IMMUNE RESPONSES IN *BURKHOLDERIA PSEUDOMALLEI* INFECTION, RELAPSE, REACTIVATION AND PROTECTION

Thesis submitted by
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DECLARATION

I declare that this thesis is my own work and has not been submitted in any other form for another degree of diploma at any university or institution of tertiary education. Reagents obtained from other organisations and researchers have been acknowledged where appropriate. Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references is given.

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DECLARATION ON ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Statement on Ethics Conduct in Research Involving Human (1999)*, the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice (1997)*, the *James Cook University Policy on Experimentation Ethics*. *Standard Practices and Guidelines (2001)*, and the *James Cook University Statement and Guidelines on Reseach Practice (2001)*. The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (approval numbers A709, A987 and H1659).

Sharon Marie Lazzaroni
January 2009

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PUBLICATIONS

Publications resulting from this Thesis:

1. McAllister J, Gauci P, Lazzaroni S, Barnes J, Ketheesan N and Proll D. 2007. Asssessment of a DNA vaccine encoding *Burkholderia pseudomallei* bacterioferritin (technical report). *Defence Science and Technology Organisation*.

2. McAllister J, Lazzaroni S, Barnes J, Ketheesan N and Proll D. 2007. Development of DNA vaccines against *Burkholderia pseudomallei* (technical report). *Defence Science and Technology Organisation*.

3. Lazzaroni S M, Barnes J L, Williams N L, Govan B L, Norton R E, LaBrooy J T and Ketheesan N. 2008. Seropositivity to *Burkholderia pseudomallei* does not reflect the development of cell-mediated immunity. *Transactions of the Royal Society of Tropical Medicine and Hygiene (in press)*.

Presentations:

1. Lazzaroni S, Inglis T, Currie B, Ketheesan N, Norton R. 2004. The characterisation of *Burkholderia pseudomallei* isolates from the Townsville region using pulsed-field gel electrophoresis. 4th World Melioidosis Congress (poster presentation).

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3. Lazzaroni S, Barnes J, Govan B, Norton R, Ketheesan N. 2006. Immune responses in melioidosis. Australian Institute of Medical Scientists 21st Annual Conference (oral presentation).

4. McAllister J, Lazzaroni S, Barnes J, Shahin S, Gauci P, Govan B, Ketheesan N, Proll D. 2006. Assessment of DNA vaccines against *Burkholderia pseudomallei*. Australian Society of Microbiology (poster presentation).

ABSTRACT

Burkholderia pseudomallei is the bacterium that causes the potentially fatal disease melioidosis. Endemic regions include South-east Asia and northern Australia. The immunopathogenesis of melioidosis is not fully understood as the bacterium can evade the host immune responses surviving within cells becoming apparent when the host is immunocompromised. Since *B. pseudomallei* is an intracellular organism, cell-mediated immune (CMI) responses are important in clearance and protection. The processes involved in the induction of a protective immune response is necessary to understand how the disease would progress and for developing vaccine strategies against *B. pseudomallei*. Therefore, the focus of the research in this thesis is to determine the extent of exposure to *B. pseudomallei* in northern Queensland and to characterise the CMI responses of seropositive healthy individuals from this endemic region. The immune responses involved in relapse and latent *B. pseudomallei* infection will also be determined by analysing the clonality of isolates from patients with recurrent melioidosis and developing a murine model of latency. The protective effect of DNA constructs of putative virulence factors will be studied in an experimental model of melioidosis.

Using the indirect haemagglutination assay, 1500 blood donors from northern Queensland were assessed for antibodies to *B. pseudomallei*. The results demonstrated 2.47% (n=37) of the population had an antibody response to *B. pseudomallei* with a titre of ≥1:40. Using these seropositive donors, the Melioidosis IgG Rapid Cassette test was evaluated as a potential screening test for melioidosis. The results found only 32% were positive using this rapid test. This study demonstrated that individuals living in endemic regions may have been exposed to *B. pseudomallei* and consequently produced an antibody response. By characterising the CMI response of these seropositive individuals it will be possible to determine whether these individuals develop protective immunity against the bacterium.

Lymphocyte proliferation assays and flow cytometry were used to characterise the CMI response in seropositive individuals (n=8). Lymphocytes were isolated and stimulated

with *B. pseudomallei* antigen (BpLy1) the proliferative response was assessed, as well as activation of T cell subsets and cytokine production. Results demonstrated no significant differences in the proliferative response of lymphocytes in seropositive individuals when compared to seronegative individuals. There were no significant differences observed in CD4+ and CD8+ populations, interferon (IFN)-γ or interleukin (IL)-4 production. These results did not demonstrate an induction of a CMI response following stimulation with BpLy1. Although these seropositive individuals may have produced an antibody response to *B. pseudomallei*, the exposure was not sufficient to produce a CMI response.

Clonality of *B. pseudomallei* isolates from patients who had relapsed with melioidosis was determined using pulsed-field gel electrophoresis (PFGE). Fragment patterns produced following PFGE demonstrated clonal isolates were responsible for each relapse episode in four patients. One patient who had relapsed with melioidosis nine times over a period of eight years was due to the same *B. pseudomallei* isolate recrudescing rather than infection with a different isolate. These results suggest that the bacterium is able to evade the immune responses and cause continual episodes of melioidosis, therefore, treatment of the disease may need to be reassessed.

The development of a latent model of melioidosis involved C57BL/6 mice being infected with a low dose of a low virulence strain of *B. pseudomallei* (NCTC13179). The mice were allowed to clear the infection over a period of 30 days with bacterial load, delayed-type hypersensitivity (DTH) and lymphocyte proliferation responses assessed at days 10, 20 and 30. These results demonstrated that a CMI response had been produced with significant differences seen in the DTH response and proliferative response of lymphocytes following stimulation with BpLy1. There was also an apparent clearance of *B. pseudomallei*, with negative culture from spleen and liver of infected animals. Mice were then immunosuppressesed with dexamethasone and monitored for survival, DTH and lymphocyte proliferation responses and bacterial load. Following immunosuppression, there was a significantly reduced immune response demonstrated by the DTH response. The proliferative response of lymphocytes in infected, immunosuppressed mice was not reflective of observations seen *in vivo* with no

differences observed in proliferation of lymphocytes, following stimulation with BpLy1, in infected, immunosuppressed mice compared to infected, non-immunosuppressed mice. Interestingly, results from bacterial loads demonstrated that by bacterial culture, *B. pseudomallei* was not present, however using real-time PCR (qPCR) *B. pseudomallei* DNA was detected in the spleens of both groups of mice. There was apparent reactivation of *B. pseudomallei* infection with a significant decrease in survival of infected mice that had been immunosuppressed and infected mice that had not been immunosuppressed. This aspect of the investigation demonstrates the initial development of an animal model of latent melioidosis.

Potential DNA vaccine constructs were evaluated using a BALB/c mouse model of experimental *B. pseudomallei* infection to determine their protective potential. The DNA vaccine constructs were developed against selective putative virulence factors of *B. pseudomallei*. Mice were vaccinated at three time points using a gene gun and subsequently challenged with a low virulence strain of *B. pseudomallei* (NCTC13179). Mice were monitored for survival and bacterial load. There were no significant differences seen in survival of mice vaccinated with different DNA constructs. When a combination of DNA constructs or CpG, an immunostimulator, was added to the vaccination regime, no significant differences were seen in survival of mice in these groups when compared to control mice. Splenic bacterial loads were not reduced in vaccinated groups when compared to control mice.

The results of this present study have provided further knowledge into host-pathogen interactions of *B. pseudomallei*. There is evidence that although there is exposure to *B. pseudomallei* in northern Queensland, detected by assessing antibody production, this exposure does not produce a CMI response which is essential in protection against the bacterium. Initial studies in determining whether *B. pseudomallei* persists within the host has been modelled in a murine model of latency. To produce a successful vaccine to protect against *B. pseudomallei* infection, further understanding of the immune responses and interaction of the bacterium is required.

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LIST OF ABBREVIATIONS

APC – antigen presenting cell
BpLy1 – *B. pseudomallei* lysate (NCTC13179)
BSA – bovine serum albumin
CDC – Centre of Disease Control and Prevention
CFA – culture filtrate antigen
cfu – colony forming units
CMI – cell-mediated immunity
CO_2 – carbon dioxide
Con A – concavalin A
Da – dalton
DC – dendritic cell
DNA – deoxyribonucleic acid
EDTA – ethylene diamine tetraacetic acid
ELISA – enzyme linked immunosorbent assays
FACS – fluorescent-activated cell sorting
FBS – foetal bovine serum
ICT – immunochromatographic test
ID – intradermal
IFA – immunofluorescent antibody assays
IFN – interferon
Ig – immunoglobulin
IHA – indirect haemagglutination assay
IL - interleukin
ip – intraperitoneal
in – intranasal
iNOS – inducible nitric oxide synthase
iv – intravenous
kb – kilobases
kDa – kilodalton
LPS – lipopolysaccharide
MAb – monoclonal antibodies
MEE – multilocus enqyme electrophoresis
MLST – multilocus sequence testing
MNL – mononuclear leukocytes
mRNA – messenger RNA
NF – nuclear transcription factor
NK – natural killer
NO – nitric oxide
OD – optical density
ODN – oligodeoxynucleotides
PAMP – pathogen-associated molecular patterns
PBS – phosphate buffered saline
PC – physical containment
PCR – polymerase chain reaction
PFGE – pulsed field gel electrophoresis
PHA – phytohaemagglutinin
PMA – phorbol myristate acetate
PMED – particle-mediated epidermal delivery
PMNL – polymorphonuclear leukocytes
PPD – purified protein derivative
PS - polysaccharide
qPCR – real-time PCR
RAPD – random amplified polymorphic DNA
RNA – ribonucleic acid
rRNA – ribosomal RNA
sc – subcutaneous
SI – stimulation index
TE – Tris-EDTA
TBE – Tris-Boric-EDTA
TES – Tris-EDTA sarcosine
Th – T helper
TLRs – toll-like receptors
TNF – tumour necrosis factor
TTSS – type III secretion system

CHAPTER 1

INTRODUCTION

Burkholderia pseudomallei is a Gram negative bacilli and the causative agent of melioidosis. Endemic to South-East Asia and northern Australia, *B. pseudomallei* is an environmental saprophyte (White, 2003). The route of infection occurs via inhalation, ingestion or direct inoculation through wounds and skin abrasions in contact with contaminated soil or surface water (Reckseidler *et al*., 2001; Ulett *et al*., 2001; Kharakul *et al*., 1999). Increased incidence of melioidosis coincides with heavy rainfall Predisposing conditions that are considered to be risk factors for the disease include diabetes mellitus, renal dysfunction and alcoholism (Ulett *et al*., 2001). Disease presentation ranges from septicaemia that can be fatal, to chronic infection that often involves suppurative abscesses (Reckseidler *et al*., 2001; Sirisinha *et al*., 2000). The fatality rate of melioidosis in the Townsville Hospital in northern Queensland is approximately 25%(Malczewski *et al.*, 2005).

The extent of exposure to *B. pseudomallei* in healthy populations within endemic regions has been widely studied (Ashdown, 1987; Wuthiekanun *et al.*, 2006a; Wuthiekanun *et al.*, 2006b). Epidemiological studies have demonstrated that people living in endemic areas of the world can be exposed to *B. pseudomallei* and produce an antibody response without evidence of clinical melioidosis. In endemic areas of Thailand, the seroprevalence to *B. pseudomallei* within a healthy population of blood donors was found to be between 20-30%, compared to less than 6% in non-endemic regions (Naigowit *et al.*, 1992). A serological study carried out in the early 1980s suggested that 5.7% of the population in northern Queensland had been exposed to *B. pseudomallei* (Ashdown and Guard, 1984) using samples that were collected in a hospital setting. By conducting a serological study involving a healthy population of blood donors from northern Queensland, the true exposure to *B. pseudomallei* in this endemic region can be determined.

As *B. pseudomallei* is a facultative intracellular organism, the cell-mediated immune (CMI) response is important in clearance of the infection. To date, host immune responses to *B. pseudomallei* have not been fully elucidated. Studies on other intracellular organisms such as *Listeria monocytogenes* and *Mycobacterium tuberculosis*, have demonstrated the importance of CD4+ helper T lymphocytes, CD8+ cytotoxic T lymphocytes and the production of IFN-γ in protection against infection (Nagata *et al.*, 2008; Xu *et al.*, 2008). Characterising the CMI response of blood donors who are seropositive to *B. pseudomallei* will further the understanding of the host immune response to this bacterium. This information will also be useful in determining whether seropositive individuals with no previous history of clinical disease produce a strong CMI response to protect against clinical disease.

Based on the high rates of seroprevalence described in endemic regions, it is possible that a proportion of individuals are exposed to *B. pseudomallei* without progressing to clinical disease. In such individuals, *B. pseudomallei* has the potential to remain dormant, only advancing to clinically apparent melioidosis when the immune system is suppressed. The longest period of dormancy reported for *B. pseudomallei* infection is 62 years in a World War II prisoner of war (Nguay *et al.*, 2005). The site where *B. pseudomallei* persists within the host while it is dormant is unclear. However, it has been demonstrated that *B. pseudomallei* can invade phagocytic cells, surviving within these host cells to avoid the immune responses (Jones *et al.*, 1996). In human, it is impossible to determine the site of persistence. There is currently no animal model available to demonstrate latency of *B. pseudomallei* infection. The development of such an animal model would be advantageous in determining the immunopathogenesis of latent *B. pseudomallei* infection and reactivation to the acute form.

Relapse of *B. pseudomallei* infection in patients with melioidosis has been widely described in the literature. Relapse occurs following the apparent resolution of initial infection in a previously sterile site. Patient non-compliance with antibiotic treatment has been suggested as a contributory factor to relapse of melioidosis (Chaowagul *et al.*, 1993). However, immunosuppression of a patient may also be a cause of relapse. This

has major implications for clinicians who may need to institute immunosuppressive therapy in a patient with a past history of melioidosis. There is currently debate in the literature as to whether relapse is due to the original infecting strain recrudescing, or whether the patient has been infected with a second strain of the organism. Molecular typing has been used to determine clonality of isolates recovered from patients who have relapsed with melioidosis. By typing isolates from relapse patients, it can be determined whether the patient is being reinfected or whether it is the recrudescence of the original infection. In the majority of studies, the original infecting isolate has been shown to be responsible for the relapse of *B. pseudomallei* infection (Desmarchelier *et al.*, 1993). This information can lead to changes in treatment of melioidosis and will also provide insight into the pathogenesis of *B. pseudomallei* and how the bacterium is able to continuously evade the host immune responses.

The immune responses to *B. pseudomallei* has not been fully characterised which has hindered the development of a successful vaccine to protect against infection. Since *B. pseudomallei* is an intracellular organism, potential vaccines would need to induce a strong CMI response, in addition to a humoral response (Healey *et al.*, 2005). Previous studies targeting various virulence factors of *B. pseudomallei* have failed to provide protection in animal models following subsequent challenge with the organism (Brett and Woods, 1996; Wiersinga *et al.*, 2006). Brett *et al* (1994) injected rats with antibodies from flagellin proteins, then infected with *B. pseudomallei* and found a prolonged survival time and reduced blood bacterial load. In another study mice were immunised with type III secretion system (TTSS) proteins (Druar *et al.*, 2008). Antibodies to the TTSS proteins were detected however these antibodies did not have a protective effect following challenge with *B. pseudomallei*. The development of a vaccine to protect against melioidosis could potentially decrease the mortality rate and protect vulnerable populations and individuals in areas endemic for *B. pseudomallei*. There is growing interest in investigating multiple strategies to develop optimal means of protection to *B. pseudomallei* in the host.

Therefore, the aims for the studies outlined in the subsequent chapters of this thesis were:

1. To determine the seroprevalence to *B. pseudomallei* in healthy blood donors from the Townsville district.

2. To evaluate the Melioidosis IgG Rapid Cassette Test as a screening method for antibody detection of *B. pseudomallei*.

3. To characterise the CMI response to *B. pseudomallei* in previously screened seropositive healthy individuals.

4. To determine the clonality of *B. pseudomallei* isolates from a selected group of patients with recurrent melioidosis.

5. To develop a murine model of latent melioidosis.

6. To determine whether DNA vaccinations against selected putative virulence factors of *B. pseudomallei* will produce the immune responses that could protect against subsequent infection.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Burkholderia pseudomallei is the causative agent of the disease melioidosis. The organism was originally described as a bacterium causing "glanders-like illness" in 1911 by Captain Alfred Whitmore and C S Krishnaswami in the pathology laboratory at Rangoon General Hospital, Burma. Glanders caused by *Burkholderia mallei* is a disease of horses rarely affecting humans. Melioidosis is derived from the Greek words "melis" meaning "a distemper of asses" and "eidos" meaning resemblance (Puthucheary and Vadivelu, 2002). The organism has had many nomenclature changes in recent years. Initially it was known as *Bacillus whitmori* then *Bacillus pseudomallei*, *Loefferella whitmori*, *Pfeirfferella whitmori* and *Malleomyces pseudomallei*. It was then reclassified based on the organisms growth, motility and metabolic characteristics to *Pseudomonas pseudomallei* and finally, in 1992, to the new genus *Burkholderia pseudomallei* based on the 16S sequences, DNA-DNA homology values, cellular lipid and fatty acid composition and phenotypic characteristics (Puthucheary and Vadivelu, 2002).

The Sanger Institute has sequenced the entire 7.25 megabase *B. pseudomallei* genome of isolate K96243 which can potentially provide new insights into the biology and evolution of the bacteria (Holden *et al.*, 2004), as well as providing opportunities for the selection of proteins to the development of vaccines.

2.2 Epidemiology

Over the past two decades, *B. pseudomallei* has been identified as an important human pathogen in South-east Asia and northern Australia with the potential to produce rapid and fatal septicaemia (White, 2003). The endemic region for melioidosis is defined as the area between latitudes 20°South and 20°North (Currie *et al.*, 2000d). Despite this, the

first case of melioidosis was described in a sheep from a subtropical location at latitude 22°S and this is considered to be the area of endemicity for animals. Documented cases of introduced melioidosis in temperate regions of Australia have been described and are supported by molecular typing of isolates which demonstrate clonality (Currie *et al.*, 2000d). The presence of clonal isolates in geographically distinct regions may have occurred as a result of environmental contamination via the movement of animals, local dissemination and the persistence of the bacteria over many years (Munckhof *et al.*, 2001).

Following the discovery of *B. pseudomallei*, there was an increase in the reporting of melioidosis cases, with 38 cases of melioidosis documented in Burma in 1917 where it was first discovered. Approximately 5% of post-mortem reports in 1917 listed *B. pseudomallei* as the causative agent (Whitmore and Krishnaswami, 1912). In Malaysia and Singapore, melioidosis was isolated from laboratory animals in 1913 with human and animal infections occurring near Kuala Lumpar. The first case of melioidosis in the Indochina (Vietnam, Laos and Cambodia) region was identified in 1925. In the 1960's, researchers demonstrated the presence of the bacteria in mud and surface waters throughout eastern and western Malaysia, Singapore and Brunei (Dance, 1991). Throughout Indochina, it is thought that melioidosis is highly prevalent within the population. With increased awareness and improved facilities more cases of melioidosis are being diagnosed. In Indonesia, the first case of melioidosis was reported in 1929, however, in the Philippines, China, Hong Kong and Korea recent data on the prevalence of the disease is scarce. Serological surveys have been carried out in China, Hong Kong and Korea and have demonstrated that there is evidence of human exposure. Throughout the years, melioidosis has become more recognised in Thailand with a survey showing more than 29% of Thai volunteer blood donors have been exposed to *B. pseudomallei* (Nigg, 1963).

Information on the prevalence of melioidosis is scant from India, the Pacific Islands, Central and South America, Africa, the Middle East and Papua New Guinea. However, there is evidence that the disease is present (Dance, 1991). With increased awareness in

recent years, cases and outbreaks of melioidosis have been reported in Papua New Guinea (Currie *et al.*, 2000d), India (Jesudason *et al.*, 2005), Africa and South America.

Over the past 35 years, sporadic cases of melioidosis have been reported in ex-servicemen who fought in World War II and the Vietnam War. These patients presented with a chronic form of the disease. During the Wars, soldiers were exposed to the organism via skin wounds, burns and inhalation. *B. pseudomallei* has the ability to remain dormant within the host for many years and can activate following suppression of the immune system. The activation of *B. pseudomallei* infection in ex-servicemen has given rise to melioidosis being called the "Vietnam time-bomb" (Dance, 1991).

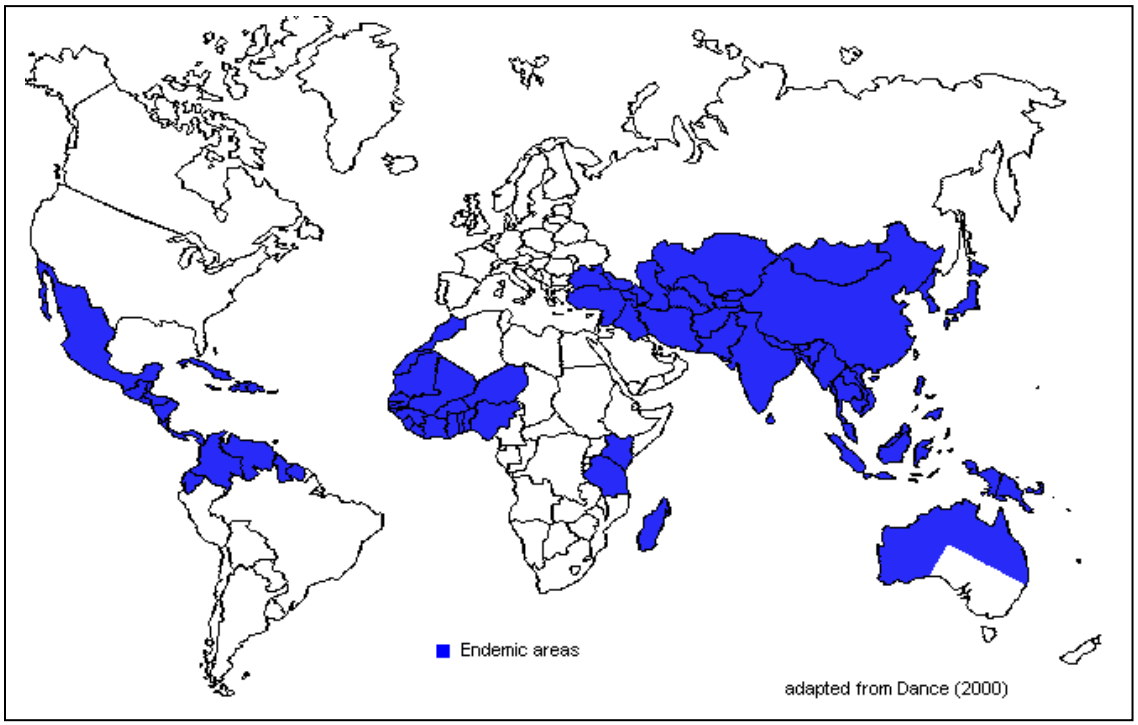


Figure 2.1 Global distribution of melioidosis. Melioidosis is endemic in the areas between latitudes 20°South and 20°North. Shaded blue areas represent regions where *B. pseudomallei* has been reported either in the environment or in patients presenting with melioidosis.

2.2.1 Epidemiology of melioidosis in Australia

The first case of melioidosis in Australia was described in a sheep in 1949 at Winton, Queensland. Following this the first human case was reported in 1950 in Townsville, Queensland in a 32 year old diabetic (Rimington, 1962; Ashdown and Guard, 1984). In 1960, the first case of melioidosis was reported in the Northern Territory (Crotty *et al.*, 1963). The disease has increasingly been recognised as an important cause of sepsis in both humans and animals in the northern regions of Australia where the reported incidence is high (Currie *et al.*, 2000d). Melioidosis in the Northern Territory is the most common cause of fatal community-acquired bacteremic pneumonia. In Western Australia, *B. pseudomallei* has been isolated from both patients and the environment (Inglis *et al.*, 1999). Serological studies conducted in north Queensland have demonstrated that the exposure to *B. pseudomallei* is more widespread then first realised (Ashdown and Guard, 1984).

The incidence of melioidosis around the world is not well documented. The awareness of the disease is low and the capacity of laboratories to be able to identify *B. pseudomallei* is limited especially in rural areas and in underdeveloped countries. In many regions, melioidosis is not classed as a notifiable disease. In Queensland however, melioidosis has been a notifiable disease since 1988 and therefore more thorough documentation on the disease exists (Ashdown, 1991). Melioidosis is also a notifiable disease in the Northern Territory and Western Australia. Over an 11 year period, from 1996 to the present in Queensland alone in excess of 130 patients with melioidosis have been documented. However a greater number of cases are seen in the Northern Territory with 364 cases of culture-confirmed melioidosis patients presenting over 14 years (Currie *et al.*, 2004). Cheng *et al* (2003) reported the incidence rate of melioidosis in the Northern Territory as 16.5 cases per 100 000 population and the rate in the Torres Strait Islands of Queensland from1995 to 2000, as 42.7 cases per 100 000 population. This incidence of melioidosis is considerably higher than rates reported for northeast Thailand (Suputtamongkol *et al.*, 1994) at 3.5 to 5.5 cases per 100 000 reported.

Cheng *et al* (2003) documented melioidosis cases from centres in Western Australia, the Northern Territory and Queensland. Data was collected from patients that presented with melioidosis between November 2001 and October 2002. Results showed Queensland and the Northern Territory had 23 patients presenting during the 2001-2002 period while only one patient presented with melioidosis in Western Australia during the same time period.

2.2.2 Ecology of *B. pseudomallei*

In 1921, Stanton and Fletcher stated that melioidosis was a zoonotic infection with the animal reservoir probably being rats, fleas or mosquitors as being the vector. However, this has not been proven. Rather *B. pseudomallei* has been recovered from soil during wet and humid weather (Asche, 1991).

B. pseudomallei is a soil saprophyte in certain tropical regions and it is presumed that it has an ecological niche similar to *Burkholderia cepacia* (Ashdown, 1991; Ashdown and Clarke, 1992). The organism can be readily isolated from rice paddies, still or stagnant waters and moist soils in predominantly tropical environments (Brett and Woods, 2000). In the dry season, the organism is thought to recede with surface water and dissipate deep into the subsoil (Ashdown, 1991). Studies have shown that *B. pseudomallei* may persist in the environment in a viable, nonculturable state during times of stress (Inglis *et al.*, 2001b). The association between rainfall and melioidosis has been recognised for many years with 75 and 85% of cases occurring during the wet season in northeast Thailand and northern Australia respectively (Currie and Jacups, 2003). It has been hypothesised that during the wet season, *B. pseudomallei* is carried to the surface as the water levels rise with heavy rain (Asche, 1991; Ashdown, 1991), therefore increasing exposure. The organism can be isolated from soil and surface water that receives filtered light rather than direct sunlight. The number of bacteria in the soil and water is directly related to rainfall, temperature and humidity (Ashdown, 1991). A study carried out by Currie and others (2003) demonstrated that the number of seasonal cases of melioidosis correlated with the total rainfall. They also observed that melioidosis patients admitted one to two weeks after heavy monsoonal rainfall are more ill and more likely to succumb to

infection than those who fall ill with melioidosis at other times of the year. This may be as a result of either a larger bacterial inoculating dose, or infection with more virulent bacteria (Currie and Jacups, 2003).

2.2.3 Seroprevalence of melioidosis

In endemic regions, the prevalence of seropositive individuals to *B. pseudomallei* has been well documented. A survey conducted in northern Queensland between 1977 and 1981 screening 9047 individuals demonstrated 512 out of 9047 (5.7%) serum samples tested produced a titre of 1:40 or greater, indicating exposure to *B. pseudomallei* (Ashdown and Guard, 1984). In this study, Indigenous donors, South Sea Islanders, hospital staff, army personnel and veterinarians were sampled for serological screening using an IHA assay which may have caused bias and skewing of results. Therefore, a survey that covers all groups living in northern Queensland would be beneficial. Of those with a positive titre, 61% were male and 39% were female. In the majority of the groups, antibody titres to *B. pseudomallei* were higher in males. Samples from predominately Indigenous or South Sea Islander populations showed a higher prevalence of seropositivity compared to samples from people of European descent from the same area. Townsville had the highest seroprevalence when compared to other areas in northern Queensland suggesting that it may be an area of higher endemicity (Ashdown and Guard, 1984). However these observations may also have been due to the larger number of individuals screened compared to other areas. Interestingly, the seroprevalence of soldiers stationed in northern Queensland was much lower than expected, which may have been as a result of the protective clothing worn by this group or from the continuous movement of soldiers from non-endemic regions to the endemic region of northern Queensland.

A survey on the prevalence of seropositivity within the population of Thailand was carried out between 1989 and 1991. Naigowit *et al* (1992) collected serum samples from healthy donors in eight locations in Thailand, including the endemic regions of Khon Kaen and Ubon Ratchathani and the non-endemic areas of Songkhla, Suratthani, Chon

Buri, Bangkok, Phisanulok and Lampang. The results demonstrated that the areas defined as non-endemic had a seroprevalence of less than 6% while in the endemic areas the seroprevalence were between 20 and 30% (Naigowit *et al.*, 1992). Norazah *et al* (1996) also conducted a survey on the prevalence of healthy seropositive donors in Malaysia. In this study healthy blood donors (n=100) were screened for antibodies to *B. pseudomallei*. At a titre equal to or greater than 1:40, the results demonstrated 26.5% of donors had been exposed to *B. pseudomallei* (Norazah *et al.*, 1996). These results suggest that more individuals in Thailand and Malaysia are exposed to the organism than in the population of Australia; however, the sample size is considerably lower than that used by Ashdown and Guard (1984) and a more thorough survey may be necessary.

2.2.4 Characterisation of *B. pseudomallei* isolates and clonality

Early methods used to characterise bacteria include antimicrobial susceptibility, bacteriocin production or sensitivity, bacteriophage susceptibility and serotyping (Sexton *et al.*, 1993). Epidemiological studies have been restricted in the past with techniques such as ribotyping and randomly amplified polymorphic DNA (RAPD), that were not sensitive enough to discriminate between strains of *B. pseudomallei* (Lew and Desmarchelier, 1993; Haase *et al.*, 1995b; Vadivelu *et al.*, 1997). To address this issue, methods have been developed that take advantage of chromosomal DNA restriction fragment heterogeneity, advancing the understanding of the epidemiology of the organism. Recent techniques such as pulsed-field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST) have allowed further discrimination between strains.

Ribotyping was one of the first molecular techniques used to differentiate *B. pseudomallei* isolates. Studies on other bacterial species, including *Yersinia enterocolitica*, *Salmonella typhi* (Hosoglu *et al.*, 2003) and *Staphylococcus aureus* (Almer *et al.*, 2002), have demonstrated ribotyping as a method capable of differentiating isolates from the same species. Ribotyping is based on the principle of the generation of specific patterns that result from the hybridisation of ribosomal ribonucleic acid (rRNA)

from *Escherichia coli* to restriction endonuclease-digested DNAs (Lew and Desmarchelier, 1993).

Lew *et al* (1993) established a ribotyping technique investigating epidemiologically related isolates of *B. pseudomallei* and demonstrated the suitability of ribotyping in epidemiological studies for this species. The study included bacteria isolated from Australia, Thailand and other reference strains. From the collection of 100 *B. pseudomallei* isolates, 22 ribotypes were identified. Ribotype 1 included 29 isolates which were collected from the regions of Thailand and Malaysia. The Australian isolates showed greater diversity having 19 different ribotypes. Some of the ribotypes were restricted to particular host species, geographic area or period of isolation. Sexton *et al* (1993) carried out an analysis of 74 clinical and 10 environmental isolates from Thailand. This study demonstrated ten distinct ribotypes and subtypes in a collection of clinical and environmental isolates. The clinical isolates were grouped mainly into ribotypes A to D with the majority of the isolates classified into ribotype A1 (64%). The lack of diversity among the clinical isolates suggests that ribotype A1 had a regional prevalence in Thailand. The presence of ribotype E and F in environmental isolates suggests that these organisms may be less pathogenic than those in the ribotypes of clinical isolates (Sexton *et al.*, 1993). To further evaluate the ribotyping technique as an epidemiological tool, Currie *et al* (1994) typed a select group of *B. pseudomallei* isolates from Western Australia (n=11) and the Northern Territory (n=3). The group found that the 10 isolates from Western Australia produced an indistinguishable ribotype pattern, however the isolates from the Northern Territory were different from the Western Australian isolates and from each other. These results suggested that there was a clonal introduction of *B. pseudomallei* into a non-tropical region of Australia that persisted and disseminated over a 25 year period. This data supports the possible environmental stability of some ribotypes. Ribotyping has been proven to be a useful epidemiological typing tool and its use can be extended to investigate disease outbreaks (Lew and Desmarchelier, 1993). However, Haase *et al* (1995) questioned the discriminatory power of ribotyping using another molecular typing technique, RAPD.

RAPD has been described as a quick and sensitive polymerase chain reaction (PCR) based typing method used to differentiate strains of bacteria. The technique involves the use of single, short, random primers that amplify parts of the genome (Haase *et al.*, 1995b). Haase *et al* (1995) found that *B. pseudomallei* isolates previously characterised into one ribotype were further discriminated using RAPD. However, both Currie *et al* (1994) and Haase *et al* (1995) found that isolates from Western Australia demonstrated strain stability with both ribotyping and RAPD.

PFGE is a more recent molecular technique used in characterising strains of *B. pseudomallei*. PFGE has been developed to separate large DNA molecules from approximately 10 to 2000 kilobases (kb) that have been digested with rare restriction enzymes. This is compared to conventional agarose electrophoresis that can only separate fragments of DNA of approximately 25 kb (Finney, 2000). With PFGE, a pulse or change of direction is introduced to an electric field. The first electric field influences the DNA fragments to elongate in the direction of that field, the electric field is then changed to a different orientation and the DNA fragments are pulled in a new direction. The process of changing the electric fields allows the separation and resolution of the DNA fragments.

Vadivelu *et al* (1997) compared PFGE and ribotyping with *B. pseudomallei* isolates from patients in Malaysia. From these results, PFGE was able to further discriminate between ribotypes, suggesting that ribotyping may only be useful to provide a broad epidemiological grouping and that PFGE provides greater discrimination between strains (Vadivelu *et al.*, 1997). The observations by Vadivelu *et al* (1997) using PFGE, correlates with Currie *et al* (1994), whereby ribotyping demonstrated broad epidemiological groups in the endemic Northern Territory, with only three ribotypes produced from clinical isolates (Vadivelu *et al.*, 1997).

Although PFGE has been shown to discriminate isolates of *B. pseudomallei*, it is a difficult and time consuming technique. Results from PFGE can not be compared between laboratories. Another technique, MLST has been utilised as a method that

allows interlaboratory comparisons. This technique uses a PCR to multiply copies of seven housekeeping genes that are conserved within the genome of the organism. The PCR products can then be sequenced and the sequence entered into a WEB-based database to allow comparison of isolates internationally for relatedness and clonality.

A study by Godoy *et al* (2003) typed a collection of *B. pseudomallei*, *Burkholderia thailandensis* and *Burkholderia mallei* isolates. The results showed that the *B. pseudomallei* isolates were grouped together and quite separate to *B. thailandensis* as expected. The isolates of *B. mallei* were identical in allelic profiles and clustered with the *B. pseudomallei* group of isolates. The group went on further to compare the discriminatory power of PFGE versus MLST. The results demonstrated that both PFGE and MLST grouped the isolates into the same major clusters. Minor differences were seen between isolates of a cluster; however, these differences are thought to be insignificant in an epidemiological study (Godoy *et al.*, 2003). This initial study shows the potential of MLST in typing *B. pseudomallei*. Although the technique allows results to be compared internationally, it is expensive.

