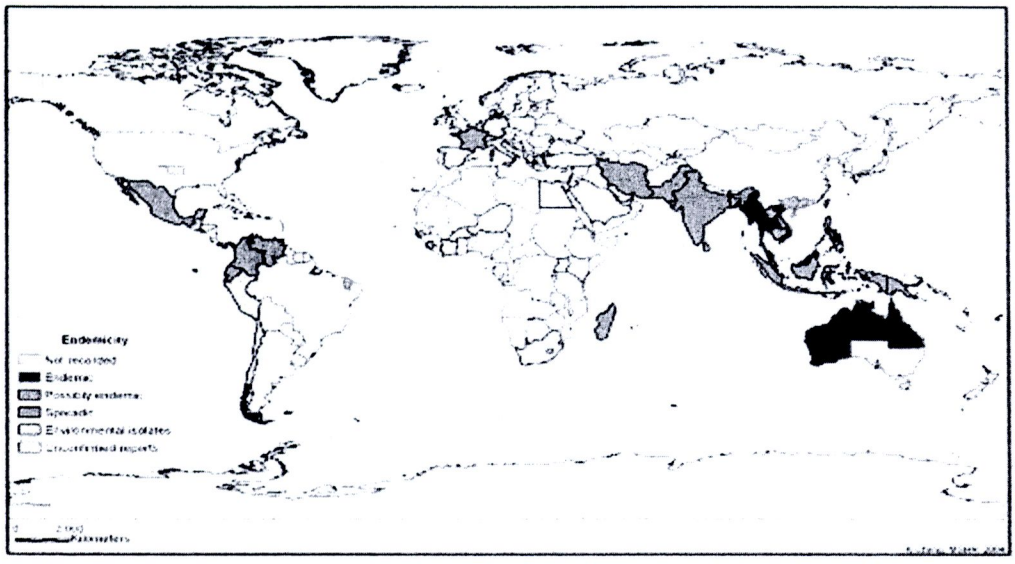

Figure 1 Worldwide distribution of melioidosis (Inglis & Sagripanti, 2006). The incidence of melioidosis is represented by the color code shown on the map. Epidemiology in Thailand as reported by Vuddhakul et al. (Vuddhakul et al., 1999) is represented. Areas showing non recorded cases (white regions) should not be assumed to be free of melioidosis since identification and reporting in the Americas, parts of Southeast Asia, and most of Africa are incomplete (Cheng & Currie, 2005). Zones with higher incidences (red regions) may correlate with a higher awareness of melioidosis and more-developed laboratory diagnostic and surveillance infrastructure.

3. Bacteriology and Pathogenesis

3.1 General bacteriology

B. pseudomallei is a saprophytic, gram-negative rod, a facultative anaerobic, 0.3-0.5 μm in width, 1-2 μm in length. The bacterium motiles by polar flagella and usually exhibit remarkable bipolar staining or "safety pin" (Stanford, 1994). *B. pseudomallei* can grow at 15-42 °C, also are able to grow at pH 5.6 (Yabuuchi & Arakawa, 1993) and can grow on simple media, including nutrient, blood and MacConkey agar. Ashdown 's selective differential agar medium has been commonly used in selective isolation of the organism from contaminated specimens (Ashdown, 1979). The selective and differential media for *B. pseudomallei* have been develop to improve the recovery of *B. pseudomallei* (Francis et al., 2006; Howard & Inglis,

2003). After 72-96 hour incubation, the colony becomes 7-10 mm in diameter, dull and wrinkled, and has a strong musty earthy odour (Salisbury & Likos, 1970). On culture, it demonstrates differing colonial morphology, with mostly smooth colonies initially and subsequent dry or wrinkled colonies on further incubation. It is oxidase positive and can be distinguished from the closely related but less pathogenic *B. thailandensis* by its ability to assimilate arabinose (Lertpatanasuwan et al., 1999; Smith et al., 1997). Whitmore distinguished it from *B. mallei* by its motility on hanging drop but in semisolid media this finding is less reliable (Finkelstein et al., 2000).

3.2 Taxonomy and Genome

The genus *Burkholderia* contains more than 30 species, the most pathogenic members of which are *B. pseudomallei* and *B. mallei* (Fig. 2). The genus also includes *Burkholderia thailandensis*, a nonpathogenic soil organism originally isolated in Thailand (Brett et al., 1997). Based on biochemical, immunological and genetic data, *B. pseudomallei* and *B. thailandensis* are closely related species. However, these two organisms differ in a number of ways and have been classified into two different species (Brett et al., 1998). The most distinct difference between these two species is their relative virulence. *B. thailandensis* is rarely causes disease and is $>10^5$-fold less virulent than *B. pseudomallei* in Syrian hamsters or mice (Brett et al., 1998).

The genome of *B. pseudomallei* (strain K96243, 170b, 668 and 1106a) has been sequenced and contain two chromosomes (Holden et al., 2004). The large chromosome I carries many genes associated with core functions such as cell growth and metabolism, and the smaller chromosome II carries more genes encoding accessory functions that could be associated with adaptation and survival in different environments. Approximately 6% of the genome is made up of putative genomic islands that have probably been acquired through horizontal gene transfer. These are mostly absent from the *B. thailandensis* genome (and are absent from the *B. mallei* genome (Nierman et al., 2004); it is unclear whether these regions have a role in disease pathogenesis.

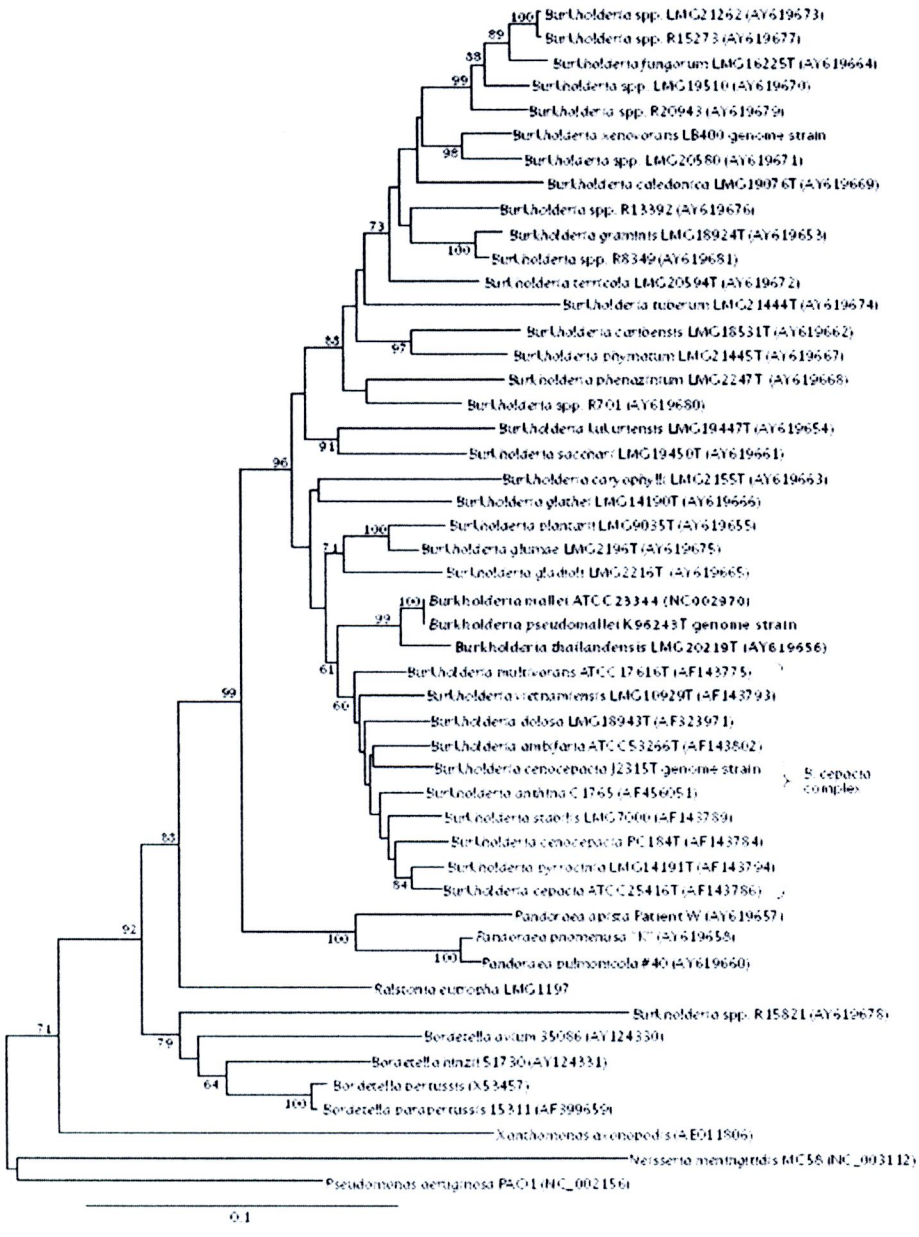

Figure 2 The phylogeny of the *Burkholderia* genus (Taken directly form Melioidosis: insights into the pathogenicity of *B.pseudomallei*) (Wiersinga et al., 2006)

