Antibody Detection of *Burkholderia pseudomallei* and *Burkholderia mallei*

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Summary

Monoclonal and polyclonal antibodies have been produced for use in immunological assays for the detection of *Burkholderia pseudomallei* and *Burkholderia mallei*. Monoclonal antibodies recognising a high molecular weight polysaccharide material found in some strains of both species have been shown to be effective in recognising *B. pseudomallei* and *B. mallei* and distinguishing them from other organisms. The high molecular weight polysaccharide material is thought to be the capsule of *B. pseudomallei* and *B. mallei* and may have important links with virulence. *B. pseudomallei* and *B. mallei* are known to be closely related, sharing many epitopes, but antigenic variation has been demonstrated within both the species. The lipopolysaccharide from strains of *B. pseudomallei* and *B. mallei* has been isolated and the silver stain profiles found to be visually very similar. A monoclonal antibody raised to *B. mallei* LPS has been found to recognise both *B. mallei* and *B. pseudomallei* strains. However, in a small number of *B. pseudomallei* strains a visually atypical LPS profile has been demonstrated. A monoclonal antibody raised against this atypical LPS showed no recognition of the typical LPS profile of either *B. mallei* or *B. pseudomallei*. This atypical LPS structure has not been reported and may be immunologically distinct from the typical LPS. Molecular biology and antibody engineering techniques have been used in an attempt to produce single-chain antibody fragments reactive to *B. pseudomallei*. Sequencing of one of the single-chain antibody fragments produced showed high homology with murine immunoglobulin genes, but none of the single-chain antibody fragments were found to be specific to *B. pseudomallei*.

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Abbreviations

A_{280}	absorbance at 280nm
A_{362}	absorbance at 362nm
A_{490}	absorbance at 490nm
ABB	antigen binding buffer
ABTS	2'2,-azino-bis{3-ethylbenzthiazoline}-6-sulphonic acid
ACDP	Advisory Committee on Dangerous Pathogens
ATCC	American Type Culture Collection
BSA	bovine serum albumin
cfu(s)	colony forming unit(s)
°C	degrees centigrade
cDNA	cyclic deoxyribonucleic acid
dH_2O	distilled water
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
FBS	foetal bovine serum
FIA	Freunds incomplete adjuvant
FITC	fluorescein isothiocyanate
HAT	hypoxanthine-aminopterin-thymidine
HRP	horse radish peroxidase
HT	hypoxanthine-thymidine
IFA	indirect fluorescence assay
Ig	immunoglobulin
IgG	immunoglobulin class G
IgG-IFA	indirect fluorescence antibody assay for IgG antibodies
IgG_1	immunoglobulin G subclass 1
IgG_{2a}	immunoglobulin G subclass 2a
IgG_{2b}	immunoglobulin G subclass 2b
IgG_3	immunoglobulin G subclass 3

IgM	immunoglobulin class M
IgM-IFA	indirect fluorescence antibody assay for IgM antibodies
IHA	indirect haemagglutination assay
kb	kilobase(s)
kDa	kilodalton(s)
LAL	limulus amoebocyte lysate
LPS	lipopolysaccharide
LSHTM	London School of Hygiene and Tropical Medicine
µg	microgramme(s)
µl	microlitre(s)
µm	micrometre(s)
mg	milligramme(s)
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
M	molar concentration
MCR	molar coupling ratio
MIC_{90}	minimum inhibitory concentration for 90% inhibition
mM	millimolar concentration
MW	molecular weight
NCTC	National Collection of Typed Cultures
ng	nanogramme(s)
nm	nanometre(s)
nmol	nanomolar concentration
OD_{600}	optical density at 600nm
OMP	outer membrane protein
PBS	phosphate buffered saline
PBST	PBS containing 0.05% tween 20
PCR	polymerase chain reaction
PHLS	Public Health Laboratory Service
pmol	picomolar concentration
rpm	revolutions per minute

rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	second(s)
TAE	tris-acetate-EDTA buffer
U	units of enzyme activity
v/v	volume per volume
w/v	weight per volume

1. Introduction

1.1 History

Burkholderia pseudomallei and *Burkholderia mallei* are closely related, both antigenically and epidemiologically. Taxonomically they have been classified together in a variety of genera (Rogul, M., Brendle, J.J. *et al.* 1970). In 1992, both species were reclassified into the newly approved genus *Burkholderia* with five other related species (Yabuuchi, E., Kosako, Y. *et al.* 1992; IUMS. 1993). Limited studies with polyclonal antisera have suggested that there are no serologic tests that can differentiate melioidosis from glanders (Ashdown, L.R., Johnson, R.W. *et al.* 1989).

In the 19th century glanders/farcy was a common disease of horses and related animals. The causative organism, *B. mallei*, was not isolated until 1882 when Loeffler and Schutz succeeded in culturing it from an infected animal (Smith, G.R., Pearson, A.D. *et al.* 1990). Human disease occurs primarily in persons in close contact with horses, mules or donkeys (Sanford, J.P. 1985). *B. pseudomallei* is the causative agent of melioidosis, a disease endemic in the tropics. It was first described in 1912 by Whitmore and Krishnaswami who reported a glanders-like illness in Rangoon. However, the bacterium isolated was not the glanders bacillus as expected but an unknown, actively motile bacterium which was designated *Bacillus whitmori*, now classified as *B. pseudomallei* (Whitmore, A. and Krishnaswami, C.S. 1912).

Cases of melioidosis and glanders are rare in the Western world. However, a number of United States servicemen developed melioidosis after serving in Vietnam (Weber, D.R., Douglass, L.E. *et al.* 1969; Rubin, H.L., Alexander, A.D. *et al.* 1963).

1.2 Growth and cultural conditions

B. pseudomallei is a Gram negative, motile, non spore-forming bacillus, 0.4 - 0.6μm in width and 2 - 5μm in length. The bacteria exhibit a characteristic bipolar staining (Figure 1) and are seen singly, in pairs or very occasionally in chains (Pitt, T.L. 1990). The organism grows aerobically in many simple media and will grow under strictly anaerobic conditions in a complex medium containing nitrate. It has been suggested that the organism can switch from aerobic to anaerobic metabolism in response to environmental conditions (Kanai, K. and Kondo, E. 1994). The range of organic compounds which may be used as sole sources of carbon and energy is exceptionally wide, making *B. pseudomallei* one of the most nutritionally versatile members of the *Burkholderia* genus (Redfearn, M.S., Palleroni, N.J. *et al.* 1966).

The form and colour of growth on solid media is highly variable and colonies can range in structure from extreme rough to mucoid and in colour from cream to bright orange (Redfearn, M.S., Palleroni, N.J. *et al.* 1966). No particular colonial type appears associated with virulence (Howe, C., Sampath, A. *et al.* 1971). However, Velyanov (1982) reported that rough colonies were more virulent in guinea pigs than smooth, which were only slightly virulent or avirulent (Velyanov, D., Kharbov, D. *et al.* 1982).

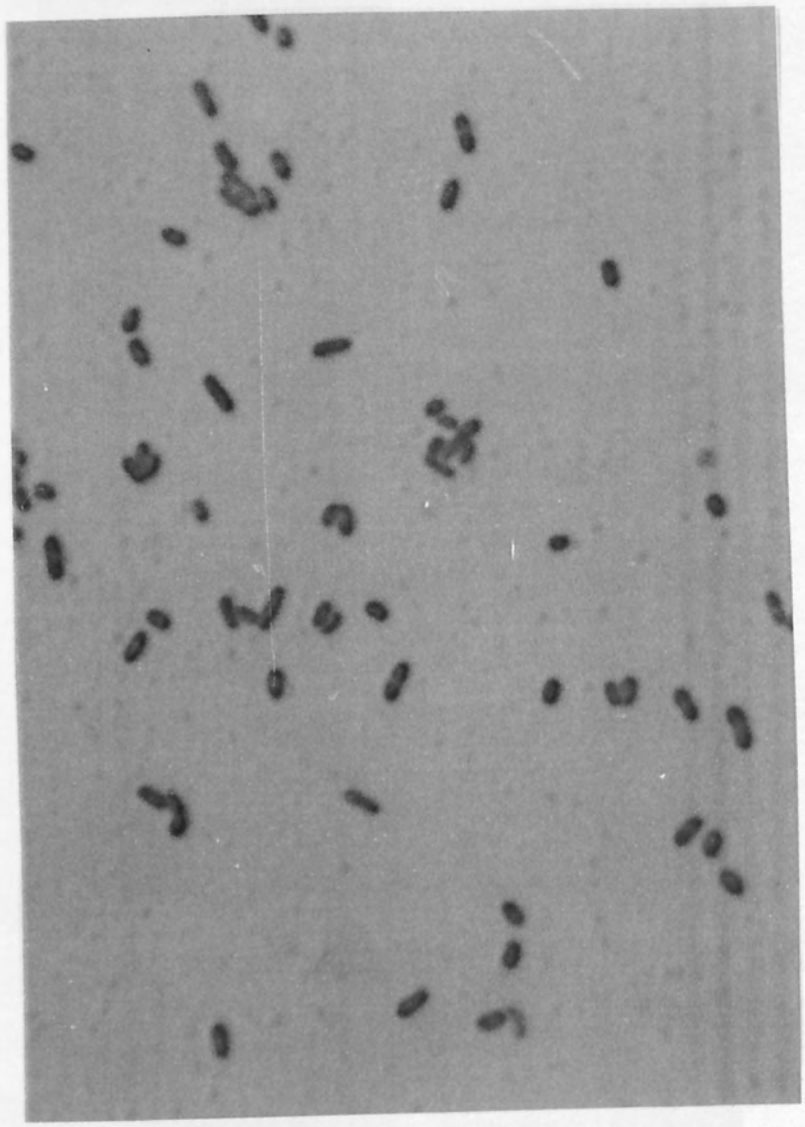


Figure 1. Gram stain of *B. pseudomallei* grown on nutrient agar plates (magnification x600).

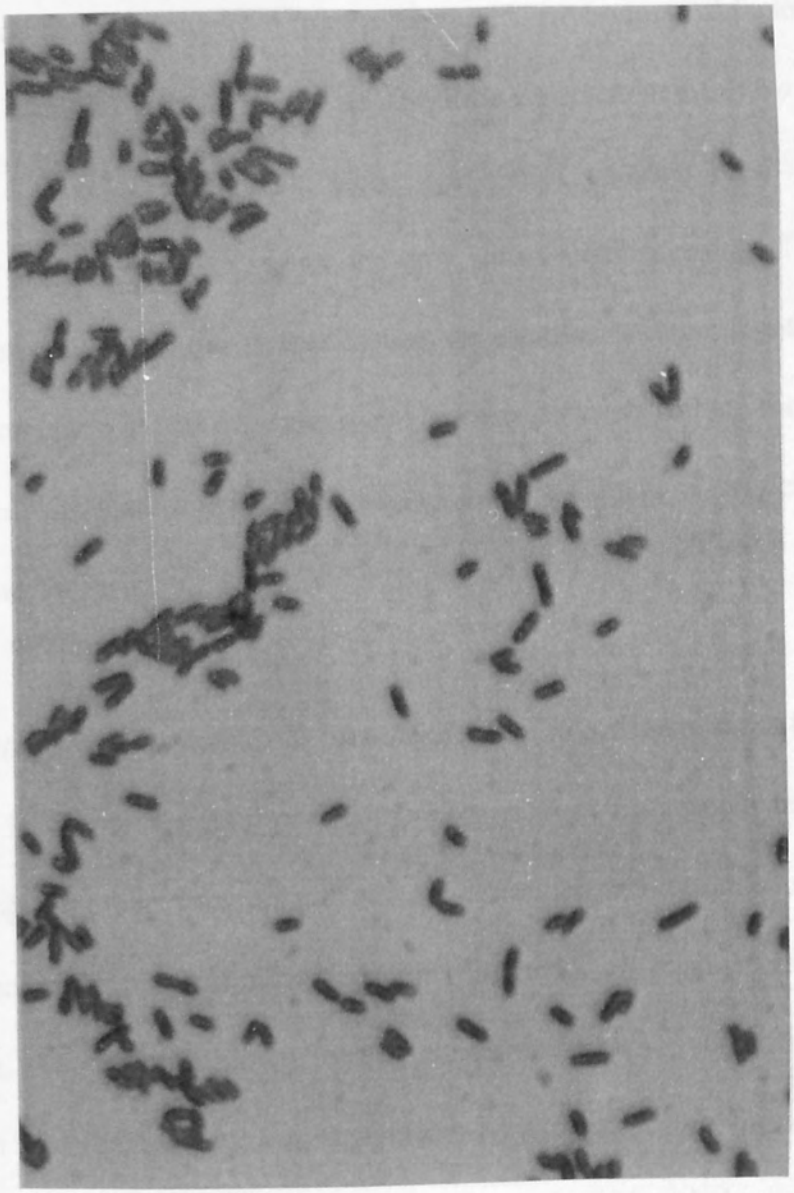


Figure 2. Gram stain of *B. mallei* grown on nutrient agar plates (magnification x600)

Chambon and Fournier (1956) also suggested that virulence may be dependent on the presence of the capsular antigen (Chambon, L. and Fournier, J. 1956). *B. pseudomallei* has been shown to secrete an abundant slime layer made up of polysaccharides. This layer has been termed the "glycocalyx" and it is thought that bacterial cells may live and divide within it, protected from unfavourable environmental factors (Kanai, K. and Kondo, E. 1994). This capsule has also been demonstrated *in vivo* (Mel'nikov, B.I., Popov, S.F. *et al.* 1990). On blood agar plates, colonies of *B. pseudomallei* are transparent, shiny, convex, entire and grey with a slight greening of the blood around confluent growth. After further incubation colonies become slightly raised, circular with a regular crenated edge and a dull surface. In broth culture, moderate turbidity is obtained with surface growth becoming a definite pellicle in forty eight hours (Green, R.N. and Tuffnell, P.G. 1968).

B. mallei closely resembles *B. pseudomallei*. The organism lacks the polar flagellum found in *B. pseudomallei* and is therefore non-motile. Some cells exhibit bipolar staining and others show minute alternating stained and unstained segments similar to the beading of the tuberculosis bacilli (Figure 2). *B. mallei* grows well on simple media, although more slowly than *B. pseudomallei*. The range of organic compounds which may be used by the organism is similar but more limited than that for *B. pseudomallei*. *B. mallei* is also capable of growth under strictly anaerobic conditions in a complex medium containing nitrate (Redfearn, M.S., Palleroni, N.J. *et al.* 1966). The form and colour of growth of *B. mallei* on solid medium does not show the variations found with *B. pseudomallei*. Colonies range from smooth cream to smooth white, with occasional rough white colonies being seen. In contrast to *B. pseudomallei*, *B. mallei* is

typically homogeneous in liquid culture and produces a modest turbidity (Egmed, T. and Ipatenko, N.G. 1967).

1.3 Epidemiology

Melioidosis has been detected in the tropics in the region from 20° north to 20° south of the equator (Figure 3) (Leelarasamee, A. and Bovornkitti, S. 1989). The disease is an important cause of human morbidity and mortality in Thailand and this may be true throughout south-east Asia. In Australia, melioidosis causes a smaller number of human infections but among livestock the disease has important economic and public health implications (Currie, B. 1993). Increasing numbers of reports of the infection indicate its presence in other tropical regions such as the Indian subcontinent, Africa, China and Central and South America (Dance, D.A.B. 1991; Li, L. and You-wen, H. 1992).

Cases of melioidosis have been considered rare in the Western world (Barnes, P.F., Appleman, M.D. *et al.* 1986) but in the 1960's, a large number of cases were reported in soldiers who became ill after returning home from the Vietnam War (Weber, D.R., Douglass, L.E. *et al.* 1969; Sanford, J.P. 1977). With improvements in diagnostic techniques the number of reported cases in developing countries is increasing (Arakawa, M. 1990a). The number of reported cases of travellers to equatorial regions developing the disease during their visit or after their return has also increased (Jenkins, D.R., Lewis, A.M. *et al.* 1990; Wilks, D., Jacobson, S.K. *et al.* 1994; Beeker, A., Van de Stadt, K.D. *et al.* 1999; Dance, D.A.B., Smith, M.D. *et al.* 1999; Torrens, J.K., McWhinney, P.H.M. *et al.* 1999).

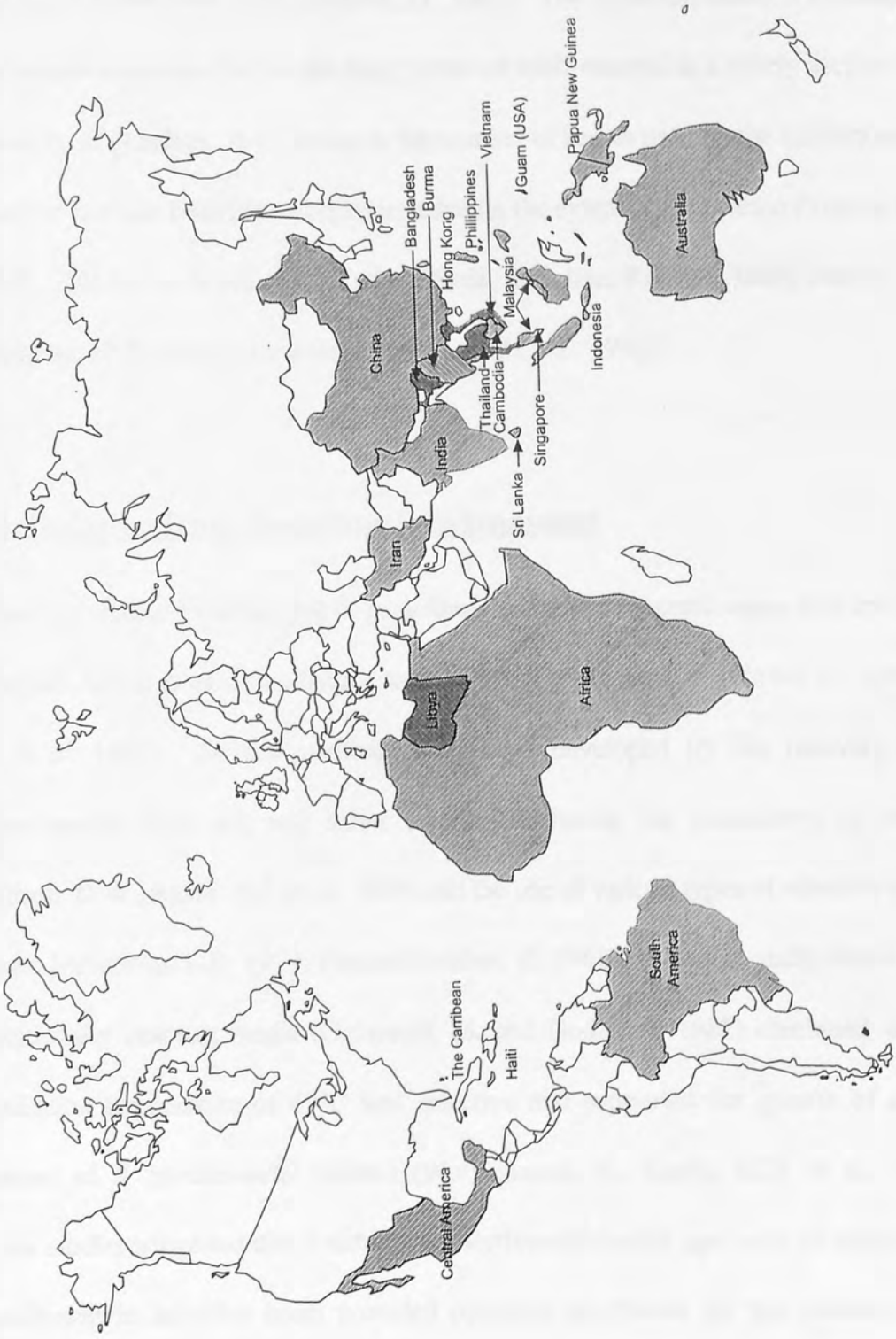


Figure 3. World map showing areas endemic for *B. pseudomallei.*

Glanders was once distributed throughout the world but now appears to have been eradicated except in Asia. There have been no naturally acquired infections reported in the United States since 1938 (Sanford, J.P. 1985). The implementation of eradication and control measures such as the Farcy Order of 1907 resulted in a steady decline in the incidence of glanders. A reduction in the number of horses used by the military and for transport has also been instrumental in reducing the extent of the disease (Verma, R.D. 1981). The disease is present in Turkey, Syria, Iraq, Iran, Pakistan, India, Burma, Indonesia, Philippines, China and Mongolia (Pitt, T.L. 1990).

1.4 Isolation from the natural environment

There is abundant evidence that *B. pseudomallei* exists in endemic areas as a free living organism which is easily cultivated from soil and water samples (Howe, C., Sampath, A. *et al.* 1971). Several methods have been developed for the recovery of *B. pseudomallei* from soil and water samples including the inoculation of animals (Ellison, D.W., Baker, H.J. *et al.* 1969) and the use of various types of selective culture media (Ashdown, L.R. 1979; Farkas-Himsley, H. 1968). A recent study found that a nutritionally exacting media (Galimand, M. and Dodin, A. 1982) combined with an incubation temperature of 42°C was selective and supported the growth of a large number of *B. pseudomallei* isolates (Wuthiekanun, V., Smith, M.D. *et al.* 1995a). Other studies suggested that Ashdown's selective-differential agar with or without pre-enrichment in selective broth provided optimum conditions for the isolation of *B. pseudomallei* from soil (Ashdown, L.R. and Clarke, S.G. 1992; Brook, M.D., Currie, B. *et al.* 1997). It has been suggested that *B. pseudomallei* can survive nutrient

starvation by forming viable but non-culturable cells which would aid its long term survival in the environment (Thibault, F.M., Paucod, J.C. *et al.* 1997).

B. mallei is considered an obligate animal parasite as it has not been isolated from environmental reservoirs. Culture is usually on nutrient or glycerol agar. If the material is contaminated, it may be incubated for 3 hours at 37°C in physiological saline containing 1,000 units/ml of benzyl penicillin and then plated onto agar containing crystal violet. A more sensitive method is to inject the material, preferably after incubation with penicillin into a guinea pig or hamster and recover the organism from enlarged glands (Parker, M.T., Smith, G.R. *et al.* 1990).

1.5 Identification

1.5.1 Microbiological methods

Isolation of the causative organism remains a vital part of the diagnosis of melioidosis. The use of selective agar and pre-enrichment in broth facilitates the isolation and identification of *B. pseudomallei* (Wuthiekanun, V., Dance, D.A.B. *et al.* 1990), particularly in mixed isolates. One problem has been the tendency to disregard isolates because of their resemblance to insignificant pseudomonads such as *Pseudomonas aeruginosa* or *Bacillus spp.* (Walsh, A. and Wuthiekanun, V. 1996). Table 1 shows the characteristics used to differentiate between *B. mallei*, *B. pseudomallei* and *Pseudomonas aeruginosa.*

Characteristics	*B. mallei*	*B. pseudomallei*	*P. aeruginosa*
Growth on simple media	slow	rapid	rapid
Motility	-	+	+
Production of soluble pigment	none	none	frequent
Susceptibility to *B. pseudomallei* phage	+	+	-
Phosphatase activity	no data	heat stable	heat labile
Growth at 42°C	-	+	+

Table 1. Characteristics used to differentiate between *B. pseudomallei*, *B. mallei* and *P. aeruginosa* (Howe, C., Sampath, A. *et al.* 1971).

Thomas (1983) evaluated the use of the API 20E (Biomerieux, Lyon, France) and the Microbact 24E (Disposable Products Ltd., Ridleyton, Australia) systems for the identification of *B. pseudomallei* and found the Microbact system to be consistently reliable with few false identifications (Thomas, A.D. 1983). The use of simple biochemical screening tests and the API 20NE system has been reported as a cheap, simple and accurate means of identifying *B. pseudomallei* from clinical specimens (Dance, D.A.B., Wuthiekanun, V. *et al.* 1989b). The Minitek disc system has also been recommended for rapidly differentiating *B. pseudomallei* from *B. cepacia* (Ashdown, L.R. 1992b). A latex agglutination test has been developed using polyclonal rabbit serum which was 100% sensitive and specific for the identification of *B. pseudomallei* and offers a rapid and effective method for the early screening of suspect *B. pseudomallei* colonies (Smith, M.D., Wuthiekanun, V. *et al.* 1993). Cultures of

B. pseudomallei were routinely "sniffed" to detect the characteristic earthy odour. However, in 1992 a recommendation was made to prohibit the "sniff" test to reduce the risk of laboratory acquired infection (Ashdown, L.R. 1992a).

1.5.2 Serological methods

B. pseudomallei and *B. mallei* are antigenically similar and currently there is no serological test that can differentiate melioidosis from glanders (Ashdown, L.R., Johnson, R.W. *et al.* 1989). Serum specimens can be screened by indirect haemagglutination (IHA) assay. The IHA is sensitive, with false positives occurring infrequently. However sera from culture-positive patients were sometimes non-reactive in the IHA suggesting that false negative reactions may occur in culture-negative patients with sub-clinical melioidosis. Nigg (1963) suggested that the haemagglutination test was not sufficiently specific for the diagnosis of sub-clinical melioidosis in man (Nigg, C. 1963).

Bacterial agglutination and precipitation tests are unreliable for the detection of *B. mallei*. Indirect haemagglutination and complement fixation are believed to give better results (Parker, M.T., Smith, G.R. *et al.* 1990).

1.5.3 Immunological methods

Fluorescent-labelled antibodies are a useful tool in identification and can be used either directly or indirectly on cultures and contaminated specimens. Indirect fluorescence assays (IFA) have been developed for specific immunoglobulin classes (IgG-IFA and

IgM-IFA). Sub-clinical melioidosis can be distinguished from active infection with the IgM-IFA. High titres of IgM antibody (1/40 or higher) with appropriate signs and symptoms are indicative of clinical melioidosis (Ashdown, L.R. 1981b; Manson-Bahr, P.E.C. and Bell, D.R. 1991). *B. mallei* and *B. pseudomallei* are found to cross react but are distinguishable from pseudomonads (Ashdown, L.R. 1981a).

Direct immunofluorescence has also been used and is reported to be both sensitive and specific. This technique is believed to give a confident diagnosis of melioidosis within two hours of admission to hospital providing appropriate samples are available (Walsh, A.L., Smith, M.D. *et al.* 1994). Enzyme linked immunosorbent assay (ELISA) based methods have been developed to detect antibodies to *B. pseudomallei* and *B. mallei* in underlying sub-clinical infections (Ashdown, L.R., Johnson, R.W. *et al.* 1989; Verma, R.D., Sharma, J.K. *et al.* 1990). Other ELISA methods have been developed for the detection of *B. pseudomallei* antigens (Wongratanacheewin, S., Tattawasart, U. *et al.* 1990) and the detection of antibodies to the 31kDa exotoxin produced by *B. pseudomallei* has been suggested as a means of diagnosis (Ismail, G., Noor Embi, M. *et al.* 1987). A monoclonal antibody to a 30kDa outer membrane protein has also been used to identify *B. pseudomallei* in blood cultures (Pongsunk, S., Thirawattanasuk, N. *et al.* 1999).

1.5.4 Deoxyribonucleic acid (DNA) based methods

DNA probes have been developed for the rapid and specific identification of *B. pseudomallei*. Sermswan *et al* developed a specific DNA probe for *B. pseudomallei* (based on the sequence of 1.5kb chromosomal DNA) which in Southern blot

hybridisation could detect 1.5ng of genomic DNA or 40,000 *B. pseudomallei* cells. The probe did not cross-react with DNA prepared from other related bacteria although the specificity with *B. mallei* was not tested (Sermswan, R.W., Wongratanacheewin, S. *et al.* 1994).

A molecular method for the detection and identification of *B. pseudomallei* would be a useful tool in the diagnosis of *B. pseudomallei* infection. Oligonucleotide probes have been developed for use in the polymerase chain reaction (PCR) for the amplification of specific DNA sequences. These probes have been based on the 23S ribosomal RNA (rRNA) gene sequence, 16S rRNA gene sequence or chromosomal DNA sequences. PCR-based detection reactions have ranged in sensitivity from trace amounts of DNA to 10^4 cells and have been used to detect *B. pseudomallei* in both clinical and soil samples (Brook, M.D., Currie, B. *et al.* 1997; Rattanathongkom, A., Sermswan, R.W. *et al.* 1997; Lew, A.E. and Desmarchelier, P.M. 1994; Dharakul, T., Songsivilai, S. *et al.* 1996). Trials carried out in Australia found PCR assays to be useful in identifying *B. pseudomallei* in environmental samples. However, there were a number of false positives in clinical samples and because of this PCR is not used in the routine diagnosis of *B. pseudomallei* infection (Haase, A., Brennan, M. *et al.* 1998).

The development of PCR-based probes to detect and differentiate between *B. mallei* and *B. pseudomallei* has proved difficult. Probes based on the 16S rRNA detected both *B. pseudomallei* and *B. mallei*, with no cross reaction with other related or commonly found organisms (Leary, S.E.C. 1995). A specific PCR assay for *B. pseudomallei* has been developed based on random DNA fragments which does not cross react with *B.*

mallei (Sura, T., Smith, M.D. *et al.* 1997) and PCR amplification of the flagellin gene followed by digestion of the products with a restriction enzyme has also differentiated between the two species (Neubauer, H., Splettstober, W. *et al.* 1999).

1.5.5 The mallein test

Following the discovery and identification of *B. mallei* in 1822, Helman and Kilning isolated a material termed mallein from cultures of the organism. When injected into horses previously exposed to *B. mallei* it caused a rise in body temperature and swelling at the injection site (McGilvray, C.D. 1944). This became known as the mallein test and is used to detect latent cases of the disease in equines (Egmed, T. and Ipatenko, N.G. 1967). In the past the mallein test has been modified to permit its safe use in humans, commercial mallein was diluted 1:10,000 before intradermal injection in the forearm. Reactions in positive individuals occurred after 24 hours, were 10-20mm in diameter and did not fade for 48 hours (Howe, C. and Miller, W.R. 1947).

1.6 Glanders and melioidosis

B. pseudomallei causes disease in humans and in a variety of animal species (Table 2) including dolphins, sheep, pigs and goats (Currie, B., Smith-Vaughan, H. *et al.* 1994; Dance, D.A.B. 1991; Vedros, N.A., Chow, D. *et al.* 1988; Dance, D.A.B., King, C. *et al.* 1992). In humans, melioidosis is acquired mainly by contamination with infectious soil and water through a pre-existing skin abrasion or ulcer or alternatively by inhalation of infectious dust particles (Leelarasamee, A. and Bovornkitti, S. 1989). The incubation period is normally 2-3 days, but there are reports of the disease developing

6-26 years after exposure (Arakawa, M. 1990b). The clinical spectrum of melioidosis is extremely diverse, consisting of four forms of disease: acute septicaemia, sub-acute illness, chronic infection and sub-clinical disease. The disease may be localised or disseminated and virtually any organ may be affected (Table 3). Depending on whether the disease is acute or chronic, melioidosis can mimic other infections including typhoid, malaria, tuberculosis and septicaemia from other common bacterial organisms (Ip, M., Osterberg, L.G. *et al.* 1995; Everett, E.D. and Nelson, R.A. 1975). The importance of early diagnosis and treatment has been stressed in the management of acute, septicaemic melioidosis. However, symptoms are so varied and the demise of the patient so rapid that unless a clinician is alert to the possibility of a *B. pseudomallei* infection, the patient is at great risk (Arakawa, M. 1990b).

Animal species	**Reporting Country**
Sheep, goats and pigs	Caribbean
Dolphins	Hong Kong
Sheep, cattle, birds and pigs	Australia
Cynomologus monkeys	Britain (imported from the Philippines)
Rats	Indonesia
Pigs	Madagascar
Goats and pigs	Africa
Horses and mules	Iran
Horses	Spain
Horses and various zoo animals	France

Table 2. Animal species which have been reported to be susceptible to infection with *B. pseudomallei*.

The acute septicaemic form is usually associated with an underlying disease or condition such as diabetes, alcoholism or immunodeficiency and despite current antibiotic regimes, still carries a high mortality rate (50-75%) (Majid, A.A. 1990).

Lesions apparent during the progression of melioidosis were shown to contain a large number of infiltrating cells, mainly macrophages many of which contained phagocytosed bacilli (Wong, K.T., Puthucheary, S.D. *et al.* 1995). Most of the bacterial cells were surrounded by an electron dense substance and had a clear halo between the substance and the cell wall (Narita, M., Loganathan, P. *et al.* 1982). Following uptake, ingested *B. pseudomallei* escape from the vacuoles, possibly as a result of bacterium mediated vacuolar membrane damage (Harley, V.S., Dance, D.A.B. *et al.* 1994).

Each clinical case of melioidosis may represent the outcome of one of three possible events: primary infection, re-infection or re-activation of latent disease. Primary infection appears almost inevitable in children in north-east Thailand where approximately 80% have developed antibodies to *B. pseudomallei* by the age of 4 years. Nearly all these infections are asymptomatic or sub-clinical. In most cases, adult melioidosis must result from re-infection or re-activation of latent infections (Suputtamongkol, Y., Hall, A.J. *et al.* 1994). Serological studies in Malaysia also found a high incidence of antibodies to *B. pseudomallei*, presumably due to mild, unrecognised or asymptomatic infections (Strauss, J.M., Alexander, A.D. *et al.* 1969).

Form	Signs and Symptoms
Septicaemic	
disseminated	abrupt onset with fever and impaired consciousness, disseminated nodular shadows in lung, septic shock, death often within 1-3 days (87% fatality).
non-disseminated	One or two organs involved (mostly lung, liver and spleen), may progress into disseminated infection if not treated appropriately.
Localised	Symptoms related to the organs involved, mostly slow and progressive course.
Transient bacteraemic	Isolation of *B. pseudomallei* from the blood without apparent symptoms and signs of infection, close observation is recommended.
Probable	Symptoms compatible with melioidosis, with positive serological test but without isolation of *B. pseudomallei*.
Sub-clinical	Serological test alone is positive, close observation for potential reactivation of dormant infection triggered by trauma, infection, alcoholism, diabetes mellitus etc.

Table 3. Typical signs and symptoms of the different forms of melioidosis (Arakawa, M. 1990b).

Glanders is primarily a disease of horses, but may occasionally affect goats, sheep, dogs and cats (Bartlett, J.G. 1992). Documented human cases of glanders are rare. The symptoms are variable and there is no clear clinical picture, so some real cases may be wrongly diagnosed. The incubation period varies from a few hours to several weeks. It may be acute or chronic and may be localised chiefly in the respiratory organs or in the skin or subcutaneous tissues. Without treatment death usually occurs in a week to ten days (Parker, M.T., Smith, G.R. *et al.* 1990). At onset the disease may be characterised by general illness (nausea, loss of appetite and elevated temperature) and undue fatigue (Howe, C. and Miller, W.R. 1947). The disease may remain active for weeks, months or even years and is usually but not invariably fatal. Sometimes, after apparent recovery, the disease may break out again and latent periods of up to 10 years have been observed (Gaiger, S.H. 1913; Gaiger, S.H. 1916).

1.7 Transmission

In humans, *B. pseudomallei* infection is usually transmitted by soil contamination of skin abrasions in endemic areas. Ingestion, nasal instillation and inhalation are other probable methods of spread (Sanford, J.P. 1987). Human to human transmission has only been reported once. The case involved a patient's wife who had raised haemagglutination titres that indicated recent infection. The woman's infection was most thought to be the result of sexual contact as she had no apparent contact with a source of *B. pseudomallei* other than the patient (McCormick, J.B., Sexton, D.J. *et al.* 1975).

Arthropod-borne infection does not occur naturally but experimentally the disease can be transmitted by the rat flea (*Xenopsylla cheopsis*) and by the mosquito (*Aedes aegypti*) (Prevatt, A.L. and Hunt, J.S. 1957). There are several reported cases of laboratory acquired infection. One occurred after sonication of supposed *B. cepacia* isolates (Schlech, W.F., Turchik, J.B. *et al.* 1981). This suggests that aerosols of the organisms are infectious to humans, although the infective dose is unknown.

Zubaidy and Al-Ani (1978) suggested that in overcrowded stables the *B. mallei* infection may spread by a highly infectious nasal discharge which contaminates surrounding harnesses, feeding and watering troughs (Zubaidy, A.J. and Al-Ani, F.K. 1978). Infection may also be spread by communal grooming equipment. Inhalation, ingestion and inoculation through breaks in the skin have been suggested as routes of infection in animals. In humans the disease occurs primarily in individuals with close contact with horses, mules or donkeys through inoculation of a break in the skin or by exposing the nasal mucosa to contaminated discharges. A number of instances of airborne infection have been reported in laboratory workers (Sanford, J.P. 1987), but again the infective dose for man is unknown.

1.8 Pathogenesis

The pathogenesis of melioidosis is poorly understood, and although several putative virulence determinants have been identified they are relatively uncharacterised. Putative extracellular virulence determinants include a thermolabile toxin and a protease. Sexton *et al.* (1994) reported on the purification and characterisation of the *B. pseudomallei* protease. The 36kDa metalloprotease was implicated in the inactivation

of host defence mechanisms and facilitating the invasion of host tissues in melioidosis pneumonias. Strains deficient in protease produced significantly less lung damage (Sexton, M.M., Jones, A.L. *et al.* 1994). Metalloproteases have been identified in many bacterial species and are thought to play a role in disease pathogenesis. *P. aeruginosa* secretes a number of metalloproteases which have been shown to degrade or inactivate several biologically important substrates such as fibrin, complement and human IgG. *B. cepacia* has also been shown to secrete two metalloproteases (Kooi, C. and Sokol, P.A. 1996).

Cell-associated virulence determinants include pili and the extracellular polysaccharide (Brett, P.J., Mah, D.C.W. *et al.* 1994). The flagellum of *B. pseudomallei* was considered to be a potential virulence determinant. However, the production of non-motile mutants appeared to have little effect on virulence *in vivo* in the diabetic rat disease model (DeShazer, D., Brett, P.J. *et al.* 1997). LPS (endotoxin) has also been considered in the pathogenesis of the disease, although its toxicity is low (Gotoh, N., White, N.J. *et al.* 1994).

B. pseudomallei resists the bactericidal activity of normal human serum. For disease-producing bacteria their initial contact with the host defence mechanism after penetration of the tissues is normal serum which is thought to play a significant role in protection against infection. It has been suggested that resistance to serum bactericidal killing is an important virulence determinant in Gram negative bacteria. There appears to be a correlation between serum resistance and the ability of a bacteria to invade and survive in the human blood stream (Ismail, G., Razak, N. *et al.* 1988).

Virulence in *P. aeruginosa* is attributed to a range of extracellular substances and cellular structures which in combination account for the pathology of infection. Exotoxin A, elastase and phospholipase C all secreted by the bacteria and structures such as LPS, alginate and pili are all thought to contribute to virulence (Doring, G., Maier, M. *et al.* 1987).

There are no references to putative virulence determinants in *B. mallei* infections. Mallein (see section 1.5.5) has been suggested as a putative virulence factor because animals previously exposed to *B. mallei* react to subsequent exposure to mallein (Parker, M.T., Smith, G.R. *et al.* 1990). The composition of mallein is unknown.

1.9 Toxins

B. pseudomallei grows luxuriantly in many simple media. Toxin production is favoured by more complex media, glycerol, heart infusion broth and mucin being important constituents (Colling, M., Nigg, C. *et al.* 1958). *B. pseudomallei* produces several toxic factors; culture filtrates contain both toxic and enzymatic activity and a heat stable molecule with classical endotoxin activity (Heckly, R.J. and Nigg, C. 1958). Further characterisation showed the presence of a heat-labile exotoxin (Heckly, R.J. 1964) which is thought to suppress cellular immune functions (Yahya, M.D. and Chui Lik, S.Y. 1995). *P. aeruginosa* is known to secrete exotoxin A which is thought to cause much of the pathology seen in *P. aeruginosa* infections (Liu, P.V. 1973).

Well isolated colonies of *B. pseudomallei* appear completely non haemolytic. However, smaller colonies in a crowded environment are haemolytic. The exact

mechanism of haemolysin production is unknown but it appears that mutual inhibition by similar organisms in a dense population alters the metabolic pattern so that haemolysin is produced (Liu, P.V. 1957). Ashdown and Koehler identified a second haemolysin occurring infrequently which is heat labile and produces clear haemolytic zones on blood agar (Ashdown, L.R. and Koehler, J.M. 1990).

1.10 Relapses in infection

The improvement in recognition of *B. pseudomallei* infection and in the treatment of acute cases has resulted in a greater number of successfully treated patients who survive to leave the hospital and, despite prolonged treatment, relapsing infection has been seen in up to 30% of survivors of severe melioidosis. Several factors influence the likelihood of relapse, including clinical severity at presentation and the type of parenteral and oral treatment given (Chaowagul, W., Suputtamongkol, Y. *et al.* 1993). *B. pseudomallei* is a facultative intracellular bacterium which is able to survive and multiply in phagocytic and non-phagocytic cells. This is thought to be a factor in the common occurrence of relapse, and is an important consideration in the choice of antibiotic treatment (Pruksachartvuthi, S., Aswapokee, N. *et al.* 1990; Jones, A.L., Beveridge, T.J. *et al.* 1996; Egan, A.M. and Gordon, D.L. 1996). DNA-based typing methods have demonstrated that in many cases patient relapse was clearly due to the original infective strain re-emerging (Haase, A., Melder, A. *et al.* 1995; Mohandas, S., Puthucheary, S.D. *et al.* 1995; Desmarchelier, P.M., Dance, D.A.B. *et al.* 1993; Vadivelu, J., Puthucheary, S.D. *et al.* 1998). Relapse in glanders infections has also been reported (Gaiger, S.H. 1916).

1.11 Vaccination

No vaccine currently exists for *B. pseudomallei*. Avirulent strains of *B. pseudomallei* have been used to vaccinate animals prior to respiratory challenge with variable results (Levine, H.B. and Maurer, R.L. 1958). Dannenberg (1958) reported that the inoculation of mice and hamsters with live avirulent strains induced significant protection against respiratory challenge (Dannenberg, A.M. and Scott, E.M. 1958). More recently both the LPS and the flagellin proteins of *B. pseudomallei* have been identified as possible candidates for use as protective immunogens (Brett, P.J., Mah, D.C.W. *et al.* 1994; Bryan, L.E., Wong, S. *et al.* 1994).

An acellular vaccine has been used successfully in captive marine mammals to protect against *B. pseudomallei* infection. The vaccine contains protein and polysaccharide (1:3), induces high levels of specific antibodies and has significantly reduced mortality (Vedros, N.A., Chow, D. *et al.* 1988). No vaccine exists for *B. mallei*.

1.12 Chemotherapy

Early positive laboratory diagnosis and early appropriate chemotherapy are essential for the successful management of melioidosis (Table 4). If these prerequisites are met then the prognosis of this disease is good and fatalities are limited to those patients who present with overwhelming septicaemia which cannot be halted by current medical treatment (Ashdown, L.R., Duffy, V.A. *et al.* 1980). *B. pseudomallei* can produce a chronic form of disease. Organisms are often situated intracellularly and persist over a

long period of time suggesting that infection may be very difficult to eliminate and necessitating long courses of treatment (Hobby, G.L., Lenert, T.F. *et al.* 1969).

In 1969 the US Armed Forces recommended treatment for melioidosis was 12g chloramphenicol, 4g kanamycin and 6g novobiocin daily for four weeks (Heckly, R.J. 1969). Chloramphenicol was also recommended by Hezebicks and Nigg (1958) as being effective against *B. pseudomallei*. However, Prevatt and Hunt (1957) reported toxic side effects from prolonged treatment with chloramphenicol (Prevatt, A.L. and Hunt, J.S. 1957; Hezebicks, M.M. and Nigg, C. 1958). Tetracycline was also highlighted as being valuable in the treatment of melioidosis (Eickhoff, T.C., Bennett, J.V. *et al.* 1970). In Thailand, where melioidosis is an endemic disease, the conventional maintenance treatment regimen for *B. pseudomallei* infections is a combination of chloramphenicol, doxycycline and cotrimoxazole (Dance, D.A.B., Wuthiekanun, V. *et al.* 1989a; Chaowagul, W., Simpson, A.J. *et al.* 1999). The fluoroquinolone antibiotics were found to be inappropriate for the treatment of melioidosis (Winton, M.D., Everett, E.D. *et al.* 1988; Chaowagul, W., Suputtamongkul, Y. *et al.* 1997).

Trials carried out in the 1980s recommended ceftazidime as the antibiotic of choice to replace the use of high doses of chloramphenicol, doxycycline, trimethoprim-sulphamethoxazole and sometimes kanamycin (Chau, P.Y., Ng, W.S. *et al.* 1986). In other antibiotic trials *B. pseudomallei*, *P. aeruginosa* and *B. cepacia* strains were found to be more resistant to the action of chemotherapeutic compounds than *B. mallei* strains. *B. pseudomallei* strains were sensitive to a number of chemotherapeutic agents

including ceftazidime, imipenem and doxycycline (Antonov, Y.V., Ilyukhin, V.I. *et al.* 1991). Ceftazidime, widely used as a single drug treatment against *P. aeruginosa* septicaemia, is safe, well tolerated and highly active against *B. pseudomallei in vitro* and its use in the treatment of melioidosis has halved the mortality of severe cases (White, N.J., Dance, D.A.B. *et al.* 1989). The case fatality rate is still high (approximately 40%) with an increasing number of reports of resistance to currently used drug treatments (Dance, D.A.B., Wuthiekanun, V. *et al.* 1991; Godfrey, A.J., Wong, S. *et al.* 1991). In this respect, recent studies indicate that imipenem is a safe and effective treatment for acute melioidosis and may be used as an alternative to ceftazidime (Smith, M.D., Wuthiekan, V. *et al.* 1994; Simpson, A.J., Suputtamongkol, Y. *et al.* 1999).

Antibiotic	MIC $_{90}$ (μg/ml)	Antibiotic	MIC $_{90}$ (μg/ml)
imipenem	0.1 - 4.0	ceftazidime	0.4 - 0.8
carumonan	0.8 - 2.0	piperacillin	1.0 - 1.6
cefuzonam	1.6	amoxicillin-clavulanic acid	1.0 - 8.0
ceftizoxime	3.2	doxycycline	4.0
ciprofloxacin	1.0 - 4.0	cefotaxime	6.5 - 16.0
minocycline	6.5	cefoperazone	12.5
ofloxacin	8.0 - 12.5	tetracycline	8.0 - 12.5
chloramphenicol	16.0 - 25.0	trimethoprim-sulfamethoxazole	16.0
kanamycin	32.0 - 50.0	tobramycin	32.0
nalidixic acid	50.0	amoxicillin	64.0
amikacin	64.0 - 100.0		

Table 4. Reported minimum inhibitory concentrations (MIC_{90}) of some antibiotics with *B. pseudomallei* (Leelarasamee, A. and Bovornkitti, S. 1989; Arakawa, M. 1990b).

A study of 34 isolates of *B. mallei* found all strains to be sensitive to sulfamethizole, gentamicin, tetracycline, sulphathiazole, kanamycin, streptomycin and a combination of trimethoprim and sulphamethoxazole. Gentamicin was recommended as the treatment of choice for glanders (Al-Izzi, S.A. and Al-Bassam, L.S. 1989). A more recent antibiotic study carried out on *B. mallei* found the bacteria to be highly sensitive

to ciprofloxacin and ofloxacin. In experimentally infected animals both these drugs were highly effective, with ciprofloxacin being the most active *in vitro* and effective *in vivo*. It is considered that successful treatment of glanders in man could be expected using ofloxacin and ciprofloxacin at daily doses of 1.6-2.4g and 1.5-2.1g respectively (Batmanov, V.P. 1991). After chemotherapy, treatment of laboratory animals with immunosuppressants did not result in re-infection (Batmanov, V.P., Ilyukhin, V.I. *et al.* 1996).

1.13 Survival in the environment

B. pseudomallei is a free-living bacterium in soil and water and is endemic in Africa, the Indian subcontinent, Iran, Northern Australia and Central and South America. Sporadic reports have indicated the presence of *B. pseudomallei* in Hong Kong, Hawaii, Fiji, Haiti, Puerto Rico, France and North America (Dance, D.A.B. 1991). The isolation of *B. pseudomallei* is markedly influenced by the environment. High temperature, increased humidity and consistent rainfall allows for the accumulation of stagnant pools and muddy water courses and the isolation rates of *B. pseudomallei* are increased (Strauss, J.M., Groves, M.G. *et al.* 1969).

B. pseudomallei is endemic in Northern Australia and most human cases have been reported from the Townsville area, the Northern Territory and the Torres Straits Islands. In 1981 Thomas and Forbes-Faulkner collected soil samples containing *B. pseudomallei*, stored them in their original collecting bags on a shaded shelf in the laboratory at ambient temperature. *B. pseudomallei* survived for up to 30 months in

soil kept under the above conditions with the average time for survival being six months (Thomas, A.D. and Forbes-Faulkner, J.C. 1981).

