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JAMES COOK
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THE EPIDEMIOLOGY OF MELIOIDOSIS IN PAPUA NEW GUINEA

A Thesis submitted by
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in December, 2004

for the degree of Doctor of Philosophy in
the discipline of Microbiology and Immunology
of the School of Biomedical Science at
James Cook University, Townsville

DECLARATION

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 Information derived from the published or unpublished work of others has been acknowledged in the text and a list of references given.

J M Warner
December 2004

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STATEMENT ON THE CONTRIBUTION OF OTHERS

I acknowledge the help and support of Mr Daniel Gal, Mr Mark Mayo and Prof. Bart Currie in the preparation of the macro restriction digest gels. Also, the help of Dr Catriona McElene in the preparation of the PCR gels. Finally I acknowledge the help of Dr Bryant Allen for access to the PNGRIS and preparation of biogeographical maps.

J M Warner
December 2004

ACKNOWLEDGMENTS

kabigibega - this is as much yours as mine. To my Australian-based family and friends, thanks for sticking by me at a time of acute selfishness.

But to my long suffering wife, ▮▮▮▮ and our other important project of the last five years, our son ▮▮▮▮. To them I dedicate this work in the memory of the sacrifices they both have endured during their support of me as I have attempted to complete this work. I am coming home now!

ABSTRACT

Melioidosis has only been sporadically reported in PNG and its contribution to the disease burden of Papua New Guineans has been questioned. The rural district of Balimo, located within the Aramia flood plain of the Western province, was chosen to test the hypothesis that melioidosis is under recognised in rural PNG due to a lack of clinical awareness and a poorly resourced laboratory sector. A prospective clinical screening program conducted at Balimo Health Centre revealed melioidosis as the cause of a previously recognised fatal febrile illness affecting children. The implementation of diagnosis and treatment protocols reduced the apparent case fatality rates from 100% to 45%. Although case numbers were small, features of melioidosis in this community include childhood predilection (average age 12-years), a lack of traditional co-morbidity and regional clustering.

Simple methods of isolate identification were tested against gold standards of phenotypic and genotypic techniques and found to be sensitive and sustainable.

An IHA serological study of 747 children demonstrated a correlation between sero-reactivity and clinical incidence. Furthermore, selective culture of 374 soil samples taken from the environment within this region revealed autochthonous *B. pseudomallei* from village communities demonstrated to be melioidosis endemic. Of the 191 samples taken from areas within these villages where children play, 3.7% were found to harbour the organism. DNA macro restriction analysis demonstrated clonality between clinical and environmental strains further substantiating the hypothesis that a driver of childhood predilection is behaviour typical of children which encourages exposure to *B. pseudomallei* from permanently saturated soil and/or water, most likely through preexisting abrasions or pernasal inoculation.

A lack of genetic diversity of *B. pseudomallei* revealed by DNA macro restriction analysis is a feature. This may represent recent importation or the comfortable niche of environment - host cycling of this virulent saprophyte. This is in contrast to the diversity demonstrated in the analysis of the avirulent PNG derived *B. thailandensis*.

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In a geographical analysis of the Balimo region, the environmental attributes of low altitude (<600 m), inundation and extent of inundation and hydraquents as the predominate soil type are typical of this melioidosis implicated region. The subsequent mapping of PNG in terms of these attributes revealed only isolated regions which share these features. If the rare reports of melioidosis elsewhere in PNG is an accurate reflection of the national burden of the disease, these environmental attributes may represent important biogeographical boundaries for melioidosis in PNG. These data may serve in the remote sensing of melioidosis in PNG and throughout the Pacific-Australasian region.

To further substantiate the importance of these geographic boundaries, an indirect IgG ELISA-based sero-epidemiological assay was developed using antigen derived from PNG *B. pseudomallei* and used on samples taken from individuals from 16 regions throughout PNG. The assay was able to detect sero-reactivity that was dependent on region which varied according to degrees of melioidosis prevalence. The true sero-prevalence ranged from 0 - 55%, demonstrating significant spatial sero-clustering. Further, when regions were classified into risk-localities based on sero-reactivity, a correlation was revealed between regions determined high-risk by population sero-reactivity and biogeography.

A prospective study in Port Moresby where 3561 samples were selectively screened for *B. pseudomallei* demonstrated melioidosis to be endemic in the empirically diagnosed tuberculosis (TB) patient cohort and patients presenting with sepsis associated type 2 diabetes, although the incidence is low.

In demonstrating endemic melioidosis in rural PNG for the first time, it is hoped this work will contribute to decreasing the fatality rates of pneumonia and sepsis in this rural subsistence community and may aid in the uncovering of the submerged iceberg that is melioidosis within this region.

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ABBREVIATIONS

<	less than
>	greater than
ACD	acid citrate dextrose
AFB	acid fast bacilli
ALP	alkaline phosphatase
ALT	analine transaminase
ANOVA	analysis of variance
ARA	arabinose
ASH	Ashdown agar
ASHEB	Ashdown environmental selective broth
AST	aspartate transaminase
ATCC	American Type Culture Collection
B. thailandensis	*Burkholderia thailandensis*
B. cepacia	*Burkholderia cepacia*
B. mallei	*Burkholderia mallei*
B. pseudomallei	*Burkholderia pseudomallei*
BD	Becton Dickinson
bp	base pair
CF	cystic fibrosis
cfu	colony forming unit
CHEF	contour-clamped homogenous electric field
CI	confidence limit
cm	centimeter
CMI	cell mediate immunity
CPHL	Central Public Health Laboratory
CTAB	hexadecyltrimethy ammonium bromide
df	degrees of freedom
dl	decilitre
DM	diabetes mellitus
DNA	deoxyribonucleic acid
EDTA	ethyl diamine tetra acetic acid
ELISA	enzyme linked immunosorbant assay
ESP	East Sepik province
fl	femtolitres
g	gram
g	gravity
G-CSF	granulocytic colony stimulating factor
GASP	growth advantage in stationary phase
GIS	geographic information system
GP	soil from garden place
GPS	global positioning system
Hb	haemoglobin
HLA	human leukocyte antigen
hr	hour(s)
ICT	immuuno chromatography test
IFA	immuno fluorescent assay

IgG	immunoglobulin G
IgM	immunoglobulin M
IHA	immunohaemoagglutination
IMI	intra muscular injection
IV	intra venous
kb	kilobase
kg	kilogram
km	kilometer
l	litre
Lat	latitude
Long	longitude
LPS	lipopolysaccaride
m	meter
Mb	megabase
MCV	mean cell volume
mg	milligram
min	minute
ml	millilitre
MLA	Medical Laboratory Assistant
MLT	Medical Laboratory Technology
MLST	multi locus sequencing typing
mm	millimeter
mM	millimolar
mmol	millimoles
MPN	most probably number
N. fowleri	*Naegleria fowleri*
NA	nucleic acid(s)
NCCLS	National Committee of Clinical Laboratory Standards
NCTC	National Collection of Type Cultures
N–PtC	soil from the body of village (not points of land) frequented by children
NPV	negative predictive value
NT	not tested
p	probability
P. fluorescens	*Pseudomonas fluorescens*
PaLMS	Pacific Laboratory Medical Services
PCR	polymerase chain reaction
PFGE	pulse field gel electrophoresis
PNG	Papua New Guinea
PNGRIS	Papua New Guinea Resource Information System
POM	Port Moresby
POMGH	Port Moresby General Hospital
POMGHP	Port Moresby General Hospital Pathology
PPV	positive predictive value
pt	point
PtC	soil from points of land frequented by children
PUO	pyrexia of unknown origin
QID	*quarter in die* (four times a day)

RAPD	random amplified polymorphic DNA
RMU	Resource Mapping Unit
RNA	ribonucleic acid
RT	room temperature
S. aureus	*Staphylococcus aureus*
SBps	suspected *B. pseudomallei*
SD	standard deviation
SDS	sodium dodecyl sulfate
SNH	soil from near or under houses
spp	species
TB	tuberculosis
TBE	tris boric acid EDTA
TBps	typical *B. pseudomallei*
TE	tris-EDTA
TP	true prevalence
TSA	tryptone soya agar
TTS	type III secretion
UFM	Unevangelised Fields Mission
UK	United Kingdom
URT	upper respiratory tract infection
UV	ultra violet
vs.	versus
WBC	white blood count
WCC	white cell count
WELLS	soil adjacent to wells
°C	degrees Celsius
μl	micro litre
μm	micro metre

CHAPTER 1 - AN INTRODUCTION

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1.1 Introduction

The context of this study is the health expectations of 4.5 million Papua New Guineans, 80% of whom live in rural districts. These people experience the highest childhood mortality, lowest life expectancy and have the lowest health care expenditure per capita of any other people in the immediate pacific region (Anon, 2000a). Within this context, it is unlikely that the aetiology of morbidity and mortality is reported accurately.

Melioidosis is an infection caused by the saprophytic Gram-negative bacillus originally described by Whitmore and Krishnaswami in Burma during 1912 (Whitmore and Krishnaswami, 1912). After much taxonomic controversy it is currently widely accepted as *Burkholderia pseudomallei*, (Yabuuchi *et al.*, 1992) a member of the order *Burkholderiaceae*. Melioidosis has since been emerging as a significant cause of community acquired sepsis in the tropics (Dance, 2000b). A feature of the disease is the diversity of the continuing clinical spectrum of presentations which has led clinicians to describe melioidosis as a great mimic of infectious diseases (Leelarasamee and Bovornkitti, 1989).

The "tip of the iceberg" metaphor is often used in describing evidence of melioidosis endemicity (Dance, 1991a; John *et al.*, 1996; Dance, 2000b). Factors that contribute to the under-recognition of melioidosis are linked with the ecology of the causative microorganism, a lack of clinical awareness of the protean manifestations of infection and poor access to well resourced rural laboratory facilities (Dance, 2000b; Dance, 2000c). These factors together constitute melioidosis as a disease of rural subsistence communities that lack quality health care facilities (Dance, 1991a).

1.2   The Purpose and Hypothesis

The purpose of this study is to gather and present evidence in the form of clinical and epidemiological data describing the distribution and determinates of melioidosis in Papua New Guinea (PNG). In doing so, an attempt is made to establish sustainable methods of diagnosis and treatment in the PNG context. The underlying hypothesis is that melioidosis contributes to the high rates of mortality due to respiratory and infectious disease, particularly apparent in rural PNG (Anon, 2000b), but through miss diagnosis is under-recognised. Clinical awareness of the extent or potential for endemicity and the strengthening of laboratory services with appropriate diagnostic skills will lead to increased diagnosis rates and more successful treatment strategies and thus improved patient outcomes.

1.3   Rationale for the Study

Although melioidosis has previously been reported from PNG, the sporadic clinical reports have been based in the urban capital of Port Moresby (Rowlands and Curtis, 1965; De Buse *et al.*, 1975; Lee and Naraqi, 1980; Currie, 1993). In some cases travel history has implicated PNG as the source of exposure with recrudescence of latent infection diagnosed elsewhere (Newland, 1969; Kingston, 1971). This sporadic and sometimes unsubstantiated evidence has led melioidosis to be described as only a rare disease in PNG. Its manifestations are not widely recognised and its significance has been questioned (Currie, 1993).

Papua New Guinea has led the world in establishing standard treatment manuals for common diseases and these, used by rural health workers working in rural communities, is the mainstay of patient management in rural communities in rural provinces (Currie, 1992). However, the antibiotic regimes for the treatment of sepsis and pneumonia do not include antimicrobial therapy appropriate for melioidosis (Anon, 1993; Anon, 1997). Therefore if melioidosis is prevalent in rural PNG but miss-diagnosed it may contribute to the high mortality rates in PNG.

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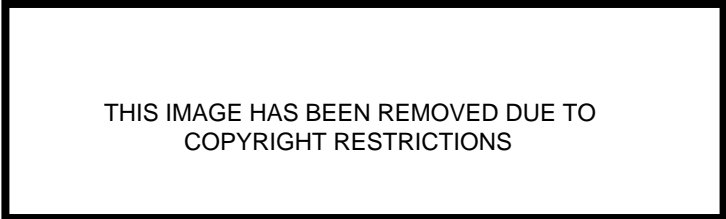
1.4   The Study

The study area chosen centred on the Balimo region of the Western province of PNG mostly through serendipity. The genesis and most clinical aspects of this project where conducted whilst the author was employed at Balimo Health Centre in the early 1990's with the responsibility, among other duties, of helping to develop the capacity of the medical laboratory service within a rural provincial health service. During this time an outbreak of a fatal febrile disease in children presented and one case was culture confirmed to be melioidosis. The hypothesis for this study was developed from this experience.