2.2.5 Disease association and clonality of *B. pseudomallei* isolates

Whether there is a relationship between disease presentation and clonally-related *B. pseudomallei* isolates has been studied extensively. Using the ribotyping technique, an association between ribotypes of isolates and survival of the patient or between the ribotype and manifestations of disease was not demonstrated (Sexton *et al.*, 1993). RAPD analysis also did not provide evidence that RAPD patterns were related to the clinical outcome of melioidosis (Haase *et al.*, 1995b), with clonal strains of *B. pseudomallei* being isolated from both fatal and mild infections. This finding correlates with clinical observations that the outcome of melioidosis is determined largely by other bacterial and host factors (Haase *et al.*, 1995b) rather than the RAPD profiles.

In contrast, Norton *et al* (1998) used RAPD and multilocus enzyme electrophoresis (MEE) to characterise 18 *B. pseudomallei* isolates to determine whether there was an

association between clonal isolates and geographical location. The results demonstrated an association between clonal isolates which clustered on the basis of disease presentation rather than geographical location (Norton *et al.*, 1998). This was contradicted by two recent studies (Cheng *et al.*, 2004; Cheng *et al.*, 2005) that used MLST and PFGE to characterise a larger numbers of isolates. Both of these studies looked at the relationships between clonal *B. pseudomallei* isolates and disease severity, presentation and geographical location. The results demonstrated that, unlike the observations made by Norton *et al* (1998), there was no correlation between clonal isolates and disease presentation (Cheng *et al.*, 2004; Cheng *et al.*, 2005). A relationship between clonal isolates and tropism of the organism or geographical location also was not demonstrated. Therefore severity of the disease may not be associated with the clonally related strains of *B. pseudomallei* but instead could be reliant on host factors, the route of infection and the inoculum size (Cheng *et al.*, 2005).

2.3 Route of Infection

The route of infection of *B. pseudomallei* is only one factor in influencing disease outcome (Barnes and Ketheesan, 2005). *B. pseudomallei* can penetrate host defences through skin abrasions, ulcers and burns, or can be ingested through contaminated particles. The organism can also enter the body through the inhalation of contaminated dust or through aspiration of contaminated water in near-drowning episodes (Pruekprasert and Jitsurong, 1991). Cases of aspiration of *B. pseudomallei* have been seen more recently following the tsunami disaster of December 2004 with cases of melioidosis appearing as a result of near-drowning events (Allworth, 2005; Kongsaengdao *et al.*, 2005).

The highest incidence of melioidosis is observed in the monsoon and wet seasons (Brett and Woods, 2000). During the tropical wet season, the organism is thought to rise through the underlying surfaces with the water table, increasing the risk of exposure to humans and animals (Brett and Woods, 2000). This route of infection explains the high prevalence of melioidosis among rice farmers and labourers who work in the paddies

without protective clothing. Heavy monsoonal rainfall with heavy winds has also been associated with increased episodes of inhalation of the organism. Currie and Jacups (2003), in a prospective study, found an increase in patients presenting with pneumonia and severe disease following heavy rainfall suggesting a possible shift to inhalation as the route of infection (Currie and Jacups, 2003).

In a recent study, Barnes and Ketheesan (2005) compared the route of infection of *B. pseudomallei* using a murine model and two strains of the organism (a highly virulent strain and a less virulent strain). Mice were inoculated with the organism via four routes; intravenous (*iv*), intraperitoneal (*ip*), intranasal (*in*), oral, and subcutaneous (*sc*) routes of infection. The results indicated varying levels of virulence depending on the route of entry. For example, *iv* inoculation required only a small number of organisms compared to the oral route (Barnes and Ketheesan, 2005) to initiate infection.

2.4 Host Risk Factors

Particular underlying conditions predispose individuals to *B. pseudomallei* infection (Dance *et al.*, 1989a). Conditions such as diabetes mellitus, renal failure, systemic lupus, cirrhosis or alcoholism have been identified (Chaowagul *et al.*, 1993; Ip *et al.*, 1995) as risk factors. These conditions have been shown to interfere with phagocytic function (Inglis *et al.*, 2001a), therefore, enhancing the ability of *B. pseudomallei* to survive within the host.

Studies have demonstrated that certain populations are over represented in the total number of cases of melioidosis. This may be as a result of lifestyle or occupation, and hence an increased exposure to *B. pseudomallei*. The male population represented over 72% in a study of 364 melioidosis patients from the Northern Territory (Currie *et al.*, 2004) and 63% of cases from Townsville patients (Malczewski *et al.*, 2005). Factors that increase the exposure of *B. pseudomallei* to this particular population include environmental factors, such as contaminated soil or water as a result of daily activities (Puthucheary and Vadivelu, 2002). Therefore, exposure may be increased in males that

predominantly work in occupations that require contact with soil and water. Within Australia, over-representation of the Indigenous population with melioidosis cases was observed by Ashdown and Guard (1984) and has more recently been reported in a prospective study with Malczewski *et al* (2005). The subsequent study reported 30% of 57 patients with melioidosis were Indigenous Australians. This is significant as Indigenous Australians make up only 3.5% of the population in Queensland (Malczewski *et al.*, 2005). As previously suggested, this high rate of infection may be as a result of increased exposure to the organism due to lifestyle factors such as remote aboriginal communities, being in frequent contact with soils and surface water (Currie *et al.*, 2004). Other risk factors, such as, diabetes mellitus and alcoholism are also more common in this population.

2.5 Pathogenesis and Associated Virulence Factors

Despite numerous studies, little is known about the pathogenesis of *B. pseudomallei*. In vitro studies using cell lines have determined that *B. pseudomallei* is a facultative intracellular pathogen (Harley *et al.*, 1998). A study looking at the effect of *B. pseudomallei* infection in mice and hamsters found that macrophages play a role in the pathogenesis of melioidosis (Dannenberg and Scott, 1958). Kishimoto *et al* (1976) further investigated the interaction between macrophages from the rabbit peritoneum and *B. pseudomallei*. The macrophages were able to internalise and kill *B. pseudomallei*, however survival of the bacteria was influenced by the virulence of the strain.

A number of virulence determinants have been studied and shown to be involved in the pathogenesis of the organism, however the extent to which each factor is involved is still not clear. The virulence determinants can be divided into two groups; cell-associated virulence determinants and secretory virulence determinants (Table 2.1 and 2.2).

Table 2.1 Cell-associated virulence factors of *B. pseudomallei*

Factor	Features	References
Lipopolysaccharide (LPS)	➔ endotoxic activities ➔ macrophage activation by LPS weak ➔ unique acid-stable structures in inner core attached to lipid A moiety ➔ highly conserved between strains	(Kawahara *et al.*, 1992; Pitt *et al.*, 1992; Matsuura *et al.*, 1996; Anuntagool *et al.*, 2000a; Reckseidler *et al.*, 2001)
Flagella	➔ motility allows the establishment of infection	(Brett *et al.*, 1994; Winstanley *et al.*, 1998; Chua *et al.*, 2003)
Exopolysaccharide capsule	➔ >150 kiloDalton (kDa) ➔ inhibit complement activation and phagocytosis	(Steinmetz *et al.*, 1995; Masoud *et al.*, 1997)
Acid phosphatase	➔ 2 different acid phosphatases – thermolabile and thermostable ➔ role of phosphatases still unclear	(Kondo *et al.*, 1991; Kondo *et al.*, 1994; Kondo *et al.*, 1996)

While these factors are important in the pathogenesis of the organism, other factors such as the inoculating dose, mode of infection, host risk factors and variations in virulence of *B. pseudomallei* strains may possibly influence the incubation period, severity of the disease and the disease outcome (Currie *et al.*, 2000a).

Table 2.2 Secretory virulence determinants of *B. pseudomallei*

Factor	Features	References
Proteases	➔ metalloprotease – sequesters iron ➔ inactivates host defence mechanisms	(Heckly and Nigg, 1958; Sexton *et al.*, 1994; Lee and Liu, 2000)
Phospholipase C	➔ lecithinase ➔ endotoxin – damages cell membranes ➔ potential roles in virulence reported	(Yeo *et al.*, 1998; Korbsrisate *et al.*, 1999; Puthucheary and Vadivelu, 2002; Korbsrisate *et al.*, 2007)
Siderophore	➔ low-molecular-weight specific iron chelators to extract iron released by bacteria ➔ malleobactin – 1000 Dalton (Da)	(Yang *et al.*, 1991; Yang *et al.*, 1993)
Type III Secretion System (TTSS)	➔ 3 TTSS recognised ➔ delivery of virulence factors direct to host cell ➔ influences intracellular behaviour of organism	(Winstanley and Hart, 2000; Attree and Attree, 2001; Stevens *et al.*, 2002; Stevens *et al.*, 2004)

2.6 Immune Responses to *B. pseudomallei*

B. pseudomallei is a facultative intracellular bacteria and therefore CMI responses would be the main host defence against the organism. However, it has been demonstrated that both the innate and adaptive immune responses are essential in successfully clearing *B. pseudomallei* infection.

2.7 Innate Immune Response

The innate immune response is the first line of defense against invading pathogens. *B. pseudomallei* is able to evade different mechanisms of the innate response in order to safely enter host cells and multiply. The complement system is an essential component

of the innate immune response, in particular the alternative pathway. To achieve complement-mediated lysis of bacteria, the activation of the complement pathway, fixation of C3 fragments to the cell wall of the organism and assembly of the membrane attack complex needs to occur. Another important feature of this defense mechanism is the opsonisation of bacteria to enhance phagocytosis by professional phagocytes. Some bacteria have the ability to evade destruction by opsonic, chemotactic and lytic functions of the complement system. This may be achieved due to surface components of the bacteria or the organisms may also secrete molecules to evade or interrupt the cascade. The bacteria may also compromise complement receptors allowing direct access into the cell (Joiner, 1988).

Resistance to lysis in human sera was demonstrated by Ismail *et al* (1988) and further examined by Egan and Gordon (1996). Egan and Gordon (1996) evaluated the mechanisms by which *B. pseudomallei* resists complement. Following incubation in human sera there was no decrease in viable counts of *B. pseudomallei*. These results demonstrated that *B. pseudomallei* is a powerful activator of complement, with a rapid response seen in haemolytic assays (Egan and Gordon, 1996). Phagocytosis of *B. pseudomallei* by polymorphonuclear leukocytes (PMNL) was also studied demonstrating a significant difference in phagocytosis between bacteria that had been opsonised with pooled human serum compared to heat-inactivated human. These results suggest the involvement of complement in the cellular uptake of *B. pseudomallei* (Egan and Gordon, 1996). This has been observed in other intracellular organisms such as *M. tuberculosis*, where the organism is ingested following opsonisation with complement through the C3 receptor, to gain entry into the cell and multiply (Schlesinger *et al.*, 1990).

When *B. pseudomallei* enters a host cell several microbicidal mechanisms are activated. However, Pruksachartvuthi *et al* (1990) and Egan and Gordon (1996) demonstrated that *B. pseudomallei* was resistant to killing by PMNL. Macrophages are essential in killing bacteria, therefore by evading these mechanisms the immune response is undermined. *B. pseudomallei* resists killing in one of two ways: either by resisting the phagocytic oxidative process or suppressing the oxidative metabolic burst. The phagocytic oxidative

processes employed by the cell to kill intracellular organism is through the production of nitric oxide (NO) by the macrophages inducible nitric oxide synthase (iNOS). In a study by Miyagi *et al* (1997), the involvement of the microbicidal mechanisms reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) in destroying *B. pseudomallei* were examined. The results demonstrated that interferon (IFN)-γ activated murine macrophages inhibited the growth of intracellular *B. pseudomallei* through both ROI and RNI mechanisms, with RNI being a more dominant factor (Miyagi *et al.*, 1997). These results however do not explain how *B. pseudomallei* is able to evade killing by macrophages. Utaisincharoen *et al* (2001) also looked further into the ability of the organism to evade killing by macrophages, by monitoring the expression of iNOS and production of NO in cell lines infected with *B. pseudomallei*. However, the observations from these experiments differed to those of Miyagi *et al* (1997), with *B. pseudomallei* interfering with the cellular defense mechanisms thereby invading and multiplying within macrophages. Cell lines infected with *B. pseudomallei* did not express iNOS, however cell lines infected with *E. coli* and *S. typhi* as controls did express iNOS (Utaisincharoen *et al.*, 2001). Macrophages also did not produce a detectable amount of NO following infection unlike the control cell lines. These results suggest a unique mechanism by which *B. pseudomallei* is able to enter macrophages without significant activation and multiply within the cell (Utaisincharoen *et al.*, 2004). Utaisincharoen *et al* (2003) studied the ability of macrophages infected with *B. pseudomallei* to produce IFN-β. IFN-β, which enhances iNOS and NO production, is produced by macrophages that are infected with pathogens or are exposed to products of the organisms such as lipopolysaccharide (LPS). Following in vitro experiments in a mouse macrophage cell line, it has been found that IFN-β is not stimulated by *B. pseudomallei*. Therefore, with *B. pseudomallei*-infected cells blocking the production of IFN-β which in turn decreases iNOS production, the bacteria may be able to survive and therefore multiply inside the macrophages.

2.7.1 Toll-like receptors (TLRs) in *B. pseudomallei* infection

Toll-like receptors are recently discovered transmembrane receptors that are involved in innate immunity against pathogens and provide a crucial link between the innate and adaptive immune responses. These receptors are activated by pathogen-associated molecular patterns (PAMP) which leads to the production of nuclear transcription factor (NF)-κB translocation and the induction of the pro-inflammatory response. TLR2 in conjunction with TLR1 or TLR6 are activated by bacterial cell wall lipopeptides and peptidoglycan while TLR4 recognises the lipid A component of LPS. Wiersinga *et al* (2007) analysed expression of TLRs and messenger RNA (mRNA) levels in patients with septic melioidosis. They demonstrated an increase in expression of TLR1, TLR2 and TLR4 on the cell surfaces of circulating monocytes and granulocyes. There were also increased levels of mRNA for TLR1 through to TLR5, TLR8 and TLR10 in the peripheral blood mononuclear cells of these patients. The study also demonstrated that TLR2 and TLR4 contribute to cellular responsiveness to *B. pseudomallei* through experiments with TLR knockout mice, although TLR4 was not involved in protective immunity to the organism. However, TLR2 was found to be important in the control and dissemination of *B. pseudomallei* in investigations carried out on TLR2 knockout mice (Wiersinga *et al.*, 2007). West *et al* (2008) demonstrated the activation of TLR2 with TLR1 or TLR6 and TLR4 following stimulation of cell lines with heat-killed *B. pseudomallei*. The production of cytokines as a result of stimulation of primary cells was found to be dependent on TLR4 while TLR2 with TLR1 or TLR6 was demonstrated to initiate the inflammatory response to the heat-killed *B. pseudomallei*. These studies demonstrate the importance of TLRs in immune responses to *B. pseudomallei* infection.

2.7.2 The role of cytokines in innate immunity

The role of cytokines in a bacterial infection can be either beneficial or detrimental to the host depending on the time of production and numbers of bacteria. A correlation between high systemic levels of IFN-γ, tumour necrosis factor (TNF)-α, interleukin (IL)-6, IL-8 and IL-12 and septic shock and mortality has been observed (Lauw *et al.*, 1999;

Santanirand *et al.*, 1999; Cheng and Currie, 2005), however these cytokines are also necessary for the containment of infection and determining the host's innate resistance to the organism (Ulett *et al.*, 2000). Simpson *et al* (2000) studied concentrations of TNF-α, IL-6 and IL-10 to determine whether proinflammatory cytokine levels could be used as predictors of mortality. Analysis of serum samples from patients with melioidosis demonstrated significantly higher proinflammatory cytokine concentrations in patients that did not survive. The level of IL-6 and IL-10 were shown to be independent predictors of mortality. However, these results did not indicate that there was an increased mortality as a result of high levels of IL-6 or IL-10 or whether these cytokines were indicative of the severity of the disease (Simpson *et al.*, 2000).

The importance of IFN-γ and other proinflammatory cytokines in *B. pseudomallei* infection was studied by Santanirand *et al* (1999) in a murine model of melioidosis. IFN-γ is produced by activated natural killer (NK) cells, T helper 1 (Th1) cells and CD8+ cytotoxic T cells. The production of IFN-γ in the early response to infection with *B. pseudomallei* plays an essential protective role. The significance of IFN-γ in protection and clearance of the infection was demonstrated in mice treated with anti-mouse IFN-γ monoclonal antibodies (MAb) following infection with *B. pseudomallei*. The control mice survived while mice treated with the MAb died within 40 hours of infection. With MAb and a low dose infection of 2 cfu (colony forming units), the mortality in treated mice suggests that macrophages are unable to kill *B. pseudomallei* in the absence of IFN-γ. With the anti-IFN-γ MAb eliminating resistance in an animal model, the rapidness of the onset of disease and death suggests that clonal expansion of antigen-specific T cells could not take place in the short time frame. Instead, it would be more likely that the NK cells are the source of IFN-γ which may be important to stabilise the infection (Santanirand *et al.*, 1999).

In a similar experiment, mice were infected with *B. pseudomallei* and treated with either neutralising MAb specific for TNF, IL-12 or IFN-γ. The results demonstrated that IL-12 and TNF-α are also important in host resistance to acute infection, with 60-80% mortality

of the animals occurring in 3-4 days following treatment (Santanirand *et al.*, 1999). Utaisincharoen *et al* (2001) demonstrated that in cells infected with *B. pseudomallei*, IFN-γ increased the expression of iNOS and the release of TNF-α. This result decreased the intracellular survival of *B. pseudomallei* within the macrophages.

IL-8 is a strong neutrophil chemoattractant and is therefore likely to play an important role in defense against bacterial pathogens. An elevated level of IL-8 is associated with poor patient prognosis (Friedland *et al.*, 1992). Friedland *et al* (1992) studied plasma levels of IL-8 in patients presenting with melioidosis. The IL-8 concentrations were elevated in 8 out of 18 patients; of whom 50% died. The authors concluded that IL-8 was a significant predictor of mortality. Although, IL-8 has been investigated, the analogue cytokine in mice, KC, has not been researched extensively.

An elevated IL-6 serum levels is another indicator of poor prognosis. Serum IL-6 levels were monitored in patients with melioidosis following admission. Friedland *et al* (1992) found elevated IL-6 levels in 4 patients; 3 of whom died. These IL-6 levels were found to be significantly higher when compared to patients who survived. The results demonstrated a high correlation between IL-6 levels and fatal sepsis as a result of infection with *B. pseudomallei*.

The production of early cytokines in a murine model has also been studied. Ulett *et al* (2000) used two models; one model (C57BL/6 mice) represents the chronic form of melioidosis and is relatively resistant to *B. pseudomallei* infection and the other model (BALB/c) represents the acute form and is susceptible to infection. The results demonstrated that in BALB/c mice, high levels of TNF-α and IL-6 in the liver correlated with the development of acute infection.

Unmethylated CpG oligodeoxynucleotides (CpG ODNs) induce an effective protective immunity in chronic infectious diseases and is a potent immunomodulator of the innate immune response. There are a number of functions of CpG ODN including an increase of resistance in mice against acute sepsis and the use of CpG ODN as an adjuvant to

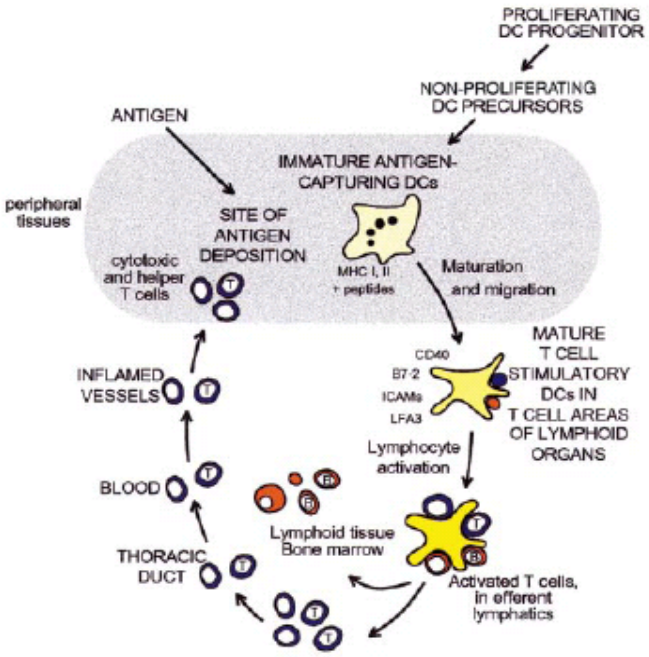
boost the immune system following vaccination. CpG ODN stimulate macrophages, DC, NK cells and some populations of lymphocytes, inducing proinflammatory cytokines TNF-α, IL-12 and IFN-γ. Utaisincharoen *et al* (2003) studied the cytokine production in response to CpG ODN and bacterial uptake by macrophages. To determine whether bacterial uptake by macrophages is enhanced with CpG ODN, murine macrophages were stimulated with CpG ODN prior to being infected with *B. pseudomallei*. The results demonstrated that uptake of *B. pseudomallei* by macrophages was enhanced. With CpG ODN stimulating macrophages to produce proinflammatory cytokines such as TNF-α and IL-12, NK cells produce IFN-γ which is essential in an early immune response. Wongratanacheewin *et al* (2004) studied the effect of CpG ODNs in acute sepsis as a result of infection with *B. pseudomallei*. A murine model of acute *B. pseudomallei* infection was used, with animals pre treated with CpG ODN prior to infection. When compared to controls the pre treated animals conferred between 90 – 100% survival. Bacterial loads were also reduced considerably compared to the control mice. These results suggest that CpG ODN in acute melioidosis can clear the infection. IFN-γ levels in the mice pre treated with CpG ODN demonstrated lower levels of the cytokines compared to the control mice (Utaisincharoen *et al.*, 2003). While IFN-γ is known to be important in protection against *B. pseudomallei* infection, excessive production can also be detrimental. *B. pseudomallei* is not a potent stimulator of the immune response which therefore leads to a rapid multiplication of bacteria leading to the excessive production of cytokines and death as a result of overwhelming sepsis. It has been demonstrated in this study that the administration of CpG ODN will enhance the innate immune response prior to exposure to *B. pseudomallei*. CpG ODN needs to be administered at least 48 hours prior to exposure but no more than 10-15 days, therefore its suitability as a therapeutic agent as suggested by Wongratanacheewin *et al* (2001) may not be valid.

2.7.3 The role of dendritic cells

Dendritic cells are potent stimulators of the adaptive immune response. As antigen-presenting cells (APCs), DCs initiate and modulate the immune response. DCs have

been described as mobile sentinels, presenting antigens to T cells and expressing co-stimulators for the induction of immunity (Banchereau and Steinman, 1998).

The role of DCs following infection with *B. pseudomallei* has not been extensively studied. Healey *et al* (2005) studied the ability of DCs to generate a CMI response to *B. pseudomallei*. In early stages of the experiment, results demonstrated that immature DCs were responding to *B. pseudomallei*, maturing and presenting the antigens to naïve T cells. These studies indicate that the early activation of the immune system, with the activation of macrophages producing cytokines and DCs to initiate an adaptive immune response is critical. However as described, *B. pseudomallei* is able to evade the immune response in various ways, for example, surviving within macrophages and evading the complement cascade (Figure 2.2). There is potential for DCs to be developed as a modality to deliver *B. pseudomallei* vaccine preparations.



(Banchereau and Steinman, 1998)

Figure 2.2 Activation and role of DCs. Immature DCs capture and process antigens presenting them on their cell surface. Following maturation, DCs prime T cells which in turn interact with other cells, such as, B cells that produce antibodies or macrophages that release cytokines and target cells for lysis.

2.8 Adaptive Immune Response

Recent studies have demonstrated that both the CMI and the humoral response are important in protecting the host (Healey *et al.*, 2005). However, with the organism being a facultative, intracellular organism it is thought the CMI responses are the main defense mechanism employed by the host.

2.8.1 Antibody response to *B. pseudomallei*

The innate immune response activates T cells which in turn help B cells to produce antibodies specific to the pathogen. Antibodies produced as a result of this response destroy the pathogen primarily via two mechanisms. One is to coat the surface or opsonise the pathogen to enhance phagocytosis and secondly, the complement cascade can be activated to enhance opsonisation. However, the humoral immune response to *B. pseudomallei* has not been studied extensively. Studies have suggested, however, that although the CMI responses are important in clearing the infection, antibodies that destroy the organism also play a role in the initial clearance of *B. pseudomallei* (Chenthamarakshan *et al.*, 2001).

Chenthamarakshan *et al* (2001) determined the Ig isotype and IgG subisotype distribution in patients with melioidosis against *B. pseudomallei* culture filtrate antigen (CFA). The results demonstrated that all immunoglobulin isotypes, except IgE, were involved in the humoral response. However IgG was the predominant antibody isotype in patients with septicaemia, localised infection and clinically suspected melioidosis patients. The IgG subisotypes were also analysed, with the subisotypes IgG1 and IgG2 being the predominant. These two subisotypes are thought to activate the classical complement pathway and IgG1 also binds to mononuclear cells (Chenthamarakshan *et al.*, 2001). *B. pseudomallei* infects macrophages and monocytes and these results suggest that the levels of IgG1 being higher than other subisotypes may facilitate internalisation. With elevated levels of IgG1, it remains unclear why antibodies are unsuccessful at destroying the infection. Vasu *et al* (2003) further studied the antibodies produced in response to

B. pseudomallei CFA, monitoring patients with melioidosis undergoing long-term treatment or eradication therapy ranging from 7 months to 4 years. The results also demonstrated that the presence of IgG antibodies were much higher than other immunoglobulins. The subtypes of IgG that predominated were also IgG1 and IgG2, as described by Chenthamarakshan *et al* (2001). Vasu *et al* (2003) also commented on the elevated level of IgG1 antibodies and the patients not being able to clear *B. pseudomallei*.

Levels of IgM antibody varied between patients. Vasu *et al* (2003) suggested that persistence of antibody levels in the patients may have been as a result of varying polysaccharide antigens present in the patients. Polysaccharides persist for a period of time in the lymphoid tissues and can therefore continue to stimulate B cells to produce IgM. However, the fluctuation of IgM levels observed in some of the patients may also have been due to relapse episodes throughout the sampling period.

2.8.2 Cell-mediated immune response to *B. pseudomallei*

With *B. pseudomallei* being an intracellular pathogen, the CMI is most likely to be involved in killing the invading organism, however, the response is yet to be fully characterised and understood. Defining the role of the CMI response would allow for the development of vaccines and treatment strategies against *B. pseudomallei*.

The production of IFN-γ in *B. pseudomallei* infection through the activation of CD8+ T cells was demonstrated by Lertmemongkolchai *et al* (2001). The results of flow cytometry, following intracellular staining of splenocytes stimulated with *B. pseudomallei* for IFN-γ, demonstrated that 40% of IFN-γ-producing cells were CD8+ T cells. However, the activation of the cytotoxic (CD8+) T cells was not as a result of antigen-specific activation but as a result of cytokine-mediated bystander activity in response to IL-12 and IL-18. This was further demonstrated in another intracellular pathogen, *L. monocytogenes* showing production of IFN-γ by CD8+ T cells and not CD4+ T cells. These results of rapid production of IFN-γ suggest that the CMI response plays an important role in resistance to *B. pseudomallei*. The bystander activation of

CD8+ T cells may contribute to the immune response in three ways: firstly, as an additional source of IFN-γ in the early immune response; secondly, in the proliferation of effector T cells and finally by maintaining the memory T cells (Lertmemongkolchai *et al.*, 2001).

Brown *et al* (1990) measured the activation of the CMI response in patients with melioidosis by the quantitation of neopterin which is a metabolite excreted in the urine from the stimulation of monocytes or macrophages by IFN-γ produced by activated T cells. The results demonstrated elevated levels of urinary neopterin in patients with an active infection and relapse patients when compared to the control group. In patients suffering from severe sepsis, the urinary neopterin levels were greatly elevated. The elevated levels of neopterin indicated the activation of the CMI response. In sepsis patients where there are high levels of neopterin, the CMI response does not appear to be adequate to control the *B. pseudomallei* infection and the high levels may be as a result of secondary, non-specific T cell-macrophage activation due to the overwhelming infection. Whether a CMI response occurs in response to *B. pseudomallei* was studied by Ketheesan *et al* (2002), in patients who had recovered from melioidosis. An antigen-specific memory T cell response of lymphocytes following antigen stimulation with *B. pseudomallei* was demonstrated with marked lymphocyte proliferation and IFN- γ production. CD4+ and CD8+ T cells were identified by flow cytometry. T cell populations from patients were significantly greater following stimulation with *B. pseudomallei* antigen when compared to controls. The expression of CD69 on activated T cells (CD4+ and CD8+) was higher in lymphocytes taken from patients when compared to controls and this could indicate the possible role of CD4+ and CD8+ T cells in the CMI response against *B. pseudomallei* (Ketheesan *et al.*, 2002).

Barnes *et al* (2004) investigated the possible role of T cells in seropositive individuals with subclinical *B. pseudomallei* infection. Lymphocytes taken from recovered, culture confirmed patients with melioidosis and seropositive individuals proliferated significantly compared to controls. However, lymphocyte proliferation and the production of IFN-γ by lymphocytes in seropositive individuals were significantly greater than culture

confirmed patients. The differences in IFN-γ production in seropositive individuals and culture confirmed patients with melioidosis may be a reflection of differences in populations of antigen-specific memory T cells. The production of a strong CMI response in seropositive individuals suggests that these responses may be essential in preventing the progression of *B. pseudomallei* infection (Barnes *et al.*, 2004). A weaker CMI in patients with melioidosis response may be indicative of patients being unable to mount an adequate response and therefore succumb to the disease.

The development of a CMI response in healthy individuals possibly exposed to *B. pseudomallei* was studied by Govan and Ketheesan (2004). The healthy volunteers were Vietnam veterans who had no history of melioidosis or other diseases presenting with pyrexia of unknown origin and had possibly been exposed to the organism whilst serving in South-East Asia. Lymphocytes from the Vietnam veterans proliferated significantly when stimulated with *B. pseudomallei* antigens compared to controls. This demonstrates the development of a CMI response to *B. pseudomallei* due to exposure and not as a result of the disease (Govan and Ketheesan, 2004). This response may be sufficient to protect against any future infections with *B. pseudomallei* but could become a problem should the individual become immunosuppressed.

Cytotoxic lymphocytes (CD8+) lyse cells infected with intracellular bacteria, viruses and parasites. Cell lysis occurs through Fas ligation through the granule exocytosis pathway. This pathway releases perforin that targets cell entry and directs granzymes which induces apoptosis of the target cell. Granzyme A and B have two different methods to initiate apoptosis. Granzyme B induces rapid apoptosis, on the other hand, granzyme A acts slower using a different pathway and only becomes apparent if granzyme B is inhibited (Lauw *et al.*, 2000). Levels of granzyme in septicaemic and non septicaemic patients with melioidosis were measured. Elevated levels of granzyme A and B were determined in septicaemic patients compared to control subjects, however there was no increase in levels of granzyme in non septicaemic patients. Lauw *et al* (2000) also analysed the role of cytokines, TNF-α, IL-12, IL-18 and IFN-γ, in the release of granzyme following *B. pseudomallei* infection. The results demonstrated a significant

decrease in granzyme release in patients with melioidosis following the neutralisation of either TNF-α, IL-12, or IL-18 (Lauw *et al.*, 2000). Suppression of IFN-γ did not have an affect on the release of granzyme suggesting that IL-12 and IL-18 which are mediated by IFN-γ induce the release of granzyme through an IFN-γ independent pathway. These results demonstrate the interaction between cytokines and granzymes may be important in both the innate and CMI responses.

2.9 Clinical presentation

Defining the clinical presentation of melioidosis is difficult due to the broad spectrum of clinical symptoms that patients present with from benign skin and soft tissue infections to a fulminant and fatal septicaemia (Puthucheary and Vadivelu, 2002). Cases of melioidosis have been described as presenting with diverse clinical features, severity and duration (Kingston, 1971).

The majority of melioidosis patients present with acute fulminating septicaemia which can have a mortality rate of over 90%. A report by Kingston (1971) described a broad spectrum of clinical features such as chills, fever and prostration, bronchopneumonia, dysentery or septicaemia with death following within a few days.

As outlined above, melioidosis may present as either a localised or disseminated infection in any organ. Another difficulty associated with diagnosing melioidosis is that the disease mimics a number of other acute and chronic infections with non-specific signs and symptoms (Table 2.3).

The broad clinical spectrum of melioidosis could be divided into sub-groups including:

1. acute melioidosis
2. chronic infection
3. relapse of melioidosis
4. latent melioidosis

Table 2.3 Clinical presentations of melioidosis

Sub-group	Clinical Presentations
Acute	• septicaemia • pneumonia • abscesses • multiple organ involvement
Chronic	• abscesses • infiltration of lungs • chronic osteomyelitis
Relapse	• initial infection presenting as acute or chronic • can recrudesce repeatedly following immunosuppression presenting as either an acute or chronic infection
Latent/Subclinical	• no apparent infection • can remain dormant for years until immunosuppression • varying presentations including acute septicaemia

2.9.1 Acute infection

Acute infections present in many clinical forms, from acute pneumonia to septicaemia and multiorgan involvement. Septicaemia is most often seen in endemic regions. In Thailand melioidosis forms 19% of community-acquired septicaemia and is responsible for 40% of fatalities from septicaemia (Ip *et al.*, 1995). The most common acute clinical presentation of melioidosis is pneumonia. In Australia's Northern Territory, melioidosis is the most common cause of fatal bacteraemic pneumonia (Currie *et al.*, 2000c). In a retrospective study of Queensland patients, Malczewski *et al* (2005) found that 55% of patients (n=57) with melioidosis presented with lung involvement. This figure is similar to that of the Northern Territory where 50% of patients (n=252) presented with pneumonia (Malczewski *et al.*, 2005). A less common clinical presentation of acute melioidosis is the neurological form involving the brainstem, cerebellum and spinal cord (Currie *et al.*, 2000b).

2.9.2 Chronic infection

Chronic melioidosis presents as enduring suppurative abscesses. Chronic infections often present as usually present as subcutaneous or soft-tissue abscesses, infiltration of the lungs and chronic osteomyelitis (Kingston, 1971) in contrast to an acute infection with duration from three months.

2.9.3 Relapse

Chaowagul *et al* (1993) defined relapse of melioidosis as the development of new symptoms and signs of the bacterial infection together with the isolation of *B. pseudomallei* from bloods or sites that previously were negative following culture. Relapse of melioidosis has been associated, in the majority of cases, due to result of non-compliance with antimicrobial therapy (Haase *et al.*, 1995a). In understanding relapse of *B. pseudomallei* infections learning whether the isolates are as a result of a recurrence of primary infection or whether a secondary inoculation has occurred is essential (Desmarchelier *et al.*, 1993). Molecular techniques have been used in studies to try and determine this; whether the initial organism is responsible for the recurring infection or whether the patient has been exposed to a new strain.

In a study comparing ribotype patterns with clinical and environmental isolates, Sexton *et al* (1993) found that in two cases of melioidosis where patients clinically relapsed, the ribotype of the organism was identical to that isolated during the initial infection. It has been suggested that relapse is likely to be due to resurgence of the original infecting organism rather than as a result of reinfection (Sexton *et al.*, 1993).

Similarly, Desmarchelier *et al* (1993) conducted a study which monitored patients following initial diagnosis of melioidosis to track cases of relapse. The study involved 602 patients with 25 of these patients relapsing. Ribotyping was carried out on the isolates and showed that of the 25 patients, 23 had identical or very similar ribotype patterns. The remaining two patients displayed a different ribotype pattern from the

initial isolateThe study demonstrated that, in the majority of cases, primary and recurrent infections with *B. pseudomallei* occurred as a result of the persistence of the initial isolate (Desmarchelier *et al.*, 1993).

A smaller study (n=10) was carried out by Haase *et al* (1995) characterising relapse isolates cultured from melioidosis patients. The results demonstrated that 80% of the relapse patients were infected with *B. pseudomallei* showing identical RAPD patterns. Of the two remaining patients, one produced two different patterns and the other was possibly due to concurrent infection with two strains of *B. pseudomallei* (Haase *et al.*, 1995a).