Studies carried out in Townsville showed that the organism survives in the clay layer of the soil 25-30cm from the surface. Bacteria and water are electrostatically attracted to clay particles. This means that the clay layer retains some of the moisture that would normally be pulled down into the water table. It is possible that in this way the organism is well adapted to survive in areas experiencing a long, dry season (Guard, R.W., Khafagi, F.A. *et al.* 1984). During the rainy season the water table rises to the surface, carrying with it bacteria which then multiply in the favourable conditions (Figure 4).

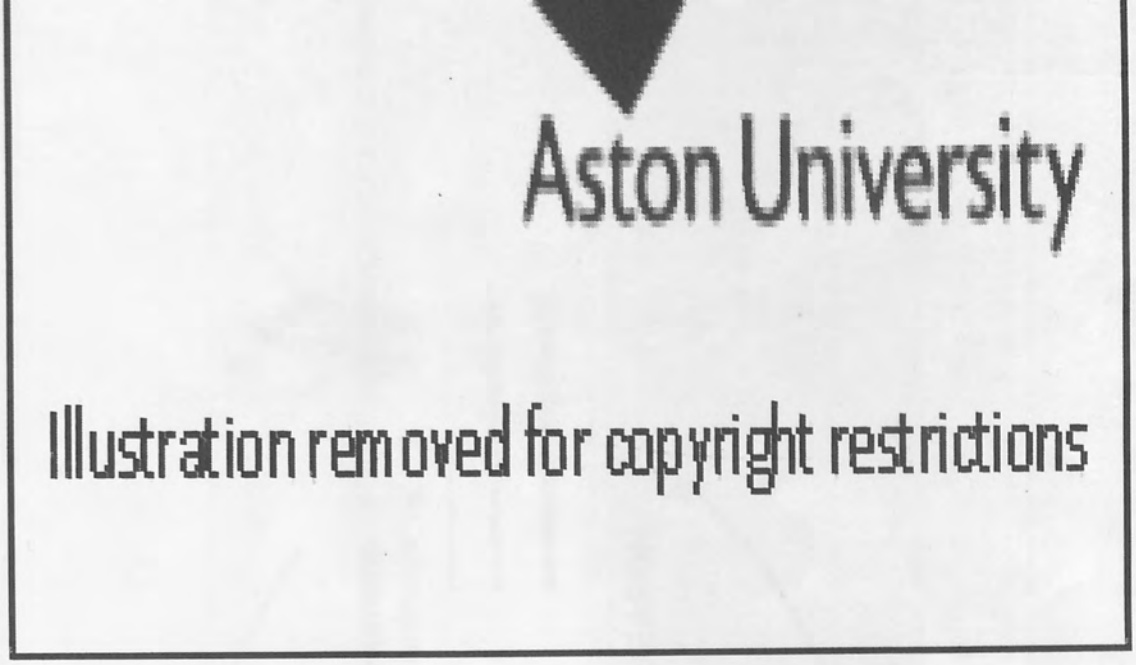
It is during the rainy season that *B. pseudomallei* is recognised as a hazard to animal and human health (Thomas, A.D., Forbes-Faulkner, J. *et al.* 1979). A more recent study has shown that cases of *B. pseudomallei* in temperate south-west Western Australia over a 25 year period are caused by a distinct ribotype, unlike those commonly seen in Northern Australia. It is thought that an imported animal may have introduced the organism which subsequently survived in soil, infecting local animals including sheep and goats. Movement of animals between farms is likely to account for the local spread of the disease (Currie, B., Smith-Vaughan, H. *et al.* 1994; Haase, A., Smith-Vaughan, H. *et al.* 1995).

Studies in China have indicated that distribution of *B. pseudomallei* appeared closely associated with climate. *B. pseudomallei* was easily isolated from rice fields in areas

with high temperatures all year round. In mainland China the distribution appeared restricted to coastal areas where atmospheric temperatures remained above 12°C throughout the year (Yang, S.Y., Tong, S. *et al.* 1995). In laboratory tests, bacteria maintained in soil samples with more than 40% water were still viable after 726 days (Tong, S., Yang, S. *et al.* 1996).

In Thailand, the presence of *B. pseudomallei* was also linked with high temperatures and consistently high moisture levels found in rubber plantations and rice fields (Nachiangmai, N., Patamasucon, P. *et al.* 1985). There is seasonal variation in the incidence of melioidosis, the majority of cases occurring during the rainy season, this reflects the exposure of the population working the rice paddies and parallels the isolation of *B. pseudomallei* from top soil (Suputtamongkol, Y., Hall, A.J. *et al.* 1994). *B. pseudomallei* survives in tap water for 44 days (Strauss, J.M., Groves, M.G. *et al.* 1969) and in one case a bacterial suspension in water has remained viable for over 3 years (Wuthiekanun, V., Smith, M.D. *et al.* 1995b).

B. mallei is considered to be an obligatory parasite with an equine species as a normal host. While it is theoretically possible that given the correct conditions the organism could propagate in the outside world, competition with hardier and more rapidly growing saprophytic bacteria means it is highly likely to be crowded out of existence (M'Fadyean, J. 1904). The remarkable success of eradication and control measures for glanders indicate that soil and water do not act as reservoirs for *B. mallei* (Redfearn, M.S., Palleroni, N.J. *et al.* 1966).



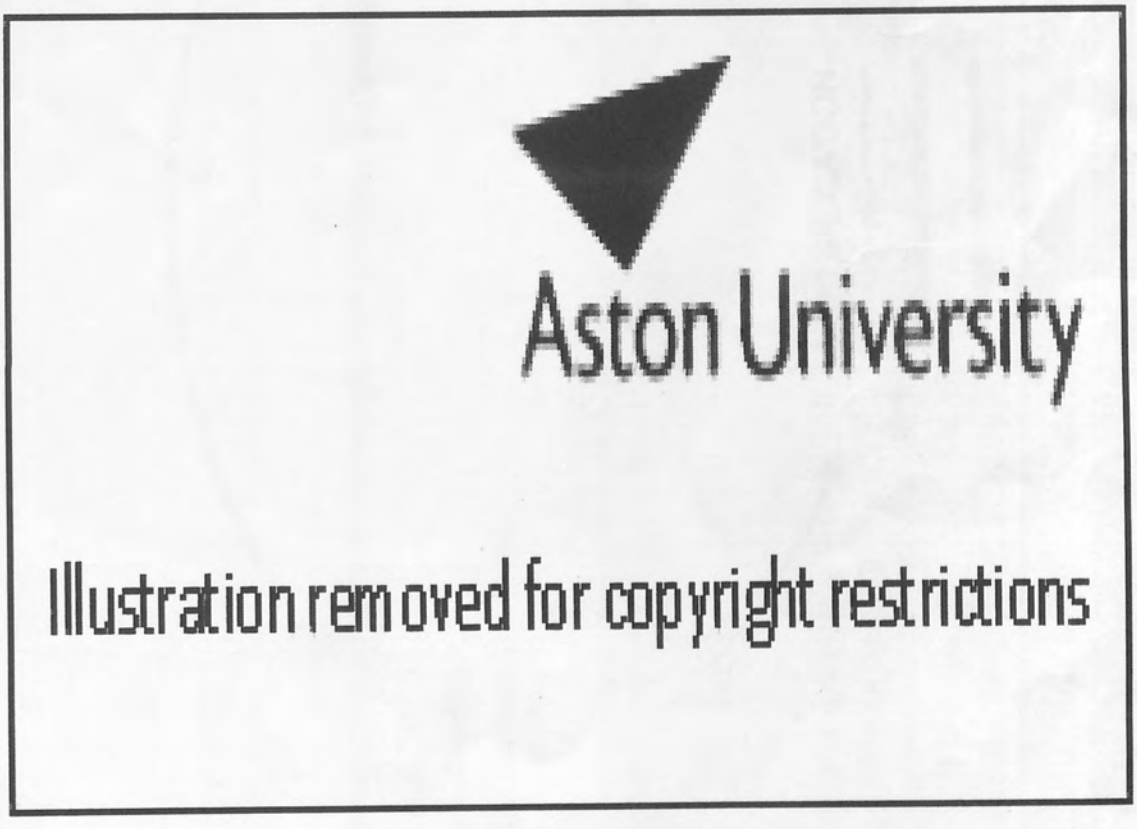


Figure 4. Proposed environmental survival and cycle of infection of *B. pseudomallei*.

1.14 Laboratory safety

Human infection with *B. pseudomallei* typically produces sub-clinical disease and an asymptomatic carrier state, occasionally clinical illness frequently with a fatal outcome may occur. Consequently to protect staff from laboratory-acquired melioidosis microbiological laboratories must have adequate facilities for safe work procedures and safe work practices (Ashdown, L.R. 1992a). Consideration must also be given for protection of the environment, as evidence from an outbreak in France in the 1970's suggests that *B. pseudomallei* may become established in the soil and water of temperate climates (Mollaret, H.H. 1988; Dodin, A. and Galimand, M. 1986). Laboratories in non-endemic areas should attempt, therefore, not only to protect their workers, but also their environment, from colonisation with *B. pseudomallei* (Dance, D.A.B., Smith, M.D. *et al.* 1992).

Laboratory acquired infection has been reported with both *B. pseudomallei* and *B. mallei*. In the 1940's, six cases of glanders were reported in laboratory workers, all were successfully treated with sulphadiazine although six months after treatment two cases still had a positive skin test. Cases of laboratory acquired *B. pseudomallei* infection have also been reported. One case occurred during the sonication of a apparent *B. cepacia* isolate from a Vietnam veteran, which was later identified as *B. pseudomallei* (Schlech, W.F., Turchik, J.B. *et al.* 1981). A second case occurred during a DNA preparation from *B. pseudomallei* when a continuous flow centrifuge developed a leak and grossly contaminated the laboratory. Three days later the microbiologist was admitted to hospital with melioidosis (Green, R.N. and Tuffnell, P.G. 1968). Both *B. pseudomallei* and *B. mallei* represent a risk to laboratory

workers and additionally *B. pseudomallei* has the potential to colonise the environment. Consequently, full Category III containment procedures should continue to be employed for handling the organism in non-endemic areas (Dance, D.A.B., Smith, M.D. *et al.* 1992).

1.15 Aims

The aim of the project was to investigate the surface antigens of *B. pseudomallei* and *B. mallei* and produce immunological reagents for use in the detection of these organisms. Work was to be carried out to identify antigens on the surfaces of the organisms which could be used to produce monoclonal and polyclonal antibodies. Antibody reagents used in detection assays need to be both sensitive and specific for the agent of concern and therefore any antibodies produced would have to be tested for sensitivity and cross reactivity.

A further part of the project was to try and differentiate between these two closely related organisms, by identifying antigens which were specific to a single species. Molecular methods were also studied as an alternative means of antibody production.

2. Materials and Methods

Both *B. pseudomallei* and *B. mallei* are listed as Category III pathogens by the Advisory Committee on Dangerous Pathogens (ACDP). *B. mallei* is also listed as a Specific Animal Pathogen by the Ministry of Agriculture, Fisheries and Food (MAFF) and may only be handled under licence. All manipulations of live *B. pseudomallei* and *B. mallei* strains were carried out in Class 3 microbiological safety cabinets located in a designated ACDP Category 3 laboratory.

All reagents and materials were supplied by Sigma-Aldrich Company Ltd. (Poole) unless otherwise stated.

2.1 Bacterial growth and manipulation

2.1.1 Media preparation

Nutrient broth and nutrient agar (Oxoid, Basingstoke) was prepared according to the manufacturer's instructions.

2.1.2 Bacterial cultivation

B. pseudomallei and *B. mallei* strains were obtained from the National Collection of Typed Cultures (NCTC, London). Other strains were obtained from Dr David Dance at the London School of Hygiene and Tropical Medicine (LSHTM, London), Dr Ty Pitt at the Public Health Laboratory Service (PHLS, London) and the American Type

Culture Collection (ATCC, Maryland, USA) (Tables 5 & 6). Both species were grown either on nutrient agar or in nutrient broth.

Organism	Reference	Origin	Source	Notes
B. mallei	ATCC 23344	China	Human	ATCC type strain
B. mallei	NCTC 10230	Hungary 1961		
B. mallei	NCTC 120	London 1920		
B. mallei	NCTC 3708	India 1931	Mule	
B. mallei	NCTC 3709	India 1932	Horse	
B. mallei	NCTC10229	Hungary 1961		
B. mallei	NCTC 10245	China 1942	Horse	
B. mallei	NCTC 10247	Turkey 1960		
B. mallei	NCTC 10248	1950	Human	
B. mallei	NCTC 10260	1949	Human	

Table 5. *B. mallei* strains.

Organism	Reference	Origin	Source	Notes
B. pseudomallei	ATCC 23343		Human	ATCC type strain
B. pseudomallei	NCTC 4845	Singapore	Monkey	1935
B. pseudomallei	USAMRU-1	Malaysia	Soil	
B. pseudomallei	UBOL-2	Thailand	Soil	
B. pseudomallei	4889	Equador	Man	
B. pseudomallei	USAMRU-21	Malaysia	Soil	
B. pseudomallei	SEARLE	Australia	Human	
B. pseudomallei	123		Human	
B. pseudomallei	O	Australia	Man	
B. pseudomallei	PA	Australia	Man	
B. pseudomallei	BRI			
B. pseudomallei	E8		Environmental	Arabinose -ve †
B. pseudomallei	E25		Environmental	Arabinose -ve †
B. pseudomallei	E27		Environmental	Arabinose +ve †
B. pseudomallei	E82		Environmental	Arabinose +ve †
B. pseudomallei	204	Thailand	Human	Arabinose -ve †
B. pseudomallei	576	Thailand	Human	Arabinose -ve †
B. pseudomallei	551a	Thailand	Human	LSHTM
B. pseudomallei	603a	Thailand	Human	LSHTM
B. pseudomallei	708a	Thailand	Human	Gentamicin sensitive LSHTM
B. pseudomallei	E38	Thailand	Environmental	LSHTM
B. pseudomallei	Mal 6	Malaysia		LSHTM
B. pseudomallei	Human Australia	Australia	Human	LSHTM

† indicates strain characterisation by T. Pitt (PHLS)

Table 6. *B. pseudomallei* strains.

2.1.3 Gram stain

Bacterial cultures were routinely checked for purity by culturing on nutrient agar plates and by Gram staining. Single bacterial colonies were resuspended in 20µl distilled water (dH_2O) on glass microscope slides (BDH, Poole) and allowed to dry. Slides were flame fixed and immersed in crystal violet solution (2% crystal violet, 0.1% ammonium oxalate in 20% ethanol) for 30secs. Slides were briefly rinsed in dH_2O before incubating in Gram's iodine solution (0.3% iodine, 0.6% potassium iodide in dH_2O) for 30secs. A few drops of decolouriser solution (75% propanol, 25% acetone) was placed on the slides which were rinsed in dH_2O before incubating in safranin solution (6% safranin O in 20% ethanol) for 1min. Slides were rinsed in dH_2O and allowed to dry before examination under oil immersion.

2.1.4 Preparation of stock cultures

Freeze-dried vials of *B. pseudomallei* and *B. mallei* were resuspended in 200µl nutrient broth then streaked onto a dried nutrient agar plate. After incubation at 37°C a single colony from the plate was used to inoculate 100ml of nutrient broth, which was incubated overnight at 37°C with shaking (150rpm). Bacterial cells were harvested by spinning at 10,000g for 15min, and then washed three times in 5mM phosphate buffered saline (PBS) pH 7.3 (Oxoid) by resuspending the cells and then repeating the centrifugation. The cells were finally resuspended in 25% sterile glycerol in PBS, aliquoted into sterile polythene cryotubes (Nalgene, Hereford) and frozen at -70°C.

2.1.5 Inactivation of *B. pseudomallei* and *B. mallei*

Bacterial cells were grown overnight in nutrient broth. Cells were harvested by centrifugation, and washed three times in PBS, before resuspending in one tenth the original volume of PBS. For heat inactivation, the bacterial cell suspension was placed in o-ring sealed centrifuge tubes (Sorvall, Connecticut, USA) and placed in a water bath at 70°C for 3 hours, with occasional gentle shaking. After inactivation the suspension was checked for viability by inoculating 10ml volumes of nutrient broth with 0.5ml aliquots of the suspension to a total of 5ml or 10% of the original volume (whichever is smaller) and incubating at 37°C for seven days. Nutrient agar plates were then inoculated with the total volume of the broth cultures to check for bacterial growth, and incubated for a further seven days. If no growth occurred on the agar plates then the bacterial suspension was assumed to be inactivated.

For chemical inactivation, formaldehyde (final concentration 1% v/v) was added to the bacterial suspension and incubated overnight at room temperature. After incubation, cells were washed five times in PBS to remove all traces of formaldehyde and viability checks carried out as above.

Prior to inactivation, viable counts (section 2.1.6) were carried out on cell suspensions allowing an estimate to be made of the number of colony forming units (cfu) per ml. When inactivated suspensions were later used to coat ELISA plates these viable counts were used to dilute cell suspensions to required concentrations.

2.1.6 Viable counts

The sample to be counted was serially diluted an appropriate number of times in sterile PBS. Three 0.25ml aliquots were taken from each dilution and spread over the surface of dried nutrient agar plates by rotation. Petri dishes were inverted and incubated overnight at 37°C. Colonies on each plate were counted and the viable count calculated from the dilution which gave between 30 and 300 colonies per plate.

2.1.7 Total counts

Samples to be counted for the total number of cells were stored in PBS containing 1% formaldehyde in order to preserve the cells whilst inhibiting growth. A drop of appropriately treated suspension was then placed in a 0.02mm haemocytometer (Thoma, Weber Scientific International Ltd., Teddington) and the chamber left in a humid atmosphere for 30 minutes to allow cells to settle in one plane of vision. Cell counts were made in 80 small squares and the total number of cells was calculated, taking the volume into consideration.

2.1.8 Growth curves

Three 100ml volumes of broth in 250ml conical flasks were inoculated with 1ml stock culture and incubated with shaking at 37°C. At 30min intervals the optical density at 600nm (OD_{600}) of each flask was determined using a Colorimeter 254 (Corning, Stone) using disposable plastic cuvettes (1cm light path).

In order to correlate viable cell counts with OD_{600}, serial dilutions into sterile PBS were prepared from suspensions of known optical densities and 250µl aliquots spread onto nutrient agar plates. After overnight incubation at 37°C, the number of cfus was counted and it was found that OD_{600} readings of 0.5 and 1.0 indicated concentrations of approximately 1.5 x 10^9cfu/ml and 3.3 x 10^9cfu/ml respectively.

2.1.9 Polymerase chain reaction (PCR) assay

A PCR assay was used for the identification of colonies and for the screening of blood samples for the presence of *B. mallei* and *B. pseudomallei*. The primer set for this assay were 16S rRNA gene primers PM2F and PM4R (Cruachem, Glasgow) developed as specific gene probes for the detection of *B. pseudomallei* and *B. mallei*. All PCR reactions were performed in a GeneAmp PCR system 9600 (Perkin Elmer Ltd., Beaconsfield) according to the manufacturers instructions.

PCR reactions were performed in 100µl volumes containing 5µl template DNA and 95µl of the following mix: 1 x PCR buffer (Boehringer Mannheim, Lewes), 0.2mM PCR nucleotide mix (Boehringer Mannheim), 1.5mM $MgCl_2$, 5% (v/v) dimethylsulphoxide, 1µg of each primer and 2.5U of Taq polymerase (Boehringer Mannheim). Two minutes denaturation at 95°C was followed by 35 cycles of 30sec at 95°C, 1min at 55°C, 1min at 72°C. Annealing was completed with 10min at 72°C.

PCR products were separated at 100V on 2% agarose in TAE buffer (0.04M Tris-acetate buffer pH 8.3 containing 1mM ethylenediaminetetraacetic acid {EDTA}). Gels contained 1μg/ml ethidium bromide to visualise the bands. Molecular weight (MW) was calculated by comparison with a range of standards. The presence of a band migrating at a molecular weight equivalent to 0.42kb confirms the presence of either *B. mallei* or *B. pseudomallei*.

2.2 Production of antibodies against heat inactivated *B. pseudomallei*

2.2.1 Antigen preparation

Heat inactivated *B. pseudomallei* was prepared by inoculating 100ml nutrient broth with a single colony from a nutrient agar plate. The broth was incubated overnight at 37°C with shaking (180rpm). Bacterial cells were harvested and heat inactivated according to section 2.1.5.

2.2.2 Polyclonal antibody production

Six female Dunkin Hartley guinea pigs (Harlan UK Ltd., Loughborough) were immunised once every two weeks over an eight week period with $1x10^8$ cfu/ml heat inactivated *B. pseudomallei* NCTC 4845 cells. The first injection was given subcutaneously with Freund's incomplete adjuvant (FIA). Subsequent injections were given intramuscularly with Freund's incomplete adjuvant. Two weeks after the final injection, guinea pigs were terminally anaesthetised and blood taken by cardiac

puncture. The serum was separated from red blood cells using serum separation tubes (Becton Dickinson UK Ltd., Cowley) and centrifuging at 1,200g for 10min.

2.2.3 Polyclonal antibody purification

Polyclonal antibody was purified by ammonium sulphate precipitation. An equal volume of 80% saturated ammonium sulphate, pH 7.0, was added to the serum and the mixture stirred for 30min at 4°C. The precipitate was harvested by centrifuging at 15,000g for 15min at 4°C and the pellet resuspended in PBS (approximately 50ml for every 10ml of original serum). The precipitation step was repeated twice and the final pellet resuspended in PBS (approximately 5ml for every 10ml of original serum) and dialysed overnight against 75mM phosphate buffer, pH 6.8 at 4°C. The dialysed solution was then absorbed batchwise onto equilibrated DE-52 (20g DE-52 per 100ml original serum, Whatman International Ltd., Maidstone) and stirred gently for 10min at 4°C. The supernatant was then recovered after centrifugation at 15,000g for 10min at 4°C. The antibody content of the supernatant was estimated by measuring the absorbance at 280nm and applying Equation 1.

$$A_{280} \times 0.6 = \text{antibody concentration in mg/ml}$$

Equation 1. Determination of approximate antibody concentration.

2.2.4 Immunisation schedule for monoclonal antibody production

Ten female Balb/c mice (6 - 9 weeks old)(Charles River UK Ltd., Margate) were immunised by intraperitoneal injection once every two weeks over an eight week period with 2 x 10^8 cfu heat inactivated *B. pseudomallei* NCTC 4845 cells. The first four injections were given with incomplete Freund's adjuvant. For the final injection, cells were suspended in PBS and injected intravenously.

2.2.5 Production of hybridoma cells producing antibody

Fusions were carried out according to the method of Harlow and Lane (Harlow, E. and Lane, D. 1988). Three days after the final immunisation, mice were sacrificed and their spleens removed aseptically. Splenocytes were obtained by gently pressing the mouse spleen through a sterile tea strainer and washing with serum-free medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin and 25µg/ml amphotericin B. The splenocytes were centrifuged at 1,000g for 5min in preparation for fusion. Myeloma cells P3/X63 Ag8.653 and SP2/0-Ag14 (European Collection of Animal Cell Cultures, Salisbury), grown in serum-free medium supplemented with 10% foetal bovine serum (FBS) were harvested and centrifuged at 1,000g for 5min. Splenocytes were mixed with 1x10^7 myeloma cells at an approximate ratio of 1 myeloma cell to 5 splenocytes, and the mixture centrifuged at 1,000g for 5min. The supernatant was removed and the cell pellet loosened by tapping. 1ml of 30% PEG 1500 in serum-free medium was added and, after one minute the cells centrifuged at 1,000g for 5min. After a further one minute incubation, 5ml of serum-free medium

was slowly added, followed by 5ml serum-free medium supplemented with 20% FBS and the cells centrifuged at 1,000g for 5min. The supernatant was removed and the cells gently resuspended in 30ml of serum-free medium supplemented with 20% FBS and hypoxanthine-aminopterin-thymidine (HAT) medium. 50µl of cell suspension was added to each well of a 96-well tissue culture plate (Falcon Biomedical Products, Cowley) and incubated at 37°C in 5% CO_2 and at 100% humidity. Cell suspensions were supplemented with an additional 50µl and 100µl of HAT medium 24 and 120 hours after fusion. The cell suspensions were examined for the presence of hybridoma cells between five and fourteen days after fusion using an inverted microscope. Culture supernatants were assayed for antibody by ELISA (section 2.2.8) using goat anti-mouse polyvalent immunglobulin (Ig) conjugated to horse radish peroxidase (HRP) (Harlan SeraLab, Loughborough) as the secondary antibody.

2.2.6 Cloning by limiting dilution

Cloning was carried out according to the method of Liddell and Cryer (Liddell, J.E. and Cryer, A. 1991). Cloning was carried out in serum-free medium supplemented with 20% FBS and hypoxanthine-thymidine (HT) medium. Hybridoma cells were counted and diluted to 2,000 cells/ml. 100µl from this suspension was added to 4ml HT medium and 100µl of the resulting suspension added to 24 wells of a 96-well tissue culture plate at a calculated dilution of 5 cells/well. The remainder of the suspension was added to 2.4ml HT medium and 100µl of the resulting suspension added to a further 24 wells (2 cells/well). This process was repeated adding 100µl to 24 wells at calculated concentrations of 1 and 0.5 cells/well. Plates were incubated at

37°C in 5% CO_2 and 100% humidity for 14 days before screening for antibody by ELISA (section 2.2.8). Cells from positive wells were taken and subjected to two further rounds of cloning. Following cloning, cells were grown in 24-well tissue culture plates in serum-free medium supplemented with 10% FBS before expanding into tissue culture flasks for eventual introduction into the miniPERM bioreactor (section 2.2.7).

2.2.7 Production of antibody using the miniPERM bioreactor

For production of monoclonal antibody, each hybridoma cell line was expanded into six 75cm^2 tissue culture flasks containing DMEM supplemented with 2mM L-glutamine, 100U/ml penicillin, 100μg/ml streptomycin and 10% FBS (miniPERM medium). The miniPERM bioreactor (Hereaus Instruments GmbH., Osterlode am Harz, Germany) was assembled by clipping together the nutrient and production modules, adding 400ml of miniPERM medium into the nutrient module and allowing the bioreactor to equilibrate at 37°C for 10min. Hybridoma cells were harvested, centrifuged at 1,000g for 5min and resuspended in 35ml of miniPERM medium supplemented with 20% FBS. The cell suspension was then introduced into the production module of the bioreactor. The bioreactor was then placed on the rotating platform and turned at 9rpm in 5% CO_2, 100% humidity at 37°C. Samples were taken periodically to check the antibody titre and that the cells were healthy. The medium in the nutrient module was changed every 3-5 days.

After approximately 10 days, 15-20ml was harvested from the production module and replaced with 15-20ml of miniPERM medium supplemented with 20% FBS.

Cells were removed from the harvested medium by centrifugation at 1,000g for 5min and the supernatant used for the purification of antibody (section 2.2.10).

2.2.8 Enzyme linked immunosorbent assay (ELISA) for hybridoma screening

100µl of inactivated *B. pseudomallei* NCTC 4845 (1 x 10^7 cfu/ml) in antigen binding buffer (ABB) was added to each well of a Immulon II 96-well plate (Dynex Technologies Inc., Virginia, USA) and incubated overnight at 4°C. Plates were aspirated, 1% milk powder in PBS (150µl/well) was added to prevent non-specific binding and incubated at 37°C for 1-2 hours. Plates were washed three times with PBS containing 0.05% Tween-20 (PBST), hybridoma culture supernatant (100µl/well) added and incubated for 1 hour at 37°C. Plates were washed three times with PBST and goat anti-mouse polyvalent Ig conjugated to HRP diluted 1:5,000 in 1% milk powder was added (100µl/well) and incubated for 1 hour at 37°C. Plates were aspirated and washed five times in PBST. Substrate solution was made by dissolving 10mg 2,2'-azino-bis{3-ethylbenzthiazoline-6-sulphonic acid} (ABTS) in 50ml ABTS buffer (28ml 0.1M Na_2HPO_4 and 22ml 50mM citric acid, pH 4.3) and adding 10µl H_2O_2 just before use. Substrate was added (100µl/well) and plates incubated for 15min at room temperature. The optical density at 414nm was determined using a Titertek Multiskan plate reader (ICN Biomedicals Ltd., Thame).

Polyclonal serum taken from mice prior to fusion (at a dilution of 1:100) was used as a positive control and HAT medium was used as a negative control.

2.2.9 Determination of antibody isotype

Antibody classes were determined using a mouse monoclonal antibody isotyping kit (Gibco BRL Life Technologies Ltd., Paisley) according to the manufacturer's instructions.

2.2.10 Purification of antibody from hybridoma culture supernatant

Hybridoma culture supernatant samples were dialysed overnight at 4°C against 20mM phosphate buffer pH 7.0, before filtering through a 0.2μm filter. Samples were then loaded onto a pre-equilibrated protein A column (Amersham Pharmacia Biotech UK Ltd., Little Chalfont) and washed with phosphate buffer until the absorbance at 280nm returned to zero. Antibody was then eluted by washing the column with 0.1M citric acid pH 2.7. Fractions containing antibody were immediately dialysed against 0.1M Tris-HCl pH 8.0 before estimating the protein content by measuring the absorbance at 280nm and applying Equation 1.

2.2.11 Biotin-labelling of antibodies

Antibody samples were exhaustively dialysed against PBS and the protein concentration adjusted to 1mg/ml. For routine biotin labelling, a molar coupling ratio (MCR) of 10 moles NHS-biotin to 1 mole of antibody was used. Biotin ester (h-NHS-biotin, Molecular Devices Corporation, Wokingham) was dissolved in fresh, anhydrous dimethylformamide at a concentration of 5mg/ml just before use. For an MCR of 10, 9.6µl of ester was added to 1mg of antibody, mixed immediately and incubated in the dark for 2 hours at room temperature. For other MCRs, see Table 7 .

To separate labelled antibody from free biotin, a PD-10 Sephadex G-25 column (Amersham Pharmacia Biotech) was equilibrated with 30ml of PBS and loaded with sample. 1ml fractions were collected and assayed for labelled antibody by measuring absorbance at 280 and 362nm. Fractions which contained both protein and biotin were pooled. The concentration of biotin labelled antibody and the molar incorporation ratio were calculated using Equation 2 and Equation 3. Labelled antibody was stored frozen in small aliquots.

MCR	µl of NHS-biotin
2	1.9
5	4.8
10	9.6
15	14.4
20	19.1
40	38.2

Table 7. Amount of NHS-biotin stock solution required to label 1mg protein at different molar coupling ratios.

$$\text{mg/ml} = \frac{A_{280}^{bp} - 0.32\, A_{362}^{bp}}{A_{280}^{1mg/ml\ of\ p}}$$

where bp is the biotin labelled protein and p is the unlabelled protein.

For mouse IgG $A_{280}^{1mg/ml\ of\ p}$ is equal to 1.4.

Equation 2. Determination of the concentration of biotin-labelled protein.

$$b/p = \frac{\text{nmol b}}{\text{nmol p}}$$

$$b/p = \frac{(58 \times A_{362}^{bp})\,(A_{280}^{1mg/ml\ of\ p} \times MW^{p} / 10^{6})}{A_{280}^{bp} - 0.32\, A_{362}^{bp}}$$

For mouse IgG the equation simplifies to:

$$b/IgG = \frac{13\, A_{362}^{bp}}{A_{280}^{bp} - 0.32\, A_{362}^{bp}}$$

Equation 3. Determination of the molar incorporation of biotin.

2.2.12 Labelling of antibodies with fluorescein isothiocyanate (FITC)

Antibody samples were exhaustively dialysed against PBS and the protein concentration adjusted to 1mg/ml. For routine FITC labelling an MCR of 40 moles NHS-fluorescein isothiocyanate to 1 mole of antibody were used. Fluorescein ester (h-NHS-biotin, Molecular Devices) was dissolved in fresh, anhydrous dimethylformamide at a concentration of 5mg/ml just before use. For an MCR of 30,

29.4µl of ester was added to 1mg antibody, mixed immediately and incubated in the dark for 2 hours at room temperature. In order to separate labelled antibody from free FITC a PD-10 Sephadex G-25 column (Amersham Pharmacia Biotech) was equilibrated with 30ml of PBS and loaded with sample. 1ml fractions were collected and assayed for labelled antibody by measuring absorbance at 280 and 490nm. Fractions which contained both protein and FITC were pooled. The concentration of biotin labelled antibody and the molar incorporation ratio were calculated using Equation 4 and Equation 5. Labelled antibody was stored frozen in small aliquots.

$$\text{mg/ml} = \frac{A_{280}^{fp} - 0.18\, A_{490}^{fp}}{A_{280}^{1\text{mg/ml of p}}}$$

where fp is the FITC-labelled protein and p is the unlabelled protein. For mouse IgG $A_{280}^{1\text{mg/ml of p}}$ is equal to 1.4.

Equation 4. Determination of the concentration of FITC-labelled protein.

$$f/p = \frac{\text{nmol f}}{\text{nmol p}}$$

$$f/p = \frac{(18 \times A_{490}^{fp})\,(A_{280}^{1mg/ml\ of\ p} \times MW^{p} / 10^{6})}{A_{280}^{fp} - 0.18\, A_{490}^{fp}}$$

For mouse IgG the equation simplifies to:

$$f/IgG = \frac{4\, A_{490}^{fp}}{A_{280}^{fp} - 0.18\, A_{490}^{fp}}$$

Equation 5. Determination of the molar incorporation of FITC.

2.2.13 Capture ELISA

100µl/well of antibody (5µg/ml) in PBS was added to 96-well ELISA plates and incubated overnight at 4°C. Non-specific binding was blocked by adding 1% milk powder (150µl/well) and incubating for 1-2 hours at 37°C. Plates were washed three times in PBST, bacterial suspensions added (100µl/well, diluted in 1% milk powder) and plates incubated for a further hour at 37°C. Secondary antibody (biotin labelled)

was added (100µl/well, 10µg/ml in 1% milk powder) and plates incubated for 1 hour at 37°C, before aspirating and washing three times in PBST. 100µl/well of streptavidin-HRP conjugate (1µg/ml in 1% milk powder) was added and incubated for 1 hour at 37°C. Plates were aspirated and washed five times in PBST before adding 100µl/well of ABTS substrate solution and incubating for 15min at room temperature (section 2.2.8). The optical density at 414nm was determined using a Titertek Multiskan plate reader. The background was determined by using a negative control which contained all the reagents except the bacterial suspension. Optimisation of the capture assay gave the following: plates were coated with 4IIIA11 monoclonal antibody at 5µg/ml (100µl per well). After blocking and addition of bacteria, biotin labelled 4VIH12 (4.4 biotin molecules per IgG) was applied at 1µg/ml (100µl per well), followed after incubation and washing by streptavidin-HRP polymer at 0.5µg/ml (100µl per well). ABTS was used as the substrate and the plates read at 414nm after incubation at 37°C for 15min.

2.2.14 ELISA to demonstrate antibody competition

100µl/well of inactivated *B. pseudomallei* NCTC 4845 (1 x 10^7 cfu/ml) in ABB was added to 96-well plates and incubated overnight at 4°C. Non-specific binding was blocked by adding 1% milk powder (150µl/well) and incubating for 1-2 hours at 37°C. After three washes with PBST, 50µl of purified monoclonal antibody (10µg/ml) was added to the first row of wells and double dilutions (in 1% milk powder) prepared down to row 11. Biotin-labelled monoclonal antibody (50µl) at 1µg/ml was added to all wells except negative controls and the plates incubated for 1

hour at 37°C. The plates were washed three times with PBST and 100µl/well of streptavidin-HRP polymer (0.5µg/ml) added before incubating for 1 hour at 37°C. The plate was washed 5 times in PBST and 100µl/well of ABTS substrate solution added (section 2.2.8). The optical density at 414nm was determined using a Titertek Multiskan plate reader.

A decrease in absorbance (a minimum of 0.2 units compared with the control using biotin labelled antibody only) at high concentrations of unlabelled antibody was taken as an indication of competition between the two antibodies tested.

2.2.15 Strain variation assays

100µl/well of inactivated *B. pseudomallei* and *B. mallei* (1×10^7 cfu/ml) in ABB was added to 96-well plates and incubated overnight at 4°C. Cell suspensions were aspirated, 150µl/well 1% milk powder in PBS added to block non-specific binding and the plates incubated at 37°C for 1-2 hours. After 3 washes with PBST, 100µl/well of monoclonal antibody (1µg/ml) was added to the first row of wells and double dilutions (in 1% milk powder) prepared down to row 11. The plates were then incubated for 1 hour at 37°C. The plates were washed 3 times with PBST and 100µl/well goat anti-mouse polyvalent Ig-HRP conjugate (1:5,000 in 1% milk powder) added and the plates incubated for 1 hour at 37°C. The plate was washed 5 times in PBST before adding 100µl/well of ABTS substrate solution (section 2.2.8). The optical density at 414nm was determined using a Titertek Multiskan plate reader after incubating plates for 15min at room temperature. Positive wells were taken as those with an absorbance at least 0.1 absorbance units above the control wells.

2.2.16 Cross reactivity assays

Cross reactivity assays were carried out according to the strain variation assay method in section 2.2.15 and the capture ELISA method in section 2.2.13. In the first case cells from other species of bacteria were immobilised on the ELISA plate and screened for binding of the antibodies. In the second case a sample containing *B. pseudomallei* was double diluted down the plate in 1% milk powder containing other organisms or toxins.

2.2.17 Biosensor assays

2.2.17.1 Theory

Manual Threshold (Molecular Devices Corporation) is the specific detector currently used in the Ministry of Defence's biological detection programme. Assays are made up of two specific antibodies; one labelled with biotin and one labelled with FITC. Detection is carried out using an anti-FITC antibody labelled with urease and a substrate solution containing urea (section 3.1.9).

2.2.17.2 Manual threshold assays

Manual threshold assays were conducted using both biotin-labelled and FITC-labelled monoclonal antibodies. Antibody cocktail was prepared by mixing assay buffer (10mM phosphate buffer pH 7.0 containing 154mM NaCl, 0.005% sodium azide, 0.025% Triton X-100 and 0.1% bovine serum albumin), biotin-labelled antibody (1µg/ml), streptavidin (1mg/ml) and FITC-labelled antibody (2.5µg/ml).

Antibody cocktail (100µl) was mixed with 100µl of cell suspension (10^7, 10^6 and 10^5 cfu/ml). Each mixture (100µl) was simultaneously transferred to the assay stick and incubated for 1min before drawing the liquid through the membrane using a low vacuum. Anti-FITC-urease conjugate (100µl, Molecular Devices) was then added and drawn through the membrane. Wash buffer (500µl, 10mM phosphate buffer pH 6.5 containing 100mM NaCl, 0.005% sodium azide, 0.05% Tween 20 and 0.5mM EDTA), used to reduce non-specific background, was added to all the spots on the assay stick before being drawn through the membrane using a high vacuum. When the stick was fully drained it was rinsed in wash buffer, before inserting in the reader containing substrate solution (0.6% urea in wash buffer).

Readings for each concentration were then compared with the two blank negative controls. The detection factor was calculated by dividing the signal given in the presence of *B. pseudomallei* or *B. mallei* by the mean of the negative controls. A detection factor of over two represented a positive result.

2.2.18 Protein assays

Protein assays were carried out according to the method of Smith (Smith, P.K., Krohn, R.I. *et al.* 1985) using the BCA protein assay kit (Pierce and Warriner, Chester) according to the manufacturers instructions.

2.3 Visualisation of bacterial antigens

2.3.1 Sodium dodecyl sulphate-polyacylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (Laemmli, E.K. 1970) using a 12.5% separating gel (pH 8.45) which was allowed to set under iso-butanol, and a 4.5% stacking gel (pH 8.45) applied with various combs after the iso-butanol had been removed and the gel surface rinsed with dH_2O. Both separating and stacking gels contained 0.1% SDS (w/v). The Mini-Protean II (Bio-Rad Laboratories Ltd., Hemel Hempstead) dual slab system was used according to the manufacturers instructions with 6 x 9 x 0.1cm sized gels being used. Samples were diluted 1:2 with sample buffer (4% SDS, 10% 2-mercaptoethanol, 125 mM tris-HCl pH 6.8, 10% glycerol and 0.004% bromophenol blue), boiled for 5min and separated at 100V for 90min using tris-glycine running buffer (50mM Tris, 380mM glycine pH 8.3 containing 0.2% w/v SDS).

2.3.2 Coomassie blue staining of SDS-PAGE gels

SDS-PAGE gels were placed in coomassie blue staining solution (0.1g coomassie blue R-250 dissolved in 100ml of 25% v/v methanol, 10% v/v acetic acid) for 1 hour with gentle shaking followed by destaining (25% v/v methanol, 10% v/v acetic acid) until background staining was negligible.

2.3.3 Silver staining of SDS-PAGE gels

Silver staining was carried out according to the method of Chart (Chart, H. 1994c). SDS-PAGE gels were placed in fixative solution (50% v/v methanol) for a minimum of 1 hour. In a fume cupboard, 21ml 0.1M sodium hydroxide was mixed with 1.4ml ammonium hydroxide (BDH, specific gravity 0.88). With vigorous stirring silver nitrate solution (0.8g silver nitrate dissolved in 4ml of dH_2O) was added dropwise before making up the volume to 100ml with dH_2O. Fixative solution was poured off and freshly prepared staining solution added to the gels before incubating with gentle shaking for 15min. The staining solution was removed and the gels washed in dH_2O for 5min. Developing solution was prepared by mixing 2.5ml 1% (w/v) citric acid and 0.25ml formaldehyde and making up the volume to 500ml with dH_2O. Developing solution was added to the gel and gently mixed until bands became visible. The reaction was stopped by placing the gel in 5% (v/v) acetic acid solution for a minimum of 20min.

2.3.4 Polysaccharide silver stain

Silver staining of polysaccharides was carried out using the BioRad Silver stain kit according to the manufacturer's instructions.

2.3.5 Immunoblotting (Western transfer)

2.3.5.1 Activation of membrane

Immobilon-P transfer membranes (Millipore (UK) Ltd, Watford) were activated before use by immersing in methanol and then dH_2O for 1min.

2.3.5.2 Equilibration of gel and membrane

After SDS-PAGE and prior to transfer, the gel, activated membrane and filter paper were soaked in transfer buffer (25mM tris, 150mM glycine, 10% methanol pH 8.3) for 5min.

2.3.5.3 Blotting

Blotting was carried out using a semi-dry blotting system (Figure 5) according to the manufacturers instructions (Biometra, Göttingen, Germany). Following transfer, membranes were incubated in 5% bovine serum albumin (BSA) in PBS for one hour at 37°C or overnight at 4°C. Following three 5min washes in 0.1% BSA in PBS, the membrane was incubated, at room temperature for 1 hour with tissue culture supernatant (diluted 1:10 in 0.1% BSA containing 1% normal goat serum) or purified antibody at 1μg/ml before washing a further three times with 0.1% BSA. The membrane was then incubated with goat anti-mouse IgG (Harlan SeraLab, diluted 1:2,000 in 0.1% BSA) for a further hour at room temperature. After five 5min washes in 0.1% BSA and three 5min washes in dH_2O the substrate solution (6mg 4-chloro-1-naphthol in 20ml 20mM triethanolamine buffer pH 7.5 containing 125mM NaCl plus 5μl 30% H_2O_2) was applied and colour allowed to develop. The reaction was stopped when appropriate by washing the membrane in dH_2O. The membrane was dried and stored away from direct light.

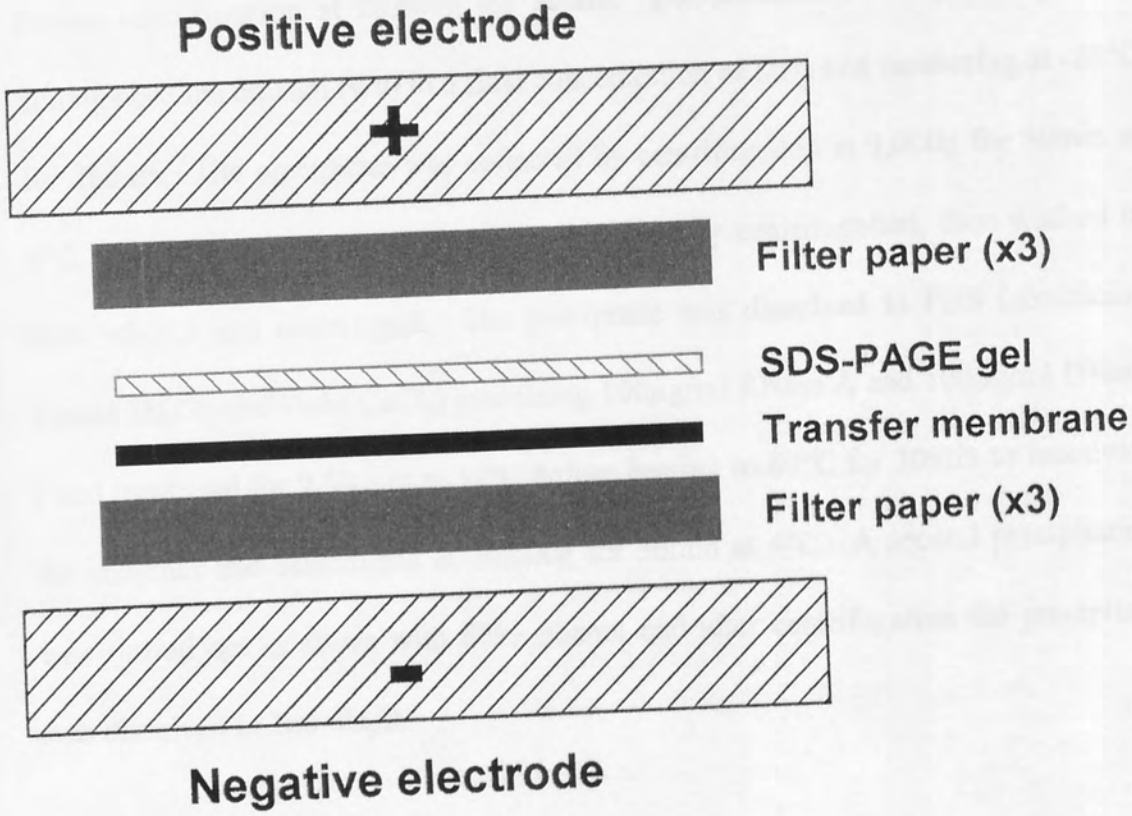


Figure 5. Assembly of immunoblotting apparatus.

2.3.6 Extraction of exopolysaccharide

Exopolysaccharide extractions were carried out according to the method of Steinmetz (Steinmetz, I., Rohde, M. *et al.* 1995). Bacteria grown on nutrient agar plates were harvested into PBS and stirred vigorously for 1 hour. The solution was centrifuged for 4 hours at 20,000g, the supernatant decanted, heated to 80°C for 30min before a further centrifugation at 20,000g for 30min. Polysaccharide was precipitated by adding absolute ethanol (v/v) to a final concentration of 80% and incubating at -20°C for 2hours. The precipitate was collected by centrifugation at 3,000g for 30min at 4°C, washed in 80% ethanol for 30min followed by centrifugation, then washed in 96% ethanol and centrifuged. The precipitate was dissolved in PBS (containing 10mM $MgCl_2$ and 1mM $CaCl_2$) containing 100µg/ml RNase A and 100µg/ml DNase I and incubated for 2.5hours at 37°C before heating to 80°C for 30min to inactivate the enzymes and centrifuged at 20,000g for 30min at 4°C. A second precipitation was carried out as above with 80% ethanol and after centrifugation the precipitate was dissolved in 2ml dH_2O.

2.3.7 Immunofluorescent staining

Inactivated bacterial suspensions were diluted to a concentration of 1 x 10^9cfu/ml in dH_2O. 40µl of this suspension was placed on a glass coverslip (BDH) and allowed to dry. Coverslips were washed briefly in PBS before immersing in acetone for 2min to fix the bacteria. After fixing, coverslips were washed briefly with PBS before immersing in 5% FBS in PBS for 2 hours, to block non-specific binding. After three 2min washes in PBS, coverslips were incubated with specific antibody (1µg/ml in

5% FBS in PBS) for 1 hour at 37°C. After a further three 2min washes in PBS, coverslips were incubated with anti-species FITC conjugate (1:64, as recommended by the manufacturer) for 1 hour at 37°C. Coverslips were washed in PBS (three 2min washes) and mounted onto glass slides with 92% glycerol in deionised water. Slides were examined using phase contrast and fluorescence microscopy on a Olympus Fluoview Laser Scanning Microscope (Japan).

2.3.8 Sodium periodate treatment

Sodium periodate treatment was carried out according to the method of Oyston and Handley (Oyston, P.C.F. and Handley, P. 1991). *B. pseudomallei* NCTC 4845 was cultured overnight in nutrient broth before harvesting by centrifugation (10,000g for 10 minutes). The cells were washed with PBS before being resuspended in PBS and the optical density (OD_{600}) adjusted to 0.1 absorbance units. An equal volume of 100mM sodium periodate was added to the cell suspension and incubated for 1 hour at 37°C. Cells were washed with PBS before being treated with SDS-PAGE sample buffer (section 2.3.1).