The thesis is presented in essentially a chronological presentation of events and experimentation in an attempt to address the hypothesis. The rationale for determining melioidosis endemicity is based on finding evidence of clinical disease from patients presenting with febrile disease and isolating the organism, also through identifying a reservoir of infection from the environment. Bacteriology and serological techniques are based on methodology previously reported (Alexander *et al.*, 1970; Thomas, 1977; Ashdown, 1979b; Ashdown *et al.*, 1989; Wuthiekanun *et al.*, 1995a) but the development of a sero-epidemiological assay using an antigen derived from locally isolated organism and used as a national mapping tool is a feature. Insights into modes of transmission were determined by molecular epidemiology using DNA macro restriction analysis and host predisposition is speculated based on case descriptions and links to the reservoir of infection.

The resultant data collected from the Balimo experience has enabled further study into the biogeography of *B. pseudomallei* within PNG which may have relevance in furthering studies into the ecology of *B. pseudomallei* and thus the environmental microbiology of melioidosis. The development of a sero-epidemiological tool, applied nationally may also be used to aid in the mapping of potentially endemic regions. Both the biogeography and sero-epidemiological data may be used for subsequent geographic information system analysis (GIS) and the development of risk models.

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With the Church-run health services receding from this region of PNG and with a Government attempting to fill the gap, a fundamental aspect of this work was an attempt to provide sustainable solutions to a local problem in an underdeveloped world environment, thus the *"maybe melioidosis"* campaign at Balimo Health Centre. Much of the funding that has been sourced for this project has been utilised in training local technicians and health care workers in the bacteriology of *B. pseudomallei* and the treatment of melioidosis so that the disease could be better managed. Although, it is with these people and their government's ability to support them, on which the sustainable outcome of this work depends.

CHAPTER 2 - A REVIEW OF THE LITERATURE

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2.1 The Microorganism

2.1.1 Discovery

Burkholderia pseudomallei was first reported by the British pathologist Whitmore and his colleague Krishnaswami in 1912 when a clinical syndrome resembling glanders, found mostly in morphia addicts, was described by them in Rangoon, Burma (Whitmore and Krishnaswami, 1912). Culture of lung tissue from the index case after autopsy revealed a microorganism similar to *Bacillus mallei* (now known as *Burkholderia mallei*), the causative agent of glanders, a pulmonary disease mostly of *equidae*. Later he published a review of the 38 cases and proposed the name for the causative organism *Bacillus pseudomallei* recognising the similarity but differences from glanders (Whitmore, 1913).

2.1.2 Nomenclature and taxonomy

The microorganism, since its first description, has been the subject of taxonomic controversy (Brindle and Cowan, 1951; Fournier, 1965) and reported with various names (Stanton and Fletcher, 1921; Breed, 1939; Brindle and Cowan, 1951).This has been fuelled, to some extent, by the complexities of bacterial taxonomy and a lack of consensus until recently on how phenotypic and genotypic taxonomic tools should be used (Vandamme *et al.*, 1996). Brindle and Cowan judged that the bacillus belonged in the *Pseudomonadaceae* but, based on the observation that the colonial pigment was not soluble in water, proposed that it could not belong in the *Pseudomonas* genus (Brindle and Cowan, 1951) and proposed a new genus, the *Loefferella*. Notwithstanding this observation Haynes and Burkholder in 1953 in a reorganisation of the *Pseudomonadaceae* re-described members of the *Loefferella* genus, including *L. pseudomallei* as species within the *Pseudomonas* genus (Haynes and Burkholder, 1957).

Although widely accepted as a member of the *Pseudomonas* genus since then, the organism was only formally recognised as *Pseudomonas pseudomallei* in the latest

review of bacterial nomenclature in1980 by Sherman and colleagues (Skerman *et al.*, 1980).

A review of the Pseudomonads using molecular taxonomy divided the *Pseudomonas* genera into five homology groups and proposed *P. pseudomallei* into group II (Palleroni *et al.*, 1972). Further analysis of the genome of these groups resulted in new genera being proposed and group II was placed in the *Burkholderia* genus, named in memory of the eminent plant microbiologist and taxonomist Prof.W. H Burkholder (Yabuuchi *et al.*, 1992). The causative agent of melioidosis now rests within this genus as *Burkholderia pseudomallei* with a growing number of new species still the focus of some nomenclature controversy as phenotypically similar organisms are discovered and described (Woods, 1999; Yabuuchi *et al.*, 2000).

2.1.3 Description

Burkholderia pseudomallei is an aerobic non-spore-forming small bipolar staining Gram-negative bacillus motile by 1 - 4 polar flagellae (Brindle and Cowan, 1951; Haynes and Burkholder, 1957). Motility and disease presentation are the major phenotypic characteristics that differentiate it from *B. mallei.* It is catalase and oxidase positive and utilises carbohydrates oxidatively. There is increasing evidence that it can utilise nitrate under anaerobic conditions (Wongwanich *et al.*, 1996), and thus may have the capacity of anaerobic oxidative phosphorylation. It is nutritionally non-fastidious, being able to utilise a variety of carbon and nitrogen sources (Levine *et al.*, 1954) and grows on most traditionally used media within a wide temperature range, but optimally at 35°C.

Wuthiekanun and colleagues' ongoing experiment describe the organism's survival in triple distilled water (Wuthiekanun *et al.*, 1995b), a character which presumably enables it to survive in its relatively nutrient-poor saprophytic environment. This experiment might not simply describe a physiological adaption to starvation stress but could have uncovered an example of a growth-advantage-in-stationary-phase phenotype (GASP) capability of *B. pseudomallei* caused by the development of

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"waves" of beneficial mutant subpopulations (Storz and Hengge-Aronis, 2000). Further molecular studies of this population of organisms derived from this experiment could help elucidate key ecological survival mechanisms.

Its colonial morphology varies even within strains. Three types have been reported, a traditional rugose appearance, smooth and a mucoid form (Nigg *et al.*, 1956). Small colony variants have also been described (Haussler *et al.*, 1999) that may be linked to antimicrobial resistance development. Clearly if ongoing selection pressure causes certain genetic mutations to survive and flourish, this may result in phenotypic divergence which may manifest in colonial variation (Storz and Hengge-Aronis, 2000). The addition of glycerol in media enhances wrinkling, which is a useful diagnostic observation. These aspects and a broad review of the utility of the laboratory in the identification of the bacterium will be reviewed later in this chapter.

2.2   Melioidosis

2.2.1 The name

As the clinical condition first reported by Whitmore (Whitmore and Krishnaswami, 1912) was similar to glanders, Stanton and Fletcher in 1921 coined the name meli*oidosis* derived from Greek *melis* meaning "a dystemper of asses" and the suffix *oid*, like that of (Stanton and Fletcher, 1921).

2.2.2 Host range

Burkholderia pseudomallei has a wide host range and has been extensively reported as an animal pathogen in both domestic and wild animals, birds, marine mammals, reptiles and marsupials (Egerton, 1963b; Sencer, 1971; Stedham, 1971; Moe *et al.*, 1972; Ketterer *et al.*, 1975; Saroja, 1979; Thomas *et al.*, 1980; Britt *et al.*, 1981; Ladds *et al.*, 1981; Thomas, 1981; Ketterer *et al.*, 1986; Sheikh-Omar and Muda, 1986; Lloyd *et al.*, 1988; Thomas *et al.*, 1988; Ladds *et al.*, 1990; Bergin and

Torenbeeck, 1991; Choy *et al.*, 2000). As a pathogen is it most commonly associated with infections in man, sheep, goats and pigs.

2.2.3 Clinical presentation

A feature of the disease in humans is its protean manifestations and ability to remain latent with recrudescence after decades of presumed initial contact (Kingston, 1971; Sheppard *et al.*, 1990). Melioidosis has a wet season predilection in most endemic regions (Suputtamongkol *et al.*, 1999; Currie *et al.*, 2000d). The extensive diversity of clinical presentations has led clinicians to describe melioidosis as the great mimicker of other infectious diseases (Carruthers, 1981; Yee *et al.*, 1988; Wilairatana and Wilairatana, 1994; Howe *et al.*, 1997; Khoo *et al.*, 2000; Reechaipichitkul *et al.*, 2000).This feature of melioidosis almost certainly contributes to its miss diagnosis and under-recognition in regions where clinical awareness and appropriate bacteriology are lacking (Dance, 2000c).

The spectrum of disease manifestation probably represents a dynamic clinical continuum. However, for convenience, clinical descriptions of melioidosis has been grouped into three broad categories: acute fulminating disease (characterised by bacterial sepsis); subacute localised infection and subclinical latency (Leelarasamee and Bovornkitti, 1989). Melioidosis chronicity has also been observed and defined when symptoms persist for greater than two months duration (Currie *et al.*, 2000b). It has been suggested by Puthuchery and Vadivelu that a more useful clinical classification is simply septicaemic and non-septicaemic melioidosis (Puthucheary and Vadivelu, 2002) as this reflects better the clinical prognosis.

Many variables may influence the manifestation of the clinical presentation (Ashdown *et al.*, 1980) and the apparent incubation period (Currie *et al.*, 2000e). Innate genetic factors have been demonstrated in animals (Leakey *et al.*, 1998; Hoppe *et al.*, 1999; Woods, 2002) and are suggested to be relevant in human infection (Dharakul *et al.*, 1998).Co-morbidity factors are also well documented, in particular diabetes, renal disease and alcoholism in Australia (Chofnas, 1972;

Jackson *et al.*, 1972; Currie *et al.*, 1993; Suputtamongkol *et al.*, 1994a; Suputtamongkol *et al.*, 1999). How one encounters the microbe (through which portal of entry), for example inhalation - pulmonary infection, subcutaneous inoculation - skin lesion, may also constitute a particular manifestation or at least contribute to the clinical manifestation of the disease as might the perceived tropism of the organism for glandular and pulmonary tissue. However, regardless of how a susceptible host acquires the organism, without treatment intervention, acute sepsis usually results.

2.2.3.1   Acute melioidosis

The acute presentation is characterised by typical Gram-negative sepsis with abrupt onset and dissemination with endotoxin induced shock and organ failure (Puthucheary *et al.*, 1981; Atisook and Panyathanya, 1988; Puthucheary *et al.*, 1992; Scott *et al.*, 1997; Inglis *et al.*, 1999; Simpson, 2001). This may be an exacerbation of subacute or chronic disease or be the first presentation from initial exposure of the organism in a particularly susceptible host. High case fatality rates are a feature, particularly if the patient's treatment does not include ceftazidime (White *et al.*, 1989). Complications also arise when empirical therapy for pneumonia and sepsis includes only an aminoglycoside and penicillin, as *B. pseudomallei* is resistant to both class of antimicrobials. This has led to a change of empirical therapy for community acquired pneumonia in Australia when the patient has a history of acquiring the disease in the tropics (Anon, 2000c).

Pulmonary melioidosis is the most common clinical manifestation and often the primary site where acute exacerbation leads to sepsis. Pneumonia may be present secondary to sepsis (Leelarasamee and Bovornkitti, 1989; Ip *et al.*, 1995) where acute respiratory failure with high mortality rates usually results (Puthucheary *et al.*, 2001).Currie describes melioidosis as the most common fatal form of community-acquired bacteremic pneumonia at the Royal Darwin Hospital (Currie *et al.*, 2000e) and thus reminds clinicians to included melioidosis in the differential

diagnosis of a patient presenting with pneumonia and sepsis with a history of travel or residence in a melioidosis endemic region.

2.2.3.2   Subacute melioidosis

Localised infection has been reported from a wide range of organ systems, including skin (McGovern *et al.*, 1999; Currie and Carapetis, 2000) and preexisting ulcers (Rowlands and Curtis, 1965), the eye (Nussbaum *et al.*, 1980; Siripanthong *et al.*, 1991), bone and joints as septic arthritis (Diamond and Pastore, 1967; Borgmeier and Kalovidouris, 1980; Morgan *et al.*, 1996), localised osteomyelitis (Greenawald *et al.*, 1969; Kosuwon *et al.*, 1993; Subhadrabandhu *et al.*, 1995) and mimicking TB spondylitis (Wilairatana and Wilairatana, 1994). Neurological melioidosis, a particular feature of melioidosis in northern Australia, has been reviewed by Currie *et al.* (Currie *et al.*, 2000f). Manifestations include cerebral abscess (Lee and Chua, 1986; Pit *et al.*, 1988; Pelekanos and Appleton, 1989; Kasantikul *et al.*, 1992; Lath *et al.*, 1998; Padiglione *et al.*, 1998; Peetermans *et al.*, 1999; Atapattu *et al.*, 2001) and brainstem encephalitis (Woods *et al.*, 1992; Howe *et al.*, 1997) with varying manifestations including facial and cranial nerve palsy and peripheral motor weakness.