Another technique, PFGE, was used by Vadivelu *et al* (1998) to characterise *B. pseudomallei* isolates from five relapse patients with melioidosis. The results demonstrated 100% of the subsequent isolates had indistinguishable PFGE patterns from the original infecting strain. Koonpaew *et al* (2000) also used PFGE to characterise *B. pseudomallei* isolates from 35 melioidosis relapse patients. Four of these patients did not produce identical macrorestriction patterns suggesting possible simultaneous infection with two strains of *B. pseudomallei* or a subsequent infection with a second organism (Koonpaew *et al.*, 2000). Currie *et al* (2000) conducted a study involving 207 patients that had "cleared" the infection following antibiotic therapy. Of these patients, 27 (13%) relapsed once and another five of these relapsed twice. PFGE was used to characterise the *B. pseudomallei* isolates to demonstrate that 96% (26/32) produced similar macrorestriction patterns to the original isolate.

A number of factors have been raised in the characterisation of isolates from relapse patients. One issue is whether the initial strain is identical to the recurrent strain, that is, whether the initial *B. pseudomallei* infection was only with one strain or perhaps more than one (Desmarchelier *et al.*, 1993; Haase *et al.*, 1995a). However, this may be overlooked when the isolate is typed with only a single colony being chosen from the culture plate. The studies, previously mentioned, demonstrated that a small percentage of isolates taken from relapse patients displayed different ribotype patterns (Desmarchelier

et al., 1993; Sexton *et al.*, 1993; Haase *et al.*, 1995a; Currie *et al.*, 2000a; Koonpaew *et al.*, 2000). The fact that some of the isolates are not identical in restriction patterns suggest that the strain may be the same initial strain but subsequent rearrangements of genes may have resulted in what appeared to be a different strain of *B. pseudomallei*. This hypothesis was reiterated in the study by Haase *et al* (1995) with the antimicrobial sensitivity of two of the relapse isolates changing which suggested genetic mutation. It can not be ruled out that 'recurrent' patients are not in fact being re-infected with the same strain of *B. pseudomallei*, however, the probability of this is low. A table summarising some of these studies has been included (Table 2.4).

Table 2.4 Characterisation of *B. pseudomallei* isolates from relapse patients

Location	Number of relapse patients	Number of patients with recurring isolate	%	Method of Typing	Reference
Thailand	25	23	92%	Ribotyping	(Desmarchelier *et al.*, 1993)
Thailand	34	33	97%	Ribotyping	(Sexton *et al.*, 1993)
Northern Territory	10	8	80%	RAPD	(Haase *et al.*, 1995b)
Malaysia	5	5	100%	PFGE	(Vadivelu *et al.*, 1998)
Thailand	35	31	89%	PFGE	(Koonpaew *et al.*, 2000)
Northern Territory	27	26	96%	PFGE	(Currie *et al.*, 2000a)

2.9.4 Latent/subclinical infection

Latent or subclinical infections do not produce any signs of infection. Latent infections of *B. pseudomallei* are able to remain dormant in host cells until the individual becomes immunosuppressed. The disease can then recrudesce to produce an acute septicaemia. One of the longest latency period for melioidosis documented is 62 years (Nguay *et al.*, 2005).

There is evidence that *B. pseudomallei* has the potential to remain latent and recrudesce with varying severity of clinical infection (Mays and Ricketts, 1975). The activation of a latent infection due to an immunosuppressed state can lead to an acute, fulminating and possibly fatal infection (Jones *et al.*, 1996). The ability of *B. pseudomallei* to survive intracellularly in phagocytic cells, such as macrophages, has been demonstrated in cell lines. This defence mechanism is a method by which the organism evades clearance from the host and may be important in the pathogenesis of activation of melioidosis (Jones *et al.*, 1996).

The infection has been described in returned Vietnam veterans following the activation of latent *B. pseudomallei* infection. Serological studies have also shown an estimated 225 000 ex-servicemen could have potentially have been exposed to *B. pseudomallei* (Currie *et al.*, 2000a).

2.10 Bacteriology and Isolation

B. pseudomallei is a Gram negative bacilli, 40-60µm wide and 2-5µm long (Puthucheary and Vadivelu, 2002) and is a facultative intracellular pathogen surviving within host phagocytic cells (White, 2003). The organism is motile, aerobic, non-spore forming and oxidative positive (Forbes *et al.*, 1998).

B. pseudomallei is not fastidious but can be cultured on most agar media, producing visible colonies after incubation for 24 hours at 37°C, aerobically (Puthucheary and

Vadivelu, 2002). A Gram stain of the organism produces bi-polar staining of the Gram-negative rods and is often described as resembling safety pins (White, 2003). The most commonly used selective medium for the isolation of *B. pseudomallei* is Ashdown's Agar containing crystal-violet, glycerol and gentamicin (Ashdown, 1979). Absorption of neutral red by the organism allows differentiation of *B. pseudomallei* from other bacteria. The addition of gentamicin to the media selects *B. pseudomallei* from organisms that are commonly found in clinical material and the addition of glycerol produces the characteristic morphology of the organism (Ashdown, 1979). The characteristic colonial morphology of *B. pseudomallei* are purple-pink wrinkled colonies following 48 hours of incubation at 37ºC, however this varies significantly between isolates. Recognition and identification of *B. pseudomallei* depends on awareness and familiarity with the cultural characteristics. The varying colonial morphology occurs as a result of the medium and the source of the strain. The morphology of *B. pseudomallei* can range from colonies that are rough to those that are mucoid and colours from cream to bright orange (Puthucheary and Vadivelu, 2002). Ashdown's agar is useful when culturing clinical specimens. Samples, such as throat swabs and sputum are heavily contaminated by flora that would otherwise overgrow *B. pseudomallei* due to its extended generation time (Sirisinha *et al.*, 2000).

2.11 Diagnosis of *B. pseudomallei*

Diagnosis of melioidosis remains a problem with clinical presentation ranging from acute fatal septicaemia to mild localised infection. The current 'gold standard' for the diagnosis of melioidosis involves culturing the organism from a clinical specimen followed by phenotypic identification. The process of culturing and subculturing the organism, however, can take up to four days with the bacterium taking from 24-48 hours to grow on each occasion.

Early diagnosis of melioidosis is essential to increase patient survival rates, with delays in diagnosis leading to high mortality. Approximately 50% of patients with acute septicaemic melioidosis die within 48 hours of admission (Anuntagool *et al.*, 2000b).

The difficulty in diagnosing septicaemia caused by *B. pseudomallei* compared to septicaemia caused by other organisms becomes particularly relevant in non-endemic areas or where the frequency of encountering *B. pseudomallei* is low. Other rapid tests such as the slide agglutination test using specific polyclonal antibodies and the API20NE are rapid and cheap tests that can be used as a preliminary identification of *B. pseudomallei* in endemic areas, however the organism still needs to be cultured prior to using these techniques. A comparison of diagnostic techniques was carried out using conventional methods versus the API 20NE (Dance *et al.*, 1989b). There was 100% accuracy using conventional methods of identifying *B. pseudomallei*. Conventional methods included culturing onto a modified Ashdown's agar to look for the characteristic morphology of *B. pseudomallei* followed by Gram stain, oxidase test and antibiotic sensitivities. The API 20NE tests demonstrated 97.5% (n=390) accuracy in identifying *B. pseudomallei* with an initial screen. Following the second screen, the accuracy increased to 99.8% (n=399) and only one organism was misidentified by the API. These results suggested that this test is a simple, accurate method for identifying *B. pseudomallei* (Dance *et al.*, 1989b). However, the API 20NE still does not have the ability to identify the organism more rapidly than conventional methods, with both tests requiring the organism to have been grown in culture prior to the test being carried out.

Other techniques for the detection of the organism have been developed including immunological and molecular techniques attempting to identify the organism more rapidly than culturing the organism. However, these techniques are not yet considered to be sensitive or specific enough to replace the 'gold standard' (Sirisinha *et al.*, 2000).

2.11.1 Immunological diagnostic assays

There have been a number of immunological approaches that have been developed to detect *B. pseudomallei* from a clinical specimen. Immunological methods of identifying the organism can be divided into two areas, the detection of antibodies and the detection of antigens. The detection of antibodies employs tests such as IHA, immunofluorescent

antibody assays (IFA), enzyme linked immunosorbent assay (ELISA) and immunochromatographic test (ICT).

The IHA is a simple and moderately sensitive test. However, in endemic regions it has limited value as background antibodies present in healthy individuals interfere with the test to produce false positive results. The test is also not indicative of the infectious activity or the stage of the infection, with antibodies circulating for several years following infection or exposure. The IHA, however, is valuable in non-endemic areas and as an epidemiological, screening tool in endemic areas. Studies have been carried out on populations of healthy individuals living in endemic regions have been discussed previously (Section 2.2.3).

The IgM-ELISA uses antigens from *B. pseudomallei* to detect IgM antibodies in patient serum. This method of detection has not been evaluated on a large scale. One of the problems with the assay is the production of false negative results due to the insignificant antibody levels produced in patients with acute sepsis. The IFA is a rapid, highly sensitive assay. The test indicates current infections and is more suitable than IHA as a test for melioidosis in endemic areas (Puthucheary and Vadivelu, 2002) however, as with other immunological assays it can produce incorrect results due to variability and specificity of the antigen (Vadivelu and Puthucheary, 2000).

The detection of *B. pseudomallei* antigens is superior to antibody detection because it is indicative of active infection, however assays are still being trialled. Detection of soluble secreted product in blood and urine and the detection of whole organisms in other clinical specimens is possible. A number of assays have been described to detect *B. pseudomallei* antigens, Ismail *et al* (1987) developed a monoclonal antibody-based assay for the quantitation of exotoxins produced by the organism. This assay has not been evaluated in a clinical setting. Wongratanacheewin *et al* (1990) developed a polyclonal antibody-based avidin-biotin ELISA, which also has not been evaluated clinically. An assay has been developed that detects *B. pseudomallei* antigen in urine from patients with either a

systemic or localised infection (Desakorn *et al.*, 1994). However this system has not been incorporated into the standard diagnosis of melioidosis.

2.11.2 Molecular diagnosis of *B. pseudomallei*

With the identification of *B. pseudomallei* using the conventional 'gold standard' technique being time consuming, studies have been carried out in an attempt to replace these methods with molecular tools. PCR is a sensitive, rapid and a simple method. It commonly involves the amplification of a specific sequence of DNA from the *B. pseudomallei* genome that is conserved throughout all strains. This technique has the ability to detect the presence of a single copy of the target sequence which is important in the early diagnosis of melioidosis.

Rattanathongkom *et al* (1997) developed a PCR demonstrating the technique to be sensitive and specific. However, this study did not use clinical specimens, such as blood, to extract the bacterial DNA from, but instead cultured the organisms first. This PCR would therefore require an extra 48 hours to allow the organism to grow before DNA could be extracted. However, the challenge is bring able to take samples from suspected patients with melioidosis and test them directly for the presence of *B. pseudomallei* DNA. Haase *et al* (1998) attempted to achieve this and compared various published PCR techniques using clinical specimens including sputum, blood, swabs and fluids. The study developed a 162 rRNA-based PCR that produced close to 100% sensitivity. There was no cross-reaction between the other organisms tested or the various specimens used.

Real-time PCR (qPCR) is in the early stages of development for the detection of *B. pseudomallei* and has a very high sensitivity and specificity. The technique is a PCR method that allows the quantitation of DNA with results being collected in 'real-time'. Two studies have targeted the TTSS genes on the genome of *B. pseudomallei* (Thibault *et al.*, 2005; Tomaso *et al.*, 2005). These two studies used DNA extracted from cultured organisms to determine the sensitivity and specificity of the assay, however, there is potential to be able to detect *B. pseudmallei* from blood or other specimen samples.

Novak *et al* (2006) designed a qPCR to also target the TTSS. The primers designed where unique to *B. pseudomallei* and did not appear to have any cross-reactivity with *B. mallei* or *B. thailendensis*. The study used blood spiked with *B. pseudomallei* and other organisms to extract DNA. Results demonstrated that the assay was able to distinguish between *B. pseudomallei* and other organisms and was not inhibited by the presence of human blood products or DNA. The assay was able to detect the presence of *B. pseudomallei* at a low threshold of 500 cfu/ml (Novak *et al.*, 2006). Therefore, early bacteraemia was not able to be detected using these primers. A retrospective study targeted the genes that code 16S rRNA. However following the detection of *B. pseudomallei* from various clinical specimens, the sensitivity was only found to be 61% (Chantratita *et al.*, 2007). Supaprom *et al* (2007) developed two qPCR assays that targeted genes from the TTSS. To evaluate the sensitivity and specificity of the assays, DNA was extracted from clinical specimens taken from patients diagnosed with septicaemia caused by infection with *B. pseudomallei* and other organisms. Two primers were designed, 8653 and 9438, both of which were highly conserved within the *B. pseudomallei* genome. Results demonstrated that both assays produced 100% specificity for the detection of *B. pseudomallei*, as there was no cross-reactivity with isolates of other species. The sensitivity was found to be 71% and 54%, for assays 8653 and 9438 respectively (Supaprom *et al.*, 2007). Like the previous study (Novak *et al.*, 2006), the detection of *B. pseudomallei* was also not low enough to be able to diagnose melioidosis in the early stages of infection. However, qPCR has the potential to reduce diagnosis time and therefore increase survival through early and rapid treatment with antibiotics.

2.12 Treatment

Early diagnosis and treatment is essential in reducing the mortality of melioidosis. Antibiotics used in the treatment of other Gram-negative infections are often not effective against *B. pseudomallei* infections. Drugs that have been recommended for the treatment of melioidosis can be toxic to the patient (Ashdown and Frettingham, 1984). *B. pseudomallei* is susceptible to a wide range of antibiotics including chloramphenicol,

the tetracyclines, carbapenems and third generation cephalosporins (White, 2003). The recommended treatment of melioidosis requires combination antibiotic therapy consisting of chloramphenicol, doxycycline, trimethoprim and sulphamethoxazole. This treatment regime, however, still results in mortality of greater than 70% in septicaemic patients with melioidosis (White *et al.*, 1989). Acute infection requires more intense antimicrobial therapy while chronic melioidosis is resistant to most antibiotics but is sensitive to prolonged use of the antibiotics, chloramphenicol and tetracycline (Mays and Ricketts, 1975). White *et al* (1989) studied the use of ceftazidime as a potential antibiotic in the treatment of severe melioidosis. The results demonstrated that the ceftazidime (23/38) halved the overall mortality compared to the conventional antibiotic regime (34/43). The study recommended that ceftazidime be adopted as the antibiotic of choice in the treatment of severe melioidosis (White *et al.*, 1989). Compliance with the antibiotic regime also decreases the chances of relapse and reactivation of the organism (Haase *et al.*, 1995a).

2.14 Vaccines

To date a vaccine to protect against melioidosis has not been developed. As an intracellular organism to develop an effective vaccine against *B. pseudomallei* it would need to stimulate both humoral and cell-mediated immunity. To date, candidate vaccines that target various factors of *B. pseudomallei* have been unsuccessful. Brett and Wood (2002) designed a conjugate vaccine that included two protective antigens, flagellin and polysaccharide antigens. By using two antigens it would possibly enhance the immune response further than if only one antigen had been used. The study used a rabbit model that involved monitoring of antibody levels to the vaccine pre and post immunisation. The results demonstrated successful conversion of the polysaccharide antigens to T-cell-dependent antigens (Brett and Woods, 1996). Juvenile diabetic rats were challenged with *B. pseudomallei* following immunisation and monitored for survival for eight days. However, to have an active immunisation study, a longer period of time would be required to monitor the animals and the model used would no longer be susceptible to challenge with *B. pseudomallei*, as only juvenile rats are susceptible to the organism

(Brett and Woods, 1996). Although the vaccine showed some protection without having an appropriate animal model the vaccine could not be fully investigated.

The mutant 2D2 has previously (Atkins *et al.*, 2002b) been shown to be significantly attenuated compared to the wild strain, differing in nutritional requirements. Haque *et al* (2006) used a live, attenuated mutant (2D2) in an immunisation study using a murine model. This study demonstrated that following immunisation with 2D2, mice were able to generate CD4+ T cell-mediated immunity. Following further analysis, the study demonstrated that the CD4+ T-cell response was specific to two antigens of the TTSS. Although, challenge studies with *B. pseudomallei* failed to demonstrate complete protection, the results show the crucial role of CD4+ T cells in protection. Therefore, developing a vaccine that induces a CD4+ T cell-mediated immune response may be critical in protection against *B. pseudomallei*.

DCs have also been studied to determine their effectiveness as a vaccine vector. Healey *et al* (2005) used DCs as a delivery vector to generate a CMI response to *B. pseudomallei*. Reponses were compared, in a murine model, following immunisation with *B. pseudomallei*-pulsed DCs or heat-killed *B. pseudomallei*. Following exposure to *B. pseudomallei*, DCs matured and presented epitopes to naïve T cells. Proliferation data demonstrated a significant increase in the T-cell response of lymphocytes taken from mice immunised with antigen-pulsed DCs compared to those from mice immunised with heat-killed *B. pseudomallei*. However, there was no antibody response, so the immunised was altered to include an injection of heat-killed *B. pseudomallei* in adjuvant. The results demonstrated an increase in antibody production when compared to the previous immunisation regime. This was also reflected in the survival of immunised mice, with mice that were injected with both pulsed-DCs and heat-killed *B. pseudomallei* having a 60% rate compared to other immunisation combinations of just DC's or heat-killed *B. pseudomallei* alone with survival of between 10-40% (Healey *et al.*, 2005). DCs may be effective in invoking an immune response to *B. pseudomallei*, however, it's efficacy as a vaccine is limited. A vaccine would need to be manufactured for each individual following the isolation, culture and exposure of DCs to *B. pseudomallei*, therefore, mass

immunisations would not be cost effective and time consuming. However, the results from this study (Healey *et al.*, 2005) reflect that both a CMI response and antibody response are important in *B. pseudomallei* infection. Therefore, a vaccine that would elicit both of these responses may be effective in providing protection against the organism.

CHAPTER 3

GENERAL MATERIALS & METHODS

3.1 Bacterial Isolates

3.1.1 Handling Category 3 pathogen, *B. pseudomallei*

B. pseudomallei is classed as a category 3 pathogen, therefore, all experimental procedures that involved manipulation of the organism were carried out in a Class II biosafety cabinet in the Physical Containment Level 3 (PC3) facility located in the School of Veterinary and Biomedical Sciences, James Cook University, Townsville.. Experimental animals were housed in the PC3 facility in a positive pressure isolation unit.

3.1.2 Origin of *B. pseudomallei* isolates

B. pseudomallei isolate NCTC 13179 from the Infectious Diseases and Immunopathogenesis Research Group culture collection was used to prepare a crude *B. pseudomallei* antigen. NCTC 13179 is a low virulence strain of *B. pseudomallei* previously characterised by Leakey *et al* (1998).

3.1.3 Preparation of *B. pseudomallei* lysate (BpLy1)

A crude BpLy1 was prepared using isolate NCTC 13179, using the method previously described by Ketheesan *et al* (2002). Briefly, NCTC 13179 was grown for 48 hours at 37ºC on blood agar (Appendix 1). A 0.9 McFarland standard suspension of *B. pseudomallei* was made in 30ml of phosphate-buffered saline (PBS; Appendix 1). The weight of bacteria was estimated and an equal amount of 0.1mm diameter glass beads (Sigma-Aldrich, Australia) was added. The bacteria and beads were suspended in breaking buffer (Appendix 1) and sonicated on ice for 4 x 10 minute bursts of 60W

(Biosonik III; Bronwill Scientific, USA) allowing 10 minutes resting between each burst. The suspension was centrifuged at 9000g for 12 minutes at room temperature and the supernatant was filtered using 0.22µm filters (Sarstedt, Australia) and then dialysed in PBS overnight at 4ºC. The resulting lysate was filtered again using 0.22µm filters. The protein concentration was estimated using a BCA protein assay kit (Progen, Australia) as per manufacturer's instructions. Sterility was confirmed following incubation of BpLy1 aliquots on blood and Ashdown's agar (Appendix 1) at 37ºC for 48 hours. BpLy1 (0.9mg/ml) was aliquoted into microcentrifuge tubes and stored at -70ºC. Prior to use, the lysate was thawed at room temperature and diluted to the required concentration in RPMI 1640 medium (Invitrogen, Australia) or PBS.

3.2 Experimental animals

3.2.1 Ethics approval

Experiments that involved laboratory animals required approval by the James Cook University Animal Ethics Committee and were carried out under Ethics Approval numbers A709 and A987. The experiments were performed in accordance with the guidelines defined by the National Health and Medical Research Council (NH&MRC) of Australia.

3.2.2 Maintenance of animals

BALB/c or C57BL/6 mice were bred by single pair sibling mating at the School of Veterinary and Biomedical Sciences small animal facility, James Cook University. Approximately equal numbers of male and female mice aged between eight and ten weeks were used for all experiments. Animals were housed in plastic, wire-topped boxes with wooden shavings and bedding and were supplied protein-enriched pellets and water, *ad libitum*. During the experimental period, animals were monitored twice daily. Animals that were moribund were euthanased via CO_2 asphyxiation.

3.2.3 Preparation and delivery of bacteria

B. pseudomallei NCTC 13179 was plated onto blood agar and incubated for 48 hours at 37ºC. A 1 x 10^8cfu/ml bacterial suspension was made in sterile PBS by preparing a suspension to an absorbance of optical density $(OD)_{650}$0.198. This suspension was then diluted to the desired concentration. The dose was further diluted in serial ten-fold dilutions and 20µl was plated in triplicate onto Ashdown's agar to calculate the exact inoculating dose given. Plates were incubated for 24-48 hours and colonies were enumerated. Inoculation of animals was carried out by injecting 200µl of the bacterial suspension either through *iv* or *ip* routes of infection using a 27 gauge needle.

3.2.4 Bacterial loads on animal organs

To determine bacterial loads in experimental animals, mice were euthanised using carbon dioxide (CO_2) asphyxiation. Organs were removed aseptically and placed into a stomacher bag (Sarstedt, Australia) with 1ml of sterile PBS. The spleens were then homogenised using a stomacher. The homogenate was then serially diluted in 10-fold dilutions in sterile PBS and 20µl of each dilution was plated onto Ashdown's agar in triplicate. Plates were incubated at 37°C for 24 to 48 hours. Colonies were counted and the mean bacterial loads for the organ were calculated. To determine the number of bacteria in the homogenate the following calculation was used:

Concentration (cfu/ml) = mean no. of colonies in 20µl sample x 50 x dilution factor

3.2.5 Lymphocyte proliferation assay

Mononuclear leukocytes (MNL) were separated from either heparinised blood or mouse spleens on 3ml Ficoll-Paque PLUS (GE Healthcare Bio-Sciences, NSW) in a 12ml culture tube (Sarstedt, South Australia). To separate the MNL the blood or homogenate was centrifuged at 500g for 20 minutes at room temperature. Sterile Pasteur pipettes were used to collect the layer of MNL which were then washed three times (500g; 10 minutes; room temperature) in RPMI. A cell count was performed using a

haemocytometer and the cells were suspended to a concentration of 10^6cells/ml in RPMI. The MNL were plated into 96 well U-bottom (Invitro technologies, Queensland) in culture media (Appendix 1). Cells were then stimulated with appropriate antigens and incubated at 37°C in 5% CO_2. . All tests were performed in triplicate and all work was carried out under sterile conditions. Following incubation from 48 to 168 hours, the cells were pulsed with 1.25μCi/ml ^{3}H-thymidine (GE Healthcare Biosciences, NSW) for 4 hours. Results from lymphocyte proliferation assays were expressed as a stimulation index (SI) which is defined as counts per minute from stimulated wells divided by counts per minute from unstimulated wells. The maximum SI for each stimulant was used in subsequent analyses.

CHAPTER 4

DETERMINATION OF THE SEROPREVALENCE TO *B. PSEUDOMALLEI* IN THE NORTHERN QUEENSLAND POPULATION

4.1 Introduction

The many clinical presentations of melioidosis is diverse but can be broadly categorised into acute and chronic infection, latent disease and relapse. Acute infections present in many clinical forms from pneumonia to septicaemia and multiorgan involvement, while chronic melioidosis presents as enduring suppurative abscesses. Latent or subclinical infections do not produce any symptomatic signs of disease. In the latter infections, *B. pseudomallei* is able to remain dormant in host cells until the individual becomes immunosuppressed. The disease can then recrudesce to produce an acute septicaemia. There is considerable evidence that *B. pseudomallei* has the potential to remain latent for long periods of time with recrudescence resulting in varying degrees of clinical severity (Mays and Ricketts, 1975). Relapse of melioidosis has been associated, in the majority of cases, with non-compliance of antimicrobial therapy (Haase *et al.*, 1995a).

The current 'gold standard' for the diagnosis of melioidosis involves culturing the organism from a clinical specimen followed by phenotypic identification. This method can lead to delays of up to four days while the bacterium is being identified. With approximately 50% of patients with acute septicaemic melioidosis dying within 48 hours of admission (Anuntagool *et al.*, 2000b) rapid diagnosis and treatment is essential. Immunological and molecular techniques to detect *B. pseudomallei* have been developed to prevent delays in treatment. Immunological methods can be divided into the detection of antibodies and the detection of antigens. The detection of antibodies employs tests such as the IHA, IFA, ELISA and ICT. The detection of *B. pseudomallei* antigen is superior to antibody detection because it is indicative of active infection. However these assays are still being refined for clinical use. Molecular techniques such as PCR and real-time PCR are also being developed to detect *B. pseudomallei* in clinical specimens.

These techniques are not yet considered to be sensitive or specific enough to replace the 'gold standard' (Sirisinha *et al.*, 2000).

Immunological assays are problematic for a number of reasons. Firstly, the IHA is not indicative of the infectious activity or the stage of the infection, with antibodies capable of circulating for several years following infection or exposure. Secondly, ELISAs can produce false negative results due to the low antibody levels that are produced in patients with acute sepsis. Thirdly, immunological assays can produce incorrect results due to variability and specificity of the antigen used (Vadivelu and Puthucheary, 2000).

A proportion of the population living in endemic regions is exposed to *B. pseudomallei* through environmental factors such as inhalation, direct inoculation of contaminated soil and surface water or ingestion of the organism. Not all of these individuals progress to fulminating disease, but instead produce an antibody response to the organism.

The IHA has been widely used in studies as both a diagnostic tool and an epidemiological tool to determine antibody levels to *B. pseudomallei* within endemic regions. This assay involves the incubation of heat-inactivated serum samples with antigen-sensitised blood cells to determine the presence of antibodies with haemagglutination being a positive result.

The use of the IHA as a diagnostic tool is problematic as it produces a high background reactivity in endemic regions as a result of environmental exposure (Appassakij *et al.*, 1990). A positive IHA test may not in itself diagnose melioidosis, as the IHA is not a measure of disease activity (Ashdown, 1987). The IHA measures both IgG and IgM antibodies. Therefore, IHA test results in endemic regions need to be assessed in conjunction with clinical evaluation. As an epidemiological tool the IHA has proven to be useful in evaluating the extent of exposure to *B. pseudomallei* in a healthy population.

Rapid serological diagnosis of melioidosis is important in endemic areas where there are minimal resources. These tests provide an accurate and rapid presumptive diagnosis that allows antibiotic treatment to begin whilst cultures are taken and the aetiological agent identified. Due to problems associated with the IHA, commercially available IgM and IgG ICT prototypes have been evaluated for diagnosis of melioidosis (Cuzzubbo *et al.*, 2000; O'Brien *et al.*, 2004; Wuthiekanun *et al.*, 2004; Chuah *et al.*, 2005; Cheng *et al.*, 2006b). An ICT involves the antigen of interest being embedded onto a nitrocellulose membrane. When the antibody joins to a colloidal gold-labelled protein present it binds to the antigen producing a visible pink/purple line. The sensitivity of the IgG ICT ranges from 50-100% with a specificity range from 47-95% whilst the IgM ICT had a sensitivity ranging from 67-93% and a specificity range from 47-95% (add ref). IHA sensitivity and specificity varies between 72-80% (add ref) and 64-91% (add ref), respectively depending on the cut-off titre used.

Melioidosis is endemic to northern Queensland. Ashdown and Guard (1984) surveyed over 9000 individuals in northern Queensland for antibodies to *B. pseudomallei* using the IHA method. The survey found that 5.7% of the population in this region had been exposed to the organism, producing an antibody response. Within the Townsville district, which included Townsville, Ingham, Ayr, Bowen, Home Hill, Charters Towers and Mt Isa, the seroporevalence was 4.9%. However, true exposure in North Queensland remains unclear as the study utilised serum collected from samples provided for unrelated blood tests to pathology laboratories and this may have introduced bias. Therefore, the purpose of this study was to determine exposure to *B. pseudomallei* within a healthy population of blood donors from the Townsville district.

The specific aims for the work described in this chapter were:

1. To determine the seroprevalence to *B. pseudomallei* in healthy blood donors from the Townsville district.

2. To evaluate the Melioidosis IgG Rapid Cassette Test as a screening method for antibody detection of *B. pseudomallei*

4.2 Materials and Methods

4.2.1 Subjects

Between 2003 and 2004 following informed consent, serum samples (n=1500) were collected from healthy blood donors who attended the Red Cross Blood Bank in Townsville, northern Queensland. Blood was collected into 6ml tubes with no anticoagulant and allowed to clot. It was then spun at 500g for 20 minutes at room temperature and the serum was removed and stored at -70ºC until needed. Ethics approval was obtained from the Australian Red Cross Blood Service Ethics Committee (2003#04) and James Cook University Human Research Ethics Committee (H1659) on condition that donor personal information and demographic details remained confidential.

4.2.2 Antigen preparation

To determine antibody levels in the donor serum, an IHA was used as previously described by Ashdown *et al* (1989). Antigen was prepared using five *B. pseudomallei* isolates cultured from patients with melioidosis presenting to the Townsville Hospital. The isolates were grown in protein-free liquid media for 2 weeks and the supernatant was collected (Gilmore *et al.*, 2007). The supernatant was heat-treated, preserved in phenol (Alexander *et al.*, 1970) and stored at -70ºC.

4.2.3 Indirect haemagglutination assay

Serum samples (4.2.1) were heat-inactivated by incubation at 54ºC for 30 min and then incubated with saline-washed non-sensitised sheep erythrocytes, to remove non-specific agglutinins, for 5 min at room temperature. The samples were then centrifuged at 11000g for 5 min at room temperature. The supernatants from each sample were diluted two-fold across 96-well microtitre plates in isotonic saline (1:5–1:40). Sensitised saline-washed sheep cells that had been incubated with antigen (4.2.2) at 37 º C for 1 hour were added to each well. For each sample, a negative control well was used where non-sensitised

cells were added. Each plate also contained control samples: a serum sample of a patient with melioidosis who presented to the Townsville Hospital and had produced a positive response in the IHA, as well as a serum sample from a volunteer who had not presented previously with melioidosis. The plates were then mixed gently and incubated at room temperature for 2-4 hours. Haemagglutination was indicated by a positive antibody response where the highest dilution was the end-point titre. An IHA titre was considered positive at ≥1:40 (Ashdown *et al.*, 1989).

4.2.4 Melioidosis IgG Rapid Cassette Test

Samples that demonstrated a titre of 1:40 were serially diluted to a titre of 1:5120. These samples were also used to assess the performance of PanBio's Melioidosis IgG Rapid Cassette Test (PanBio Ltd, Brisbane, Australia), which was designed to detect the presence of IgG antibodies to *B. pseudomallei*. An equal number of IHA negative samples were included in the assay. The assays were carried out in accordance with the manufacturer's recommendations. Briefly, 5µl of serum was placed on the cassette with 3 drops of buffer. The results were read 15 minutes after the buffer was added. A positive result was indicated by the formation of a pink/purple line on both the control and test areas (Figure 4.1).

Figure 4.1 Example of Melioidosis IgG Rapid ICT Cassette tests. The 1st test (left) demonstrates a faint positive, the 2nd test (middle) is indicative of a positive result and the 3rd test (right) indicates a negative result for antibodies to *B. pseudomallei*.

4.3 Results

4.3.1 Serum antibody levels

Of the 1500 donor serum samples that were tested for antibodies to *B. pseudomallei* using the IHA, 37 were found to have a positive titre of ≥ 1:40 indicating that 2.5% of the population tested had developed antibodies to *B. pseudomallei* (Figure 4.2). The 37 donor samples that were positive in the IHA were used to assess the Melioidosis IgG Rapid Cassette test and 32% (n=12) were found to be positive using this assay.

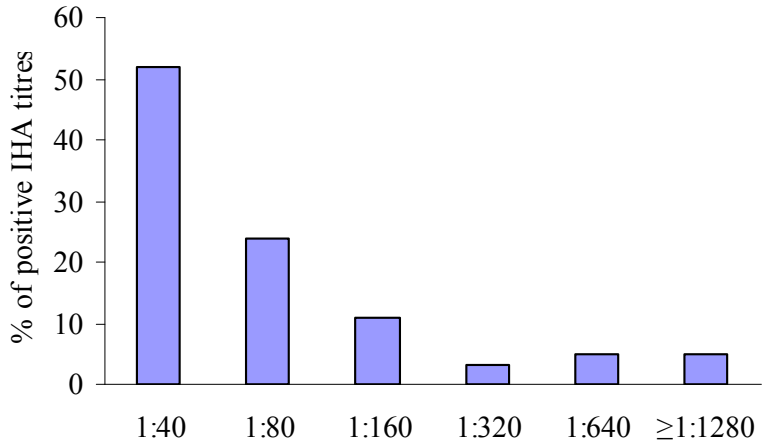


Figure 4.2 Positive IHA titres of ≥1:40. Of the 1500 serum samples screened, 37 had a positive result; 19 (52%) had a titre of 1:40, 9 (24%) had a titre of 1:80, 4 (11%) had a titre of 1:160, 1 (3%) had a titre of 1:320, 2 (5%) had a titre of 1:640 and the remaining 2 (5%) had a titre of equal to or greater than 1:1280.

Table 4.1 Exposure to *B. pseudomallei* as assessed by indirect haemagglutination assay

	Negative (<1:40)	Positive (≥1:40)	Seroprevalence within population
Male (n=711)	693	18	2.53%
Female (n=789)	770	19	2.41%
TOTAL (n=1500)	1463	37	2.47%

4.4 Discussion

The rate of seroprevalence reported in the current study is less than half that described in a previous report carried out over 20 years ago in the same region (Ashdown and Guard, 1984). This may be partially explained by sample bias in the earlier study. Serological surveys carried out in Malaysia and Thailand have used samples obtained from healthy volunteers to determine the extent of exposure to *B. pseudomallei*. In a study carried out

in Thailand, 20-30% of donors (study size of n=835) were seropositive in highly endemic regions in comparison to non-endemic regions where the seroprevalence was 6% (Naigowit *et al.*, 1992). In Malaysia, another endemic region, using 200 donor samples from two distinct regions, the seroprevalence was found to be 26.5% (Norazah *et al.*, 1996). The rate of seroprevalence in northern Queensland is lower than other endemic regions and may reflect differences in environmental, lifestyle and occupational factors. Extensive investigations involving demographic and past medical history data from donors may have helped to determine some of the factors that affect seroprevalence. However, obtaining ethical approval for such data collection involving blood donors was not possible in the current study.

In consecutive clinical samples collected from a pediatric population in a highly endemic region in Thailand, it was found that there was a sharp increase in antibody titres during the first four years of life (Wuthiekanun *et al.*, 2006a). The authors suggested that this result could be attributed to environmental exposure. Another study reported a higher seroprevalence rate in children from rural rice farming families (Kanaphun *et al.*, 1993) which provides further support that children are exposed to *B. pseudomallei* in endemic regions in their early years of life.