3.3 Virulence factors

A little is known about *B. pseudomallei* virulence factors compared with other gram negative bacteria. In the present the factors described are included on the basis of a known role in virulence for other pathogens, or virulence in experimental models. Like many saprophytic organisms, *B. pseudomallei* is a resilient bacterium that can survive in a variety of hostile conditions, including nutrient deficiency, acid and alkaline pH, in disinfectant and antiseptic solutions including detergents and chlorine, exposure to many antibiotics and at extremes of temperature. Shed organisms can survive for months or years in soil and water. *B. pseudomallei* is also well adapted to its many hosts, producing proteases, lipases, lechithinase, catalase, peroxidase, superoxide dismutase, haemolysins, a cytotoxic exolipid and a siderophore. It is resistant to complement lysosomal defensins and cationic peptidases and can survive within many eukaryotic cell lines including professional phagocytes such as neutrophils and macrophages (White, 2003).

3.3.1 Quorum sensing

Quorum sensing is a cell-density-dependent communication system in gram negative bacteria that uses N-acyl-homoserine lactones (AHL) for the coordination of gene expression (Fuqua et al., 1994). LuxI protein are responsible for AHL biosynthesis while LuxR transcriptional regulators, following association with their cognate AHL(s), mediate gene repression or expression (Schuster et al., 2003; Wagner et al., 2003). The *B. pseudomallei* contain three LuxI and five LuxR quorumsensing homologues (Ulrich et al., 2004). Mass-spectrometry analysis of *B. pseudomallei* culture supernatants has demonstrated the presence of many signalling molecules, including *N*-decanoyl-homoserine-lactone and *N*-(3-oxotetradecanoyl)- l-homoserine lactone (Ulrich et al., 2004). Mutagenesis of the *B. pseudomallei* quorum-sensing system leading to a significant increase in the 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).

A LuxI-LuxR homologue termed PmlI-PmlR, which directs the synthesis of *N*-decanoylhomoserine-lactone and is involved in regulation of a metalloprotease, is essential for full virulence in a mouse model (Valade et al., 2004). A homologue termed BpsI-BpsR is also required for optimal expression of virulence and the

secretion of exoproducts in *B. pseudomallei* (Song et al., 2005). Some quorum-sensing-controlled candidate virulence factors and processes such as siderophores, phospholipase C and biofilm formation are probably partially dependent on BpeAB-OprB, a multidrug efflux pump in *B. pseudomallei* that is also known to be responsible for conferring antimicrobial resistance to aminoglycosides and macrolides (Y. Y. Chan & Chua, 2005). The *bpeAB-oprB* operon in turn might be regulated by quorum sensing, as *N*-decanoylhomoserine-lactone and *N*-octanoyl-homoserine lactone can induce *bpeAB-oprB* expression (Y. Y. Chan & Chua, 2005). BpeAB mutants are also associated with attenuated cell invasion and cytotoxicity of human lung epithelial (A549) and human macrophage (THP-1) cells (Y. Y. Chan & Chua, 2005).

3.3.2 Type III secretion system

B. pseudomallei contains three type III secretion system (TTSS) gene clusters, which contain between 16 and 18 reported genes (Attree & Attree, 2001; Holden et al., 2004; Rainbow et al., 2002; Stevens et al., 2002; Warawa & Woods, 2005). One of these clusters; the TTSS3 cluster shares homology with the *inv/spa/prg* TTSS of *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) and the *ipa/mxi/spa* TTSS cluster of *Shigella flexneri* (Attree & Attree, 2001; Rainbow et al., 2002; Stevens et al., 2002). Type III secretion system are key virulence determinants of Salmonella and Shigella, and other gram negative intracellular pathogen. TTSS encodes a secretion apparatus that functions like a molecular syringe and serve to inject 'effector' proteins into the target-cell cytosol (reviewed by Cornelis and Van Gijsegem (Cornelis & Van Gijsegem, 2000). The gene cluster in *B. pseudomallei* (termed *bsa, Burkholderia* secretion apparatus) encodes proteins that are very similar to the *S. typhimurium* and *S. flexneri* type-III-secreted proteins required for invasion, escape from endocytic vacuoles, intercellular spread and pathogenesis (Stevens et al., 2002). *B. pseudomallei* mutants lacking components of the Bsa secretion and translocation apparatus have reduced replication in murine macrophage-like cells, an inability to escape from endocytic vacuoles and a complete absence of membrane protrusions and actin tails (Stevens et al., 2002).

Inactivation of BopE, a TTSS protein that secreted by the Bsa type III secretion apparatus and is homologous to *Salmonella enterica* SopE/SopE2 leads to

impaired bacterial entry into Hela cells, indicating that BopE facilitates invasion (Stevens et al., 2003), most likely by acting as a guanine nucleotide exchange for RhoGTPases that regulate the actin network (Stevens et al., 2003). Other bsa-encoded effector proteins have been identified, including BopA and BopB (Stevens et al., 2002). BopA is a homologue of the *Shigella* type III secreted protein IscB, which mediates cell-to-cell spread of Shigella by lysing the double membrane surrounding actin-based protrusions that project the bacteria into adjacent cells (Allaoui et al., 1992). BopB is predicted to be encoded at one end of the bsa locus that homologue with the type III secreted protein of *Salmonella* (SopB), witch influences inositol phosphate signalling pathways in eukaryotic cells, bacterial invasion and Salmonella-induced enteritis (Norris et al., 1998; Zhou et al., 2001).

The *B. pseudomallei bsa* locus encodes homologues of *Salmonella* Sip translocator proteins (BipB, BipC and BipD) (Stevens et al., 2002). Salmonella SipB, SipC and SipD proteins are required for injection of effector proteins and invasion of epithelial cells in vitro (Collazo & Galan, 1997; Kaniga et al., 1995), mutation of the *B. pseudomallei bipD* gene impairs invasion of epithelial cells in vitro (Stevens et al., 2003). In addition, BipD is required for replication of *B. pseudomallei* in murine macrophage-like cells and for bacterial escape from endocytic vesicles and subsequent actin tail formation (Stevens et al., 2002). *B. pseudomallei* BipD mutants that lack 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 in early stages of infection (Stevens et al., 2004). *B. pseudomallei* BipB has been shown to mediate the formation of multinucleated giant cells, cell-to-cell spreading of bacteria and apoptosis of infected host cells (Suparak et al., 2005). The *bipB* mutant is unable to deliver the effector proteins into the host cell cytoplasm and was impaired in invasion efficiency and ability to induce apoptosis. Deletion of BipB clearly also reduces the efficiency of MNGC formation; however, the relationship between BipB protein and the fusion process is still under investigation.