Urogenital system infection has been represented mostly by prostatitis (Kan and Kay, 1978; Morrison *et al.*, 1979; Woo *et al.*, 1987; Arakawa *et al.*, 1993; Munns and Thompson, 1999; Yip *et al.*, 2001), with one case implicated in human to human transmission during sexual contact although the evidence of transmission was based on sero-conversion and not clinical disease or culture confirmation and is therefore speculative (McCormick *et al.*, 1975). Epididymo-orchitis has also been reported (Jayanetra *et al.*, 1983).

Gastrointestinal tract infection reports have been limited to the oral cavity with parotitis being the most common. Indeed parotitis is the most frequent presentation in children in Thailand (Dance *et al.*, 1989a; Lumbiganon and Viengnondha, 1995). It may be also associated with disseminated melioidosis in adults (Arakawa *et al.*,

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1993). Interestingly, parotitis seems a rare localised manifestation in Australia (Faa and Holt, 2002). It is therefore unlikely that this manifestation in children will serve as a sensitive indicator to the extent of melioidosis endemicity in Australia or Papua New Guinea (Edmond *et al.*, 1998; Warner *et al.*, 1998; Currie and Brewster, 2001; Edmond *et al.*, 2001), as Dance *et al.* suggest it might be in Thailand (Dance *et al.*, 1989a).

Localised subacute melioidosis of the respiratory tract includes pneumonitis and bronchitis (Spotnitz *et al.*, 1967; Everett and Nelson, 1975) and even pharyngitis (Tan and Sethi, 1997). Pharyngeal carriage is unlikely in individuals not suffering melioidosis (Kanaphun *et al.*, 1993) but is apparent in a diversity of clinical manifestations, not simply localised respiratory disease (Wuthiekanun *et al.*, 2001). It has been noted that even when the infection results from an inoculation incident, pulmonary infection is frequent. This may be explained by a proposed tropism for pulmonary epithelium by *B. pseudomallei* (Dance, 2000c).Given that human respiratory tract melioidosis is a significant reservoir of infection in endemic regions, its seems unusual that person to person transmission via the respiratory tract is not apparent. This might be explained by an inability of *B. pseudomallei* to attach to the epithelium of the upper respiratory tract (URT) (Ahmed *et al.*, 1999). The organism is either unable to achieve critical numbers for transmission from an infected host or a failure to attach to transmitted host URT prevents invasion.

Melioidosis is emerging as a cause of respiratory infection in cystic fibrosis sufferers (Schulin and Steinmetz, 2001; Holland *et al.*, 2002) where person to person transmission may be a feature.

Medical imaging technology has aided the diagnosis of visceral organ abscesses manifesting in the kidney, spleen, liver and adrenal glands (Singcharoen, 1989; Lee *et al.*, 1999; Wibulpolprasert and Dhiensiri, 1999). In endemic regions without this technology, pyrexia of unknown origin (PUO) with or without serological evidence may be the only evidence for deep organ abscesses and unpalpable masses elsewhere.

2.2.3.3 Chronic melioidosis

Chronic infection presents in individuals who, compared to those who develop sepsis upon initial exposure, process the organism more successfully. This has been demonstrated in animal modelling (Leakey *et al.*, 1998). Patients may present with a typical Gram-negative endotoxin induced wasting, presumably linked to cytokine hyper-induction, although this has recently been challenged and linked simply to a protein-calorie malnutrition (Paton *et al.*, 2001).

Chronic pulmonary melioidosis may mimic TB in radiological findings and clinical presentation (Sweet *et al.*, 1968; Newland, 1969; Puthucheary *et al.*, 1992). Given the diversity of clinical forms of TB and the difficulty in obtaining a clinical diagnosis based on retrieving the microorganism, it is also likely that melioidosis may mimic both the localised and miliary forms of extra-pulmonary TB and therefore may be missed in countries where a strong medical culture of tuberculosis diagnosis exists but may swamp other forms of bacteriological investigation. The radiological findings of pulmonary melioidosis in its diversity of presentations have been reviewed elsewhere (Dhiensiri *et al.*, 1988; Pui and Tan, 1995; Tan *et al.*, 1995; Chong and Fan, 1996).

Although there has been no evidence of TB/melioidosis co-infection (Kanai *et al.*, 1992), it is interesting to speculate how many slide negative treatment-refractory cases of TB presenting in rural regions of developing countries, might be undiagnosed melioidosis.

2.2.3.4 Latent melioidosis

A worrying feature of the disease is its ability to reactivate after apparent extended periods of asymptomatic infection (Newland, 1969; Kingston, 1971). The longest period of incubation leading to activation has been reported to be 29 years (Chodimella *et al.*, 1997). Reactivation is usually exacerbated by co-morbidity factors such as late onset diabetes and renal disease, predisposing the host through

suppression of cellular immunity, similar to recrudescence of miliary or localised extra-pulmonary TB. Other less traditional factors such as neoplasia may also be responsible (Mays and Ricketts, 1975; Read *et al.*, 2001). The clinical complications of this presentation is that the period of latency may be decades and the patient may have no history of exposure to a traditional endemic countries or regions (Morrison *et al.*, 1988). This highlights the importance of obtaining an accurate patient travel history in determining the differential diagnosis of pneumonia and sepsis and in the investigation of PUO (Peetermans *et al.*, 1999; Schwarzmaier *et al.*, 2000; Visca *et al.*, 2001) and also the continued mapping and reporting of global melioidosis endemicity.

The organism is an intracellular pathogen (Wong *et al.*, 1995; Jones *et al.*, 1996). It is possible that a proportion of asymptomatic individuals that demonstrate serological evidence of exposure to *B. pseudomallei* antigen are latently infected and therefore prone to reactivation illness (Ashdown and Guard, 1984; Kanaphun *et al.*, 1993). Given the lack of understanding on the immunopathogenesis of melioidosis, it is equally possible that the presence of detectable antibody indicates successful processing of the organism and a decreased chance of reactivation (Charuchaimontri *et al.*, 1999). Indeed, antibodies produced against cell wall components that have been implicated as virulence determinants, have been shown to improve survival in acute melioidosis (Charuchaimontri *et al.*, 1999). Supporting this view, Thompson and Ashdown found in a sample of individuals from a high-risk population no correlation between the presence of antibody and the rate of prospective clinical activation (Thompson and Ashdown, 1989). Currie also notes the high sero-prevalence in children in Thailand may provide protection and explain the relatively low incidence of the disease (cases per population per year) - for a hyperendemic region - in North Eastern Thailand compared to the Northern Territory where sero-prevalence is low (Currie *et al.*, 2000e).

Detection of an antibody response in individuals therefore may not provide a defined marker for latency or subclinical infection - perhaps the reverse. Perhaps those unable to mount a detectable immune response, but are at risk of exposure, are those

more likely to develop disease when exposed. Latency therefore may only be accurately described retrospectively, that is, after an episode of melioidosis where there was no chance of recent acquisition of the organism and where the individual has a strong past exposure history, this may be very difficult to determine. Currie *et al.* in an attempt to define latency determined that patients who developed melioidosis during hospital admission, unrelated to melioidosis, with a history of past sero-positive status were examples of reactivation illness from a period of latency (Currie *et al.*, 2000b).

2.2.2 Treatment

Regardless of the initial clinical presentation, melioidosis will continue inexorably on a fatal course if not treated aggressively and thoroughly. Early observations noted resistance to penicillin and gentamicin (Eickhoff *et al.*, 1970) which contributes to the high mortality of melioidosis when not suspected, as this combination is commonly used as empirical treatment for pneumonia and sepsis throughout the world (Reece, 1984; Anon, 1993; Anon, 2000c; White, 2003). Early attempts at treatment included high dose chloramphenicol, doxycycline, trimethoprim-sulphamethoxazole and kanamycin for up to 6 month and were based on *in vitro* sensitivity results of isolates uncovered as cases were described (Rowlands and Curtis, 1965; Johnson, 1967; Magee *et al.*, 1967; Newland, 1969; Beaumont, 1970; De Buse *et al.*, 1975; Kan and Kay, 1978; Ashdown, 1979e). Ashdown confirmed the sensitivity to kanamycin but only in toxic doses was it mostly effective (Ashdown, 1979a).

2.2.2.1   Directed therapy based on prospective trials

Two forms of treatment are now standard directed therapy, based on a series of randomised trials of both parenteral and oral antibiotics shown to have *in vitro* activity. The traditional regimen of combination doxycycline, chloramphenicol and cotrimoxazole for 20 weeks was more effective than doxycycline on its own (Chaowagul *et al.*, 1999). Amoxicillin-clavulanate was also found to be less effective

than this combination, but more appropriate for children and pregnant women because of fewer side affects (Rajchanuvong *et al.*, 1995). The combination of azithromycin and ciprofloxacin had a 22% relapse rate compared to 3% using the traditional combination (Chetchotisakd *et al.*, 2001). Following encouraging reports of some third generation cephalosporins in the treatment of severe melioidosis, White *et al* demonstrated a halving of mortality with the use of ceftazidime compared to the traditional combination of doxycycline, chloramphenicol and co-trimoxazole (White *et al.*, 1989). The addition of cotrimozaxole decreased mortality further compared to the same combination (Sookpranee *et al.*, 1992). Both imipenem alone (Simpson *et al.*, 1999) and amoxicillin-clavulanic acid alone (Suputtamongkol *et al.*, 1994b)in separate trials have demonstrated similar mortalities to each other but less effectiveness than combination therapy with ceftazidime.

These studies have provided the basis for the accepted directed therapy for severe melioidosis of either ceftazidime (40 mg/kg IV every 8 hr, total 120 mg/kg/day or 19 mg/kg IV followed by a continuous infusion) or imipenem (20 mg/kg IV every 8 hr, total 60 mg/kg/day) for at least 10 days followed by oral antibiotics chloramphenicol (40 mg/kg/day) plus doxycycline (4 mg/kg/day) plus trimethoprim-sulfamethoxazole (10 mg/kg and 50 mg/kg/day respectively) for 20 weeks as maintenance therapy although chloramphenicol should be discontinued after 8 weeks to limit side affects. This oral regimen can be used for subacute disease when parenteral antibiotics are not available (White, 2003). Currie *et al* suggest that monotherapy of high dose trimethoprim-sulfamethoxazole could be used to replace the combination therapy for maintenance (Currie *et al.*, 2000d). This may also be a workable treatment for subacute disease in resource poor, rural regions where patient compliance and costs are issues affecting successful treatment. If available, amoxicillin-clavulanic acid (amoxicillin 30 mg/kg/day, clavulanic acid 15 mg/kg/day) plus amoxicillin (30 mg/kg/day) can be used in children and pregnant women.

An obvious problem with the ideal therapies is that they are expensive and communities that are at-risk of melioidosis are poor. In these regions at least the conventional regimen of doxycycline, chloramphenicol and cotrimoxazole is often available (Anon, 1993; Anon, 1997). And in spite of its relative lack of efficacy compared to the optimal therapy, the cost benefit may justify its use. Particularly if subacute disease can be identified and treated before exacerbation of acute sepsis.

2.2.2.2   Cytokine therapy and vaccination

Cytokines that modulate functional activity of cells during haematopoiesis and immune stimulation, have been used for therapy for an array of clinical conditions including infection, inflammation, neoplasia and autoimmune diseases (Rodak, 1995). Granulocyte colony stimulating factor (G-CSF) has a role in the stimulation of polymorphonuclear neutrophils from myeloblasts and their differentiation from the monocyte and macrophage line. Given the observation that co-morbidity factors are linked with neutrophil function (i.e. diabetes) it has recently been observed that G-CSF can be used successfully in the treatment of melioidosis patients in septic shock (Stephens *et al.*, 2002). Although its beneficial activity has not been demonstrated in animal experimentation (Powell *et al.*, 2003) and therefore the scientific basis for the clinical outcomes observed require further study.

Vaccine candidates exist but as melioidosis is a disease of individuals living in subsistence rural communities there is unlikely to be a market to justify pharmaceutical company investment, evaluation and mass production (Warawa and Woods, 2002). Concerns of biowarfare have driven vaccine research and this may progress government sponsored vaccine initiatives in the future.

2.3   Epidemiology

2.3.1 Geographic distribution

2.3.1.1   Known regions of endemicity are the tip of the iceberg

The tip of the iceberg metaphor has been commonly used to describe the evidence for global melioidosis distribution (Dance, 1991a; John *et al.*, 1996; Warner *et al.*, 1998; Dance, 2000b). The detection of melioidosis is often linked with the strengthening of medical and laboratory services in developing countries (White and Dance, 1988; Chaowagul *et al.*, 1989).