A recent survey of *B. pseudomallei* seroprevalence in East Timorese refugees using IHA was found to be 17% (Armstrong *et al.*, 2005). Since the majority of those who were positive were female, the authors proposed that these differences may reflect environmental factors related to exposure to *B. pseudomallei*. In the East Timorese culture, females do most of the physical activity and are therefore exposed to the environment more than males (Armstrong *et al.*, 2005). The study also found differences in socio-economic groups of East Timorese, with a higher seroprevalence in rural dwellers compared to people living in urban regions (Armstrong *et al.*, 2005). This is in contrast to the endemic regions of Taiwan where a 3:1 ratio of male to females with antibodies to *B. pseudomallei* was observed (Chen *et al.*, 2005). This difference was also attributed to occupational exposure, with males being in contact with the environment more frequently than females in this culture. In the current study the sex ratio was equal

which indicates that lifestyle and occupational factors including living conditions and socio-economic status, which may vary from one endemic region to another, should be considered when interpreting seroprevalence data.

It has been observed that not all *B. pseudomallei* culture-positive patients who develop melioidosis have a positive antibody test. This could be attributed to a failure to mount an antibody response to *B. pseudomallei*, or a more likely explanation is that the antibodies produced in these individuals are not recognised by the antigen preparations used in detection assays. One of the limitations in using the IHA is the uncharacterised nature of the antigenic epitopes and the antibodies involved (Cheng *et al.*, 2006a). The antigens incorporated in the IHA in this study were from five different *B. pseudomallei* isolates derived from patients presenting to the regional hospital. Variations in strains of *B. pseudomallei* that individuals are exposed to could possibly produce antibodies that are not directed against the crude antigen and are therefore undetected by the IHA. However, Gilmore *et al* (2007) found that the geographical source of the *B. pseudomallei* isolates used in the antigen preparations for the IHA did not have an effect on the resultant IHA titre. In this study, *B. pseudomallei* isolates from various locations including Papua New Guinea, Sri Lanka and Australia were used in the antigen preparation for the IHA. The results demonstrated similar IHA titres in the sera of culture-positive individuals (n=48). These results suggest that it may not be necessary to pool the antigens from different isolates to increase the specificity and sensitivity of the assay.

The study by Gilmore *et al* (2007) also used an antigen prepared from *B. thailandensis* to compare IHA titres of sera from culture-positive individuals. *B. thailandensis* has not yet been isolated from the environment in northern Queensland, however, exposure to the organism in endemic regions of Thailand has been suggested as the reason for high background seropositivity. The study found that 88% of the culture-positive sera tested cross reacted with *B. thailendensis* (Gilmore *et al.*, 2007). However it was noted that the antigen dilution was much lower than that used for the *B. pseudomallei* isolates.

Of the 37 donor samples that were positive in the IHA, only 32% were found to be positive using the ICT. The antigens used in the ICT assay are a commercial culture filtrate preparation of *B. pseudomallei*. Previous studies in northern Queensland have found the sensitivity of the IHA to be 85-92% (Ashdown *et al.*, 1989) and the specificity to be as high as 100% using a positive cutoff of ≥1:40 . While an initial study performed has shown the sensitivity and specificity of IgG ICT to be 100% (Cuzzubbo *et al.*, 2000), a study carried out in northern Queensland (Chuah *et al.*, 2005) found the sensitivity was 50.6% and specificity 97.4%. Antigenic differences between isolates from various regions could also account for some of the discrepancies observed. As a rapid diagnostic test, the the Melioidosis IgG Rapid Cassette Test together with clinical presentation would be useful to provide a presumptive diagnosis while the organism is being isolated and identified allowing antibiotic treatment to be initiated. However, results from the ICT alone should be used with caution considering the high seroprevalence rates in healthy populations of endemic regions.

This study has demonstrated that within a healthy population of an endemic region, the seroprevalence to *B. pseudomallei* is 2.5% which is considerably lower than the previously reported 5.7% (Ashdown and Guard, 1984). Although northern Queensland is considered to be an endemic region for melioidosis, these results suggest that subclinical infection may not be as widespread as previously considered. The differences observed in endemic regions of southeast Asia and northern Queensland may be due to varying levels of exposure to *B. pseudomallei* associated with occupational and recreational activities, together with varying environmental and weather patterns. These differences in endemic regions may also be as a result of the ability of the IHA to detect antibodies to *B. pseudomallei* and the cross-reactivity of the assay.

The results of this study show that individuals living in endemic areas can be exposed to *B. pseudomallei* and produce an antibody response. However *B. pseudomallei* is an intracellular pathogen and the development of protection would depend on the adaptive CMI responses that develop. Therefore it is also essential to determine CMI responses in individuals that have been exposed to *B. pseudomallei* and have a positive antibody response.

CHAPTER 5

CHARACTERISATION OF CELL-MEDIATED IMMUNE RESPONSE IN SEROPOSITIVE DONORS

5.1 Introduction

The innate immune response is the first line of defence against invading microorganisms. However, if this immune response is unable to control and eliminate the infection, the adaptive immune response is activated. Activation depends on interactions with various receptors and co-stimulatory molecules and secretion of cytokines and chemokines during the innate immune response (Li and Yang, 2008). The adaptive immune response is further divided into a CMI response and a humoral response. CMI responses are mainly involved in eliminating viral and intracellular bacterial pathogens. The CMI response involves the clonal selection and expansion of T cells following recognition of antigens from intracellular pathogens leading to the production of cytokines and activated T cells (both CD4 and CD8) that ultimately bring about the elimination of the pathogen. The main cytokine produced by CD4+ cells of the Th1 response is IFN-γ which activates macrophages. Other cells involved are CD8+ cytotoxic T cells which are recruited to kill infected cells. The Th2 response is primarily involved in removing extracellular infections through the activation of antigen-specific B cells to produce antibodies that opsonise bacterial pathogens. But this response is not effective in intracellular pathogenic infections.

B. pseudomallei is a facultative intracellular bacteria. Studies have shown that both the CMI and humoral response are important in clearing *B. pseudomallei* infection (Healey *et al.*, 2005). However, as *B. pseudomallei* is an intracellular organism, it is likely that the CMI response is the most efficacious immune response employed by the host. However, the immune responses to *B. pseudomallei* are not fully understood and have not been fully characterised. To date, studies characterising the CMI responses to *B. pseudomallei* have been limited.

L. monocytogenes and *M. tuberculosis* are also intracellular organisms, however, the immune responses produced by a host following infection have been more widely studied than in melioidosis. CD4+ helper T lymphocytes and CD8+ cytotoxic T-lymphocytes have been shown to play a critical role in protection following infection with *L. monocytogenes* where populations of these specific cells expand (Nagata *et al.*, 2008). Studies involving *M. tuberculosis* have also demonstrated the critical role of Th1 CMI responses in protection against the organism (Xu *et al.*, 2008). In support of this, IFN-γ has also been recognised as a major cytokine in the activation of the CMI response following infection with both of these pathogens (Nagata *et al.*, 2008; Xu *et al.*, 2008).

Further characterisation of T cell responses to *B. pseudomallei* will help determine the role of this response in the outcome of disease. Specifically, we examined whether seropositive individuals produce a strong cellular immune response and were therefore protected from *B. pseudomallei* infection. By characterising the immune responses and understanding which T cell subsets are involved, we hoped to determine the role of T cell subsets in protection.

The specific aim for the work described in this chapter is:

1. To characterise the CMI response to *B. pseudomallei* in seropositive donors previously screened (Chapter 4).

5.2 Materials & Methods

5.2.1 Subjects

Donors (n=37) who had a positive IHA titre (≥1:40) for *B. pseudomallei* and age-matched IHA negative donors (n=37; ≤1:40) were recalled from the 1500 donors screened (Chapter 4) to participate in this study. Of these age and sex matched donors, 8 donors agreed to participate. Ethics approval was obtained from the Australian Red Cross Blood Service Ethics Committee (2003#04) and James Cook University Human Research Ethics Committee (H1659) for these studies.

5.2.2 Lymphocyte proliferation assay

Heparinised blood collected from each donor was diluted 1:1 in RPMI (Invitrogen, Victoria) and layered onto 3ml Ficoll-Paque PLUS (Chapter 3; GE Healthcare Bio-Sciences, NSW) in a 12ml culture tube (Sarstedt, South Australia). Dilutions of the lymphocyes were carried out to achieve the required concentration of 10^6cells/ml. Cells were left unstimulated or stimulated with *B. pseudomallei* lysate (20µg/ml; Appendix 1), phytohemagglutinin (PHA; 10µg/ml; Sigma Aldrich, NSW) or Purified Protein Derivative (PPD; 15µg/ml; CSL, Queensland). Plates were incubated and harvested as previously described (Chapter 3).

5.2.3 Intracellular cytokine staining

Heparinised blood collected from each donor was divided into 3 separate 1ml aliquots and incubated for one hour at 37ºC in a 5% CO_2 incubator as unstimulated, stimulated with 1µg/ml BpLy1 or stimulated with phorbol myristate acetate (PMA; 50ng/ml; P1585; Sigma-Aldrich, NSW, Australia) and calcium ionophore (1µg/ml; C5149; Sigma-Aldrich, NSW, Australia). Brefeldin A (10µg/ml; 340512; Becton Dickinson, NSW, Australia) was added to inhibit secretion of cytokines and cells were incubated for a further 5 hours at 37ºC in a 5% CO_2 incubator. Ethylene diamine tetraacetic acid (EDTA; 2mM/ml; E26282; Sigma-Aldrich, Asutralia) was added to arrest activation and remove adherent cells. Red blood cells were lysed with FACSlyse (555899; Becton Dickinson, Australia) and the cells were fixed in 2% paraformaldehyde (Appendix 1) for 30 minutes. Cells were washed in sodium azide buffer (Appendix 1) and left overnight at 4ºC. The following day, the cells were divided into aliquots and stained with monoclonal antibodies for cell surface markers (Table 1; as per manufactuer's instructions). Cells were then permeabilized with FACS Permeabilizing Solution 2 (554722; Becton Dickinson, Australia) and stained with specific cytokine antibodies (Table 5.1).

Table 5.1. Combinations of monoclonal antibodies used in the study

Monoclonal antibody combination	Purpose for stain
IgG1 FITC / IgG1 PE / IgG1 PerCP	Isotype control
CD4 FITC / CD3 PE / CD8 PerCP	Different T cell subsets
CD69 FITC / IL-4 PE / CD4 PerCP	Activated T helper cells producing IL-4
CD69 FITC / IFN-γ PE / CD4 PerCP	Activated T helper cell producing IFN-γ
CD69 FITC / IL-4 PE / CD8 PerCP	Activated cytotoxic IL-4 producing T cells
CD69 FITC / IFN-γ PE / CD8 PerCP	Activated cytotoxic IFN-γ producing T cells

IgG1 FITC (#349041); IgG1 PE (#559320); IgG1 PerCP(#349044); CD4 FITC (#555346); CD3 PE (#555333); CD8 PerCP (#347314); CD69 FITC (#347823); IL-4 PE (#340451); CD4 PerCP (#347324); IFN-γ PE (#559326)

5.2.4 Fluorescent-activated cell scanning (FACS) analysis

Following staining of the cells, data from the various T cell lymphocyte populations and cytokines produced was collected using the FACScan™ (Becton Dickinson, Australia). Lymphocytes were gated on a dot plot and further analysed for the presence of specific surface markers and intracellular cytokines using dot plots and histograms. Percentages of cells of interest were determined (Appendix 2) using CellQuest Pro software (Becton Dickinson Immunocytometry Systems, California, USA).

5.2.5 Statistical analysis

Statistical analysis was performed using SPSS statistical software version 14.0. Results from the FACS analysis and lymphocyte proliferation assays were assessed by univariate analysis of variance. Data was assessed for normality using QQ plots. Maximum SI values were transformed by using the log function. The data from FACS analysis was calculated as percentage difference of unstimulated cells compared to BpLy1 stimulated cells. Data was log transformed. Data was considered significant if the probability of a type I error was less than 0.05%.

5.3 Results

5.3.1 Lymphocyte proliferation

The maximum SIs in response to BpLy1 were compared between seronegative controls (n=8) and seropositive donors divided into two groups based on titres of ≤1:80 (n=5) and ≥1:80 (n=3). No significant differences were observed between SI on seronegative and either of the seropositive groups (Figure 5.1a). There were also no significant differences in maximum SI of seronegative and seropositive groups in response to PPD (Figure 5.1b).

5.3.2 FACS analysis of lymphocytes from donors

No significant difference were observed in populations of CD3+/CD4+ (Figure 5.2a) and CD3+/CD8+ (Figure 5.2b) cells of seropositive donors when compared to lymphocytes isolated from seronegative donors. Following stimulation with BpLy1, there were no significant differences in IL-4 production (Figure 5.3a) or IFN-γ production (Figure 5.3b) by CD4+ lymphocytes isolated from seropositive donors compared to lymphocytes isolated from seronegative donors. There were also no differences seen in IL-4 production (Figure 5.4a) or IFN-γ production (Figure 5.4b) by CD8+ lymphocytes of seropositive donors compared to lymphocytes of seronegative donors.

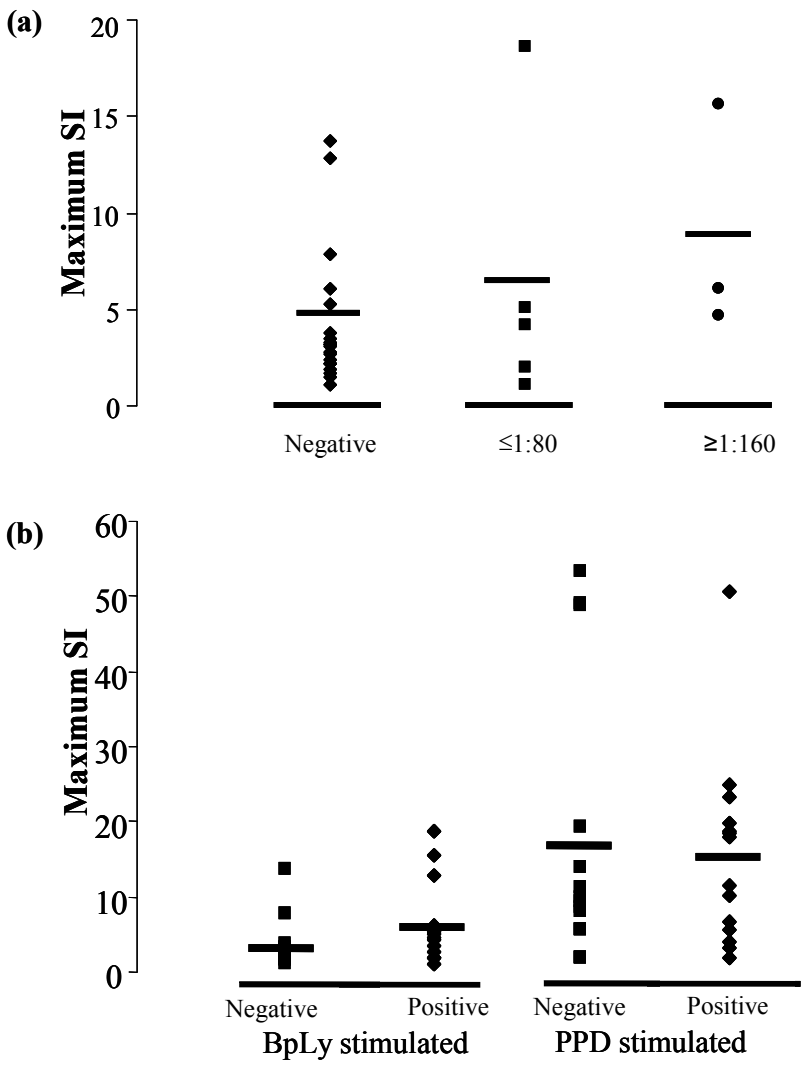


Figure 5.1 Proliferation of lymphocytes from seropositive and seronegative donors in a) response to BpLy1 and b) in response to BpLy1 and PPD. There was no significant difference between maximum SI of seronegative (n=16) and seropositive donors in either groups of ≤1:80 (n=5) and ≥1:80 (n=3). No significant difference in lymphocyte proliferation (maximum SI) between seronegative (n=16) and seropositive donors (n=16) was observed following stimulation with either BpLy1 or PPD. Mean values are displayed (horizontal lines).

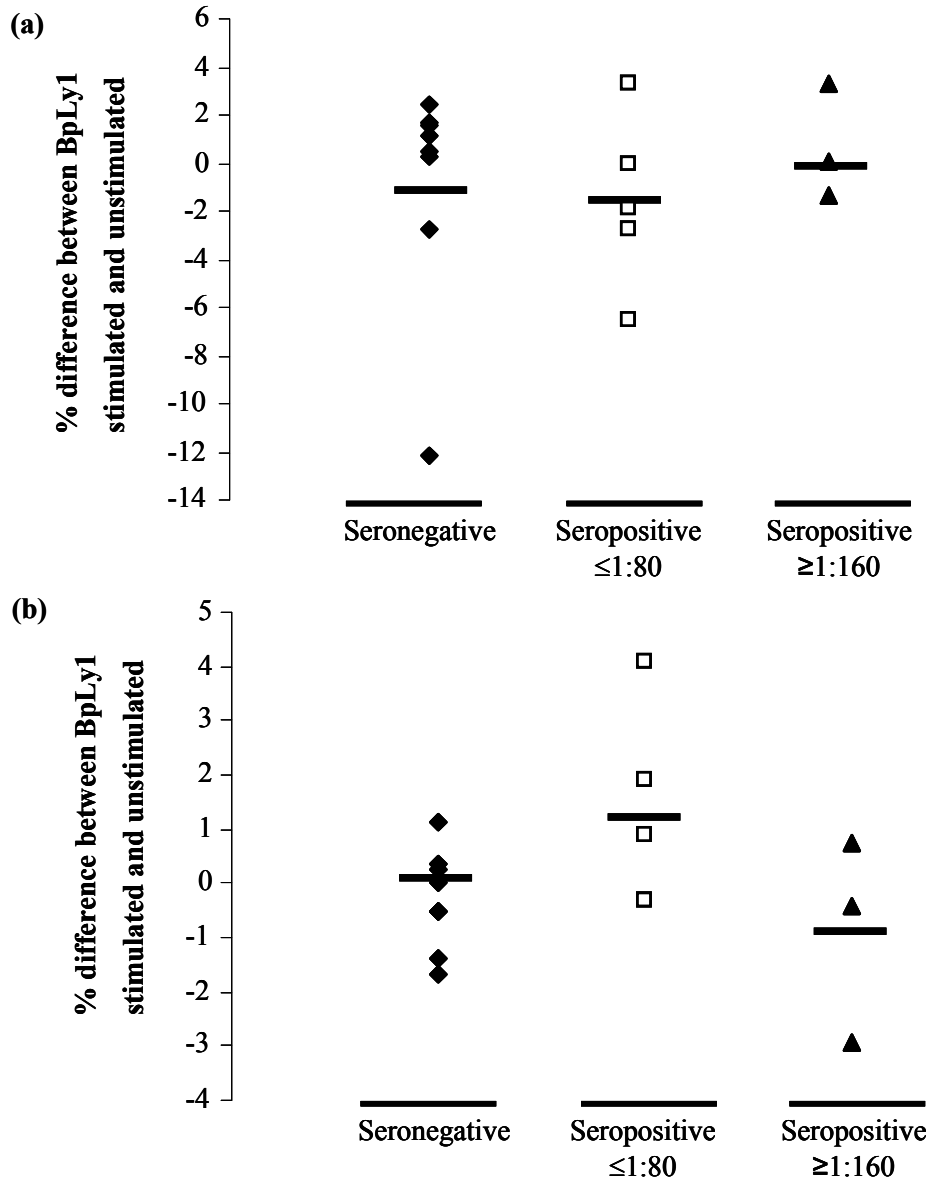


Figure 5.2 Percentage difference between BpLy1 stimulated and unstimulated a) CD3+/CD4+ cells and b) CD3+/CD8+ cells. No significant difference in CD3+/CD4+ populations following stimulation with BpLy1 between seronegative and seropositive donors was observed. No significant difference in CD3+/CD8+ populations following stimulation with BpLy1 between seronegative and seropositive donors was observed. Mean values are displayed (horizontal lines).

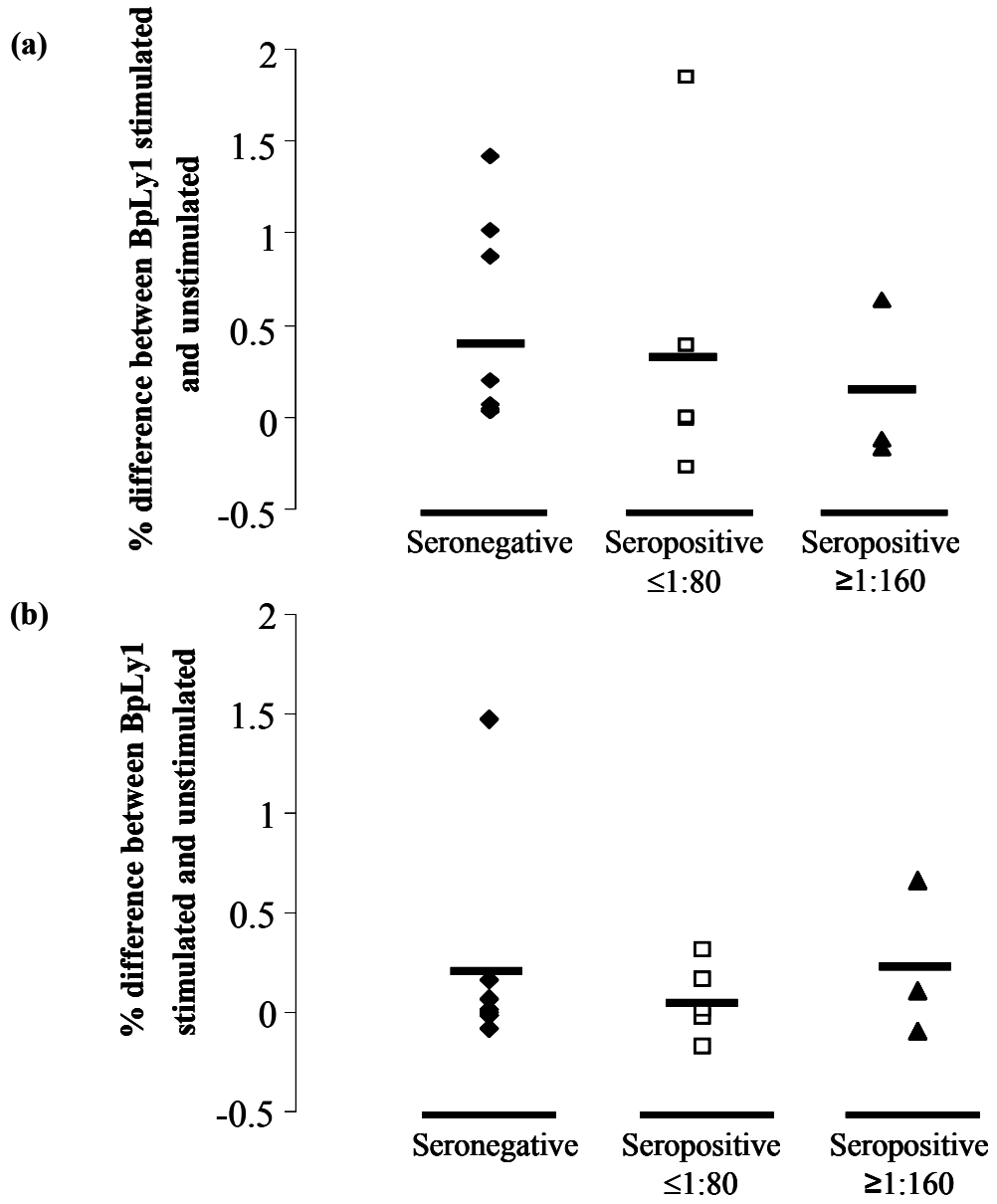


Figure 5.3 Percentage difference between BpLy1 stimulated and unstimulated a) activated CD4+ cells producing IL-4 and b) activated CD4+ cells producing IFN-γ. No significant difference in production of IL-4 by activated CD4+ populations following stimulation with BpLy1 between seronegative and seropositive donors was observed. No significant difference in production of IFN-γ by activated CD4+ populations following stimulation with BpLy1 between seronegative and seropositive donors was observed. Mean values are displayed (horizontal lines).

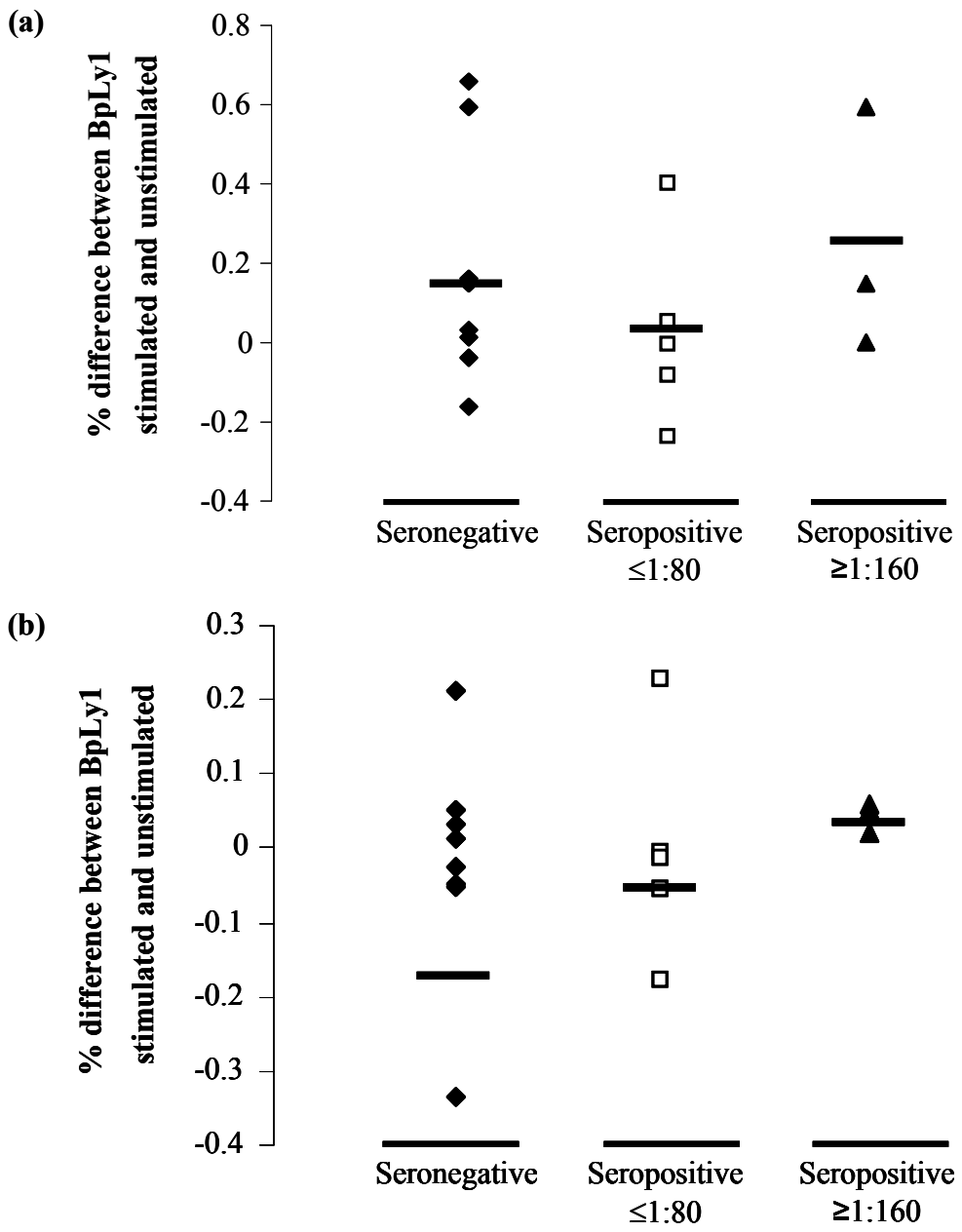


Figure 5.4 Percentage difference between BpLy1 stimulated and unstimulated a) activated CD8+ cells producing IL-4 and b) activated CD8+ cells producing IFN-γ. No significant difference in production of IL-4 by activated CD8+ populations following stimulation with BpLy1 between seronegative and seropositive donors was observed. No significant difference in production of IFN-γ by activated CD8+ populations following stimulation with BpLy1 between seronegative and seropositive donors was observed. Mean values are displayed (horizontal lines).

5.4 Discussion

Determining how the immune system responds to the specific pathogens is important in understanding how *B. pseudomallei* can remain latent and recrudesce, as well as being important in the future development of vaccines. Healthy individuals with antibodies to *B. pseudomallei* may have had a subclinical infection or may have been previously exposed to the organism. An individual with a subclinical infection does not present with symptoms typical of *B. pseudomallei* infection, however, should the immune system become compromised, clinical symptoms of melioidosis may occur. Subclinical infection in war veterans has been well documented, with the longest incubation period prior to reactivation reported as 62 years (Nguay *et al.*, 2005).

Lymphocyte proliferation and cytokine profiling using FACS analysis were used in the current study to monitor the immune response to *B. pseudomallei* in seropositive donors. The data demonstrated that seropositive donors did not have a significantly higher CMI following stimulation with BpLy1 antigen when compared to seronegative controls.

A lymphocyte proliferation assay was used to determine a memory T-cell response to *B. pseudomallei* antigens. The SI reflects the extent of the response. Proliferation of lymphocytes and IFN-γ production by lymphocytes of antigen stimulated cells, demonstrates recognition of *B. pseudomallei* antigens by memory T cells (Ketheesan *et al.*, 2002). No significant differences in lymphocyte proliferation were seen between seropositive and seronegative donors. However these results are not consistent with those observed by Ketheesan *et al* (2002) who found that patients who had recovered from melioidosis had significantly higher proliferation of lymphocytes in the presence of BpLy1 compared to seronegative donors. As there was no significant difference in lymphocyte proliferation between seropositive and seronegative donors following stimulation with PPD, a ubiquitous T cell antigen, the donors did not have defective immune responses.

Govan & Ketheesan (2004) studied the CMI responses in Vietnam veterans. The results demonstrated a significantly higher lymphocyte proliferation following stimulation with a *B. pseudomallei* antigen when compared to negative controls (Govan and Ketheesan, 2004). The study demonstrated the development of a CMI response to *B. pseudomallei* even though an IHA titre was not present in the majority of the veterans. By excluding the veteran with a positive IHA titre, the results were still significantly higher than controls.

Barnes *et al* (2004) compared CMI responses in 3 groups including individuals who were 1) patients who were symptomatic, culture positive; 2) culture negative, seropositive and 3) culture negative and seronegative. The results demonstrated proliferating lymphocytes from seropositive individuals in response to stimulation with *B. pseudomallei* antigen were significantly greater than culture positive individuals. IFN-γ production was also significantly greater in seropositive individuals compared to culture positive individuals. These results suggest that seropositive individuals may be producing a stronger CMI response and are able to clear the infection more efficiently.

Ketheesan *et al* (2002) analysed the CMI response in patients who had recovered from melioidosis and found that compared to controls the proliferation of lymphocytes in response to *B. pseudomallei* antigen was significantly greater. IFN-γ production was also significantly greater in patients and activated (CD69+) CD4+ and CD8+ populations were significantly larger than populations in controls. The increase in IFN-γ and presence of CD69 are indicative of both an activated CD4+ and CD8+ T cell response to *B. pseudomallei*.

Previous studies have demonstrated the importance of CD4+ and CD8+ responses in *B. pseudomallei* infection (Haque *et al.*, 2006a; Haque *et al.*, 2006b). However, this current study failed to demonstrate either a CD4+ or CD8+ response or the production of cytokines in response to BpLy1 in seropositive donors. This may reflect issues, such as, the reliability of the IHA and the antigen used in the IHA to be specific to *B. pseudomallei*. It has been previously found in animal models that the infecting isolate

(Ulett *et al.*, 2001), the route of infection (Barnes and Ketheesan, 2005) and the antigens used (Haque *et al.*, 2006a) influence the immune response produced by the host following infection with *B. pseudomallei*.

In previous studies (Ketheesan *et al.*, 2002; Govan and Ketheesan, 2004), the IHA titre was not consistent. Patients who had recovered from melioidosis had varying IHA titres from titres of <1:40 through to >1:5120 (Ketheesan *et al.*, 2002). In patients who had recovered from melioidosis it would be expected that clearing the infection would not only produce an effective CMI response but would also produce relatively high antibody levels. This, however, was not demonstrated. The Vietnam veterans from the study by Govan & Ketheesan (2004) had a negative titre except for one who had a titre of 1:320 and in the study by Barnes *et al* (2004) the IHA titres also varied from <1:40 to >1:5120. These observations are consistent with immune responses to intracellular bacteria where a CMI response is more effective in clearing the infection rather than a humoral response.

The antigens used in both the IHA and the immunoassays could also influence the lymphocyte responses. Previous studies have found that antigens have different immunising potential (Haque *et al* 2006). As discussed previously (Chapter 4) there are disadvantages with the IHA and the sensitivity of the assay, due to the use of uncharacterised antigens. It is possible that individuals who have been exposed to *B. pseudomallei* would produce antibodies that are not directed against the crude antigen and therefore are undetected. Therefore, some of the individuals used as controls in this study may have previously been exposed to *B. pseudomallei* without their knowledge at an extent too limited to produce a detectable immune response. On the other hand, it is also possible that positive IHA titres are a result of cross-reactivity to other related *Burkholderia* species. Gilmore *et al* (2007) observed that sera from *B. pseudomallei* culture-positive patients also cross-reacted with *B. thailandensis* antigen preparation.

This study demonstrated that there were no significant differences in proliferation of lymphocytes, activation of T cell subsets or production of cytokines between *B. pseudomallei* seropositive and seronegative blood donors. The lack of difference

between the two groups may have been a result of the detection assay (IHA) used or the antigens used. To gain further insight into the immune responses to *B. pseudomallei,* extending the study to include patients who have recovered from melioidosis, those who have had melioidosis and relapsed would allow comparison of the CMI response between the groups. Further understanding of the immunopathogenesis of *B. pseudomallei* and knowledge of the immune responses that develop will provide basic scientific data that would also be useful in developing vaccine strategies.

CHAPTER 6

RELAPSE AND LATENCY IN *BURKHOLDERIA PSEUDOMALLEI* INFECTION

6.1 Introduction

Melioidosis has a broad spectrum of clinical presentations. Clinical presentations include acute, chronic and latent infection. Relapse has been widely described in patients who have apparently recovered from melioidosis (Haase *et al.*, 1995a; Vadivelu *et al.*, 1997). It has been suggested that the recurrence of melioidosis is a result of the initial organism recrudescing rather than a new strain infecting the patient (Desmarchelier *et al.*, 1993). Relapse is defined as the development of new signs and symptoms of melioidosis following apparent recovery (Limmathurotsakul *et al.*, 2006) and a positive culture of *B. pseudomallei* from a previously culture negative site (Chaowagul *et al.*, 1993).

Risk factors for relapse of melioidosis include non-compliance with antibiotic treatment (Chaowagul *et al.*, 1993) and the immune system becoming compromised (Vadivelu *et al.*, 1998). Clinical presentations and the severity of the disease may also contribute to relapse with systemic disease more likely to relapse when compared to localised infections (Chaowagul *et al.*, 1993). In addition, since *B. pseudomallei* infection requires prolonged antibiotic treatment, it is also possible that the bacterium becomes resistant to the antibiotics, therefore increasing the chance of relapse. Chaowagul *et al* (1993) monitored melioidosis patients for relapse and found that clinical severity and choice of initial antibiotic treatment were factors that contribute to recurrence.