2.4 Extraction and characterisation of lipopolysaccharide (LPS)

2.4.1 Extraction of LPS by aqueous phenol

Phenol hot water extraction was carried out according to the method of Westphal and Jann (Westphal, O. and Jann, K. 1965). Heat killed lyophilised bacteria (3g) were suspended to a concentration of approximately 5% (w/v) in dH_2O and heated to 67°C in a water bath. An equal volume of 90% aqueous phenol was also heated to 67°C.

The pre-warmed bacterial suspension and the phenol were mixed and stirred at 67°C for 15min. The mixture was carefully transferred to 30ml Corex centrifuge tubes (Beckman, California, USA) and cooled on ice until phase separation occurred. The tubes were centrifuged at 10,000g for 15min to complete phase separation. The upper phenol phase was carefully removed and discarded. The lower aqueous phase was transferred to dialysis cassettes (Pierce & Warriner) and exhaustively dialysed against dH_2O until there was no smell of phenol. The solution was then centrifuged at 10,000g for 15min and then supernatant concentrated by rotary evaporation to approximately one fifth of its original volume. The solution was centrifuged at 100,000g for 3 hours to pellet LPS. The pellet was resuspended in dH_2O and lyophilised.

2.4.2 Proteinase K preparation of LPS (mini-prep)

Proteinase-K mini-preparations were carried out according to the method of Chart (Chart, H. 1994b). Heat-killed suspensions of bacteria were dispensed into pre-weighed micro-centrifuge tubes (Costar Corporation, Massachusetts, USA) and spun at 12,000g for 5min. The supernatant was removed, the tubes re-weighed and the wet-weight of the bacteria calculated. SDS-solubilisation buffer (62.5mM Tris-HCl pH 6.8 containing 10% v/v glycerol, 5% v/v 2-mercaptoethanol, 3% w/v SDS and 0.01% w/v bromophenol blue) was added to give a final concentration of 1mg bacteria per 30µl of solubilisation buffer. Tubes were mixed with a vortex mixer and incubated in a boiling water bath for 10min. Tubes were removed and carefully mixed. Using a background light source, the tubes were examined for clarity. If the suspension remained cloudy the tubes were incubated in the water bath for a further 10min. A suspension of proteinase-K was prepared (3.3mg in 1ml of solubilisation

buffer) and 30µl dispensed into new micro-centrifuge tubes. 30µl of the clear, boiled bacterial suspension was added to the proteinase-K and the tubes incubated in a water bath at 60°C for a minimum of one hour. Samples were then separated by SDS-PAGE according to section 2.3.1.

2.4.3 Proteinase-K preparation of LPS (small scale)

Inactivated suspensions of bacterial cells were centrifuged in pre-weighed tubes at 10,000g for 10min. The supernatant was removed, the tubes reweighed and the wet-weight of the bacteria calculated. The bacteria were suspended in extraction buffer (62.5mM Tris-HCl pH 6.8 containing 10% v/v glycerol, 5% v/v β-mercaptoethanol and 3% w/v SDS) at a concentration of 160mg bacteria/ml buffer. Tubes were mixed and placed in a boiling water bath for 15min after which they were removed, mixed and examined for clarity. Cloudy suspensions were replaced in the water bath for a further 15min. Proteinase-K was prepared (2mg/ml in extraction buffer). After cooling to approximately 60°C, equal volumes of boiled bacteria and proteinase-K were mixed and incubated in a 60°C water bath for a minimum of 2 hours. After incubation, tubes were replaced in the boiling water bath for 10min to inactivate the proteinase-K. The solution was transferred to dialysis tubing and dialysed exhaustively against dH_2O until no smell of 2-mercaptoethanol remained. The solution was then dispensed into glass freeze-drying vials (Wheaton Scientific, New Jersey, USA) and frozen at -70°C before lyophilisation.

2.4.4 Silver staining for LPS

After SDS-PAGE (section 2.3.1) gels were carefully placed in clean plastic boxes and fixed in 40% v/v methanol, 5% v/v acetic acid for a minimum of two hours. The fixing solution was removed and oxidising solution added (0.7% w/v periodic acid in fixing solution) and the gels incubated with gentle mixing for 5min. The oxidising solution was removed and gels washed in dH_2O for 15min, three times. During the last washing step fresh silver stain was prepared. In a fume cupboard, 28ml 0.1M sodium hydroxide was mixed with 2ml ammonium hydroxide (specific gravity 0.88). 5ml of 20% silver nitrate in dH_2O was added dropwise, mixing until the brown colouration disappeared before making up the volume to 150ml with dH_2O. The fresh silver staining solution was placed in a clean plastic box, the gels transferred and incubated with gentle mixing for 10min. The staining solution was discarded and the gels washed in dH_2O for 10min, three times. During the last washing step, developer was prepared (50mg citric acid in 1,000ml dH_2O containing 0.5ml formaldehyde). The gels were carefully transferred into clean plastic boxes containing developer and mixed. When bands were clearly visible the developing solution was removed and the gels placed in 5% acetic acid to stop the reaction.

2.4.5 Limulus amoebocyte lysate (LAL) assay

LAL assays were carried out using the Chromogenix LAL assay kit (Quadratech, Epsom) according to the manufacturer's instructions.

2.5 Production of monoclonal antibodies against LPS

2.5.1 Immunisation schedule using LPS isolated from *B. pseudomallei* NCTC 4845

Four groups of 5 female Balb/c mice (6 - 9 weeks old) were immunised with 50μg LPS from *B. pseudomallei* NCTC 4845 in different adjuvants on days 1, 21, 42, 84 and 197. Group I were given LPS in PBS only, group II were given LPS in Alhydrogel™ (Superfos Biosector, Vedbaek, Denmark), group III were given LPS in Hunter's Titermax® and group IV were given LPS in FIA. Groups I, II and IV were immunised by intraperitoneal injection, but group III was immunised intramuscularly, as recommended by the manufacturers. A fifth group of five mice were used as negative controls.

2.5.2 Screening of intermediate blood samples

Fourteen days after each injection intermediate blood samples were taken from the tail vein of each mouse for analysis of the immune response. Blood was placed in an microcentrifuge tube and spun in a microcentrifuge at 12,000g for 10min. The serum was removed and recentrifuged to remove residual red cells. Serum samples were stored frozen at -20°C. ELISAs were carried out 5 times for each sample, each time using different HRP conjugates specific for mouse IgM, IgG_1, IgG_{2a}, IgG_{2b} and IgG_3 (Harlan SeraLab). Control sera from the unimmunised mice were used as negative controls. In addition to individual serum samples, an aggregated serum was used for each group by pooling 5μl of serum from each of the individual mice in the group.

Each well of a 96-well plate was coated with 50µl of an inactivated suspension of *B. pseudomallei* NCTC 4845 (2 x 10^7 cfu/ml) in ABB and incubated overnight at 4°C. The plate was aspirated, 200µl/well of 1% milk powder in PBS was added to block non-specific binding sites and the plates incubated at 37°C for 1-2 hours. After 3 washes with PBST, 100µl of diluted serum was added to the first row of wells and double dilutions (in 1% milk powder) prepared down the plate before incubating for 1 hour at 37°C. Plates were washed three times with PBST and 100µl/well goat anti-mouse HRP conjugate (diluted 1:5,000 in 1% milk powder) added to each well before incubating for 1 hour at 37°C. Plates were washed five times in PBST before adding 100µl/well of ABTS substrate solution (section 2.2.8) and incubating for 15min at room temperature. The optical density at 414nm was determined using a Titertek Multiskan plate reader. Positive wells were taken as those with an absorbance 0.1 units above the negative control wells.

2.5.3 Immunisation schedule for *B. mallei* and *B. pseudomallei* atypical LPS

Two groups of 3 female Balb/c mice (6 - 9 weeks old) were immunised by intraperitoneal injection with 50µg LPS from *B. pseudomallei* HA or *B. mallei* NCTC 23344 in FIA. Boosts were given every three weeks for a total of twelve weeks. Intermediate blood samples were taken and screened for IgG and IgM isotypes as in section 2.5.2.

2.6 Production of single chain antibodies (scFv)

2.6.1 Media preparation

2YT broth was prepared by dissolving 5g tryptone (Oxoid), 2.5g NaCl (BDH) and 5g yeast extract (Difco Laboratories, Michigan, USA) in 450ml dH_2O, the pH was adjusted to pH 7.2-7.4 with 5M NaOH, the volume made up to 500ml with dH_2O and the media autoclaved at 121°C (15psi) for 15min. 2YT agar was prepared in the same way with the addition of 7.5g agar per 500ml media. SOC broth was prepared by dissolving 20g bacto-tryptone (Oxoid), 5g bacto-yeast extract (Oxoid) and 0.5g NaCl (BDH) in 950ml dH_2O. 10ml 250mM potassium chloride was added and the pH adjusted to 7.0 with 5M NaOH before making up the volume to 1,000ml with dH_2O. The media were autoclaved at 121°C (15psi) for 15min. Before use, 5ml sterile 2M $MgCl_2$ and 20ml sterile 1M glucose was added.

2.6.2 Isolation of total ribonucleic acid (RNA)

Total RNA was isolated from a portion of mouse spleen or from hybridoma cells using the Qiagen RNeasy Maxiprep kit (Crawley) according to the manufacturer's instructions.

2.6.3 Purification of messenger RNA (mRNA)

Messenger RNA was purified from total RNA using the Qiagen Oligotex mRNA Purification kit according to the manufacturer's instructions.

2.6.4 Production of cyclic deoxyribonucleic acid (cDNA)

Approximately 1μg mRNA was reverse transcribed in a reaction volume of 100μl with random hexamer primers using the cDNA synthesis kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

2.6.5 Amplification of immunoglobulin heavy (H) & light (L) chains

2.6.5.1 Theory

The cDNA is used as a template for the primary PCR amplification of the V_H and V_L domains. The PCR primers, described in Tables 8-11, have been optimised, incorporating all presently known mouse sequences and has been shown to generate a diverse library when used to prepare a repertoire of antibodies from an immunised mouse. The primers also encode a 20 amino-acid (Gly_4-Ser)-linker which allows the V_H and V_L to pair effectively but avoids dimerisation or aggregation of scFv fragments. The incorporation of the linker sequence during the PCR also has the advantage that scFv assembly can then be carried out using two fragments instead of three.

2.6.5.2 Amplification of heavy and light chains

Heavy and Light chains were amplified separately by PCR using PCR beads (Amersham Pharmacia Biotech). PCR reactions were performed in 25μl volumes containing 0.5μl cDNA, 1μl VHfor and VHback primer mixes (100pmol/μl, Cruachem) for amplification of heavy chains or 1μl VLfor and VLback primer mixes (100pmol/μl, Cruachem) for amplification of light chains. Three minutes

denaturation at 92°C was followed by 7 cycles of 1min at 92°C, 30sec at 63°C, 50sec at 58°C, 1min at 72°C and 23 cycles of 1min at 92°C, 30sec at 63°C, 1min at 72°C. Products were analysed by agarose gel electrophoresis (1.5%, low melting point agarose) and the appropriate bands excised.

2.6.6 Purification of PCR products from excised gel fragments

DNA was purified from gel fragments using the Qiagen Gel Extraction kit according to the manufacturer's instructions.

2.6.7 Assembly of scFv fragment (Ligation of H & L chains)

2.6.7.1 Theory

Equal concentrations of V_H and V_L fragments are mixed and thermal cycling is carried out in the absence of primers to allow the two fragments to align via the 20 amino-acid linker. To avoid any wrong overlap during assembly PCR, the (Gly_4-Ser) repeats are encoded by different codons. Primers scfor and scback (described below) are added and the whole scFv fragment amplified.

Primer scfor 5' ggaattcggcccccgag 3'

Primer scback 5' ttactcgcggcccagccggccatggcggactacaaag 3'

Primer VHback	5' (Gly$_4$Ser)-linker BamHI V_H 3'	µl in mix (100pmol/µl)
HB1	**ggcggcggcggctcc**ggtggtggtggatccGAKGTRMAGCTTCAGGAGTC	4
HB2	**ggcggcggcggctcc**ggtggtggtggatccGAGGTBCAGCTBCAGCAGTC	4
HB3	**ggcggcggcggctcc**ggtggtggtggatccCAGGTGCAGCTGAAGSASTC	3
HB4	**ggcggcggcggctcc**ggtggtggtggatccGAGGTCCARCTGCAACARTC	4
HB5	**ggcggcggcggctcc**ggtggtggtggatccCAGGTYCAGCTBCAGCARTC	7
HB6	**ggcggcggcggctcc**ggtggtggtggatccCAGGTYCARCTGCAGCAGTC	2
HB7	**ggcggcggcggctcc**ggtggtggtggatccCAGGTCCACGTGAAGCAGTC	1
HB8	**ggcggcggcggctcc**ggtggtggtggatccGAGGTGAASSTGGTGGAATC	2
HB9	**ggcggcggcggctcc**ggtggtggtggatccGAVGTGAWGYTGGTGGAGTC	5
HB10	**ggcggcggcggctcc**ggtggtggtggatccGAGGTGCAGSKGGTGGAGTC	2
HB11	**ggcggcggcggctcc**ggtggtggtggatccGAKGTGCAMCTGGTGGAGTC	2
HB12	**ggcggcggcggctcc**ggtggtggtggatccGAGGTGAAGCTGATGGARTC	2
HB13	**ggcggcggcggctcc**ggtggtggtggatccGAGGTGCARCTTGTTGAGTC	1
HB14	**ggcggcggcggctcc**ggtggtggtggatccGARGTRAAGCTTCTCGAGTC	2
HB15	**ggcggcggcggctcc**ggtggtggtggatccGAAGTGAARSTTGAGGAGTC	2
HB16	**ggcggcggcggctcc**ggtggtggtggatccCAGGTTACTCTRAAAGWGTSTG	5
HB17	**ggcggcggcggctcc**ggtggtggtggatccCAGGTCCAACTVCAGCARCC	3.5
HB18	**ggcggcggcggctcc**ggtggtggtggatccGATGTGAACTTGGAAGTGTC	0.7
HB 19	**ggcggcggcggctcc**ggtggtggtggatccGAGGTGAAGGTCATCGAGTC	0.7

NB: Ambiguity codes for primer sequences are K= T or G, R= A or G, M= A or C, B= C, G or T, S= G or C, Y= T or C, V= A, C or G, W= A or T

Table 8. Primer sequences for VHback used in the amplification of immunoglobulin V_H domains (see section 2.6.5).

Primer VHfor	5'EcoRI SfiI V_H 3'	µl in mix (100pmol/µl)
HF1	ggaattc<u>ggcccccgaggcC</u>GAGGAAACGGTGACCGTGGT	1
HF2	ggaattc<u>ggcccccgaggcC</u>GAGGAGACTGTGAGAGTGGT	1
HF3	ggaattc<u>ggcccccgaggcC</u>GCAGAGACAGTGACCAGAGT	1
HF4	ggaattc<u>ggcccccgaggcC</u>GAGGAGACGGTGACTGAGGT	1

NB: Ambiguity codes for primer sequences are K= T or G, R= A or G, M= A or C, B= C, G or T, S= G or C, Y= T or C, V= A, C or G, W= A or T

Table 9. Primer sequences for VHfor used in the amplification of immunoglobulin V_H domains (see section 2.6.5).

Primer VLfor	5'(Gly_4Ser)-linker V_L 3'	µl in mix (100pmol/µl)
LF1	**ggagccgccgccgcc** $(agaaccaccaccacc)_2$ ACGTTTGATTTCCAGCTTGG	1
LF2	**ggagccgccgccgcc** $(agaaccaccaccacc)_2$ ACGTTTTATTTCCAGCTTGG	1
LF3	**ggagccgccgccgcc** $(agaaccaccaccacc)_2$ ACGTTTTATTTCCAACTTTG	1
LF4	**ggagccgccgccgcc** $(agaaccaccaccacc)_2$ ACGTTTCAGCTCCAGCTTGG	1
λF	**ggagccgccgccgcc** $(agaaccaccaccacc)_2$ ACCTAGGACAGTCAGTTTGG	0.25

NB: Ambiguity codes for primer sequences are K= T or G, R= A or G, M= A or C, B= C, G or T, S= G or C, Y= T or C, V= A, C or G, W= A or T

Table 10. Primer sequences for VLfor used in the amplification of immunoglobulin V_L domains (see section2.6.5).

Primer VLback	5' FLAG V_L 3'	µl in mix (100pmol/µl)
LB1	gccatggcg*gactacaaaGAY*ATCCAGCTGACTCAGCC	1
LB2	gccatggcg*gactacaaaGAY*ATTGTTCTCWCCCAGTC	2
LB3	gccatggcg*gactacaaaGAY*ATTGTGMTMACTCAGTC	5
LB4	gccatggcg*gactacaaaGAY*ATTGTGYTRACACAGTC	3.5
LB5	gccatggcg*gactacaaaGAY*ATTGTRATGACMCAGTC	4
LB6	gccatggcg*gactacaaaGAY*ATTMAGATRAMCCAGTC	7
LB7	gccatggcg*gactacaaaGAY*ATTCAGATGAYDCAGTC	6
LB8	gccatggcg*gactacaaaGAY*ATYCAGATGACACAGAC	1.5
LB9	gccatggcg*gactacaaaGAY*ATTGTTCTCAWCCAGTC	2
LB10	gccatggcg*gactacaaaGAY*ATTGWGCTSACCCAATC	3.5
LB11	gccatggcg*gactacaaaGAY*ATTSTRATGACCCARTC	8
LB12	gccatggcg*gactacaaaGAY*RTTKTGATGACCCARAC	8
LB13	gccatggcg*gactacaaaGAY*ATTGTGATGACBCAGKC	6
LB14	gccatggcg*gactacaaaGAY*ATTGTGATAACYCAGGA	2
LB15	gccatggcg*gactacaaaGAY*ATTGTGATGACCCAGWT	2
LB16	gccatggcg*gactacaaaGAY*ATTGTGATGACACAACC	1
LB17	gccatggcg*gactacaaaGAY*ATTTTGCTGACTCAGTC	1
λB	gccatggcg*gactacaaaGAT*GCTGTTGTACTCAGGAATC	1

NB: Ambiguity codes for primer sequences are K= T or G, R= A or G, M= A or C, B= C, G or T, S= G or C, Y= T or C, V= A, C or G, W= A or T

Table 11. Primer sequences for VLback used in the amplification of immunoglobulin V_L domains (see section 2.6.5).

2.6.7.2 Assembly and amplification of scFv fragment

Approximately 10ng of each heavy and light chain were combined and amplified using PCR beads. An initial denaturation step (3min, 92°C) was followed by 2 cycles of 1min at 92°C, 30sec at 63°C, 50sec at 58°C, 1min at 72°C in the absence of primers. After adding primers scfor and scback (1pg each, Cruachem), 5 cycles of 1min at 92°C, 30sec at 63°C, 50sec at 58°C, 1min at 72°C and 23 cycles of 1min at 92°C, 30sec at 63°C, 1min at 72°C were performed. Products were analysed by agarose gel electrophoresis (1.5%, low melting point agarose) and appropriate bands excised.

2.6.8 *SfiI* digest and cloning into pAK100

The phagemid vector pAK100 was obtained from A. Pluckthun at the University of Zurich. The gel purified scFv fragment and the phage display vector pAK100 were both digested with *SfiI* (Boehringer Manneheim). Approximately 1µg DNA was digested with 20U *SfiI* at 50°C in 50µl of reaction buffer provided by the manufacturer. After digestion the scFv fragment was purified using the Qiagen PCR product clean up kit before ligating into the cut vector pAK100 (insert:vector ratio of 1:10) using 1U T4 ligase (Boehringer Manneheim) at 16°C overnight.

2.6.9 Transformation

Transformation of the vector into *E. coli* XLI-blue cells (Stratagene Ltd., Cambridge) was carried out using the Stratagene transformation kit according to the manufacturers instructions. After transformation cells were plated onto 2YT agar

(containing 30μg/ml chloramphenicol, 10mM $MgSO_4$ and 1% glucose) and incubated at 37°C overnight. Colonies were counted for an estimation of library size, and random colonies selected for verification (section 2.6.10). Remaining colonies were harvested and stored at -70°C in SOC broth containing 25% glycerol.

2.6.10 Verification of clones

2.6.10.1 Theory

Verification was carried out to check that random colonies selected after transformation contained the scFv insert. PCR was carried out using primers scfor and scback to amplify the scFv insert, which was then digested with the restriction enzyme *BstNI*. *BstNI* fingerprinting gives a rough guide to diversity in a panel of clones.

2.6.10.2 Amplification and *BstNI* fingerprinting

Random colonies selected after transformation were grown up in 4ml 2YT broth containing 30μg/ml chloramphenicol and 1% glucose. 100μl of culture was harvested by centrifugation at 6,000g for 5min, cells resuspended in 1ml dH_2O and boiled for 3min. 5μl of each suspension was used for PCR amplification of the scFv insert (section 2.6.7). 10μl of the PCR reaction was analysed by agarose gel electrophoresis using a 2% agarose gel. The remainder of the PCR reaction was subjected to a *BstNI* digestion using 20U *BstNI* (Boehringer Manneheim) in reaction buffer provided by the manufacturer. After incubation for 3 hours at 60°C the products were analysed by agarose gel electrophoresis.

2.6.11 Selection of phage by panning

2.6.11.1 Preparation of phage

500µl of the *E. coli* library was added to 10ml 2YT broth (containing 100mM glucose, 30µg/ml chloramphenicol and 30µg/ml tetracycline) and incubated for 5 hours at 37°C on an orbital shaker (200rpm). The cells were harvested by centrifugation at 3,000g for 10min and resuspended in 2YT broth containing 30µg/ml chloramphenicol, 30µg/ml tetracycline, 1mM IPTG (Boehringer Manneheim) and 1 x 10^{10} pfu VCSM13 helper phage (Stratagene). The suspension was cultured overnight at 37°C with shaking. Cells were harvested by centrifugation (3,000g for 10min) and the supernatant decanted. Phage in the supernatant (approximate volume 10ml) was precipitated by adding 2ml of 20% PEG 8000 in 2.5M NaCl and incubating on ice for 60min. The precipitate was recovered by centrifugation at 10,000g for 30min and resuspended in 5ml blocking buffer (2% milk powder,1% BSA in PBST).

2.6.11.2 Solid-phase selection of phage antibodies

Immunotubes (Nalgene) were coated with 5ml of inactivated *B. pseudomallei* 4845 (2 x 10^7 cfu/ml) in ABB and incubated overnight at 4°C. Tubes were washed three times with 5ml volumes of PBST. Non-specific binding was blocked by adding 5ml of blocking buffer and incubating at room temperature for 1-2 hours. Tubes were washed three times with PBST, the phage suspension added and incubated at 37°C for 2 hours. The phage suspension was discarded and the tubes washed twenty times with PBS followed by twenty washes with PBST. Bound phage was eluted by

adding 1ml of 100mM triethylamine and gently mixing the suspension for 10min. Triethylamine was neutralised with 500µl of 1.5M Tris-HCl, pH 8.0. The eluted phage was added to growing *E. coli* XLI-Blue cells in 2YT broth (containing 30µg/ml tetracycline) and allowed to infect for 2 hours. An aliquot of the culture was removed for analysis by ELISA (section 2.6.12) and the remainder used to produce the selected phage (section 2.6.11.3).

2.6.11.3 Production of selected phage

The *E. coli* XLI-Blue cells were harvested by centrifugation (3,000g for 10min) and resuspended in 2YT broth (containing 30µg/ml chloramphenicol, 30µg/ml tetracycline, 1mM IPTG and 1 x 10^{10} pfu VCSM13 helper phage) and grown overnight at 37°C with shaking. Phage were harvested from the overnight culture by centrifugation of the cells and PEG precipitation of the supernatant. After centrifugation the phage pellet was resuspended in 2ml of 2YT broth, filtered through a 0.2µm filter and stored at 4°C for the next round of panning.

2.6.12 Analysis of phage-derived antibodies by ELISA

2.6.12.1 Production of clones for analysis

A 100µl aliquot of the phage infected cells (section 2.6.11.2) was serially diluted three times. 100µl of each dilution was then plated out onto 2YT agar (containing 30µg/ml chloramphenicol and 30µg/ml tetracycline) in duplicate and the plates incubated overnight at 37°C. Individual colonies were picked and grown up in 400µl of 2YT broth (containing 100mM glucose, 30µg/ml chloramphenicol and 30µg/ml

tetracycline). Phage production was then induced by harvesting cells, resuspending them in 2YT broth (containing 30μg/ml chloramphenicol, 30μg/ml tetracycline, 1mM IPTG and 1 x 10^{10} pfu VCSM13 helper phage) and incubating at 37°C overnight with shaking. Cells were harvested by centrifugation and the supernatant screened using ELISA (section 2.6.12.2).

2.6.12.2 Screening for binding by ELISA

Each well of a 96-well plate was coated with 50μl of an inactivated suspension of *B. pseudomallei* NCTC 4845 (2 x 10^7 cfu/ml) in ABB and incubated overnight at 4°C. An extract from baby hamster kidney (BHK) cells prepared by precipitating cell culture fluid (7% PEG 8000 in 0.5M NaCl) was diluted 1:400 in ABB and used to coat negative control plates (50μl/well). The plates were aspirated, 200μl/well of blocking buffer was added to block non-specific binding sites and the plates incubated at room temperature for 2 hours. Supernatant from section 2.6.12.1 (400μl) was mixed with 100μl blocking buffer. The mixture was incubated at room temperature for 30min before 100μl was added (in duplicate) to ELISA plates and incubated at room temperature for 2 hours. The plates were washed a further three times in PBST before adding 100μl/well anti-M13 HRPO conjugate (Amersham Pharmacia Biotech, 1:5,000 in blocking buffer) and incubating at room temperature for 1 hour. Plates were washed five times in PBST before adding 100μl/well of ABTS substrate solution (section 2.2.8) and incubating for 15min at room temperature. The optical density at 414nm was determined using a Titertek Multiskan plate reader. Positive wells had an absorbance greater than that of the corresponding well on the negative control plate.

2.6.13 Sequencing

Plasmid DNA for sequencing was prepared using the Qiagen Plasmid Miniprep kit according to the manufacturer's instructions. DNA yield was quantified by spectrophotometry and templates were then sequenced using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq DNA polymerase (Perkin Elmer), a CATALYST 800 Molecular Biology Work Station and Applied Biosystems automated Sequencer according to the manufacturer's instructions.

3. Detection of *B. pseudomallei* and *B. mallei*

3.1 Introduction

3.1.1 The immune system

The principal function of the immune system is to protect animals from infectious organisms and from their toxic products. The system has evolved a powerful range of mechanisms to locate foreign cells, viruses or macromolecules: to neutralise these invaders and to eliminate them from the body. These mechanisms can be divided into two broad categories non-adaptive (innate) and adaptive immunity.

Non adaptive immunity is mediated by cells which respond non-specifically to foreign molecules and includes systems such as phagocytosis by macrophages and cell lysis by natural killer cells. Adaptive immunity is directed against specific molecules, it is mediated by lymphocytes, which synthesise cell surface receptors or secrete proteins that bind specifically to foreign molecules. Adaptive immune responses are generally divided into two classes, humoral and cell-mediated. Cell-mediated responses are typified by the binding of cytotoxic T lymphocytes to foreign or infected cells and the subsequent lysis of these cells and is carried out primarily by cytotoxic T lymphocytes with some involvement by helper T lymphocytes. The humoral response results in the generation of circulating antibodies that bind to foreign antigens. It is mediated by B lymphocytes in conjunction with helper T cells. Antibodies have several functions, including acting as flexible adaptors which bring about the adherence of infectious agents to phagocytes and activation of the

complement system (Harlow, E. and Lane, D. 1988; Roitt, I., Brostoff, J. *et al.* 1989; Kuby, J. 1992).

The specificity of the adaptive immune system is based on the specificity of the antibodies and lymphocytes. Each lymphocyte is capable of recognising only one particular antigen. Antigen binds to the small number of cells which can recognise it and induces them to proliferate so they constitute sufficient cells to mount an adequate immune response (clonal selection). This initial response has a lag of approximately 5-7 days before antibody levels begin to rise. Antibody levels peak in the primary response at about day 14 and then begin to drop off as the antibody-secreting cells begin to die. After the antigen has been eliminated, some cells from the expanded pool of antigen-specific lymphocytes remain in circulation. These cells are primed to respond to any subsequent exposure to the same antigen and are known as memory cells. Re-exposure to the same antigen produces a secondary response, this response is much quicker with a lag time of only 1-2 days. Secondary response antibody levels are much higher and sustained for a longer period of time (Roitt, I., Brostoff, J. *et al.* 1989; Kuby, J. 1992).

3.1.2 Antigens

Any molecule that can bind to an antibody is known as an antigen. Antigens, which are generally very large and complex, are not recognised in their entirety by lymphocytes. Both B and T lymphocytes recognise discrete sites on the antigen called antigenic determinants or epitopes. A single antigen may have many different epitopes or several identical epitopes. Each antigenic determinant has a size

corresponding to 6-22 amino acid or sugar residues. However, they are three-dimensional defined sites and the number of combinations of shape and charge on a single antigen means that the potential number of determinants is very large (Catty, D. 1988; Kuby, J. 1992).

When a molecule is used to induce an adaptive response it is called an immunogen. Natural immunogens are usually macromolecules of protein or carbohydrate composition with a molecular weight greater than 1,000 and normally above 5,000kDa. Highly immunogenic molecules usually have a molecular weight greater than 100,000kDa and some structural complexity (Catty, D. 1988).

3.1.3 Antibodies and antibody binding

Antibodies are glycoprotein molecules of approximately 160,000 daltons, composed of four protein chains, two heavy chains (55kDa) and two light chains (25kDa). These heavy and light chains are made up of different peptide regions, known as constant and variable regions based on their amino acid sequence. Each light chain consists of one variable and one constant region while each heavy chain contains one variable and three constant regions (Figure 6). The variable regions of the light and heavy chains associate and form a cleft which encloses the antigen binding region of the antibody. The constant region of the light chain then associates with the first constant region of the heavy chain via a disulphide bond to form what is known as a Fab fragment. The two heavy chains then associate via two disulphide bonds linking the second constant region of the heavy chains forming the Fc part of the antibody. There is a small proline-rich region between the first and second constant regions of

the heavy chain, known as the hinge. This gives the antibody flexibility and enables the antigen binding domains to interact with a large number of antigen conformations (Amzel, L.M. and Poljak, R.J. 1979; Harlow, E. and Lane, D. 1988; Kuby, J. 1992).

Sequence analysis has revealed five different types of heavy chain (μ, δ, γ, ε, and α). Each of the different heavy chains is called an isotype (section 3.1.5). Amino acid sequences of both heavy and light chains have been found to contain a highly variable region (100-110 amino-acids) at the amino-terminal end. This sequence variability was found to be concentrated into three hypervariable regions, consisting of 15-20% of the variable region. These hypervariable regions form the antigen-binding site of the antibody, and because the antigen-binding site is complementary to the structure of the epitope, the hypervariable regions are also known as complementarity-determining regions (CDRs). The wide range of specificities exhibited by antibodies is a function of the six hypervariable regions (Kabat, E.A., Wu, T.T. *et al.* 1977).

The binding of an antibody to an epitope involves weak noncovalent interactions, which operate only over short distances. For a strong interaction to occur, the antibody's binding site and the epitope must have a complementary conformation. The size of the epitope recognised is therefore determined by the size, shape and amino-acid residues of the antibody's binding site. Smaller ligands such as carbohydrates, nucleic acids and peptides often bind to an antibody within the deep, concave pocket or cleft. Large globular antigens interact differently and protrusions on the epitope are matched by depressions in the antibody's binding site. Studies

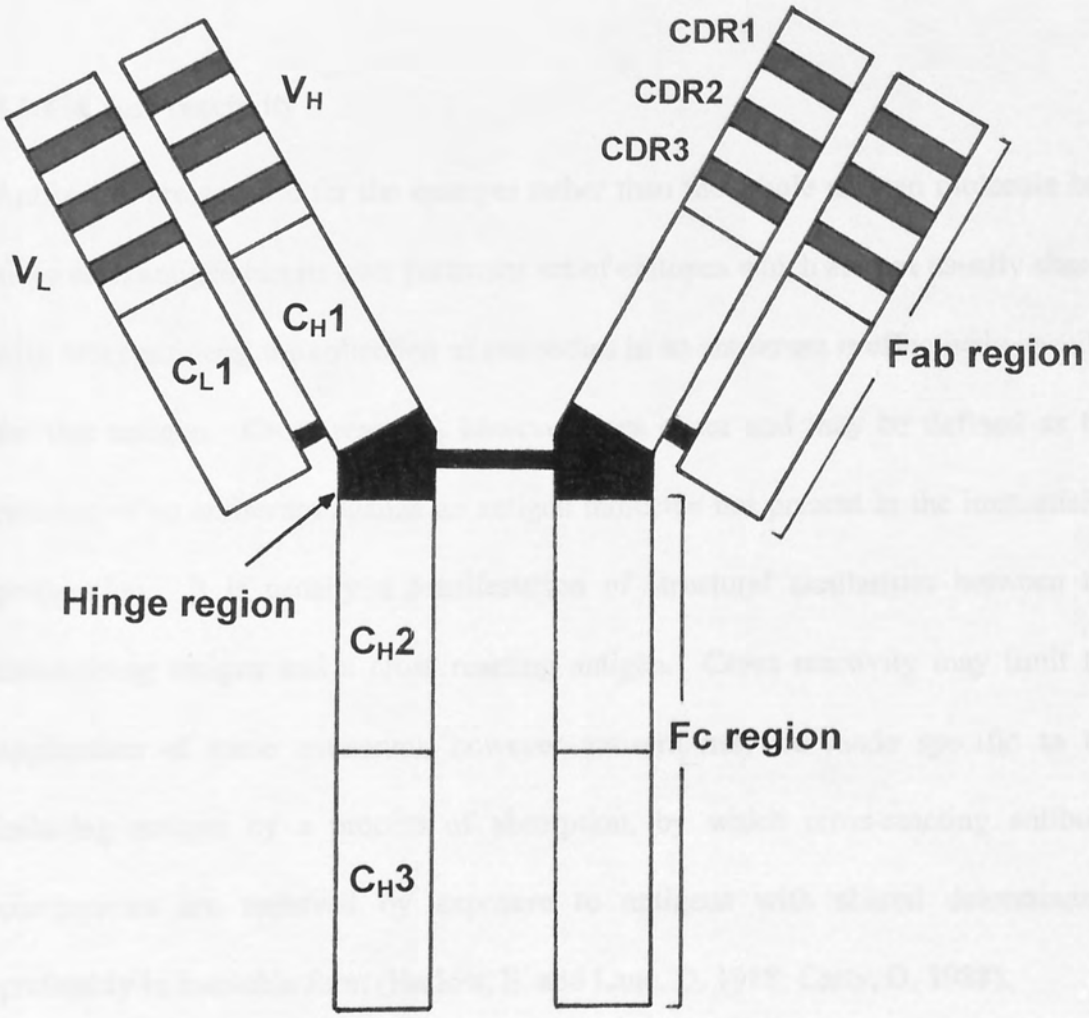


Figure 6. Structure of an immunoglobulin G molecule.

have shown that 15-22 amino-acids on the surface of the antigen make contact with a similar number of residues in the antibody's binding site (Kuby, J. 1992). For these globular antigens the epitope is dependent on the tertiary conformation of the native antigen.

3.1.4 Cross reactivity

Antibodies are specific for the epitopes rather than the whole antigen molecule but, since each antigen has its own particular set of epitopes which are not usually shared with other antigens, the collection of antibodies in an antiserum is effectively specific for that antigen. Cross reaction, however does occur and may be defined as the reaction of an antiserum against an antigen molecule not present in the immunising preparation. It is usually a manifestation of structural similarities between the immunising antigen and a cross reacting antigen. Cross reactivity may limit the application of some antiserum, however antisera may be made specific to the inducing antigen by a process of absorption, by which cross-reacting antibody components are removed by exposure to antigens with shared determinants, preferably in insoluble form (Harlow, E. and Lane, D. 1988; Catty, D. 1988).

3.1.5 Antibody isotypes

Five distinct classes of immunoglobulin molecule are recognised in most higher mammals, namely IgG, IgA, IgM, IgD and IgE. They differ from each other in size, charge, amino-acid composition and carbohydrate content (Roitt, I., Brostoff, J. *et al.* 1989).

IgG is the major immunoglobulin in normal human serum accounting for 70-75% of the total immunoglobulin pool. IgG is a monomeric protein with a molecular weight of 146,000kDa. Studies of IgG suclasses have shown that IgG_3 proteins are slightly larger than the other subclasses due to a slightly heavier γ_3 chain. The IgG class is the major antibody of secondary immune responses and the exclusive anti-toxin class.

IgM accounts for 10% of the immunoglobulin pool. The molecule has a pentameric structure in which individual heavy chains have a molecular weight of 65,000kDa and the whole molecule has a molecular weight of 970,000kDa. IgM is the predominant early antibody frequently seen in the immune response to antigenically complex infectious organisms. IgM antibodies are usually of low affinity and their affinity does not seem to rise with time. However the low affinity may be compensated by their multivalency.

IgA represents 15-20% of the human immunoglobulin pool and in most mammals occurs most commonly as a dimer. IgA is the predominant immunoglobulin in seromucous secretions such as saliva, milk and intestinal secretions. Secretory IgA exists mainly in the dimeric form and has a molecular weight of 385,000kDa.

IgD accounts for less than 1% of the total immunoglobulin pool but is known to be present in large quantities on the membrane of circulating B lymphocytes. The

precise biological function of this class is not clear but it may play a role in antigen-triggered lymphocyte differentiation.

IgE, though a trace serum protein, is found on the surface membrane of basophils and mast cells. This class of immunoglobulin may play a role in active immunity to helminthic parasites, but in developed countries it is more commonly associated with hypersensitivity diseases such as asthma and hay fever.

3.1.6 Polyclonal antibodies

The specificity of conventional polyclonal serum is the result of a consensus of thousands of clonal products which bind to antigenic determinants covering most or all of the external surface of the antigen. As a result, small changes in the structure of the antigen due to genetic polymorphism, heterogeneity of glycosylation or slight denaturation will usually have little effect on polyclonal antibody binding. Similarly, a larger or smaller subset of antibodies from polyclonal serum will usually bind to antigens which have been modified or denatured. Another advantage of polyclonal antiserum lies in its capacity to form large insoluble complexes important in some types of immunoassay. However, polyclonal antisera have certain limitations for exploitation in immunoassays, the main one being their heterogeneity in specificity, even when reacting to small antigens and their variability between animals and batches. Without affinity purification specific antibodies in antisera represent 20-30% of the immunoglobulin in most cases. This may reduce their efficiency in some procedures and may lead to high background readings in others (Catty, D. 1988; Goding, J.W. 1983).

3.1.7 Monoclonal antibodies

For most research, diagnostic and therapeutic purposes, monoclonal antibodies, derived from a single clone, and specific for a single epitope are preferable. Monoclonal antibodies, first described by Köhler and Milstein in 1975, are prepared by fusing a normal, activated, antibody-producing B cell with a cancerous cell line. The resulting hybrid cell, or hybridoma, possesses the immortal growth properties of the cancerous cell line and secretes the antibody produced by the B cell. These hybridoma cells can be cultured indefinitely, the secreted antibody is homogeneous in specificity, affinity and isotype and each monoclonal product is specific to a single antigenic determinant of the immunogen. When prepared in vitro, monoclonal antibody is the exclusive or predominant protein in the culture medium and each antibody can be prepared in theoretically unlimited amounts (Kuby, J. 1992; Goding, J.W. 1983).

The specificity of monoclonal antibodies for a single epitope means that if for any reason this epitope is altered, the antibody may or may not continue to bind. Whether this is seen as a problem or an advantage will depend on circumstances. Their special value lies in their ability to select a unique feature of an antigen that may for example allow definition and separation of cell populations and analysis of bacterial antigens (Kuby, J. 1992; Goding, J.W. 1983).

3.1.8 Immunoassays

A number of techniques have been developed based on the antigen-antibody interaction. One of the first observations of antigen-antibody reactions was the precipitation reaction when antigen and antibody are mixed in similar proportions. This reaction formed the basis of techniques such as immunodiffusion and immunoelectrophoresis. Immunofluorescence assays are used extensively to detect autoantibodies and antibodies to tissue and cellular antigens. Furthermore immunofluorescent tests may be used to identify antigens on live cells using a fluorescence activated cell sorter (FACS). This technique permits the isolation of different cell populations with different surface antigens (stained with different fluorescent antibodies) (Radbruch, A. 1992).

Radioimmunoassay (RIA) and enzyme linked immunosorbent assay (ELISA) are exquisitely sensitive for detecting antigens and antibodies, and are extremely economical in the use of reagents. RIA and ELISA are probably the most widely used of all immunological assays since large numbers of tests can be performed in a relatively short time. RIA has a number of important disadvantages, the use of expensive equipment, the potential health hazards and the need for specialist disposal. For ELISA, several enzymes can be easily conjugated to antibodies (alkaline phosphatase, β-D-galactosidase and horse radish peroxidase) and substrates are cheap and easily available (Tijssen, P. 1985; Roitt, I., Brostoff, J. *et al.* 1989; Johnstone, A. and Thorpe, R. 1982; Kuby, J. 1992).

3.1.9 Use of immunological reagents in biosensors

Manual Threshold is a biodetector (Molecular Devices Ltd) used primarily by the pharmaceutical industry to screen products for contamination as part of the quality control procedure. The technique has been adapted for use in the detection of microorganisms.

Manual Threshold is a light addressable potentiometric biosensor system which can provide specific identification using labelled antibody reagents in approximately 5 minutes. A biotin-labelled capture antibody is linked to a BSA-biotin nitrocellulose membrane via streptavidin. The bacteria are then bound by the capture antibody and are detected via a second specific antibody labelled with fluorescein isothiocyanate (FITC) and an anti-fluorescein antibody conjugated to urease (Figure 7).

The membrane is then placed in a reader containing a urea substrate, urease releases ammonium ions from the urea which build up on the surface of the reader, the sensor measures the amount of current required to neutralise the potential difference created by these ions in μV/s. Detection factors are calculated by dividing the reading of an assay by the reading of the negative control. Detection factors of over 2 are considered to be positive.

3.1.10 Aims

One of the key aims of the project was to produce antibody reagents for the detection and identification of *B. mallei* and *B. pseudomallei* and to demonstrate their effectiveness and reliability. It is important that the antibodies do not cross react

with other species of bacteria which could cause false positives, an unreliable detection system and in some cases the administration of inappropriate medical countermeasures. The requirement is therefore, for antibody reagents which are specific and can be incorporated into sensitive detection assays. In the case of *B. mallei* and *B. pseudomallei* it is also desirable to produce reagents which can differentiate between the two species.

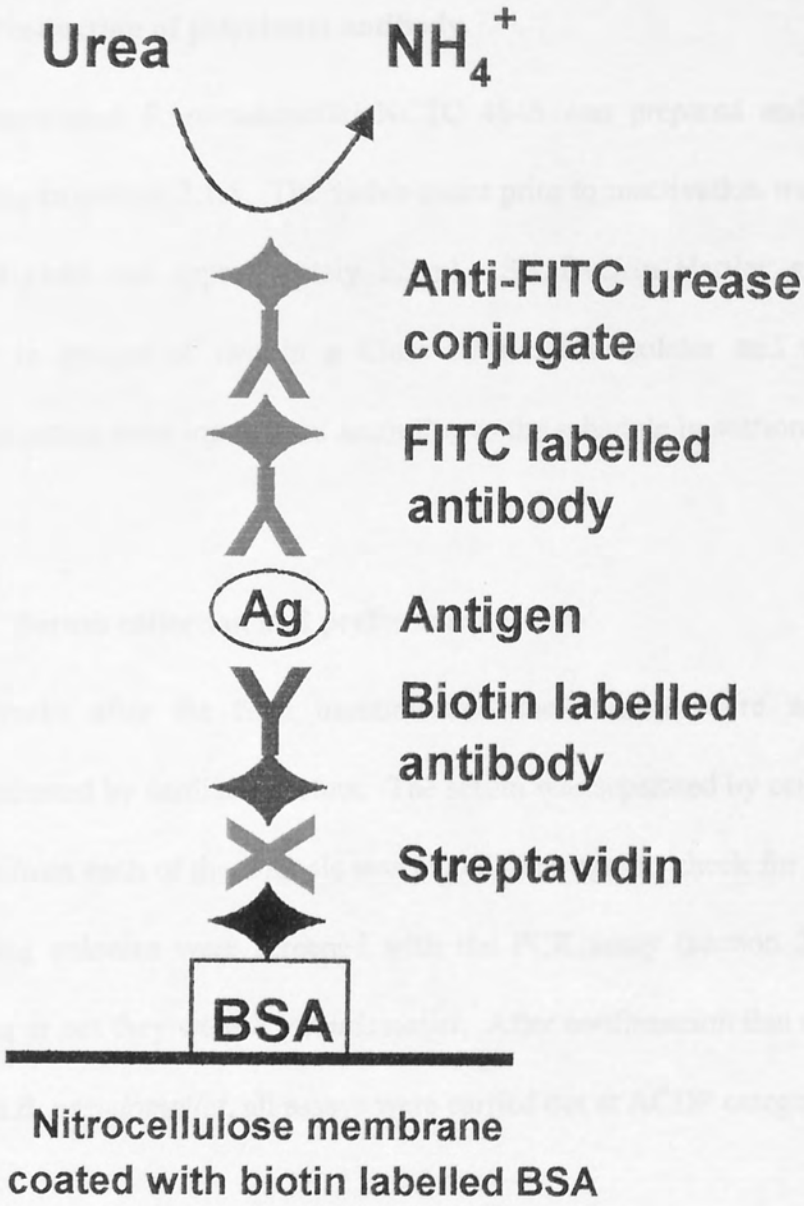


Figure 7. The manual threshold biosensor detection assay system.

3.2 Detection of *B. pseudomallei* and *B. mallei* using antibodies raised against heat killed whole cells

3.2.1 Production of polyclonal antibody

Heat inactivated *B. pseudomallei* NCTC 4845 was prepared and safety checked according to section 2.1.5. The viable count prior to inactivation was 1 x 10^{10} cfu/ml and the yield was approximately 120ml. Six Dunkin Hartley guinea pigs were housed in groups of two in a Class III flexible isolator and after one weeks acclimatisation were immunised according to the schedule in section 2.2.2.

3.2.1.1 Serum collection and preliminary assays

Two weeks after the final immunisation the animals were anaesthetised and exsanguinated by cardiac puncture. The serum was separated by centrifugation and a sample from each of the animals was plated onto agar to check for bacterial growth. Resulting colonies were screened with the PCR assay (section 2.1.9) to confirm whether or not they were *B. pseudomallei*. After confirmation that the serum did not contain *B. pseudomallei*, all assays were carried out at ACDP category II.

Initial experiments carried out to optimise the immobilisation of bacterial cells on the microtitre plate showed that coating the plate in antigen binding buffer (pH 9.6) was comparable with drying the organisms onto the plate and both these methods were better than coating in PBS. Using antigen binding buffer overnight at 4°C was simpler and easier than drying and was adopted as the coating method for all immobilised antigen ELISAs.

Serum samples from each of the individual animals (initially diluted 1:5,000 in blotto) showed good titres against immobilised *B. pseudomallei* with all samples showing positive recognition at a dilution of approximately 1:1,000,000. Normal guinea pig serum used as a control showed no activity against the immobilised bacteria (Figure 8). The six individual serum samples were pooled and polyclonal antibody purified by ammonium sulphate precipitation (section 2.2.3). In ELISA, antibody diluted to a final concentration of 40ng/ml showed positive recognition of immobilised *B. pseudomallei* NCTC 4845.

3.2.2 Biotin-labelling of polyclonal antibody and formulation of capture assay

Guinea pig polyclonal antibody was labelled with biotin (section 2.2.11) so that a polyclonal antibody capture assay could be formulated. The ability of the labelled antibody to recognise immobilised *B. pseudomallei* NCTC 4845 was determined to check that labelling had not affected antibody binding and that the biotin label had been conjugated to the antibody molecule. In ELISA, biotin labelled antibody diluted to a final concentration of 80ng/ml showed positive recognition of immobilised *B. pseudomallei* NCTC 4845.