The extent of the melioidosis endemic range was originally reported to be within 20° north and 20° south latitude (Redfearn *et al.*, 1966) this includes most of South East Asia - particularly North Eastern Thailand and Northern Australia (the regions of highest reported incidence) and parts of equatorial Africa and the Americas. Reports of animal melioidosis, in particular, have extended this region to beyond 40° latitude. Importation of melioidosis and subsequent maintenance of the reservoir of infection in temperate regions seems possible (Currie *et al.*, 1994).

It perhaps betrays a lack of knowledge of the geographic and demographic diversity of PNG that resulting from only a handful of reported melioidosis cases from Port Moresby (the urban capital) the whole island of New Guinea, including West Papua is often highlighted as endemic for melioidosis (Guard, 1987).This may be the case, but prior to this study no case of melioidosis has been reported from or traced to provinces outside Port Moresby. Details of the history of melioidosis in PNG will be presented later.

It is interesting to note that since the first reports of melioidosis by Whitmore, (Whitmore and Krishnaswami, 1912; Whitmore, 1913) only a handful of cases have since been reported from Burma (Lee *et al.*, 1999; Leeuwenburgh *et al.*, 2002). It seems unlikely that the index cases reported from Rangoon resulting from the work

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of Whitmore and Krishnaswami represents an isolated outbreak that does not reflect national endemicity. Since the withdrawal of its colonial administration many countries may have struggled to maintain the facilities that were established by expatriate colonisation and therefore relative boutique causes of febrile disease (as apposed to malaria, TB and typhoid) that may have provided scientific curiosity to expatriates, have since been overlooked.

2.3.1.2 Melioidosis is hyperendemic in some Asian countries

Most melioidosis cases from Asia have been reported from Thailand where it has been extensively studied and reviewed (White, 2003). Features include regional clustering with a hyperendemic focus in the north east province with only sporadic cases being reported from Bangkok and elsewhere (Chaowagul *et al.*, 1989). Melioidosis has also been reported from Vietnam where the US and French military encountered the organism and, given its potential for latency, was described as the Vietnamese time bomb (Brundage *et al.*, 1968; Stedham, 1971; Moe *et al.*, 1972; Worthington and McEniry, 1990; Van Phung *et al.*, 1993; Parry *et al.*, 1999; Finkelstein *et al.*, 2000).

Melioidosis has long been recognised in Malaysia (Strauss *et al.*, 1969a) where it also has been extensively studied (Ellison *et al.*, 1969; Strauss *et al.*, 1969a; Strauss *et al.*, 1969b; Cheong *et al.*, 1987; Yee *et al.*, 1988; Puthucheary *et al.*, 1992; Radua *et al.*, 2000; Vadivelu and Puthucheary, 2000; Chenthamarakshan *et al.*, 2001; Puthucheary *et al.*, 2001) and recently in Laos (Phetsouvanh *et al.*, 2001). Other regions of Asia where it seems endemic but less prevalent include Hong Kong and mainland China where regional clustering is also a feature (So *et al.*, 1984; Woo *et al.*, 1987; Li and He, 1992; Yang *et al.*, 1995; Yang, 2000), Taiwan (Lee *et al.*, 1985; Hsueh *et al.*, 2001), Japan (Arakawa *et al.*, 1993) and Singapore (Heng *et al.*, 1998). Animal and human cases have been reported from Indonesia and the Philippines (Trakulsomboon *et al.*, 1994).

It seems clear that not withstanding the feature of regional clustering, it is likely that if one looked hard enough in a well funded prospective study of sepsis and atypical pneumonia in any of the countries within Asia which share basic similarities of subsistence farming and geography, melioidosis could be revealed. The fact that there is *bona fide* regional clustering in some of these regions may aid an understanding into the pathogenesis and epidemiology of infection through studies in preferred habitat and host predisposition.

In the Indian subcontinent melioidosis has been reported from Bangladesh (Struelens *et al.*, 1988) and India (Kang *et al.*, 1996), but was subject to an interesting debate when suggestions arose that melioidosis might have caused an outbreak of a plague-like illness in Maharashtra (Bharadwaj *et al.*, 1994; Bharadwaj *et al.*, 1995; Cherian *et al.*, 1995; Dance *et al.*, 1995; John, 1995). It seems that consensus now rests with melioidosis not being implicated although this case highlights the problem of confirming melioidosis in regions where the disease and organism characteristics are not well known.

An imported case identified in the UK had India implicated as the source of infection (Hoque *et al.*, 1999). Sri Lanka has been implicated in an imported case of melioidosis also (Peetermans *et al.*, 1999) but surveys in the urban capital demonstrated little evidence for the potential for clinical disease (Van Peenen *et al.*, 1976). This again may not reflect the endemicity in rural regions of the country.

2.3.1.3 Africa and the Americas

In studies undertaken in Africa, cases seem genuinely sporadic (Batchelor *et al.*, 1994) although clinical cases and environmental prevalence of the organism have been reported from Western Africa indicating a true endemicity (Wall *et al.*, 1985). There has been a number of reports from the Americas, with those from Northern America being mostly imported from regions of known endemicity. Although, a case was reported from the USA from a patient with a travel history to Mexico (Barnes

et al., 1986). Sporadic cases have been reported from Central America and South America (Perez *et al.*, 1997; Dorman *et al.*, 1998).

In a review of melioidosis geographic distribution Dance also reported cases described from Fiji, Pakistan, Iran and Turkey (Dance, 1991a). This author also has personal experience of a fatal case imported from Fiji to Sydney in 1982.

2.3.1.4 Melioidosis in temperate regions

The importation of melioidosis to regions outside the tradition zones of endemicity is a common feature and may be on the rise (Dance *et al.*, 1999; Peltroche-Llacsahuanga and Haase, 1999; Heyse *et al.*, 2003). Factors associated with this observation may be associated with increased global travel but also may simply reflect increased awareness by clinicians and laboratory scientists throughout the world as the global significance becomes more apparent. It is also possible that importation of the organism carried by animals may provide a vehicle for environmental colonisation which could provide a reservoir of infection for human disease as is the case described by Currie *et al.* (Currie *et al.*, 1994). This case has resulted in persistent autochthonous infection in temperate regions for over 25 years.

2.3.1.5 Melioidosis in Australia

In Australia the first reported cases were from animals (Cottew *et al.*, 1952; Laws and Hall, 1964). The index cases resulted from an outbreak in a flock of 4000 Merino sheep in Winton, north western Queensland in 1949. In total 80 - 100 head died but only three were confirmed on autopsy with culture confirmed melioidosis. In April the following year Rimmington began observing the first of his six human cases from the Townsville region between 1950 - 1960 (Rimmington, 1962). Five of the cases presented with co-morbidity of diabetes, renal impairment and one pregnant 17-year-old who appeared on autopsy to be suffering congenital lung cysts. All patients, five of the six, that presented with septicaemia died. Soon after,

melioidosis was reported for the first time in the Northern Territory (Crotty *et al.*, 1963) where it is now extensively studied (Currie *et al.*, 2000d).

Two major foci of human melioidosis have since been identified: north eastern Queensland, including the Torres Strait where a high incidence has recently been reported (Faa and Holt, 2002) and the Northern Territory where the incidence has been calculated to be 16.5/100 000 annually, increasing to 34.5/100 000 during heavy wet seasons (Currie *et al.*, 2000e). Cases of autochthonous disease have also been reported from northern and southern Western Australia (Currie *et al.*, 1994; Inglis *et al.*, 1998b; Inglis *et al.*, 1999) where approximately five cases are reported annually. Southern Queensland has also been implicated (Magee *et al.*, 1967; Munckhof *et al.*, 2001). Further reports of melioidosis in NSW and other southern ports have been imported either from an overseas endemic region or elsewhere in Australia (Wilson *et al.*, 1987; Chan, 1992).

All forms of disease are presented, but the chronic forms may attract more interest in their unravelling and therefore are more represented in the literature. As with the index cases reported by Rimmington most individuals with melioidosis reported in Australia share the co-morbidity factors of either diabetes or renal failure with alcoholism also apparent. The recent increase in incidence in the Torres Strait may be due to the parallel increase of type 2 diabetes in the community (Faa and Holt, 2002).

Interesting aspects of the disease in Australia include the low prevalence of acute parotitis seen in childhood melioidosis - only one case reported - (Edmond *et al.*, 1998; Edmond *et al.*, 2001; Faa and Holt, 2002) and the relatively high pervalence of prostatic abscesses in men and neurological melioidosis reported in both adults and children (Currie *et al.*, 2000d).

Melioidosis has only recently been classified as a notifiable disease in parts of Australia, so prevalence and annual incidence is open to underestimation. In

Townsville, 15-25 cases per year can be expected with a fatality rate of 25% (Norton, 2003).

The diversity of geographic distribution and regional clustering within endemic regions highlights the importance of concerted prospective studies into the cause of atypical pneumonia and sepsis in developing countries within the tropics in determining the global significance of melioidosis. Perhaps also this situation provides tangible evidence of the direct link between the quality of medical laboratory services in developing countries and community health outcomes.

2.3.2   Reservoir of infection, habitat and ecology

2.3.2.1 Early observations

Early reports investigating a reservoir of infection implicated animals, particularly rodents, and hypothesised that the mode of transmission was faeces contaminating food (Whitmore, 1913). Ellison *et al* cite Stanton and Fletcher's description of an outbreak of melioidosis in an experimental guinea pig colony. This was attributed to wild rodent contamination of vegetables used as food (Ellison *et al.*, 1969). Washings of the food inoculated into disease-free guinea pigs resulted in signs consistent with melioidosis although a subsequent attempt to isolate the organism from the wild rodents failed. Magee *et al* cite early reports of blood feeding insects being considered as reservoirs and vectors (Magee *et al.*, 1967). Not withstanding the difficulties in the diagnosis of melioidosis, leading to under recognition, the lack of apparent widespread epidemics seems inconsistent with the vector borne transmission model suggested in these early reports.

2.3.2.2 Soil and water

Ellison cites that Chambon isolated the organism for the first time from the soil and water of swamps and rice fields in Vietnam 30 years later. This provided the basis for current opinion that *B. pseudomallei* is indeed a saprophyte. Although, the

prevalence in his study was low from a small numbers of samples (Ellison *et al.*, 1969). Limited isolation rates were demonstrated in Townsville in the late 1950's when Laws and Hall recovered *B. pseudomallei* from a local swamp. They found that *B. pseudomallei* survived for months when inoculated in water and kept at room temperature, thus confirming the status of *B. pseudomallei* as nutritionally non-fastidious (Laws and Hall, 1964).

Thomas successfully isolated the organism from a paddock in Townsville associated with clinical disease in sheep (Thomas, 1977) and later went on to study the association of soil type and depth with the prevalence of *B. pseudomallei* isolation (Thomas *et al.*, 1979). This work has been the basis for the general consensus that *B. pseudomallei* survives best in moist clay at approximately 30 cm below the surface where it may be sequestered during times of drought, to be brought to the surface during rainfall where, if finding shelter from UV light and in persistent water may multiply to numbers sufficient to successfully cause disease when transmitted to a host. These observations have been broadly supported by *in vitro* studies of the physio-chemical requirements of the organism in soil (Tong *et al.*, 1996).

2.3.2.3 Interactions with the environment

Inglis in his review of the environmental microbiology of melioidosis points out that the organism's relationship with plants and protozoa and ability to form bioflims shed light on the complexity of the ecology of the organism (Inglis *et al.*, 2001). It may be possible as with *Legionella pneumophilia*, that passage through free living amoeba may not only provide a reservoir of infection but also may enhance virulence and thus prepare *B. pseudomallei* for a parasitic intracellular life in more complex animal hosts. Passage through animals has been shown to enhance virulence (Nigg *et al.*, 1956). There has been anecdotal association between presence of free living amoeba in the environment associated with a melioidosis outbreak. This perhaps should not be surprising, given the presence of free living amoeba in water in the tropics is likely to be as ubiquitous and uncharacterised as soil bacteria. Interestingly though, meningoencephalitis in children caused by *Nagleria fowleri* has been

reported in the same location of PNG where melioidosis has been implicated (Tucker and Zerk, 1991; Warner *et al.*, 1998). It was hypothesised in this report that the amoebae made their way through the cribiform plate when introduced during bathing in stagnant, warm swamp water. It is possible that regions with an environment that is conducive to this bacteria/protozoa interaction, are breeding grounds for conveying virulence to otherwise relatively avirulent saprophytes.