Previous studies on *B. pseudomallei* isolates from patients who had relapsed have demonstrated that infection with a new strain of *B. pseudomallei* is uncommon (Desmarchelier *et al.*, 1993). Rather, it is more like that the original infecting strain has recrudesced. Molecular typing methods, including PFGE and MLST have been used to determine clonality of *B. pseudomallei* isolates from patients who have relapsed with melioidosis.

Latent *B. pseudomallei* infection occurs when an individual has been exposed to the organism and does not produce any signs of clinical infection, but produces an immune response. If the individual later becomes immunocompromised, *B. pseudomallei* is able to recrudesce to produce clinically apparent disease. Latent *B. pseudomallei* infection has been described in ex-servicemen who have developed melioidosis years after primary exposure. To date the longest incubation period is 62 years in a prisoner of war from World War II (Nguay *et al.*, 2005). It is not known where *B. pseudomallei* persists while it is dormant in the host.

Latency has also been described in other intracellular infections including *Mycobacterium tuberculosis* (Parrish *et al.*, 1998). Similarly it is unclear as to where this organism remains latent in the host, but two possible hypotheses have been proposed: 1) *M. tuberculosis* remains in old pulmonary granulomatous lesions or pulmonary lymph nodes, and 2) the organism is maintained in widely disseminated sites from the early bacteraemic phase following initial exposure (Parrish *et al.*, 1998).

While there are no published animal models of latency in melioidosis, attempts have been made to develop an animal model for latent tuberculosis. One early study describes, the Cornell model, (McCune *et al.*, 1966a; McCune *et al.*, 1966b) where mice are infected with *M. tuberculosis*, and then treated with antimycobacterial drugs. The mice reached a 'sterile-state' that included negative bacterial culture in tissues and rare bacilli observed in smears. Following cessation of antibiotic treatment, the mice subsequently developed active infection. Other groups have attempted a guinea pig model (Kashino *et al.*, 2008) and a rabbit model (Manabe *et al.*, 2008) to develop latent tuberculosis infection.

B. pseudomallei is a facultative intracellular organism. As such, if the CMI response is impaired, the ability for the host to successfully clear the organism will be reduced increasing the possibility for relapse and latent infection. *B. pseudomallei* is able to invade phagocytic cells and survive within host cells, avoiding typical host clearance mechanisms (Jones *et al.*, 1996). Development of a murine model of latent melioidosis will aid in understanding the immunopathogenesis of this disease. Using traditional

culture methods in conjunction with real-time PCR (qPCR), detection of the site of *B. pseudomallei* persistence in a murine latency model may be possible.

The specific aims for the work described in this chapter are to:

1. To characterise *B. pseudomallei* isolates from patients with recurrent melioidosis using PFGE

2. To develop a murine model of latent melioidosis

6.2 Materials & Methods

6.2.1 Characterisation of *B. pseudomallei* isolates from patients with recurrent melioidosis

6.2.1.1 Collection and recovery of *B. pseudomallei* isolates from patients

B. pseudomallei isolates (n=39) were cultured from patients with melioidosis (n=12) following the collection of blood, sputum, urine, CSF or wound swabs (Table 6.1). Relapse of melioidosis was defined as the presence of *B. pseudomallei* in a previously culture negative site by the Infectious Diseases Clinician at the Townsville Hospital. Each isolate was identified using Vitek 1 (Biomerieux, Australia) and stored at -70°C. All isolates were cultured on blood agar and grown at 37°C for 48 hours. Each plate was visually checked for the presence of a pure culture.

Table 6.1 *B. pseudomallei* isolates cultured from patients with recurrent melioidosis

Relapse patient	Date *B. pseudomallei* cultured	Relapse patient	Date *B. pseudomallei* cultured
A	01/09/1996	**D**	22/03/1996
	05/06/1997		17/06/1997
	12/07/1999		29/09/1998
	02/09/1999		12/10/1998
	01/03/2000		21/02/2000
	24/07/2001		
	22/04/2002	**F**	08/04/2003
	07/10/2002		03/05/2003
	30/03/2004		15/05/2003
E	30/03/1999		
	10/06/1999		
	02/07/1999		

6.2.1.2 Preparation of plugs for PFGE

A suspension of each *B. pseudomallei* isolate of OD_{550}1.00-1.25 was prepared in TE buffer (Appendix 1). Each suspension (130µl) was pipetted into a sterile microcentrifuge tube and washed by centrifuging at 4,000g for 5 minutes at 4°C. The supernatant was removed and the pellets stored at -70°C overnight. Proteinase K (10µl of 1mg/ml; P2308, Sigma Aldrich, Australia; Appendix 1) and 100µl Tris-EDTA (TE) buffer (Appendix 1) was added to each pellet with 100µl molten 1.8% low melt agarose held at 60°C. The suspension was mixed thoroughly and loaded into pre-chilled (-20°C) plug moulds. Plugs were allowed to set at 4ºC for 20 minutes before gently pushing them into tubes with 500µl Tris-EDTA sarcosine (TES) buffer (Appendix 1) and 20µl Proteinase K (20mg/ml). Plugs were incubated overnight at 50°C.

6.2.1.3 Washing and enzymatic treatment

The TES buffer was removed from each tube and plugs were washed as per the following protocol:

- 1 x 30 minutes in TE buffer at room temperature
- 2 x 1 hour in TE buffer at room temperature
- 2 x 1 hour in TE buffer at 50°C
- 1 x 30 minutes in 0.1x TE buffer (Appendix 1) at room temperature
- 1 x 30 minutes in 300µl restriction enzyme buffer (Appendix 1)

The restriction enzyme buffer was replaced with 500µl fresh buffer with 3µl Bovine Serum Albumin (BSA; Promega, Australia; Appendix 1) and 5µl of restriction enzyme (*Xba1*; R6185; Promega Corporation, United States of America) and incubated overnight at 37°C. This step was repeated 24 hours later, with the restriction enzyme buffer being replaced, 3µl BSA and 5µl restriction enzyme added and incubating the plugs again overnight at 37°C. After further 24 hour incubation, the buffer was removed and TE buffer added. The plugs were stored at 4°C until use.

6.2.1.4 Preparation of gel for PFGE

A 1% agarose gel was made by heating 0.8g PFGE grade agarose (X174, Amresco, United States of America) and 80ml of 0.5x Tris-Boric-EDTA (TBE) buffer (Appendix 1). The molten agarose was allowed to cool slightly by placing in a 56°C water bath for 20 minutes. The gel casting stand was assembled and the gel was poured in and left to set for 30 minutes. Once the gel was set, the casting stand was removed.

6.2.1.5 Loading and electrophoresis

Each agarose plug was cut in half and loaded into the wells of the gel. The tops of the wells were then filled with 1.8% molten agarose and left to set for 5minutes. The gel was placed into the electrophoresis tank with 2.5 litres of 0.5x TBE buffer pre-cooled to 14°C. Thiourea (final concentration 50µM; T8656; Sigma-Aldrich, Australia; Appendix 1) was added to the electrophoresis buffer. Pulsed-field electrophoresis of the isolates was carried out on Bio-Rad's CHEF Mapper XA Pulsed Field Electrophoresis System at 6V/cm at an angle of 120° with a ramping of 5.5-52 seconds for 20.2 hours at 14°C.

6.2.1.6 Staining of agarose gel

Each gel was stained with ethidium bromide (Appendix 1). The gel was left in the ethidium bromide solution for 2 minutes and then destained in TE buffer overnight. The macrorestriction patterns were visualised using Bio-Rad's Geldoc scanner system.

6.2.2 Murine model of latent melioidosis

6.2.2.1 Infection of mice

C57BL/6 mice (n=60) aged between 8-9 weeks were divided into two groups: control (n=18) or infected (n=42). Control mice were injected *iv* in the tail vein with 200µl PBS (pH 7.4) using a 27 gauge needle. Infected mice were injected *iv* with 0.003 x LD_{50} NCTC13179 (1.62 x 10^4 cfu/200µl). The dose was calculated following serial dilutions (Chapter 3).

6.2.2.2 Delayed-type hypersensitivity (DTH) response to *B. pseudomallei*

On days 10, 20 and 30 post-infection, control (n=3) and infected (n=6) mice were assessed to evaluate the CMI response to *B. pseudomallei*. Antigen prepared from *B. pseudomallei* (BpLy1; 5µg/40µl) was injected into the right hind footpad

(subcutaneous; *sc*) and PBS (pH7.4) injected into the left hind footpad of each mouse using a 27 gauge needle. Footpad thickness (mm) was measured, with seven replicates for each footpad, using electronic callipers at 48 hours. The difference in footpad thickness between BpLy1 injected and PBS injected was calculated and recorded.

6.2.2.3 Bacterial loads in mice

On days 10, 20 and 30 post-infection, splenic and liver bacterial loads were enumerated in infected mice (n=3) (Chapter 3) to demonstrate clearance or otherwise of *B. pseudomallei*.

6.2.2.4 Lymphocyte proliferation assays

On days 10, 20 and 30 post-infection, spleens were aseptically removed from control (n=3) and infected (n=3) mice. Spleens were homogenised through a strainer, resuspended in RPMI and the suspension allowed to settle for 20 minutes. The supernatant was then layered onto 3ml Ficoll-Paque (Chapter 3). Dilutions of control and infected cells were carried out to achieve the required concentration of cells (1×10^6 cells/ml) and stimulated with BpLy1 (20µg/ml) or Concavalin A (Con A; Appendix 1; Sigma Aldrich, NSW). Plates were incubated and harvested as previously described (Chapter 3).

6.2.2.5 Storage of organs and extraction of DNA for qPCR

At days 10, 20 and 30 post-infection, sections of spleen and liver from infected and control animals were removed using a scalpel blade and frozen at -70ºC until required. Tissue samples were homogenised through a strainer and resuspended in PBS (pH 7.4). The homogenate was sonicated on ice using 2 x 30 second bursts of 60W (Biosonik III; Bronwill Scientific, USA) with a 5 minute rest between each burst. The sonicate was then centrifuged at 4,000g for 5 minutes and the supernatant was removed. The pellet was resuspended in 1ml of PBS and DNA was extracted using the RBC DNA extraction

kit (DKSH, Victoria, Australia) for cultured/bacterial cells as per the manufacturer's instructions. Extracted DNA was stored at 4°C until qPCR was performed.

6.2.2.6 Immunosuppression of mice

At day 43, control (n=6) and infected (n=12) were injected with 1.5mg/200µl dexamethasone (Sigma Aldrich, NSW). Dexamethasone was prepared by diluting in PBS (pH7.4) to 7.5mg/ml. Mice were injected *ip* once per day over six days. Animals were monitored for survival and any moribund animals were euthanized by CO_2. At day 60, control/untreated (n=3), control/dexamethasone treated (n=3), infected/untreated (n=3) and infected/dexamethasone treated (n=3) were evaluated for DTH response as described in section 6.2.2.2. Bacterial loads (6.2.2.3), lymphocyte proliferation (6.2.2.4) and extraction of DNA (6.2.2.5) were also carried out. The experiment was terminated at day 90 at which time bacterial loads and DNA extractions were completed on the remaining animals.

6.2.2.7 Real-time PCR

Real-time PCR was carried out following the extraction of DNA from spleens and livers of mice. The assay used previously published primers that target the TTSS1 gene cluster (Supaprom *et al.*, 2007): forward-ATCGAATCAGGGCGTTCAAG, reverse-CATTCGGTGACGACACGACC. The assay was optimised at the School of Veterinary and Biomedical Sciences using Rotor-Gene RG-3000 (Corbett Research, Australia)

6.2.2.8 Statistical analysis

Statistical analysis was performed using SPSS statistical software version 14.0. Results from DTH and lymphocyte proliferation assays were assessed by univariate analysis of variance. Data was assessed for normality using QQ plots. The data for DTH responses was calculated as change in footpad thickness (mm) between PBS injected footpads subtracted from BpLy1 injected footpads (±SEM). Analysis of mouse survival rates was

carried out using GraphPad Prism 5 Kaplan-Meier statistical analysis. Data was considered significant if the probability of a type I error was less than 0.05%.

6.3 Results

6.3.1 Clonality of isolates from patients with recurrent melioidosis

Of the 12 patients with recurrent melioidosis, isolates from four (A, D, E and F) were characterised following digestion with restriction enzyme *XbaI*. The fragment patterns produced from PFGE demonstrated clonally-related isolates for each patient (Figure 6.1 & Figure 6.2).

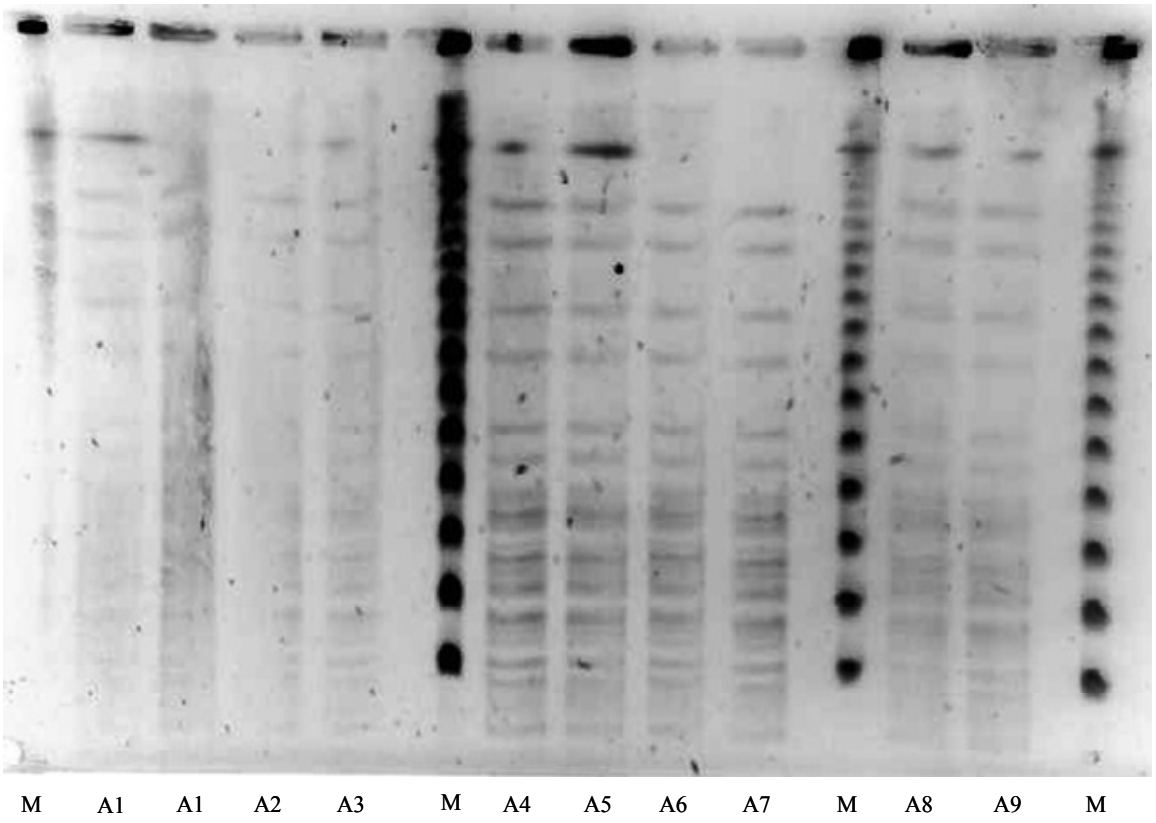


Figure 6.1 Example of PFGE image of isolates cultured from patient A with recurrent melioidosis. Clonally-related fragment patterns were produced following digestion with restriction enzyme, *XbaI*, of 9 isolates cultured from patient A. M: marker; A1: 01/09/1996; A2: 05/06/1997; A3: 12/07/1999; A4: 02/09/1999; A5: 01/03/2000; A6: 24/07/2001; A7: 22/04/2002; A8: 07/10/2002; A9: 30/03/2004.

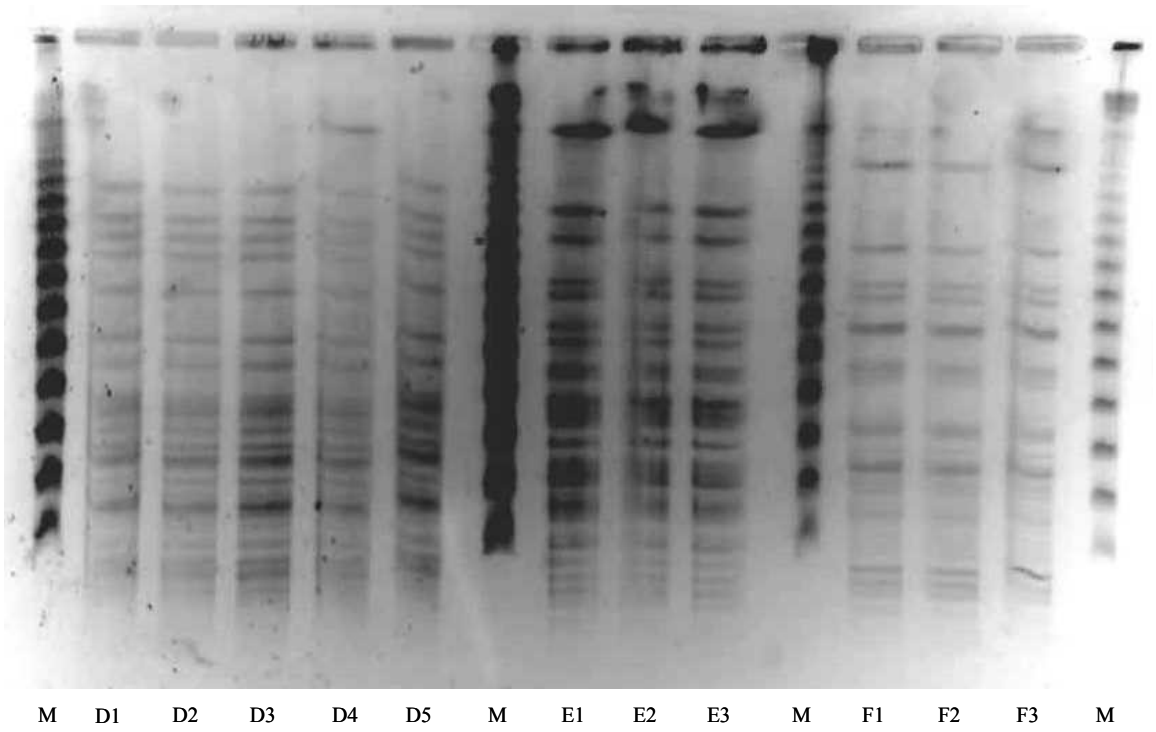


Figure 6.2 Example of PFGE image of isolates cultured from patients D, E & F with recurrent melioidosis. Clonally-related fragment patterns were produced following digestion with restriction enzyme, *XbaI*, isolates cultured from patient D (n=5), E (n=3) and F (n=3). M: marker; D1: 22/03/1996; D2: 17/06/1997; D3: 29/09/1998 D4: 12/10/1998; D5: 21/02/2000; E1: 30/03/1999; E2: 10/06/1999; E3: 02/07/1999; F1: 08/04/2003; F2: 03/05/2003; F3: 15/05/2003.

6.3.2 DTH responses following infection with *B. pseudomallei*

At days 10, 20 and 30 post-infection, footpad swelling (mm) 48 hours following injection of BpLy1 and PBS in control and infected mice was monitored. Significant differences ($p<0.01$) were observed between footpad swelling of control mice and infected mice (Figure 6.3).

6.3.3 Lymphocyte proliferation following infection with *B. pseudomallei*

Lymphocyte proliferation in response to BpLy1 of infected and control mice were assessed at days 10, 20 and 30 post-infection. There was a significant increase ($p<0.01$)

in proliferation of lymphocytes of infected mice at days 10, 20 and 30 when compared to control mice (Figure 6.4).

6.3.4 Bacterial loads following infection with *B. pseudomallei*

Following infection with *B. pseudomallei*, bacterial loads in the spleen and liver were enumerated at days 10, 20 and 30. No detectable *B. pseudomallei* was observed following the culture of the spleen and liver of infected animals on days 10, 20 or 30.

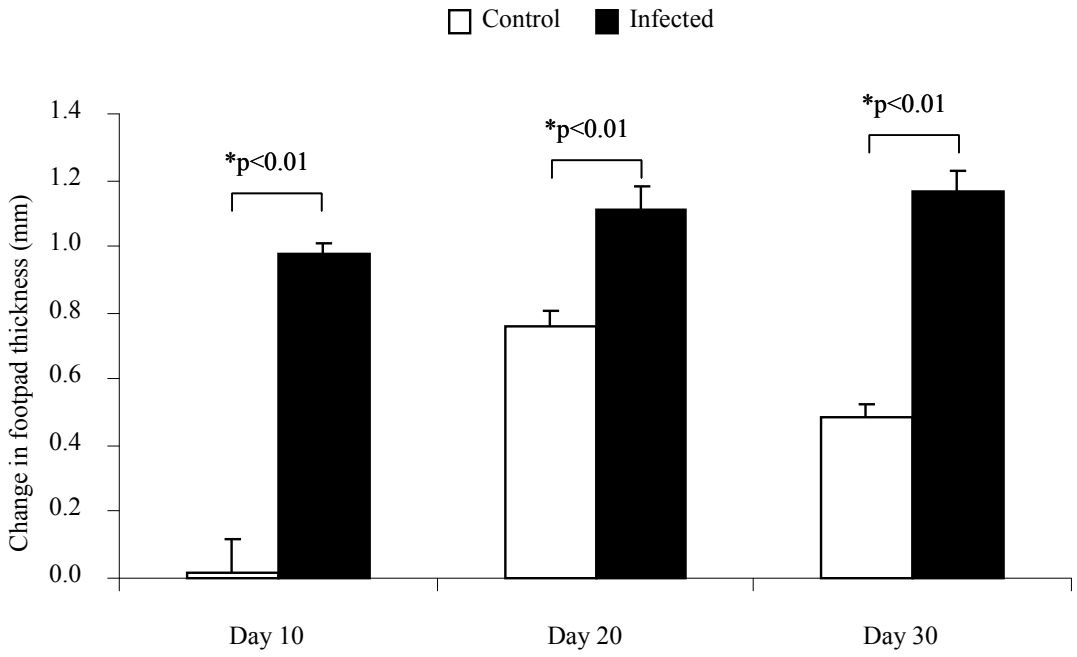


Figure 6.3 DTH responses at days 10, 20 and 30 following low dose infection with *B. pseudomallei*. There was a significant increase ($p<0.01$) in footpad thickness between control and infected mice at days 10, 20 and 30 post-infection. Results are expressed as change in footpad thickness (mm) ± SEM.

6.3.5 DTH responses following immunosuppression

At day 60 post-infection, change in swelling (mm) at 48 hours between BpLy1 and PBS injected footpads of the 4 groups of mice: 1) control, untreated; 2) control, dexamethasone treated; 3) infected, untreated mice and 4) infected, dexamethasone treated mice was monitored. Significant differences ($p<0.01$) were observed between

footpad swelling of control mice and infected mice following low dose infection with *B. pseudomallei* (Figure 6.5).

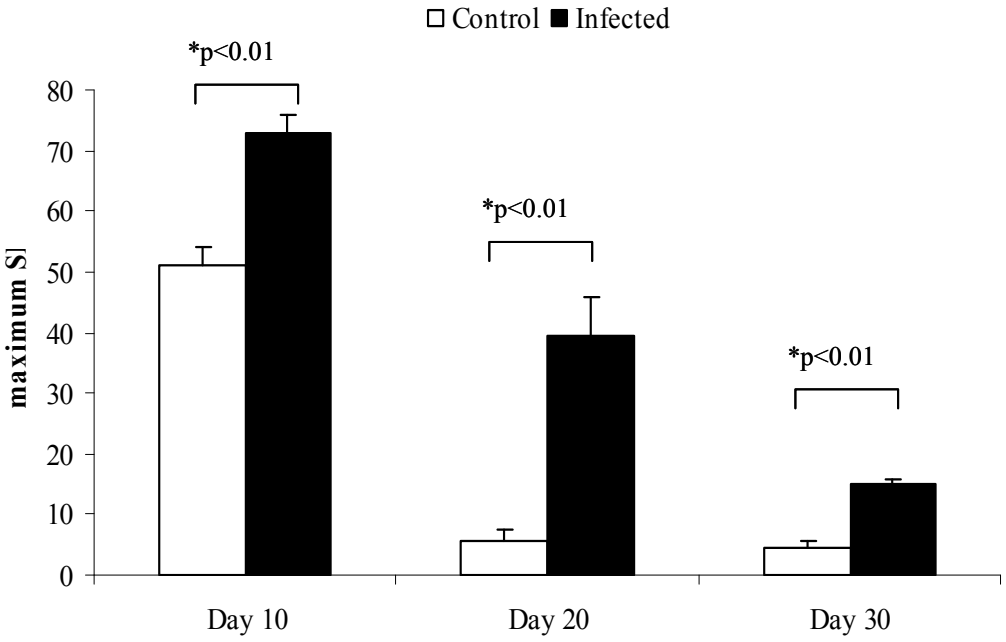


Figure 6.4 Lymphocyte proliferation responses at days 10, 20 and 30 following low dose infection with *B. pseudomallei*. There was a significant increase ($p<0.01$) in lymphocyte proliferation in infected mice compared to control mice following stimulation with BpLy1. The significant differences were seen at days 10, 20 and 30. Results are expressed as maximum SI ± SEM.

6.3.6 Lymphocyte proliferation following immunosuppression

No significant differences were observed in the proliferative response of lymphocytes from infected, untreated and infected, dexamethasone treated mice following stimulation with BpLy1 (Figure 6.6).

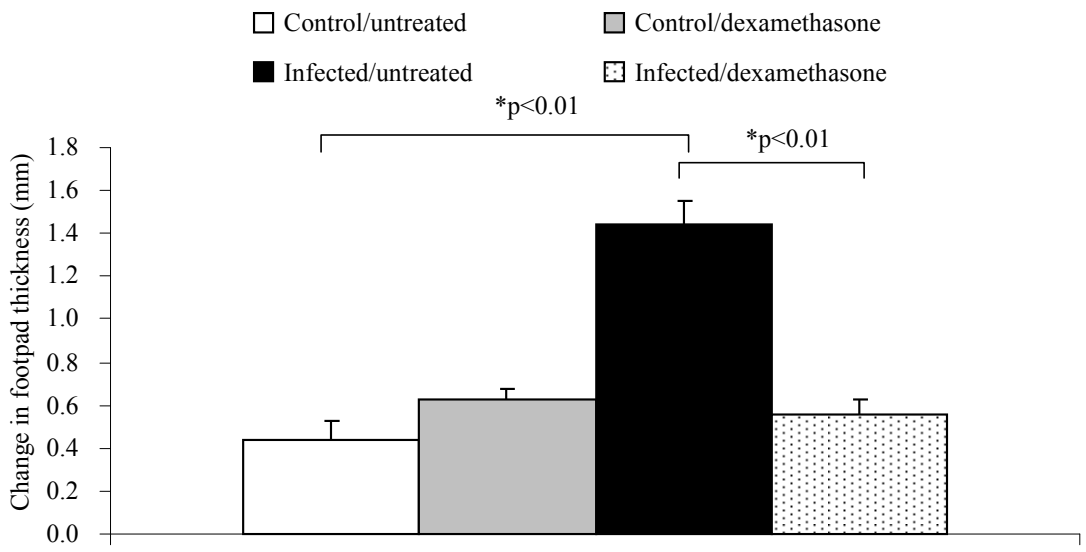


Figure 6.5 DTH responses at day 60 post infection following immunosuppression of mice. A significant increase ($p<0.01$) in footpad thickness was observed in infected, untreated mice when compared to control, untreated mice. There was a significant decrease ($p<0.01$) in footpad swelling between infected, dexamethasone treated mice and infected, non-immunosuppressed mice.

6.3.7 Bacterial loads following immunosuppression

Following immunosuppression, bacterial loads were carried out on the spleens and livers of infected, untreated mice and infected, dexamethasone treated mice on day 60 post-infection. *B. pseudomallei* was not detected following bacterial culture in either the spleen or liver of either group of mice. However, using qPCR, *B. pseudomallei* was detected in the spleens of infected, untreated and infected, dexamethasone mice (Table 6.2).

6.3.8 Monitoring survival following immunosuppression

Survival of mice following immunosuppression was monitored. There was a significant decrease ($p<0.05$) in survival of infected, dexamethasone treated mice when compared to infected, non-immunosuppressed mice by the end of the experimental period (Figure 6.7).

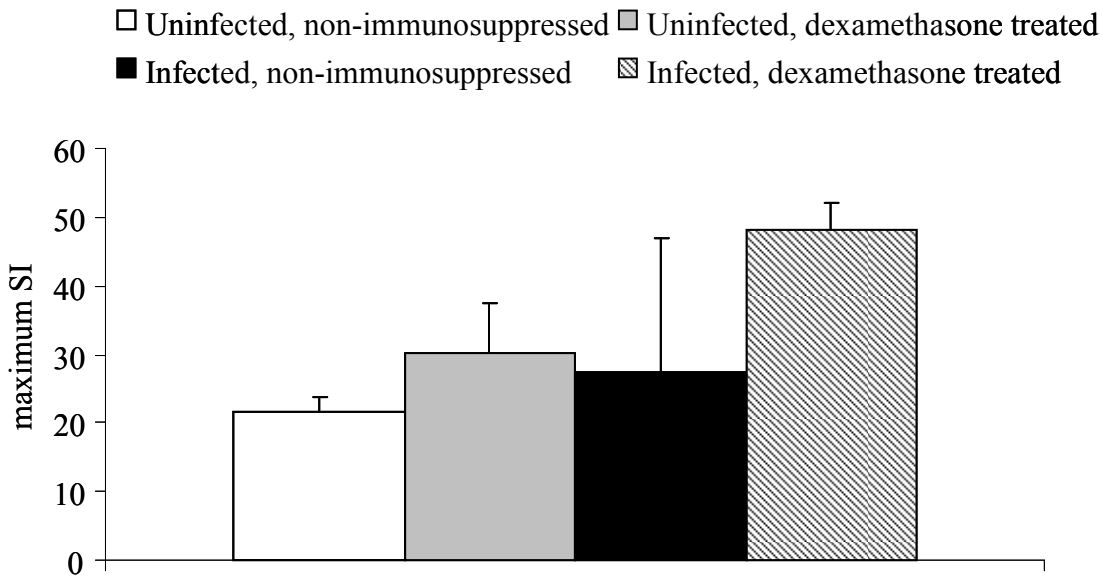


Figure 6.6 Lymphocyte proliferation at day 60 post infection with *B. pseudomallei* following immunosuppression with dexamethasone. No significant differences were observed in proliferation of lymphocytes following stimulation with BpLy1 between infected, non-immunosuppresed mice and infected, dexamethasone treated mice.

Table 6.2 Bacterial loads and qPCR following immunosuppression of mice

	Day 60													
	Uninfected mice		**Infected, non-immunosuppressed mice**						**Infected, dexamethasone treated mice**					
	spleen	**liver**	**spleen**			**liver**			**spleen**			**liver**		
Bacterial culture	-	-	-	-	-	-	-	-	-	-	-	-	-	-
qPCR	-	-	+	+	+	-	-	-	+	+	+	-	-	-

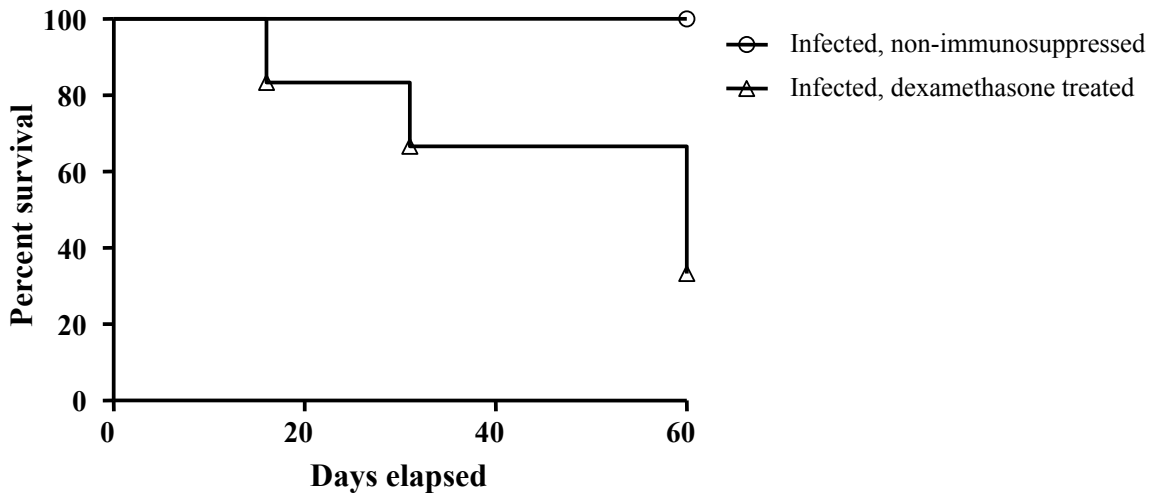


Figure 6.7 Survival of infected mice following immunosuppression with dexamethasone. There was a significant decrease in survival in infected, dexamethasone treated mice when compared to infected, non-immunosuppressed mice ($p<0.05$). Day 0 indicates where dexamethasone treatment was started.

6.4 Discussion

When a patient relapses with melioidosis it is difficult to establish whether the patient has been infected with a different isolate of *B. pseudomallei* or whether the original isolate is recrudescing. This has important implications for treatment. Determining how *B. pseudomallei* is able to recrudesce is essential for the development of improved therapeutics and management of this disease. There are a number of factors that increase the chance of relapse. Clinical severity of melioidosis on original presentation, the duration and choice of antibiotic treatment and survival of *B. pseudomallei* within phagocytic cells have been associated with influencing relapse incidences (Chaowagul *et al.*, 1993). Patient non-compliance with antibiotic treatment and a lowered immune status also increase relapse rates (Currie *et al.*, 2000a; Limmathurotsakul *et al.*, 2006).

Molecular typing methods have been used in previous studies to determine whether the original *B. pseudomallei* isolate is recrudescing or whether a second or subsequent isolate has caused infection. These methods include ribotyping (Desmarchelier *et al.*, 1993), RAPD (Haase *et al.*, 1995a), PFGE (Vadivelu *et al.*, 1998). The current study used

PFGE as a molecular typing tool to analyse isolates cultured from four patients who had relapsed with melioidosis. The results demonstrated that for each patient the original isolate was recrudescing, with clonally-related fragment patterns produced from PFGE.

Desmarchelier *et al* (1993) used ribotyping to analyse *B. pseudomallei* isolates cultured from patients who had relapsed with melioidosis. The results demonstrated that from 25 patients with relapse, 23 patients had relapsed with the original isolate and two patients were infected with different *B. pseudomallei* isolates (Desmarchelier *et al.*, 1993). Another study used random amplification of polymorphic DNA (RAPD) to analyse isolates from melioidosis patients who had relapsed. This study also found that two patients from ten had isolates that produced different ribotype and RAPD patterns (Haase *et al.*, 1995a).

Differences in fragment patterns are assumed to be a result of re-infection with a different *B. pseudomallei* isolate. However, differences may also be a result of molecular changes in the original infecting isolate. Changes in antibiotic sensitivity have been observed in clonal isolates recovered from patients who have relapsed (Haase *et al.*, 1995a). It is also possible that a patient may be infected with more than one *B. pseudomallei* isolate at one time. However, as molecular typing techniques only select one colony from a plate for analysis, such an event may go undetected.

A large number of studies have compared the efficacies and discriminatory power of different molecular typing techniques. PFGE has been shown to allow the resolution of isolates within a ribotype (Vadivelu *et al.*, 1998). However MLST, a sequence based technique, has been optimised for *B. pseudomallei* (Godoy *et al.*, 2003) and is reported to allow greater discrimination of isolates than PFGE. Maharjan *et al* (2005) used both PFGE and MLST in a study to characterise *B. pseudomallei* isolates from patients who had relapsed with melioidosis. The results demonstrated that MLST was able to better differentiate isolates by discriminating between isolates found to be clonally-related by PFGE (Maharjan *et al.*, 2005).