3.3.3 Capsular polysaccharide

Capsular polysaccharides or exopolysaccharide were among the first known bacterial virulence determinants in gram-negative and gram-positive bacteria. Capsular material isolated from *B. pseudomallei* consists of an unbranched

homopolymer with the structure -3)-2-*O*-acetyl-6-deoxy-β-d-*manno*-heptopyranose-(1- (Knirel et al., 1992; Perry et al., 1995). The role of capsular polysaccharides in virulence is unknown, but sera from patients with melioidosis have been shown to contain antibodies against EPS (Steinmetz et al., 1995). The addition of purified *B. pseudomallei* capsule to serum bactericidal assays increases the survival of *B. pseudomallei* SLR5, a serum-sensitive strain, by 1,000-fold in normal human serum. The capsule-deficient mutant compared to the wild type in the present of normal human serum demonstrated an increase phagocytosis. These results suggest that capsule might act as a barrier contributes to resistance phagocytosis by reducing C3b deposition on the surface of the bacterium (Reckseidler-Zenteno et al., 2005).

3.3.4 Lipopolysaccharide

The lipopolysaccharide (LPS) of *B. pseudomallei* (formally termed type II O-antigenic polysaccharide) have been demonstrated both a virulence determinant and protective antigen (Charuchaimontri et al., 1999; DeShazer et al., 1998; Ho et al., 1997). *B. pseudomallei* LPS seems to be largely conserved across this species. Analysis of the LPS >700 *B. pseudomallei* isolates using proteinase-K digestion and SDS-PAGE silver-stained gels, a technique that examines the O-side chain, demonstrated that most isolates exhibited 'typical' ladder pattern of extracted LPS, 3% exhibite 'atypical' pattern, and 0.1% did not exhibit a ladder appearance (Anuntagool et al., 2000a). The different LPS preparations have similar endotoxic activity in the Limulus amoebocyte lysate assay. However, there were no immunological cross reactivity between typical and atypical LPS evident from immunoblot reactivity against pooled serum from patients whom the typical or atypical LPSs were isolate (Anuntagool et al., 2000a). The level of antibody to LPS on admission to hospital is higher in patients with melioidosis who survive compared with those who die, and in patients with non-septicaemic versus septicaemic melioidosis (Charuchaimontri et al., 1999). These antibodies might protect the host against death; alternatively, there could be an association between a raised anti-LPS antibody titre and a more efficient host immune response, including cell-mediated killing.

3.3.5 Flagella

B. pseudomallei is flagellated and motile. Flagella are commonly recognized as important virulence determinants expressed by bacterial pathogens since the motility phenotype imparted by these organelles often correlates with the ability of an organism to cause disease. The *fliC* gene that has a variable region compared between *B. pseudomallei* and closely related to avirulent *B. thailandensis* (Wajanarogana et al., 1999). The difference between these two species is their relative virulence. The ability of invasion, internalization and intracellular replication of both phagocytic and non-phagocytic cells of *B. pseudomallei fliC* knockout mutant was compared with those of the wild-type strains of *B. pseudomallei*, flagella are involved in macrophage invasion and facilitate invasion in non-phagocytic cell (Chuaygud et al., 2008). Unlike Syrian hamster or diabetic rat models of infection, demonstrate no difference between mutant and wild-type *B. pseudomallei* (DeShazer et al., 1997). The bacterial numbers were markedly reduced in the lung and spleen of BALB/c mice following intranasal infection of an aflagellate mutant compared with the wild type, and the mutant was less virulent following intraperitoneal infection of BALB/c mice, based on the LD_{50} (Chua et al., 2003).

3.3.6 Type IV pili-mediated adherence

Adherence of pathogens to host surfaces is prerequisite step in the pathogenesis of almost all infectious diseases. Bacterial adherence requires the specific interaction of bacterial molecules, termed adhesions, with host cell membrane molecules or extracellular matrix proteins such as carbohydrate molecules, pilus and nonpilus adhesions (Beachey, 1981; Hultgren et al., 1993). Adherence of *B. pseudomallei* to cultured cells increases dramatically following prior growth at 30° C compaired with growth at 37° C (N. F. Brown et al., 2002), this occurs as the result of microcolony formation (bacterium-bacterium interaction) following growth at 27° C but not at 37° C (Boddey et al., 2006). Type IV pili are important for virulence in many Gram-negative bacteria. The *B. pseudomallei* K96243 genome contains multiple type IV pilin-associated loci, including one encoding a putative pilus structural protein (PilA) (Essex-Lopresti et al., 2005), *pilA*, is essential for microcolony development by *B. pseudomallei* but is not required for direct adherence(bacterium-cell interaction (Boddey et al., 2006). A *pilA* deletion mutant has reduced adherence to human

epithelial cells and is less virulent in the the murine model of melioidosis, suggesting a role for type IV pili in *B. pseudomallei* virulence (Essex-Lopresti et al., 2005).

3.3.7 Secretory products

The role of secreted products is unclear. Many, including proteases (Lee & Liu, 2000; Sexton et al., 1994), phospholipase C (Korbsrisate et al., 1999), haemolysin, lecithinase and lipase (Ashdown & Koehler, 1990) are probably secreted via the general secretory pathway (type II secretion system) (DeShazer et al., 1999). Transposon mutations in the general secretory pathway, resulting in a failure to secrete protease, lipase or lecithinase, does not appear to result in an attenuation of virulence in an animal model (Brett & Woods, 2000). However, the finding that the relationship between the density of bacteraemia and mortality is similar in melioidosis compared to other gram negative bacteraemias suggests that exotoxins do not play a significant role in determining outcome (White, 2003). In addition, less virulent *B. thailandensis* do not contain some TTSS (Rainbow et al., 2002) and *B. pseudomallei* with mutations involving the TTSS translocator BipE and putative effectors have attenuated virulence (Stevens et al., 2004). Microarray studies have determined that growth of *B. thailandensis* in the presence of arabinose results in downregulation of the TTSS3 via the putative positive regulator *bsaN*, suggesting that the loss of the ability to assimilate arabinose is linked to the increased virulence of *B. pseudomallei* in humans and animals (Moore et al., 2004).

3.4 *B. pseudomallei* - Host cell interactions

B. pseudomallei is a saprophyte that is commonly isolated from soil and surface water throughout the endemic regions. Transmission is believed to occur by inhalation of contaminated dust, ingestion of contaminate water, and contact with contaminated soil or water especially through abraded skin. As with other gram-negative facultative intracellular pathogens, *B. pseudomallei* has evolved mechanisms to enter eukaryotic cells and subvert cellular process. *B. pseudomallei* can promote its uptake by non-phagocytic cells (Jones et al., 1996) and inaddition can survive and proliferate within phagocytic cells for prolonged periods (Jones et al., 1996; Pruksachartvuthi et al., 1990). The intracellular life style of *B. pseudomallei* to shown in Fig. 3, *B. pseudomallei* can invade phagocytic or nonphagocytic cells and can survive, proliferate for prolonged periods within phagocytic cells. The *Burkholderia*

secretion apparatus (*bsa*) system, encodes proteins that are required for invasion, escape form phagosomes and intracellular spread (Fig 3;a). Following internalization, *B. pseudomallei* can escape from endocytic vacuoles into the cytoplasm of infected cells by lysing the endosome membrane (Fig 3;b) (Harley et al., 1998; Stevens et al., 2002). Once insides the cytoplasm, *B. pseudomallei* induced continuous polymerization of actin at one pole of the bacterial cell (Fig 3;c) (Breitbach et al., 2003; Kespichayawattana et al., 2000; Stevens et al., 2002). This process results in the formation of membrane protrusions and cell to cell spread. In addition, uniquely among intracellular bacterial pathogens so for described *B. pseudomallei* is able to induce the fusion of adjacent cells leading to the formation of giant multinucleated cells (Fig 3;d) (Kespichayawattana et al., 2000).