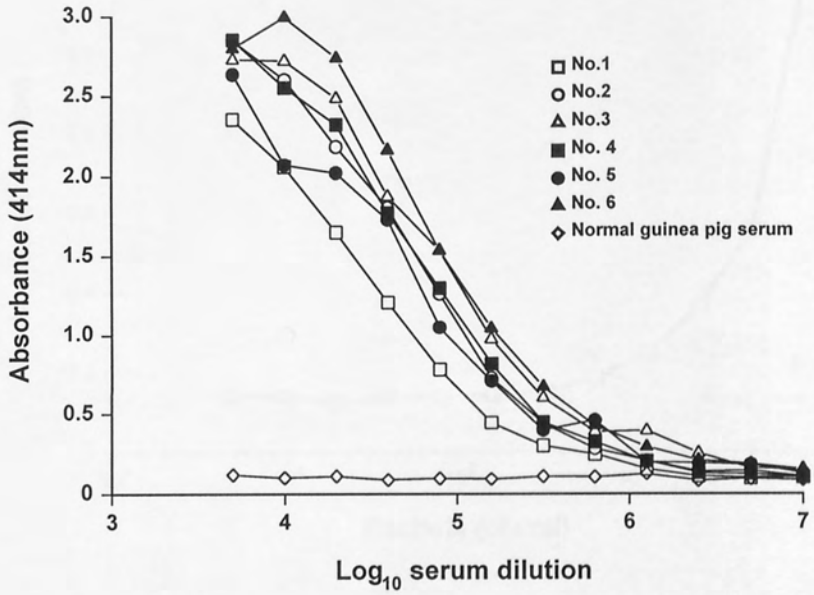


Figure 8. Titration of guinea pig polyclonal serum in ELISA using immobilised *B. pseudomallei* NCTC 4845

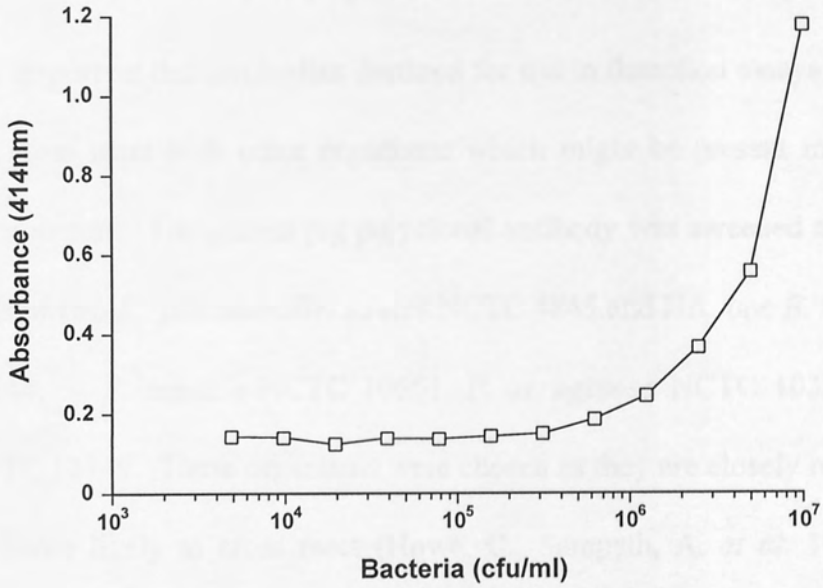


Figure 9. Capture assay using guinea pig polyclonal antibody as both capture and detection (biotin-labelled) antibodies. The lower limit of detection of *B. pseudomallei* is at approximately 10^6 cfu/ml.

An assay was formulated with 20μg/ml of purified polyclonal antibody as the capture antibody and 20μg/ml of biotin labelled polyclonal antibody as the detection antibody. The lower limit of detection of inactivated *B. pseudomallei* was at approximately 7 x 10^5 cfu/ml (Figure 9).

3.2.3 Cross reactivity of polyclonal antibody

It is important that antibodies destined for use in detection assays are specific and do not cross react with other organisms which might be present in samples or in the environment. The guinea pig polyclonal antibody was screened against immobilised cells of two *B. pseudomallei* strains NCTC 4845 and HA, one *B. mallei* strain ATCC 23344, *B. cepacia* NCTC 10661, *P. aeruginosa* NCTC 10332 and *B. pickettii* NCTC 11149. These organisms were chosen as they are closely related and therefore are more likely to cross react (Howe, C., Sampath, A. *et al.* 1971; Yabuuchi, E., Kosako, Y. *et al.* 1992; Mohandas, S., Puthucheary, S.D. *et al.* 1994).

In ELISAs the polyclonal serum reacted well with *B. pseudomallei* 4845 and *B. mallei* 23344, but less well with *B. pseudomallei* HA (Figure 10). The polyclonal serum also reacted well with *B. pickettii* and weakly with *B. cepacia* and with *P. aeruginosa*. The importance of specificity in future detection assays meant that the polyclonal antibody was likely to be of limited use.

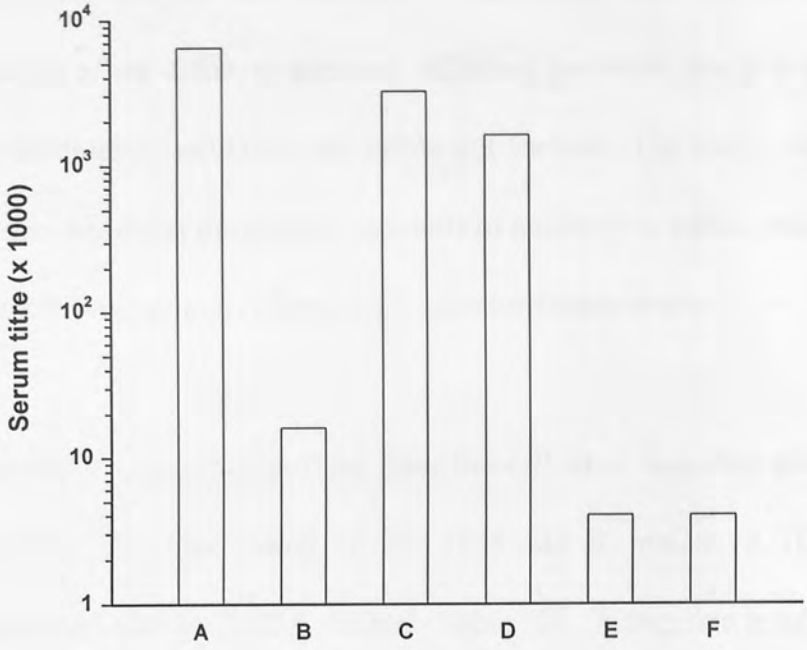


Figure 10. Cross reactivity of guinea pig polyclonal serum with different species of bacteria. A: *B. pseudomallei* NCTC 4845, B: *B. pseudomallei* HA, C: *B. mallei* ATCC 23344, D: *B. pickettii* NCTC 11149, E: *P. aeruginosa* NCTC 10332 and F: *B. cepacia* NCTC 10661.

3.2.4 Production of monoclonal antibodies

Mice were immunised with heat inactivated *B. pseudomallei* NCTC 4845 and monoclonal antibodies produced according to the methods in section 2.2.4 and 2.2.5. After screening and cloning 19 hybridoma cell lines were identified and isotyped. Tissue culture supernatant fluid from each of the 19 cell lines was used as the detection antibody in a capture assays using *B. pseudomallei* NCTC 4845 as the antigen. The titre of the monoclonal antibody was defined as the highest dilution of tissue culture supernatant which showed positive recognition of *B. pseudomallei* (ie. an absorbance 0.1units above the corresponding negative control). If all the culture

supernatants had the same antibody concentration then this assay could give some indication of the different antibody affinities, however, this is not the case and high concentrations of antibody will dilute out furthest. The assay will show which cell lines are producing the greatest amounts of antibody in culture and allow selection of a small number of monoclonal antibodies for further study.

Tissue culture supernatant fluid from the cell lines was also screened against both inactivated *B. pseudomallei* NCTC 4845 and *B. mallei* NCTC 10247 using the immobilised antigen ELISA method (Table 12). A negative result was defined as an absorbance reading of less than the corresponding negative control plus 0.1units. A positive result was defined as an absorbance reading greater than the corresponding negative control plus 0.2units. Any reading between the two was defined as borderline (±).

Antibody	Isotype	Titre in capture *	Detection of *B. pseudomallei* †	Detection of *B. mallei* †
3VIE5	IgG_{2b}	1:1024	+	+
4IG11	IgM	1:128	+	+
4IIIB12	Mixed	1:16	+	+
4IIIH6	IgG_1	1:256	+	+
4IIIA11	IgG_1	1:1024	+	+
4IIIE4	IgG_3	nd	-	-
4IIIF11	IgG_3	1:1024	+	+
4IIIH2	IgM	1:64	+	+
4IVA5	IgM	1:4	+	+
4IVH2	IgG_{2a}	1:256	+	+
4IVE2	IgM	1:128	+	+
4VF5	IgG_{2a}	1:64	+	+
4VA5	IgG_1	1:1024	+	+
4VD3	IgM	1:1024	+	+
4VE11	IgM	1:64	+	+
4VA4	IgG_1	1:1024	+	+
4VH7	Mixed	1:128	+	+
4VIH12	IgG_{2b}	1:1024	+	+
4VIE7	IgG_1	1:256	+	+

* The titre of the monoclonal antibody was defined as the highest dilution of tissue culture supernatant which showed positive recognition of *B. pseudomallei* NCTC 4845 (ie. an absorbance 0.1units above the corresponding negative control). nd indicates the titre was not determined.

†A negative result was defined as an absorbance reading of less than the corresponding negative control plus 0.1units. A positive result was defined as an absorbance reading greater than the corresponding negative control plus 0.2units.

Table 12. Isotypes of the nineteen monoclonal antibodies. Titres in capture assays using inactivated *B. pseudomallei* NCTC 4845 and recognition of immobilised *B. pseudomallei* NCTC 4845 and *B. mallei* ATCC 23344.

All of the antibodies recognised both *B. pseudomallei* NCTC 4845 and *B. mallei* NCTC 10247 except 4IIIE4. This lack of recognition may be due to low levels of antibody in the culture supernatant fluid used for the ELISA, rather than non-recognition of the organism as this antibody was positive throughout the fusion screening process. Six antibodies (3VIE5, 4IIIA11, 4IIIF11, 4VA4, 4VA5 and 4VIH12) were selected for further study as they gave high titres (1:1024) when used in the capture assay. 4IIIF11 was later abandoned as the hybridoma cells stopped secreting antibody in culture. 4VD3 was not selected despite having a good titre in capture (1:1024) because the antibody had an IgM isotype and therefore would be difficult to purify and likely to be a low affinity antibody (Kenney, J.S., Hughes, B.W. *et al.* 1989; Liddell, J.E. and Cryer, A. 1991).

3.2.5 Production of monoclonal antibody in miniPERM bioreactor

The five monoclonal hybridoma cell lines selected (3VIE5, 4IIIA11, 4VA4, 4VA5 and 4VIH12) were all cultured and introduced into the miniPERM bioreactor. All 5 cell lines were successfully maintained in the bioreactor for between 4 and 8 weeks and culture supernatant showed high titres of antibody in all cases. Monoclonal antibody was purified from all 5 cell lines with yields ranging from 15 to 50mg of antibody.

3.2.6 Biotin-labelling of monoclonal antibodies

Biotin-labelling was carried out over a range of MCRs to determine the optimum level of labelling. Table 13 shows the number of biotin molecules incorporated per IgG molecule for different MCRs and different antibodies.

The three 4VIH12-biotin conjugates were screened by ELISA to see if biotin labelling had affected the binding of the antibody to immobilised *B. pseudomallei* NCTC 4845. Each antibody was screened using an anti-mouse HRPO conjugate and the titres compared (Figure 11). It was found that more than 3 biotin molecules per IgG molecule began to affect antibody binding when compared with the unlabelled antibody. However 3-4 biotin molecules per IgG molecule was found to give the best performance in ELISA, less than 3 biotin molecules per IgG molecule gave low absorbances and therefore lower detection limits.

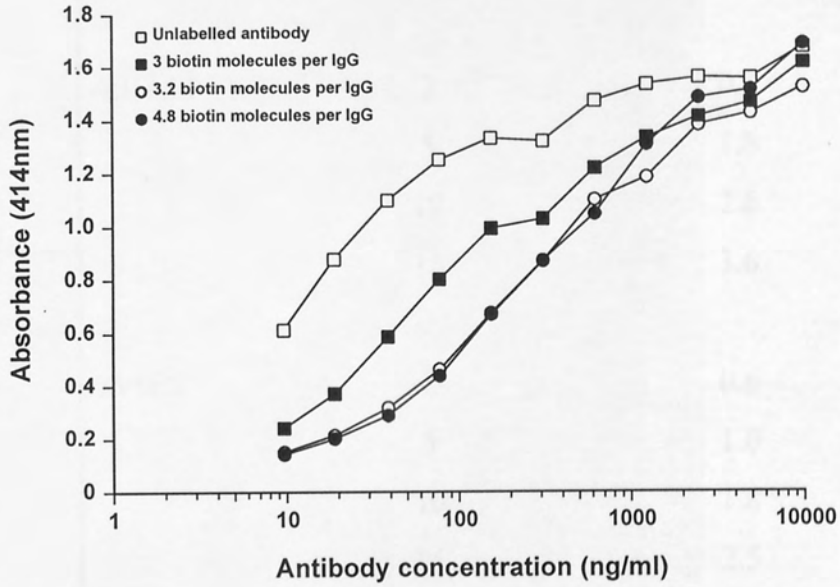


Figure 11. The effect of biotin labelling on the binding of 4VIH12 to immobilised *B. pseudomallei* NCTC 4845.

Antibody	MCR used	No. of biotin molecules per IgG molecule
4VIH12	10	1.7
	15	3.0
	20	3.2
	40	4.8
4IIIA11	2	0.7
	5	1.6
	10	2.6
	15	3.6
3VIE5	2	0.6
	5	1.0
	10	1.8
	15	2.5

Table 13. Labelling of antibodies with biotin at different molar coupling ratios (MCR).

3.2.7 Labelling antibodies with fluorescein isothiocyanate

Two antibodies (3VIE5 and 4VIH12) were labelled with FITC so a number of antibody combinations could be tested on the Manual threshold biosensor. Manual threshold assays require the detection antibodies to be labelled with approximately

six FITC molecules per IgG molecule so an MCR of 30 was used (as recommended by the manufacturer). The results were that 4VIH12 was labelled with 6 FITC molecules per IgG molecule and 3VIE5 was labelled with 8 FITC molecules per IgG molecule.

3.2.8 Formulation of capture assays

A number of monoclonal antibody combinations were used in ELISA to find a capture assay configuration (Figure 12). Monoclonal antibody was used to coat the ELISA plate, acting as the capture antibody. After blocking, a dilution series of inactivated *B. pseudomallei* NCTC 4845 was applied and allowed to bind. After further washing, biotin labelled monoclonal antibodies (4VIH12, 4IIIA11 and 3VIE5) were used as detection antibodies followed by incubation with streptavidin labelled with horse radish peroxidase (SA-HRPO). After a final washing step, the enzyme substrate ABTS was applied and the colour allowed to develop. Lower limits of detection were defined as the lowest number of organisms which gave an absorbance reading greater than the corresponding negative control plus three standard deviations. The lower limit of detection obtained with different antibody combinations are given in Table 14.

Optimisation of the assay was carried out by using different concentrations of both capture and detection antibody. A new product, streptavidin-horse radish peroxidase polymer (SA-HRPO polymer), made up of a number of peroxidase molecules conjugated to a streptavidin molecule was also found to increase sensitivity when compared with the standard streptavidin-horse radish peroxidase conjugate. The

optimised capture assay, using 4IIIA11 as the capture antibody (5μg/ml) and biotin labelled 4VIH12 (4.4 biotin molecules per IgG molecule, 1μg/ml) was found to have a lower limit of detection of approximately 1-5 x 10^4 cfu/ml depending on the negative controls for each assay (Figure 13).

Capture assays were carried out using a number of *B. mallei* and *B. pseudomallei* strains to determine lower limits of detection for these strains (Table 15). The detection limits were found to be between 1 x 10^4 and 5 x 10^4 cfu/ml. Some variation may be due to the accuracy of viable counts carried out during the preparation of inactivated bacteria. However, the detection limits for the *B. mallei* and *B. pseudomallei* strains tested were similar.

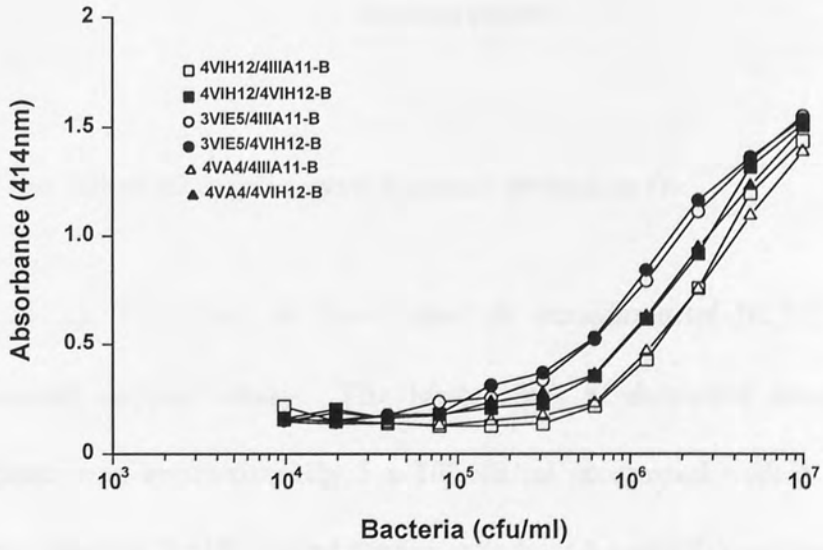
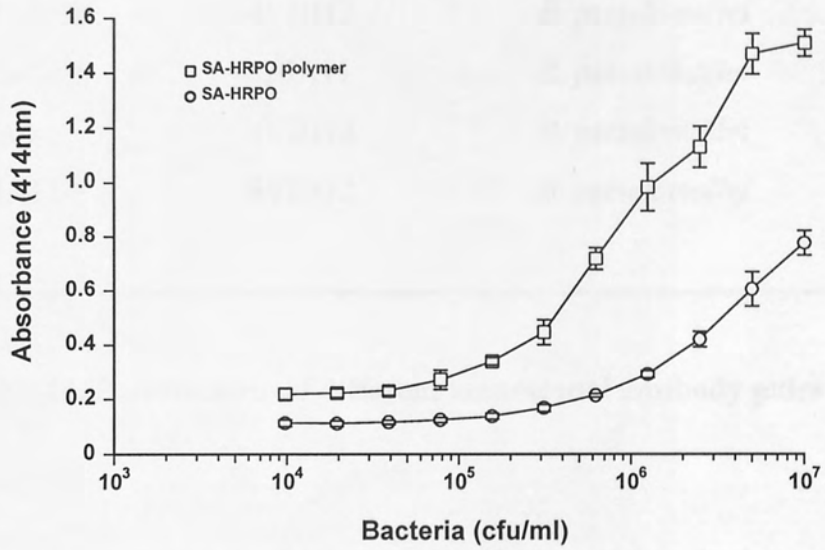


Figure 12. Capture ELISA for *B. pseudomallei.* Capture antibodies are listed first and biotin-labelled detection antibodies are denoted by -B.



Error bars indicate the negative control ± standard deviation (n-1)

Figure 13. Detection of inactivated *B. pseudomallei* NCTC 4845 using the optimised capture assay. The lower limit of detection using the SA-HRPO polymer was approximately 5 x 10^4 cfu/ml compared with a detection limit of approximately 7 x10^5 cfu/ml for the standard SA-HRPO conjugate.

Capture Ab	Detection Ab	Organism	Detection limit
4VIH12	4IIIA11	*B. pseudomallei*	2-7 x 10^5 cfu/ml
4VIH12	4IIIA11	*B. mallei*	2 x 10^5 cfu/ml
4VIH12	4VIH12	*B. pseudomallei*	2 x 10^5 cfu/ml
3VIE5	4IIIA11	*B. pseudomallei*	2 x 10^5 cfu/ml
3VIE5	4VIH12	*B. pseudomallei*	2 x 10^5 cfu/ml
4VA4	4IIIA11	*B. pseudomallei*	7 x 10^5 cfu/ml
4VA4	4VIH12	*B. pseudomallei*	3 x 10^5 cfu/ml
4IIIA11	4VIH12	*B. pseudomallei*	1 x 10^5 cfu/ml

Table 14. Performance of different monoclonal antibody pairs in capture assays.

The most sensitive combination of antibodies giving the lowest limit of detection was 4IIIA11 as the capture antibody and biotin-labelled 4VIH12 as the detection antibody which gave a lower limit of detection for inactivated *B. pseudomallei* NCTC 4845 of approximately 1 x 10^5 cfu/ml.

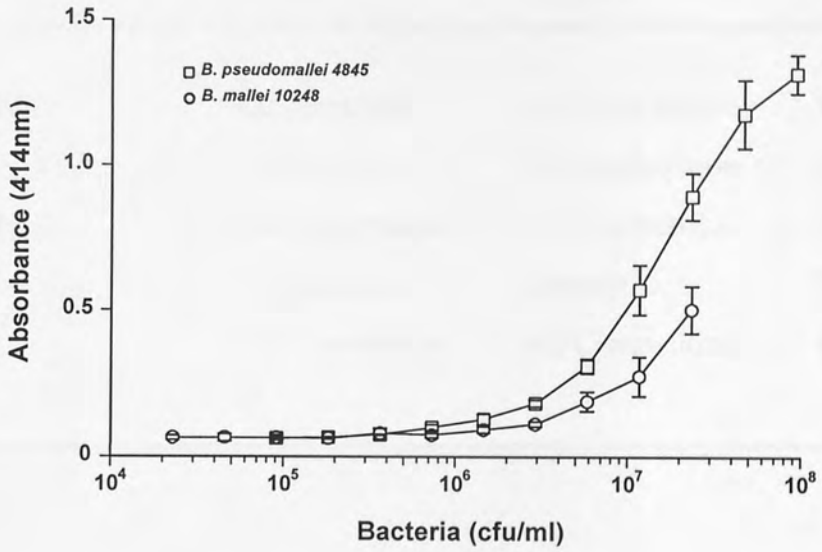
Organism	Detection Limit
B. pseudomallei NCTC 4845	5 x 10^4 cfu/ml
B. pseudomallei E38	1 x 10^4 cfu/ml
B. pseudomallei Mal 6	2 x 10^5 cfu/ml
B. pseudomallei Human Australia	less than 1 x 10^4 cfu/ml
B. pseudomallei 551a	1 x 10^4 cfu/ml
B. mallei ATCC 23344	5 x 10^4 cfu/ml
B. mallei NCTC 10247	5 x 10^4 cfu/ml
B. mallei NCTC 10248	1 x 10^4 cfu/ml

Table 15. Lower limits of detection for different strains of *B. mallei* and *B. pseudomallei* using the optimised capture assay.

3.2.9 Detection of live *B. pseudomallei* and live *B. mallei*

A suspension of live, washed cells of *B. pseudomallei* NCTC 4845, *B. mallei* NCTC 10247 and 10248 at an approximate concentration of 1 x 10^8cfu/ml were used to coat microtitre plates. The polyclonal antibody and two monoclonal antibodies (4IIIA11 and 4VIH12) were tested to ensure that these antibodies detected the live bacteria as well as the inactivated material. At an antibody concentration of 40ng/ml, both monoclonal antibodies showed positive detection of all three organisms. The polyclonal antibody, however, showed poor recognition of the three organisms.

A capture assay was then carried out with 4VIH12 as the capture antibody and biotinylated 4IIIA11 as the detection antibody with both *B. pseudomallei* NCTC 4845 and *B. mallei* NCTC 10248. The lower limits of detection of *B. pseudomallei* NCTC 4845 and *B. mallei* NCTC 10248 using this assay were 7 x 10^5 cfu/ml and 2 x 10^6 cfu/ml respectively (Figure 14).



Error bars indicate the negative control ± standard deviation (n-1)

Figure 14. Detection of live *B. mallei* and *B. pseudomallei.*

3.2.10 Competition assays

The three biotin-labelled monoclonal antibodies (4VIH12, 4IIIA11 and 3VIE5) were used in competition assays to determine whether the monoclonal antibodies shared the same binding sites on the bacterial cell. Both unlabelled and biotin labelled antibodies were titrated against immobilised *B. pseudomallei* NCTC 4845 to determine what concentrations to use. Unlabelled antibody was then mixed with biotin-labelled antibody and applied to the immobilised bacteria. A decrease in

absorbance (a minimum of 0.2units compared with the control using biotin labelled antibody only) at high concentrations of unlabelled antibody indicated that there was some inhibition to the binding of the biotin-labelled antibody, the results of these competition assays are given in Table 16.

	Biotin-labelled 3VIE5	**Biotin-labelled 4IIIA11**	**Biotin-labelled 4VIH12**
3VIE5	Competition	No Competition	Competition
4IIIA11	Competition	No Competition	Competition
4VIH12	No Competition	No Competition	No Competition
4VA4	Competition	Competition	Competition
4VA5	No Competition	No Competition	No Competition

Table 16. Results of competition assays with different pairs of antibodies.

Surprisingly 4IIIA11 was found to compete with biotin labelled 4VIH12. This antibody combination was found to be the best for use in capture assays, and gave the lowest limit of detection for inactivated *B. pseudomallei* NCTC 4845. It is possible that these antibodies only compete in the immobilised antigen format used in these competition assays but in the capture assays when antigen is captured by antibody there is no competition between these antibodies. The fact that 4VIH12 does not compete with biotin-labelled 4IIIA11 may support the argument that this is due to assay format.

3.2.11 Detection of different strains of *B. pseudomallei* and *B. mallei* using monoclonal antibodies

The five monoclonal antibodies (4IIIA11, 4VIH12, 4VA4, 4VA5 and 3VIE5) were screened against a panel of *B. pseudomallei* and *B. mallei* strains as it was important to determine the utility of these antibodies for the detection of different strains of each pathogen. Assays were carried out according to section 2.2.15 with different strains being immobilised on the plate and the antibodies diluted down. The results of these assays are summarised in Tables 17 and 18.

The five monoclonal antibodies (4VIH12, 4IIIA11, 4VA4, 4VA5 and 3VIE5) were tested for binding to ten *B. mallei* strains. Two strains 3709 and 10245 were not detected by any of the antibodies. Eight strains were detected by three of the five antibodies (4VA4, 4IIIA11 and 3VIE5), seven strains by 4VIH12 and six strains by 4VA5. Twenty two strains of *B. pseudomallei* strains were screened and of those nineteen were detected by all of the monoclonal antibodies. E8, an environmental strain was detected by four of the five antibodies but the ± symbol indicates that the result was only slightly above the negative control. E27 and E82, also environmental strains were not detected by any of the monoclonal antibodies.

Bacterial Strain	4VIH12	4VA4	4VA5	4IIIA11	3VIE5
B. mallei NCTC 10247	+	+	+	+	+
B. mallei NCTC 10248	+	+	+	+	+
B. mallei NCTC 10260	±	+	±	+	±
B. mallei NCTC 10229	±	±	±	+	±
B. mallei NCTC 120	±	±	-	+	±
B. mallei NCTC 3708	-	±	-	±	±
B. mallei NCTC 3709	-	-	-	-	-
B. mallei ATCC 23344	+	+	+	±	+
B. mallei NCTC 10245	-	-	-	-	-
B. mallei NCTC 10230	±	±	±	+	±

A negative result was defined as an absorbance reading of less than the corresponding negative control plus 0.1units. A positive result was defined as an absorbance reading greater than the corresponding negative control plus 0.2units. Any reading between the two was defined as borderline (±).

Table 17. The detection of *B. mallei* strains by five monoclonal antibodies.

Bacterial Strain	4VIH12	4VA4	4VA5	4IIIA11	3VIE5
B. pseudomallei NCTC 4845	+	+	+	+	+
B. pseudomallei USAMRU-1	+	+	+	+	+
B. pseudomallei UBOL-2	+	+	+	+	+
B. pseudomallei 4889	+	+	+	+	+
B. pseudomallei USAMRU 21	+	+	+	+	+
B. pseudomallei SEARLE	+	+	+	+	+
B. pseudomallei 123	+	+	+	+	+
B. pseudomallei O	+	+	+	+	+
B. pseudomallei PA	+	+	+	+	+
B. pseudomallei BRI	+	+	+	+	+
B. pseudomallei E8	±	±	-	±	±
B. pseudomallei E25	+	+	+	+	+
B. pseudomallei E27	-	-	-	-	-
B. pseudomallei E82	-	-	-	-	-
B. pseudomallei 204	+	+	+	+	+
B. pseudomallei 576	+	+	+	+	+
B. pseudomallei 551a	+	+	+	+	+
B. pseudomallei 603a	+	+	+	+	+
B. pseudomallei E38	+	+	+	+	+
B. pseudomallei Mal 6	+	+	+	+	+
B. pseudomallei ATCC 23343	+	+	+	+	+
B. pseudomallei HA	+	+	+	+	+

A negative result was defined as an absorbance reading of less than the corresponding negative control plus 0.1units. A positive result was defined as an absorbance reading greater than the corresponding negative control plus 0.2units. Any reading between the two was defined as borderline (±).

Table 18. The detection of *B. pseudomallei* strains by five monoclonal antibodies.

3.2.12 Cross reactivity assays

Monoclonal antibodies (4VIH12, 4IIIA11, 4VA4, 4VA5 and 3VIE5) were tested for their ability to detect immobilised bacteria from other closely related species. *B. cepacia* strains, *B. pickettii* NCTC 11149 and *P. aeruginosa* NCTC 10332 were selected as these organisms are thought to be the nearest relatives to *B. mallei* and *B. pseudomallei* and if the antibodies are cross reactive it may well be towards these organisms (Yabuuchi, E., Kosako, Y. *et al.* 1992; Howe, C., Sampath, A. *et al.* 1971; Mohandas, S., Puthucheary, S.D. *et al.* 1994). Table 19 shows the results of these screening assays.

Organism	3VIE5	4IIIA11	4VA4	4VIH12	Guinea pig Polyclonal antibody
B. cepacia NCTC 10744	-	-	-	-	+
B. cepacia NCTC 10743	-	-	-	-	+
B. cepacia NCTC 10734	-	-	-	-	±
B. cepacia NCTC 10661	-	-	-	-	+
B. pickettii NCTC 11149	-	-	-	-	+
P. aeruginosa NCTC 10332	-	-	-	-	+

A negative result was defined as an absorbance reading of less than the corresponding negative control plus 0.1units. A positive result was defined as an absorbance reading greater than the corresponding negative control plus 0.2units. Any reading between the two was defined as borderline (±).

Table 19. Cross reactivity of monoclonal and polyclonal antibodies with closely related organisms.

As seen in Figure 10, the guinea pig polyclonal antibody shows some cross reactivity detecting all three closely related species. The five monoclonal antibodies however, did not detect these other organisms.

Capture assays using 4IIIA11 as the capture antibody and biotin labelled 4VIH12 as the detection antibody were carried out including three bacterial species as background contaminants. When the *B. pseudomallei* was diluted down the plate, the diluent contained 1 x 10^7 cfu/ml of either *B. pickettii* NCTC 11149, *B. cepacia* NCTC 10661 or *P. aeruginosa* NCTC 10332. As can be seen in Figure 15, the presence of these organisms in the sample had no effect on detection levels and the monoclonal antibodies were able to discriminate between these organisms and *B. pseudomallei.*

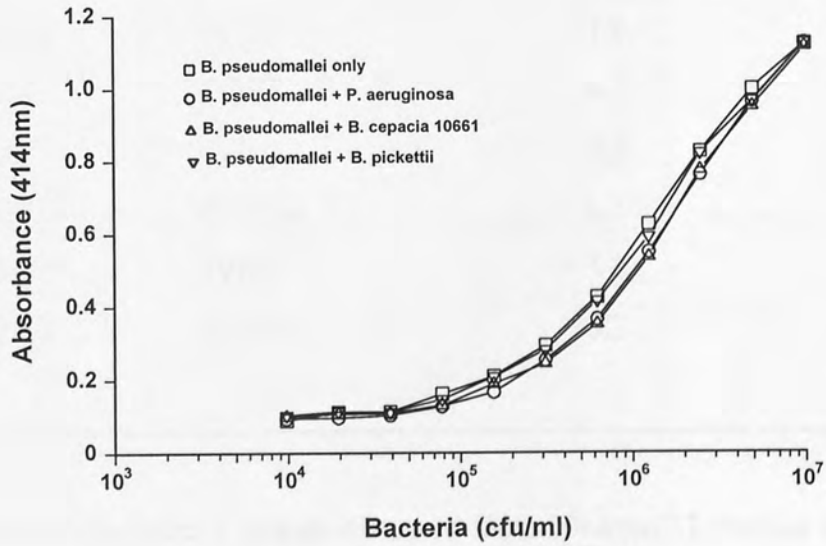


Figure 15. The performance of the capture assay in the presence of three contaminating organisms.

3.2.13 Use of monoclonal antibodies in the Manual Threshold Biosensor

Several combinations of antibodies were tested in the manual threshold biosensor to assess whether they were suitable for use in this type of detector. Biotin-labelled

antibody is used as the capture and FITC-labelled antibody as the detection antibody. The results are given as detection factors, which are calculated by dividing the result of an assay in μV/s by the result of the corresponding negative control. Detection factors of greater than 2 are positive. Table 20 shows the results of using different antibody combinations to detect both *B. pseudomallei* and *B. mallei* in the Manual Threshold biosensor.

Biotin-labelled Antibody	**FITC-labelled Antibody**	**Detection factor at 10^5 *B. mallei***	**Detection factor at 10^5 *B. pseudomallei***
4IIIA11	3VIE5	7.9	3.9
3VIE5	3VIE5	4.1	2.4
4IIIA11	4VIH12	5.5	2.6
3VIE5	4VIH12	6.3	3.2
4VIH12	3VIE5	5.8	3.1
4VIH12	4VIH12	5.7	3.9

Table 20. Summary of data obtained from Manual Threshold Biosensor assays.

All monoclonal antibody combinations showed a positive detection factor of over two showing that all the monoclonal antibody combinations tested detect both *B. pseudomallei* NCTC 4845 and *B. mallei* ATCC 23344 at a concentration of 1 x 10^5 cfu/ml. Some of the detection factors are higher than two and this may indicate the potential to detect bacterial concentrations lower than 1 x 10^5 cfu/ml. The monoclonal antibodies appeared to be suitable for use in this type of biosensor and with optimisation of the assay conditions and antibody concentrations there is the potential to detect both *B. mallei* and *B. pseudomallei* at concentrations below 1 x 10^5 cfu/ml.

3.2.14 Determination of antibody binding sites

To investigate the possible identity of the antigen recognised by the five monoclonal antibodies (4VIH12, 4IIIA11, 4VA4, 4VA5 and 3VIE5), *B. pseudomallei* NCTC 4845 was treated with SDS sample buffer before gel electrophoresis (1mg wet weight bacteria in 30µl SDS sample buffer). Figure 16 shows the appearance of the a 12.5% gel stained with the BioRad silver stain which is optimised to stain polysaccharide. Western blotting carried out with the monoclonal antibodies showed that all five bound to high molecular weight material found in the same region of the gel. Western blotting carried out with a non-specific anti-botulinum toxin antibody also suggested that this binding was specific to the five (4VIH12, 4IIIA11, 4VA4, 4VA5 and 3VIE5) monoclonal antibodies (Figure 17). The high molecular weight substance to which the antibodies bound was not made up of protein as it did not stain with Coomassie Blue and remained after a sample was treated with proteinase-K. A second proteinase-K preparation was carried out and this time the gel was

stained with a method optimised for staining lipopolysaccharide (LPS). As can be seen from Figure 18, the LPS is very different to the band in the first gel, and therefore the monoclonal antibodies are not binding the LPS. It appears that the monoclonal antibodies are binding to a high molecular weight polysaccharide material which may be the exopolysaccharide or capsule of *B. pseudomallei*.

Western blotting carried out with the guinea pig polyclonal antibody showed recognition of a large number of bands. However, it would appear that the polyclonal antibody may be recognising the LPS, the high molecular weight polysaccharide material and other bands in the bacterial cell preparation. This would not be surprising given the variety of epitopes recognised by polyclonal antibodies.

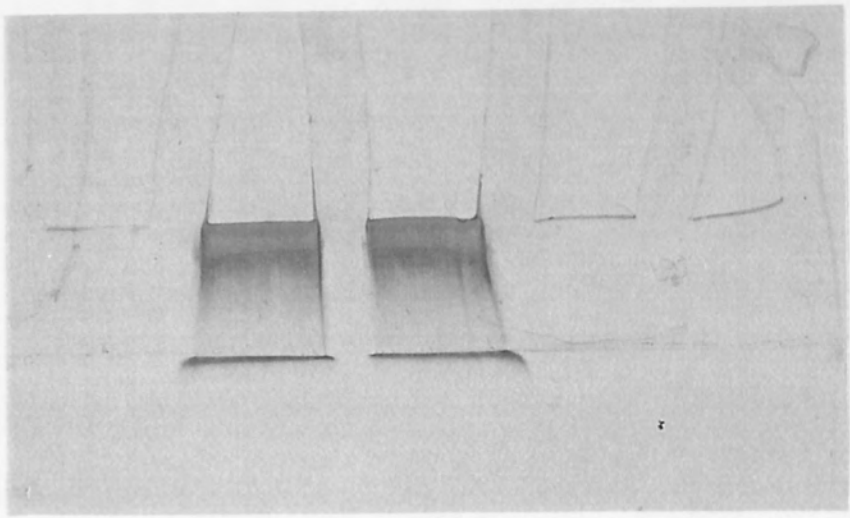


Figure 16. 12.5% SDS polyacrylamide gel of *B. pseudomallei* NCTC 4845 cells stained with BioRad polysaccharide silver stain. Lanes 1 & 2 are duplicates.

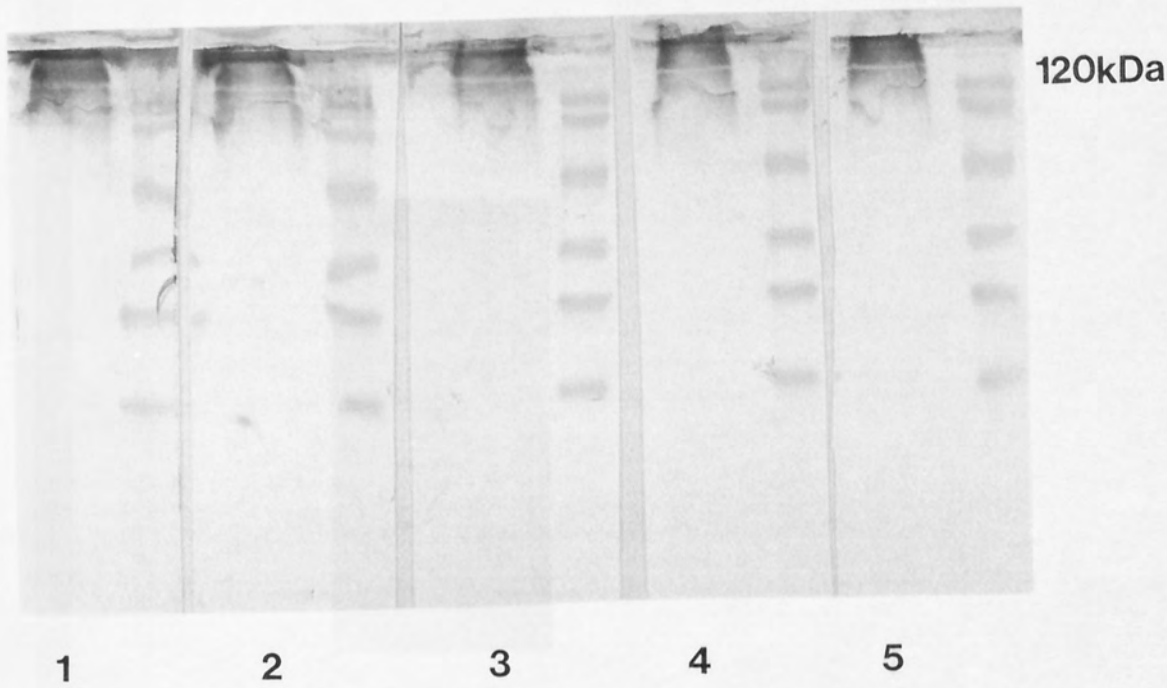


Figure 17. Western blot of *B. pseudomallei* NCTC 4845 cells with five monoclonal antibodies. All five antibodies recognise high molecular weight material.

Lane	Sample
1	4IIIA11
2	4VA4
3	4VA5
4	4VIH12
5	3VIE5

Figure 18. 12.5% SDS-PAGE gel of the LPS of *B. pseudomallei* NCTC 4845 (see chapter four). Lanes 1 and 2 are duplicates.

3.2.15 Extraction of exopolysaccharide

The extraction was carried out according to the method of Steinmetz given in section 2.3.6. In the first extraction the bacterial cells were grown on normal nutrient agar plates. Electrophoresis followed by polysaccharide silver staining (BioRad) of the crude extract gave surprising results with a single band of low molecular weight, approximately 30kDa which was not detected in a blot by the monoclonal antibody 3VIE5.

A second extraction was carried out using bacterial cells grown on MacConkey agar, which is thought to promote exopolysaccharide production. Electrophoresis of the crude product gave three relatively pure bands at approximately 14-19kDa, 31-36kDa and 55kDa (Figure 19). Silver staining showed the presence of some LPS also in the sample.

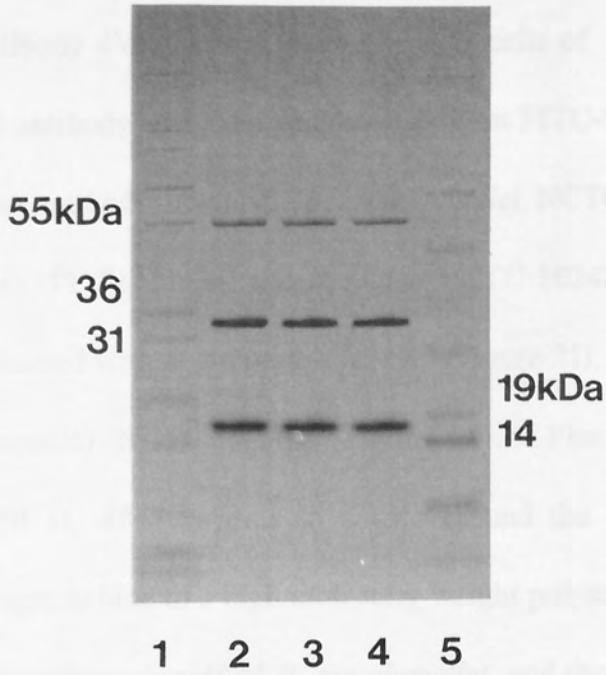


Figure 19. Analysis of exopolysaccharide extraction material. SDS-PAGE gel showing the material stained with Coomassie blue after exopolysaccharide extraction. The proteins visualised had apparent molecular weights of 14-19kDa, 31-36kDa and 55kDa.

Lane	Sample
1	Molecular weight markers
2	Exopolysaccharide preparation
3	Exopolysaccharide preparation
4	Exopolysaccharide preparation
5	Molecular weight markers

3.2.16 Immunofluorescent staining

To investigate whether the monoclonal antibodies recognised a surface antigen, the monoclonal antibody 4VIH12 was incubated with cells of *B. pseudomallei* and *B. mallei*. Bound antibody was then detected using an FITC-labelled anti-mouse IgG conjugate. The antibody bound to *B. pseudomallei* NCTC 4845 (Figure 20), *B. mallei* ATCC 23344 (Figure 24) and *B. mallei* NCTC 10248 (Figure 22). Negative results were obtained with *B. pseudomallei* E82 (Figure 21), *B. mallei* 10260 (Figure 23) and *B. cepacia* NCTC 10661 (Figure 25). Fluorescent staining of *B. pseudomallei* NCTC 4845 appears in a halo around the outside of the bacteria. 4VIH12 is thought to bind to a high molecular weight polysaccharide material which could be the exopolysaccharide of *B. pseudomallei*, and the results would appear to support this.

These results correlate with those obtained in ELISA (Tables 17 & 18). Positive binding was found with *B. pseudomallei* NCTC 4845, *B. mallei* NCTC 10248 and *B. mallei* ATCC 23344. No binding was found with *B. pseudomallei* E82 and *B. cepacia* strains and the binding to *B. mallei* NCTC 10260 was borderline.

3.2.17 Effect of sodium periodate treatment

To investigate whether the antigens recognised by the monoclonal antibodies are carbohydrate based, cells of *B. pseudomallei* NCTC 4845 were treated with sodium periodate. Samples of untreated *B. pseudomallei* NCTC 4845 cells were compared with sodium periodate treated cells by SDS-PAGE and western blotting. Western

blotting was carried out using the monoclonal antibody 4VIH12 and an anti-mouse horse radish peroxidase conjugate. A clear high molecular weight band was seen in the untreated lane compared with no band after sodium periodate treatment (Figure 26).

Periodate treatment oxidises carbohydrate residues present on bacterial cells (Vel, W.A.C., Namavar, F. *et al.* 1986). The inhibition of antibody binding after periodate treatment indicates that carbohydrate residues are important in the binding site of the antibody 4VIH12. These results coupled with the immunofluorescent microscopy would suggest that the antibody is binding to high molecular weight polysaccharide material which could be the capsule or exopolysaccharide of *B. pseudomallei* and *B. mallei*.

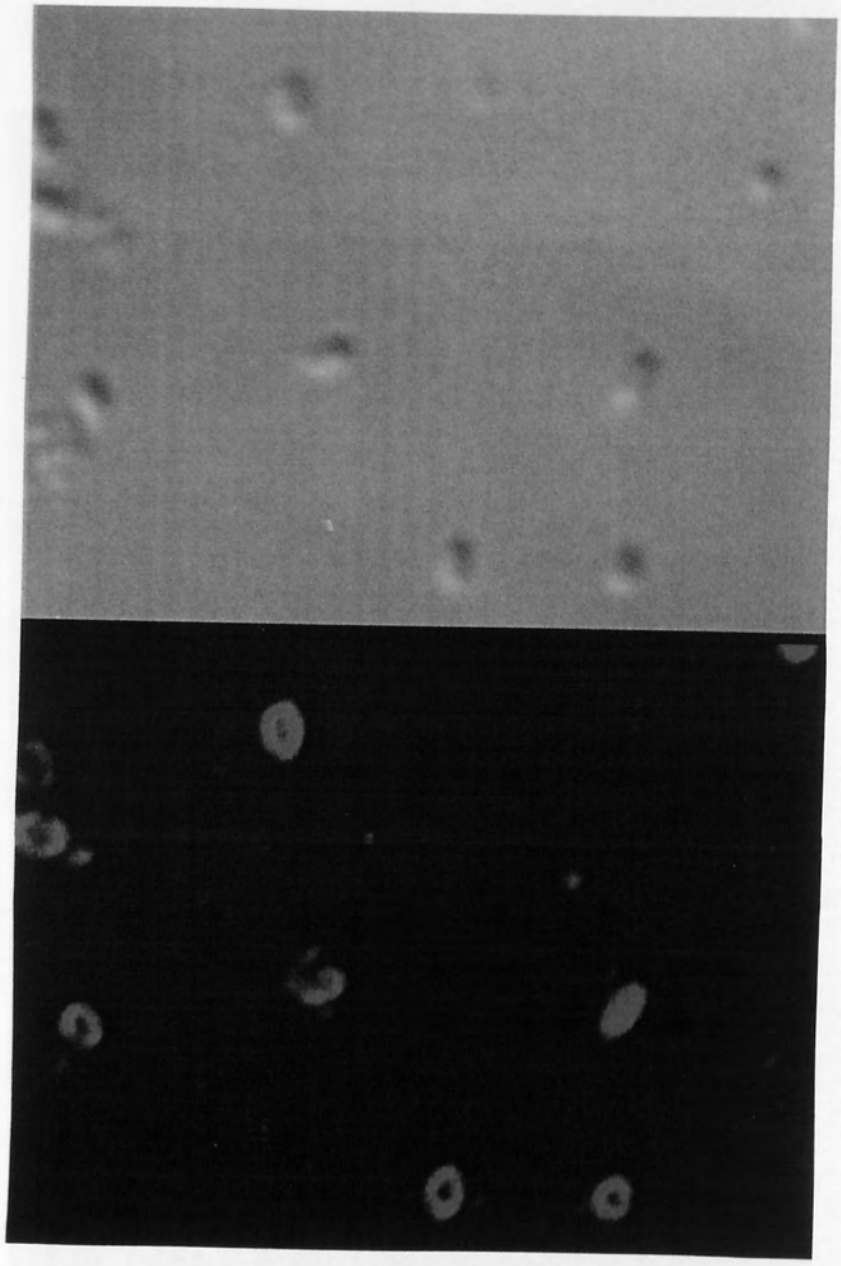


Figure 20. Immunofluorescent staining of *B. pseudomallei* NCTC 4845 with monoclonal antibody 4VIH12 (magnification x300).