Determining where the organism remains dormant in the host and how the immune response is able to keep the infection under control is important for the development of improved therapeutics and vaccines for melioidosis. Such information is particularly important in a clinical setting, as physicians are eager to know whether immunosuppressive therapies for patients with a history of melioidosis or those with positive serology for *B. pseudomallei* may trigger recrudescence.

In the current study, C57BL/6 mice were infected with a low dose of *B. pseudomallei* and infection was allowed to proceed. At days 10, 20 and 30, the DTH response and lymphocyte proliferation following stimulation with BpLy1, was used to determine whether the mice had produced a CMI response to *B. pseudomallei*. Bacterial loads in the spleens and livers of mice were also assessed. There was a significant increase in footpad thickness in infected mice when compared to control mice at each time point. There was also a significant increase in the proliferative response of lymphocytes from infected mice compared to control mice. Bacterial culture of spleen and liver from infected mice did not yield *B. pseudomallei* at days 10, 20 and 30. These results suggest that the infected mice had developed *B. pseudomallei*-specific lymphocytes may have cleared the infection.

Attempts to develop animal models of latency in *M. tuberculosis* have demonstrated the development of an immune response and the gradual clearance of the organism following infection. Manabe *et al* (2008), using an aerosol rabbit model of tuberculosis, demonstrated a decrease in bacterial loads as determined by bacterial culture and a positive DTH response to *M. tuberculosis* antigen. Another study used a guinea pig model of latent tuberculosis infection and demonstrated the resolution of infection using bacterial culture together with a positive DTH response and an proliferation of lympocytes from infected guinea pigs when compared to control guinea pigs (Kashino *et al.*, 2008).

The current study attempted to induce immunosuppression in mice using dexamethasone treatment. The broad immunosuppressant, dexamethasone, is a glucocorticoid which also

results in T cell depletion and therefore a decreased T cell response (Sierra-Honigmann and Murphy, 1992),. Mice were treated with dexamethasone for six days then the DTH response, lymphocyte proliferation and the presence of *B. pseudomallei* in the spleen and liver was assessed. Results of the DTH response in mice that had been infected and treated with dexamethasone, demonstrated a significantly depressed immune response when compared to the response produced in mice that had been infected but had not received any immunosuppressant. The reduced DTH response indicates a reduction in immune response to *B. pseudomallei*. There was a significant increase in footpad thickness of infected, non-immunosuppressed mice when compared to control, non-immunosuppressed mice which indicates that an immune response to *B. pseudomallei* was still present in this group of animals and that the decreased DTH response in infected mice treated with dexamethasone was as a result of the administration of the immunosuppressant.

No significant differences were observed in the proliferative response of lymphocytes from infected, dexamethasone treated and infected, non-immunosuppressed mice following stimulation with BpLy1. While processing the lymphocytes from these groups of mice, a low total cell count was observed. However, when setting up the lymphocyte proliferation assay, 10^5 cells are placed into each well regardless of the initial cell count. It is possible that the low cell count was compensated for, as a result of this and therefore the results that were observed were not reflective of the *in vivo* situation. Another explanation may be the delay in carrying out the assay following the dexamethasone treatment. It is possible that the immunosuppressive effects of the drug had diminished and therefore the immune system was no longer depressed. This is unlikely since the DTH response which was carried out in conjunction with the lymphocyte proliferation assay did not corroborate this observation.

Bacterial loads as determined by culture were negative for *B. pseudomallei* in liver and spleen of infected animals. However, using qPCR, *B. pseudomallei* DNA was detected in the spleens of both groups of infected mice. These results suggest that bacterial culture may not be sensitive enough to detect the presence of low numbers of *B. pseudomallei* in

the organs of infected mice. A study (de Wit *et al.*, 1995) replicating the Cornell model used PCR in conjunction with bacterial culture to determine whether *M. tuberculosis* was being cleared or whether it was still present within the mouse. Using PCR, *M. tuberculosis* was detected in the organs of mice even though the animals had reached an apparent 'sterile' state using conventional bacterial culture. However, it is possible that a positive PCR or qPCR does not indicate the presence of live *B. pseudomallei*. As qPCR amplifies and detects the presence of DNA of *B. pseudomallei* it is also possible that the technique is detecting DNA from dead or non-viable bacteria. When *B. pseudomallei* is in a dormant state it may not only remain in the spleen but may also be present in other organs within the mouse. This study only carried out bacterial loads on the spleen and liver since these organs typically have the highest loads during acute experimental melioidosis. Further investigations detecting the presence of *B. pseudomallei* in other organs would aid in the understanding of latent *B. pseudomallei* infection.

There was a significant decrease in survival of infected mice treated with dexamethasone compared to infected mice that did not receive dexamethasone. In a rabbit model of tuberculosis, a cellular immune response to *M. tuberculosis* developed and there was apparent clearance of the organism. When the animals were immunosuppressed using dexamethasone, reactivation of the *M. tuberculosis* infection was demonstrated using bacterial culture and the demonstration of an increase of granulomas present in the lungs of infected rabbits (Manabe *et al.*, 2008).

Further characterisation of this latent model of *B. pseudomallei* will aid in understanding the immunopathogenesis of relapse, activation and latency in melioidosis. Different strategies may need to be undertaken in the initial treatment of melioidosis to prevent relapse of *B. pseudomallei* infection. Also understanding where *B. pseudomallei* remains latent may help in the development of vaccine candidates to target the correct virulence factors and prime an effective immune response.

CHAPTER 7

EVALUATION OF DNA VACCINES FOR PROTECTION AGAINST EXPERIMENTAL *B. PSEUDOMALLEI* INFECTION

7.1 Introduction

B. pseudomallei has been classed as a Category B biothreat agent by the Centre of Disease Control and Prevention (CDC), USA. Currently, there is no vaccine available to protect against infection with this organism or subsequent development of the disease melioidosis. Melioidosis has a high mortality rate, as evidenced by the fact that even with antibiotic treatment patients with septic shock have a mortality of over 80% (Currie *et al.*, 2000c). If diagnosed early, melioidosis can be successfully treated with antibiotics, however, treatment needs to occur over extended time periods and there is a high possibility of relapse as a result of non-compliance with the prescribed antibiotic regime.

As *B. pseudomallei* is a facultative intracellular organism, an effective vaccine should invoke both a strong cellular and humoral immune response. Cytokines such as IFN-γ, TNF-α and IL-12 have been shown to play critical roles in clearing *B. pseudomallei* infections (Warawa and Woods, 2002). However, balancing the production of cytokines as a result of immunisation is important, with overproduction of cytokines leading to septic shock (Warawa and Woods, 2002).

There are a number of virulence factors of *B. pseudomallei* that are potential vaccine targets. These include genes associated with the TTSS, which delivers virulence factors into the target host cells. Although developing a vaccine against the TTSS would not target the actual pathogen, the proteins can be recognised by the host. On the other hand, immune responses against cell-associated antigens such as LPS, the capsule, flagella and pili, which are also potential vaccine targets, could directly interact with *B. pseudomallei*.

There are various approaches to developing vaccine constructs which include conjugate, attenuated, heterologus and DNA vaccines. Conjugate vaccines are generated by joining

a carrier molecule together with a less immunogenic pathogen molecule, such as a small peptide (Brett and Woods, 1996). The carrier molecule can act to either direct the immune response towards the immunogenic molecule or to enhance the immune response due to its larger structure. An attenuated vaccine containing a non-pathogenic variant of the organism, including a capsular mutant strain of *B. pseudomallei* have been examined to determine whether protective immunity is mounted (Atkins *et al.*, 2002a). Heterologous vaccines such as a non-pathogenic relative, of *B. pseudomallei* such as *B. thailandensis* can also be used. DCs are highly efficient vectors for generating CMI responses (Healey *et al.*, 2005). *B. pseudomallei* primed DCs have been used in vaccine studies.

Inducing both humoral and CMI responses has is crucial role for a protective vaccine(Healey *et al.*, 2005). A CMI response is considered to be more effective, however, with *B. pseudomallei* being an intracellular organism (Haque *et al.*, 2006a). Therefore, the aim to producing a successful vaccine against *B. pseudomallei* should be to induce immunity that stimulates a Th1 profile. Previous studies, have demonstrated the DNA vaccines are capable of provoking the innate immune response, producing antibodies (Laddy and Weiner, 2006) and inducing a Th1-type response (Warawa and Woods, 2002). Construction of a DNA vaccine involves introducing an expression vector carrying the bacterial gene of interest to the host cells. Another advantage of using a DNA vaccine is the ability to stimulate a CMI response without using adjuvants (Sharma and Khuller, 2001). DNA vaccines have been studied to test their safety and immunogenicity against infectious diseases including HIV, influenza and malaria (Laddy and Weiner, 2006). However to date, no phase III trials have been conducted in humans. Currently only two licensed DNA vaccines are available in the veterinary field, those being vaccines against West Nile Virus for horses and Infectious Haemopoetic Necrosis for salmon.

Currently, mortality due to melioidosis is unacceptably high. The development of a vaccine that would provide immunity to *B pseudomallei* would decrease this mortality rate and protect vulnerable populations and individuals from infection. This would

include both individuals susceptible to *B. pseudomallei* such as immunocompromised individuals and also personnel who may be exposed as a result of work related exposure or biowarfare.

The specific aim for the work in this chapter is:

1. To determine whether DNA vaccinations against selected putative virulence factors of *B. pseudomallei* will produce the immune responses that could protect against subsequent infection.

7.2 Materials & Methods

7.2.1 Preparation of candidate vaccines

Candidate antigens for DNA vaccination were selected and DNA constructs prepared by the Defence Science & Technology Organisation, Melbourne (Table 7.1). Methods on preparation of the DNA vaccines are outlined in McAllister *et al* (2008).

7.2.2 Vaccination studies

BALB/c mice aged between 8-9 weeks were used for all vaccination experiments. For each antigen being trialled, mice (n=13) were identified for either monitoring of survival (n=10) or for the determination of bacterial loads (n=3) were included. BALB/c mice were intradermally (ID) immunised on days 0, 14 and 28 with either a single or combination of vaccine candidates containing DNA from *B. pseudomallei* (Table 7.1) with or without the addition of CpG, or vaccinated with the pcDNA3.1start vector that did not contain any *B. pseudomallei* genes was used as control (sham). Immunisation was carried out using the Helios gene gun (Biorad, Australia), which delivered 2μg DNA at 300psi of pressurised helium to the shaved abdomen of each mouse.

Table 7.1 Oligonucleotides used for amplification of *B. pseudomallei* gene targets

DNA vaccine construct	Gene construct number	Vaccine gene target	Sense 5'- 3'	Antisense 5'- 3'	Size of the expected product (base pairs)
pBps130	3	AimA	atctagaggctcgatc gtcgacaag	ggtctagagatcgaggta cccgtgccg	882
pBps160	6	Heat Shock Protein	atctagagctaaagac gtcgtattcgg	ggtctagacggagcgtct tccttcg	1584
pBps170	7	FliC (flagellin)	acggctctagaggaat caacagcaacattaac tcg	agaggtctagattgcagg agcttcagcacttg	1151
pBps090	9	Bacterioferritin	acggctctagacaag gcgacaagaaagtca	agaggtctagattccggc gagcccatcatc	500
pBps1100	10	OMPA (outer membrane protein A)	acggctctagagcgttt ccgcaaggtattccg	agaggtctagacttttgctt gatgcggatttcc	770
pBps1200	12	Hag2 (haemagglutinin)	acggctctagaagccc agcctggcgcttag	agaggtctagagtcgagc agatgtgatcggg	560
pBps1130	13	OPC (porin protein)	acggctctagagttatg ctcgcaacatggctg	agaggtctagagaaagc atgttgaatccccgc	1070
pBps1140	14	PsA (adhesin)	atctagagcctcttgttc gtcgcgctcg	agaggtctagaggcgcc gagcgcgtcgagctg	900
pBps1150	15	Hag1 (haemagglutinin)	acggctctagaagga atgaggtcgtgaacag g	agaggtctagattccacct gctggaagagtc	2745
pBps1160	16	Urease	acggctctagaaggg cgtacgcggaaatgta cgg	agaggtctagagaacag gaagtagcgttgcgcc	1707
pBps1170	17	Cardiolipin Synthase	acggctctagaaccct cgactggctccatctc gg	gaggtctagagaacagc cgtgcgacgtgcatcgcc	1440
pBps1180	18	Transmembrane Protein	acggctctagacagca ccgatatctgacccag g	agaggtctagagccgctc gcgctcgcgttgac	702

(McAllister *et al.*, 2008)

7.2.3 Challenging with *B. pseudomallei* following vaccination

The *B. pseudomallei* strain NCTC 13179 (Barnes and Ketheesan, 2005) was plated onto blood agar for 48 hours prior use. On day 42 post immunisation, animals were challenged with 9.4 X 10^5cfu/ml (2 X LD_{50}) *B. pseudomallei* NCTC 13179 (Barnes and Ketheesan, 2005) in a volume of 200µl was delivered via the intraperitoneal route using a

27 gauge needle (Chapter 3). Doses given were enumerated and varied between experiments from 2 to 3
X LD_{50}.

7.2.4 Monitoring mice for survival following challenge with *B. pseudomallei*

Following challenge, mice were monitored for survival daily. Moribund animals were euthanased using CO_2 asphyxiation as per NH&MRC guidelines. Mice surviving at the end of 10 days were euthanised.

7.2.5 Bacterial loads following challenge with *B. pseudomallei*

Splenic bacterial loads were carried out on mice (n=3) between one and three days post challenge (Chapter 3). Mice were euthanised using CO_2 asphyxiation and spleens were removed aseptically and placed into stomacher bags with sterile PBS. The spleens were then homogenised using a Lab-Blender 80 Stomacher (Townson & Mercer, Australia), diluted in sterile PBS and volumes of 20µl plated onto Ashdown's agar in triplicate. Plates were incubated at 37°C for 24 to 48 hours, colonies were counted and the splenic load calculated.

7.2.6 Statistical analysis

Statistical analysis was performed using SPSS statistical software version 14.0. Results from bacterial loads were assessed by univariate analysis of variance using LSD as the post-hoc test. Data was assessed for normality using QQ plots. Bacterial loads are expressed as $\log_{10}$ cfu per ml. Analysis of survival was carried out using GraphPad Prism 5 Kaplan-Meier statistical analysis. Data was considered significant if the probability of a type I error was less than $<0.05\%$.

7.3 Results

7.3.1 Survival of vaccinated mice

To determine whether the DNA vaccine candidates were effective, vaccinated mice were monitored for survival for ten days following challenge with *B. pseudomallei* NCTC 13179 (Figure 7.1). No differences in survival were observed between the different vaccine candidates used. When a DNA vaccine using a combination of DNA constructs, including DNA targeting against antigens 6, 12, 15 and 16, was used (Figure 7.1a) there was no significant increase in survival following challenge with *B. pseudomallei*. Likewise, with the addition of CpG, survival was also not increased following challenge (Figure 7.1c).

7.3.2 Bacterial loads

Bacterial loads in the spleens of infected, immunised mice were determined (Figure 7.2). Bacterial loads were carried out on day 2 post-challenge. Some mice succumbed to infection at a very early stage of infection, some bacterial loads were not enumerated as the number of mice left was insufficient for statistical analysis. Bacterial loads that were not completed included, mice vaccinated with DNA targeting antigens 6, 7, 9, 15, 16 and the combinations 5, 12, 15 and 16 and 12 + CpG and 15 + CpG. A univariate analysis of variance was carried out on bacterial loads in mice immunised with some of the constructs. The analysis demonstrated no significant differences between splenic bacterial loads in mice vaccinated with DNA constructs compared to sham vaccinated mice.

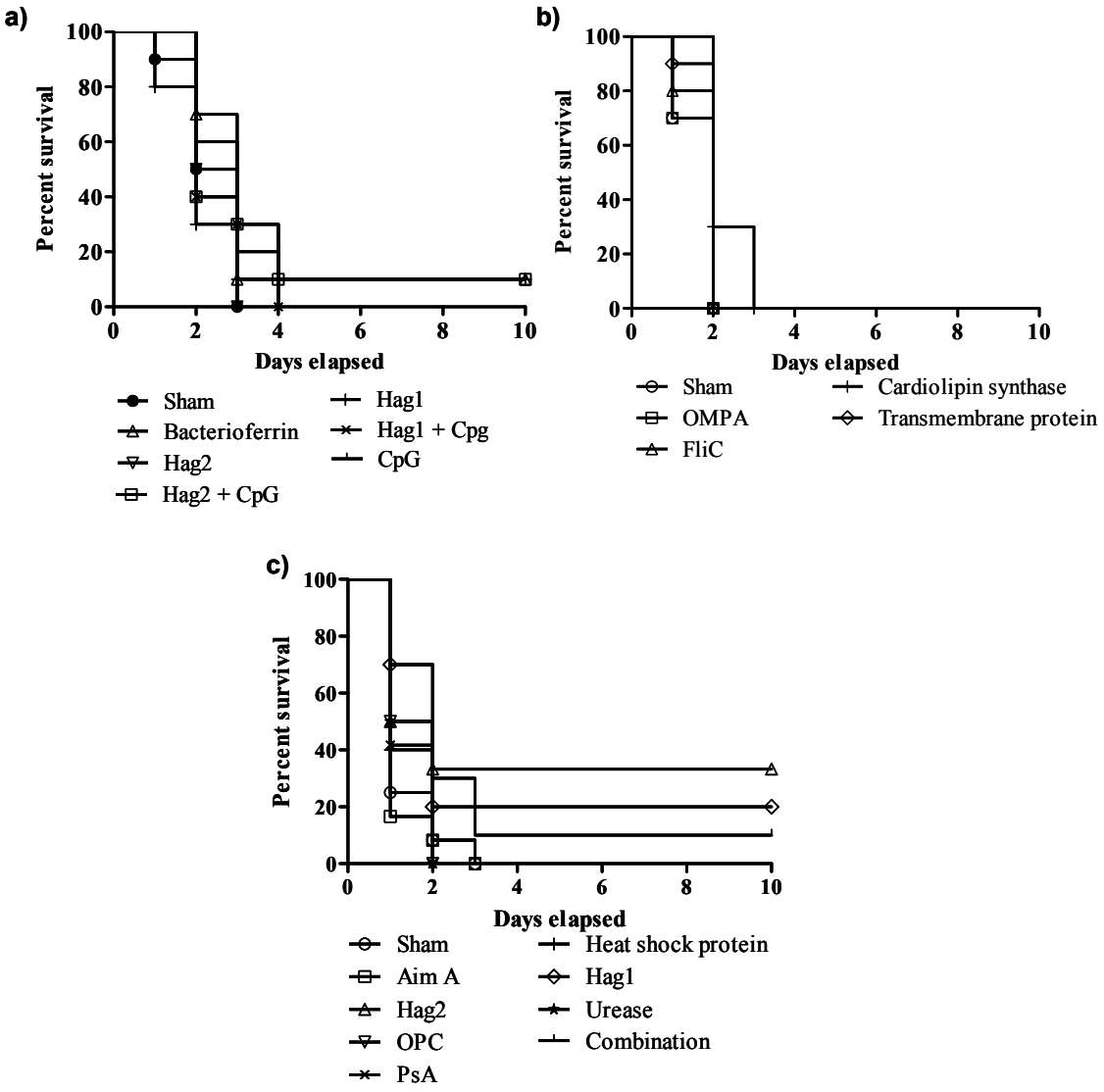


Figure 7.1. Survival rates of vaccinated BALB/c mice following challenge with NCTC 13179 at a dose of a) 3X LD_{50}, b) 2.5X LD_{50} and c) 2X LD_{50}. No significant differences were observed in survival of mice vaccinated with specific *B. pseudomallei* DNA constructs targeting genes for different virulence factors compared with mice that received only the sham vaccination. (a) Following immunisation with a combination of DNA constructs 6, 12, 15 and 16 no significant increase in survival was observed when compared to sham vaccinated mice and mice vaccinated with individual DNA vaccines. (c) There was no increase in survival of mice when CpG was added to the DNA constructs compared to sham vaccinated mice and mice vaccinated with individual DNA constructs.

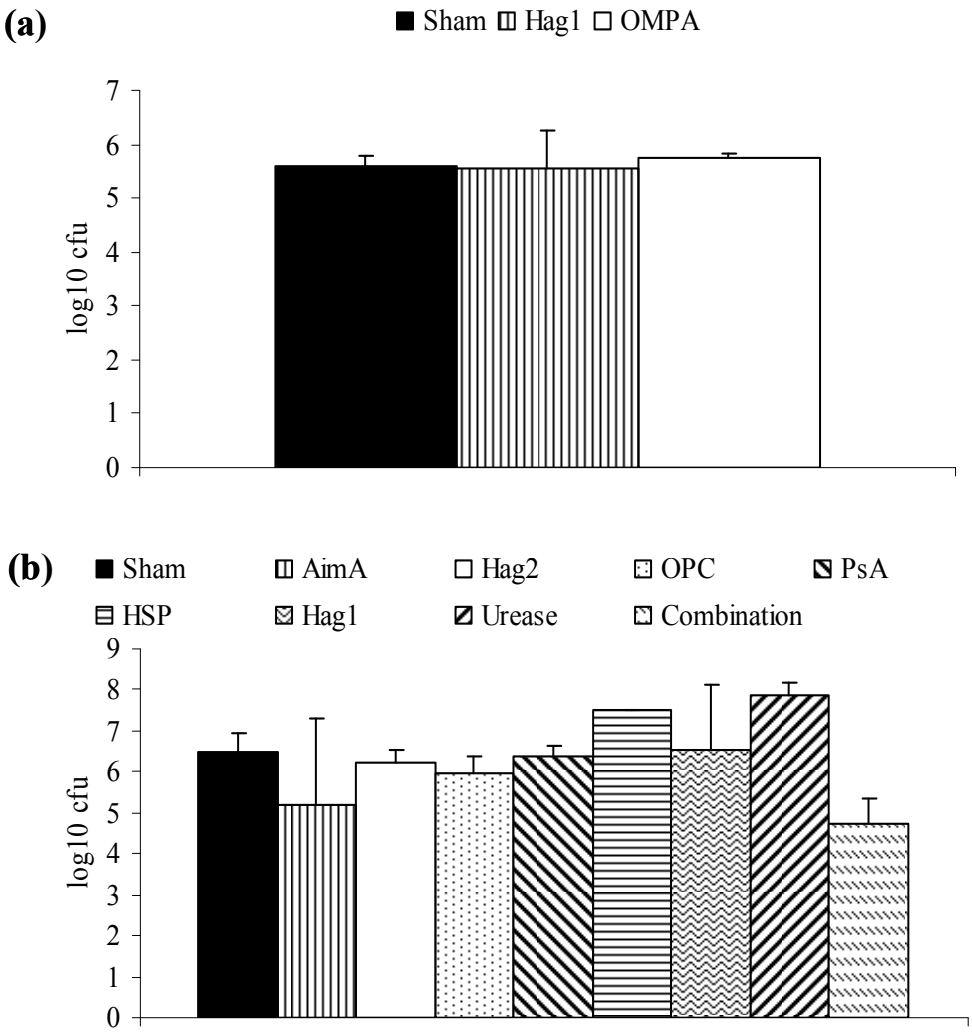


Figure 7.2 Day two splenic bacterial loads of vaccinated BALB/c mice challenged with NCTC 13179 at a dose of a) 2.5X LD_{50} and b) 3X LD_{50}. No significant differences (>0.05) were observed between mice vaccinated with different antigens when compared to sham vaccinated mice. Values are expressed as the mean ± SEM.

7.4 Discussion

Currently, there is no vaccine to provide protection against *B.pseudomallei*, an organism classed as a Category B biothreat agent by the CDC. The potential aerosol route through which *B. pseudomallei* could infect the host is considered to be a highly effective strategy to bypass some of the innate/protective mechanisms that may prevent progression to

disease. Therefore some consider *B. pseudomallei* as a potential biowarfare agent. Treatment failure and high mortality rates in melioidosis have also prompted attempts to develop an effective vaccine. Treatment of melioidosis involves an extended regimen of antibiotics to eradicate the organism. However, this can sometimes be unsuccessful and the patient can potentially relapse. Mortality rates of melioidosis are also high, with prompt identification and diagnosis essential but not always successful. A vaccine against such a facultative intracellular organism needs to not only induce a humoral response, but more importantly induce a cell-mediated immune response.

In this study several potential DNA vaccines for protection against *B. pseudomallei* in a murine model were investigated. Each vaccine candidate targeted a virulence factor gene present on the organism's genome that could potentially produce a protective immune response against *B. pseudomallei*. The results demonstrated no significant increases in survival with any of the DNA constructs used for vaccination when compared to the sham vaccinated mice. This trend did not change when a combination of DNA candidates was used and when CpG was added to the vaccination. In splenic bacterial loads no significant differences were observed in DNA vaccinated or sham vaccinated mice once again demonstrating the ineffectiveness of the DNA constructs. Although bacterial loads were not enumerated for all DNA constructs tested, the survival of these mice indicates that the bacterial load would have been high.

There have been a number of studies that have examined potential vaccines for protection against *B. pseudomallei*. These studies have targeted virulence factors of *B. pseudomallei* that could potentially be used in the development of a vaccine, including LPS, the capsular protein, flagella, pili and TTSS (Brett and Woods, 1996; Charuchaimontri *et al.*, 1999; Sarkar-Tyson *et al.*, 2007; Druar *et al.*, 2008). Brett & Woods (1996) developed a conjugate molecule that incorporated flagellin protein and polysaccharide (PS) antigens. The group hypothesised that by using two target antigens, the immune response would be enhanced. The results demonstrated successful conversion of the polysaccharide antigens to T-cell-dependent antigens (Brett and Woods, 1996). Juvenile diabetic rats were challenged with *B. pseudomallei* following

immunisation and monitored for survival for eight days. Immunised rats had a 60% survival rate compared to control rats with a survival rate of 20%. A longer period of time to monitor survival of the animals would have enhanced analysis of the data, however this was not possible in this study as only juvenile rats are susceptible to *B. pseudomallei* infection. Although the vaccine did seem to be a reasonable candidate for protection, the animal model used was not able to be fully validated in demonstrating protection against *B. pseudomallei*. Haque *et al* (2006) immunised mice with a live attenuated mutant of *B. pseudomallei*.

Another study used antibodies against flagellin proteins as a potential candidate in providing protection against *B. pseudomallei* (Brett *et al.*, 1994). Following challenge with *B. pseudomallei*, diabetic rats were passively immunised with the antibodies raised against flagellin proteins and monitored for survival for six days. The results demonstrated that compared to control animals, rats that had been immunised with the antibodies had a prolonged survival time and a lower blood bacterial load. The authors suggested that these results may have been due to the antibodies disrupting the function of flagella, therefore, attenuating the pathogenicity of *B. pseudomallei* (Brett *et al.*, 1994). However, the survival time was minimal and would need to be extended to determine protection over a prolonged period of time.

Druar *et al* (2008) studied the protective effect of three TTSS proteins; BipB, BipC and BipD against *B. pseudomallei* infection. Mice were immunised with the proteins and challenged with *B. pseudomallei*. Although, antibodies to the proteins were detected in the mice, survival time was not prolonged when compared to unvaccinated mice (Druar *et al.*, 2008). The current study used DNA vaccines encoding several *B.pseudomallei* antigens. These antigens included heat shock protein, flagellin and a transmembrane protein. No vaccine constructs tested provided statistically significant differences between the vaccinated mice when compared to control mice. A combination vaccination was trialed that included several DNA constructs in the vaccine schedule. However, this also failed to show either prolonged survival times or reduced bacterial numbers in the spleens following challenge with *B. pseudomallei*.

There are various routes of administration that have been studied to improve the potency of DNA vaccines. Intramuscular injections allow the DNA to transfect into the muscle and infiltrating APCs (Chattergoon *et al.*, 1998). The APCs subsequently migrate to the draining lymph nodes and prime the immune response. Electroporation has also been trialled as a method to increase the potency of DNA vaccines (Babiuk *et al.*, 2002). Following injection, electrode needles are inserted in the site surrounding the injection and electric pulses are delivered. This has the ability to momentarily increase the membrane permeability, increasing both the number of cells transfected and the number of plasmids that enter each cell. However, there are concerns that there is also an increased risk of plasmid integration into the host genome as well as pain involved in the method. The current study involved Particle-mediated Epidermal Delivery (PMED) with the use of a gene gun. PMED is thought to be the ideal method of introducing the vaccine due to the number of APCs in the skin that potentially aid in the priming of cellular and humoral immune responses. This is the only method to date that has produced seroconversion in humans following immunisation (Drape *et al.*, 2006). Intradermal vaccination also requires less DNA than intramuscular vaccination (Laddy and Weiner, 2006). Therefore, the method chosen to vaccinate the animals should have provided the best opportunity for protection when compared to other routes of administration.

CpG is a potent immunostimulator that consists of either bacterial genomic DNA or synthetic oligonucleotide containing unmethylated cytosine followed by guanine that can induce protective immunity. CpG has been demonstrated to produce a variety of proinflammatory cytokines including TNF-α, IL-12 and IFN-α (Utaisincharoen *et al.*, 2003) as well as stimulating a Th1 response by activating macrophages, dendritic cells, NK cells and some subpopulations of lymphocytes (Wongratanacheewin *et al.*, 2004). Utaisincharoen *et al* (2003) demonstrated an increase in bacterial uptake in a mouse macrophage cell line as well as an increase in production of iNOS and NO which are essential in destroying intracellular organisms. Further investigations with CpG also demonstrated an upregulation of iNOS and NO production (Wongratanacheewin *et al.*, 2004). Wongratanacheewin *et al* (2004) challenged mice with *B. pseudomallei* followed

by administration of CpG. The results demonstrated a significantly prolonged survival time when compared to control groups not given the CpG. There was also a decreased bacterial load in the blood and a decrease in IFN-γ levels. The study also found that after 30 days, mice had no demonstrable *B. pseudomallei* present following culture of blood, spleen, liver and lungs. The findings suggest that treatment with CpG could possibly affect the outcome of acute infection and also have an effect on the progression of melioidosis to chronic forms. Harland *et al* (2007) immunised mice with proteins of the ATP-binding cassette system (LolC) of *B. pseudomallei* in conjunction with CpG. There was a significant increase in survival of mice immunised with LolC plus CpG when compared to unimmunised mice. Therefore in the present study, to enhance the protective immunity in mice of the DNA constructs, CpG was included with vaccinations of constructs 12 and 15 which encoded haemagglutinins, Hag2 & Hag1 respectively. The results did not demonstrate a significant difference in survival in mice vaccinated between the constructs by themselves or mice vaccinated with the constructs plus CpG. Splenic bacterial loads were not able to be fully completed in all groups as the mice succumbed to infection following challenge before the spleens could be removed and bacteria enumerated. However, it can be assumed that any differences in bacterial loads would not have been significant due to the rapid death of the animals.

The proliferative response of lymphocytes and DTH response was used to detect the presence of *B. pseudomallei*-specific T cells following vaccination with DNA construct bacterioferritin. The results demonstrated that there was no significant differences in proliferation of lymphocytes or DTH response between vaccinated and control mice (McAllister *et al.*, 2007). However, the presence of antibodies in mice vaccinated with the DNA construct bacterioferritin was demonstrated. Serum was collected prior to vaccination and again prior to challenge. The presence of antibodies demonstrates the production of a humoral immune response to the DNA vaccine. However, as observed in survival studies, mice vaccinated with the DNA construct did not produce a protective immunite response against *B. pseudomallei* following challenge.

This study tested a number of DNA constructs encoding for genes of putative virulence factors in *B. pseudomallei* in a murine model. The results demonstrated that the vaccinations did not significantly increase survival times or reduce bacterial loads in any of the mice vaccinated with the DNA constructs when compared to control animals. The pathogenesis of *B. pseudomallei* is still not fully understood. Determining which virulence factors are important in protection and progression of *B. pseudomallei* infection is essential for the successful development of an effective vaccine.

CHAPTER 8

GENERAL DISCUSSION

Melioidosis has an unacceptably high mortality rate even with early antibiotic treatment (Currie *et al.*, 2000c). With *B. pseudomallei* being classed as a Category B biothreat agent by the CDC, the need to understand the pathogenesis and immune responses to *B. pseudomallei* have become increasingly important. Currently, the processes by which *B. pseudomallei* is able to cause infection and evade host immune responses are poorly understood. The ability of *B. pseudomallei* to recrudesce after apparent recovery from melioidosis and to remain dormant for years following exposure furthers the requirement to understand the pathogenesis of this tropical infection. This is of particular importance due to the possibility that individuals residing in areas that are endemic may have been exposed to *B. pseudomallei* and may still harbour the bacterium. How the organism can also evade the immune responses of the host are of particular interest in the development of a vaccine to protect against the infection.

Serological studies using the IHA have determined that healthy populations living in endemic regions of *B. pseudomallei* are exposed to the organism and produce an antibody response (Naigowit *et al.*, 1992; Norazah *et al.*, 1996). In endemic regions of Thailand, the seroprevalence was found to be between 20-30% of healthy donors (Naigowit *et al.*, 1992). Similarly in Malaysia, another endemic region the seroprevalence was 26.5% (Norazah *et al.*, 1996). In the present study, exposure to *B. pseudomallei* was determined in a healthy population of blood donors from northern Queensland. Although an earlier study from northern Queensland demonstrated a seroprevalence of 5.7% (Ashdown and Guard, 1984), this study found the seroprevalence to be 2.5%. Northern Queensland has been identified as a region that is endemic for melioidosis. The differences observed in the seroprevalence from the northern Queensland studies may be a result of sample bias, with the earlier study using serum that had been sent to the pathology laboratory for other tests. Differences observed in the rate of seroprevalence between northern Queensland and other endemic regions may be due to lifestyle, environmental and occupational factors. Kanaphun *et al* (1993) found a higher rate of seroprevalence in children from

rice farming families, reflecting that environmental exposure to *B. pseudomallei* affects antibody production. In East Timor where females do most of the physical activity within the family unit, the seroprevalence to *B. pseudomallei* was higher in females than males again reflecting environmental exposure as a factor in higher seroprevalence rates (Armstrong *et al.*, 2005). In comparison to the current study which did not identify gender differences in seroprevalence to *B. pseudomallei*. This suggests similar exposure levels for males and females through lifestyle and occupational factors within the northern Queensland population.

The Melioidosis IgG Rapid Cassette test (ICT) was evaluated as a screening method for detecting antibodies to *B. pseudomallei*. Using the 37 donor samples that were positive by the IHA, 32% were found to be positive using the Melioidosis IgG Rapid Cassette test. There are a number of disadvantages in using the IHA and the ICT to determine seroprevalence. The major disadvantage of these assays is the uncharacterised nature of the antigen used. It is possible that antibodies produced by the individual may not be directed against the antigen used. Cross-reactivity with other organisms is also possible with a study demonstrating cross-reactivity with *B. thailandensis* (Gilmore *et al.*, 2007). However, the IHA is currently the accepted serolocial technique that is used to determine the presence of antibodies against *B. pseudomallei*. While it is possible to develop an ELISA to detect antibodies against *B. pseudomallei*, similar problems may arise with the antigen used and cross-reactivity. The findings of the current study suggest the ICT may be useful in a diagnostic setting when used in conjunction with a patient's clinical presentation. However due to high background seropositivity for *B. pseudomallei* in endemic regions, the rapid test would not be an adequate replacement for conventional diagnostic tests.