The nitrogen fixation capacity of members of the genus *Burkholderia* have been documented (Estrada-De Los Santos *et al.*, 2001). Although it was widely accepted that *B. vietnamensis* alone possesses this characteristic, a more diverse group has been uncovered with a wide distribution and capacity to colonise host plants. Further work on determining this capacity - and thus the organism's association with plants - may aid in mapping the geographical distribution of melioidosis and may provide control strategies through reforestation and land management of at-risk localities. Bioremediation is possible in the control of disease if an environmental preference for a non-virulent species, which competes successfully against *B. pseudomallei*, could be determined. Although the sustained maintenance of bacteria seeded into soil requires a detailed understanding of the interactions between the bacteria and aspects of soil (van Veen *et al.*, 1997).

2.3.2.4 Environmental isolation techniques

In studies of the environmental microbiology of melioidosis both direct inoculation of environmental samples into susceptible animals (guinea pigs or hamsters) and direct inoculation of samples into MacConkey broth with or without crystal violet and neutral red subcultured onto similarly formulated agar have been used.

Galimand and Dodin developed a medium, based on selection rather than enrichment, using threonine as the sole source of nitrogen and carbon (Galimand and Dodin, 1982). In an *in vitro* study Ashdown and Clark tested the sensitivity of the techniques including those used by Galimand and earlier investigators against trypticase soy broth incorporating 5 mg/l of crystal violet and 20 mg/l of colistin.

After incubation supernatant was subcultured to Ashdown agar which had been previously described (Ashdown, 1979c). The Ashdown based media alone recovered all strains tested (Ashdown and Clark, 1992).

Both the Ashdown media and threonine media have been compared in a field study in north-eastern Thailand. Samples of soil for culture where suspended into sterile water and left to settle, samples of supernatant were used to inoculate the preferred media. The threonine based media with the addition of 50 mg/l colistin was determined to be more sensitive, although some isolates recoverable by the Ashdown method were not recovered by the threonine broth (Wuthiekanun *et al.*, 1995a). It was also noted during this study that isolation rates increased the deeper the samples were taken. This was in contrast to Thomas's observations that 30 cm was the optimal depth for recovery of *B. pseudomallei* (Thomas *et al.*, 1979). The polymerase chain reaction (PCR) has also been used and found to be highly sensitive when used on samples of soil following enrichment. Ashdown broth was more effective than Galimand's Threonine broth (Brook *et al.*, 1997). Samples of water for selective culture can be passed through 0.45 or 0.22 µm filters and either the filter is placed onto solid media or immersed into broth.

The preferred micro habitat of *B. pseudomallei* is presumably more complicated than mere depth of soil. It is more likely that depth may reflect a change in soil profile with a change in physio-chemical and biological factors that affect *B. pseudomallei* survival. Water may either be the endogenous habitat with or without an association with aquatic plants or protozoa or simply the vehicle of transmission from a terrestrial source with or without plant and animal association. A detailed analysis of these interactions and associations remains to be undertaken and would provide information for GIS which may aid in the mapping of potential clinical endemcity.

2.3.3   Transmission

2.3.3.1 Environmental exposure

Evidence supports the association between environmental exposure and incidence of clinical disease (Merianos *et al.*, 1993). Studies in Thailand have demonstrated an association between clinical and environmental prevalence. In the north eastern provinces, *B. pseudomallei* was recovered from 50.1% of soil samples analysed. with the incidence of clinical disease at 137.9 per 100,000 inpatients. This is compared to 13.8% and 18 per 100,000 in the north, 24.5% and 13.4 per 100,000 in central regions, 18.4% and 14.4 per 100,000 in the south (Vuddhakul *et al.*, 1999). Although, Finkelstein and colleagues in a belatedly reported study conducted in the 1960's describes the highest soil prevalence of *B. pseudomallei* in southern regions of Thailand (Finkelstein *et al.*, 2000). This led to a prospective investigation into the clinical prevalence in patients suffering respiratory disease in this region where no evidence was found of clinical disease. This created doubt in investigator's minds that melioidosis was significant in Thailand. Perhaps variation in sampling and selective culture produced this anomaly. Regardless, this highlights the difficulties in obtaining meaningful results from random soil sampling and the problems of comparing results when the techniques used are not standardised, and that these results can lead to misleading interpretations.

2.3.3.2 Arabinose utilisation

The discovery of a soil isolate with similar phenotypic characteristics to *B. pseudomallei* but avirulent in an animal model may help to explain anomalies in past studies in the environmental microbiology of melioidosis (Wuthiekanun *et al.*, 1996). All soil isolates from central Thailand were subsequently found to be this avirulent type, now characterised as a separate species, *B. thailandensis*. Whereas in North Eastern Thailand 75% of soil isolates were the arabinose utilising, *B. pseudomallei* (Smith *et al.*, 1997). Therefore the relative prevalence of *B. thailandensis* compared to *B. pseudomallei* in the environment may aid in the

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classification of melioidosis endemicity. It is interesting to note the discovery of
B. uboniae, a *B. thailandensis*-like isolate, similar in terms of arabinose utilisation
but with considerable genetic divergence (Yabuuchi *et al.*, 2000).

2.3.3.3 Rainfall association

Time-space clustering is a recognised feature of melioidosis (Suputtamongkol *et al.*,
1994a). This is likely to be caused by the complexity of the relationship between the
microbe, the environment and the host but epidemiological studies have shed light on
consistent environmental and host risk factors These may in turn aid in broadening
the understanding of the microbiology of melioidosis. The rationale proposed by
Thomas (Thomas, 1977; Thomas *et al.*, 1979) and others for the association with
saturated soil (Tong *et al.*, 1996) is consistent with the incidence of clinical cases
associated with rainfall, best represented in data from Darwin (Merianos *et al.*, 1993;
Currie and Jacups, 2003). Currie and Jacups (Currie and Jacups, 2003) also make the
observation that not only does case incidence increase during times of heavy rain, the
virulence of the disease rises with the intensity of rainfall. The increased rainfall may
make available a larger inoculum (compared to inocula from soil or dust alone) thus
increasing virulence. This is suggested by the observation of increased virulence of
water as a sample (as opposed to soil) in isolation models using animals (Ellison
et al., 1969).

The index outbreaks in Australia may also have been associated with rainfall.
Cottew's first case reports in sheep during May 2-weeks after tail docking in Winton
was preceded by March rainfall four times the monthly average (data kindly supplied
by Lesley Rowland, Bureau of Meteorology, Melbourne).

Increased rainfall may facilitate a respiratory tract portal of entry through increased
aerosolisation which may initiate a more virulent course as transmission experiments
in animals via the nasopharyngeal route suggest (Jeddeloh *et al.*, 2003). In contrast,
the problems of weak adherence to upper respiratory epithelium have been
demonstrated, this may explain the lack of natural transmission by this route as in

human to human respiratory tract transmission, particularly when the inoculum is below a critical number. This may be the case in non-aerosolised water encounter by this route because this characteristic weakness can be overcome by increasing the inoculum size (Ahmed *et al.*, 1999).

The flooded rice fields of Thailand and Vietnam with farming practices that aerate the soil/water slurry during cultivation have been widely implicated (Suputtamongkol *et al.*, 1994a; Smith *et al.*, 1995b; Parry *et al.*, 1999) as a reservoir of infection for melioidosis and provide the best evidence of clinical and environmental association. Early observations of outbreaks in Vietnam war veterans have implicated dust disturbed by helicopters as a source (Howe *et al.*, 1971) but this could just as likely be aerosolised water. Outbreaks where rainfall has not been a feature have been attributed to alteration of articulated water providing an environment to best harbour significant numbers of organism and/or the means with which to transmit the organism to the animal host (Ketterer *et al.*, 1986; Inglis *et al.*, 1999; Inglis *et al.*, 2000a; Currie *et al.*, 2001).

Limited case control studies have not been able to clearly define a disease correlation with any particular mode of transmission (Merianos *et al.*, 1993; Suputtamongkol *et al.*, 1999), other than the broad correlation with environmental exposure. This supports clinical observations where indigenous northern Australians and rice farmers of north eastern Thailand have a higher sero-prevalence and higher incidence of disease than other groups (Suputtamongkol *et al.*, 1994a; Currie *et al.*, 2000d). The difficulties arise in obtaining accurate information on inoculation incidents, retrospective to onset of symptoms. Although it is widely accepted that subcutaneous inoculation is the most likely mode of transmission, in a Thailand study of 63 cases only four had a history that suggested direct inoculation (Chaowagul *et al.*, 1989). As is likely to be the case in other rural, subsistence communities where the source of infection in not clear, it was noted that almost all individuals had minor abrasions to limbs.

2.3.3.4 Human to human transmission

Human to human transmission appears not to be a feature of melioidosis
epidemiology. Studies of outbreaks have not been able to identify a common human
source for the infection (Merianos *et al.*, 1993). Cases have been reported within
outbreaks to share genotypically identical isolates, but this is just as likely to be
explained by a shared environmental reservoir (Inglis *et al.*, 1999). Although, it
seems possible that in patients with a close familial association who share chronic
respiratory disease, that the factors required for successful respiratory droplet
transfer and establishment of infection is possible. This may have been the case in
the reports of siblings with cystic fibrosis who were shown to be infected with the
same genotype of *B. pseudomallei*. Again, a common environmental source could
not be ruled out (Holland *et al.*, 2002). Past reports of human to human transmission
have been defined by sero-conversion evidence alone (McCormick *et al.*, 1975),
although the relatively high incidence of genito-urinary tract infections in northern
Australia may result from true sexual transmission.

In traditional cultures in the developing world, numbers of individuals per house is
high. Where cooking fires are kept inside houses smoke particle-induced chronic
respiratory tract disease is common. Where TB is endemic, transmission within
families in common. Not withstanding the current evidence suggesting shared
environmental exposure results in outbreaks, one feels that in these environments
horizontal familial transmission of melioidosis may also be possible.

2.3.3.5 Nosocomial and iatrogenic transmission

Although rarely reported, it should be noted that iatrogenic and nosocomial
transmission has been noted (Ashdown, 1979d; Markovitz, 1979) as has transmission
due to laboratory accidents (Schlech *et al.*, 1981), particularly when aerosols of
suspensions are produced.

2.3.4   Molecular epidemiology

2.3.4.1 Ribotyping

Molecular typing methods have been employed in an attempt to reveal quantifiable association between environmental and clinical clones so to provide conclusive evidence for a reservoir of infection and a mode of transmission. Ribotyping was first reported for use in melioidosis epidemiology by Lew and Desemarchelier using *Bam*HI digests of chromosomal DNA (Lew and Desmarchelier, 1993). Fragments were separated by agrose gel electrophoresis and transferred to a nylon membrane. The membrane was subjected to hybridisation with a cDNA copies of *Escherichia coli* rRNA probes labelled with horseradish peroxidase, based on studies by Stull (Stull *et al.*, 1988). Twenty-two rDNA patterns resulted which demonstrated greater diversity than phenotypic typing schemes such as serology and therefore provided the basis for an epidemiological tool. Clonality is determined, rather arbitrarily, if the patterns are not able to differentiated visually and this provides evidence of relatedness between isolates. The tool was used to reveal probable endogenous relapse in recurrent infection (Desmarchelier *et al.*, 1993) and clonal introduction and persistence of the clone being responsible for endemic melioidosis in a temperate zone (Currie *et al.*, 1994). However more than 60% of isolates from an endemic region in northern Australia fell into only three ribotypes and therefore the technique was considered to have poor discriminating sensitivity. Sexton *et al.* used *Eco*RI digests to achieve similar results (Sexton *et al.*, 1993).

2.3.4.2 Random amplified polymorphic DNA

Haase *et al* showed that ribotypes could be further discriminated using random amplified polymorphic DNA (RAPD) PCR, where short arbitrary primers under low-stringency conditions bind and produce products displayed as a pattern when separated by agarose gel electrophoresis (Haase *et al.*, 1995b). This technique proved to be faster and able to discriminate the unrelated isolates within a single ribotype from isolates from the Northern Territory. The technique was also used to confirm

the findings by Currie of clonal persistence in southern Western Australia (Currie *et al.*, 1994). Secondary infection rather than reactivation was demonstrated using this technique (Haase *et al.*, 1995a). Norton and colleagues reported an association between isolates in terms of the clinical presentation they produced in humans using RAPD and multilocus enzyme electrophoresis (Norton *et al.*, 1998) although this observation seems to be dispelled by Ulett and colleagues where, in a more detailed study, no association between virulence and genotype could be described. (Ulett *et al.*, 2001). This suggests that host factors rather than differences in microbial pathogenesis (as demonstrated by broad genotypic differences) play a more significant role in the diversity of disease presentation.