The lower half of the picture shows the image when viewed under fluorescent light and the upper half shows the same image under phase contrast.

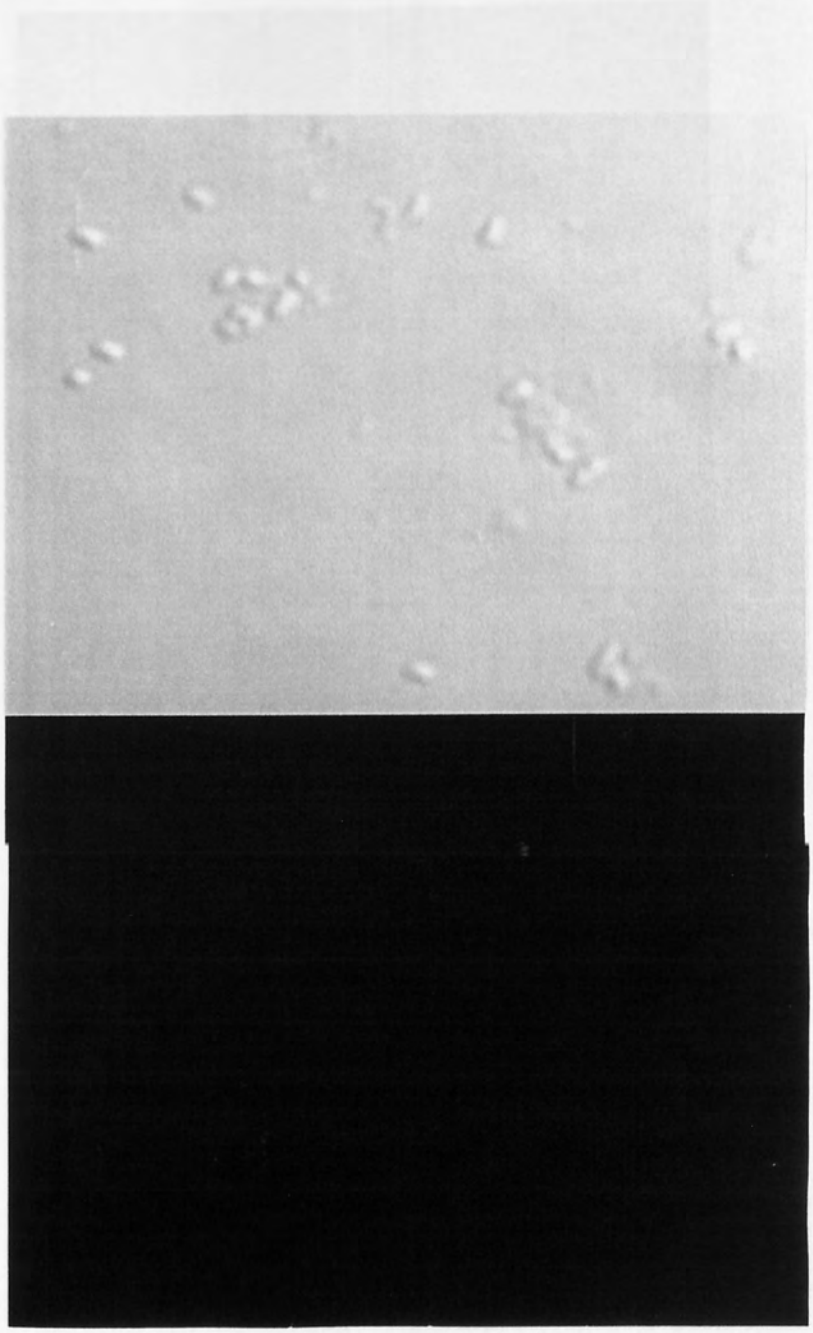


Figure 21. Immunofluorescent staining of *B. pseudomallei* E82 with monoclonal antibody 4VIH12 (magnification x300).

The lower half of the picture shows the image when viewed under fluorescent light and the upper half shows the same image under phase contrast.

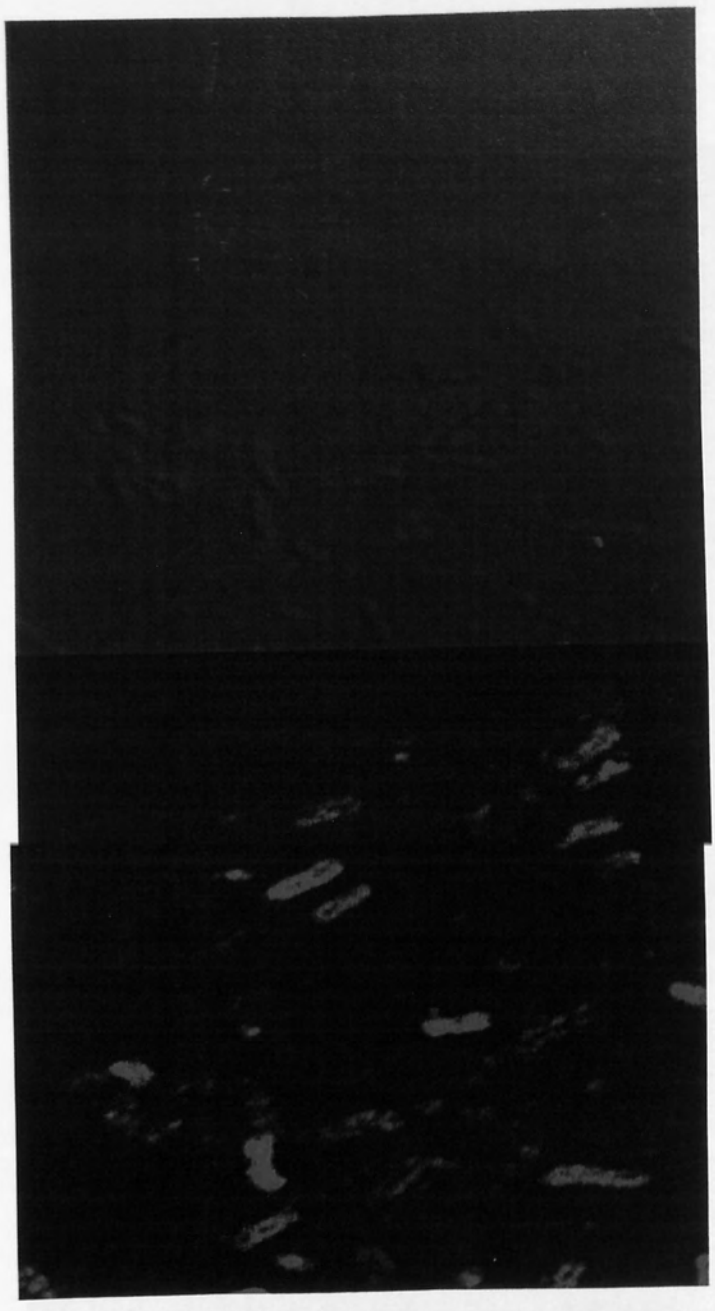


Figure 22. Immunofluorescent staining of *B. mallei* NCTC 10248 with monoclonal antibody 4VIH12 (magnification x300).

The lower half of the picture shows the image when viewed under fluorescent light and the upper half shows the same image under phase contrast.

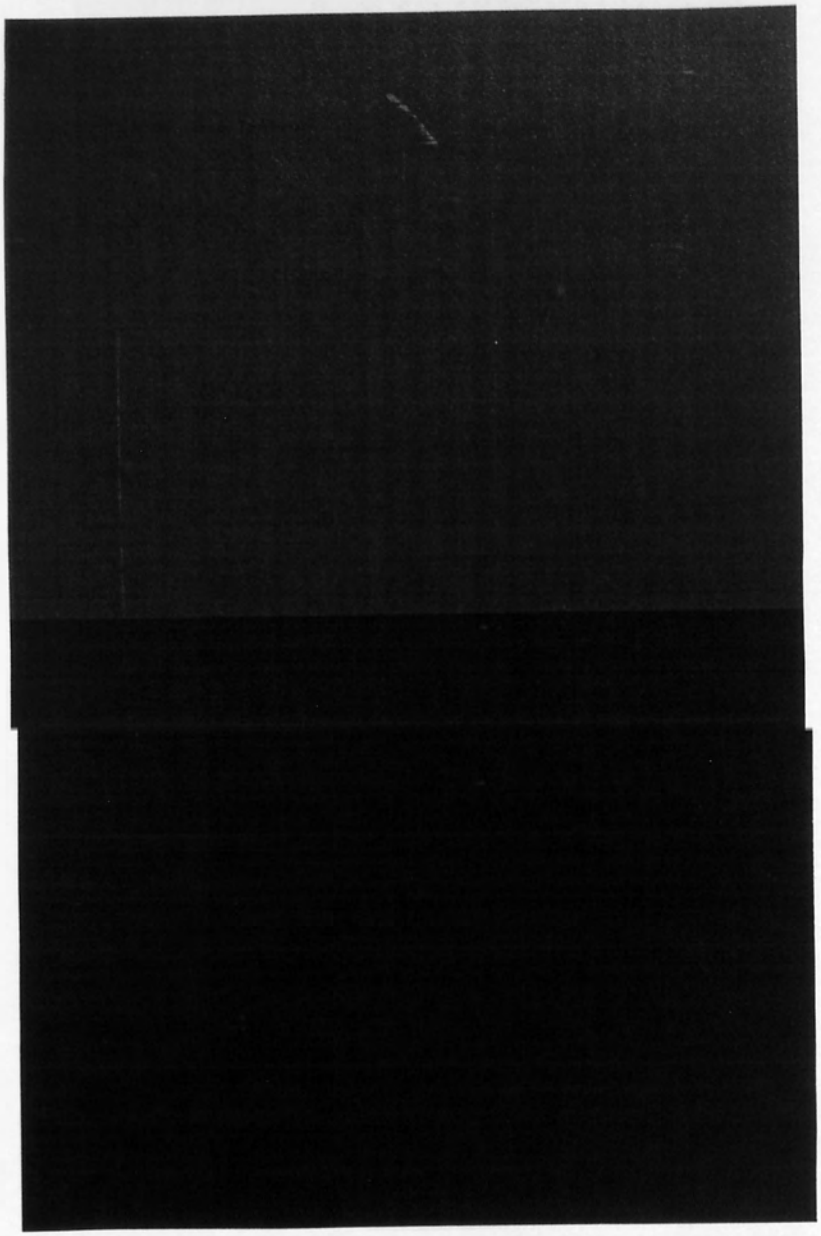


Figure 23. Immunofluorescent staining of *B. mallei* NCTC 10260 with monoclonal antibody 4VIH12 (magnification x300).

The lower half of the picture shows the image when viewed under fluorescent light and the upper half shows the same image under phase contrast.

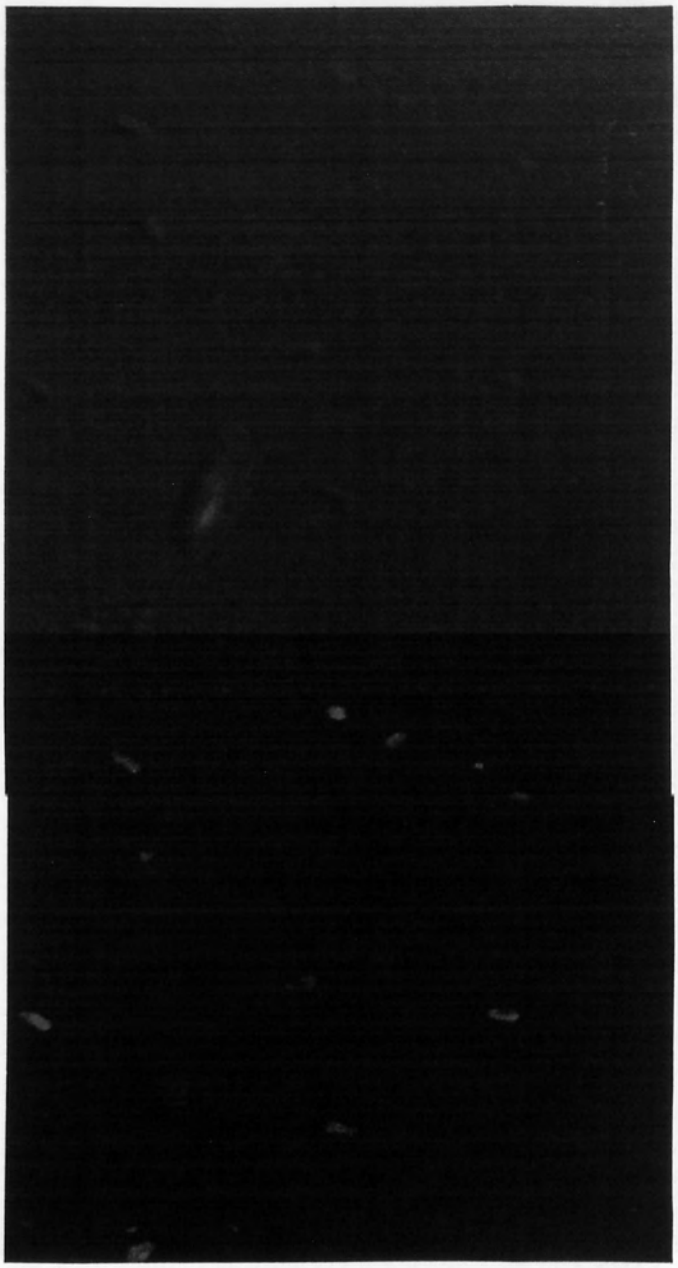


Figure 24. Immunofluorescent staining of *B. mallei* ATCC 23344 with monoclonal antibody 4VIH12 (magnification x300).

The lower half of the picture shows the image when viewed under fluorescent light and the upper half shows the same image under phase contrast.

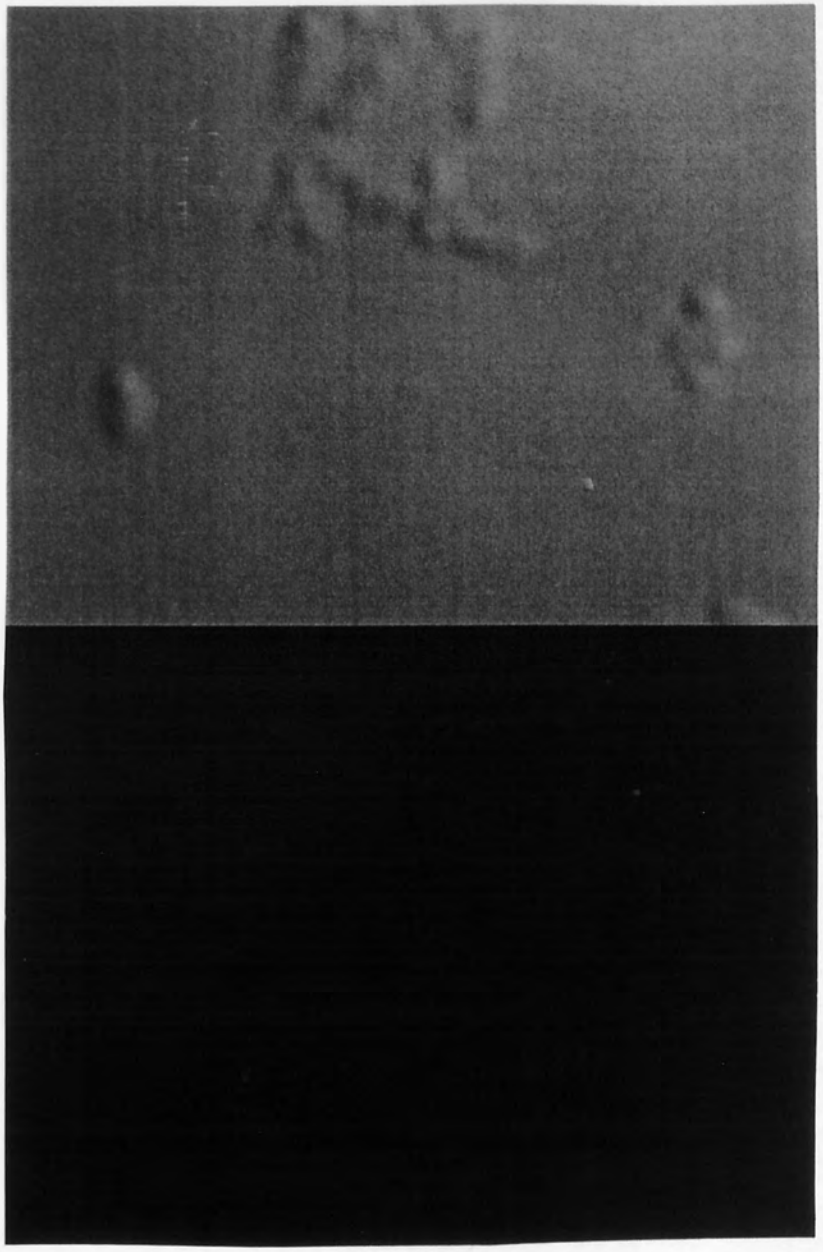


Figure 25. Immunofluorescent staining of *B. cepacia* NCTC 10661 with monoclonal antibody 4VIH12 (magnification x300).

The lower half of the picture shows the image when viewed under fluorescent light and the upper half shows the same image under phase contrast.

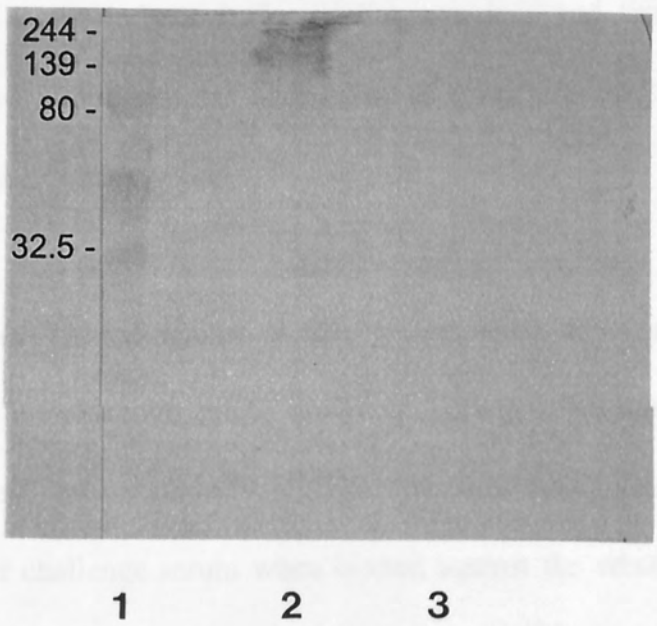


Figure 26. Western blot showing the effect of treating *B. pseudomallei* cells with sodium periodate on the binding of the monoclonal antibody 4VIH12.

Lane	Sample
1	Kaleidoscope markers
2	Untreated *B. pseudomallei* NCTC 4845
3	Treated *B. pseudomallei* NCTC 4845

3.2.18 Identification of antigens recognised *in vivo*

Western blotting was carried out using serum taken from animals which had been infected with both *B. mallei* and *B. pseudomallei*. Porton out-bred mice were challenged with an intraperitoneal injection of approximately 1 x 10^6cfu/ml of either *B. mallei* ATCC 23344 or *B. pseudomallei* NCTC 4845. Blood was taken before death, at the humane end-point (3-5 days for *B. pseudomallei* infection and 10-14 days for *B. mallei* infection), by cardiac puncture and the serum separated by centrifugation. Blood samples were safety checked (section 2.1.5) before using at a 1:200 dilution in western blots.

The serum was blotted against *in vitro* grown whole cell lysates and proteinase-K LPS preparations (section 2.4.2) of two *B. pseudomallei* strains (NCTC 4845 and HA) and one *B. mallei* strain (ATCC 23344). Few bands were obtained with the *B. pseudomallei* challenge serum when blotted against the whole cell extracts (Figure 27) and no bands at all with the LPS preparations. The *B. mallei* challenge serum highlighted a number of protein bands present in all three *B. pseudomallei* and *B. mallei* strains used (Figure 28). When blotted against the proteinase-K LPS preparations, the *B. mallei* challenge serum clearly recognised the LPS ladder of *B. mallei* and faintly recognised the LPS of *B. pseudomallei* NCTC 4845 (Figure 29).

The differences in blotting profiles may be due to differences in the diseases caused by *B. pseudomallei* and *B. mallei*. *B. pseudomallei* infection is rapidly fatal with death occurring 2-3 days after challenge. *B. mallei* causes a more protracted illness, death occurring after 10-14 days. The lag phase for a primary antibody response is approximately 5-7 days, which means in the case of a *B. pseudomallei* challenge, antibody titres will not have developed before death, however, animals challenged with *B. mallei* will have developed some primary antibody response prior to death. Figure 30 shows the difference in serum titres of the two challenge serum when tested in ELISA against their respective antigens. The *B. mallei* challenge serum clearly has higher antibody levels.

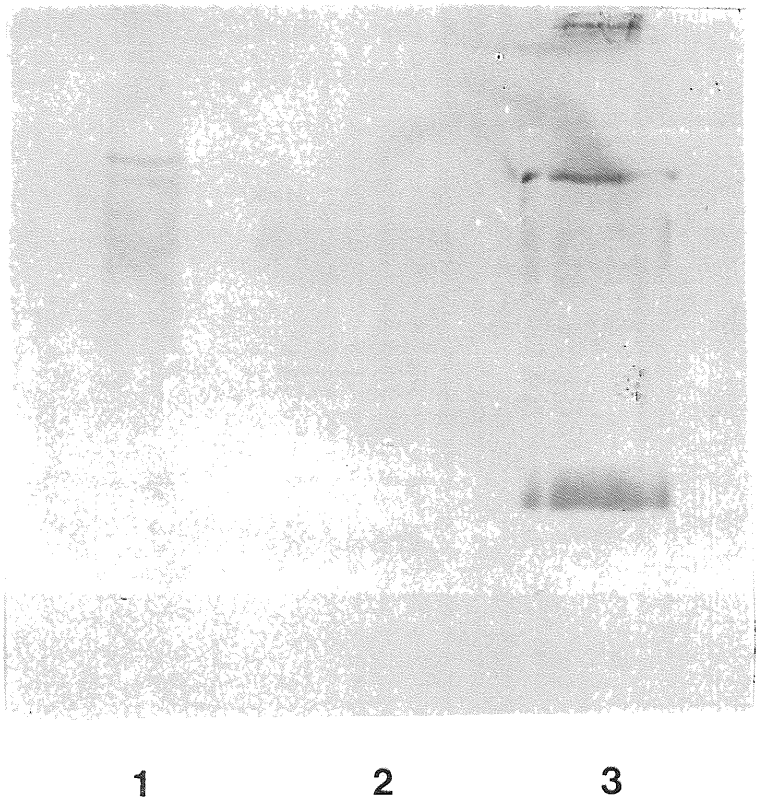


Figure 27. Western blot using mouse serum from a *B. pseudomallei* infection (1:200) against boiled whole cell lysates.

Lane	Sample
1	*B. pseudomallei* NCTC 4845
2	*B. pseudomallei* HA
3	*B. mallei* ATCC 23344

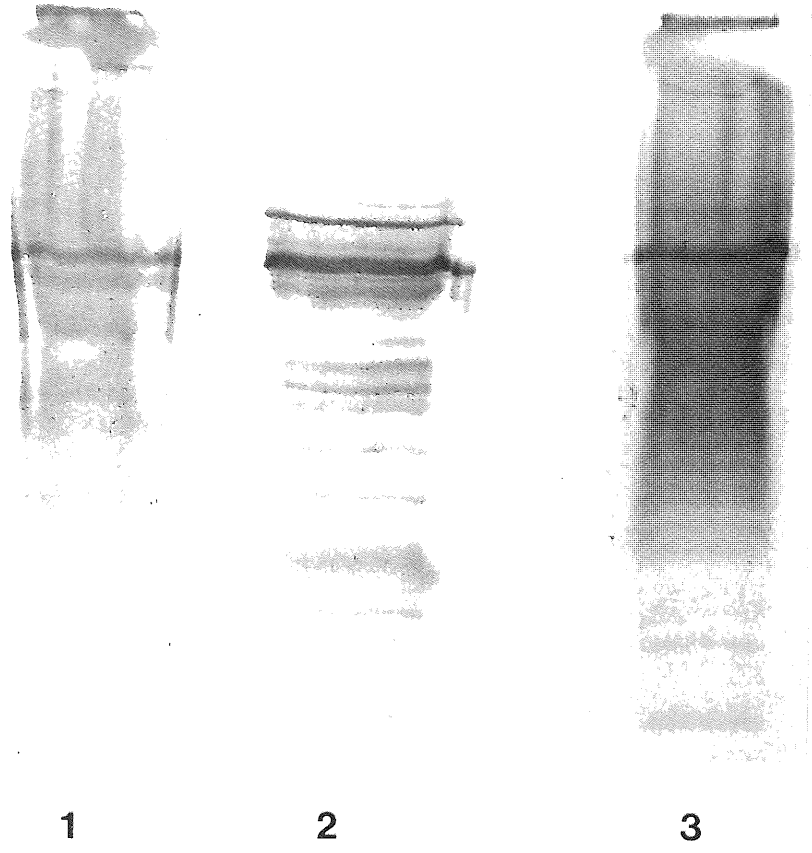


Figure 28. Western blot using mouse serum from a *B. mallei* infection (1:200) against boiled whole cell lysates.

Lane	Sample
1	*B. pseudomallei* NCTC 4845
2	*B. pseudomallei* HA
3	*B. mallei* ATCC 23344

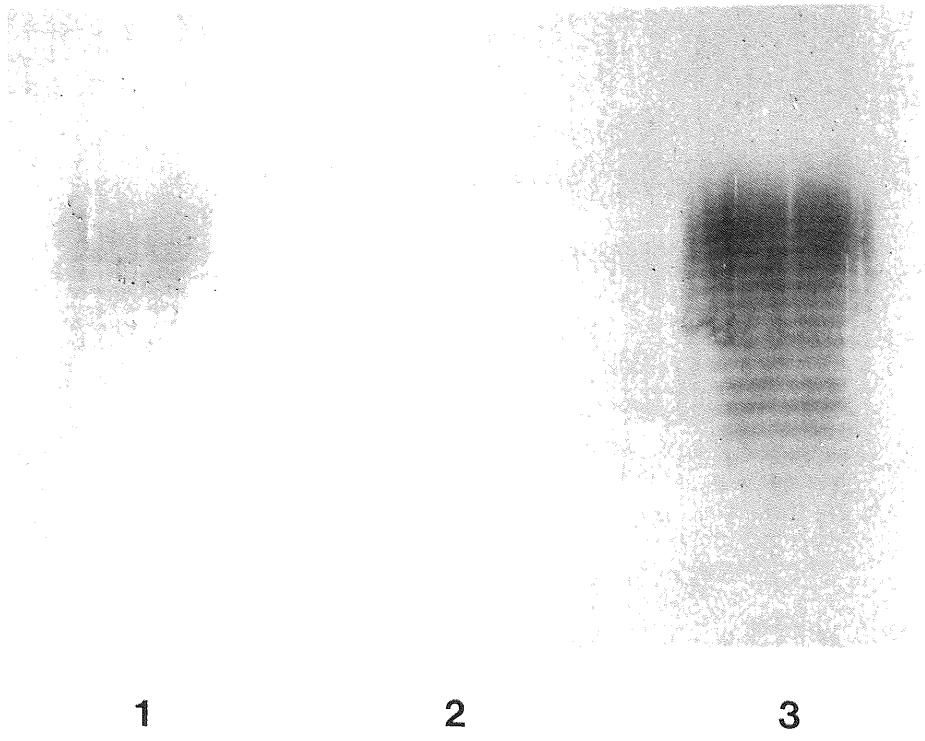


Figure 29. Western blot using mouse serum from a *B. mallei* infection (1:200) against proteinase-K LPS preparations.

Lane	Sample
1	*B. pseudomallei* NCTC 4845
2	*B. pseudomallei* HA
3	*B. mallei* ATCC 23344

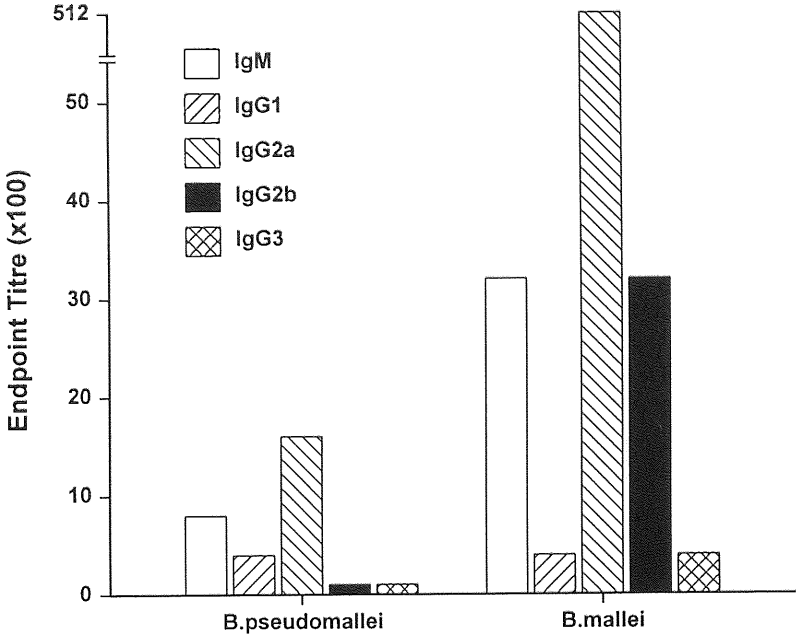


Figure 30. Serum antibody titres in mouse serum from *B. pseudomallei* and *B. mallei* infections.

3.3 Discussion

3.3.1 Detection assays

Antibody reagents, both polyclonal and monoclonal, have been produced for use in detection assays for *B. pseudomallei* and *B. mallei*. Antibodies have been labelled with both biotin and FITC and the performance of these antibodies in ELISA and biosensor assays has been characterised. The cross reactivity of the antibodies has also been investigated and their potential for use in detection assays for both *B. pseudomallei* and *B. mallei* has been assessed. The binding site of the monoclonal antibodies has also been identified.

The polyclonal antibody raised against heat killed whole cells of *B. pseudomallei* has been labelled with biotin and a capture assay formulated. It has, however, been found to cross react with other bacterial species such as *B. pickettii* and *B. cepacia*. These other species are taxonomically related (Yabuuchi, E., Kosako, Y. *et al.* 1992; Howe, C., Sampath, A. *et al.* 1971; Mohandas, S., Puthucheary, S.D. *et al.* 1994) and are likely to share common epitopes, and it would appear that these shared epitopes are recognised by the polyclonal antibody. It is possible to absorb out cross reactive antibodies from polyclonal serum using whole cells of *B. pickettii* or *B. cepacia* (Goding, J.W. 1983). However, removal of these cross reactive antibodies could affect the performance of the polyclonal serum in immunoassays. If these antibodies are to be incorporated into a biological detection system, there will be an ongoing requirement for antibodies to be produced. Polyclonal antibodies are inherently heterogeneous, and subject to variation between different animal species and different immunisations (Catty, D. 1988). There is also an ongoing requirement to

use laboratory animals. A reliance on absorption to ensure the polyclonal antibody is specific coupled with the inherent heterogeneity could lead to large differences in the specificity and sensitivity of different batches of polyclonal antibodies.

Monoclonal antibodies, once produced, can be cultured *in vitro* indefinitely producing large quantities of antibody of known specificity (Harlow, E. and Lane, D. 1988). The monoclonal antibodies against *B. pseudomallei* would appear to be specific for the two bacterial species of interest. Limited cross reactivity studies indicate that the other organisms tested are not recognised including *B. pickettii* and *B. cepacia*. To be sure of the specificity of these monoclonal antibodies a much larger cross reactivity study would need to be carried out using a larger number of strains from each species.

ELISAs formulated with the monoclonal antibodies allowed the detection of approximately 1 x 10^5 cfu/ml of *B. pseudomallei* and *B. mallei*. These monoclonal antibodies have also been shown to work in the Manual Threshold biosensor with a lower detection limit of approximately 1 x 10^5 cfu/ml for both *B. pseudomallei* and *B. mallei*. Assays carried out with a number of strains of these organisms suggests that the assays are capable of detecting several different of strains from each of the two species. The assays do not however distinguish between the two organisms.

B. mallei and *B. pseudomallei* were thought to be serologically indistinguishable (Cravitz, L. and Miller, W.R. 1950; Ashdown, L.R., Johnson, R.W. *et al.* 1989). However, studies have shown that while there are a number of epitopes shared by

these species, it may be possible with the specificity of monoclonal antibodies to distinguish between them, although the identity of the discriminatory binding sites of these monoclonal antibodies is unclear (Jianzhong, Z., Zi, L. *et al.* 1990; Yakovleva, I.V., Sviridov, V.V. *et al.* 1995).

Ideal detection assays must be sensitive, specific and able to detect the majority of strains in a species of bacteria. The monoclonal antibodies generated here appear to meet the requirements in terms of sensitivity and specificity and did detect the majority of strains against which they were screened. Six out of ten *B. mallei* strains were detected and nineteen out of the twenty two *B. pseudomallei* strains used. E27 and E82, two of the undetected *B. pseudomallei* strains are arabinose-positive soil isolates. These arabinose-positive strains have been shown to be avirulent and antigenically different from the virulent arabinose-negative strains (Sirisinha, S., Anuntagool, N. *et al.* 1998; Wuthiekanun, V., Smith, M.D. *et al.* 1996; Trakulsomboon, S., Dance, D.A.B. *et al.* 1997; Smith, M.D., Angus, B.J. *et al.* 1997). It has been proposed that these arabinose-positive strains may constitute a separate species which has been proposed as *B. thailandensis* (Brett, P.J., DeShazer, D. *et al.* 1997; Brett, P.J., DeShazer, D. *et al.* 1998). It would appear that the specificity of the monoclonal antibodies is such that they do not recognise this new species. This illustrates the importance of using a positively identified bacterial strain when carrying out research of this kind. Had one of these strains, given as *B. pseudomallei* been used for immunisation then the antibodies produced would have been useless. *B. pseudomallei* 4845, whilst not the type strain (NCTC 23343) had been used previously and was a recognised strain of *B. pseudomallei.*

A number of the published detection assays for *B. pseudomallei* are for clinical use and detect the presence of patient antibodies to the organism rather than the organism itself (Ashdown, L.R. 1981a; Ashdown, L.R., Johnson, R.W. *et al.* 1989; Ismail, G., Noor Embi, M. *et al.* 1987; Wongratanacheewin, S., Amornpunt, S. *et al.* 1995). Detection assays which are based on specific antigens of *B. pseudomallei* have in general not been tested for cross reactivity with *B. mallei* (Walsh, A.L., Smith, M.D. *et al.* 1994; Wongratanacheewin, S., Tattawasart, U. *et al.* 1990; Anuntagool, A., Intachote, P. *et al.* 1996; Anuntagool, N., Rugdech, P. *et al.* 1993; Pongsunk, S., Ekpo, P. *et al.* 1996). An assay based on the detection of antibodies to *B. mallei* in equines has also been reported but again the cross reactivity of this assay with *B. pseudomallei* has not been investigated (Verma, R.D., Sharma, J.K. *et al.* 1990).

3.3.2 Monoclonal antibody specificity

It would appear that the monoclonal antibodies are binding a high molecular weight polysaccharide material which may be the exopolysaccharide of both *B. pseudomallei* and *B. mallei*. Treatment of *B. pseudomallei* with sodium periodate inhibited the binding of the monoclonal antibody 4VIH12. Sodium periodate treatment is known to modify carbohydrate residues and blotting carried out on treated and untreated cells shows an absence of binding after treatment. This suggests that carbohydrate residues are vital in the binding of 4VIH12 to *B. pseudomallei*. Immunofluorescent microscopy using 4VIH12 showed bacterial cells surrounded by a halo of fluorescent stained material.

These results would support the theory that the monoclonal is binding to polysaccharide material which is distributed around the outside of the cell. Steinmetz (Steinmetz, I., Rohde, M. *et al.* 1995) reported the purification and characterisation of the exopolysaccharide of *B. pseudomallei* and the production of a specific monoclonal antibody. Cross reactivity testing showed reactivity only with two strains of *B. mallei.* It would appear that the five monoclonal antibodies produced have very similar reactivity to the monoclonal antibody (MAb 3015) produced by Steinmetz. However the isolation of two further capsular polysaccharides *from B. pseudomallei* makes the identification of the exact monoclonal antibody binding site difficult (Kawahara, K., Dejsirilert, S. *et al.* 1998). Only further investigation coupled with chemical analysis could identify which polysaccharide the monoclonal antibodies are binding to.

The binding of the monoclonal antibodies to both *B. pseudomallei* and *B. mallei* suggests that this antigen is common to both organisms but is not shared by other members of the *Burkholderia* genera such as *B. cepacia* or *B. pickettii.* The difference in detection levels between the different strains of the two species may suggest that the levels of polysaccharide present in different strains is variable. This reasoning has been confirmed by Khrapova (1998) who reported a wide variation in the levels of exopolysaccharide between strains of *B. pseudomallei* and *B. mallei* and in the levels produced by a single strain under different culture conditions. The exopolysaccharide or capsular polysaccharide was first noted by Chambon and Fournier (Chambon, L. and Fournier, J. 1956) and is thought to be produced as a means of protection from the environment (Kanai, K. and Kondo, E. 1994), from

detection and phagocytosis by the immune system (Popov, S.F., Kurilov, V.I. *et al.* 1995; Mel'nikov, B.I., Popov, S.F. *et al.* 1990; Puthucheary, S.D., Vadivelu, J. *et al.* 1996; Vorachit, M., Lam, K. *et al.* 1995; Piven, N.N., Smirnova, V.I. *et al.* 1991) or as a possible means of attachment to mammalian cells (Ahmed, K., Enciso, H.D.R. *et al.* 1999).

Extraction of the exopolysaccharide using a published method (Steinmetz, I., Rohde, M. *et al.* 1995) proved unsuccessful. The product when analysed by gel electrophoresis showed three protein bands at approximately 14-19kDa, 31-36kDa and 55kDa and there was also some LPS present in the sample. The presence of LPS in the sample is not surprising as some of the LPS is only loosely associated with the membrane and shearing forces during stirring will cause it to become dissociated (Hammond, S.M., Lambert, P.A. *et al.* 1984). The three proteins are likely to be surface associated as they were harvested by stirring. It is possible that these proteins are outer membrane proteins, two of which are known to have molecular weights of 17 and 31kDa (Gotoh, N., White, N.J. *et al.* 1994). A 19.5kDa antigen has also been reported in *B. pseudomallei* (Anuntagool, N., Rugdech, P. *et al.* 1993).

3.3.3 Different biotypes and virulence

It would appear from the results of both the strain variation ELISAs and the immunofluorescence microscopy that the monoclonal antibodies do not bind to the avirulent soil isolates of *B. pseudomallei* E27 and E82. It has been reported that highly virulent strains of *B. pseudomallei* have increased level of exopolysaccharide (Peters, M.K., Piven, N.N. *et al.* 1983), whilst strains deficient in exopolysaccharide

have reduced or no virulence for laboratory animals (Piven, N.N., Smirnova, V.I. *et al.* 1991). It is possible that these two avirulent isolates lack the exopolysaccharide or capsule antigen.

Studies have shown the exopolysaccharide to be a complex structure of glycoprotein with a molecular weight of 750,000kDa. It is composed of 10% protein and 90% carbohydrate and it is thought that the carbohydrate and protein components have different biological functions. A 200kDa carbohydrate component is reported as having an immunodepressant effect, whilst a 34kDa protein has been shown to be partially protective in rats (Piven, N.N., Smirnova, V.I. *et al.* 1996).

It has also been reported that the presence or absence of a capsule or exopolysaccharide has a pronounced effect on phagocytosis. Bacterial cells without exopolysaccharide were actively phagocytosed and lysed by macrophages. Capsulated *B. pseudomallei* and *B. mallei* cells were actively taken up by phagocytes but those which were taken up were not damaged by the lysosomal contents (Popov, S.F., Tikhonov, N.G. *et al.* 1994; Popov, S.F., Mel'nikov, B.I. *et al.* 1990).

Structurally the exopolysaccharide has been shown to consist of a repeating tetrasaccharide unit of three galactose residues and a 3-deoxy-D-manno-2-octulosonic acid residue. It has been identified in a clinical strain of *B. pseudomallei* and is considered an important antigen for diagnostic purposes and as a potential vaccine candidate (Nimtz, M., Wray, V. *et al.* 1997; Masoud, H., Ho, M. *et al.* 1997). Two further capsular polysaccharides (CP-1a and CP-2) have been identified which

are thought to play a role in the virulence of *B. pseudomallei*, (Kawahara, K., Dejsirilert, S. *et al.* 1998).

3.3.4 Other target surface antigens

Western blots using serum from animals challenged with *B. pseudomallei* and *B. mallei* indicated that the LPS was also a potential target for detection antibodies. Located on the surface of the bacteria the LPS forms an integral part of the bacterial outer membrane and is thought to be conserved within the species.

Outer membrane proteins were not considered as first choice targets because of the potential for variability. Gram negative bacterial cells have been shown to alter the protein components in the outer membrane in response to stresses such as low iron availability, exposure to subinhibitory concentrations of antibiotics and nutrient limitation (Kadurugamuwa, J.L., Anwar, H. *et al.* 1985; Kadurugamuwa, J.L., Anwar, H. *et al.* 1988; Poole, K. and Hancock, R.E.W. 1986). *B. pseudomallei* has also been shown to have a wide antigenic variability between isolates (Lertmemongkolchai, G., Manmontri, W. *et al.* 1991) and between culture conditions (Wongratanacheewin, S., Tattawasart, U. *et al.* 1993).

4. Characterisation and immunological properties of the LPS of *B. pseudomallei* and *B. mallei*

4.1 Introduction

4.1.1 The outer membrane and LPS of Gram-negative bacteria

The outer membrane of Gram-negative bacteria is composed of a bimolecular leaflet (Figure 31). Its composition is different to the cytoplasmic membrane and confers special properties upon Gram-negative bacteria. The outer membrane, unlike the cytoplasmic membrane has no role in electron transport, limited enzymic activity and is distinguished by the presence of a novel series of proteins and LPS. The outer membrane constitutes a barrier to a wide variety of molecules, making the surface of Gram-negative organisms less permeable than that of Gram-positive bacteria. Electron microscopy shows LPS to be present in the outer leaflet of the membrane and to extend up to 30nm beyond it. Treatment with EDTA causes a non-specific increase in the general permeability of the outer membrane and in enteric bacteria releases between one third and a half of the LPS component (Hammond, S.M., Lambert, P.A. *et al.* 1984).

4.1.2 Structure of the LPS of Gram-negative bacteria

LPS is composed of three covalently linked regions, each with a distinctive composition and biological function (Figure 32). Integrated into the outer membrane bilayer is the lipid A region, consisting of fatty acid chains linked to glucosamine. Extending outwards from the lipid A is the core region consisting of a short

carbohydrate chain and from the core a longer carbohydrate polymer, the O-side chain. The composition of lipid A is relatively invariable, its structure being highly conserved in a wide range of Gram-negative bacteria. The sugars comprising the core are similar in many different bacterial species. In contrast the polysaccharide of the O-side chains where present, exhibit gross differences in composition and structure, often within a single species. The O-side chain is the serologically dominant part of the LPS molecule. It consists of repeating oligosaccharide units, often containing rare sugars and it is in these sugars that the serological determinants reside.

Polysaccharides are capable of a considerable degree of diversity per unit structure as a result of variation in the sugar composition and in the configuration of the glycosidic linkage. This accounts for the large number of O-serotypes in Gram-negative bacteria (Hammond, S.M., Lambert, P.A. *et al.* 1984).

Monoclonal antibodies raised against the O side chain of representative species of Gram-negative genera such as *Bordetella* and *Vibrio* have been shown to be species specific, whereas those against the core and the lipid A show extensive cross reaction. *Francisella tularensis* is a potentially capsulated Gram-negative bacterium but the capsule does not appear to mask the O antigen of the LPS, allowing antibodies specific for the O antigen to be used in the detection of the organism (Fulop, M.J., Webber, T. *et al.* 1991).

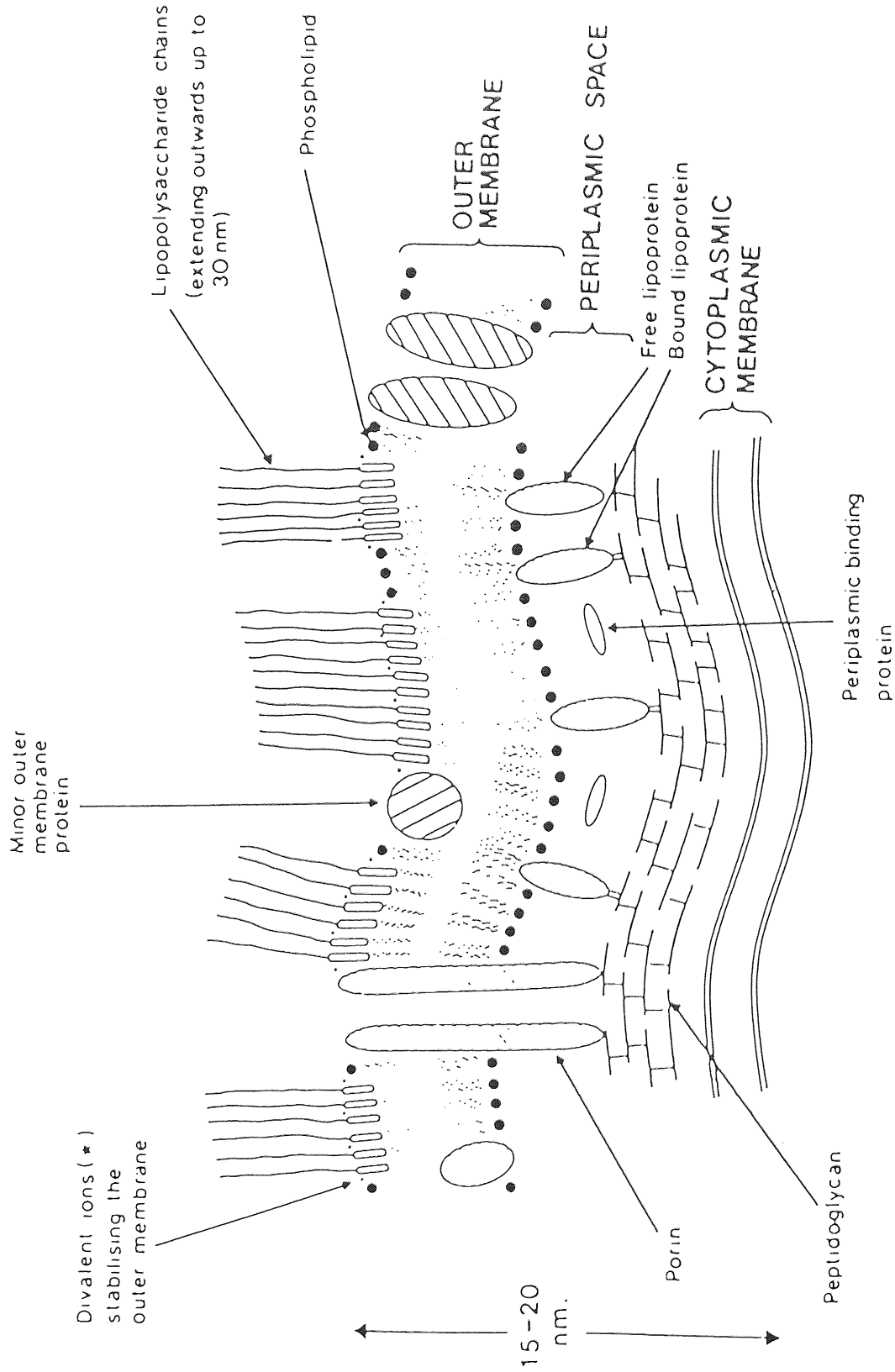


Figure 31. The cell envelope of a generalised Gram-negative bacterium.

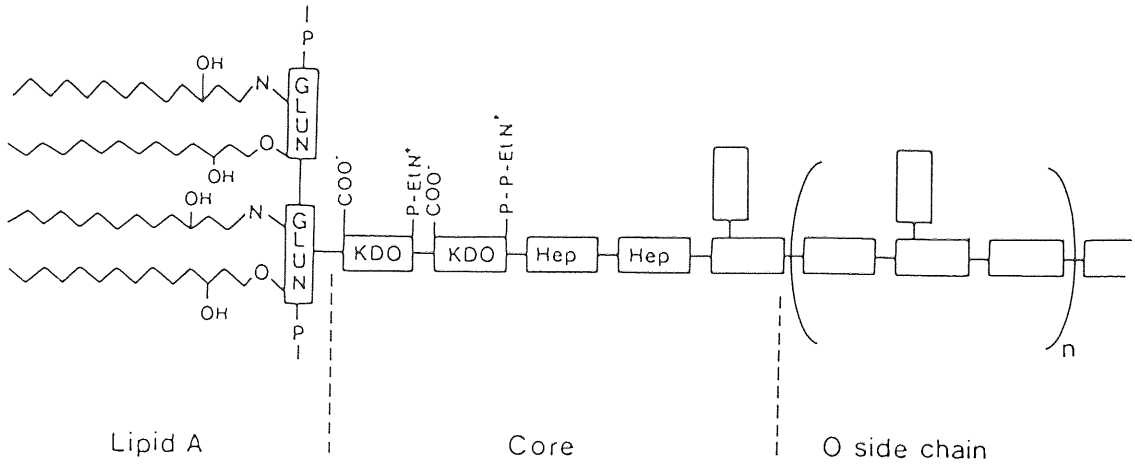


Figure 32 Generalised structure of bacterial LPS where GLUN is glucosamine, KDO is 3-deoxy-D-manno-octulosonic acid and Hep is heptose.