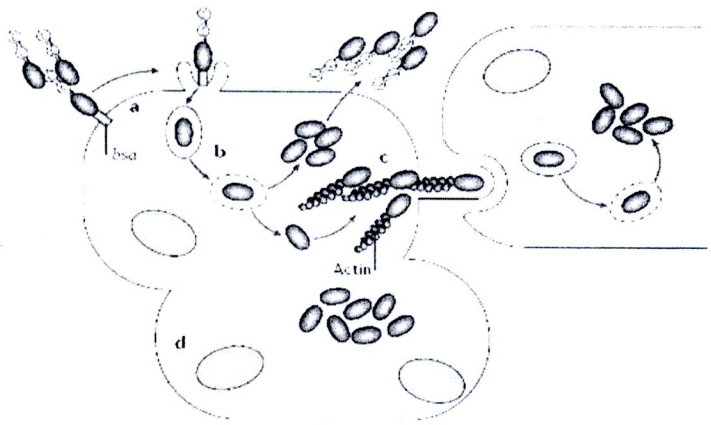

Figure 3 The intracellular lifestyle of *Burkholderia pseudomallei.* (Taken directly form Melioidosis: insights into the pathogenicity of *B. pseudomallei*) (Wiersinga et al., 2006)

3.4.1 Innate immune response

The innate immune response constitutes the frontline defense of the body against pathogens. The first barrier-to-interaction is physical barriers like the epithelial or mucosal surfaces. *B. pseudomallei* senses and captures pathogens through different classes of pattern recognition receptors (PRR) that recognize conserved surface motifs termed 'pathogen-associated molecular patterns' or PAMPs on microbes. The family of TLRs 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).

Upon entry, *B. pseudomallei* will also have to contend with key components of the innate immune response such as the complement cascade and phagocytosis by macrophages and neutrophils in order to survive. One of the virulence factors *B. pseudomallei* possesses is a capsular polysaccharide which inhibits killing of the bacteria by complement. The capsule inhibits the deposition of complement factor 3b, which is needed to start the cascade in the alternative complement pathway. Without the deposition of complement, the bacteria are less likely to be phagocytosed (Reckseidler-Zenteno et al., 2005).

Upon phagocytosis, *B. pseudomallei* is able to inhibit the up-regulation of inducible nitric oxide synthase (iNOS) (Utaisincharoen et al., 2001). This could prevent the killing of the bacteria by oxygen dependent mechanisms. Furthermore, *B. pseudomallei* was found to be resistant to anti-microbial peptides like defensins and protoamine, a cationic peptide (Jones et al., 1996). This could enable it to survive the oxygen independent killing mechanisms inside neutrophils.

The pro-inflammatory cytokine interferon (IFN)-γ has an important role in early resistance against *B. pseudomallei* infection. Inhibition of IFN-γ expression in mice lead to increase the bacterial load in liver and spleen (Santanirand et al., 1999). Inhibition of IL-12 and IL-18, the predominant endogenous inducers of IFN-γ production resulted in increased the mortality mice. IFN-γ has been shown to induce nitric oxide synthesis in infected macrophages which could kill the intracellular bacteria (Utaisincharoen et al., 2001). The pro-inflammatory cytokine tumour-necrosis factor (TNF)-α is also likely to be an important element of the early immune response, as passive immunization against this mainly macrophage derived cytokine

increased mortality in experimental murine melioidosis (Santanirand et al., 1999). Serum IFN-γ, IL-12 and TNF-α concentrations are elevated in melioidosis patients (A. E. Brown et al., 1991; Lauw et al., 1999). The release of these pro-inflammatory cytokines in an early stage of the disease shows that it is mediated by the innate immune response. In fact, in BALB/c mice, high levels of IFN-γ and TNF-α were present 24 hours after the infection (Liu et al., 2002; Ulett et al., 2000). The mice failed to control the disease and died subsequently due to overwhelming septicaemia.

3.4.2 Adaptive immune response

In patients with melioidosis, the higher levels of IgG, IgA and IgM were presented in patients throughout the infection (Vasu et al., 2003), IgG1 and IgG2 were the predominant antibodies response to the culture filtrate antigen (CFA) of *B. pseudomallei* was examined in the serum of melioidosis patients (Chenthamarakshan et al., 2001; Vasu et al., 2003). In Thailand, a higher frequency of patients with DRB1*1602 allele was found to have severe melioidosis, while DRB1*0701was found in higher frequency amongst cases with fatal melioidosis (Dharakul et al., 1998). DRB1*0701, DQA1*0201, and DQB1*0201 were detected in higher frequency amongst cases with higher relapses of melioidosis (Dharakul et al., 1998). These alleles encode major histocompatibility complex class II (MHC II) molecules, which are involved in the capturing of exogenous peptides and presentation to CD4 T cells. This could be an indirect indication of the importance of activating the appropriate T cell response against the bacteria. However, since the study is limited and there could be underlying confounding factors, larger scale studies will have to be done to conclude the significance of MHC association with severe melioidosis.

Patients who recovered from melioidosis showed evidence for an antigen-specific cell-mediated immune response, as reflected by enhanced lymphocyte proliferation and IFN-γ production in response to *B. pseudomallei* antigens (Ketheesan et al., 2002). In addition, asymptomatic seropositive individuals showed a stronger cell-mediated adaptive immune response as measured by *Burkholderia* specific lymphocyte reactions compared with subjects with a history of clinical melioidosis (Barnes et al., 2004), suggesting that a strong cell-mediated immune response might protect against disease progression, the production of IFN-γ by CD4+ T cells activates macrophages to become more bactericidal, that is supported by the

finding that IFN-γ increases the intracellular killing activity of macrophages *in vitro* (Utaisincharoen et al., 2004). Interestingly, a very recent study has suggested that *B. pseudomallei*-specific CD4+ T cells are important for late host resistance against murine melioidosis (Haque et al., 2006). However, the importance of CD4+ T cells in the control of infection is open to debate, as there does not seem to be an association between HIV infection and melioidosis (Chierakul et al., 2005b).

Antibodies also play a part in controlling the *B. pseudomallei* infection (Healey et al., 2005). Inoculation of *B. pseudomallei* O-polysaccharide-flagellin conjugated proteins elicited an IgG antibody response (Brett & Woods, 1996). The antibody response was able to increase the survival rate in rats as well as convert a T-independent response to a T-dependent response, which may be the reason for its success. The use of lipopolysaccharide as antigens for subunit vaccines generated an IgG3 antibody response (Nelson et al., 2004), while study in BALB/c mice immunized with plasmid DNA encoding flagellin exhibit the IgG production; dominated in IgG2a over IgG1 in the sera (Chen et al., 2006), which is protective in mouse models. Antibodies to the GroEL chaperonin protein was also found in patients with melioidosis (Woo et al., 2001). Collectively, *B. pseudomallei* possesses many immunogenic antigens and the response to *B. pseudomallei* could be seen to be an IgG response. As one of the routes of entry into the body is via the respiratory route, a mucosal IgA antibody response to *B. pseudomallei* will be helpful as IgA is normally found in mucosal secretions. In fact, this was observed in protective immunity in C57BL/6 mice (Liu et al., 2002) and has implications in vaccine development. In BALB/c mice, a mixed humoral and cell mediated immune response was generated by immunising animals with dendritic cells pulsed with heat-killed bacteria followed by killed *B. pseudomallei* in adjuvant (Healey et al., 2005). The survival rates of mice were found to be increased with the use of this combination vaccine. This indicates that both humoral and cell mediated immunity play an important role in the control of the infection as pulsed dendritic cells activate a good cell-mediated response while bacteria in adjuvant enhance antibody response. Humoral immunity probably helps to control infection through opsonisation of the bacteria and triggering the classical complement pathway (Casadevall, 1998). Cell-mediated immunity is useful in clearing the infection by activating infected

macrophages to kill intracellular bacteria. Therefore, both arms of the adaptive immune response are likely to be complementary in controlling the infection. This understanding will be important in the development of vaccines as well as immunotherapy.