2.3.4.3 DNA macro restriction analysis and pulse field gel electrophoresis

 Fragment patterns resulting from the digest of whole DNA with rare restriction enzymes, cutting at only a minimal number of sites, are demonstrated through pulsing and ramping the electrical field over an extended time (contour-clamped homogenous electrical field - CHEF), resulting in macro restriction patterns (Kaufmann and Pitt, 1994). In optimising the technique for *B. pseudomallei* epidemiology, it was used to dispel the explanation of animal to animal outbreak transmission in a collection of imported captive primates from Indonesia and the Phillippines (Trakulsomboon *et al.*, 1994). This technique, mostly using the restriction enzyme *Xba*I has been reported to not only reflect ribotype groups but also to discriminate within them, thus providing increased sensitivity. However it was also noticed that unrelated isolates occasionally could not be discriminated (Vadivelu *et al.*, 1997). This demonstrates the imperfection of determining clonality when only a small proportion of the genome is compared.

Not withstanding the imperfections in this technique, explanations of outbreaks, sources of infection and modes of transmission have been reported using this technique by the demonstration of indistinguishable strains using both *Xba*I and *Spe*I restriction digests (Inglis *et al.*, 1999; Currie *et al.*, 2001). These investigations have led current thinking in Australia that water *per se* may be a more significant reservoir

of infection, as opposed to soil, wet or otherwise, and that ingestion may be a mode of transmission.

2.3.4.4 Multi locus sequencing typing

Godoy *et al.* optimised a multilocus sequence typing (MLST) scheme for melioidosis epidemiology and phylogenetic analysis that may be used to type related strains and provide information at least as sensitive at PFGE macro restriction patterns (Godoy *et al.*, 2003). Given that the interpretation is based on sequence data, rather than restriction digest fragment patterns, there can be unambiguous interpretation and comparison. This technique applied to a collection of matched isolates from a geographically isolation region may shed more light on transmission and phylogenetic relationships. Interestingly, an outcome of this study using sequence data based on housekeeping genes, suggested that *B. mallei* could be considered a clone of *B. pseudomallei.* Given that basic phenotypic differences separate these organisms, including motility and manifestation of disease, this again highlights the importance of a poly phasic approach to taxonomy, that is using phenotypic and genomic data in microbial classification.

2.3.5   Prospective studies reveal risk factors and epidemiology

In the largest prospective study outside Thailand, Currie *et al.* identified 252 cases over 10 years (Currie *et al.*, 2000d). Indigenous Australians predominated (55%). The overall mortality was 19%. Those that presented with bacteraemia (46%) had the highest mortality (37%). The cause of death in most cases was septic shock (86%). In 50% of the cases pneumonia accounted for the primary diagnosis. In addition to these, 8% developed pneumonia even though the initial diagnosis did not implicate the respiratory tract.

The major differences highlighted in this study compared to the experiences of Thailand are the absence of parotitis in children (in Thailand this presentation represents up to 40% of paediatric cases) and the extent of genito-urinary infection in

Australia, 15% of all cases. The latter may be explained by the high number of Aborigines in the study with behavioural characteristics specific to them as highlighted by Webling (Webling, 1980).The lack of parotitis in children in Australia may be an aberration of the low numbers, with only nine cases of childhood melioidosis over the ten years. Another highlight is the presence in Australia of the distinct encephalomyelitis syndrome (Currie *et al.*, 2000c) although this may be an aberration of the increased sensitivity of advanced medical imaging not available in most other regions. This may also apply to the increased numbers of prostatic cases identified.

All case control studies and other prospective and retrospective studies identify co-morbidy in the host as the most important risk factor for the development of severe melioidosis (Chaowagul *et al.*, 1989; Puthucheary *et al.*, 1992; Merianos *et al.*, 1993; Suputtamongkol *et al.*, 1994a; Suputtamongkol *et al.*, 1999). The correlation is not as clear in localised infection or infection in children (Lumbiganon and Viengnondha, 1995). Diabetes, particularly late onset (type 2) has been consistently implicated as the most common risk factor (Chaowagul *et al.*, 1989; Puthucheary *et al.*, 1992; Suputtamongkol *et al.*, 1994a). In Australia, alcoholism and renal damage are also significant. In periurban regions of developing countries around the world, where traditional social practices are being overwhelmed by convenience food and social decay and where predisposition to glucose and ethanol intolerance exists, melioidosis may become more apparent.

It is possible that in regions where traditional co-morbidity is not apparent, other acquired factors, particularly co-infection will predispose to melioidosis (Enwere *et al.*, 1999; Baird *et al.*, 2002).

Although much is yet to be revealed about the ecology of *B. pseudomallei*, the boundaries of the geographic distribution of the organism are broadening. This is partly due to importation but also due to increased recovery from increased clinical suspicion and laboratory surveillance. It is clear that individuals with a heavy exposure to the environment are predisposed to infection. This represents the

generally higher incidence of disease in indigenous and subsistence farming communities. Individuals not normally exposed to the environment but who are exposed to climatic extremes such as heavy and prolonged rainfall will be exposed, as will those who are exposed to soil and water in isolated work environments. This represents the sporadic cases in urban endemic regions. In both these groups, those with co-morbidity, particular diabetes, renal disease and alcoholism (in Australia at least) will be selected out as they are more predisposed to infection. Prospective studies in these groups may provide a sensitive indicator of the relative endemicity of melioidosis.

As it is possible for the organism to lay dormant within the host for decades, attempts to implicate recent history in transmission may be misleading in some cases. Therefore determining a clear correlation between cases, controls and transmission using case control studies will always be difficult. Importation from traditional melioidosis-endemic regions will continue to be a feature and should no longer be considered a surprise when the disease is diagnosed.

2.4 The Laboratory Diagnosis of Melioidosis: Phenotypic Characteristics

A definitive diagnosis of melioidosis should only be made when the organism is isolated from an individual during periods of active infection (Suputtamongkol *et al.*, 1994a).Therefore the capacity of the laboratory scientist to isolate and identify *B. pseudomallei* and the extent to which the laboratory services are available in developing countries is a crucial factor in determining the significance of melioidosis (White, 2003).

Early methods for isolating the organism from both clinical and environmental samples used the innate susceptibility to infection of certain animals to select out the organism (Rimmington, 1962; Thomas, 1977). Traditional media such as MacConkey and Salmonella and Shigella agar were used to isolate the organism from spleen, occasionally with the addition of colistin to enhance selectivity. In acute melioidosis the organism is reliably cultured using standard blood culture techniques,

although it must be emphasised that an appropriate volume of blood must be cultured to achieve adequate sensitivity (Hall *et al.*, 1976; Li *et al.*, 1994). There can be expected a 60% reduction in sensitivity if only 10 ml of blood is cultured, compared to a 30 ml volume (Ilstrup and Washington, 1983). The author's personal experience in PNG is that often only 5 ml of blood is collected into less than 30 ml of brain heart infusion broth, this is likely to lead to a gross underestimate of the incidence of cultivatable clinical bacteraemia.

2.4.1   Selective culture colonial and cellular morphology

The organism is often not isolated in pure culture from clinical samples. Latent melioidosis has been reported to reactivate secondary to bacterial sepsis of non-*B. pseudomallei* origin and therefore dual infection from blood culture is also possible (Read *et al.*, 2001). Samples from abscesses have also resulted in dual infection, particularly with *Staphylococcus aureus* (De Buse *et al.*, 1975).Thus it is possible under these circumstances in laboratories without a high level of suspicion or familiarity of *B. pseudomallei* colonial morphology that only the familiar pathogen is considered significant.

Ashdown developed a simple selective and differential agar which was shown to increase isolation rates (Ashdown, 1979c). Independent studies in Thailand confirmed Ashdown's results and also showed that pre-enrichment/selection in a broth form of the formulation enhanced isolation from samples with high normal flora contamination (Wuthiekanun *et al.*, 1990). The colonial features of *B. pseudomallei* take up the characteristics of both the crystal violet (5 mg/l) and neutral red (50 mg/l). The addition of glycerol (4%) increases the colony's tendency to wrinkle and the gentamicin provides selection. Originally a 4 mg/l concentration was used, now often increased to 8 mg/l to enhance selection. The result is a rugose textured mauve (red/blue) coloured colony, present after 48 hr incubation at 35°C.

Within a single strain, morphological heterogenicity has been noted and is a common feature. An excellent series of photomicrographs that describe this and the varying morphotypes (on 4% beef extract agar with glycerol) can be found in the study of *B. pseudomallei* virulence by Nigg *et al.* which demonstrated a relationship with colonial morphological change through passage in mice which correlated to virulence (Nigg *et al.*, 1956). Even though the colonial morphology is mostly distinct on selective and differential agar, it is reasonable to assume that the lack of uniformity in colonial morphology will also interfere with correct identification, particular in areas where melioidosis is only sporadic.

Howard *et al* recently developed a new formulation of selective and differential agar to increase the isolation of atypical morphotypes, particularly those that exhibit a smooth or mucoid appearance (Howard and Inglis, 2003). The fact that this formulation seems to have increased selection capacity to closely related *Pseudomonadaceae* may make this a particularly useful media for environmental isolation. Although as Ashdown's agar is commercially available and has been in use for many years, it is unlikely this new formulation will be used for routine clinical sampling unless independent prospective trials conclude it produces a significant increase in sensitivity.

A feature of the growth of the organism is the earthy, sweet odour (Jayanetra *et al.*, 1975; Dance *et al.*, 1989c). The odour is pathognomic and has been traditionally used as a very sensitive indicator. Although the organism is formally classified as only a PC2 risk in Australia, specific mention has been given to exclude odour as an identification criterion in a draft of the latest standards (Australian/New Zealand Standard Part 3: Microbiology ASNZS 2243.3:200X).

The cellular morphology is often reported as small Gram-negative bacilli with safety pin appearance. This bipolar staining characteristic is a reasonably reliable feature, although a link with carbon storage in times of substrate excess may explain, that under certain growth conditions, this may not be apparent (Redfearn *et al.*, 1966).

2.4.2   Substrate utilisation tests

As *B. pseudomallei* can utilise a wide arrange of substrates, it has an unusually broad utilisation rate in commercial phenotypic systems for a member of the *Pseudomonadaceae*. Traditionally it is been identified as being motile, oxidase and catalase positive, indole negative, citrate positive, ONPG negative, gelatin positive, producing acid from glucose and maltose with no gas. It is lactose negative, arginine and ornithine are positive as is lysine. Urease is not produced. It can be differentiated from the *Pseudomonas aeruginosa* group by the lack of typical pigment;

Alcaligenes spp by the rapid oxidation of glucose and from *B. cepacia* by the rapid reduction of nitrate to nitrogen with the production of amylase (Smith *et al.*, 1975).

2.4.2.1 Commercial phenotypic identification systems

Since the introduction of commercial phenotypic identification systems, the API 20E and 20 NE (bioMerieux, Baulkham Hills, NSW) have been traditionally used, although the lack of diversity of isolates in the analytical index, has provided uncertainty in the past (Dance *et al.*, 1989c). Ashdown found the API 20E to be useful with a combination of other simple tests (Ashdown, 1979b), although this was questioned by Thomas who found the Microbact 24E (MedVet, Adelaide, South Australia) more reliable (Thomas, 1983) as did Inglis (Inglis *et al.*, 1998a), who with Lowe (Lowe *et al.*, 2002) found misidentification with *Chromobacterium violaceum* a feature, although the stark violet pigment produced by this organism should aid in their differentiation. The new generation of automated phenotypic identification systems have demonstrated diverse results. The Vitek 2 (bioMerieux, Baulkham Hills, NSW) was only able to correctly identify 19% of isolates that the Vitek 1 and API systems agreed upon (Lowe *et al.*, 2002). Commercial phenotypic identification systems are as only as accurate as the database allows, and it should not be surprising that a system that does not include *B. pseudomallei* in the analytical profile would mis-identify the isolate as the closest available (Koh *et al.*, 2003). Perhaps the emerging global prevalence of melioidosis should encourage manufactures of such systems as the Phoenix (BD) to include *B. pseudomallei*. More importantly, the microbiologist should be aware of the limitations of systems available and choose wisely and not use these systems simply as a "black box".

Not withstanding the aberration of the Vitek 2 system, it is reasonably clear that most systems which include *B. pseudomallei* in the profile database all are useful and mostly accurate in the identification of *B. pseudomallei* particularly when reactions are read at 48 hr (Dance *et al.*, 1989c) and when they are used by skilled microbiologists. The systems may legitimately struggle with the identification of bacteria from the environment. This perhaps should not be surprising considering the

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ecology of *B. pseudomallei* is so uncertain there may be any number of closely related species newly recovered with characteristics which are similar but are not represented on commercial databases. The history of *B. thailandensis*, an organism originally identified as a biotype of *B. pseudomallei* but differentiated by a limited number of substrate utilisation tests, including the ability to utilise arabinose, and its relative avirulence but similar serology, highlights this well (Wuthiekanun *et al.*, 1996; Smith *et al.*, 1997; Brett *et al.*, 1998; Dharakul *et al.*, 1999b).