4.1.3 Biological activity of the LPS from gram-negative bacteria

Bacterial extracts have long been known to be toxic to man and animals. LPS, unique to Gram-negative bacteria is, or contains within it, what has been designated as endotoxin. Endotoxin is thought to play a role in a variety of bacterial diseases and is a possible causative agent of shock arising from post operative sepsis or other forms of traumatic injury. It is commonly accepted that lipid A is the primarily toxic part of the LPS molecule but that the O-side chain has an important role in conferring

solubility on the molecule and affecting biological activity. In bacterial infections endotoxin is thought to play a part in pyrogenicity, the Schwartzman reaction, abortion and shock (Stephen, J. and Pietrowski, R.A. 1986). Early experiments with a crude *B. pseudomallei* extract showed the extract to contain classical endotoxin activity which caused generalised Schwartzman reaction and haemorrhage in experimental animals (Rapaport, F.T., Millar, J.W. *et al.* 1961).

4.1.4 Aims

LPS forms an integral part of the outer membrane of gram negative bacteria and will therefore be present in all strains of a single species. Antibodies raised against the LPS have the potential to detect a large number of strains from a particular species, depending on whether the O antigen is subject to variation within the species (i.e. the number of different serotypes).

The aim of this part of the project was to isolate the LPS from both *B. pseudomallei* and *B. mallei*, raise antibodies against the LPS of both species and characterise the binding of those antibodies.

4.2 Isolation and characterisation of the LPS from *B. pseudomallei* and *B. mallei*

4.2.1 Proteinase-K mini-preparations

To visualise the LPS after gel electrophoresis LPS mini-preparations from whole cells were carried out using proteinase-K. Initial proteinase-K preparations were carried out with *B. pseudomallei* NCTC 4845 according to the method of Hitchcock and Brown and stained using the BioRad Silver Stain Plus kit (Hitchcock, P.J. and Brown, T.M. 1983). The results were disappointing with no evidence of the characteristic LPS O-antigen ladder. Coomassie blue stained preparations treated with proteinase-K were compared with preparations without proteinase-K: there were a number of bands in the untreated preparations compared with no visible bands in the proteinase-K treated preparations. Gels were also stained with the BioRad Silver Stain kit which is optimised to stain polysaccharides and in these gels there was a faint band on the bottom of the gel which may have been lipid A. However, the O-antigen ladder was undetected.

Western blotting of the *B. pseudomallei* proteinase-K preparations using mouse polyclonal serum as the primary detection antibody did visualise the ladder effect characteristic of LPS. It is possible that the LPS minipreparations had worked in terms of isolation and separation but that the silver stains were not effective at staining the O-antigen.

Using an alternative silver staining method (section 2.4.4) using periodic acid oxidation followed by ammoniacal silver nitrate treatment (Tsai, C.M. and Frasch, C.E. 1982), the O-antigen stained and the characteristic ladder was visualised (Figure 33, lane 1). This alternative method is believed to reduce the loss of band visibility due to excessive washing (Fomsgaard, A., Freundenberg, M.A. *et al.* 1990), however it is more likely that differences in oxidation may be responsible. The silver stain of Tsai and Frasch is assumed to be dependent on the presence of periodate-sensitive cis-glycols in the LPS molecule, and these groups would appear to be present in *B. pseudomallei* LPS. The Bio-Rad silver staining kits may use a different oxidising agent which, in the case of *B. pseudomallei* LPS, appears ineffective. The effectiveness of silver staining methods appear to be dependent on the presence of reactive groups in the LPS molecules under examination. The LPS from *P. aeruginosa* PAO is very poorly stained due to the absence of these reactive groups (Kropinski, A.M., Berry, D. *et al.* 1986).

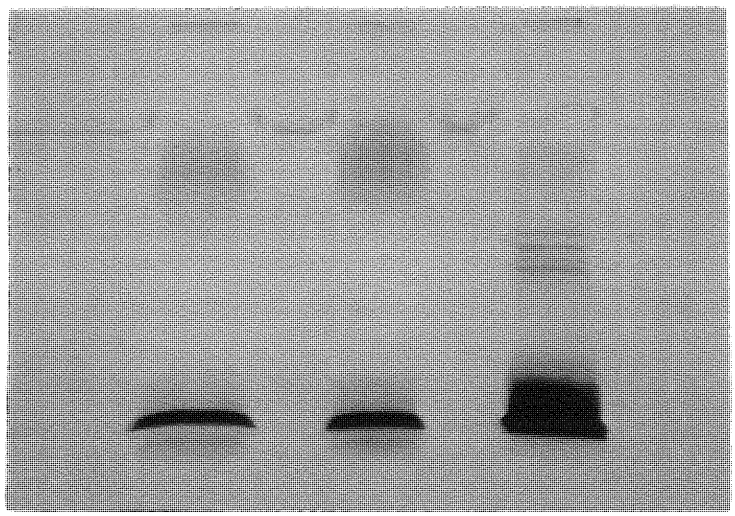


Figure 33. Comparison of LPS extracted from *B. pseudomallei* NCTC 4845 by different methods. 10μg of material has been loaded in lanes 2 and 3.

Lane	Sample
1	Mini proteinase-K preparation
2	Small scale proteinase-K preparation
3	Phenol/water extraction

4.2.2 Comparison of the LPS of *B. pseudomallei* NCTC 4845 with other strains of *B. pseudomallei*

Proteinase-K minipreparations were carried out on a number of different strains of *B. pseudomallei*. Initially minipreparations were carried out on six strains of *B. pseudomallei*. Silver staining showed the profiles of the extracts from different *B. pseudomallei* strains to be very similar apart from the extract from strain HA (Figure 34). The O antigen bands in the profile of this strain were of a slightly higher molecular weight and spaced further apart. This profile was termed the atypical LPS. Further proteinase-K minipreparations were carried out on a total of 22 strains of *B. pseudomallei* (Figures 35-37). Seventeen strains showed the typical LPS profile, four strains (HA, 123, PA and 576) were found to have the atypical LPS profile and one strain (E8) had no visible O antigen at all. All PK minipreps were carried out with the same amount of starting material (1mg {wet weight} bacterial cells in 30µl sample buffer) so the absence of a visible O antigen in E8 is not due to large differences in the amount of bacteria used in the preparation of LPS.

4.2.3 Comparison of the LPS of *B. pseudomallei* with the LPS of *B. mallei* strains

Previous workers have shown that *B. mallei* and *B. pseudomallei* are antigenically very similar (Jianzhong, Z., Zi, L. *et al.* 1990). To compare the silver stain profiles of *B. mallei* and *B. pseudomallei* proteinase-K minipreparations were carried out on seven different *B. mallei* strains. Silver stained profiles showed great visual similarity between the strains (Figures 38 & 39) and to the typical LPS profile of *B.*

pseudomallei. The exceptions were *B. mallei* 10245 and 120 which showed no visible O-antigen. As with the *B. pseudomallei* strains each proteinase-K minipreparation was carried out with the same amount of starting material (1mg {wet weight} bacterial cells in 30µl sample buffer) and the same volume was loaded in each case. Given the antigenic similarities between these two species of bacteria, it is possible that the LPS of *B. mallei* is very similar to the typical *B. pseudomallei* LPS. The structure of *B. mallei* LPS has not been reported, however the visual similarity of the silver stained profiles of the LPS from the two species would support this theory.

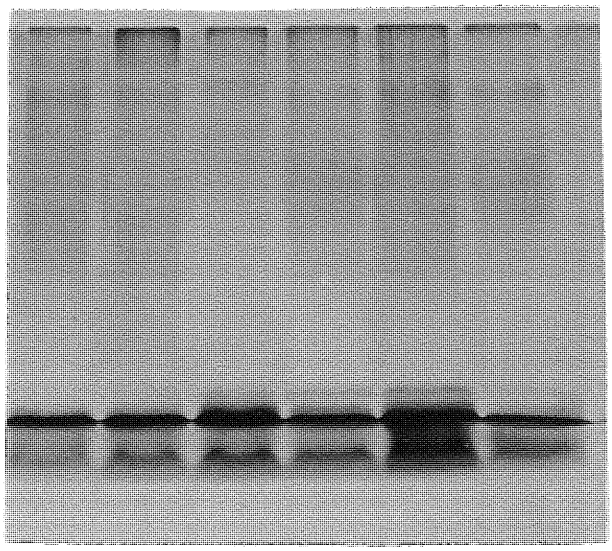


Figure 34. Appearance of proteinase-K extracts of whole cells of *B. pseudomallei* after SDS-PAGE and silver staining. The material loaded in lane 3 has an atypical profile.

Lane	Sample
1	*B. pseudomallei* E38
2	*B. pseudomallei* Mal 6
3	*B. pseudomallei* HA
4	*B. pseudomallei* 551a
5	*B. pseudomallei* 603a
6	*B. pseudomallei* NCTC 4845

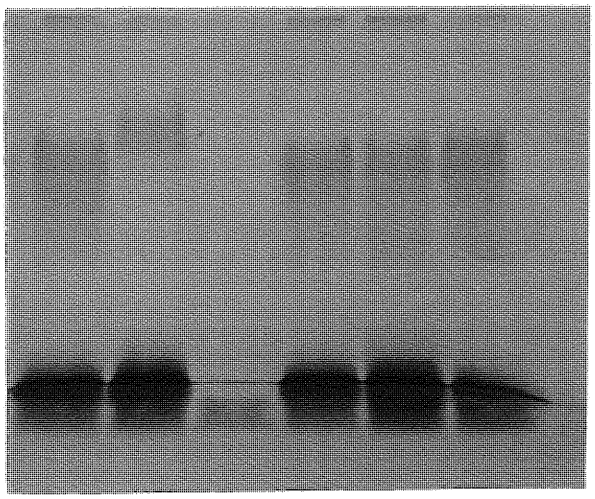


Figure 35. Appearance of proteinase-K extracts of whole cells of *B. pseudomallei* after SDS-PAGE and silver staining. The material loaded in lane 2 has an atypical profile and lane 3 has no visible O-antigen.

Lane	Sample
1	*B. pseudomallei* 204
2	*B. pseudomallei* 576
3	*B. pseudomallei* E8
4	*B. pseudomallei* E25
5	*B. pseudomallei* E27
6	*B. pseudomallei* E82

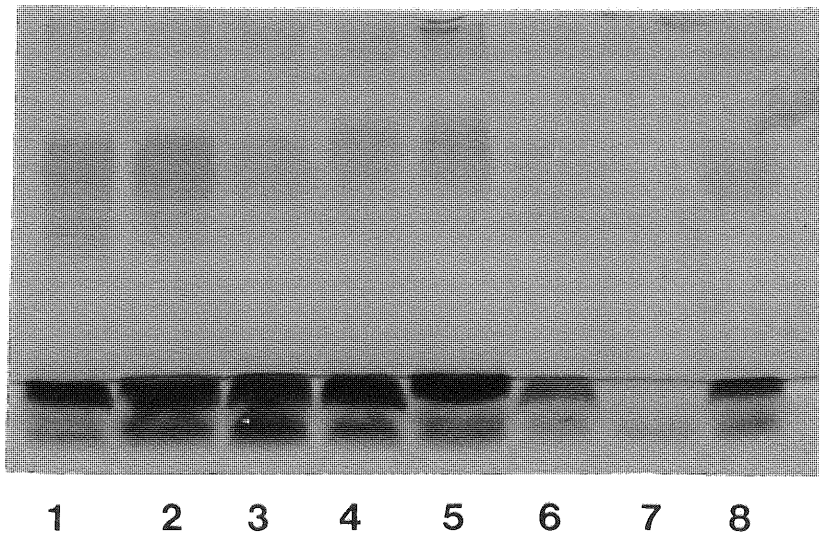


Figure 36. Appearance of proteinase-K extracts of whole cells of *B. pseudomallei* after SDS-PAGE and silver staining. The material loaded in lanes 4 and 5 has an atypical LPS profile and lane 7 shows no visible O-antigen.

Lane	Sample
1	*B. pseudomallei* ATCC 23343
2	*B. pseudomallei* 4889
3	*B. pseudomallei* O
4	*B. pseudomallei* 123
5	*B. pseudomallei* PA
6	*B. pseudomallei* Mal 6
7	*B. pseudomallei* E8
8	*B. pseudomallei* BRI

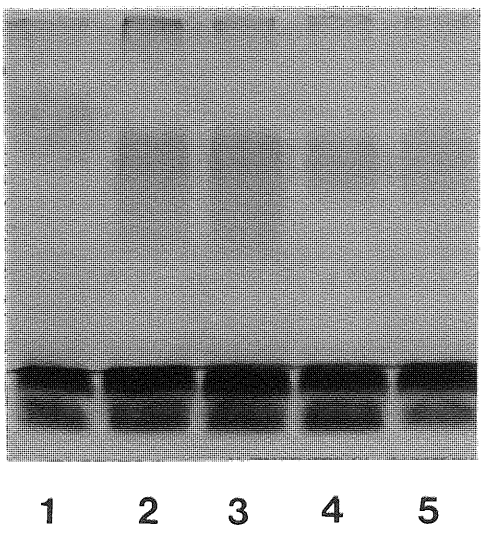


Figure 37. Appearance of proteinase-K extracts of whole cells of *B. pseudomallei* after SDS-PAGE and silver staining. The material loaded in lane 1 has an atypical LPS profile.

Lane	Sample
1	*B. pseudomallei* 576
2	*B. pseudomallei* UBOL-2
3	*B. pseudomallei* USAMRU-21
4	*B. pseudomallei* SEARLE
5	*B. pseudomallei* USAMRU-1

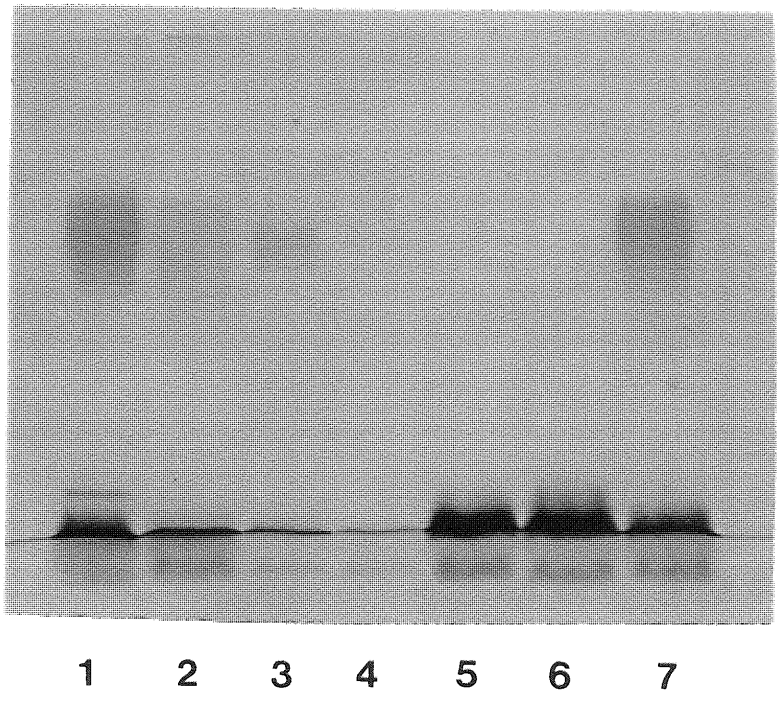


Figure 38. Appearance of proteinase-K extracts of whole cells of *B. mallei* after SDS-PAGE and silver staining. The material loaded in lanes 4, 5 and 6 shows no visible O-antigen.

Lane	Sample
1	*B. pseudomallei* NCTC 4845
2	*B. pseudomallei* Mal 6
3	*B. mallei* NCTC 3708
4	*B. mallei* NCTC 3709
5	*B. mallei* NCTC 10245
6	*B. mallei* NCTC 120
7	*B. mallei* NCTC 10229

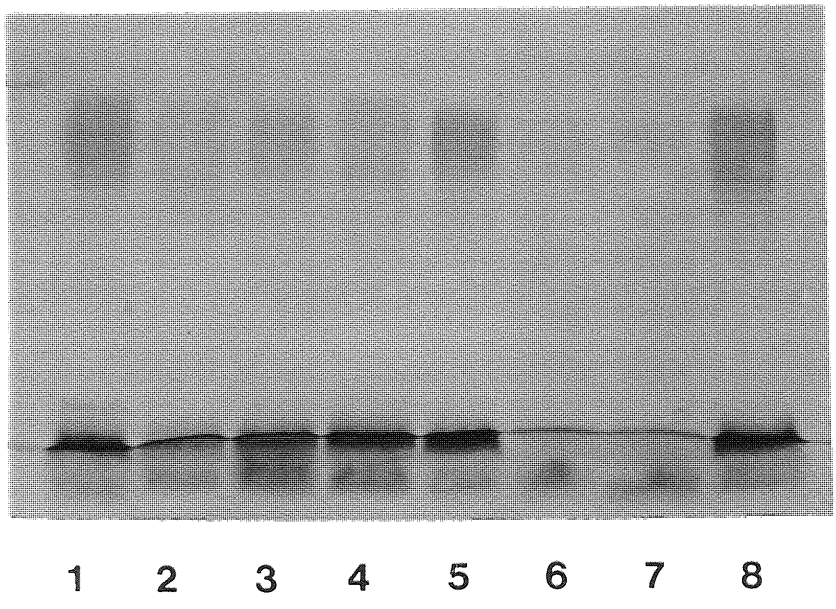


Figure 39. Appearance of proteinase-K extracts of whole cells of *B. mallei* after SDS-PAGE and silver staining.

Lane	Sample
1	*B. pseudomallei* NCTC 4845
2	*B. pseudomallei* Mal 6
3	*B. mallei* NCTC 10247
4	*B. mallei* NCTC 10248
5	*B. mallei* NCTC 10230
6	*B. mallei* NCTC 10260
7	*B. mallei* NCTC 10260
8	*B. mallei* ATCC 23344

4.2.4 Comparison of the LPS of *B. pseudomallei* and *B. mallei* with that of other bacterial species

To compare the LPS of *B. pseudomallei* and *B. mallei* with the LPS of other bacterial species, proteinase-K minipreparations were carried out with other species known to be closely related to *B. pseudomallei*. Heat killed cells of *B. pickettii* NCTC 11149, *P. aeruginosa* NCTC 10332 and *B. cepacia* NCTC 10661 were treated. Electrophoresis followed by silver staining showed different LPS profiles (Figure 40). The ladders of *B. mallei* and *B. pseudomallei* were very similar and unlike those of *P. aeruginosa* and *B. pickettii*. There is, however, some visual similarity between the LPS profiles of *B. cepacia* NCTC 10661, *B. mallei* NCTC 10247 and *B. pseudomallei* NCTC 4845.

4.2.5 Phenol hot water extraction

To produce purified LPS for endotoxin assays and immunisations an LPS extraction using phenol/hot water was carried out. A small scale LPS extraction was carried out on *B. pseudomallei* NCTC 4845 heat inactivated cells utilising the phenol hot water method of Westphal and Jann (Westphal, O. and Jann, K. 1965). Extraction of the LPS was carried out using phenol at 67°C. However, after dialysis and ultracentrifugation there was no visible pellet. The failure to isolate any LPS may have been due to the use of insufficient bacteria as the starting material. The original method uses 20g (dry weight) bacterial cells, however, due to the technical problems of producing large amounts of inactivated Category III pathogen this first extraction was carried out using 0.5g of bacteria.

A second experiment was carried out using 3g of lyophilised bacteria made up to 5% (w/v) in dH_2O. Extractions were carried out using hot phenol followed by dialysis and ultracentrifugation. There was no evidence of a pellet but resuspension was carried out using 1ml of dH_2O. The preparation was then frozen and lyophilised overnight. The yield was low (0.5mg) and when analysed by SDS-PAGE there was a band in the lower part of the gel which could be lipid A but no characteristic LPS ladder effect (Figure 33).

4.2.6 Small scale PK extraction of *B. pseudomallei* LPS

The lack of success using the traditional phenol/hot water method for the extraction of LPS meant that a second method was required for producing adequate quantities of purified LPS for immunisations. The proteinase-K minipreparation method was scaled up by removing the bromophenol blue from the solubilisation buffer, lengthening the incubation time with proteinase-K followed by exhaustive dialysis against water before lyophilisation.

260mg (wet weight) of heat inactivated bacteria were treated with PK using this method. After lyophilisation the yield of LPS was 12mg. Electrophoresis followed by silver staining showed the O-antigen to be clearly present. LPS produced in this way, when silver stained, appeared identical to that produced in the minipreparations (Figure 33).

4.2.7 Small scale PK extraction of *B. mallei* LPS

The small scale PK method was used to produce LPS from *B. mallei* 23344. 360mg of heat inactivated bacteria were treated with proteinase-K by this method. After lyophilisation the yield of LPS was 11mg. Electrophoresis followed by silver staining showed the O-antigen to be clearly present. The LPS produced by this method from *B. mallei* appeared identical to that produced in proteinase-K minipreparations when silver stained and showed close similarity to the typical LPS of *B. pseudomallei*.

4.2.8 Small scale PK extraction of atypical *B. pseudomallei* LPS

The small scale PK method was used to produce LPS from *B. pseudomallei* HA. 144mg of heat inactivated *B. pseudomallei* HA was treated with proteinase-K by this method. After lyophilisation the yield of LPS was 7mg. Electrophoresis followed by silver staining showed the O-antigen to be clearly present. The LPS produced by this method from *B. pseudomallei* HA appeared identical to that produced in proteinase-K minipreparations when silver stained and shows the wider spaced O-antigen bands of the atypical LPS.

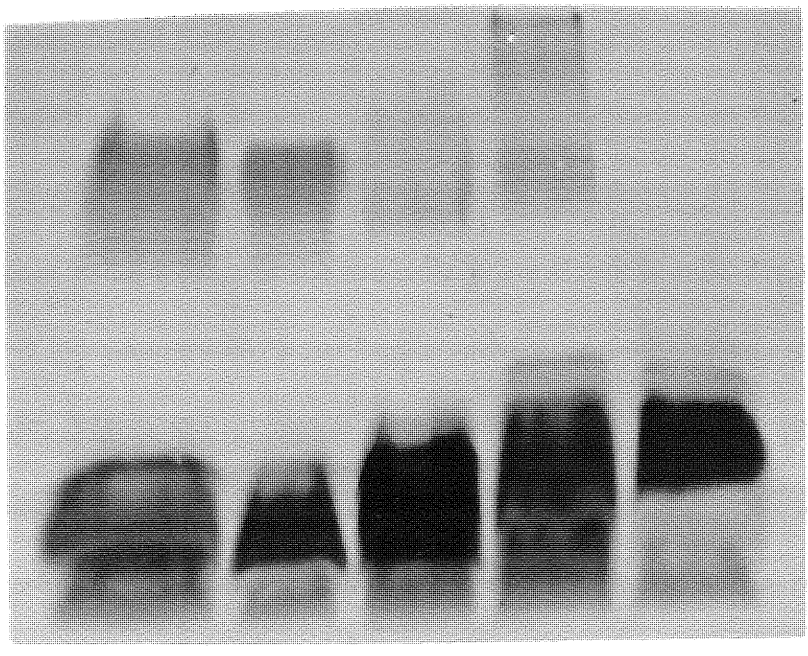
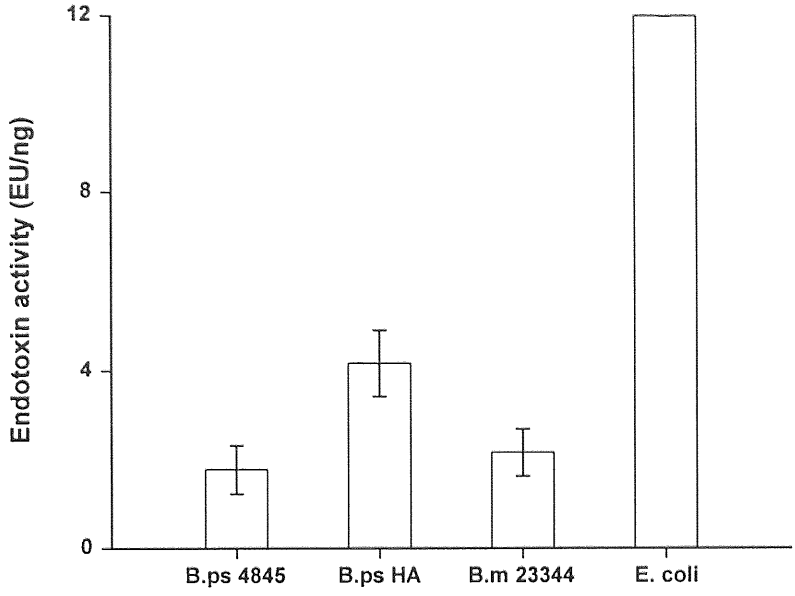


Figure 40. Comparison of proteinase-K extracts of whole cells of *B. pseudomallei* and *B. mallei* with those of closely related bacterial species and SDS-PAGE and silver staining.

Lane	Sample
1	*B. pseudomallei* 204
2	*B. mallei* ATCC 23344
3	*B. cepacia* NCTC 10661
4	*P. aeruginosa* NCTC 10332
5	*B. pickettii* NCTC 11149

4.2.9 Limulus amoebocyte lysate (LAL) assays

LAL assays were carried out to assess the endotoxin activities of the different LPS preparations. Small scale preparations of *B. pseudomallei* 4845, *B. pseudomallei* HA and *B. mallei* 23344 at 2mg/ml were diluted in endotoxin-free water for use in the assay. The endotoxin activities of the typical *B. pseudomallei* LPS and the *B. mallei* LPS appear to be similar at approximately 2 endotoxin units per ng LPS, but the atypical *B. pseudomallei* LPS has higher activity at approximately 4 endotoxin units per ng LPS (Figure 41). However, protein assays showed there to be some low level protein contamination (approximately 5-10%) in the three LPS preparations, so these figures are only an indication of the endotoxin activity. It is possible that the differences in endotoxin activity between the LPS types may be due to the protein contaminants or to slight differences in the amounts of LPS tested due to the presence of the protein contamination. However, all three samples are low when compared with that of the control *E. coli* LPS.



Error bars represent the mean endotoxin activity ± standard deviation (n-1)

Figure 41. Endotoxin activity of each of the LPS preparations from *B. pseudomallei* NCTC 4845 (typical), *B. pseudomallei* HA (atypical) and *B. mallei* NCTC 23344.

4.3 Production of antibodies to LPS

4.3.1 Effect of adjuvants on the immune response to *B. pseudomallei* LPS

One of the aims of the project was to produce antibodies for the detection of *B. mallei* and *B. pseudomallei.* Antibodies of the isotype IgM were unsuitable for use in the Manual Threshold Biosensor and are also difficult to produce and purify in quantity (Kenney, J.S., Hughes, B.W. *et al.* 1989). Antibodies of the isotype IgG are therefore preferable. LPS usually induces an IgM response (Hadjipetrou-Kourounakis, L. and Moller, E. 1984). However, the use of different adjuvants may produce different immune responses with the same antigen.

LPS was administered in four different ways to see if one method promoted an IgG response. The different adjuvants were Alhydrogel™, Titremax® and Freund's incomplete adjuvant, LPS was also administered without any adjuvant. Fourteen days after each immunisation, intermediate blood samples were taken and analysed for titre and isotyping against immobilised *B. pseudomallei*. After four boosts at three week intervals the blood samples showed a difference in immune response according to the adjuvant (Figure 42).

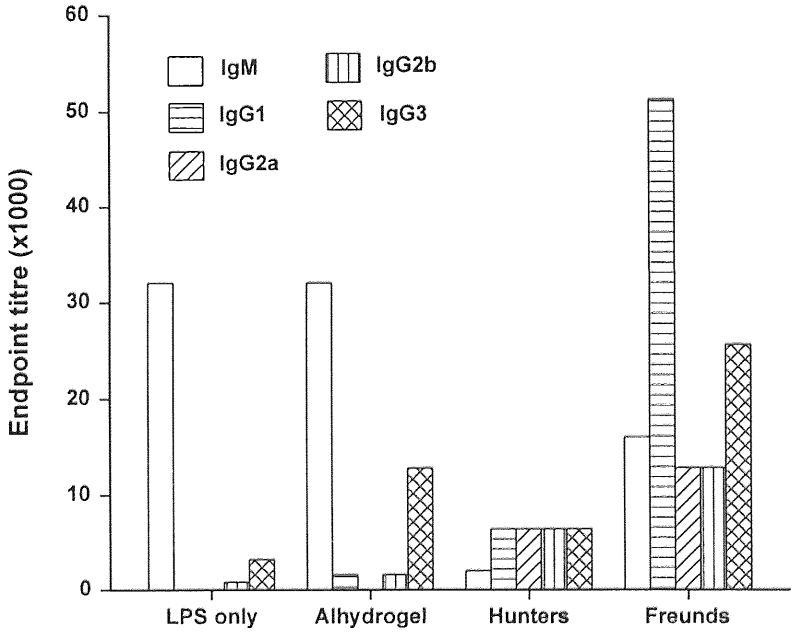


Figure 42. Serum titres from mice immunised with LPS alone and in three different adjuvants. Endpoint titre was taken as the last serum dilution which was a minimum of 0.1 absorbance units above the negative control.

LPS alone showed the weakest response with a high IgM titre, low levels of IgG_{2b} and IgG_3, and no detectable levels of IgG_1 and IgG_{2a}. LPS in combination with

Alhydrogel™ also gave a predominantly IgM and IgG_3 response, but also gave low levels of IgG_1 and IgG_{2b}. The greatest responses were from the LPS in FIA and the LPS in Titremax® groups. LPS in FIA gave high titres of all antibody isotypes whilst the LPS in Titremax® group had lower titres overall but of particular interest was the very low IgM titre (<1:4000).

4.3.2 Production of monoclonal antibodies

Only those animals in the groups immunised with *B. pseudomallei* NCTC 4845 LPS in FIA and in Titremax® were considered for fusions. Pre-fusion boosts were either 50μg *B. pseudomallei* NCTC 4845 LPS or 2 x 10^7cfu inactivated *B. pseudomallei* NCTC 4845 given intravenously three days prior to fusion. Fusions were carried out according to the method in section 2.2.5 and blood was collected from each animal to assess polyclonal antibody titre. After 14 days the fusion plates were examined for the presence of clones.

In total eight fusions were carried out. However, fusion frequency was low, producing few clones, none of which were positive when screened against immobilised *B. pseudomallei* NCTC 4845. Screening was carried out against immobilised bacteria rather than purified LPS as it is important that any antibodies produced recognise LPS as part of the whole bacterial cell rather than as purified material. It was thought that immobilising LPS onto an ELISA plate might alter its conformation.

One positive clone was identified (BA2) from a mouse immunised with LPS from *B. pseudomallei* NCTC 4845, which when tested in ELISA with immobilised *B. mallei*

and *B. pseudomallei* strains was found to bind to both species. When tested in western blot against boiled whole cell preparations it was found to bind to a 14-21kDa protein present in both species. When used in western blots against the exopolysaccharide extraction material (section 3.2.15) it appeared to recognise the 16-17kDa protein present in the preparation (Figure 43). BA2 was found in ELISA, to bind to twelve out of the twenty two *B. pseudomallei* strains (Table 21) and one of the ten *B. mallei* strains (Table 22). It would appear that this protein is found in both *B. mallei* and *B. pseudomallei* but is not present in all strains of the two species.

Fusions were also carried out using animals immunised with *B. mallei* NCTC 23344, one of which yielded a monoclonal hybridoma cell line (LF7). LF7 was found to bind to a 31-45kDa protein found in boiled whole cell preparations of both *B. pseudomallei* and *B. mallei*. When used in western blots against the exopolysaccharide extraction material (section 3.2.15) LF7 was found to bind to the 31-36kDa protein (Figure 44). LF7 was found in ELISA, to bind to ten out of the twenty *B. pseudomallei* strains screened (Table 21) and five of the ten *B. mallei* strains (Table 22). It would appear that this protein is also found in both *B. mallei* and *B. pseudomallei* but is not present in all strains of the two species.

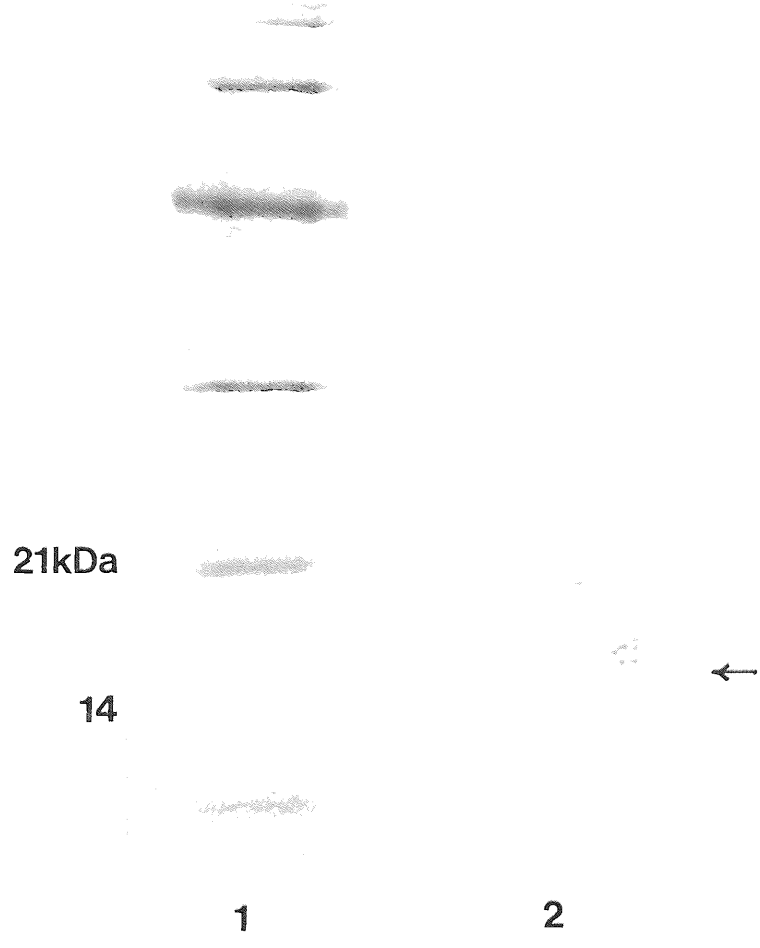


Figure 43. Western blot showing the binding of monoclonal antibody BA2 to a 14-21kDa protein found in the exopolysaccharide extraction material.

Lane	Sample
1	Molecular weight markers
2	Exopolysaccharide preparation

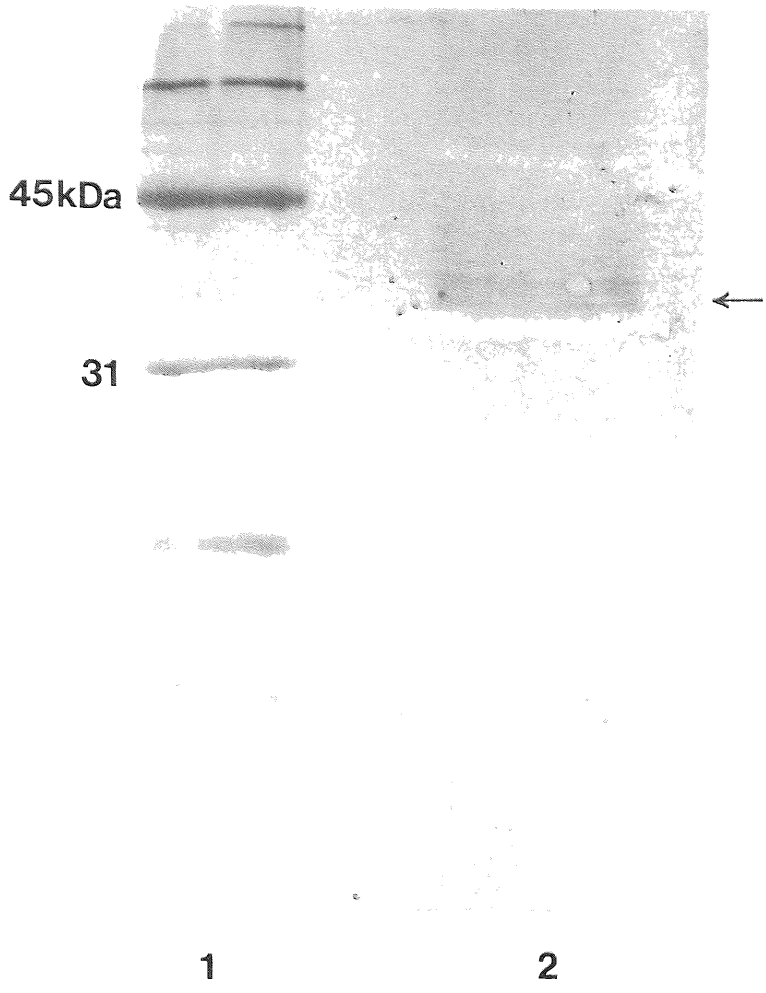


Figure 44. Western blot showing the binding of monoclonal antibody LF7 to a 31-45kDa protein found in the exopolysaccharide extraction material.

Lane	Sample
1	Molecular weight markers
2	Exopolysaccharide preparation

Strain	BA2	LF7
B. pseudomallei NCTC 4845	+	+
B. pseudomallei ATCC 23343	+	-
B. pseudomallei USAMRU-1	+	+
B. pseudomallei UBOL-2	-	+
B. pseudomallei 4889	+	-
B. pseudomallei USAMRU-21	+	+
B. pseudomallei SEARLE	-	+
B. pseudomallei 123	-	-
B. pseudomallei O	+	+
B. pseudomallei PA	+	-
B. pseudomallei BRI	-	+
B. pseudomallei E8	-	-
B. pseudomallei E25	-	+
B. pseudomallei E27 †	-	+
B. pseudomallei E82 †	-	+
B. pseudomallei 204	+	-
B. pseudomallei 576	+	-
B. pseudomallei 551a	+	nd
B. pseudomallei 603a	+	-
B. pseudomallei E38	-	-
B. pseudomallei HA	+	nd
B. pseudomallei Mal 6	-	-

† Arabinose-positive avirulent isolates

A negative result was defined as an absorbance reading of less than the corresponding negative control plus 0.1units. A positive result was defined as an absorbance reading greater than the corresponding negative control plus 0.2units. Any reading between the two was defined as borderline (±). nd indicates the result was not determined.

Table 21. Reactivity of the monoclonal antibodies BA2 and LF7 with *B. pseudomallei* strains.

Strain	BA2	LF7
B. mallei ATCC 23344	+	-
B. mallei NCTC 10230	-	+
B. mallei NCTC 120	-	-
B. mallei NCTC 3708	-	+
B. mallei NCTC 3709	-	-
B. mallei NCTC 10229	-	±
B. mallei NCTC 10245	-	-
B. mallei NCTC 10247	-	+
B. mallei NCTC 10248	-	+
B. mallei NCTC 10260	-	-

A negative result was defined as an absorbance reading of less than the corresponding negative control plus 0.1units. A positive result was defined as an absorbance reading greater than the corresponding negative control plus 0.2units. Any reading between the two was defined as borderline (±). nd indicates the result was not determined.

Table 22. Reactivity of the monoclonal antibodies BA2 and LF7 with *B. mallei* strains.

It would appear that the monoclonal hybridoma cell lines produced in fusions do not recognise LPS. Western blots were carried out with boiled whole cell preparations and pre-fusion serum from one of the mice immunised with LPS from *B. pseudomallei* NCTC 4845. The blot indicates that the animal has not only raised antibodies to the LPS, but also to the low level protein contaminants in the LPS preparations (Figure 45).

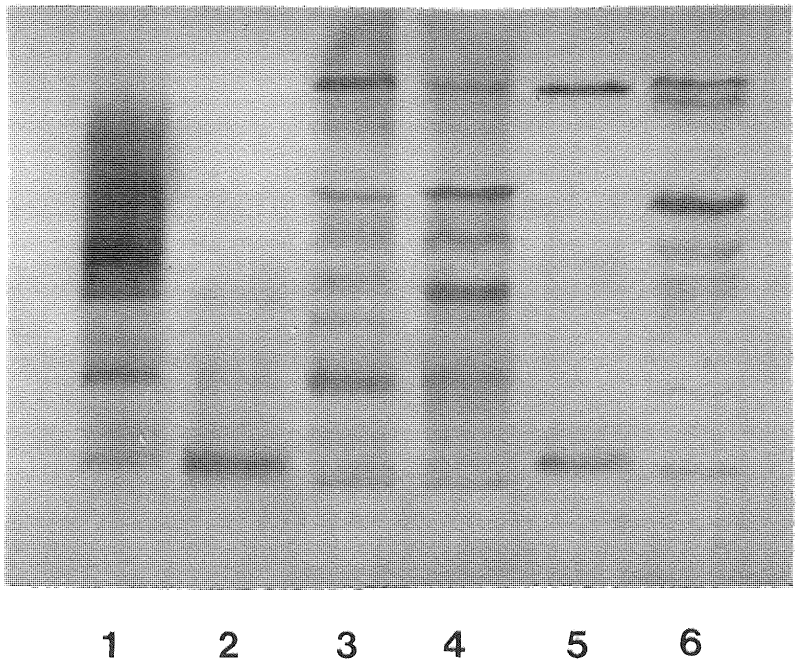


Figure 45. Western blot showing the binding of serum antibodies from a mouse immunised with *B. pseudomallei* NCTC 4845 LPS to boiled whole cell preparations.

Lane	Sample
1	*B. pseudomallei* NCTC 4845
2	*B. pseudomallei* HA
3	*B. mallei* ATCC 23344
4	*B. cepacia* NCTC 10661
5	*P. aeruginosa* NCTC 10332
6	*B. pickettii* NCTC 11149

4.3.3 Cross reactivity of polyclonal immune serum

Polyclonal serum from mice immunised with LPS was used in western blots to assess the cross-reactivity between different LPS types. Proteinase-K minipreparations from *B. pseudomallei* NCTC 4845, *B. pseudomallei* HA and *B. mallei* ATCC 23344 were used in the cross reactivity experiments.

The pre-fusion polyclonal serum from one mouse immunised with LPS from *B. pseudomallei* NCTC 4845 in Titremax® appeared only to detect the typical LPS O-antigen found in *B. pseudomallei* 4845 (Figure 46). It was anticipated that the polyclonal serum would cross react with the *B. mallei* 23344 LPS which on gels appears very similar to that of *B. pseudomallei* 4845, however this did not appear to be the case. Polyclonal sera from this animal recognised the lipid A band, which appeared to be conserved for all three LPS types (Figure 46). This is not surprising as it has been reported that lipid A part of the LPS molecule shows little variation within species and this would appear to be the case (Hammond, S.M., Lambert, P.A. *et al.* 1984). In further blots the pre-fusion sera from a second animal immunised in the same way, was found to recognise 12 strains of *B. pseudomallei*, all having the typical LPS profile, there was however no recognition of the 3 strains with the atypical profile (Figures 47 & 48).

Western blots with pre-fusion serum from a number of other animals showed different blotting profiles, some with cross reactivity between *B. mallei* LPS and the *B. pseudomallei* typical LPS. This suggests that the LPS molecules of *B. mallei* and typical *B. pseudomallei* have some shared epitopes. The pre-fusion serum, however,

did not show any cross reaction with the atypical LPS of *B. pseudomallei* which appears to be immunologically unique.

Western blots carried out with the guinea pig polyclonal serum raised against heat killed whole cells gave different results. This serum appears to recognise the LPS profiles of *B. pseudomallei* NCTC 4845 (typical), *B. mallei* NCTC 23344 and *B. pickettii* NCTC 11149, but not those of *B. pseudomallei* HA (atypical), *P. aeruginosa* NCTC 10332 and *B. cepacia* NCTC 10661 (Figure 49). ELISAs carried out with this polyclonal serum had shown detection of all six organisms, although detection of *B. pseudomallei* HA , *P. aeruginosa* and *B. cepacia* 10661 was weak (Figure 10).

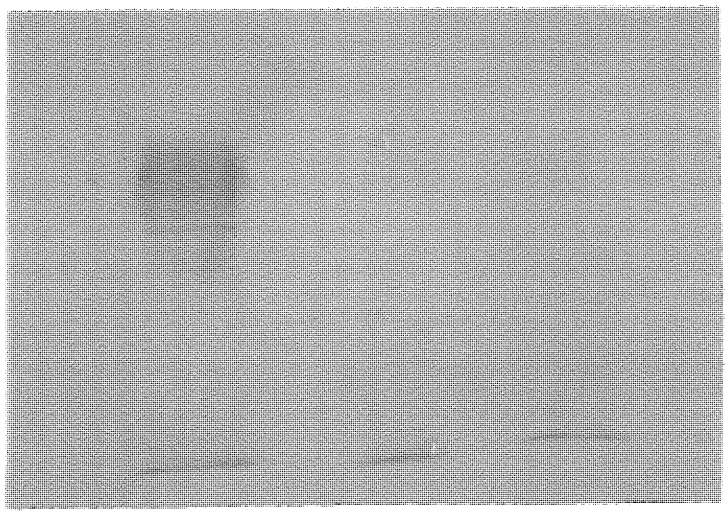


Figure 46. Western blot of pre-fusion serum from a mouse immunised with LPS from *B. pseudomallei* 4845 against proteinase-K minipreparations from *B. pseudomallei* and *B. mallei.*

Lane	Sample
1	*B. pseudomallei* NCTC 4845
2	*B. pseudomallei* HA
3	*B. mallei* ATCC 23344

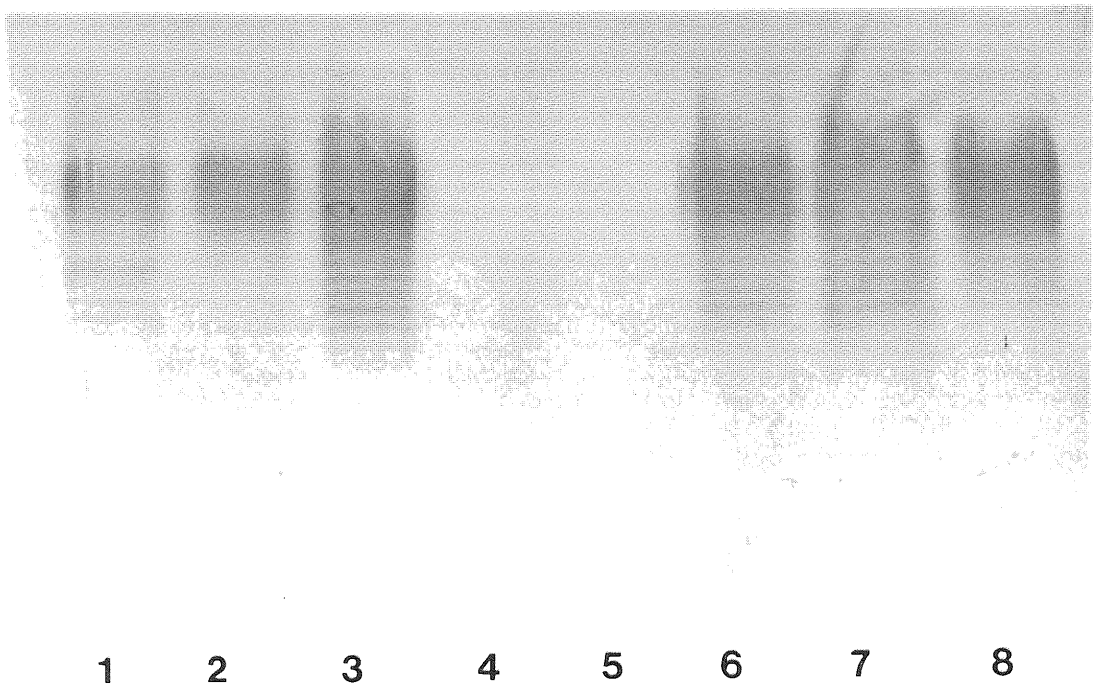


Figure 47. Western blot of pre-fusion serum from a mouse immunised with LPS from *B. pseudomallei* NCTC 4845 against proteinase-K minipreparations from different *B. pseudomallei* strains. The material loaded in lane 4 has an atypical LPS profile and lane 5 had no visible O-antigen when silver stained.