4. Animal models of disease susceptibility

Current animal models of infection focus primarily on acute disease. Diabetic rats and Syrian hamsters have been shown to susceptible to *B. pseudomallei* infection, which results in acute disease and rapid death (Brett et al., 1994; DeShazer et al., 1997). In the BALB/c and C57BL/6 inbred mouse models, BALB/c mice are susceptible, whereas C57BL/6 are relatively more resistant via intravenous (Hoppe et al., 1999; Leakey et al., 1998) and intranasal (Liu et al., 2002) routes of infection. BALB/c mice died from septicemia with overwhelming bacterial loads in organs and blood, accompanied by organ inflammation and necrosis a few days after infection. It reflected a failure of the host innate immune response to control the infection (Hoppe et al., 1999; Leakey et al., 1998). Infected BALB/c mice produced higher levels of proinflammatory cytokines, such as TNF-α, IL-1β, and interferon (IFN)–γ, than infected C57BL/6 mice at 1–2 days after infection (Liu et al., 2002; Ulett et al., 2000). Despite the presence of inflammation, the host innate immune response failed to control the infection. Although IFN-γ is necessary for resistance to acute infection (depletion of IL-12 and IFN-γ by the use of neutralizing antibodies results in sepsis and early death of Taylor outbred mice (Santanirand et al., 1999), in susceptible BALB/c mice were found to hyperproduce IFN-γ compared to resistant C57BL/6 mice (Liu et al., 2002), and they died perhaps as a result of septic shock. IFN-γ is also involved in isotype switching of antibodies to IgG2a, as evidenced by higher ratios of IgG2a as compared to IgG1 in C57BL/6 mice, which has lower bacterial loads than BALB/c mouse (Hoppe et al., 1999). IgG2a is indicative of a cell mediated immune response as it promotes opsonisation of antibody coated bacteria. These experiments suggest that a moderate amount of IFN-γ is needed to induce cell mediated immunity in acute melioidosis during the early stages of infection whereas hyper-production of IFN-γ and other pro-inflammatory cytokines leads to sepsis and death. *B.*

pseudomallei can induce NK cells as well as bystander CD8 T cells to rapidly make IFN-γ, which likely contributes to the early increase in IFN-γ level (Lertmemongkolchai et al., 2001). The initial dependence on IFN-γ is important, because IFN-γ has been shown to prime macrophages to increase their bactericidal activity to *B. pseudomallei* (Utaisincharoen et al., 2001). The infection in BALB/c mice was similar to that which occurs in acute human infection, susceptible BALB/c mice demonstrate the resistance mechanisms or controls are inadequate, resulting in bacterial growth and inflammation. Same in patients with melioidosis, whom markedly elevated plasma IFNγ levels correlate with disease severity (Lauw et al., 1999). In contrast the studied infection in C57BL/6 mice as a model of chronic human disease. All of the mice died of disease 2–6 weeks after infection, compared with 96 h after infection for BALB/c mice, at an infective dose of 37 cfu. It is doubtful whether this represents true chronicity, because the animals progressed to disease and death, althought with delayed kinetics (Leakey et al., 1998). In other reports, C57BL/6 mice seemed to be able to clear infection (Hoppe et al., 1999; Liu et al., 2002), which more resembled an asymptomatic infection with seroconversion. In C57BL/6 mice that cleared infection, antibodies specific to the pathogen could be detected (Hoppe et al., 1999; Liu et al., 2002), including IgG2a (Hoppe et al., 1999). In some C57BL/6 mice, infection was not completely cleared, but the animals were able to effectively establish a latent infection until the control was disrupted. It would be interesting to examine these mice in detail to see if disease could be reactivated through various forms of stress and to determine any residual reservoirs of hidden bacteria.

Another interestingly animal model is the diabetic mouse, because the strongest risk factor associated with severe melioidosis is diabetes. It is known that patients with type 2 diabetes have higher infection-related mortality rates than do the general population(Shah & Hux, 2003). One of the common explanations for the association between melioidosis and diabetes is that the innate immunity of diabetic patients is suppressed, particularly neutrophil function (Cheng & Currie, 2005). This is certainly possible, because high alcohol consumption and chronic renal failure, both risk factors for melioidosis, also present with similar defects. As reviewed by Geerlings et al. (Geerlings & Hoepelman, 1999), most studies with peripheral blood

polymorphonuclear cells of diabetic patients have shown a somewhat decreased chemotactic and phagocytic function, with lower cytokine production on stimulation. However, other studies have not found these differences, and in one diabetic patients had elevated baseline levels of TNF-α, IL-6, and IL-8 (Geerlings & Hoepelman, 1999). A recent publication indicated that mice rendered diabetic with streptozotoxin (type 1 diabetes) and diabetic *db/db* (type 2) mice exhibited greater inflammation, characterized by prolonged TNF-α expression, when infected with *P. gingivalis* than did controls (Graves et al., 2005). In the *db/db* mice, intraperitoneal injection of lipopolysaccharide triggered greater sickness behavior mediated by IL- 1β (O'Connor et al., 2005). Macrophages from these *db/db* mice secreted more IL-1β than did macrophages from heterozygous controls when stimulated with lipopolysaccharide, and peritoneal fluid from these mice showed reduced levels of IL-1 receptor antagonist (O'Connor et al., 2005).

5. Clinical features and management of melioidosis

5.1 Host risk factors and disease acquisition

Melioidosis is found only in individuals who have been exposed to environments containing *B. pseudomallei*; infection is acquired through cutaneous inoculation, inhalation and aspiration. The factors associated with disease acquisition in endemic regions include environmental and host factors. There is no evidence that some isolates of *B. pseudomallei* are intrinsically more infectious than others.

5.1.1 Environmental exposure

The organism can be readily isolated from environmental niches such as rice paddies, still or stagnant waters and moist soil, which predominate in the tropics and it is believed that these habitats are the primary reservoir from which susceptible hosts acquire infection (Dance, 2000b). There is a positive association between disease incidence and the extent of environmental contamination with *B. pseudomallei* (Parry et al., 1999; Vuddhakul et al., 1999; Wuthiekanun et al., 2005). However, few environmental-sampling studies have been published, and a more complete picture of the geographical distribution of *B. pseudomallei* can be derived from the reported cases of melioidosis. This topic has recently been reviewed by Cheng and Currie

(Cheng & Currie, 2005). The disease is endemic in parts of Thailand, Northern Australia, Malaysia, Singapore, Vietnam and Burma. Possible endemic areas include Southern India, Southern China, Hong Kong, Taiwan, Brunei, Laos and Cambodia. Sporadic cases and occasional clusters have been reported in large areas of Asia, the Americas (notably Brazil), the Caribbean, the Pacific, Africa and the Middle East. Thailand reports the largest number of cases of melioidosis in the world annually, with an incidence rate of 3.6-5.5/100,000 population (Suputtamongkol et al., 1994). Melioidosis accounts for 19% of hospitalizations and 40% of death from community-acquired septicemia (Chaowagul et al., 1989; Leelarasamee & Bovornkitti, 1989). Mild or subclinical infections are common with 80% of Thai children seropositive by age 4 years (Dance, 2000b). Male:female ratio is 4:1 with age range of 16 months-91 years (mean: 48 years old) (Currie et al., 2000c). There is no genetic predisposition. It is simply an occupational factor.

5.1.2 Weather condition, route of acquisition and inoculum.

Melioidosis is primarily acquired via the inoculation of compromised surface tissues by soil and water contaminated with *B. pseudomallei* (Dance, 2000a) with the highest incidence of disease occurring during rainy seasons (Currie et al., 2000c; Merianos et al., 1993; Suputtamongkol et al., 1994). There is a close association between rainfall and melioidosis. In northeast Thailand (Currie et al., 2000b; Suputtamongkol et al., 1994) and northern Australia (Currie et al., 2000b), 75 and 85% of cases, respectively, occur in the wet season. This explain the prevalence of disease among thai rice farmers and their families who labor in the rice paddies without the benefit of protective clothing (Chaowagul et al., 1989). Aerosols are created during heavy rain, and this can result in repeated inhalation of the organism. Heavy rainfall and winds consistently cause a shift towards more pneumonia in patients presenting with melioidosis in Northern Australia (Currie & Jacups, 2003). Severe or penetrating injury and near-drowning are known risk factors for melioidosis, as highlighted by a study of a cluster of melioidosis cases in Southern Thailand following the 2004 tsunami (Chierakul et al., 2005a).