This current serological screening has demonstrated that individuals living in endemic regions of Queensland may have been exposed to *B. pseudomallei* and subsequently produce an antibody response. *B. pseudomallei* is an intracellular pathogen and therefore the CMI response is important in protection and clearance of the infection. The CMI response against intracellular pathogens involves the clonal expansion of T cells to

produce cytokines and activated T cells. IFN-γ produced by CD4+ cells of the Th1 response has been identified as the major cytokine involved in activating macrophages, while CD8+ T cells are recruited to kill infected cells. Studies characterising the CMI response against *B. pseudomallei* have been limited. CMI responses against infections with other intracellular pathogens including *M. tuberculosis* and *L. monocytogenes* have demonstrated the critical role of CD4+ and CD8+ T cells in protection (Nagata *et al.*, 2008; Xu *et al.*, 2008). Individuals who have been exposed to *B. pseudomallei* and who have produced an antibody response could potentially have a subclinical infection. By determining the CMI response of seropositive individuals who have never developed clinically apparent disease, we may be able to understand the role of these immune responses in disease outcome. In the present studies, analysis of T cells isolated from seropositive donors was carried out using lymphocyte proliferation assays and flow cytometry. The results demonstrated that there were no significant differences in lymphocyte proliferation, activation of T cell subsets (CD4+ and CD8+) and production of IFN-γ and IL-4 in seropositive donors when compared to seronegative donors. Previous studies have demonstrated differences in lymphocyte proliferation, activation of T cell subsets and production of various cytokines in 1) Vietnam veterans with previous exposure to *B. pseudomallei* (Govan and Ketheesan, 2004); 2) seropositive individuals with no previous history of melioidosis (Barnes *et al.*, 2004) and 3) patients who had recovered from melioidoisis (Ketheesan *et al.*, 2002). Although differences were not seen in this current study between seropositive donors and seronegative donors, it is possible that some of these tests induced false negative and false positive results due to the IHA methodology, as previously mentioned. The study that demonstrated an increase in lymphocyte proliferation in Vietnam veterans also had inconsistencies in IHA titres, with the majority of veterans not producing a positive IHA titre (Govan and Ketheesan, 2004). The antigen used to stimulate the lymphocytes may also not have produced a response as it may have varied from the original *B. pseudomallei* that the individual was exposed to. Characterising and comparing the CMI responses in patients with melioidosis and patients who have relapsed would allow further understanding of the immune responses involved in *B. pseudomallei* infection. Until the immune responses to

B. pseudomallei have been characterised, the development of a successful vaccine may be delayed.

The ability of *B. pseudomallei* to remain latent for years following exposure and activating and relapsing after apparent recovery has been well documented (Desmarchelier *et al.*, 1993; Haase *et al.*, 1995a; Nguay *et al.*, 2005). *B. pseudomallei* is able to invade and survive within phagocytic cells (Jones *et al.*, 1996), therefore avoiding host CMI responses. Characterising the immune responses that are involved in protection against the pathogen is important in understanding the pathogenesis of *B. pseudomallei*. Relapse of *B. pseudomallei* have been associated with immunosuppression of the host. Non-compliance with antibiotic treatment has also been seen as a risk factor for relapse of melioidosis. Using molecular techniques to characterise isolates recovered from patients who have relapsed with melioidosis can determine whether the patient is being infected with the original isolate or is being re-infected with a different isolate. In the current study, PFGE was used to characterise isolates from patients with more than one episode of clinical melioidosis. Fragment patterns produced demonstrated that the patients who had relapsed were infected with the original infecting *B. pseudomallei* isolate. Other studies (Desmarchelier *et al.*, 1993; Haase *et al.*, 1995a) have demonstrated differences in fragment patterns of *B. pseudomallei* isolates. These differences may be a result of infection with a different *B. pseudomallei* isolate, however, is also possible that the original isolate had undergone molecular mutations which have been identified with changes in antibiotic sensitivities (Haase *et al.*, 1995a).

An animal model of latent melioidosis will not only further our understanding of the immune responses involved in latency but will also aid in determining where the organism resides within the host. This knowledge would also be advantageous in the treatment of patients who experience relapse. In the current study, following injection with a low dose of *B. pseudomallei*, C57BL/6 mice produced a CMI that was detected at days 10, 20 and 30 post infection. The mice also demonstrated an apparent clearance of *B. pseudomallei* as determined by bacterial culture of tissues. Immunosuppression with dexamethasone produced a depressed DTH response in infected mice when compared to

infected mice that had received no immunosuppression. Following culture, bacterial loads in spleens and livers of both infected groups of mice were negative. However using qPCR, *B. pseudomallei* DNA was detected in the spleens of both groups of mice. This result demonstrates the sensitivity of qPCR compared to bacterial culture. Survival studies of mice demonstrated that following reactivation of *B. pseudomallei*, infected, dexamethasone treated mice had a significant negative effect on survival when compared to infected and non-immunosuppressed mice. Activation of infection has also been demonstrated following immunosuppression in a *M. tuberculosis* model in rabbits using histological studies and bacterial culture (Manabe *et al.*, 2008). The development of a latent model of melioidosis is important in understanding the immunopathogenesis of *B. pseudomallei* in relapse, activation and latency. Relapse and activation of latent melioidoisis are problems that will continue to emerge until an effective treatment regime against *B. pseudomallei* is developed. To develop a successful vaccine against *B. pseudomallei* the immune responses and pathogenesis of the organism need to be understood to allow the correct virulence factors to be targeted in order to prime an appropriate immune response.

To produce an effective vaccine against *B. pseudomallei*, both a cellular and humoral immune response needs to be induced (Healey *et al.*, 2005). While potential vaccines have been developed against various virulence factors including TTSS, LPS and flagella, they have not been successful in mounting a protective immune response following subsequent challenge with virulent *B. pseudomallei*. DNA vaccines have previously demonstrated the induction of an innate immune response, production of antibodies (Laddy and Weiner, 2006) and the induction of a Th1 response (Warawa and Woods, 2002). The current study determined whether DNA vaccinations against selected virulence factors were able to produce protective immunity in a murine model of melioidosis. The DNA vaccine candidates tested did not affect splenic bacterial loads or survival time when compared with control animals. Even with combinations of DNA constructs and the addition of CpG, a potent immunostimulator, no differences were observed in survival time or bacterial loads. While DNA vaccines have been shown to induce a CMI response (Warawa and Woods, 2002), McAllister *et al* (2007) found no

significant differences in proliferation of *B. pseudomallei*-specific T cells or DTH responses to a DNA construct that encoded bacterioferritin. Antibodies to bacterioferritin were detected demonstrating a humoral response was induced. However, increased survival was not observed in vaccinated mice suggesting that without the production of an effective CMI response a humoral response is not sufficient to provide protection against subsequent challenge with *B. pseudomallei*. While DNA vaccines have shown potential in other infections to produce an effective immune response, the constructs tested in the current study failed to demonstrate the development of protective immune responses in a murine model of melioidosis. Determining virulence factors of *B. pseudomallei* that can be targeted to produce protective immune responses is essential in the development of a successful vaccine for melioidosis.

The results presented in this thesis provide further knowledge on the immunopathogenesis of *B. pseudomallei*. Exposure to *B. pseudomallei* in the endemic region of northern Queensland has been determined. Although this study failed to demonstrate that seropositive individuals produce a CMI response, further studies with specific *B. pseudomallei* antigens are warranted. Future studies focusing on characterising CMI responses in patients with melioidosis and patients who have relapsed will further the understanding of the immune responses involved in eradication and protection against *B. pseudomallei*. *B. pseudomallei* has the ability to relapse. Understanding whether patients who have relapsed with melioidosis are becoming ill from the original isolate or from a different isolate is important. The development of a latent model of melioidosis will also add to understanding of the pathogenesis. Currently, it is not clear where *B. pseudomallei* persists following exposure to the organism, however, this study has been able to identify the presence of the organism in the spleen using qPCR when bacterial cultures were not positive. Further investigations involving detection of *B. pseudomallei* in other organs will contribute to our understanding of the pathogenesis of this disease. Characterisation of the immunopathogenesis of *B. pseudomallei* infection is essential for development of a successful vaccine. Although the DNA vaccines used in the current study did not induce protection against *B. pseudomallei* infection, it did show the potential of one of the

vaccine constructs to produce a humoral immune response. Future studies could utilise this construct in combination with other immunomodulatory molecules that induce strong CMI responses to investigate their efficacy as a potential multicomponent vaccine for melioidosis. However, for the development of successful therapeutic and protection strategies against *B. pseudomallei* infection, continued research at the basic levels of host-pathogen interactions are required.

REFERENCES

Alexander, A.D., Huxsoll, D.L., Warner, A.R., Shepler, V. and Dorsey, A. (1970) Serological Diagnosis of Human Melioidosis with Indirect Hemagglutination and Complement Fixation Tests. *Appl Microbiol* **20**: 825-33

Allworth, A.M. (2005) Tsunami lung: a necrotising pneumonia in survivors of the Asian tsunami. *Med J Aust* **182**: 364

Almer, L.S., Shortridge, V.D., Nilius, A.M., Beyer, J.M., Soni, N.B., Bui, M.H., Stone, G.G. and Flamm, R.K. (2002) Antimicrobial susceptibility and molecular characterization of community-acquired methicillin-resistant *Staphylococcus aureus*. *Diagno Microbiol Infect Dis* **43**: 225-32

Anuntagool, N., Aramsri, P., Panichakul, T., Wuthiekanun, V., Kinoshita, R., White, N.J. and Sirisinha, S. (2000a) Antigenic heterogeneity of lipopolysaccharide among *Burkholderia pseudomallei* clincal isolates. *Southeast Asian J Trop Med Public Health* **31**: 146-52

Anuntagool, N., Naigowit, P., Petkanchanapong, V., Aramsri, P., Panichakul, T. and Sirisinha, S. (2000b) Monoclonal antibody-based rapid identification of *Burkholderia pseudomallei* in blood culture fluid from patients with community-acquired septicaemia. *J Med Microbiol* **49**: 1075-8

Appassakij, H., Silpapojakul, K.R., Wansit, R. and Pornpatkul, M. (1990) Diagnostic value of the indirect hemagglutination test for melioidosis in an endemic area. *Am J Trop Med Hyg* **42**: 248-53

Armstrong, P.K., Anstey, N.M., Kelly, P.M., Currie, B.J., Martins, N., Dasari, P. and Krause, V. (2005) Seroprevalence of *Burkholderia pseudomallei* in East Timorese refugees: implications for healthcare in East Timor. *Southeast Asian J Trop Med Public Health* **36**: 1496-502

Asche, V. (1991) Melioidosis - a disease for all organs. *Todays Life Science*: 34-40

Ashdown, L.R. (1979) An improved screening technique for isolation of *Pseudomonas pseudomallei* from clinical specimens. *Pathol* **11**: 293-7

Ashdown, L.R. (1987) Indirect haemagglutination test for melioidosis. *Med J Aust* **147**: 364-5

Ashdown, L.R. (1991) Epidemiological aspects of melioidosis in Australia. *Clin Infect Dis* **15**: 272-3

Ashdown, L.R. and Clarke, S.G. (1992) Evaluation of culture techniques for isolation of *Pseudomonas pseudomallei* from soil. *Appl Environ Microbiol* **58**: 4011-5

Ashdown, L.R. and Frettingham, R.J. (1984) *In vitro* activity of various cephalosporins against *Pseudomonas pseudomallei*. *J Infect Dis* **150**: 779-80

Ashdown, L.R. and Guard, R.W. (1984) The prevalence of human melioidosis in northern Queensland. *Am j Trop Med Hyg* **33**: 474-8

Ashdown, L.R., Johnson, R.W., Koehler, J.M. and Cooney, C.A. (1989) Enzyme-linked immunosorbent assay for the diagnosis of clinical and subclinical melioidosis. *J Infect Dis* **160**: 253-60

Atkins, T., Prior, R., Mack, K., Russell, P., Nelson, M., Prior, J., Ellis, J., Oyston, P.C., Dougan, G. and Titball, R.W. (2002a) Characterisation of an acapsular mutant of *Burkholderia pseudomallei* identified by signature tagged mutagenesis. *J Med Microbiol* **51**: 539-47

Atkins, T., Prior, R.G., Mack, K., Russell, P., Nelson, M., Oyston, P.C.F., Dougan, G. and Titball, R.W. (2002b) A mutant of *Burkholderia pseudomallei*, auxotrophoic in the branched chain amino acid biosynthetic pathway, is attenuated and protective in a murine model of melioidosis. *Infect Immun* **70**: 5290-4

Attree, O. and Attree, I. (2001) A second type III secretion system in *Burkholderia pseudomallei*: who is the real culprit? *Microbiol* **147**: 3197-9

Babiuk, S., Baca-Estrada, M.E., Foldvari, M., Storms, M., Rabussayk, D., Widera, G. and Babiuk, L.A. (2002) Electrophoration improves the efficacy of DNA vaccines in large animals. *Vaccine* **20**: 3399-408

Banchereau, J. and Steinman, R.M. (1998) Dendritic cells and the control of immunity. *Nature* **392**: 245-52

Barnes, J.L. and Ketheesan, N. (2005) Route of Infection in Melioidosis. *Emerg Infect Dis* **11**: 638-9

Barnes, J.L., Warner, J., Melrose, W., Durrheim, D., Speare, R., Reeder, J.C. and Ketheesan, N. (2004) Adaptive immunity in melioidosis: a possible role for T cells in determining outcome of infection with Burkholderia pseudomallei. *Clin Immunol* **113**: 22-8

Brett, P.J., Mah, D.C.W. and Woods, D.E. (1994) Isolation and characterisation of *Pseudomonas pseudomallei* flagellin proteins. *Infect Immun* **62**: 1914-9

Brett, P.J. and Woods, D.E. (1996) Structural and immunological characterization of *Burkholderia pseudomallei* O-polysaccharide-flagellin protein conjugates. *Infect Immun* **64**: 2824-8

Brett, P.J. and Woods, D.E. (2000) Pathogenesis of and immunity to melioidosis. *Acta Trop* **74**: 201-10

Chantratita, N., Wuthiekanun, V., Limmathurotsakul, D., Thanwisai, A., Chantratita, W., Day, N.P.J. and Peacock, S.J. (2007) Prospective clinical evaluation of the accuracy of 16S rRNA real-time PCR assay for the diagnosis of melioidosis. *Am J Trop Med Hyg* **77**: 814-7

Chaowagul, W., Suputtamongkol, Y., Dance, D.A.B., Rajchanuvong, A., Pattara-arechachai, J. and White, N.J. (1993) Relapse in melioidosis: incidence and risk factors. *J Infect Dis* **168**: 1181-5

Charuchaimontri, C., Suputtamongkol, Y., Nilakul, C., Chaowagul, W., Chetchotisakd, P., Lertpatanasuwun, N., Intaranongpai, S., Brett, P.J. and Woods, D.E. (1999) Antipolysaccharide II: an antibody protective against fatal melioidosis. *Clin Infect Dis* **29**: 813-8

Chattergoon, M.A., Robinson, T.M., Boyer, J.D. and Weiner, D.B. (1998) Specific Immune Induction following DNA-based Immunization through in vivo Transfection and Activation of Macrophages/Antigen-Presenting Cells. *Journal of Immunology* **160**: 5707-18

Chen, W.-T., Chen, Y.-S., Chye, S.-M., Wu, T.-R., Hong, W.-G., Lin, Y.-N., Bair, H.-T., Yang, S.-C. and Chen, Y.-L. (2005) Seroprevalence of melioidosis in diabetic patients in Taiwan. *J Microbiol Immunol Infect* **38**: 267-70

Cheng, A.C. and Currie, B.J. (2005) Melioidosis: epidemiology, pathophysiology, and management. *Clin Microbiol Rev* **18**: 383-416

Cheng, A.C., Day, N.P., Mayo, M.J., Gal, D. and Currie, B.J. (2005) *Burkholderia pseudomallei* strain type, based on pulsed-field gel electrophoresis, does not determine disease presentation on melioidosis. *Microbes Infect* **7**: 104-7

Cheng, A.C., Godoy, D., Mayo, M., Gal, D., Spratt, B.G. and Currie, B.J. (2004) Isolates of *Burkholderia pseudomallei* from Northern Australia are distinct by Multilocus Sequence Typing, but strain types do not correlate with clinical presentation. *J Clin Microbiol* **42**: 5477-83

Cheng, A.C., O'Brien, M., Freeman, K., Lum, G. and Currie, B.J. (2006a) Indirect hemagglutination assay in patients with melioidosis in northern Australia. *Am J Trop Med Hyg* **74**: 330-4

Cheng, A.C., Peacock, S.J., Limmathurotsakul, D., Wongsuman, G., Chierakul, W., Amornchai, P., Getchalarat, N., Chaowagul, W., White, N.J., Day, N.P.J. and Wuthiekanun, V. (2006b) Prospective evaluation of a rapid immunochromogenic

cassette test for the diagnosis of melioidosis in northeast Thailand. *Trans R Soc Trop Med Hyg* **100**: 64-67

Chenthamarakshan, V., Kumutha, M.V., Vadivelu, J. and Puthucheary, S.D. (2001) Distribution of immunoglobulin classes and IgG subclasses against a culture filtrate antigen of *Burkholderia pseudomallei* in melioidosis patients. *J Med Microbiol* **50**: 55-61

Chua, K.L., Chan, Y.Y. and Gan, Y.H. (2003) Flagella are virulence determinants of *Burkholderia pseudomallei*. *Infect Immun* **71**: 1622-9

Chuah, S.C., Gilmore, G. and Norton, R.E. (2005) Rapid serological diagnosis of melioidosis: an evaluation of a prototype immunochromatographic test. *Pathol* **37**: 169-71

Crotty, J.M., Bromwich, A.F. and Quinn, J.V. (1963) Melioidosis in the Northern Territory: a report of two cases. *Med J Aust* **Feb**: 274-5

Currie, B.J., Fisher, D.A., Anstey, N.M. and Jacups, S.P. (2000a) Melioidosis: acute and chronic disease, relapse and re-activation. *Trans R Soc Trop Med Hyg* **94**: 301-4

Currie, B.J., Fisher, D.A., Howard, D.M. and Burrow, J.N.C. (2000b) Neurological melioidosis. *Acta Trop* **74**: 145-51

Currie, B.J., Fisher, D.A., Howard, D.M., Burrow, J.N.C., Lo, D., Selva-nayagam, S., Anstey, N.M., Huffam, S.E., Marks, P.L.S.P.J., Stephens, D.P., Lum, G.D., Jacups, S.P. and Krause, V.L. (2000c) Endemic melioidosis in tropical Northern Australia: a 10-year prospective study and review of the literature. *Clin Infect Dis* **31**: 981-6

Currie, B.J., Fisher, D.A., Howard, D.M., Burrow, J.N.C., Selvanayagam, S., Snelling, P.L., Anstey, N.M. and Mayo, M.J. (2000d) The epidemiology of melioidosis in Australia and Papua New Guinea. *Acta Trop* **74**: 121-7

Currie, B.J. and Jacups, S.P. (2003) Intensity of rainfall and severity of melioidosis, Australia. *Emerg Infect Dis* **9**: 1538-42

Currie, B.J., Jacups, S.P., Cheng, A.C., Fisher, D.A., Anstey, N.M., Huffam, S.E. and Krause, V.L. (2004) Melioidosis epidemiology and risk factors from a prospective whole-population study in northern Australia. *Trop Med Int Health* **9**: 1167-74

Cuzzubbo, A.J., Chenthamarakshan, V., Vadivelu, J., Puthucheary, S.D., Rowland, D. and Devine, P.L. (2000) Evaluation of a new commercially available immunoglobulin M and immunoglobulin G immunochromatographic test for diagnosis of melioidosis infection. *J Clin Microbiol* **38**: 1670-1

Dance, D.A. (1991) Melioidosis: the tip of the iceberg? *Clin Microbiol Rev* **4**: 52-60

Dance, D.A.B. (2000) *Burkholderia pseudomallei* infections. *Clin Infect Dis* **30**: 235-6

Dance, D.A.B., Davis, T.M.E., Wattanagoon, Y., Chaowagul, W., Saiphan, P., Looareesuwan, S., Wuthiekanun, V. and White, N.J. (1989a) Acute suppurative parotitis caused by *Pseudomonas pseudomallei* in children. *J Infect Dis* **159**: 654-60

Dance, D.A.B., Wuthiekanun, V., Naigowit, P. and White, N.J. (1989b) Identification of *Pseudomonas pseudomallei* in clinical practice: use of simple screening tests and API20NE. *J Clin Pathol* **42**: 645-8

Dannenberg, A.M. and Scott, E.M. (1958) Melioidosis: pathogenesis and immunity in mice and hamsters. I. Studies with virulent strains of *Malleomyces pseudomallei*. *J Exp Med* **107**: 153-66

de Wit, D., Wootton, M., Dhillon, J. and Michison, D.A. (1995) The bacterial DNA content of mouse organs in the Cornell model of dormant tuberculosis. *Tubercle and Lung Disease* **76**: 555-62

Desakorn, V., Smith, M.D., Wuthiekanun, V., Dance, D.A.B., Aucken, H., Suntharasamai, P., Rajchanuwong, A. and White, N.J. (1994) Detection of *Pseudomonas pseudomallei* antigen in urine for the dianosis of melioidosis. *Am J Trop Med Hyg* **51**: 627-33

Desmarchelier, P.M., Dance, D.A.B., Chaowagul, W., Suputtamongkol, Y., White, N.J. and Pitt, T.L. (1993) Relationships among *Pseudomonas pseudomallei* isolates from patients with recurrent melioidosis. *J Clin Microbiol* **31**: 1592-6

Drape, R.J., Macklin, M.D., Barr, L.J., Jones, S., Haynes, J.R. and Dean, H.J. (2006) Epidermal DNA vaccine for influenza is immunogenic in humans. *Vaccine* **24**: 4475-81

Druar, C., Yu, F., Barnes, J.L., Okinaka, R.T., Chantratita, N., Beg, S., Stratilo, C.W., Olive, A.J., Soltes, G., Russell, M.L., Limmathurotsakul, D., Norton, R.E., Ni, S.X., Picking, W.D., Jackson, P.J., Stewart, D.I.H., Tsvetnitsky, V., Picking, W.L., Cherwonogrodzky, J.W., Ketheesan, N., Peacock, S.J. and Wiersma, E.J. (2008) Evaluating Burkholderia pseudomallei Bip proteins as vaccines and Bip antibodies as detection agents. *FEMS Immunol Med Microbiol* **52**: 78-87

Egan, A.M. and Gordon, D.L. (1996) *Burkholderia pseudomallei* activates complement and is ingested but not killed by polymorphonuclear leukocytes. *Infect Immun* **64**: 4952-9

Finney, M. (2000) Pulsed-Field Gel Electropheresis. In: F.M. Ausubel (Ed), Current Protocols in Molecular Biology, pp. Unit 2.5B1-9. John Wiley & Sons, Inc, United States of America.

Forbes, B.A., Sahm, D.F. and Weissfeld, A.S. (1998) Bailey and Scott's Diagnostic Microbiology. 10th edition ed. Mosby Inc, United States of America.

Friedland, J.S., Suputtamongkol, Y., Remick, D.G., Chaowagul, W., Strieter, R.M., Kunkel, S.L., White, N.J. and Griffin, G.E. (1992) Prolonged elevation of interleukin-8 and interleukin-6 concentrations in plasma and of leukocyte interleukin-8 mRNA levels during septicemic and localized *Pseudomonas pseudomallei* infection. *Infect Immun* **60**: 2402-8

Gilmore, G., Barnes, J., Ketheesan, N. and Norton, R. (2007) Use of antigens derived from *Burkholderia pseudomallei*, *B. thailandensis*, and *B. cepacia* in the indirect hemagglutination assay for melioidosis. *Clin Vaccine Immunol* **14**: 1529-31

Godoy, D., Randle, G., Simpson, A.J., Aanensen, D.M., Pitt, T.L., Kinoshita, R. and Spratt, B.G. (2003) Multilocus sequence typing and evolutionary relationships among the causative agents of melioidosis and glanders, *Burkholderia pseudomallei* and *Burkholderia mallei*. *J Clin Microbiol* **41**: 2068-79

Govan, B. and Ketheesan, N. (2004) Exposure to *Burkholderia pseudomallei* induces cell-mediated immunity in healthy individuals. *Clinical Microbiology and Infection* **10**: 585-587

Haase, A., Melder, A., Smith-Vaughan, H., Kemp, D. and Currie, B. (1995a) RAPD Analysis of Isolates of *Burkholderia pseudomallei* from patients with recurrent melioidosis. *Epidemiol Infect* **115**: 115-121

Haase, A., Smith-Vaughan, H., Melder, A., Wood, Y., Janmaat, A., Gilfedder, J., Kemp, D. and Currie, B. (1995b) Subdivision of *Burkholderia pseudomllei* ribotypes into multiple types by random amplified polymorphic DNA analysis provides new insights into epidemiology. *J Clin Microbiol* **33**: 1687-90

Haque, A., Chu, K., Easton, A., Stevens, M.P., Galyov, E.E., Atkins, T., Titball, R. and Bancroft, G.J. (2006a) A Live Experimental Vaccine against *Burkholderia pseudomallei* Elicits CD4+ T Cell-mediated Immunity, Priming T cells specific for 2 Type III Secretion System Proteins. *J Infect Dis* **194**: 1241-8

Haque, A., Easton, A., Smith, D., O'Garra, A., Rooijen, N.V., Lertmemongkolchai, G., Titbull, R.W. and Bancroft, G.J. (2006b) Role of T cells in Innate and Adaptive Immunity against Murine Burkholderia pseudomallei Infection. *J Infect Dis* **193**: 370-9

Harley, V.S., Dance, D.A.B., Drasar, B.S. and Tovey, G. (1998) Effects of *Burkholderia pseudomallei* and other *Burkholderia* species on eukaryotic cells in tissue culture. *Microbios* **96**: 71-93

Healey, G.D., Elvin, S.J., Morton, M. and Williamson, E.D. (2005) Humoral and Cell-Mediated Adaptive Immune Responses Are Required for Protection against *Burkholderia pseudomallei* Challenge and Bacterial Clearance Postinfection. *Infect Immun* **73**: 5945-51

Heckly, R.J. and Nigg, C. (1958) Toxins of *Pseudomonas pseudomallei*. II. Characterization. *J Bacteriol* **76**: 427-36

Holden, M.T.G., Titball, R.W., Peacock, S.J., Cerdeno-Tarraga, A.M., Atkins, T., Crossman, L.C., Pitt, T., Churcher, C., Mungall, K., Bentley, S.D., Sebaihia, M., Thomson, N.R., Bason, N., Beacham, I.R., Brooks, K., Brown, K.A., Brown, N.F., Challis, G.L., Cherevach, I., Chillingworth, T., Cronin, A., Crossett, B., Davis, P., DeShazer, D., Feltwell, T., Fraser, A., Hance, Z., Hauser, H., Holroyd, S., Jagels, K., Keith, K.E., Maddison, M., Moule, S., Price, C., Quail, M.A., Rabbinowitsch, E., Rutherford, K., Sanders, M., Simmonds, M., Songsivilai, S., Stevens, K., Tumapa, S., Vesaratchavest, M., Whitehead, S., Yeats, C., Barrell, B.G., Oyston, P.C.F. and Parkhill, J. (2004) From the Cover: Genomic plasticity of the causative agent of melioidosis, Burkholderia pseudomallei. *PNAS* **101**: 14240-5

Hosoglu, S., Loeb, M., Geyik, M.F., Ucmak, H. and Jayaratne, P. (2003) Molecular epidemiology of invasive *Salmonella typhi* in southeast Turkey. *Clin Microbiol Infect* **9**: 727-30

Inglis, T.J., Garrow, S.C., Adams, C., Henderson, M., Mayo, M. and Currie, B.J. (1999) Acute melioidosis outbreak in Western Australia. *Epidemiol Infect* **123**: 437-43

Inglis, T.J., Golledge, C.L., Clair, A. and Harvey, J. (2001a) Case report: recovery from persistent septicemic melioidosis. *Am J Trop Med Hyg* **65**: 76-82

Inglis, T.J., Mee, B.J. and Chang, B.J. (2001b) The environmental microbiology of melioidosis. *Reviews in Medical Microbiology* **12**: 13-20

Ip, M., Osterberg, L.G., Chau, P.Y. and Raffin, T.A. (1995) Pulmonary melioidosis. *Chest* **108**: 1420-4

Jesudason, M.V., Balaji, V., Sirisinha, S. and Sridharan, G. (2005) Rapid identification of Burkholderia pseudomallei in blood culture supernatants by a coagglutination assay. *Clin Microbiol Infect* **11**: 930-33

Joiner, K.A. (1988) Complement evasion by bacteria and parasites. *Ann Rev Microbiol* **42**: 201-30

Jones, A.L., Beveridge, T.J. and Woods, D.E. (1996) Intracellular survival of *Burkholderia pseudomallei*. *Infect Immun* **64**: 782-90

Kanaphun, P., Thirawattanasuk, N., Suputtamongkol, Y., Naigowit, P., Dance, D.A.B., Smith, M.D. and White, N.J. (1993) Serology and carriage of *Pseudomonas pseudomallei*: a prospective study in 1000 hospitalized children in northeast Thailand. *J Infect Dis* **167**: 230-3

Kashino, S.S., Napolitano, D.R., Skobe, Z. and Campos-Neto, A. (2008) Guinea pig model of *Mycobacterium tuberculosis* latent/dormant infection. *Microbes and Infection* **10**: 1469-76

Kawahara, K., Dejsirilert, S., Danbara, H. and Ezaki, T. (1992) Extraction and characterization of lipopolysaccharide from *Pseudomonas pseudomallei*. *FEMS Microbiol Lett* **96**: 129-34

Ketheesan, N., Barnes, J.L., Ulett, G.C., VanGessel, H.J., Norton, R.E., Hirst, R.G. and LaBrooy, J.T. (2002) Demonstration of a cell-mediated immune response in melioidosis. *J Infect Dis* **186**: 286-9

Kingston, C.W. (1971) Chronic or latent melioidosis. *Med J Aust* **2**: 618-21

Kondo, E., Kurata, T., Naigowit, P. and Kanai, K. (1996) Evolution of cell-surface acid phosphatase of *Burkholderia pseudomallei*. *Southeast Asian J Trop Med Public Health* **27**: 592-9

Kondo, E., Naigowit, P., Phanichruttiwong, P., Petkanchanapong, W., Chetanachan, P., Thirawattanasuk, N. and Kanai, K. (1991) Fatty acid profile and acid phosphatase activity of fresh isolates of *Pseudomonas pseudomallei*. *Jpn J Med Sci Biol* **44**: 195-211

Kondo, E., Wangroongsaub, P., Naigowit, P. and Kanai, K. (1994) Separation of antigenic glycoprotein fractions from cell-free homogenate of *Pseudomonas pseudomallei* and characterization as tyrosinephosphatase. *Southeast Asian J Trop Med Public Health* **25**: 436-42

Kongsaengdao, S., Bunnag, S. and Siriwiwattnakul, N. (2005) Treatment of survivors after the tsunami. *N Engl J Med* **352**: 2654-5

Koonpaew, S., Na-Ubol, M., Sirisinha, S., White, N.J. and Chaiyaroj, S.C. (2000) Genome fingerprinting by pulsed-field gel electrophoresis of isolates of *Burkholderia pseudomallei* from patients with melioidosis in Thailand. *Acta Trop* **74**: 187-91

Korbsrisate, S., Suwanasai, N., Leelaporn, A., Ezaki, T., Kawamura, Y. and Sarasombath, S. (1999) Cloning and characterization of a nonhemolytic

phospholipase C gene from *Burkholderia pseudomallei*. *J Clin Microbiol* **37**: 3742-5

Korbsrisate, S., Tomaras, A.P., Damnin, S., Ckumdee, J., Srinon, V., Lengwehasatit, I., Vasil, M.L. and Suparak, S. (2007) Characterization of two distinct phospholipase C enzymes from *Burkholderia pseudomallei*. *Microbiol* **153**: 1902-1915

Laddy, D.J. and Weiner, D.B. (2006) From Plasmids to Protection: A Review of DNA Vaccines against Infectious Diseases. *International Review of Immunology* **25**: 99-123

Lauw, F.N., Simpson, A.J., Prins, J.M., Smith, M.D., Kurimoto, M., Deventer, S.J.v., Speelman, P., Chaowagul, W., White, N.J. and Poll, T.v.d. (1999) Elevated plasma concentrations of interferon (IFN)-gamma and the IFN-gamma-inducing cytokines interleukin (IL)-18, IL-12, and IL-15 in severe melioidosis. *J Infect Dis* **180**: 1878-85

Lauw, F.N., Simpson, A.J.H., Hack, C.E., Prins, J.M., Wolbink, A.M., Deventer, S.J.H.v., Chaowagul, W., White, N. and Poll, T.v.d. (2000) Soluble granzymes are released during human endotoxemia and in patients with severe infection due to gram-negative bacteria. *J Infect Dis* **182**: 206-13

Lee, M.-A. and Liu, Y. (2000) Sequencing and characterization of a novel serine metalloprotease from *Burkholderia pseudomallei*. *FEMS Microbiol Lett* **192**: 67-72

Lertmemongkolchai, G., Cai, G., Hunter, C.A. and Bancroft, G.J. (2001) Bystander activation of CD8+ T cells contributes to the rapid production of IFN-g in response to bacterial pathogens. *J Immunol* **166**: 1097-105

Lew, A.E. and Desmarchelier, P.M. (1993) Molecular typing of *Pseudomonas pseudomallei*: restriction fragment length polymorphisms of rRNA genes. *J Clin Microbiol* **31**: 533-9

Li, B. and Yang, R. (2008) The interaction between *Yersinia pestis* and host immune system. *Infection and Immunity* **76**: 1804-11

Limmathurotsakul, D., Chaowagul, W., Chierakul, W., Stepniewska, K., Maharjan, B., Wuthiekanun, V., White, N.J., Day, N.P.J. and Peacock, S.J. (2006) Risk factors for recurrent melioidosis in Northeast Thailand. *Clin Infect Dis* **43**: 979-86

Maharjan, B., Chantratita, N., Vesaratchavest, M., Cheng, A., Wuthiekanun, V., Chierakul, W., Chaowagul, W., Day, N.P. and Peacock, S.J. (2005) Recurrent melioidosis in patients in northeast Thailand is frequently due to reinfection rather than relapse. *J Clin Microbiol* **43**: 6032-4

Malczewski, A.B., Oman, K.M., Norton, R.E. and Ketheesan, N. (2005) Clinical presentation of melioidosis in Queensland, Australia. *Trans R Soc Trop Med Hyg* **99**: 856-60

Manabe, Y.C., Kesavan, A.K., Lopez-Molina, J., Hatem, C.L., Brooks, M., Fujiwara, R., Hochstein, K., Pitt, M.L., Tufariello, J., Chan, J., McMurray, D., Bishai, W.R., Dannenberg, A.M. and Mendez, S. (2008) The aerosol rabbit model of TB latency, reactivation and immune reconstitution inflammatory syndrome. *Tuberculosis* **88**: 187-96

Masoud, H., Ho, M., Schollaardt, T. and Perry, M.B. (1997) Characterization of the capsular polysaccharide of *Burkholderia* (*Pseudomonas*) *pseudomallei* 304b. *J Bacteriol* **179**: 5663-9

Matsuura, M., Kawahara, K., Ezaki, T. and Nakano, M. (1996) Biological activities of lipopolysaccharide of *Burkholderia* (*Pseudomonas*) *pseudomallei*. *FEMS Microbiology Letters* **137**: 79-83

Mays, E.E. and Ricketts, E.A. (1975) Melioidosis: recrudescence associated with bronchogenic carcinoma twenty-six years following initial geographic exposure. *Chest* **68**: 261-2

McAllister, J., Gauci, P., Lazzaroni, S., Barnes, J., Ketheesan, N. and Proll, D. (2007) Assessment of a DNA vaccine encoding *Burkholderia pseudomallei* bacterioferritin (technical report). Defence Science and Technology Organisation, Australia.

McAllister, J., Lazzaroni, S., Barnes, J., Ketheesan, N. and Proll, D. (2008) Development of DNA vaccines against *Burkholderia pseudomallei* (technical report). DSTO Defence Science and Technology Organisation, Australia.