2.4.2.2 Simple identification criteria can be used in developing countries

Regions where melioidosis is endemic are often resource poor and unable to support commercial bacterial identification systems. A simple set of criteria can be used with close positive correlation with commercial systems when used in laboratories where melioidosis is endemic (Dance *et al.*, 1989b). These criteria include: colonial morphology on Ashdown agar (mostly rugose, mauve colonies - particularly after 48 hr incubation at 35°C with earthy, sweet odour; Gram stain (small Gram-negative rods with bipolar staining); oxidase (positive); gentamicin and colistin susceptibility (both resistant). The additional criterion of Augmentin susceptibility (sensitive) will aid in the discrimination of *B. cepacia* (resistant) (Dance *et al.*, 1989b). In laboratories outside traditional zones of endemicity, a small oxidase positive, Gram-negative rod which exhibits resistance to gentamicin and sensitivity to Augmentin, doxycycline and cotrimoxizole, should be enough to garner suspicion.

The rapid diagnosis of bacteraemia is crucial in the successful management of sepsis (Beekmann *et al.*, 2003). This is particularly the case with melioidosis in regions where empirical treatment regimens for pneumonia and sepsis do not include third generation cephalosporin coverage (White *et al.*, 1989; Puthucheary *et al.*, 1992). Almost half of the acute melioidosis cases in Thailand die within the first two days (Dharakul *et al.*, 1996) therefore the incubation times required to provide a diagnosis using traditional phenotypic characteristics do not often provide a satisfactory outcome.

2.4.3   Serology

2.4.3.1 The clinical use of serology

Serological results without culture has been used to provide evidence of infection when accompanied with a high level of clinical suspicion particularly associated with an outbreak (Johnson, 1967; Currie *et al.*, 1993). Serology has also been reported to provide evidence of human to human transmission (McCormick *et al.*, 1975). Not withstanding these reports, the use of serology is complicated by the high background titres apparent in areas of high endemicity (Kanaphun *et al.*, 1993), particularly in areas of Thailand where samples from up to 80% of children at four years of age when analysed demonstrate significant sero-reactivity. This is in contrast to Australia where the highest background antibodies titres, in endemic regions is not over 13% (Currie *et al.*, 2000e). Problems of high background titres may be alleviated by the local optimisation of the assay, with higher cut-off values, resulting in workable sensitivity and specificity (Appassakij *et al.*, 1990).

2.4.3.2 Antibodies and antibody assays

Polyclonal antisera was commercially available (Dance *et al.*, 1989c) and has been used for rapid direct colonial identification and the detection of antigens in clinical samples (Smith *et al.*, 1993; Smith *et al.*, 1995a). Monoclonal antibodies have also been developed with a wide application including direct and indirect immunofluorescence, ELISA and antigen affinity chromatography in the development of indirect ELISA (Rugdech *et al.*, 1995; Dharakul *et al.*, 1999a; Pongsunk *et al.*, 1999; Samosornsuk *et al.*, 1999; Steinmetz *et al.*, 1999; Anuntagool *et al.*, 2000; Wuthiekanun *et al.*, 2002). These seem to provide the basis for sound in-house assays.

The indirect haemagglutination (IHA) and compliment fixation tests were applied early to melioidosis diagnostics (Alexander *et al.*, 1970). Ashdown developed an in-house indirect ELISA and an indirect immunofluorescence assay based on a crude

whole cell sonicated antigen, able to detect IgG and IgM (Ashdown *et al.*, 1989). When the IgM ELISA was used on individuals who tested positive with the IgG assays, he was able use the combination to assess the stage of infection. Other methods include antibody capture assays (Kunakorn *et al.*, 1990), use of presumptive exotoxins (Smith *et al.*, 1991) and endotoxins (Petkanjanapong *et al.*, 1992), specific glycolipid (Phung *et al.*, 1995) as an antigen and monoclonal antibodies for antigen affinity capture and other applications (Rugdech *et al.*, 1995; Steinmetz *et al.*, 1995; Dharakul *et al.*, 1997). Generally these methods achieve sensitivity and specificity in the order of 80 - 90%. Only when these validation data exceed that of the IHA to the point that the increased expenditure is clinically justified, will the cheap and robust IHA be replaced as the standard screening serological assay.

Recently a commercial immuno chromatographic test (ICT) has been developed by PanBio based on a culture filtrate antigen originally based on a dot blot assay (Wongratanacheewin *et al.*, 1995). The assay provides low reader variability and high specificity, although this may be attributed to by the relatively small and non-representative disease control cohort, mostly sourced from a non-endemic region. Regardless of its validation in hyperendemic regions, the price per test may still prohibit use in resource poor regions (Cuzzubbo *et al.*, 2000).

Ashdown and Guard suggested in their study that the presence of antibody was an indication of subclinical melioidosis (Ashdown and Guard, 1984). They noted that the prevalence of sero-reactivity was higher in the traditionally at-risk groups of indigenous Australians and South East Asian immigrants, although later Thompson and Ashdown found no correlation with past antibody status and infection incidence (Thompson and Ashdown, 1989). This suggests that the assays used either had poor specificity in endemic regions or that antibody titres remain after successful clearance of the organism.

2.4.3.3 Validation and clinical utility of a sero-diagnostic assay

A serological assay must be used within the boundaries of its validation. The utility of an assay to accurately predict a true positive from a disease group is described by the positive predicative value, which is a function of sensitivity and specificity of the assay plus prevalence of the disease in the community (Rogan and Gladen, 1978). As the prevalence of melioidosis differs significantly in regions and within endemic regions, how the assay is used will determine its efficacy. This can be demonstrated using the validation of Ashdown's in-house serological tools used in Townsville as an example (Ashdown *et al.*, 1989) when the predictive values are calculated.

The assays achieved the following validation for acute phase sera if a cutoff of >2SD from the mean of control sera is used. The varying prevalence values represent the likely true prevalence of melioidosis in Australia*, the overall sero-prevalence in North Queensland** (Ashdown and Guard, 1984) and the prevalence of melioidosis in patients admitted into hospital with community acquired bacteremic pneumonia in Royal Darwin Hospital between 1986 - 1998*** (Currie *et al.*, 2000a).

Positive Predictive Value

Test	Sensitivity	Specificity	PPV*	PPV**	PPV***
Prevalence			<1%	5.7%	24%
ELISA IgG	90%	99%	<48%	85%	97%
IHA	74%	99%	<43%	82%	96%
IFA-IgG	91%	99%	<48%	85%	97%

Negative Predictive Value

Test	Sensitivity	Specificity	NPV*	NPV**	NPV***
Prevalence			<1%	5.7%	24%
ELISA IgG	90%	99%	100%	99%	97%
IHA	74%	99%	100%	98%	92%
IFA-IgG	91%	99%	100%	99%	97%

It is clear that if either assay is used indiscriminately, i.e. in regions were the true clinical prevalence of melioidosis is <1%, serology is not a good predictor of disease (represented by the low PPV if used within a population where the true prevalence is <1%). This perhaps explains the observations of Thompson and Ashdown in their study of latent melioidosis (Thompson and Ashdown, 1989) where reactive serology in individuals with no signs or symptoms of disease could not predict the recrudescence of active infection in individuals.

In contrast, if used on samples from patients where a high clinical suspicion of melioidosis exists, such as those presenting with community acquired pneumonia in an endemic region, they represent a sound aid in the diagnosis of melioidosis (PPV of between 96-97%). The negative predictive values demonstrate that when used in any region to confirm true negative, a negative result can be confidently interpreted to equate to a true negative status.

2.4.3.4 Serology as an epidemiological tool

Although serology may be problematic for use in diagnostics, there is anecdotal evidence that the prevalence of sero-reactivity in a population relates to the potential for clinical disease in that community. This can be seen in the high sero-prevalence and higher incidence of disease correlation in indigenous Australians living in the Northern Territory (Currie *et al.*, 2000d; Currie *et al.*, 2000e) and Queensland (Ashdown and Guard, 1984; Guard *et al.*, 1984; Faa and Holt, 2002) and the lack of serological evidence in regions where only sporadic cases have been reported (Currie, 1993).

This correlation may not be so clear in some regions where the presence of the avirulent, antigenically similar *B. thailandensis* may contribute to cross-reactivity, although monoclonal antibodies have been developed that will discriminate between antibody responses from these two organisms (Sirisinha *et al.*, 1998). The fact that *B. thailandensis* has never been isolated in Australia may explain the relatively low background antibody titres from an endemic region. Although as *B. thailandensis* is

avirulent, it may not invade to the extent that the host will mount a detectable immune response in nature. And even if immuno dominant epitopes are shared with *B. pseudomallei*, they may play no role in cross reaction with *B. pseudomallei*.

2.4.4 Molecular Diagnostics

The development of molecular diagnostics has lead to rapid and specific assays, which have aided in decreasing the time required to confirm diagnosis for many infectious diseases (Yang and Rothman, 2004). Targets for the development of specific primers to rapidly identify *B. pseudomallei* have concentrated on 16S and 23S rRNA sequences (Tyler *et al.*, 1995). Results of successful clinical application has been reported from buffy coat and pus (Dharakul *et al.*, 1996; Rattanathongkom *et al.*, 1997) although reports of poor specificity in prospective trials have dampened enthusiasm (Haase *et al.*, 1998; Kunakorn *et al.*, 2000).

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

The detection of a type III secretion (TTS) gene cluster in *B. pseudomallei* and its association with virulence (Winstanley *et al.*, 1999; Winstanley and Hart, 2000) provides the basis for targets of PCR primer sets that may have clinical and environmental application. With one primer set the PCR is able to distinguish *B. pseudomallei* from *B. thailandensis*, it cannot though, discriminated between *B. thailandensis* and other non-*B. pseudomallei* species, as can the protocol of Dharakul (Dharakul *et al.*, 1999b).

The clinical complication for the use of PCR based diagnostics in blood culture systems are those of sampling, expertise required and cost. Until these issues are solved and brought into line with existing diagnostics they are not likely to be broadly used, particularly in developing countries (Sermswan *et al.*, 2000).The PCR protocols of Dharakul and the TTS gene PCR described by Winstanley together provide a sound genomic identification procedure for the confirmation of *B. pseudomallei* isolates recovered from clinical or environmental samples. It is clear though, that further scrutiny of the genome through either multilocus sequencing or when the complete annotated sequence is published, will provide more primer design options and together with the application of real time PCR techniques may enhance sensitivity, reduce cost and therefore clinical applications may be more attractive (Filion *et al.*, 2003).

2.5   Pathogenesis

2.5.1   Adherence

Virulence determinants that enable attachment, evasion of host defences and damage to cells are required by an organism to cause disease. Although respiratory disease is an apparent clinical manifestation, there has been no hard evidence to implicate human-to-human transmission via the respiratory route. This may be associated with the organism's lack of effective attachment determinants to pharyngeal respiratory epithelia (Ahmed *et al.*, 1999) preventing the development of a critical number of organisms at this site. The organism appears to have no fimbriae, with the relative weak adherence partly mediated by a polysaccharide capsule, antibodies against which provide protection (Jones *et al.*, 2002). Flagellae also seem to have an adhesion function and the means of motility they confer seems to enable spread around the respiratory tract (Chua *et al.*, 2003). This may enable increased capacity for invasion and therefore may account for the increased virulence of the intra-nasopharyngeal inoculation route compared to others in some animal models (Jeddeloh *et al.*, 2003), although macrophage uptake is likely to be involved. This evidence is supported by observations that adhesion to protozoa by *B. pseudomallei*

is flagella mediated (Inglis *et al.*, 2003). The non-motile but virulent and encapsulated *B. mallei* would provide a contrasting model to determine the role of flagellae as an adherence virulence determinant.

2.5.2 Extracellular factors

Many soluble extracellular factors have been described as possible virulence determinates, including lipases, lecithinases, proteases and other haemolysins and perhaps siderphores (Ashdown and Koehler, 1990; Sexton *et al.*, 1994; Haussler *et al.*, 1998; Woods *et al.*, 1999), although their respective roles are not clear (Brett and Woods, 2000).