5.1.3 Host immunity

The important risk factor of the disease is the occupational exposure to contaminated soil and water, exemplified by rice farmers working in paddy fields. In

northeast Thailand, 80% of the population belongs to rice-farming families. The risk is approximately 10-fold higher compared to indoor workers (Dance, 2000a). Next is diabetes mellitus. In a study done recently in Thailand, they found out that among all the risk factors, diabetes mellitus is the only significant factor associated with septicemic melioidosis and there is an interaction between diabetes mellitus and the occupational factor thereby increasing the risk of infection among diabetic farmers to 6 to 9-fold (Currie et al., 2000c). Other health related factors which appear to increase the probability of acquiring melioidosis include impaired cellular immunity, leukemia/lymphoma, renal disorder and debilitating afflictions such as alcoholism and parenteral drug abuse, history of previous trauma or surgery, chronic alcoholic liver disease, certain types of hemoglobinopathies such as E-beta thalassemia (Dance, 2000b). Most affected adults (>80%) have one or more underlying diseases, by contrast, children have an identifiable risk factor in <30% of cases (most commonly trauma). It is unclear whether affected children have a greater genetic susceptibility for disease. It is also possible that disease in childhood is caused by a subset of the bacterial population with increased pathogenic potential. HIV/AIDS, although there is little evidence that it actually renders people more susceptible to melioidosis than the general population, is also considered a risk factor.

Seroprevalence studies in northeast Thailand based on the indirect haemagglutination assay show that ~80% of people have antibodies against *B. pseudomallei* by the age of 4 years (Kanaphun et al., 1993). It is not clear whether healthy individuals with high antibody titres are infected and have a quiescent focus (analogous to a quiescent tuberculosis infection), or whether repeated environmental exposure in a primed individual maintains high antibody levels.

5.2 Clinical manifestation of melioidosis

B. pseudomallei has been called the "the Great mimicker" because the infection can present with a broad spectrum of clinical signs and symptoms and may be latent for months or years before the disease becomes clinical manifestation (Fig. 4). It particularly mimics tuberculosis not only because of its latency but also because of its ability to survive in phagocytes, production of granulomatous lesions, and nodular lesions visible on x-ray. Furthermore, recurrence of infection is common despite adequate antimicrobial therapy (White, 2003). *B. pseudomallei* is intrinsically

resistant to many antibiotics (including penicillin, first and second-generation cephalosporins, macrolides, rifamycins, colistin and aminoglycosides), but is usually susceptible to amoxicillin-clavulanate, chloramphenicol, doxycycline, trimethoprim-sulphamethoxazole, ureidopenicillins, ceftazidime and carbapenems (Cheng & Currie, 2005; White, 2003). Treatment is required for 20 weeks and is divided into intravenous and oral phases (Cheng & Currie, 2005; White, 2003). Initial intravenous therapy is given for 10–14 days; ceftazidime or a carbapenem are the drugs of choice. The overall mortality for primary disease is 50% in northeast Thailand (35% in children) and ~20% overall in Northern Australia (Cheng et al., 2003; White, 2003). Interestingly in the pathogenesis of *B. pseudomallei* has increased following their classification as category B agents by the US Centers for Disease Control and Prevention.

Human melioidosis divides into three categories based on clinical symptoms; acute, subacute and chronic (Howe et al., 1971). More recently, the Infectious Disease Association of Thailand reported a study of 345 patients with melioidosis in which 45% had disseminated septicaemia (87% mortality), 12% had non-disseminated septicaemia (17% mortality), 42% had localised infection (9% mortality) and 0.3% had transient bacteraemia (no mortality) (Currie et al., 2000c).

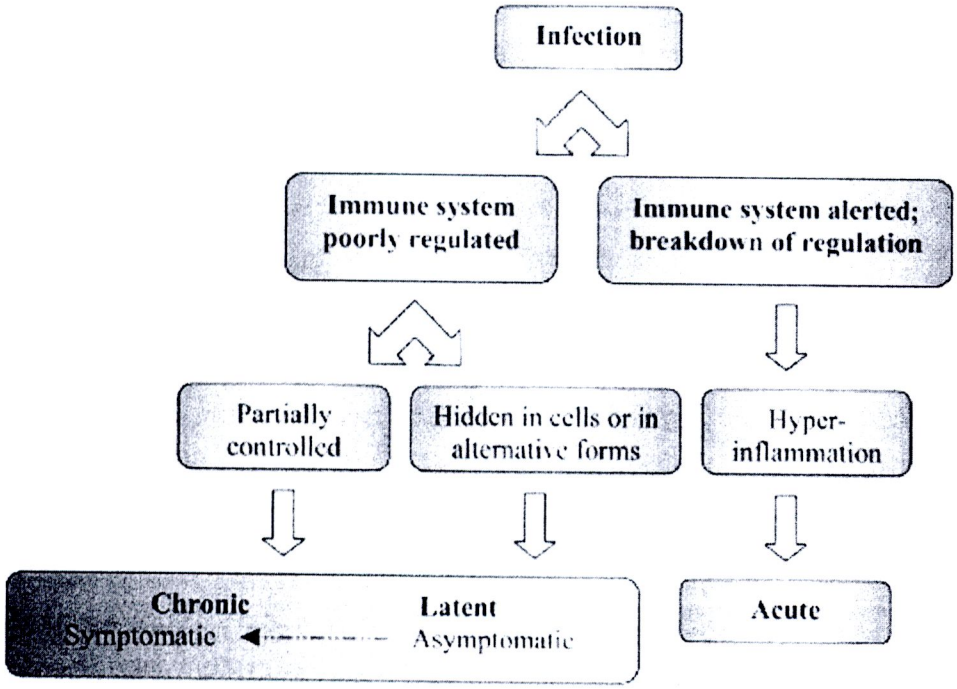

Figure 4 Various disease outcomes in *Burkholderia pseudomallei* infection. The chronic and latent states may overlap in a gradient of increasing host-pathogen interactive activity from latency to chronicity. This may be reflected in the symptoms manifested, with increasing symptoms from latency to chronicity (Taken directly form Interaction between Burkholderia pseudomallei and the Host Immune Response: Sleeping with the Enemy?) (Gan, 2005).

5.2.1 Subclinical infection /Asymptomatic infection

Most persons exposed to *B. pseudomallei* in the environment do not become ill (Ashdown & Guard, 1984; Kanaphun et al., 1993). These may eventually develop clinical disease, typically when they become immuno-suppressed from another condition. Using the indirect haemagglutination method, seroepidemiological surveys around Ubon Ratchatani, northeastern Thailand, confirm widespread seropositivity among rice farmers (Suputtamongkol et al., 1994). In endemic areas, seroconversion starts as soon as children are exposed to wet soil, and occurs at a rate

of about 25% annually between the ages of six months and four years (Kanaphun et al., 1993). Most clinical infections are therefore not primary infections with *B. pseudomallei.*

It is unknown how many of the large asymptomatic seropositive. Latent infection able to reactivate, but reactivation in the endemic areas appears to be uncommon (Currie et al., 2000a). Recent report in thailand to fond that 75% of recurrent disease were case by the same strain (relapse) and 25% were dueto infection with new strain (reinfection) (Maharjan et al., 2005).

5.2.2 Acute infection

Acute infection is the most severe, with rapid on set (24-48 h) of non-distinct symptom, and a reported 70% mortality rate within 48 h. of medical examination (Leakey et al., 1998). Melioidosis predominantly occurs in the monsoonal wet seasons of the various endemic regions; 76% of cases in northeastern Thailand occurred in the period from June to November and 85% of cases in the Northern Territory in the months of November through to April (Currie et al., 2000a).

A study in Darwin of melioidosis over 10 years to late 1999 categorised presentations as acute (symptoms of less than two months at presentation) or chronic (illness duration of greater than two months). In 252 cases of culture-confirmed melioidosis, 222 (88%) presented with acute disease, while 30 (12%) had chronic disease (Currie et al., 2000a). Two hundred and forty-four cases (97%) were considered to be from recent acquisition of *B. pseudomallei* infection, while only 8 (3%) were considered to be reactivation from a latent focus. Incubation ranged from 1 to 21 days, with a mean of 9 days, in the 244 reported cases of recent acquisition.

Pneumonia is the most common clinical presentation of melioidosis in all studies throughout all endemic areas. The symptoms usually include fever, coughing, pleuritic chest pain and, in some cases, hemoptysis. Ulcerative lesions and nodules are sometimes found in the nose, and the septum may perforate. Severe weight loss may be seen. Pulmonary signs can develop suddenly, or may occur gradually after a prodromal syndrome characterized by headache, anorexia and generalized myalgia. Complications include pneumothorax, empyema and pericarditis. Untreated cases

often progress to septicemia. Currie *et al* have observed that patients with septicaemic melioidosis pneumonia are often more systemically ill than the radiographic appearances of the lungs would suggest, indicating a spread to, rather than from, the lung (Currie et al., 2000c).