McCune, R.M., Feldmann, F.M., Lambert, H.P. and McDermott, W. (1966a) Microbial Persistance: I. The capacity of tubercle bacilli to survive sterilization in mouse tissues. *J Exp Med* **123**: 445-68

McCune, R.M., Feldmann, F.M. and McDermott, W. (1966b) Microbial Persistance: II. Characteristics of the sterile state of tubercle bacilli. *J Exp Med* **123**: 469-86

Miyagi, K., Kawakami, K. and Saito, A. (1997) The role of reactive nitrogen and oxygen intermediates in interferon-stimulated murine macrophage bactericidal activity against *Burkholderia pseudomallei*. *Infection and Immunity* **65**: 4108-13

Munckhof, W.J., Mayo, M.J., Scott, I. and Currie, B.J. (2001) Fatal human melioidosis acquired in a subtropical Australian city. *Am J Trop Med Hyg* **65**: 325-8

Nagata, T., Aoshi, T., Uchijima, M. and Koide, Y. (2008) In vivo hierarchy of individual T-cell epitope-specific helper t-cell subset against an intracellular bacterium. *Vaccine* **26**: 5123-27

Naigowit, P., Maneeboonyoung, W., Wongroonsub, P., Chaowagul, V. and Kanai, K. (1992) Serosurveillance for *Pseudomonas pseudomallei* infection in Thailand. *Jpn J Med Sci Biol* **45**: 215-30

Nguay, V., Lemeshev, Y., Sadkowski, L. and Crawford, G. (2005) Cutaneous Melioidosis in a Man Who Was Taken as a Prisoner of War by the Japanese during World War II. *J Clin Microbiol* **43**: 970-2

Nigg, C. (1963) Serologic studies on subclinical melioidosis. *J Immunol* **91**

Norazah, A., Rohani, M.Y., Chang, P.T. and Kamel, A.G.M. (1996) Indirect hemagglutination antibodies against Burkholderia pseudomallei in normal blood donors and suspected cases of melioidosis in Malaysia. *Southeast Asian J Trop Med Public Health* **27**: 263-6

Norton, R., Roberts, B., Freeman, M., Wilson, M., Ashhurst-Smith, C., Lock, W., Brookes, D. and Brooy, J.L. (1998) Characterisation and molecular typing of *Burkholderia pseudomallei*: are disease presentations of melioidosis clonally related? *FEMS Immunol Med Microbiol* **20**: 37-44

Novak, R.T., Glass, M.B., Gee, J.E., Gal, D., Mayo, M.J., Currie, B.J. and Wilkins, P.P. (2006) Development and evaluation of a real-time PCR assay targeting the type III secretion system of Burkholderia pseudomallei. *J Clin Microbiol* **44**: 85-90

O'Brien, M., Freeman, K., Lum, G., Cheng, A.C., Jacups, S.P. and Currie, B.J. (2004) Further evaluation of a rapid diagnostic test for melioidosis in an area of endemicity. *J Clin Microbiol* **42**: 2239-40

Parrish, N.M., Dick, J.D. and Bishai, W.R. (1998) Mechanisms of latency in *Mycobacterium tuberculosis*. *Trends in Microbiology* **6**: 107-12

Pitt, T.L., Aucken, H. and Dance, D.A.B. (1992) Homogeneity of lipopolysaccharide antigens in *Pseudomonas pseudomallei*. *J Infect* **25**: 139-46

Pruekprasert, P. and Jitsurong, S. (1991) Case report: septicemic melioidosis following near drowning. *Southeast Asian J Trop Med Public Health* **22**: 276-8

Puthucheary, S.D. and Vadivelu, J. (2002) Human Melioidosis. Singapore University Press, Singapore.

Reckseidler, S.L., DeShazer, D., Sokol, P.A. and Woods, D.E. (2001) Detection of bacterial virulence genes by subtractive hybridization: identification of capsular

polysaccharide of *Burkholderia pseudomallei* as a major virulence determinant. *Infect Immun* **69**: 34-44

Rimington, R.A. (1962) Melioidosis in North Queensland. *Med J Aust* **January**: 50-2

Santanirand, P., Harley, V.S., Dance, D.A.B., Drasar, B.S. and Bancroft, G.J. (1999) Obligatory role of gamma interferon for host survival in a murine model of infection with *Burkholderia pseudomallei*. *Infection and Immunity* **67**: 3593-600

Sarkar-Tyson, M., Thwaite, J.E., Harding, S.V., Smither, S.J., Oyston, P.C.F., Atkins, T.P. and Titball, R.W. (2007) Polysaccharides and virulence of *Burkholderia pseudomallei*. *J Med Microbiol* **56**: 1005-1010

Schlesinger, L.S., Bellinger-Kawahara, C.G., Payne, N.R. and Horwitz, M.A. (1990) Phagocytosis of *Mycobacterium tuberculosis* is mediated by human monocyte complement receptors and complement component C3. *J Immunol* **144**: 2771-80

Sexton, M.M., Goebel, L.A., Godfrey, A.J., Chaowagul, W., White, N.J. and Woods, D.E. (1993) Ribotype analysis of *Pseudomonas pseudomallei* isolates. *J Clin Microbiol* **31**: 238-43

Sexton, M.M., Jones, A.L. and Woods, D.E. (1994) Purification and characterization of a protease from *Pseudomonas pseudomallei*. *Can J Microbiol* **40**: 903-910

Sharma, A.K. and Khuller, G.K. (2001) DNA vaccines: Future strategies and relevance to intracellular pathogens. *Immunology and Cell Biology* **79**: 537-546

Sierra-Honigmann, M.R. and Murphy, P.A. (1992) T cell receptor-independent immunosuppression induced by dexamethasone in murine T helper cells. *J Clin Invest* **89**: 556-60

Simpson, A.J.H., Smith, M.D., Weverling, G.J., Suputtamongkol, Y., Angus, B.J., Chaowagul, W., White, N.J., vanDeventer, S.J.H. and Prins, J.M. (2000) Prognostic value of cytokine concentrations (tumor necrosis factor, interleukin-6 and interleukin-10) and clinical parameters in severe melioidosis. *J Infect Dis* **181**: 621-5

Sirisinha, S., Anuntagool, N., Dharakul, T., Ekpo, P., Wongratanacheewin, S., Naigowit, P., Petchclai, B., Thamlikitkul, V. and Suputtamongkol, Y. (2000) Recent developments in laboratory diagnosis of melioidosis. *Acta Trop* **74**: 235-45

Steinmetz, I., Rohde, M. and Brenneke, B. (1995) Purification and characterisation of an exopolysaccharide of *Burkholderia* (*Pseudomonas*) *pseudomallei*. *Infect Immun* **63**: 3959-65

Stevens, M.P., Haque, A., Atkins, T., Hill, J., Wood, M.W., Easton, A., Nelson, M., Underwood-Fowler, C., Titball, R.W., Bancroft, G.J. and Galyov, E.E. (2004) Attenuated virulence and protective efficacy of a *Burkholderia pseudomallei* bsa type III secretion mutant in murine models of melioidosis. *Microbiol* **150**: 2669-76

Stevens, M.P., Wood, M.W., Taylor, L.A., Monaghan, P., Hawes, P., Jones, P.W., Wallis, T.S. and Galyov, E.E. (2002) An Inv/Mxi-Spa-like type III protein secretion system in *Burkholderia pseudomallei* modulates intracellular behaviour of the pathogen. *Mol Microbiol* **46**: 649-59

Supaprom, C., Wang, D., Leelayuwat, C., Thaewpia, W., Susaengrat, W., Koh, V., Ooi, E.E., Lertmemongkolchai, G. and Liu, Y. (2007) Development of real-time PCR assays and evaluation of their potential use for rapid detection of *Burkholderia pseudomallei* in clinical blood specimens. *J Clin Microbiol* **45**: 2894-2901

Suputtamongkol, Y., Hall, A.J., Dance, D.A.B., Chaowagul, W., Rajchanuvong, A., Smith, M.D. and White, N.J. (1994) The epidemiology of melioidosis in Ubon Ratchatani, northeast Thailand. *Intern J Epidemiol* **23**: 1082-90

Thibault, F.M., Valade, E. and Vidal, D.R. (2005) Identification and discrimination of Burkholderia pseudomallei, B. mallei and B. thailandensis by real-time PCR targeting type III secretion system genes. *J Clin Microbiol* **42**: 5871-4

Tomaso, H., Pitt, T.L., Landt, O., Dahouk, S.A., Scholz, H.C., Reisinger, E.C., Sprague, L.D., Rathmann, I. and Neubauer, H. (2005) Rapid presumptive identification of Burkholderia pseudomallei with real-time PCR assays using fluorescent hybridization probes. *Mol Cell Probes* **19**: 9-20

Ulett, G.C., Currie, B.J., Clair, T.W., Mayo, M., Ketheesan, N., Labrooy, J., Gal, D., Norton, R., Smith, C.A., Barnes, J., Warner, J. and Hirst, R.G. (2001) *Burkholderia pseudomallei* virulence: definition, stability and association with clonality. *Microbes Infect* **3**: 1-11

Ulett, G.C., Ketheesan, N. and Hirst, R.G. (2000) Cytokine gene expression in innately susceptible BALB/c mice and relatively resistant C57BL/6 mice during infection with virulent Burkholderia pseudomallei. *Infect Immun* **68**: 2034-42

Utaisincharoen, P., Anuntagool, N., Arjcharoen, S., Limposuwan, K., Chaisuriya, P. and Sirisinha, S. (2004) Induction of iNOS expression and antimicrobial activity by interferon (IFN)-b is distinct from IFN-g in *Burkholderia pseudomallei*-infected mouse macrophages. *Clin Exp Immunol* **136**: 277-83

Utaisincharoen, P., Kespichayawattana, W., Anuntagool, N., Chaisuriya, P., Pichyangkul, S., Krieg, A.M. and Sirisinha, S. (2003) CpG ODN enhances uptake of bacteria by mouse macrophages. *Clin Exp Immunol* **132**: 70-5

Utaisincharoen, P., Tangthawornchaikul, N., Kespichayawattana, W., Chaisuriya, P. and Sirisinha, S. (2001) *Burkholderia pseudomallei* interferes with inducible nitric oxide synthase (iNOS) production: a possible mechanism of evading macrophage killing. *Microbiol Immunol* **45**: 307-13

Vadivelu, J. and Puthucheary, S.D. (2000) Diagnositic and prognostic value of an immunofluorescent assay for melioidosis. *Am J Trop Med Hyg* **62**: 297-300

Vadivelu, J., Puthucheary, S.D., Drasar, B.S., Dance, D.A.B. and Pitt, T.L. (1998) Stability of strain genotypes of *Burkholderia pseudomallei* from patients with single and recurrent episodes of melioidosis. *Trop Med Intern Health* **3**: 518-21

Vadivelu, J., Puthucheary, S.D., Mifsud, A., Drasar, B.S., Dance, D.A.B. and Pitt, T.L. (1997) Ribotyping and DNA macrorestriction analysis of isolates of *Burkholderia pseudomallei* from cases of melioidosis in Malaysia. *Trans R Soc Trop Med Hyg* **91**: 358-60

Warawa, J. and Woods, D.E. (2002) Melioidosis vaccines. *Expert Rev Vaccines* **1**: 477-82

White, N.J. (2003) Melioidosis. *The Lancet* **361**: 1715-22

White, N.J., Chaowagul, W., Wuthiekanun, V., Dance, D.A.B., Wattanagoon, Y. and Pitakwatchara, N. (1989) Halving of mortality of severe melioidosis by ceftazidime. *Lancet* **Sept**: 697-701

Whitmore, A. and Krishnaswami, C.S. (1912) An Account of the Discovery of a Hitherto Undescribed Infective Disease Occurring Among the Population of Rangoon. *The Indian Medical Gazette*: 262-7

Wiersinga, W.J., Poll, T.v.d., White, N.J., Day, N.P. and Peacock, S.J. (2006) Melioidosis: insights into the pathogenicity of Burkholderia pseudomallei. *Nat Rev Microbiol* **4**: 272-82

Wiersinga, W.J., Wieland, C.W., Dessing, M.C., Chantratita, N., Cheng, A.C., Limmathurotsakul, D., Chierakul, W., Leendertse, M., Florquin, S., de Vos, A.F., White, N., Dondrop, A.M., Day, N.P., Peacock, S.J. and van der Poll, T. (2007) Toll-like receptor 2 impairs host defense in gram-negative sepsis caused by *Burkholderia pseudomallei* (melioidosis). *PLoS Medicine* **5**: 1268-80

Winstanley, C., Hales, B.A., Corkill, J.E., Gallagher, M.J. and Hart, C.A. (1998) Flagellin gene variation between clinical and environmental isolates of *Burkholderia pseudomallei* contrasts with the invariance among clinical isolates. *J Med Microbiol* **47**: 689-94

Winstanley, C. and Hart, C.A. (2000) Presence of type III secretion genes in *Burkholderia pseudomallei* correlates with Ara(-) phenotypes. *J Clin Microbiol* **38**: 883-5

Wongratanacheewin, S., Kespichayawattana, W., Intachote, P., Pichyangkul, S., Sermswan, R.W. and Krieg, A.M. (2004) Immunostimulatory CpG oligodeoxynucleotide confers protection in a murine model of infection with *Burkholderia pseudomallei*. *Infect Immun* **72**: 4494-502

Wuthiekanun, V., Amornchai, P., Chierakul, W., Cheng, A.C., White, N.J., Peacock, S.J. and Day, N.P. (2004) Evaluation of immunoglobulin M (IgM) and IgG rapid cassette test kits for diagnosis of melioidosis in an area of endemicity. *J Clin Microbiol* **42**: 3435-7

Wuthiekanun, V., Chierakul, W., Langa, S., Chaowagul, W., Panpitpat, C., Saipan, P., Thoujaikong, T., Day, N.P. and Peacock, S.J. (2006a) Development of antibodies to *Burkholderia pseudomallei* during childhood in melioidosis-endemic northeast Thailand. *Am J Trop Med Hyg* **74**: 1074-5

Wuthiekanun, V., Langa, S., Swaddiwudhipong, W., Jedsadapanpong, W., Kaengnet, Y., Chierakul, W., Day, N.P. and Peacock, S.J. (2006b) Melioidosis in Myanmar: forgotten but not gone? *Am J Trop Med Hyg* **75**: 945-6

Xu, J., Wenhuan, X., Chen, X., Zhao, D. and Wang, Y. (2008) Recombinant DNA vaccine of the early secreted antigen ESAT-6 by *Mycobacterium tuberculosis* and Flt3 ligand enhance the cell-mediated immunity in mice. *Vaccine* **26**: 4519-25

Yang, H., Chaowagul, W. and Sokol, P.A. (1991) Siderophore production by *Pseudomonas pseudomallei*. *Infect Immun* **59**: 776-80

Yang, H., Kooi, C.D. and Sokol, P.A. (1993) Ability of *Pseudomonas pseudomallei* malleobactin to acquire transferrin-bound, lactoferrin-bound, and cell-derived iron. *Infect Immun* **61**: 656-62

Yeo, S.-H., Nihira, T. and Yamada, Y. (1998) Screening and identification of a novel lipase from *Burkholderia* sp. YY62 which hydrolyzes *t*-butyl esters effectively. *J Gen Appl Microbiol* **44**: 147-52

APPENDIX 1

SOLUTIONS & REAGENTS

A1.1 General Solutions

A1.1.1 1X PBS (pH 7.4)

NaCl (465, Ajax Finechem Pty Ltd, Australia)	8 g
KCl (393, Ajax Finechem Pty Ltd, Australia)	0.20 g
Na_2PO_4 (621, Ajax Finechem Pty Ltd, Australia)	1.15 g
KH_2PO_4 (391, Ajax Finechem Pty Ltd, Australia)	0.20 g

Make up to 800ml with single distilled water and adjust pH to 7.4. Bring the volume to 1000ml and readjust the pH if necessary. Autoclave at 121ºC for 30 mins.

A1.1.2 Breaking buffer

Leupeptin (L2023, Sigma-Aldrich, Australia)	0.2µg/ml
Pepstatin A (P5318, Sigma-Aldrich, Australia)	0.2µg/ml
DNase (D5025, Sigma-Aldrich, Australia)	50 Kunitz Units

Combine ingredients in sterile PBS and store aliquots at -20ºC until required.

A1.1.3 Blood agar

Blood agar base (CM0271, Oxoid, Australia)	20 g
Distilled water	500 ml

Weigh agar and dissolve in a boiling water bath. Autoclave at 121ºC for 30 mins. Allow to cool in 50ºC water bath overnight before adding 25ml of heparinised sheep blood. Pour into clean petri plates and allow seting. Store at 4ºC until required.

A1.1.4 Ashdown's agar

Tryptone (LP0042, Oxoid, Australia)	6 g
Glycerol (242, Ajax Finechem Pty Ltd, Australia)	16 ml
Neutral red (1%) (20078, BDH Chemicals Ltd, Australia)	2 ml
Crystal violet (0.1%) (C0775, Sigma-Aldrich, Australia)	2 ml
Agar technical no.3 (LP0013, Oxoid, Australia)	6 g
Distilled water	400 ml
Gentamicin sulphate (1mg/ml) (Gentam, Ilium, Australia)	3.2 ml

Prepare neutral red, by dissolving 1g in 100ml distilled water, and crystal violet by dissolving 0.1g in 100ml distilled water. Autoclave at 121ºC for 30 mins. Prepare gentamicin (50mg/ml) by diluting to 1mg/ml. To prepare Ashdown's agar add all ingredients, except the gentamicin, and boil for 15 minutes. Autoclave at 121ºC for 30 mins and allow to cool to 50ºC. Add 3.2ml of filter-sterilised gentamicin and pour into clean petri plates allowing setting. Store at 4ºC until required.

A1.1.5 Dexamethasone

Dexamethasone (D2915, Sigma-Aldrich, Australia)	100 mg
PBS (pH 7.4)	4 ml

Dissolve dexamethasone in PBS, aliquot and store at -70ºC. When required, dilute to 7.5mg/ml for each mouse to receive 1.5mg/200µl.

A1.2 PFGE Solutions and Reagents

A1.2.1 TE buffer

Tris-HCl pH 7.4 (10315.0500, Merck Pty Ltd, Australia)	10 mM
EDTA pH 8.0 (E26282, Sigma-Aldrich, Australia)	0.1 mM

Combine ingredients and autoclave at 121ºC for 30 mins. Store at room temperature until required.

A1.2.2 TES buffer

Tris-HCl pH 7.4 (10315.0500, Merck Pty Ltd, Australia)	50 mM
EDTA pH 8.0 (E26282, Sigma-Aldrich, Australia)	50 mM
Sarcosine (L9150, Sigma-Aldrich, Australia)	1 %

Combine ingredients and dissolve. Store at 4ºC until required. Filter-sterilise prior to use.

A1.2.3 Restriction enzyme (*XbaI*) buffer

Tris-HCl pH 7.4 (10315.0500, Merck Pty Ltd, Australia)	6 mM
$MgCl_2$ (M8266, Sigma-Aldrich, Australia)	6 mM
NaCl (465, Ajax Finechem Pty Ltd, Australia)	150 mM
Dithiothreitol (DTT) (43815, Sigma-Aldrich, Australia)	1 mM

Combine ingredients immediately before use and adjust to pH 7.9.

A1.2.4 10X TBE buffer

Tris (10315.0500, Merck Pty Ltd, Australia)	108 g
Boric acid (B7901, Sigma-Aldrich, Australia	55 g
0.5M EDTA pH 8.0	40 ml

Combine ingredients and make to 1L. Dilute solution as necessary.

A1.2.5 Ethidium bromide

Ethidium bromide (200271, Boehringer Manheim)	100 mg
TE buffer	10 ml

A1.3 Cell Culture Media & Reagents

A1.3.1 Culture medium

RPMI 1640	92 ml
(21870076, Invitrogen Corporation, Australia)	
Penicillin	10,000 Units
(15140-163, Invitrogen Corporation, Australia)	
Streptomycin	10 mg
(15140-163, Invitrogen Corporation, Australia)	
HEPES buffer	20 mM
(15630-080, Invitrogen Pty Ltd, Australia)	
L-glutamine	2 mM
(G8540, Sigma-Aldrich, Australia)	
Heat-inactivated serum	20 ml

For culture of human cells, 10% heat-inactivated pooled human serum was added and for murine cell culture, 10% heat-inactivated foetal bovine serum (FBS) was added.

A1.3.1.1 Heat-inactivated serum

Heat serum at 56ºC for 25 minutes. Allow to cool, aliquot into 10ml sterile tubes and store at -20ºC until required.

A1.3.1.2 L-glutamine stock solution

L-glutamine	15 g
(G8540, Sigma-Aldrich, Australia)	
RPMI 1640	1000 ml
(21870076, Invitrogen Corporation, Australia)	

Combine ingredients and filter-sterilise. Dispense into 2ml aliquots and store at -20ºC until required.

A1.3.2 Bovine Tuberculin purified protein derivative (PPD)

PPD stimulation antigen for BOVIGAM	20 ml
(CSL, 01201, 20901501)	

Store at 4ºC. Use 10µl of the stock solution in 200µl cell culture for a final concentration of 15µg/ml.

A1.3.3 Concanavalin A (Con A) stock solution

Concanavalin A	5 mg
(L7647, Sigma-Aldrich, Australia)	
RPMI 1640	50 ml
(21870076, Invitrogen Corporation, Australia)	

Combine ingredients and filter sterilise. Store at -70ºC until required. Use 10µl of the stock solution in 200µl cell culture for a final concentration of 2µg/ml.

A1.3.4 Phytohaemagglutinin (PHA)

PHA lectin	5 mg
(L8754, Sigma-Aldrich, Australia)	
RPMI 1640	25 ml
(21870076, Invitrogen Corporation, Australia)	

Combine ingredients and filter sterilise. Store at -70ºC until required. Use 10µl of the stock solution in 200µl cell culture for a final concentration of 10µg/ml.

A1.4 Flow Cytometry Reagents

A1.4.1 2% paraformaldehyde

Paraformaldehyde	2 %
(441244, Sigma-Aldrich, Australia)	
PBS (pH 7.4)	0.01 M

Combine ingredients and heat to 56ºC until dissolved. Store at 4ºC away from light.

A1.4.2 Sodium azide buffer

Sodium azide	0.1 %
(1222, Ajax Finechem Pty Ltd, Australia)	
FBS	1 %
(16141-079, Invitrogen Corporation, Australia)	

Combine ingredients and make in PBS (pH 7.4). Store at 4ºC until required.

APPENDIX 2

FACS DATA FOR CHARACTERISATION OF CMI RESPONSES IN SEROPOSITIVE INDIVIDUALS

Table A2.1 Data for T cell populations in seropositive and seronegative donors following stimulation with BpLy1

Donor	Titre	CD3/4/8			
		Stimulant	CD3+ cells	CD3+/4+ cells	CD3+/8+ cells
1	≤1:80	unstimulated	2780	1889	1064
		BpLy	13248	9436	5190
2	≤1:80	unstimulated	14344	8189	5840
		BpLy	13535	7347	6061
3	≤1:80	unstimulated	16542	9116	6043
		BpLy	15995	8505	6144
4	≤1:80	unstimulated	14662	11206	2891
		BpLy	14437	10096	2801
5	≤1:80	unstimulated	12813	6259	5792
		BpLy	12572	6132	5645
6	≥1:160	unstimulated	13434	8939	3629
		BpLy	13571	9032	3608
7	≥1:160	unstimulated	15376	8077	6716
		BpLy	14727	8217	5997
8	≥1:160	unstimulated	14015	10560	3033
		BpLy	13304	9847	2977
9	Negative	unstimulated	14774	10785	3865
		BpLy	13729	8351	3749
10	Negative	unstimulated	14178	12119	2782
		BpLy	13959	11965	2776
		PMA	13983	10495	2505
11	Negative	unstimulated	14737	10399	3679
		BpLy	14480	10462	3371
		PMA	17021	11885	4194
12	Negative	unstimulated	12440	9652	2264
		BpLy	11772	9185	2081
		PMA	13899	9618	2465
13	Negative	unstimulated	11100	7101	4103
		BpLy	12006	7967	4274
		PMA	17018	5741	3761
14	Negative	unstimulated	13155	8497	4464
		BpLy	13281	8784	4552
		PMA	6078	3292	2025
15	Negative	unstimulated	12874	5740	6007
		BpLy	12627	5778	5829
		PMA	3273	2546	1918
16	Negative	unstimulated	14172	10805	2977
		BpLy	14287	10497	3005
		PMA	14338	8792	2955

Table A2.2 Data for activated CD4+ T cells producing IL-4 in seropositive and seronegative donors following stimulation with BpLy1

Donor	Titre	Stimulant	CD4+ IL-4+					
			Total events	No. CD69+ cells	% CD69+ cells	No. CD69+ IL-4+	% CD69+ IL-4+	% difference CD69/IL-4+
1	≤1:80	unstimulated	4537	58	1.28	16	0.35	
		BpLy	7272	132	1.82	6	0.08	-0.27
2	≤1:80	unstimulated	6488	77	1.19	37	0.57	
		BpLy	5749	322	5.60	139	2.42	1.85
3	≤1:80	unstimulated	7219	19	0.26	3	0.04	
		BpLy	7047	97	1.38	2	0.03	-0.01
4	≤1:80	unstimulated	6937	22	0.32	3	0.04	
		BpLy	7296	219	3.00	3	0.04	0.00
5	≤1:80	unstimulated	7391	86	1.16	11	0.15	
		BpLy	7386	823	11.14	40	0.54	0.39
6	≥1:160	unstimulated	7373	140	1.90	29	0.39	
		BpLy	7320	324	4.43	20	0.27	-0.12
7	≥1:160	unstimulated	6124	147	2.40	69	1.13	
		BpLy	6230	308	4.94	60	0.96	-0.16
8	≥1:160	unstimulated	5540	35	0.63	17	0.31	
		BpLy	5159	203	3.93	49	0.95	0.64
9	Negative	unstimulated	6795	20	0.29	2	0.03	
		BpLy	6989	71	1.02	4	0.06	0.03
10	Negative	unstimulated	7196	13	0.18	3	0.04	
		BpLy	6999	78	1.11	5	0.07	0.03
		PMA	6486	4336	66.85	3	0.05	0.00
11	Negative	unstimulated	7273	151	2.08	22	0.30	
		BpLy	7224	488	6.76	36	0.50	0.20
		PMA	6886	6266	91.00	907	13.17	12.87
12	Negative	unstimulated	7529	62	0.82	12	0.16	
		BpLy	7028	354	5.04	14	0.20	0.04
		PMA	5691	5539	97.33	1855	32.60	32.44
13	Negative	unstimulated	5528	127	2.30	47	0.85	
		BpLy	5668	522	9.21	98	1.73	0.88
		PMA	1539	1506	97.86	449	29.17	28.32
14	Negative	unstimulated	5765	166	2.88	61	1.06	
		BpLy	5169	426	8.24	107	2.07	1.01
		PMA	905	809	89.39	322	35.58	34.52
15	Negative	unstimulated	4425	37	0.84	22	0.50	
		BpLy	4541	496	10.92	87	1.92	1.42
		PMA	182	170	93.41	80	43.96	43.46
16	Negative	unstimulated	6800	26	0.38	1	0.01	
		BpLy	7401	340	4.59	6	0.08	0.07
		PMA	6821	4570	67.00	5	0.07	0.06

Table A2.3 Data for activated CD4+ T cells producing IFN-γ in seropositive and seronegative donors following stimulation with BpLy1

Donor	Titre	Stimulant	CD4+ IFNγ+					
			Total events	No. CD69+ cells	% CD69+ cells	No. CD69+ IFNγ+	% CD69+ IFNγ+	% difference CD69/IFNγ+
1	≤1:80	unstimulated	5455	71	1.30	16	0.29	
		BpLy	7084	124	1.75	8	0.11	-0.18
2	≤1:80	unstimulated	6346	126	1.99	16	0.25	
		BpLy	5494	344	6.26	23	0.42	0.17
3	≤1:80	unstimulated	7230	32	0.44	4	0.06	
		BpLy	6941	134	1.93	2	0.03	-0.03
4	≤1:80	unstimulated	7382	22	0.30	3	0.04	
		BpLy	7091	204	2.88	4	0.06	0.02
5	≤1:80	unstimulated	7300	148	2.03	18	0.25	
		BpLy	7225	895	12.39	40	0.55	0.31
6	≥1:160	unstimulated	7445	105	1.41	8	0.11	
		BpLy	7343	314	4.28	16	0.22	0.11
7	≥1:160	unstimulated	5793	149	2.57	29	0.50	
		BpLy	5833	346	5.93	24	0.41	-0.09
8	≥1:160	unstimulated	5560	35	0.63	17	0.31	
		BpLy	5184	206	3.97	50	0.96	0.66
9	Negative	unstimulated	7029	24	0.34	2	0.03	
		BpLy	7271	92	1.27	2	0.03	0.00
10	Negative	unstimulated	7128	12	0.17	2	0.03	
		BpLy	6954	99	1.42	1	0.01	-0.01
		PMA	6317	3793	60.04	11	0.17	0.15
11	Negative	unstimulated	7545	199	2.64	4	0.05	
		BpLy	7304	519	7.11	16	0.22	0.17
		PMA	6985	6460	92.48	470	6.73	6.68
12	Negative	unstimulated	7554	76	1.01	1	0.01	
		BpLy	7177	428	5.96	6	0.08	0.07
		PMA	5288	5254	99.36	973	18.40	18.39
13	Negative	unstimulated	5016	248	4.94	18	0.36	
		BpLy	5264	384	7.29	18	0.34	-0.02
		PMA	1237	1157	93.53	211	17.06	16.70
14	Negative	unstimulated	5236	192	3.67	23	0.44	
		BpLy	4267	373	8.74	15	0.35	-0.09
		PMA	654	608	92.97	134	20.49	20.05
15	Negative	unstimulated	4456	39	0.88	22	0.49	
		BpLy	4586	501	10.92	90	1.96	1.47
		PMA	237	220	92.83	101	42.62	42.12
16	Negative	unstimulated	7287	37	0.51	1	0.01	
		BpLy	7065	287	4.06	2	0.03	0.01
		PMA	6934	5289	76.28	2	0.03	0.02

Table A2.4 Data for activated CD8+ T cells producing IL-4 in seropositive and seronegative donors following stimulation with BpLy1

Donor	Titre	Stimulant	CD8+ IL-4+					
			Total events	No. CD69+ cells	% CD69+ cells	No. CD69+IL-4+	% CD69+ IL-4+	% difference CD69/IFN
1	≤1:80	unstimulated	4082	93	2.28	14	0.34	
		BpLy	6778	405	5.98	7	0.10	-0.24
2	≤1:80	unstimulated	2162	41	1.90	9	0.42	
		BpLy	3798	275	7.24	31	0.82	0.40
3	≤1:80	unstimulated	7278	138	1.90	8	0.11	
		BpLy	7326	168	2.29	2	0.03	-0.08
4	≤1:80	unstimulated	4204	52	1.24	3	0.07	
		BpLy	4780	475	9.94	6	0.13	0.05
5	≤1:80	unstimulated	6514	84	1.29	6	0.09	
		BpLy	6910	751	10.87	6	0.09	-0.01
6	≥1:160	unstimulated	6249	90	1.44	14	0.22	
		BpLy	7132	339	4.75	16	0.22	0.00
7	≥1:160	unstimulated	4110	137	3.33	16	0.39	
		BpLy	3560	374	10.51	35	0.98	0.59
8	≥1:160	unstimulated	3250	63	1.94	21	0.65	
		BpLy	2774	197	7.10	22	0.79	0.15
9	Negative	unstimulated	7082	81	1.14	4	0.06	
		BpLy	6950	404	5.81	5	0.07	0.02
10	Negative	unstimulated	5320	180	3.38	7	0.13	
		BpLy	6383	478	7.49	6	0.09	-0.04
		PMA	5557	3021	54.36	8	0.14	0.01
11	Negative	unstimulated	7110	453	6.37	24	0.34	
		BpLy	6922	1132	16.35	69	1.00	0.66
		PMA	6623	5384	81.29	120	1.81	1.47
12	Negative	unstimulated	4700	90	1.91	13	0.28	
		BpLy	1594	104	6.52	7	0.44	0.16
		PMA	2156	1935	89.75	31	1.44	1.16
13	Negative	unstimulated	3267	221	6.76	57	1.74	
		BpLy	3600	277	7.69	57	1.58	-0.16
		PMA	2026	1449	71.52	35	1.73	-0.02
14	Negative	unstimulated	1131	63	5.57	11	0.97	
		BpLy	895	105	11.73	14	1.56	0.59
		PMA	1044	840	80.46	24	2.30	1.33
15	Negative	unstimulated	4419	37	0.84	17	0.38	
		BpLy	3955	274	6.93	21	0.53	0.15
		PMA	262	210	80.15	15	5.73	5.34
16	Negative	unstimulated	6339	157	2.48	3	0.05	
		BpLy	6371	658	10.33	5	0.08	0.03
		PMA	5870	3224	54.92	6	0.10	0.05

Table A2.5 Data for activated CD8+ T cells producing IFN-γ in seropositive and seronegative donors following stimulation with BpLy1

Donor	Titre	Stimulant	CD8+ IFNγ+					
			Total events	No. CD69+ cells	% CD69+ cells	No. CD69+ IFNγ+	% CD69+ IFNγ+	% difference CD69/IFNγ+
1	≤1:80	unstimulated	4607	67	1.45	11	0.24	
		BpLy	6742	366	5.43	4	0.06	-0.18
2	≤1:80	unstimulated	4098	72	1.76	4	0.10	
		BpLy	2765	110	3.98	9	0.33	0.23
3	≤1:80	unstimulated	7261	137	1.89	5	0.07	
		BpLy	7293	498	6.83	1	0.01	-0.06
4	≤1:80	unstimulated	4388	63	1.44	4	0.09	
		BpLy	4738	432	9.12	4	0.08	-0.01
5	≤1:80	unstimulated	6955	116	1.67	10	0.14	
		BpLy	6874	940	13.67	9	0.13	-0.01
6	≥1:160	unstimulated	7441	137	1.84	14	0.19	
		BpLy	6909	370	5.36	17	0.25	0.06
7	≥1:160	unstimulated	4142	73	1.76	6	0.14	
		BpLy	3539	245	6.92	7	0.20	0.05
8	≥1:160	unstimulated	3386	68	2.01	10	0.30	
		BpLy	2858	239	8.36	9	0.31	0.02
9	Negative	unstimulated	7086	82	1.16	6	0.08	
		BpLy	6844	425	6.21	4	0.06	-0.03
10	Negative	unstimulated	5362	136	2.54	1	0.02	
		BpLy	6100	438	7.18	2	0.03	0.01
		PMA	5916	3552	60.04	3	0.05	0.03
11	Negative	unstimulated	6716	345	5.14	9	0.13	
		BpLy	6361	1057	16.62	22	0.35	0.21
		PMA	6022	5310	88.18	32	0.53	0.40
12	Negative	unstimulated	6801	154	2.26	15	0.22	
		BpLy	5950	445	7.48	15	0.25	0.03
		PMA	5153	4784	92.84	84	1.63	1.41
13	Negative	unstimulated	1744	41	2.35	11	0.63	
		BpLy	2698	132	4.89	8	0.30	-0.33
		PMA	545	480	88.07	18	3.30	2.67
14	Negative	unstimulated	1505	55	3.65	10	0.66	
		BpLy	1631	164	10.06	10	0.61	-0.05
		PMA	403	338	83.87	4	0.99	0.33
15	Negative	unstimulated	4019	28	0.70	9	0.22	
		BpLy	3612	290	8.03	10	0.28	0.05
		PMA	490	457	93.27	14	2.86	2.63
16	Negative	unstimulated	6424	151	2.35	4	0.06	
		BpLy	6255	590	9.43	1	0.02	-0.05
		PMA	5928	3033	51.16	2	0.03	-0.03