Lane	Sample
1	*B. pseudomallei* NCTC 4845
2	*B. pseudomallei* 551a
3	*B. pseudomallei* 204
4	*B. pseudomallei* 576
5	*B. pseudomallei* E8
6	*B. pseudomallei* E25
7	*B. pseudomallei* E27
8	*B. pseudomallei* E82

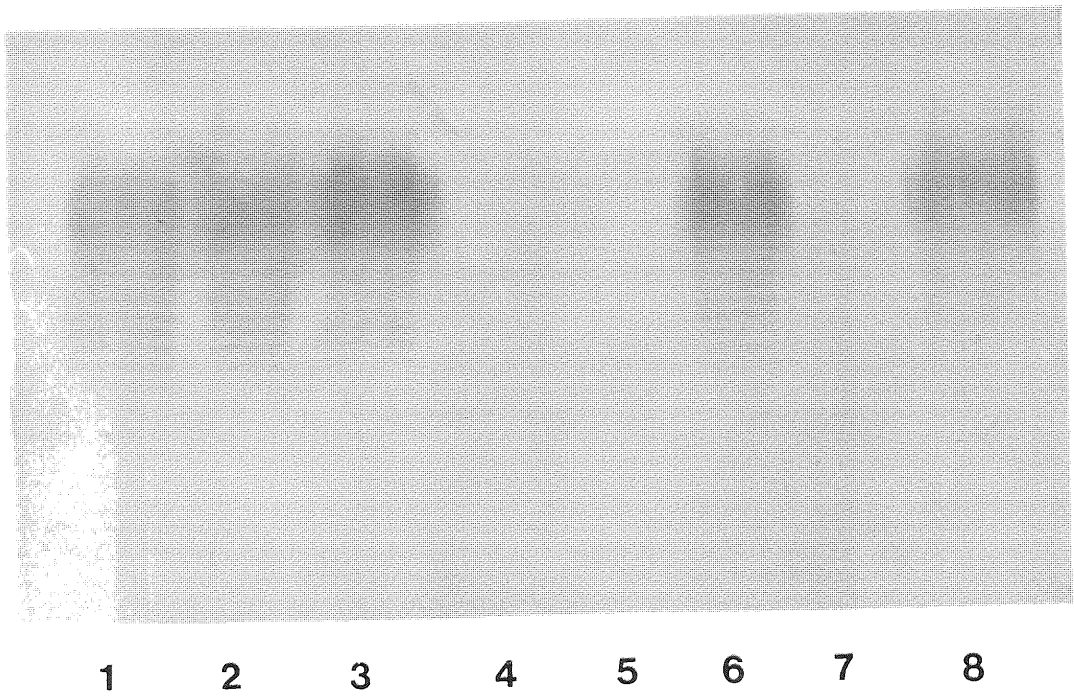


Figure 48. Western blot of pre-fusion serum from a mouse immunised with LPS from *B. pseudomallei* NCTC 4845 against proteinase-K minipreparations from different *B. pseudomallei* strains. The material loaded in lanes 4 and 5 has atypical LPS profiles and lane 7 had no visible O-antigen when silver stained.

Lane	Sample
1	*B. pseudomallei* ATCC 23343
2	*B. pseudomallei* 4889
3	*B. pseudomallei* O
4	*B. pseudomallei* 123
5	*B. pseudomallei* PA
6	*B. pseudomallei* Mal 6
7	*B. pseudomallei* E8
8	*B. pseudomallei* BRI

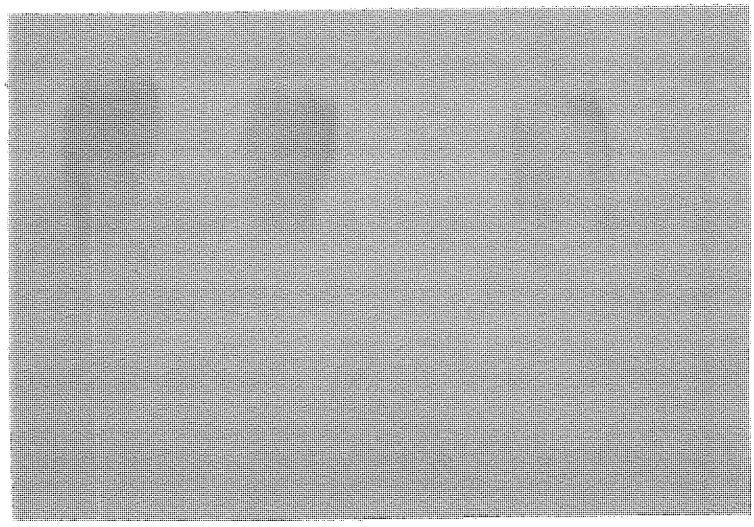


Figure 49. Western blot of guinea pig anti-*B. pseudomallei* (whole cells) polyclonal serum against proteinase-K minipreparations of different species of bacteria.

Lane	Sample
1	*B. pseudomallei* NCTC 4845
2	*B. pseudomallei* HA
3	*B. mallei* ATCC 23344
4	*B. cepacia* NCTC 10661
5	*P. aeruginosa* NCTC 10332
6	*B. pickettii* NCTC 11149

4.3.4 Production of monoclonal antibodies to LPS

Two hybridoma cell lines were produced which, when used in western blotting appear to recognise LPS.

CC6 was produced in a fusion using the spleen from a mouse immunised with LPS from *B. mallei* ATCC 23344 in Freunds incomplete adjuvant. In ELISA, CC6 was shown to recognise both *B. pseudomallei* and *B. mallei*, but did not recognise all strains of the two species. In total, CC6 bound to four of the twenty strains of *B. pseudomallei* tested (SEARLE, NCTC 4845, USAMRU-21 and UBOL-2) and six of the ten *B. mallei* strains tested (Table 23). In western blotting, the antibody showed clear recognition of LPS from *B. mallei* ATCC 23344 and faint recognition of LPS from *B. pseudomallei* NCTC 4845 (Figure 50).

Further western blots using a number of different strains of *B. mallei* showed variable recognition of the different strains (Figures 51 & 52), however, these results appear to correlate with the results found in ELISA (Table 23). Recognition of *B. pseudomallei* strains was weak both in ELISA and in western blots. The different levels of recognition may be due to differences in the quantity of LPS present in proteinase-K minipreparations loaded on the gels (10µl) compared with the number of organisms in the suspensions used to coat ELISA plates (1×10^7cfu/ml).

HC9 was produced in a fusion using the spleen from a mouse immunised with LPS from *B. pseudomallei* HA, a strain with atypical LPS. When tested in western blots, HC9 appeared to detect only the atypical LPS and shows no recognition of the typical LPS of *B. pseudomallei* NCTC 4845 or the LPS of *B. mallei* ATCC 23344 (Figure 53). This supports the theory that the atypical LPS is immunologically distinct from the typical LPS of *B. pseudomallei* and *B. mallei*.

Strain	Recognition in ELISA †	Recognition in western blots *
B. mallei ATCC 23344	+	+
B. mallei NCTC 10230	+	+
B. mallei NCTC 120	-	-
B. mallei NCTC 3708	+	±
B. mallei NCTC 3709	-	±
B. mallei NCTC 10229	±	+
B. mallei NCTC 10245	-	-
B. mallei NCTC 10247	+	±
B. mallei NCTC 10248	+	+
B. mallei NCTC 10260	+	+

† A negative result was defined as an absorbance reading of less than the corresponding negative control plus 0.1units. A positive result was defined as an absorbance reading greater than the corresponding negative control plus 0.2units. Any reading between the two was defined as borderline (±).

* A negative result was defined as no visible binding, a positive result was defined as clearly visible binding and visible but faint binding borderline (±).

Table 23. Recognition of *B. mallei* strains by the monoclonal antibody CC6 in ELISA and western blots.

1 2 3

Figure 50. Western blotting of monoclonal antibody CC6 against *B. pseudomallei* and *B. mallei* proteinase-K minipreparations.

Lane	Sample
1	*B. pseudomallei* NCTC 4845
2	*B. pseudomallei* HA
3	*B. mallei* ATCC 23344

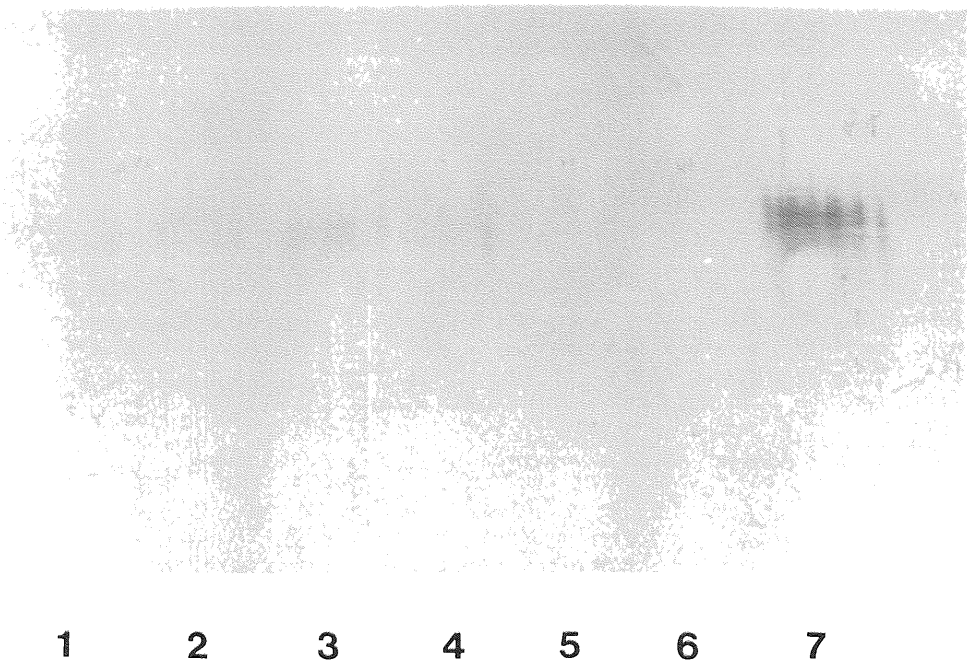


Figure 51. Western blotting of the monoclonal antibody CC6 against proteinase-K minipreparations of different *B. mallei* strains.

Lane	Sample
1	*B. pseudomallei* NCTC 4845
2	*B. pseudomallei* Mal 6
3	*B. mallei* NCTC 3708
4	*B. mallei* NCTC 3709
5	*B. mallei* NCTC 10245
6	*B. mallei* NCTC 120
7	*B. mallei* NCTC 10229

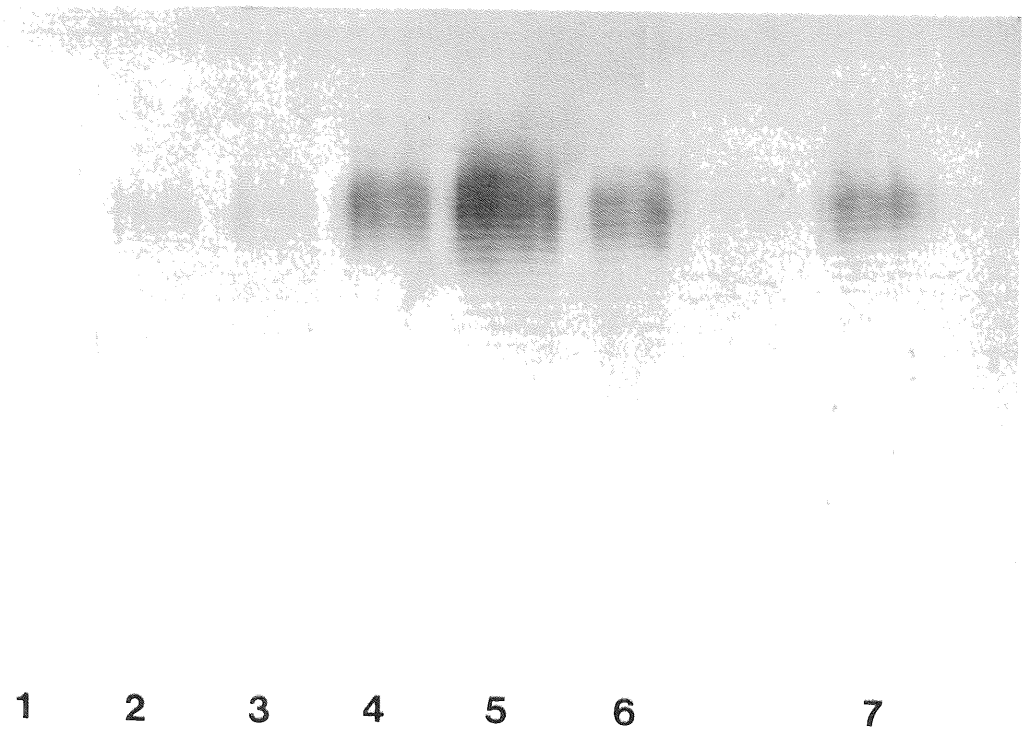


Figure 52. Western blotting of the monoclonal antibody CC6 against proteinase-K minipreparations of different *B. mallei* strains.

Lane	Sample
1	*B. pseudomallei* NCTC 4845
2	*B. pseudomallei* Mal 6
3	*B. mallei* NCTC 10247
4	*B. mallei* NCTC 10248
5	*B. mallei* NCTC 10230
6	*B. mallei* NCTC 10260
7	*B. mallei* ATCC 23344

Figure 53. Western blotting of the monoclonal antibody HC9 against *B. pseudomallei* and *B. mallei* strains.

Lane	Sample
1	*B. pseudomallei* NCTC 4845
2	*B. pseudomallei* HA
3	*B. mallei* ATCC 23344

4.3.5 Comparison of typical *B. pseudomallei* and *B. mallei* LPS

Serum taken from mice infected with *B. mallei* has been shown to clearly recognise *B. mallei* LPS and faintly recognise *B. pseudomallei* LPS (Figure 29). This difference in binding may be due to a number of factors, including differences in the amounts of LPS loaded on the gel or to differences in the structures of the LPS molecules. To determine whether *B. mallei* LPS has unique epitopes not present in the typical LPS of *B. pseudomallei*, the serum was incubated with *B. pseudomallei* cells (10^9 cells/ml serum) at 37°C. After incubation, the cells were removed by centrifugation and the serum used in western blotting.

Proteinase-K preparations of *B. pseudomallei* NCTC 4845 and *B. mallei* ATCC 23344 LPS were used to compare antibody binding before and after absorption. Figure 54 shows that after absorption with *B. pseudomallei* cells there is no residual binding to *B. mallei* LPS at all. Antibodies to unique epitopes present in *B. mallei* should not have been removed by absorption and would therefore have bound during the blotting process. There is no evidence of this, and this would suggest that the LPS molecules of *B. mallei* and *B. pseudomallei* are very similar.

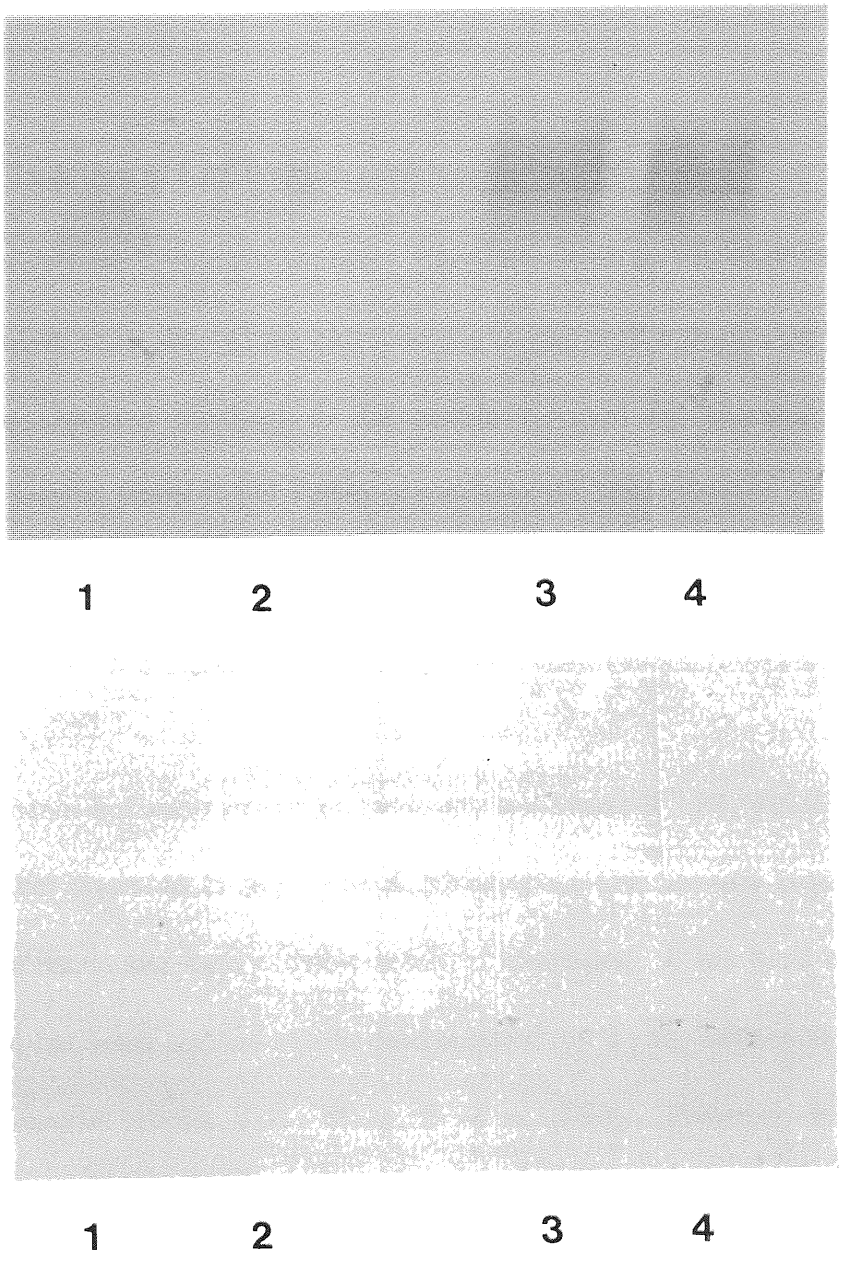


Figure 54. Western blot showing the difference in antibody binding before (upper) and after absorption (lower) with *B. pseudomallei* NCTC 4845 cells.

Lane	Sample
1	*B. pseudomallei* NCTC 4845
2	*B. pseudomallei* NCTC 4845
3	*B. mallei* ATCC 23344
4	*B. mallei* ATCC 23344

4.4 Discussion

4.4.1 Isolation of LPS from *B. pseudomallei* NCTC 4845

The proteinase-K minipreparations, when separated by electrophoresis and stained with silver stain showed the characteristic ladder profile associated with LPS. This profile appeared identical to the profile reported by Kawahara who used *B. pseudomallei* strain GIFU 12046 (Kawahara, K., Dejsirilert, S. *et al.* 1992). This similarity indicates that the material isolated and stained is the lipopolysaccharide of *B. pseudomallei*.

The phenol-hot water extraction gave very low yields of LPS, which when compared with that produced in proteinase-K minipreparations, showed large low molecular weight bands and barely visible levels of O-antigen. It has been reported that the phenol-hot water method can be used successfully for the extraction of LPS from *B. pseudomallei* (Bryan, L.E., Wong, S. *et al.* 1994). However, in the case of *B. pseudomallei* NCTC 4845 this did not appear to be the case. This agrees with the findings of Kawahara who reported that extraction of LPS with phenol/hot water resulted in a low yield with no O-antigen ladder (Kawahara, K., Dejsirilert, S. *et al.* 1992). A different extraction method, that of Galanos (Galanos, C., Luderitz, O. *et al.* 1969) normally used for the extraction of rough LPS was reported to give a better yield of LPS from *B. pseudomallei* strain (Kanai, K. and Kondo, E. 1994). Chart suggested that close association of LPS molecules with hydrophobic membrane proteins affects the phenol-hot water extraction and the LPS does not enter the aqueous phase but remains in the phenol phase (Chart, H. 1994a). It was suggested

that the extraction could be improved by carrying out an outer membrane preparation prior to phenol-hot water extraction.

4.4.2 O-antigen profiles of *B. pseudomallei* LPS

It has been reported that the LPS of *B. pseudomallei* is conserved throughout the species (Kanai, K. and Kondo, E. 1994). Twelve different strains of *B. pseudomallei* were shown to have very similar proteinase-K profiles and produced a characteristic ladder pattern with almost identical spacing and position of bands (Pitt, T.L., Aucken, H. *et al.* 1992). This characteristic ladder pattern has been found with eighteen of the twenty-two strains of *B. pseudomallei* used in these investigations. However, four of the strains were found to have a different proteinase-K profile, when silver stained. The bands appear to be of a higher molecular weight and to have a greater spacing between them (Figure 36, lanes 4 and 5). It is possible that the repeating oligosaccharide units in the atypical LPS structure are of a higher molecular weight than those found in the typical LPS profile, and this difference in molecular weight affects the banding pattern seen in silver stained gels. This atypical profile has not been described. However, one study of 214 *B. pseudomallei* isolates noted that 210 isolates had identical proteinase-K profiles and 4 appeared to be different (Anuntagool, N., Intachote, P. *et al.* 1998). Work carried out recently at the PHLS in London has confirmed that the atypical LPS is found in *B. pseudomallei* strain 576 (T. L. Pitt, personal communication).

Recent work has shown the presence of two distinct O-antigens in *B. pseudomallei* strain 304b, termed O-PS I and O-PS II, present in approximately equal proportions.

O-PS I has been shown to consist of an unbranched, high molecular weight homopolymer, whilst O-PS II is an unbranched heteropolymer with repeating D-glucose and L-talose units (Perry, M.B., MacLean, L.L. *et al.* 1995). These results correlate with those of a second group which also found two different O-antigens on a single strain of *B. pseudomallei* (Knirel, Y.A., Paramonov, N.A. *et al.* 1992). Comparison of these two O-antigens with those described here is not possible as the data in both cases is presented as mass spectroscopy profiles.

4.4.3 LPS structure and virulence

LPS is a known virulence determinant of gram-negative bacteria (Reeves, P. 1995) such as *P. aeruginosa* (Pitt, T.L. 1989), *Yersinia pestis* (Albizo, J.M. and Surgalla, M.J. 1970), *Salmonella spp.* (Islam, A.F., Moss, N.D. *et al.* 2000) and *Brucella spp.* (Eisenschenk, F.C., Houle, J.J. *et al.* 1999) and has been linked with virulence in *B. pseudomallei* (Bryan, L.E., Wong, S. *et al.* 1994). *B. pseudomallei* strains E27 and E82 are avirulent, arabinose-positive isolates and are potentially a different species, *B. thailandensis* (Brett, P.J., DeShazer, D. *et al.* 1998). When silver stained after electrophoresis, proteinase-K LPS preparations from both E27 and E82 have the same LPS profile as that of LPS from virulent *B. pseudomallei* strains. Polyclonal sera from animals immunised with LPS from *B. pseudomallei* NCTC 4845 recognises LPS from the two avirulent *B. pseudomallei (B. thailandensis)* strains as well as the other *B. pseudomallei* strains. This correlates with the findings of Anuntagool *et al.* (1998) who showed that the LPS of avirulent, arabinose-positive *B. pseudomallei* (*B. thailandensis*) isolates is immunologically indistinguishable from

virulent arabinose-negative *B. pseudomallei* clinical isolates using polyclonal serum from melioidosis patients (Anuntagool, N., Intachote, P. *et al.* 1998).

Avirulent, arabinose positive *B. pseudomallei* (*B. thailandensis*) strains are reported to only carry the O-PS II LPS side chain (DeShazer, D., Brett, P.J. *et al.* 1998). Genetic mutants deficient in O-PS II were found to be sensitive to the bactericidal effect of serum in the presence of complement and were found to be less virulent in the animal model of disease. It has been proposed therefore, that O-PS II is required for serum resistance and virulence (DeShazer, D., Brett, P.J. *et al.* 1998). A recent serological study has indicated that antibodies to O-PS II are protective against fatal melioidosis (Charuchaimontri, C., Suputtamongkol, Y. *et al.* 1999). The monoclonal antibody CC6 developed in this work recognised four strains of *B. pseudomallei*, all of which are thought to be virulent. Further studies would be needed to confirm whether CC6 binds to O-PS I or O-PS II.

The presence of the atypical *B. pseudomallei* LPS profile does not appear to be linked with virulence. *B. pseudomallei* strains 576 and HA, both of which have the atypical LPS profile are both isolates from human infection and are known to be virulent. Polyclonal serum raised against the typical LPS of *B. pseudomallei* NCTC 4845 showed no recognition of the atypical LPS of *B. pseudomallei* HA. This would suggest that the atypical LPS profile is not made up of either O-PS I or O-PS II, as antibodies to both side chains would be present in polyclonal serum. In addition, the monoclonal antibody HC9 appears only to recognise the atypical structure. These results suggest that the atypical LPS is immunologically different to the typical LPS

found in the majority of *B. pseudomallei* strains. Techniques such as mass spectroscopy could be used to further investigate the atypical LPS structure.

4.4.4 O-antigen profiles of *B. mallei* LPS

Proteinase-K minipreparations of *B. mallei* LPS, when silver stained show a profile very similar to that of the typical *B. pseudomallei* profile. This is not surprising as *B. pseudomallei* and *B. mallei* are known to be very closely related and to share many common epitopes (Yakovleva, I.V., Sviridov, V.V. *et al.* 1995). Silver staining of the proteinase-K minipreparations from the ten different strains of *B. mallei* show a conserved profile that appears to vary little between the different strains.

The O-antigen profile of *B. mallei* LPS has not been published but it would appear to be similar to that of typical *B. pseudomallei* strains. Polyclonal serum raised against the LPS of typical *B. pseudomallei* shows some cross reactivity *with B. mallei* and would, therefore suggest that the two molecules share some common epitopes.

Polyclonal sera from a number of different animals showed some variability in the levels of cross reactivity between *B. mallei* and *B. pseudomallei* LPS and this could be due to a number of reasons. It may be that the natural heterogeneity of polyclonal serum means that different batches recognise LPS to different degrees or it may be that the LPS molecules from *B. pseudomallei* and *B. mallei* have unique epitopes as well as shared ones and that the different batches of serum are recognising these different epitopes. CC6, a monoclonal antibody raised against *B. mallei* LPS also shows some recognition of the typical LPS from strains of *B. pseudomallei*.

Serum reactive against *B. mallei* LPS showed no recognition of either *B. pseudomallei* or *B. mallei* LPS after absorption with *B. pseudomallei* cells. This would suggest that the two LPS molecules are very similar and that epitopes are shared by the LPS from both species. Antibodies to unique epitopes present on *B. mallei* LPS would not have been absorbed and should have been seen in the blots carried out with the absorbed serum.

Characterisation of the LPS structure of *B. mallei* would only be possible by mass spectroscopy as the chemical techniques are not sensitive enough to show fine differences between the LPS molecules of the two species. An alternative isolation method would have to be used for the isolation of LPS for further characterisation. Mass spectroscopy studies would require a highly purified LPS sample which was not contaminated by exopolysaccharide or protein components. Studies carried out with *B. pseudomallei* LPS have used the phenol-hot water method for isolating LPS, and it is possible that this extraction technique would be more successful with *B. mallei* strains than with *B. pseudomallei* NCTC 4845.

4.4.5 Antibodies to LPS

The production of antibodies to LPS proved difficult, particularly the production of monoclonal antibodies. This may be due to difficulties raising a good immune response to LPS molecules. Adjuvants are used primarily to influence the titre and duration of an antibody response. However, they are also able to induce subtle variations in immune responses and influence the specificity of antibodies. It was

thought that the use of different adjuvants might promote the production of IgG antibodies to LPS (Hunter, R.L., Olsen, M.R. *et al.* 1995). The LPS of Gram negative bacteria can itself act as an adjuvant as it is known to function as a B cell mitogen (Allison, A.C. 1979). Mitogens are agents which activate many clones of T and B cells irrespective of their antigen specificity. This mitogenic activity is due to the lipid A moiety, which is thought to interact with the plasma membrane resulting in a cellular activation signal through as-yet-unknown mechanisms (Louis, J.A. and Lambert, P. 1979; Kuby, J. 1992).

Immunisation with LPS alone gave a poor response, mainly of IgM isotype antibodies with a low titre of IgG_3 antibody. This was to be expected as the normal antibody response to polysaccharide antigens in the mouse is IgM and IgG_3 (Hadjipetrou-Kourounakis, L. and Moller, E. 1984). The use of Alhydrogel™ as an adjuvant with LPS appeared to increase the levels of IgG_3 antibody, whilst the IgM antibody titre was similar as using LPS alone. In mice, aluminium adjuvants are reported to stimulate IgG_1 and IgE responses but do not stimulate IgG_{2a}. However, they are unable to provide a good immune response against T-independent antigens such as polysaccharides.

The presence of IgE was not investigated, and it is possible that the titres of IgM and IgG_3 were the natural response to LPS immunisations enhanced slightly by the effect of adjuvant (Lindblad, E.B. 1995). LPS in Titremax® adjuvant gave low antibody titres overall, but they were evenly spread over the IgG subclasses, and the titre of IgM antibody was very low. Titremax® is reported as having been developed for use

in antibody production and is designed to stimulate all subclasses of IgG in mice comparable with the use of FIA (Bennett, B., Check, I.J. *et al.* 1992).

Overall titres with Titremax® were lower than those obtained with FIA, however, it was recommended by the manufacturers that Titremax® be administered intramuscularly whilst the FIA emulsion was given by intraperitoneal injection. Intramuscular injections in mice proved very difficult and it is possible that the Titremax® group received lower doses than the other groups. Use of the adjuvant FIA gave high titres of IgG_1 and IgG_3 and gave the highest overall titres of all the groups. FIA is known to induce predominantly IgG_1 antibodies in Balb/c mice (Kenney, J.S., Hughes, B.W. *et al.* 1989), but both FIA and Titremax® produced titres of IgG_3 which is slightly surprising. This may be due to the powerful immunomodulatory effect of LPS and the natural immune response to polysaccharide antigens.

B cells recognise soluble antigen when it binds to their membrane bound antibody. Because the antigen is free in solution , the epitopes recognised tend to be accessible, hydrophilic sites on the surface of the immunogen (Kuby, J. 1992). It is possible that the LPS formed a suspension and was not freely available to the B cells so that low levels of specific antibody were produced. Polyclonal activation of B cells non-specifically by LPS could explain why the majority of hybridoma clones produced during the monoclonal fusions were not positive for the immunogen.

Two of the monoclonal antibodies produced (BA2 and LF7) were to protein antigens, probably present in the immunising preparation as contaminants, so it is likely that the mice reacted to these low level contaminants producing an immune response. This is confirmed in Figure 45, which shows serum from an LPS immunised mouse reacting with proteins present not only in *B. pseudomallei* and *B. mallei* but also in other closely related bacterial species.

BA2, which bound to a 14-21kDa protein, only recognised 12 of the 22 strains of *B. pseudomallei* tested. The ten strains not recognised by BA2 do not appear to be linked in any way. Six out of ten are soil isolates, two being the avirulent arabinose-positive strains, two are human isolates and two are unknown. BA2 shows recognition of only a single *B. mallei* strain of the ten tested. It would appear that the 14-21kDa protein is not present in the majority of *B. mallei* strains and therefore has the potential to discriminate between the species. However, given that it is only found in approximately 50% of *B. pseudomallei* strains, the potential use as a antigen for detection assays is limited.

LF7, binds to a 31-45kDa protein, and recognises 10 of the 20 *B. pseudomallei* strains tested. Again there appears to be no pattern in the recognition. LF7 recognises both the avirulent arabinose-positive isolates but does not recognise other environmental isolates such as E38. LF7 also does not recognise recent clinical isolates such as 204 and 576. LF7 shows clear recognition of four of the ten *B. mallei* strains tested and borderline recognition of one *B. mallei* strain. It appears

that this 31-45kDa protein is distributed throughout the two species but is not present in every strain of the two species.

The two monoclonal antibodies produced which do recognise LPS, reveal some differences in the LPS profiles of typical and atypical strains of *B. pseudomallei*. The atypical LPS appears in only a small number of strains but would appear to be antigenically different to the typical LPS profile. Polyclonal serum from animals immunised with the typical LPS does not recognise the atypical LPS in western blots. Preliminary studies carried out with the monoclonal antibody, HC9, also supports this theory. Further studies will need to be carried out to further characterise the binding of HC9.

B. mallei LPS and the typical *B. pseudomallei* LPS appear to share common epitopes and although it is possible that these LPS molecules are not identical the current evidence would appear to suggest that the two molecules are very similar. It is unknown whether *B. mallei* LPS contains the two side chains O-PS I and O-PS II found in *B. pseudomallei*. The monoclonal antibody CC6, binds to both *B. pseudomallei* and *B. mallei* strains but with varying effectiveness. This variability may be due to differences in the amounts of total LPS in strains, a difference in the proportions of the two side chains or to differences in structure.

4.4.6 Properties of the LPS from *B. pseudomallei* and *B. mallei*

The LAL assay gives some indication of the endotoxin activity of each of the LPS preparations. The atypical LPS of *B. pseudomallei* showed higher activation of the

LAL assay than the typical *B. pseudomallei* LPS. The endotoxin activity of the *B. mallei* LPS appeared very similar to that of the typical *B. pseudomallei* LPS. This similarity is, perhaps, not surprising, given the visual and immunological similarity between these two LPS preparations. However, when compared with the control LPS from *E. coli* the activity of both types of *B. pseudomallei* LPS and the *B. mallei* LPS were low.

The endotoxicity of *B. pseudomallei* LPS has been reported as lower than that of other Gram negative bacteria (Matsuura, M., Kawahara, K. *et al.* 1996). It is thought to be comparable with the endotoxicity of the LPS of *P. aeruginosa*, and to cause a loss in body weight in laboratory mice. It is thought that the elevated temperatures found in clinical cases of *B. pseudomallei* infection may be due to endotoxin although other factors may also be involved. The endotoxic portion of *P. aeruginosa* LPS is significantly less toxic than that of *E. coli* and *Salmonella typhimurium* and differs in the structure of the lipid A. Although *P. aeruginosa* lipid A is less toxic than that of enteric bacteria, it is sufficiently toxic in humans to have precluded the widespread use of LPS-based vaccines (Goldberg, J.B. and Pler, G.B. 1996).

4.4.7 Use of anti-LPS antibodies in detection and diagnosis

It has been reported that an IgM monoclonal antibody (5F8) to the LPS of *B. pseudomallei* was both specific and sensitive when used in immunofluorescence, and reacted with 56 different isolates in ELISA (Rugdech, P., Anuntagool, N. *et al.* 1995). It is assumed that this monoclonal antibody was screened against *B. pseudomallei* strains with typical LPS profiles and it is unknown whether it would

detect those strains with atypical profiles. The monoclonal antibody was found not to cross-react with other organisms such as *B. cepacia*, but the reactivity with *B. mallei* was not tested. It is likely that the similarity in profiles between *B. mallei* and *B. pseudomallei* LPS would cause cross reactivity.

LPS antigens have been used in ELISA to detect anti-*B. pseudomallei* LPS antibodies in clinical cases of infection. It was found to be sensitive and specific and to compare favourably with other published diagnostic tests (Petkanjanapong, V., Naigowit, P. *et al.* 1992).

4.4.8 Use of LPS as a vaccine candidate

Antibodies raised against tetanus toxoid-conjugated O-antigen were used passively to immunise diabetic rats (Bryan, L.E., Wong, S. *et al.* 1994). Administration of partially purified antibody at the time of infection gave high levels of protection in this model. The mechanism of protection is not known but the authors suggest it could be due to enhanced phagocytic activity. It has been suggested that this conjugate represents a potential vaccine candidate, however, one potential problem with vaccines developed from LPS is toxicity. The authors expect toxicity to be low but elevated temperatures were noted in animals used for the production of antibodies. Pyrogenicity would be expected unless the vaccine could be adequately purified, removing contaminating LPS to below detectable levels (Bryan, L.E., Wong, S. *et al.* 1994).

A protective monoclonal antibody recognising O-PS II has been found to enhance phagocytic killing in the presence of complement and it is thought that anti-LPS antibodies may play an important role in the initial clearance of the organism (Ho, M., Schollaardt, T. *et al.* 1997).

Passive protection has also been demonstrated using antibodies to the purified flagellin protein of *B. pseudomallei*. It has therefore been proposed that a conjugate molecule made up of the polysaccharide moiety from *B. pseudomallei* LPS and the flagellin protein would incorporate two protective antigens from the same organism. Initial studies have shown these conjugates to be immunogenic, producing high antibody titres and are expected to be protective (Brett, P.J. and Woods, D.E. 1996).

Polysaccharide vaccines are in use for organisms such as *Haemophilus influenzae* type B (Hib), *Neisseria meningitidis*, *Streptococcus pneumoniae* and *Salmonella typhi* (Salisbury, D.M. and Begg, N.T. 1996). However, polysaccharide antigens are poorly recognised by macrophages and T-cells in the immature immune system, so specific antibodies are not generated nor can the response be boosted (Anon 1993b). Modern *Haemophilus influenzae* type B vaccines rely upon linkage of a protein (such as diphtheria and tetanus toxoids) to enhance immunogenicity in the very young (under 18 months) (Anon 1993a).

Adverse reactions to the Hib vaccine are reported as local and mild, and reactions with typhoid polysaccharide vaccine are halved compared to the whole cell vaccine. The potential of polysaccharide vaccines is limited only by their ineffectiveness in young children and in the case of *B. pseudomallei* this may be overcome by the conjugation of the O-polysaccharide to the flagellin protein.

5. Production of single chain antibodies (scFv)

5.1 Introduction

5.1.1 Antibody engineering

Antibodies are universally used as research and diagnostic tools and any improvement in the methods used to generate them would be useful. As the structure of antibodies has been identified, ways of engineering and manipulating antibody structure in order to produce smaller fragments and to change the specific affinity or avidity of the antibody have been investigated (Garrard, L.J., Yang, M. *et al.* 1991). These engineered antibodies have a number of applications in both therapy and in the identification of biological agents. The ability to produce antibody fragments in bacterial and other novel expression systems enables these antibody fragments to be produced and purified in large quantities, eliminating the need for expensive and time consuming animal work or cell culture. The technique also has the potential of producing an enormous repertoire of antibody specificities without the limitations of immunisations and hybridoma technology that complicate the production of monoclonal antibodies (Hozumi, N. and Sandhu, J.S. 1993).

5.1.2 Antibody fragments

The structure of an IgG molecule is shown in Figure 6. Proteolytic cleavage of antibodies with papain or pepsin yields three types of antibody fragment: Fab, Fc or $F(ab)_2$. $F(ab)_2$ fragments are two Fab fragments linked by the hinge region and the

disulphide bonds (Figure 55). These fragments have been vital in determining the structure and function of the immunoglobulin molecule. The Fab region is concerned with binding to antigen, while the Fc region mediates effector functions such as complement fixation, monocyte binding and placental transmission (Kuby, J. 1992). The variable domains of the heavy (V_H) and light (V_L) chains associate tightly as an Fv fragment and bind to antigen with similar affinity to that of the parent antibody (Skerra, A. and Pluckthun, A. 1988).

Genetic manipulation techniques been developed to produce Fab fragments by cloning the genes that encode for the variable and the first constant domain of the heavy and light chains into an expression vector and expressing the fragments in *E. coli*. Single chain antibodies are novel recombinant polypeptides, composed of the V_H and the V_L amino-acid sequences. To allow expression of both domains on the same polypeptide and to stabilise the fragment, they are joined by a flexible linker $(Gly_4\text{-}Ser)_3$. The resulting antibody fragments are known as single chain antibodies or scFv (McCafferty, J., Griffiths, A.D. *et al.* 1990; Bird, R.E., Hardman, K.D. *et al.* 1988).

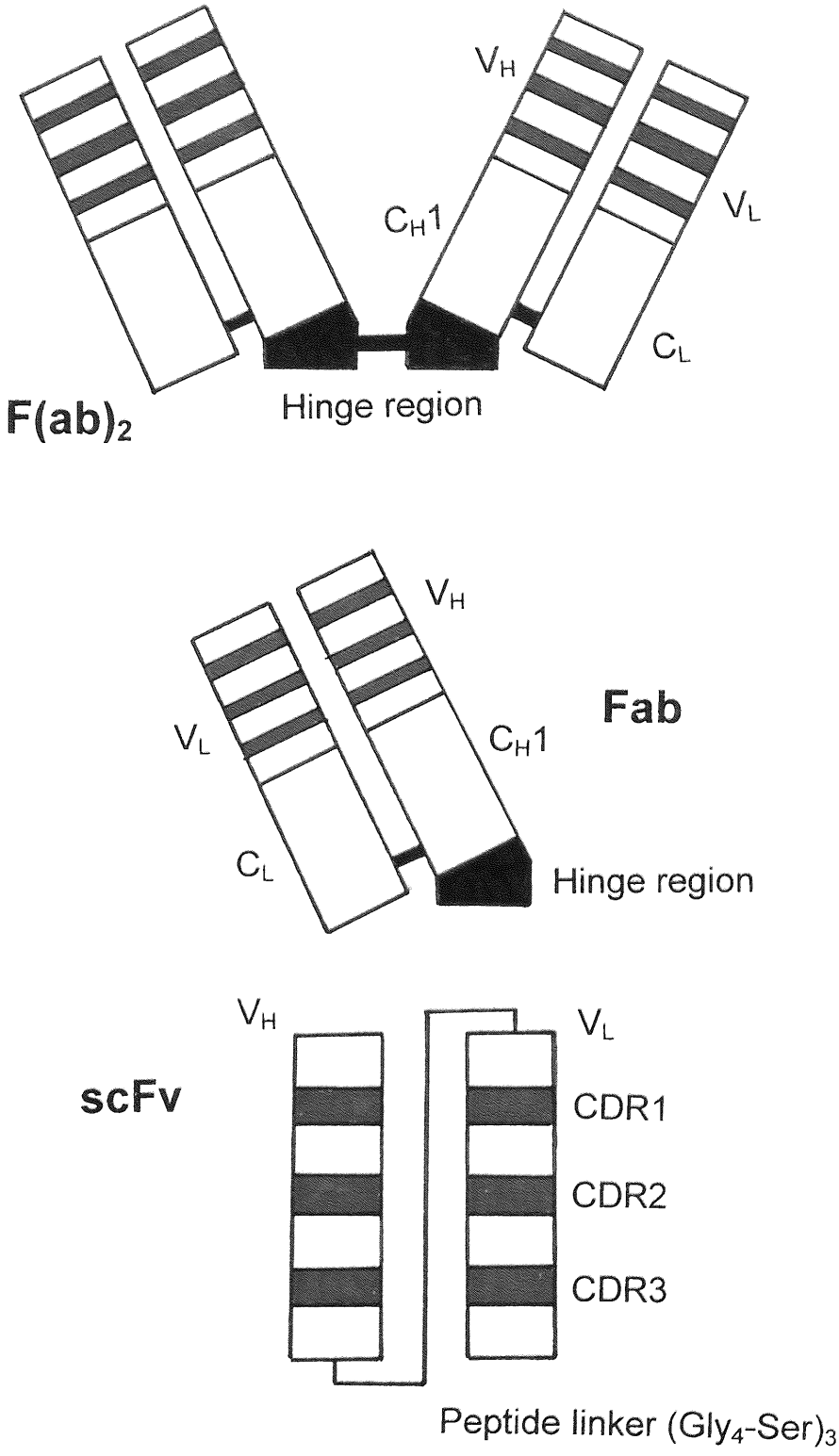


Figure 55. Types and structures of antibody fragments.

5.1.3 Generation of scFv DNA

Material for the production of scFv may be obtained from a mouse immunised in the normal way for the production of monoclonal antibodies. The spleen is removed and snap frozen in liquid nitrogen before the messenger RNA is isolated and used as a template to produce cDNA. The cDNA is then used as a template for PCR amplification of the V_H and V_L domains. Specific primers have been designed for the amplification of mouse V_H and V_L genes and have been shown to generate a diverse library when used to prepare a repertoire of antibodies from an immunised mouse. Single chain antibody DNA is then produced by ligating the V_H and V_L domains together, followed by further amplification (Krebber, A., Bornhauser, S. *et al.* 1997).

Alternatively a hybridoma cell line may be used as a source of RNA. The RNA is then used to produce cDNA and amplified using the same primers. Monoclonal antibody cell lines often contain more than a single heavy and single light chain sequence. Up to 10 different scFv clones have been obtained from a single monoclonal cell line (McCafferty, J., Hoogenboom, H.R. *et al.* 1996).

5.1.4 Phage display libraries

To allow large repertoires of antibody affinities to be cloned and produced in *E. coli*, a method was developed to produce the antibody fragments as fusion proteins with phage outer coat proteins. The scFv DNA is cloned into a phagemid vector at the N-terminal region of the gene III protein. Phagemids are plasmids that can be packaged into phage particles, but require the use of helper phage. The gene III protein is normally expressed at the tip of the phage (about three copies per virion) and is

responsible for attachment of phage to the bacterial F pilus. Phage express recombinant antibody fragments as a fusion protein with the gene III protein on the tip of the phage. The use of helper phage also reduces the valency of the gene III protein and so the phage particles then display a single (or possibly none) recombinant antibody fragment and are termed monovalent (Krebber, A., Bornhauser, S. *et al.* 1997; Hoogenboom, H.R., Marks, J.D. *et al.* 1992). Figure 56 shows the sequence of events used to clone and express scFv DNA in *E. coli.*

Phage displayed antibodies can be purified from mixtures of other phage using antigen bound to a solid surface, binding to biotinylated antigen or using a column matrix. These methods can select for high-affinity antibody fragments by limiting the amount of antigen available. Phage bound to a solid surface are eluted by dropping or raising the pH resulting in an enriched phage suspension. By growing the enriched phage and carrying out further rounds of selection, enrichments of up to a millionfold can be obtained. Even when enrichments are low, several rounds of selection can lead to the isolation of rare phage particles (McCafferty, J., Griffiths, A.D. *et al.* 1990; Hawkins, R.E., Russell, S.J. *et al.* 1992; Hoogenboom, H.R., Marks, J.D. *et al.* 1992).

5.1.5 Advantages of antibody fragments

Single-chain antibodies are expected to have advantages in clinical applications because of their small size. These proteins should be cleared from serum faster than monoclonal antibodies or Fab fragments and because they lack the Fc region of an antibody they should be less immunogenic. Single chain antibodies are likely to be

utilised in applications which currently use monoclonal antibodies such as immunotherapy, purification, detection and diagnosis (McCafferty, J., Griffiths, A.D. *et al.* 1990).

In principle the use of phage display libraries from naïve antibody genes might allow antibodies to be made entirely *in vitro*: higher affinity variants might be made by random mutation of the genes, so by-passing the need for immunisation (Hoogenboom, H.R., Griffiths, A.D. *et al.* 1991; Duenas, M., Chin, L.T. *et al.* 1996). Libraries can be constructed from unimmunised blood donors and antibody engineering techniques used to produce human antibodies. This could be of considerable importance in the production of therapeutic human monoclonal antibodies (Marks, J.D., Hoogenboom, H.R. *et al.* 1991). The production of human recombinant antibodies could widen the use of immunotherapy, a technique presently limited by the use of murine or humanised antibodies. Production of human monoclonal antibodies has been limited by the absence of a reliable fusion partner and the use of peripheral blood lymphocytes as partners. The obvious inability to immunise the human has also limited the potential of the technique (Matthews, R.C. and Burnie, J.P. 1994).

5.1.6 Aims

The production of monoclonal antibodies to the LPS of *B. pseudomallei* and *B. mallei* proved difficult using conventional methods so it was decided to attempt to produce *B. pseudomallei* reactive single chain antibody fragments using antibody engineering.