Acute septicaemic melioidosis is the most severe disease manifestation and occurs most often against a background of diabetes, renal disease, alcoholism, leukaemia and lymphoma, corticosteroid therapy or other immunosuppressive conditions. The picture is that of septic shock, with a brief incubation period and multiorgan involvement with abscess formation. The distributive shock of sepsis is characterised by a high cardiac output, a low systemic vascular resistance and low filling pressures. It is frequently complicated by the development of irreversible organ damage and the multiple organ dysfunction syndrome. A primary focus may be demonstrated in about 50% of patients, most commonly in the lung, and, less frequently, in the skin or soft tissue wounds. In spite of antibiotics, vasopressors and intravenous fluid, the mortality of melioidosis septic shock is reported to vary from 84% to 100%(Currie et al., 2000b). Since the impairment of neutrophil function may be pivotal to the spread of *B. pseudomallei*, recent preliminary work has suggested that the empirical addition of granulocyte colony stimulating factor in the management of melioidosis septic shock may be of some benefit by promoting neutrophil numbers (Currie et al., 2000b).

Suppurative parotitis is a form of acute primary disease seen almost exclusively in children and reported in up to 40% of cases of Thai childhood melioidosis but is uncommon in Australia; conversely, prostatic abscesses and brainstem encephalitis are more frequent in Australia. Recently, a syndrome of meningoencephalitis with varying involvement of brainstem, cerebellum and spinal cord has been identified (Wells et al., 1996). There is no evidence of a specific strain of *B. pseudomallei* responsible for neurological melioidosis, but further studies are required to ascertain whether the apparently higher rate of neurological disease in Australia is due to a true regional difference or results from an increased clinical awareness.

5.2.3 Subacute infection

Subacute melioidosis is much less severe, with infrequent fatalities symptoms are similar to the acute form of disease. Subacute infection is characterised pathologically by caseation necrosis and a predominantly mononuclear and plasma-cell infiltrate. This subacute suppurative form is seen most frequently within the lungs as either abscess or empyema. Like the lung, the liver may demonstrate solitary or multiple abscess formation. Abscesses within liver or spleen have a "Swiss cheese" appearance on ultrasound.

5.2.4 Chronic infection

Chronic melioidosis is believed to be the most common form of disease, this form of infection remains the least understood. Described as a 'medical time bomb' because of the ability of asymptomatic, chronic infection to flare up suddenly into a rapidly-fatal form, chronic melioidosis may persist undiagnosed for decades (Mays & Ricketts, 1975). In the subacute and chronic pulmonary form, a well-recognised presentation is an upper-lobe infiltrate, with or without cavitation, closely simulating tuberculosis.

5.3 Relapse or reactivated infection

Latent disease, quiescent over many years after primary exposure or the resolution of a limited primary infection, may reactivate in 3% of all cases, usually in association with an intercurrent illness, typically pulmonary disease, surgery or trauma. Late-onset diabetes, renal failure and immunosuppressant drugs may also contribute to reactivation. Clinical presentation in relapse or reactivated is as severe as in initial infection with mortality of about 30% (Chaowagul et al., 1993) and associated with poor compliance and prior documented infection rather than the presence of underlying disease. The ideal antimicrobial agents of melioidosis treatment should have bactericidal effect, should be able to penetrate phagocytic cells and eliminate or inhibit the production of glycocalyx.

Appropriate treatment is required to prevent relapse and failure of treatment. Despite appropriate treatment, melioidosis has a higher relapse rate. The average time between discharge from hospital and relapse is of 21 weeks. Treated patients require long-term follow up, as *B. pseudomallei* remains latent for up to 26 years in the body

(Mays & Ricketts, 1975). For maintenance therapy, Co-Amoxyclav is a safe and well-tolerated antimicrobial agent (there is some concern that it may be less effective than the conventional regimen of chloramphenicol, co-trimoxazole and doxycycline). The recommended duration for maintenance therapy is of 12 to 20 weeks (Chetchotisakd et al., 2001; Rajchanuvong et al., 1995). It has been shown that *B. pseudomallei* stays intracellularly in the body where it produces biofilms and microcolonies and is sheltered from β-Lactam antimicrobial drugs (β-Lactam drugs are unable to enter intracellular sites to kill latent *B. pseudomallei*) (Pruksachartvuthi et al., 1990). It has been suggested that a combination of ciprofloxacin and macrolides is a good alternative regimen since ciprofloxacin penetrates phagocytic cells and achieves intracellular concentrations of several times higher than extracellular concentration and kills *B. pseudomallei* while macrolides could delay or prevent production of glycocalyx (Chateau & Caravano, 1993; Vorachit et al., 2000).

5.4 Diagnosis and management of melioidosis

5.4.1 Diagnosis

Isolation of *B. pseudomallei* from patient's specimens remains to be the "gold standard" in diagnosis and requires the use of selective media for nonsterile specimens. Gram's stain and other histopathological stains are not specific for the organism. Serological tests are helpful in making a provisional diagnosis in the absence of isolation of *B. pseudomallei* in the specimen. Culture and serological methods are cost-effective and simple to perform but require experience personnal to interpret results. A number of techniques have been employed to attempt to reduce the time required to achieve a diagnosis, including antigen detection on specimens or on culture supernatant, antibody detection, molecular techniques, and rapid culture techniques. Although many of these rapid tests have been developed, only a few have been extensively tested in the field, and only IHA, latex agglutination, and immunofluorescence are currently used clinically.

5.4.1.1 Culture-based methods.

In general, the isolation of *B. pseudomallei* is using standard culture media such as blood agar, McConkey agar and routine blood culture broth. Selective media, Ashdown's agar and broth, are required for nonsterile and

environmental samples as they contained the broad antibiotic resistance of the organism (Dance et al., 1989; Farkas-Himsley, 1968; Galimand & Dodin, 1982). On blood agar, colonies developed slowly and have a characteristic of dairy-head appearance (Inglis et al., 1998b). On Ashdown's agar, there are at lest seven types of colony were developed (Fig. 5). The changes in their phenotype are associated with altered interactions with the host (Chantratita et al., 2007). The time needs to develop a blood culture positive reflecting the density of bacteremia and correlated with the mortality (73.7%) of patients. The patients died if blood cultures became positive within 24 h, compared to 40.9% of those with a time of >24 h. In that study, using the automatic BacT/Alert system, 62% of positive cultures were detected in the first 24 h and more than 90% were detected within 48 h (Tiangpitayakorn et al., 1997). Alternative blood culture methods could decrease the time to obtain a positive culture. There are conflicting opinions as to the reliability of the API 20NE test panel; two studies have reported good results with this manual system (Dance et al., 1989), as with the API 20E system (Lowe et al., 2002). However, another study found that 6 of 50 *B. pseudomallei* strains at a Western Australian laboratory were misidentified, most commonly as *Chromobacterium violaceum*, and a further 4 strains gave indeterminate results (Inglis et al., 1998a). The Vitek automated system is widely used; the Vitek 1 system, but not the Vitek 2 system, appeared to identify *B. pseudomallei* reliably (Lowe et al., 2002). These findings have significant implications for laboratories in areas where the organism is not endemic and is only occasionally encountered. Colony morphology on Ashdown's medium and where available, latex agglutination (Anuntagool et al., 2000b) and immunofluoresence (Naigowit et al., 1993; Walsh et al., 1994) are practical ways to identify *B. pseudomallei* in areas of endemicity (Dance, 1991).

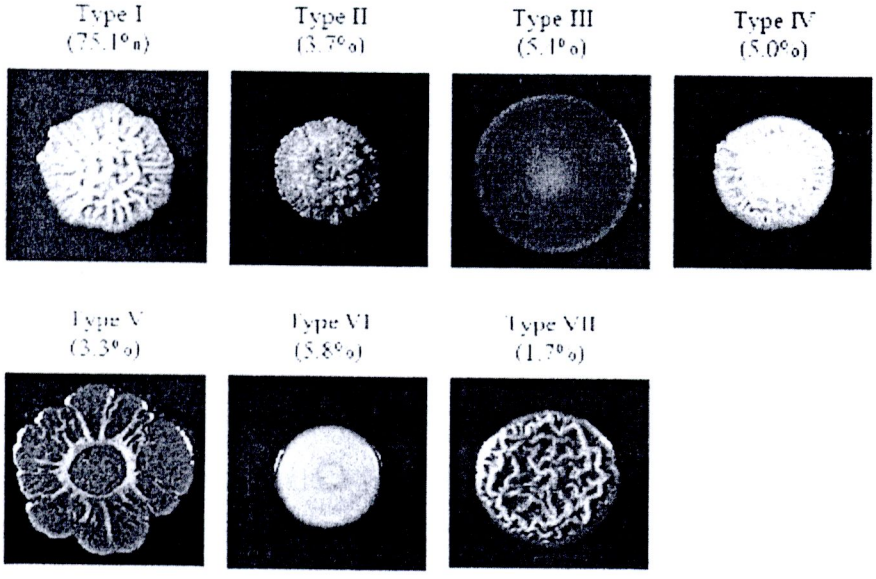

Figure 5 Seven unique *B. pseudomallei* colony morphotypes on Ashdown's agar. Colony morphology was observed for 212 samples of *B. pseudomallei* identified from 204 consecutive unselected patients presenting to a hospital in the northeast Thailand with melioidosis. Seven major morphotypes were defined after 4 days at 37°C in air. Percentages represent the proportion of each type within this population. Types II and V are distinguished by size, since type V does not always have a central crater. Types III and VI are distinguished from each other by size, since a difference in color between the two is not consistent (Taken directly form Biological Relevance of Colony Morphology and Phenotypic Switching by *Burkholderia pseudomallei* (Chantratita et al., 2007).

5.4.1.2 Antigen detections.

A variety of antigen detection methods have been studied. Antigen tests have been developed to be used on direct specimens or in blood culture supernatant; of these, latex agglutination for culture identification and direct immunofluoresence from direct specimens (such as sputum, urine, or pus) have been used in research labs in Thailand. Antigen tests for exotoxin and cell components have shown reasonable sensitivity and specificity in studies but most of them have not yet

been used in the field tested. Enzyme-linked immunosorbent assays (ELISAs), for exotoxin in culture supernatant (Ismail et al., 1987) and a 40-kDa secreted protein (Wongratanacheewin et al., 1990; Wongratanacheewin et al., 1993), and monoclonal antibodies for cell wall components, including LPS (Dharakul et al., 1999), a 30-kDa protein (Pongsunk et al., 1999), and an exopolysaccharide (Steinmetz et al., 1999). A fluorescent urinary antigen system has been developed. In initial trials, a sensitivity of 81% and a specificity of 96% were defined (Desakorn et al., 1994) but a subsequent evaluation gave poorer results (Sirisinha et al., 2000). The only test finding widespread uses in Thailand currently is a monoclonal antibody latex agglutination test against the 200-kDa protein that was evaluated in 12 centers in Thailand. It was shown to agglutinate blood culture fluid positive for *B. pseudomallei*, including strains with atypical LPS patterns, with a sensitivity of 95%. The test was also highly specific and did not agglutinate *ara+ B. thailandensis* strains, with a specificity of 99.7% (Anuntagool et al., 2000b).

Immunofluoresence from direct specimen (including sputum, urine, and pus) is the most promising way to reduce the time to diagnosis in areas of endemicity. A result can be obtained within an hour, but it requires a fluorescent microscope that is not always available in some laboratories in endemic areas.

5.4.1.3 Antibody detections.

Indirect haemagglutination test (IHA) is simple to perform as it detects the antibody against *B. pseudomallei* that appears in the blood within 1-2 weeks after the infection and reach maximal titer in 4 to 5 months (Appassakij et al., 1990). An older method is the complement fixation test (Nigg & Johnston, 1961). The use of the IHA is problematic in areas of endemicity, particularly in Thailand, where rates of background seropositivity may be up to 30 to 47% in various populations (Khupulsup & Petchclai, 1986), presumably due to subclinical exposure to *B. thailandensis* or *B. pseudomallei* early in life (Anuntagool et al., 1998; Kanaphun et al., 1993). The detection of IgM antibodies should be more specific (Ashdown, 1981; Khupulsup & Petchclai, 1986). Studies of the clinical performance of IHA are difficult to compare, as different thresholds are used for interpretation (between 1:10 and 1:160 in various studies) and the strains used to formulate the whole-cell antigen are not standardized. However, it is evident that the sensitivity of

IHA is limited in patients with acute septic illnesses (Appassakij et al., 1990). Furthermore, there is some heterogeneity between strains in LPS, a major component in the crude antigen used in the IHA; antibodies against atypical LPS may not cross-react against the IHA antigens, depending on which organisms are used to prepare the IHA assay reagent (Anuntagool et al., 2000a).

Enzyme linked immunosorbent assay (ELISA) detects specific IgG and IgM antibodies of *B. pseudomallei* in serum specimens. ELISA is more convincing in terms of sensitivity and specificity for antibody detection as it points to an active disease process (Ashdown et al., 1989). ELISAs based on LPS and 30 and 200-kDa proteins have been validated in a clinical context. IgG but not IgM appears to be more sensitive (74 to 82%) and specific (75 to 80%) than the IHA but still lack the performance necessary for clinical use (Sirisinha et al., 2000).

A rapid immunochromogenic test (PanBio Ltd., Brisbane, Queensland, 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 for comparison against IHA, rather than culture, as a gold standard (Cuzzubbo et al., 2000).

5.4.1.4 Molecular methods.

Many tests based on molecular detection of *B. pseudomallei* have been described. Molecular biology techniques such as polymerase chain reaction (PCR), dot immunoassay, pulsed field gel electrophoresis (PFGE), restricted fragmentation length polymorphism (RFLP) and random amplification of particle of deoxyribonulease (RAPD) are used for diagnosis. Molecular techniques are recommended for the rapid diagnosis of the disease and for monitoring therapy and epidemiological studies because of their high sensitivity, specificity and speed. In recent time sensitive PCR amplification techniques for detecting the DNA of *B. pseudomallei* in clinical specimens of acute melioidosis patients are useful (Kunakorn et al., 2000; Rattanathongkom et al., 1997).

Current primers targeting regions in the 23S rRNA, the 16S RNA, the junction between 16S and 23S RNA, specific sequences designed from a specific DNA probe and metalloprotease have been evaluated (Brook et al., 1997; Dharakul et al., 1996; Kunakorn & Markham, 1995; Kunakorn et al., 2000; Neubauer

et al., 2007; Rattanathongkom et al., 1997; Sirisinha et al., 2000). The use of primers to detect a region of the 16S RNA demonstrated a sensitivity of 100% but a low specificity in a small clinical study (Haase et al., 1998). Using primers from a specific DNA probe, could be amplified and detected as few as a single bacterium present in the clinical specimen (Rattanathongkom et al., 1997). More recent studies are examining the role of a PCR for the type III secretion system (Smith-Vaughan et al., 2003) in clinical specimens, as well as other 16S mRNA-specific primers (Gee et al., 2003). 16S mRNA sequencing has long been used for the identification of bacterial species; this method has been used for phylogeny (Brett et al., 1997, , 1998), as well as clinical identification (Visca et al., 2001) of *Burkholderia* spp. Sequencing of the *groEL* gene may also be useful but may not reliably differentiate *B. mallei* from *B. pseudomallei* (Woo et al., 2003; Woo et al., 2002). The *narK* and *gltB* PCR primers were chosen for PCR to identify and distinguish unambiguously *B.mallei* from *B. pseudomallei* and from *B. thailandensis / B. oklahomensis* (Wattiau et al., 2007). The PCR assay targeting the metalloprotease gene (mprA) of *B. pseudomallei* demonstrated the specific detection, all other closely related organisms including *B. mallei* and *B. thailandensis* tested negative (Neubauer et al., 2007).