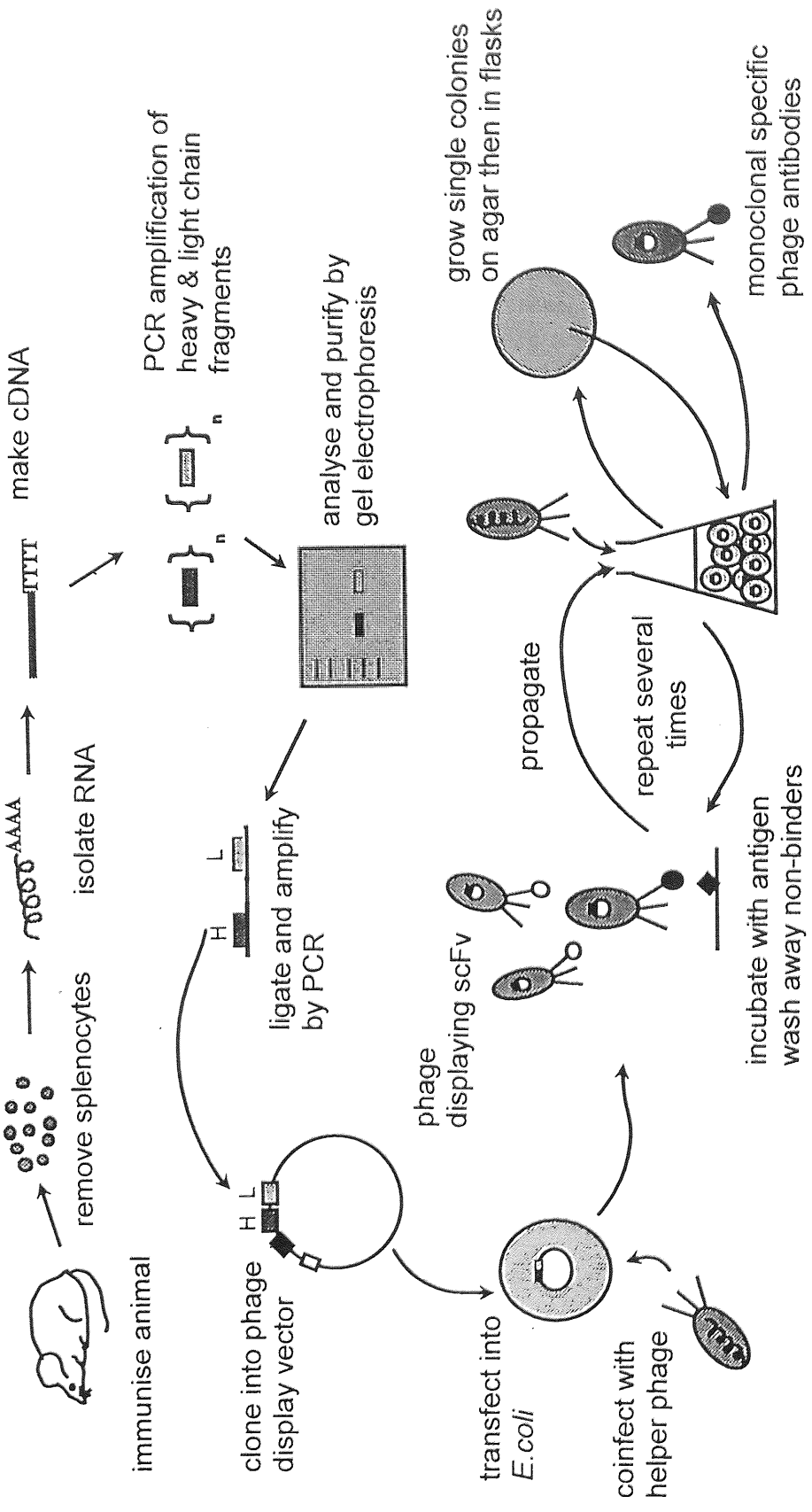


Figure 56. Flow diagram depicting the construction of a phage library.

5.2 Production of single chain antibodies from a spleen library

5.2.1 Antibody activity

The serum obtained from the mouse prior to splenectomy was screened for specific antibody activity by titrating the serum against immobilised *B. pseudomallei* NCTC 4845. Figure 57 shows that the levels of specific antibody present in the serum were not high.

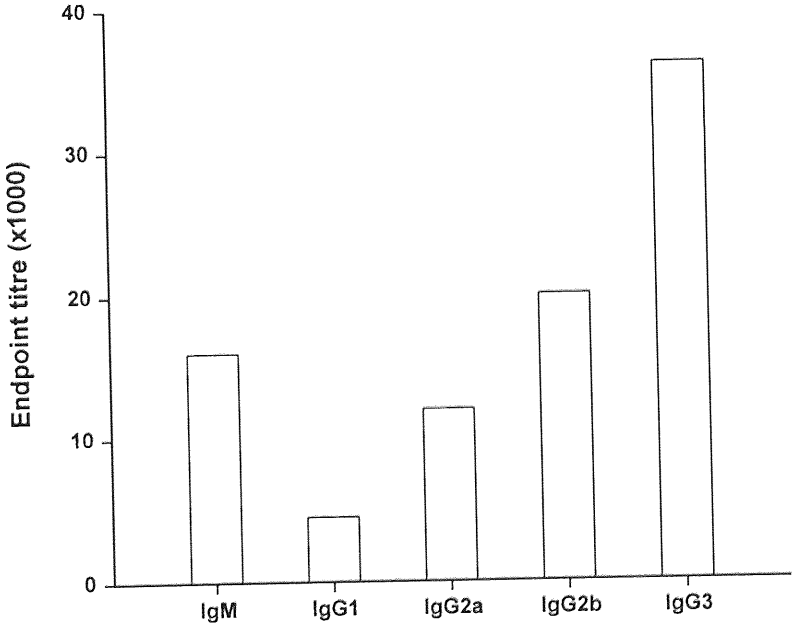


Figure 57. Antibody levels present in the serum of the mouse used to produce the spleen library.

5.2.2 Extraction of total RNA, mRNA and production of cDNA

The source of total RNA for the production of single chain antibodies was part of the spleen from a mouse immunised with LPS from *B. pseudomallei* NCTC 4845 (see section 2.5.1 for immunisation schedule). Total RNA was extracted using the RNeasy Maxiprep kit according to the manufacturers instructions. RNA yield was quantified using the Genequant spectrophotometer (Amersham Pharmacia Biotech).

Yield of total RNA:	Approximately 1ml at 491.1μg/ml
Ratio 260:280:	1.236
Protein content:	6.1mg/ml

The ideal 260:280 ratio is 1.5-1.9. However, the high level of contaminating protein meant the ratio was lower than ideal.

1μg of total RNA was then used for the purification of mRNA using the Oligotex mRNA purification kit. The yield from the purification was again quantified using the Genequant spectrophotometer.

Yield of mRNA:	Approximately 40μl at 41.6μg/ml
Ratio 260:280:	1.302
Protein content:	0.4mg/ml

Again the 260:280 ration was below ideal (1.8-2.0) but this was probably due to the quality of the total RNA starting material.

1μg mRNA was then used to synthesise single stranded cDNA using the Amersham cDNA synthesis kit. 5μl samples of total RNA, mRNA and cDNA were then analysed by agarose gel electrophoresis (Figure 58). Total RNA was seen as three bands and mainly made up of ribosomal RNA. The mRNA was seen as a blur and the cDNA was just visible as a faint blur on the gel, but is not really apparent in the photograph.

5.2.3 Amplification of immunoglobulin heavy and light chain DNA

The cDNA was used as template for the amplification of heavy and light chain DNA using the VHfor, VHback, VLfor and VLback primer mixes given in section 2.6.5. These primers incorporate the linker sequence which will be used to ligate the heavy and light chains together to form scFv DNA. Four assays were conducted for each chain. PCR products were analysed by agarose gel electrophoresis in low melting point agarose so the 300-350bp band could be excised and purified using the Qiagen gel extraction kit (Figure 59). Quantification of purified DNA was carried out using the Genequant spectrophotometer.

Yield of heavy chain DNA: Approximately 30μl at 49.2μg/ml

Yield of light chain DNA: Approximately 30μl at 169.5μg/ml

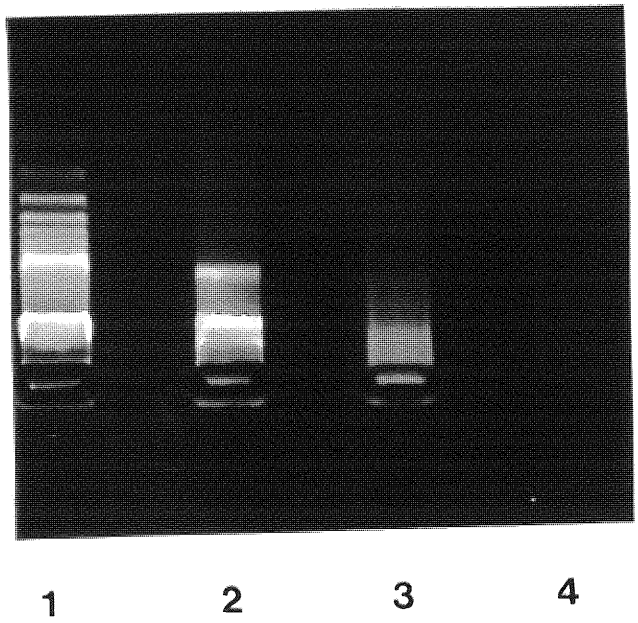


Figure 58. Agarose gel electrophoresis of total RNA, mRNA and cDNA preparations from the spleen of a mouse which had been immunised with LPS from *B. pseudomallei* NCTC 4845.

Lane	Sample
1	Molecular weight markers
2	Total RNA
3	mRNA
4	cDNA

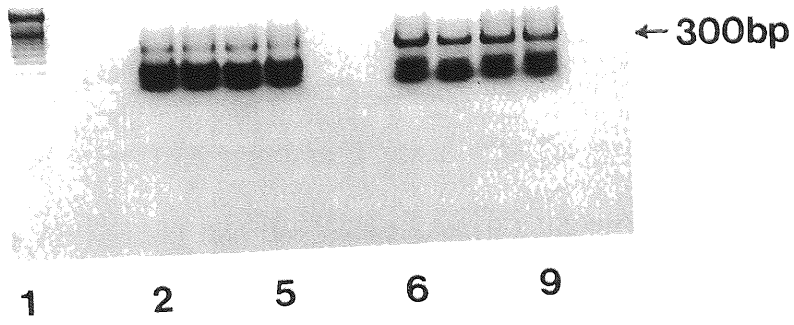


Figure 59. Agarose gel electrophoresis of PCR products from the amplification of immunoglobulin heavy and light chains from cDNA. Both bands are approximately 300bp.

Lane	Sample
1	Molecular weight markers
2	Heavy chain
3	Heavy chain
4	Heavy chain
5	Heavy chain
6	Light chain
7	Light chain
8	Light chain
9	Light chain

5.2.4 Ligation of heavy and light chains to form scFv DNA

PCR was used, first without primers to ligate the two chains together via the linker sequence and then using the scfor and scback primers (section 2.6.7) to amplify the scFv DNA. PCR products were again analysed by agarose gel electrophoresis. The scFv band at approximately 750-800bp was excised (Figure 60), purified and quantified using the Genequant spectrophotometer (Amersham Pharmacia Biotech). The yield of DNA after purification was approximately 60µl at 37.5µg/ml.

5.2.5 Digestion with *SfiI*

1µg of scFv DNA was then digested with the restriction enzyme *Sfi*I to create the sites for ligation into the vector pAK100. After digestion the DNA was cleaned using a PCR product clean-up kit and a small aliquot analysed by agarose gel electrophoresis. The phage display vector pAK100 was also digested with *Sfi*I, which removes a 2kb fragment from the vector. A faint band of scFv was obtained at approximately 800bp and the product used in the subsequent ligation step.

5.2.6 Ligation of scFv into pAK100 and transformation into *E. coli*

The *Sfi*I-digested scFv DNA was ligated with *Sfi*I-digested pAK100. Suitable controls were included: uncut vector only, cut vector only and cut vector plus the excised pAK100 2kb fragment. After overnight incubation at 16°C these ligations were then transformed into *E. coli* XL1-Blue cells using the Stratagene transformation kit and plated out onto 2YT plates containing 30µg/ml chloramphenicol, 10mM $MgSO_4$ and 1% glucose.

The results of the transformations are given in Table 24. The controls worked well with low numbers of colonies on the cut vector control. This gave some indication of the background number of unligated colonies. The number of colonies on test plates was estimated by counting the number of colonies in one eighth of plate number 4, multiplying by eight to give the number of colonies per plate and by five to give the total estimated number of colonies in the whole library. The estimated number of colonies per plate was 4800 which gave a total number of colonies of 25000. Therefore, the number of clones obtained from PCR amplified scFv DNA from a mouse spleen was approximately 2.5×10^4 clones.

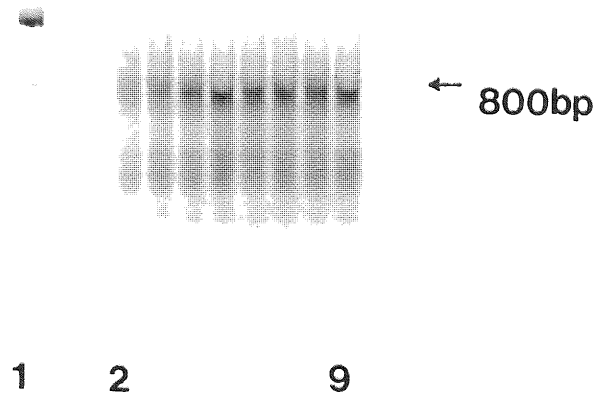


Figure 60. Agarose gel electrophoresis of PCR product from the ligation and amplification of scFv DNA. The product is approximately 800bp.

Lane	Sample
1	Molecular weight markers
2-9	scFv amplification

Experiment	No. of colonies per plate	Results
E. coli cells only	No colonies	No chloramphenicol resistance from pAK100
Uncut vector only	>1000 colonies	Transformation successful
Cut vector only	7-10 colonies	gives an indication of the background number of unligated colonies
Cut vector + 2Kb insert	>1000 colonies	Ligation reaction worked
scFv plates (nos. 1-5)	>1000 colonies	colony counts gives an indication of the library size

Table 24. Results of transformations into *E. coli* XLI-Blue.

5.2.7 Library verification

Individual colonies picked before harvesting the library were grown up and used for PCR amplification of the 800bp scFv DNA (Figure 61). The resulting PCR products were all smaller than 800bp (100-300bp). Further verification was carried out by doing plasmid preparations on six of the random clones followed by digestion of the plasmid with *Sfi*I (Figure 62) and *BstN*I restriction enzymes. The PCR products from the scFv amplification were also subjected to digestion with *BstN*I (Figure 63). The results indicated that only small fragments of DNA (100-300bp) had been cloned rather than whole 800bp scFv fragment. However, in total only 48 random clones were screened, which represents only 0.002% of the estimated total number of clones in the library, so it was decided to continue with panning and screening for any specific scFv antibodies.

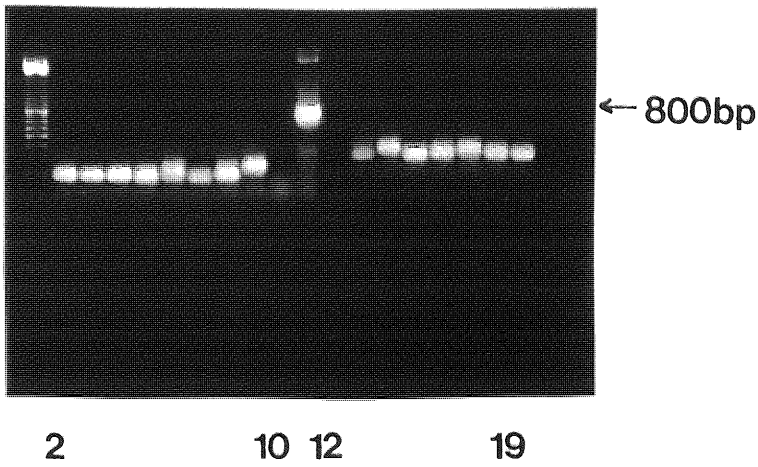


Figure 61. Agarose gel electrophoresis of PCR product from the amplification of the 800bp scFv from 16 randomly selected clones after transformation.

Lane	Sample
1	Molecular weight markers
2-10	scFv amplification
11	scFv positive control
12-19	scFv amplification

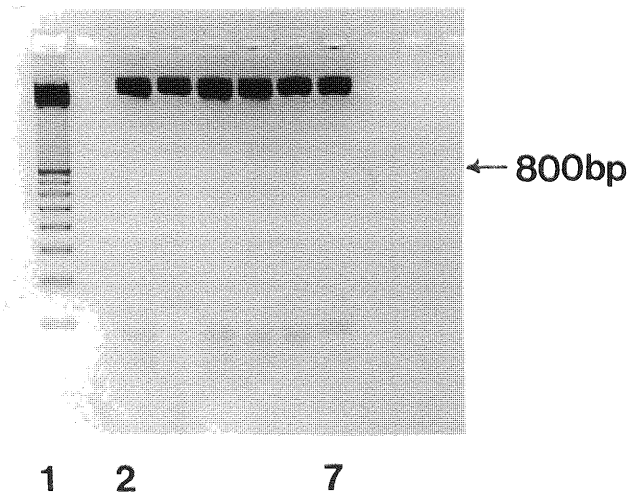


Figure 62. Agarose gel electrophoresis of products from *Sfi*I digestion of plasmid prepared from 6 random clones

Lane	Sample
1	Molecular weight markers
2-7	Plasmid digestion

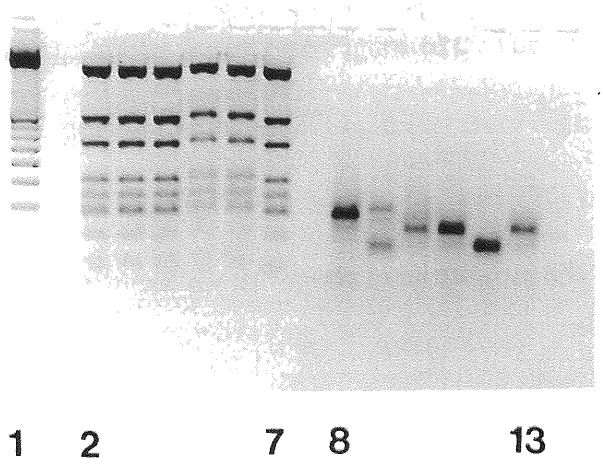


Figure 63. Agarose gel electrophoresis of products from *Bst*NI digestion of plasmid and PCR products from 6 random clones.

Lane	Sample
1	Molecular weight markers
2-7	Plasmid digestion
8-13	PCR product digestion

5.2.8 Panning

Panning was carried out against immobilised *B. pseudomallei* NCTC 4845. The library was grown overnight to produce phage which was then allowed to bind to immobilised cells of *B.pseudomallei* NCTC 4845. Bound phage was eluted and then used to reinfect *E. coli* XLI-blue. The first round of panning yielded approximately 100 individual clones. Twenty three clones were picked at random and PCR was carried out to amplify the plasmid-cloned DNA. Twenty one of the twenty three clones contained an 800bp insert (see Figure 64). The PCR products from eight of the twenty one clones containing an 800bp insert were digested with *BstN*1. Agarose gel electrophoresis of the digests showed some variation in pattern which indicated sequence variation between the different clones (Figure 65).

The 100 individual clones were screened in ELISA against *B. pseudomallei* NCTC 4845 and against BHK cell extract as a negative control. There appeared to be non-specific reactivity against the BHK negative control but no activity towards *B. pseudomallei* cells. The eluted phage from the first round was subjected to two further rounds of panning to continue the enrichment process. Clones were again isolated from these subsequent rounds and were found to contain the 800bp insert by PCR (Figure 66). BstN1 digests, however, showed the footprints to all be the same (Figure 67). It is likely that a single clone type was selected during the first round of panning which is not specific to *B. pseudomallei* and that the further panning rounds have only enriched the phage preparation for this one particular clone type.

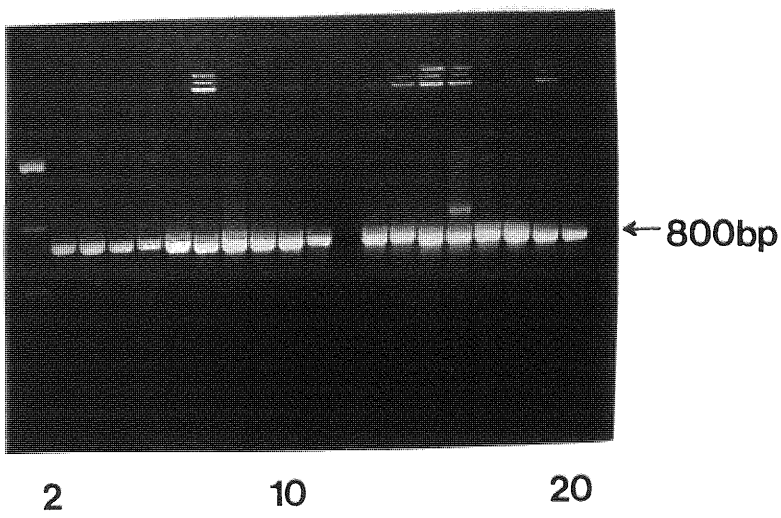


Figure 64. Agarose gel electrophoresis of PCR product from the amplification of the 800bp scFv insert from nineteen of the random clones obtained in the first round of panning.

Lane	Sample
1	Molecular weight markers
2-20	scFv amplification

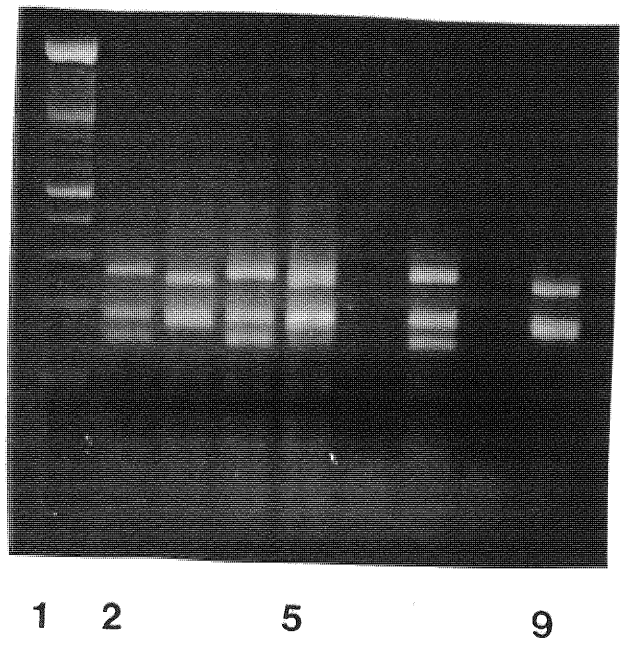


Figure 65. Agarose gel electrophoresis of the products from *BstNI* digestion of some of the PCR products in Figure 64.

Lane	Sample
1	Molecular weight markers
2-9	*BstNI* digestion

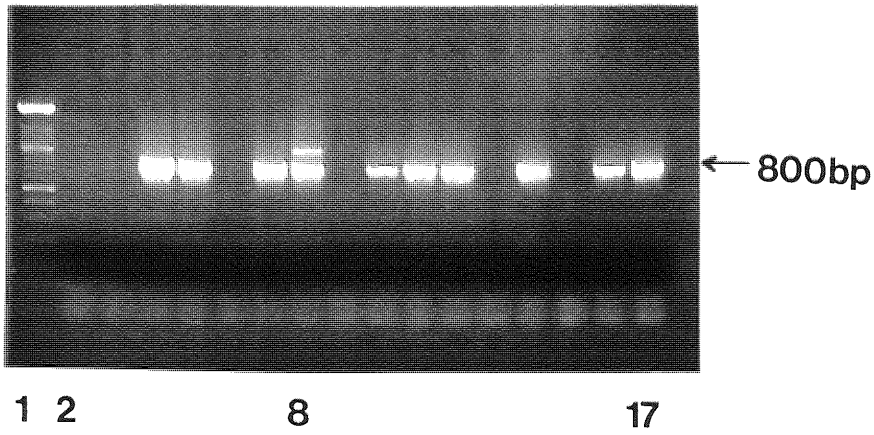


Figure 66. Agarose gel electrophoresis of PCR product from the amplification of the 800bp scFv insert from clones obtained in second and third round panning.

Lane	Sample
1	Molecular weight markers
2	Negative control
3-17	scFv amplification

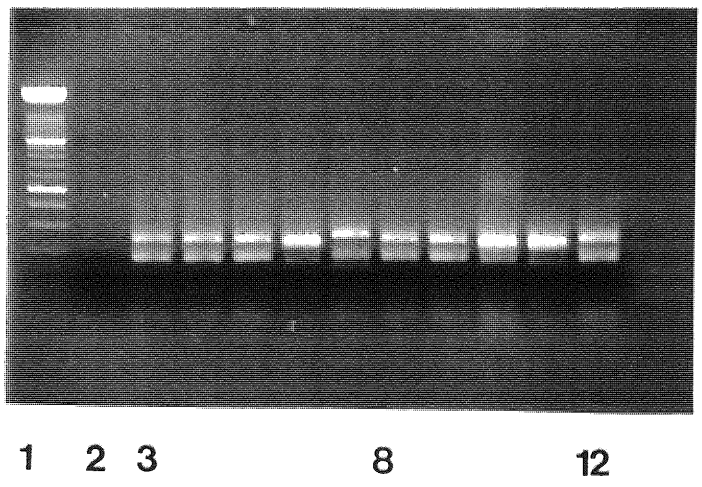


Figure 67. Agarose gel electrophoresis of products from *BstNI* digestion of some of the PCR products from Figure 66.

Lane	Sample
1	Molecular weight markers
2	Negative control
3-12	*BstNI* digestion

5.2.9 Sequencing

Plasmid was isolated from two of the clones (B2 and E5) from the first round of panning for use in DNA sequencing. Samples from both clones were analysed by agarose gel electrophoresis, but bands were only seen in the first sample (Figure 68). Insert DNA from clone B2 was sequenced twice using the scFv primers, scfor and scback.

The results of the sequencing were analysed and the two sequences were found to align. Comparison of the aligned sequence with others in the GenBank Sequence database showed a high degree of homology with both mouse immunoglobulin variable region sequences and with other single chain antibody sequences. These results would suggest that DNA encoding scFv has been incorporated into the library but that the resulting scFv produced are not recognising *B. pseudomallei.*

5.3 Production of scFv from antibody secreting monoclonal hybridoma cells

5.3.1 Isolation of mRNA

Monoclonal hybridoma cell lines can also be used as a source of mRNA for the production of single chain antibodies. 4VH7 was produced in the first round of fusions using the spleen of a mouse immunised with heat inactivated cells of *B. pseudomallei* NCTC 4845. BA2 was produced from the spleen of a mouse immunised with LPS from *B. pseudomallei* NCTC 4845. The cell lines BA2 and 4VH7 were cultured and used to isolate mRNA. Messenger RNA was extracted

using the Quickprep Micro mRNA purification kit, resuspended in 10µl RNAse-free water and the concentration was estimated using the Genequant spectrophotometer (Amersham Pharmacia Biotech) (Table 25).

Cell line	Total number of cells used	Approximate yield of mRNA	Ratio 260:280	Protein content (mg/ml)
BA2	4×10^5	219µg/ml	1.0	4.3
4VH7	1.3×10^6	294µg/ml	1.4	2.4

Table 25. Results of the extraction of mRNA from the hybridoma cells BA2 and 4VH7.

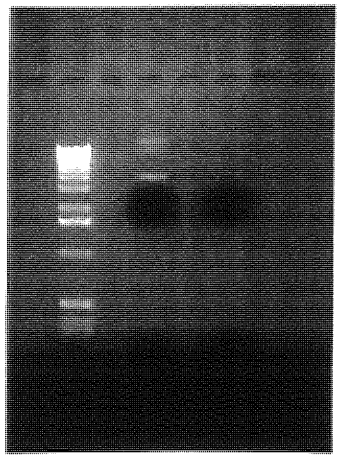


Figure 68. Agarose gel electrophoresis of plasmid DNA produced for sequencing.

Lane	Sample
1	Molecular weight markers
2	Plasmid from clone B2
3	Plasmid from clone E5

5µl of each mRNA preparation was then used to synthesise single stranded cDNA using the Amersham cDNA synthesis kit. The yields were low and the quality of the mRNA may also be low, as indicated by the 260:280 ratio. This may be due to the small numbers of cells used. Although the minimum number of cells for use in a single extraction is given as 10^5, the procedure may work better with a larger number of cells.

5.3.2 Amplification of immunoglobulin heavy and light chain DNA

The cDNA was used as template for the amplification of heavy and light chain DNA, as in section 5.2.3. PCR products were analysed by agarose gel electrophoresis in low melting point agarose and the 300-350bp band excised and purified using the Qiagen gel extraction kit (Figures 69 & 70). Quantification of purified DNA was carried out using the Genequant spectrophotometer.

Cell line	Yield of heavy chain DNA	Yield of light chain DNA
BA2	30µl at 12µg/ml	30µl at 37µg/ml
4VH7	30µl at 30µg/ml	30µl at 40µg/ml

Table 26. Yields of immunoglobulin heavy and light chain DNA after purification.

5.3.3 Ligation of heavy and light chains to form scFv

The ligation PCR was carried out with both BA2 and 4VH7 to ligate and then amplify the scFv. On analysis of the products by agarose gel electrophoresis there were no apparent bands. The PCR was repeated, but still no bands were obtained.

The failure of the ligation could be due to poor quality mRNA used as starting material. The ratio 260:280 was low in both mRNA preparations and this may have affected the reaction. These experiments were not continued as the project had come to an end but to continue this work the cells would need to be re-cultured and a fresh mRNA extraction carried out, before producing cDNA.

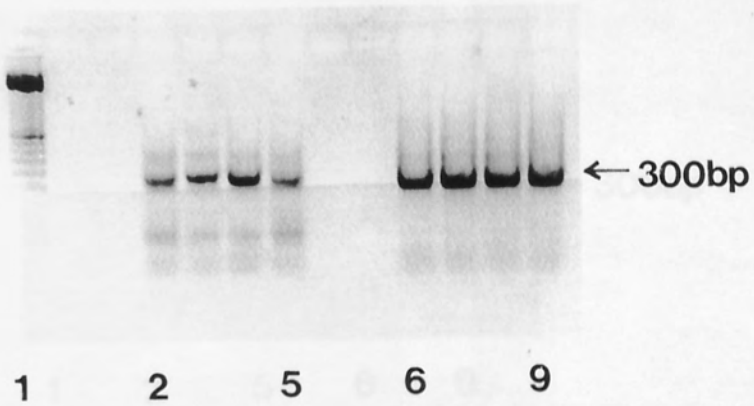


Figure 69. Agarose gel electrophoresis of PCR products from the amplification of immunoglobulin heavy and light chains from the cDNA produced from the hybridoma cell line BA2.

Lane	Sample
1	Molecular weight markers
2-5	Heavy chain
6-9	Light chain

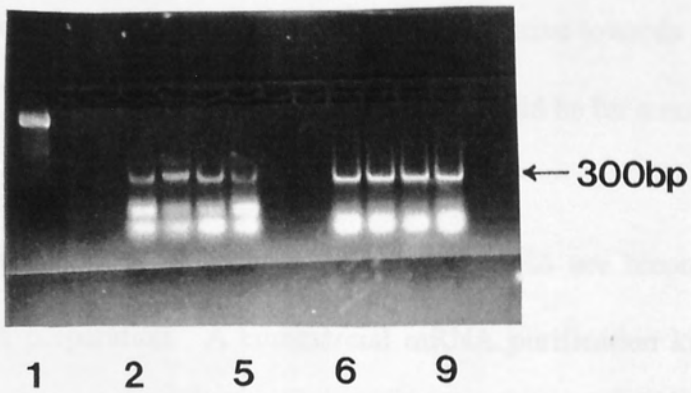


Figure 70. Agarose gel electrophoresis of PCR products from the amplification of immunoglobulin heavy and light chains from the cDNA produced from the hybridoma cell line 4VH7.

Lane	Sample
1	Molecular weight markers
2-5	Heavy chain
6-9	Light chain

5.4 Discussion

5.4.1 Production of a phage library from a spleen

The results obtained would suggest that a phage library of approximately 2.5 x 10^4 clones was produced from the spleen of a mouse immunised with the LPS of *B. pseudomallei*, the resulting scFv were not reactive towards *B. pseudomallei* bacterial cells. The failure to isolate a specific scFv could be for a number of reasons.

Commercial kits for the purification of mRNA are recommended to give a pure mRNA preparation. A commercial mRNA purification kit was used in this case. However, the absorbance ratio 260:280 for both the total RNA and the mRNA were lower than the recommended ratios. Poor quality RNA preparations can cause the subsequent PCR steps to fail. In the case of repertoire constructions, even if there are no apparent problems during construction and cloning, the diversity of the repertoire can be greatly reduced and the usefulness of the final library greatly compromised if the RNA is of poor quality (McCafferty, J., Hoogenboom, H.R. *et al.* 1996).

Panning, used as a means of selecting antigen-specific scFv appeared in this case to select a single, non-specific clone type. Panning is a powerful enrichment technique designed to greatly increase the numbers of selected phage. It appears that a single clone type selected in the first round of panning was enriched by subsequent panning rounds and became the major (or only) clone type in the phage suspension. It is

possible that any specific scFv present in the original library may have been overshadowed by the affinity of the non-specific clone and the number of particles expressing it. There are a number of reported disadvantages to panning on antigen coated plasticware: it is difficult to select for high affinity clones due to avidity effects and it is difficult to discriminate between clones of similar affinities (Griffiths, A.D. and Duncan, A.R. 1998).

The antibody titres of the animal used to produce the spleen library were not high. The mRNA purified from the spleen contains only a small fraction coding for immunoglobulins. If the animal is producing a low level of specific antibody then the chances of amplifying DNA coding for specific antibody is very much reduced. Successful scFv production from spleen libraries has in the past generally used highly immunogenic proteins or conjugated haptens which are likely to result in high antibody responses in the immunised animal. It is possible that mRNA was successfully isolated from the spleen and scFv DNA amplified but that mRNA was not coding for anti-*B. pseudomallei* antibodies, hence the resulting phage are non-specific.

The use of LPS as an immunogen may have had a detrimental effect. LPS is known to act as a B cell mitogen and activates a large number of B cells non-specifically (Kuby, J. 1992). This could mean that a proportion of the immunoglobulin mRNA isolated from the spleen was coding for non-specific antibody. This coupled with low specific antibody titres would have reduced the chances of expressing specific scFv.

5.4.2 Production of a phage library from a monoclonal hybridoma cell line

The failure to produce a library from monoclonal hybridoma cells is likely to be due to using insufficient numbers of cells. The RNA isolation kit used recommended using 1 x 10^5 cells, however other reference sources suggest using 1 x 10^7 cells. One disadvantage of using hybridoma cells is that the cell line has to be available. At the time the work was undertaken, there was no monoclonal antibody to LPS to use as a source of RNA.

Another disadvantage of using a hybridoma cell line as a starting point is that the scFv is likely to have the same binding characteristics as the parent antibody and may even have a lower affinity (Hoogenboom, H.R., Griffiths, A.D. *et al.* 1991). However, random mutagenesis of the hypervariable regions has been shown to produce variants of higher affinities or different binding activities and could be used to engineer the antibody required (Lowman, H.B., Bass, S.H. *et al.* 1991).

In the field of detection, one advantage of scFv would be the exploitation of IgM antibodies. IgM antibodies are usually considered unsuitable for detection uses and are difficult to produce and purify in quantity. However immunisation with some antigens such as LPS result in a predominantly IgM response. Production of scFv from an IgM secreting hybridoma cell line would exploit the binding activity of these antibodies without the need for complex purification schedules. One potential disadvantage of recombinant antibodies is their monovalency. This monovalency may result in a reduction in binding affinity when compared with bivalent IgG

molecules or IgM pentamers (Tout, N.L. and Lam, J.S. 1997). This may be important if an IgM antibody is used, particularly in the case of an anti-O side chain antibody, as carbohydrate binding antibodies are usually associated with relatively low affinities (Deng, S., MacKenzie, C.R. *et al.* 1994).

6. Concluding remarks

The major aim of this study was to produce antibodies which could be used to detect *B. mallei* and *B. pseudomallei* using a range of detection techniques. A batch of polyclonal antibody was raised against a heat inactivated whole cell preparation of *B. pseudomallei* NCTC 4845 in guinea pigs. Characterisation of this polyclonal antibody showed that it could be biotin-labelled and used in capture assays as both the detection and capture antibody with a lower detection limit of approximately 1 x 10^6 cfu/ml. Further characterisation showed the polyclonal antibody to cross react with closely related species such as *B. pickettii* and *B. cepacia.* It is possible to remove these non-specific antibodies from polyclonal serum by absorption using whole cells of the cross reacting species, however, the removal of antibodies may affect the binding characteristics of the polyclonal antibody as a whole and can lead to large variations in batches (Goding, J.W. 1983). The polyclonal antibody was therefore considered to have limited use as a detection reagent.

A panel of nine monoclonal antibodies were produced (Table 27) and characterised for their potential as detection reagents. Five monoclonal antibodies recognise a high molecular weight polysaccharide, two monoclonal antibodies recognise the lipopolysaccharide and two further monoclonal antibodies recognise different protein components.

Five of the monoclonal antibodies (4VIH12, 4IIIA11, 4VA4, 4VA5 and 3VIE5) were found to bind to a high molecular weight polysaccharide material present on some strains of both *B. pseudomallei* and *B. mallei*. These five antibodies were all raised in mice immunised with a heat inactivated whole cell preparation of *B. pseudomallei* NCTC 4845 and it appears that this high molecular weight, non-protein component is the dominant antigen in the whole cell preparation. It is possible that this polysaccharide material is one of the reported exopolysaccharide or capsular polysaccharides of *B. pseudomallei* (Steinmetz, I., Rohde, M. *et al.* 1995; Kawahara, K., Dejsirilert, S. *et al.* 1998). When tested, the five antibodies were found to bind to the majority of *B. pseudomallei* strains, however, there was no recognition of the avirulent, arabinose positive *B. pseudomallei* (*B. thailandensis*) strains. It is possible that these strains are lacking in the polysaccharide material, and that this may be a factor in their avirulent nature. There are many references which suggest that the presence or absence of this capsular polysaccharide may be linked to virulence of *B. pseudomallei* strains, although positive proof still has to be obtained. Increased levels of exopolysaccharide have been shown to be present on highly virulent strains of *B. pseudomallei* (Peters, M.K., Piven, N.N. *et al.* 1983) whilst strains deficient in exopolysaccharide have been shown to have reduced or no virulence for laboratory animals (Piven, N.N., Smirnova, V.I. *et al.* 1991). The presence of exopolysaccharide has also been linked with the ability to survive phagocytosis (Piven, N.N., Smirnova, V.I. *et al.* 1991).

Monoclonal antibody	Binding site
4VIH12	High molecular weight polysaccharide
3VIE5	High molecular weight polysaccharide
4VA4	High molecular weight polysaccharide
4VA5	High molecular weight polysaccharide
4IIIA11	High molecular weight polysaccharide
BA2	14-21kDa protein
LF7	36-45kDa protein
CC6	lipopolysaccharide (typical)
HC9	lipopolysaccharide (atypical)

Table 27. Monoclonal antibodies and their respective antigens.

The binding of the five monoclonal antibodies (4VIH12, 4IIIA11, 4VA4, 4VA5 and 3VIE5) to *B. mallei* strains was also tested, the antibodies binding to between 3 and 6 of the ten strains tested depending on the individual antibody. It is likely that the high molecular weight material found on the majority of *B. pseudomallei* strains is also present on some but not all *B. mallei* strains, but the possible connection with virulence is unclear. *B. mallei* strain NCTC 10248 has be shown to have the high molecular weight polysaccharide material by immunofluorescence with 4VIH12, however, when used to challenge hamsters the strain appears to be avirulent. The exopolysaccharide or capsular antigen has also been linked with virulence in *B. mallei* (Khrapova, N.P., Tikhonov, N.G. *et al.* 1998) and has been shown to protect bacterial cells against lysosomal action (Popov, S.F., Kurilov, V.I. *et al.* 1995).

It is known that *B. pseudomallei* and *B. mallei* are very closely related bacterial species and it is likely that a possible virulence factor in *B. pseudomallei* would also be present in *B. mallei* strains. DNA from strains of *B. mallei* have been found to contain large numbers of insertion sequences and it has been hypothesised that the random presence of these insertion sequences could affect the expression of different genes in *B. mallei* so disabling different functions in the organism (Dr K. Mack, personal communication).

This argument could be used in the case of *B. mallei* NCTC 10248. The production and export of exopolysaccharide would appear to be intact in this strain, however other virulence factors, as yet unidentified may have been affected making the strain avirulent.

The antigenic and genetic similarity between these two organisms is evident, both species carry the gene for flagellin protein, the DNA sequence has been shown to differ only by a single base pair, yet one species is motile and the other is not (Neubauer, H., Splettstober, W. *et al.* 1999). The production and assembly of flagella is a complex system involving many genes and it is likely that the non-motility of *B. mallei* is due to non-functional genes in another part of the locus. Whether it is possible to discriminate between these two species antigenically may not be resolved until further genetic studies are carried out. The genome sequencing of *B. pseudomallei* may go some way in answering this. However, the diseases caused by these organisms are different, as is the current antibiotic treatment for the

two diseases, so the need to detect and distinguish remains. The five monoclonal antibodies (4VIH12, 4VA5, 4VA4, 4IIIA11 and 3VIE5) work well in the different detection assays tested but do not differentiate between the two species.

Antibody reagents are not only used for detection and identification purposes, other studies have used antibodies to confer passive protection against melioidosis (Brett, P.J., Mah, D.C.W. *et al.* 1994; Bryan, L.E., Wong, S. *et al.* 1994). Preliminary studies using a cocktail of seven monoclonal antibodies has been shown to protect against a challenge of 10^4cfu *B. pseudomallei* NCTC 4845. Further work will need to be carried out to identify which individual antibodies are protective.

The LPS from both *B. pseudomallei* and *B. mallei* was characterised by proteinase-K extraction, SDS-PAGE and silver staining. The majority of *B. pseudomallei* strains were found to have highly conserved, visually identical silver-stain profiles, however four strains were found to have different profiles, the bands being spaced further apart and appeared to have a higher molecular weight. This atypical profile has not been reported, however a recent study on 214 strains of *B. pseudomallei* reported that four strains did not show the highly conserved silver stain profile (Anuntagool, N., Intachote, P. *et al.* 1998). The avirulent, arabinose positive strains (E27 and E82) were shown to have the conserved silver-stain profile and have been shown to be immunologically indistinguishable from virulent arabinose negative strains using polyclonal serum (Anuntagool, N., Intachote, P. *et al.* 1998). The silver-stain profiles of the *B. mallei* strains appeared visually very similar to each other and to the typical *B. pseudomallei* LPS profile.

Raising antibodies to the lipopolysaccharide of *B. mallei* and *B. pseudomallei* did not prove to be straightforward. This is most likely due to the poor immunogenicity of the molecule and it's mitogenic effect on the immune system. Polyclonal serum taken from animals infected with *B. mallei* and tested in western blots showed strong recognition of *B. mallei* LPS and also some activity against the typical *B. pseudomallei* LPS. Experiments have shown that while the polyclonal antibody appears to bind more strongly to the LPS of *B. mallei* strains, absorption with *B. pseudomallei* cells eliminates all LPS binding. The monoclonal antibody CC6 also bound to the LPS of *B. mallei* and to the typical LPS found in the majority of *B. pseudomallei* strains. This would suggest that the LPS of both *B. mallei* and *B. pseudomallei* share common epitopes, this would not be surprising given how closely the two species are related.

From a detection point of view, these results raise a number of potential problems. The shared epitopes on both *B. mallei* and *B. pseudomallei* LPS mean that antibodies which recognise LPS will not discriminate between the two species, as shown by the monoclonal antibody CC6. In the case of the *B. pseudomallei* strains with typical LPS profiles, the avirulent, arabinose-positive isolates are indistinguishable from the virulent arabinose-negative strains and the small number of *B. pseudomallei* strains with the atypical LPS profile would not be detected at all.

It appears that LPS profile is not linked to virulence, given that the arabinose-positive strains are indistinguishable from the virulent, arabinose-negative strains. It has been

reported that *B. pseudomallei* carries two O-antigens O-PSI and O-PSII (Perry, M.B., MacLean, L.L. *et al.* 1995) and that O-PSII is required for virulence (DeShazer, D., Brett, P.J. *et al.* 1998). It is possible that the typical LPS silver-stain profile, seen in the majority of *B. pseudomallei* strains tested is a combination of O-PS I and O-PS II, however the avirulent, arabinose positive strains are reported to carry only O-PS II (DeShazer, D., Brett, P.J. *et al.* 1998) and appear identical when silver stained. It is possible that the silver stain predominantly stains O-PS II. Polyclonal serum raised against typical *B. pseudomallei* LPS did not cross react with the atypical LPS. It is possible that the atypical LPS is immunologically distinct from either O-PS I or O-PS II. Two of the four strains found to have the atypical silver-stain profile are known to be virulent human isolates and appear not to carry O-PS II. Isolation of the atypical LPS followed by hydrolysis and mass spectroscopy would be the only means of further characterising the atypical LPS.

Attempts to produce a single chain antibody fragment reactive against *B. pseudomallei* NCTC 4845 failed. This may be for a number of reasons including the immune status of the animal prior to splenectomy, the use of partially pure LPS as an immunogen and the quality of RNA extracted from the spleen. DNA sequencing has shown that the insert carried in one of the non-specific clones isolated during panning had high homology with other single chain antibody fragments and with mouse immunoglobulin DNA. It is possible that the construction of the library had worked in principle, but that no specific scFv fragments were found. Production of scFv from monoclonal hybridoma cell lines also failed, but this is almost certainly due to the failure to extract sufficient quantity or quality RNA from the cells.

Genetic production and manipulation of antibody fragments may have a number of advantages over conventional monoclonal antibody production. Although in some cases laboratory animals are still needed for immunisation, there is no ongoing requirement to produce macrophage feeder layers, still used by some in the production and cloning of monoclonal hybridoma cells. The process may be slightly quicker, but it is more intensive. Producing the library took approximately 7-10 days, this is compared with 14 days incubation following a conventional fusion procedure. Multiple rounds of panning is time consuming as is three rounds of cloning by limiting dilution. Production of scFv, however, does not require the specialist facilities needed for conventional monoclonal antibody production and tissue culture. Recombinant antibodies have been shown to work in both ELISA and on biosensors (Duenas, M., Chin, L.T. *et al.* 1996; Emanuel, P., O'Brien, T. *et al.* 1996; Kerschbaumer, R.J., Hirschl, S. *et al.* 1997). The ability to engineer the specificity and affinity of antibodies once produced offers huge potential, particularly as existing monoclonal hybridoma cell lines can be used. These techniques may overcome the issue of antibody isotype as antibodies of the isotype IgM are considered difficult to for use in a number of detection and identification techniques. Monoclonal antibodies cell lines secreting IgMs can be used to produce scFv, retaining the antibody binding activity but overcoming the production and purification issues. HC9, the IgM monoclonal which binds to the atypical LPS of *B. pseudomallei* would be a candidate for engineering.

The use of LPS as an immunogen highlights one problem common to both conventional monoclonal antibody production and to the production of engineered antibody fragments. A poor immune response in the animal reduces the chances of producing any antibodies. Both techniques have their advantages and disadvantages, but the successful production of antibodies is still dependent on the initial immune response. Difficult immunogens such as LPS and haptens still present a challenge.

6.1 Further work

The high degree of antigenic similarity between these two species means that polyclonal antibodies are unlikely to be useful in the detection and discrimination of *B. mallei* and *B. pseudomallei* and immunological detection techniques are likely to rely on monoclonal antibodies or engineered antibody fragments. It has been shown that some antigens are not found on all the strains in a single species and it is possible that a cocktail of antibodies may eventually be needed for successful detection and discrimination of *B. mallei* and *B. pseudomallei*. It remains to be seen whether antigens can be identified which could be used to discriminate between the two species and genetic techniques such as subtractive hybridisation may be needed. The sequencing of the *B. pseudomallei* genome will be of great benefit to future studies.

The role of exopolysaccharide in the virulence of *B. mallei* could be investigated. Exopolysaccharide appears to be important in the virulence of *B. pseudomallei*, but *B. mallei* strains shown to possess high molecular weight polysaccharide material

have been shown to be avirulent. Other factors must be important in the pathogenesis *of B. mallei* infection and identification of these could lead to advances in understanding the disease profile of *B. mallei* and the possibility of formulating a potential vaccine.

Further investigation of the atypical LPS of *B. pseudomallei* could be carried out. Why do such a small number of strains have a different LPS, and what advantages/disadvantages does it bestow on the bacteria? Highly purified material could be used in mass spectroscopy to study the structure of this atypical O-antigen and allow comparisons to be made with O-PSI and O-PSII. Purification of the O-antigen from *B. mallei* followed by spectroscopic analysis would also allow comparison of the *B. mallei* and *B. pseudomallei* LPS structures.

It would be interesting to pursue the production of single chain antibody fragments from both spleens and monoclonal antibody cell lines. Any resulting scFv could be mutated in an attempt to engineer the specificity of those antibodies already in existence. Different immunisation regimes and antigens could be used to enhance the likelihood of obtaining specific scFv from a spleen library, and good quality mRNA would need to be obtained from cultured cells.

The role of monoclonal antibodies in passive protection could be further investigated. A cocktail of seven different monoclonal antibodies has been shown to confer some protection against *B.pseudomallei* infection. Investigations could be carried out to identify if one particular antibody is protective or if the cumulative effect of the cocktail is required. If a single antibody is protective, then identification of the antigen could lead to a potential vaccine candidate.

7. References

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