2.5.3 Virulence genes

Certain genes have been linked with virulence as a result of subtractive hybidisation with the avirulent but phenotypically similar *B. thailandensis* (Brown and Beacham, 2000). A gene encoding an enzyme that is vital for the production of a polysaccharide capsule was identified (Reckseidler *et al.*, 2001). Sequencing of the gene showed considerable homology with genes that encode for capsules in other pathogenic bacteria include *Haemophilus influenzae*. Similar genes were found when *B. mallei* was subject to subtractive hybidisation with *B. thailandensis* (DeShazer *et al.*, 2001).

Type III secretion gene systems that are associated with virulence and are presumably related to ancestral transposons or similar motile genetic elements have been discovered in *B. pseudomallei* (Winstanley *et al.*, 1999; Winstanley and Hart, 2000; Attree and Attree, 2001; Stevens *et al.*, 2002).

2.5.4 Surviving as an intracellular pathogen

The organism appears to activate complement but is resilient to its activity and the resultant phagocytic activity mediated by opsonisation (Egan and Gordon, 1996),

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this activity is consistent with having an antiphagocytic capsule but also the ability to survive as an intracellular pathogen (Jones *et al.*, 1996). It appears to survive intracellular killing and digestion in part through the ability to evade the phagolysozyme vacuole, with subsequent escape into the cytoplasm (Harley *et al.*, 1998). Cell to cell spread from the cytoplasm of cells may be accomplished via actin associated membrane protrusions which contain bacteria and seem to appear when cells are infected (Kespichayawattana *et al.*, 2000).

Early observations and results of prospective studies have implicated host factors as the major contributing factor to the clinical manifestation of melioidosis (Rimmington, 1962; Merianos *et al.*, 1993; Suputtamongkol *et al.*, 1999). Diabetes is the most common risk factor. A strong correlation has been demonstrated with oxidative stress and HbA1c and neutrophil and monocytes susceptibility, perhaps mediated by C-reacting protein (Yasunari *et al.*, 2002). Therefore, increased predisposition of diabetic sufferers to melioidosis, and other Gram-negative intracellular pathogens, could be explained by increased granulocytic monocyte death due to the cell's susceptibility to heightened oxidative stress during time of hyperglycaemia (Pitozzi *et al.*, 2003). This would be heightened during times of failed phagocytosis but with a maintained oxidative burst, further contributing to the oxidative stress (Pruksachartvuthi *et al.*, 1990). It is interesting to speculate that the association of leucopaenia seen in patients with acute melioidosis in a Thailand study may be related (Chaowagul *et al.*, 1989).

Experimental models and clinical observation of susceptibility in wild animals point to an innate susceptibility independent of acquired co-morbidity and behavioural risk factors (Vorachit *et al.*, 1995; Veljanov *et al.*, 1996; Vesselinova *et al.*, 1996; Leakey *et al.*, 1998). A link with melioidosis and HLA type has been demonstrated in humans (Dharakul *et al.*, 1998) suggesting that a genetic predisposition and protection may exist.

Through the use of animal models, the induction of pro-inflammatory and anti-inflammation cytokines seem to have a role in disease manifestation. In a

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murine model that contrasts both the localised/chronic and acute forms of the disease, susceptible mice that developed septic melioidosis produced a more vigorous cytokine response compared to a relatively non-susceptible strain (Ulett *et al.*, 2000). Some, particularly interleukin-6 and interleukin-10 in high concentrations, have shown to be independently implicated in mortality (Simpson *et al.*, 2000) and may provide a prognostic indicator. However, the implications of these studies are based on limited panel of cytokines. Clearly, further studies of gene induction with microarray analysis will further elucidate the role of cytokine induction in the immunopathogenesis of melioidosis.

It is clear that there are many factors at play that account for the apparent global and within-regional clustering of melioidosis. These include the ability to diagnosis the infection, the preferred ecological niche, the innate susceptibility of individuals following infection and their capacity to mount an effective cell mediated adaptive response. Regardless, the presence of co-morbidity with enhanced environmental exposure dramatically increases the risk of becoming infected. Innate factors of predisposition, independent of co-morbidity may also contribute. The severity of acute melioidosis and the likelihood that sub-acute disease will relentlessly continue along this path, highlight the need for continued work to unravel the factors at play so that management and treatment strategies can be further refined.

2.6   Melioidosis in Papua New Guinea

The first reported case of melioidosis from PNG was in a tree kanagroo *Dendrolagus spp* in 1963 (Egerton, 1963b), a species that Thomas subsequently noted was particularly susceptible (Thomas and Forbes-Faulkner, 1981). The animal presented with posterior paralysis and died five days after this sign was noticed. At autopsy, lesions in liver and spleen were cultured revealing *B. pseudomallei*. It had been a resident of a small zoo in Port Moresby for two years. The origins of the animal were not reported. It was speculated that this first case of melioidosis may provide an insight into a possible reservoir of infection for human disease (Egerton, 1963b).

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Further work by the investigator and veterinary colleagues at the time revealed both bovine (Egerton, 1963a) and porcine cases (Rampling, 1964), both from the same farm in Port Moresby. The environmental reservoir of infection was not determined although it was noted that the cow was imported from Northern Queensland. The pig, although indigenous to PNG, was held at the same farm as the infected cow. The possibility that the origin of this outbreak was imported from northern Queensland was speculated by the author but not investigated.

The first human case was recorded in 1965 from a patient referred to Port Moresby General Hospital from Gemo island, the then leprosarium, where he had been admitted two years earlier (Rowlands and Curtis, 1965). The patient's origins were not reported. The patient presented with cellulitis of the right leg, abdominal pain and eventually developed sepsis with pustular skin lesions and died soon after the administration of streptomycin four days after admission. A limited serological survey was conducted on patients at Gemo island with no positive results. It perhaps, therefore, is unlikely that the patient acquired the organism from this site and it is possible he may have suffered an activation of past latency brought about by the co-morbidity of leprosy, although this is speculative.

A case of melioidosis reported from Sydney was reported in a man with a history of World War II service in PNG. It was suggested that PNG could have provided the reservoir for infection. The illness presented in the chronic form for 19 years (Newland, 1969). Similarly Kingston, a year later, reported from the same repatriation hospital a case of presumptive reactivation illness from an individual 24 years after WW2 military service in Milne Bay, Papua New Guinea (Kingston, 1971). Both patients succumbed to the infection and were diagnosed after post mortem examination.

Since independence in 1975 three cases of culture confirmed autochthonous melioidosis have been reported from PNG (De Buse *et al.*, 1975; Lee and Naraqi, 1980; Currie, 1993). De Buse and colleagues reported a five year old boy presenting with PUO who failed to respond to malaria treatment. Two from three blood cultures

taken revealed *Staphylococcus aureus*. During exhaustive clinical work-up *B. pseudomallei* along with *S. aureus* was recovered from a pus filled nodule excised from the abdominal wall. Subcutaneous abscesses appeared which upon culture grew *B. pseudomallei.* The patient origins were traced to the Waitape region of Central province where he returned, successfully treated with kanamycin and trimethoprim-sulphamethoxazole. It was speculated that the child encountered the organism through an inoculation incident of the shoulder. Co-infection with *S. aureus* was a feature which has been subsequently reported elsewhere (Read *et al.*, 2001). Interestingly, the Waitape region may have been the origins of the tree kangaroo reported by Egerton (Egerton, 2003), although given the innate susceptibility to melioidosis by the tree kanagroo noted by Thomas (Thomas, 1981), it may be unlikely that the animal harboured latent disease acquired from this region. The authors also noted they were aware of two other cases in 1973 and 1974 and both were fatal.

Lee and Naraqi undertook a retrospective study in a review of Gram-negative pneumonia presenting in adults at Port Moresby General Hospital between February 1977 and May 1979 (Lee and Naraqi, 1980). Of the 3500 adult in-patients during this time 550 had been admitted with acute pneumonia. Of the 80 patients with Gram-negative bacteraemia, four were found to have primary pneumonia caused by the isolate. One of the cases was melioidosis pneumonia with left side consolidation and cavitation resembling pulmonary tuberculosis. The patient was a 36-year-old female who presented acutely ill with fever, cough and pleuritic chest pain. She had a past history of tuberculosis but no other traditional co-morbidity factors were described. Treatment consisted of tetracycline 2 gm/day and was ceased after 3-weeks when symptoms resolved and the patient was discharged.

Melioidosis was included in prospective study of adult atypical pneumonia at Port Moresby General Hospital over a 10-month period reported in 1991 (Barnes *et al.*, 1991). The time of the year of the study was not described. Of the 175 adults admitted to the study none revealed evidence of melioidosis using culture or IHA serology.

Currie described the latest report of melioidosis in PNG presenting in a 28-year-old highland man residing in Port Moresby with fulminating pneumonia (Currie, 1993). The patient had a history of excessive alcohol consumption, smoking and glucose intolerance; he died a day after admission.

Based on the sporadic case reports and limited serological survey results from Port Moresby it has been reported that melioidosis is less common in PNG than Northern Australia (Currie, 1993). It seems clear that the prevalence of melioidosis in the urban capital of PNG is low. As is the case with Bangkok and Thailand, it is unlikely that data sourced from the urban capital alone accurately reflects the prevalence of melioidosis throughout PNG. What's more, a feature of all reported cases of melioidosis in PNG except the retrospective case report from Lee and Naraqi, where laboratory methods were not described (Lee and Naraqi, 1980), had expatriate influence aiding in the identification of the organism. This expatriate support of a grossly under resourced laboratory sector in PNG is sporadic and rare and often not sustainable. This may suggest that miss diagnosis is possible and ongoing.

Regardless of the ability for melioidosis to be accurately diagnosed in Port Moresby, there is a case for a more extensive study into the significance of melioidosis to be undertaken outside Port Moresby. This may be particularly relevant given the recent reports of high incidence in the Torres Strait which borders the rural Western province and where imported cases have already been identified (Faa and Holt, 2002).

Regardless of the implication for Australia in the biosecurity of her borders, such a study is of particular importance given that the 80% of the population of PNG who live in rural regions experience the lowest per-capita health care support in the immediate pacific region, contributing no doubt, to the highest rates of childhood mortality and lowest life expectancy in the region (Anon, 2000a). It is interesting to speculate that given the standard methods of empirical antimicrobial therapy in PNG do not provide the coverage required to treat melioidosis, infections caused by *B. pseudomallei* may be contributing to this disease burden.

CHAPTER 3 - BALIMO PYREXIA OF UNKNOWN ORIGIN SYNDROME: MAYBE MELIOIDOSIS

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3.1   Introduction

Papua New Guinea is a country of mega diversity with 19 provinces (20 including the National capital District of Port Moresby), 4.5 million people with almost 900 linguistically unique language groups. Up to 80% of the population live in rural regions in predominately subsistence farming/hunting communities. They experience the poorest of health outcomes in the Pacific region with childhood mortality approaching 100/1000 births and an average age mortality of 52 years. Pneumonia is the leading cause of death in each province. The country has the lowest health care expenditure per capita and the lowest number of health care workers per capita in the region (Anon, 2000a).

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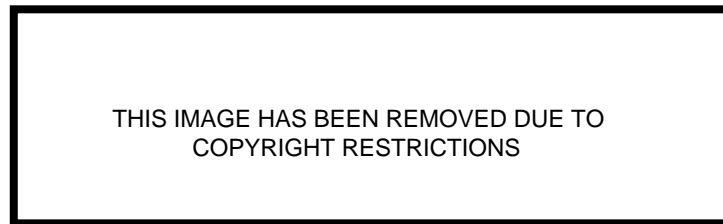
Figure 3.01   National Map of Papua New Guinea (Microsoft Encarta Atlas)

The sporadic reports of melioidosis from Port Moresby (S 9.5°; E 147°) with no evidence of sero-prevalence (Rowlands and Curtis, 1965; Newland, 1969; Kingston, 1971; De Buse *et al.*, 1975; Lee and Naraqi, 1980; Barnes *et al.*, 1991; Currie, 1993) suggests melioidosis is only a rare contributor to these poor national health statistics. However, the extent with which melioidosis is diagnosed and therefore considered clinically significant, is related to heighten clinical suspicion linked to the ability of

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the laboratory to isolate and identify *B. pseudomallei* from clinical samples (Leelarasamee and Bovornkitti, 1989). Bacteriology beyond malaria and acid fast bacilli (AFB) microcopy is rarely performed in rural provinces. Melioidosis is protean in its clinical presentation and mimics other diseases which are more widely recognised, particularly TB (Yee *et al.*, 1988). It is reasonable to assume that melioidosis may be contributing to the reported high mortality in rural regions but is under recognised due to a lack of healthcare resources.

A case of culture confirmed melioidosis was discovered during 1994 at Balimo (S 8°; E 143°) in the Western province during a project aimed at general strengthening of laboratory capacity at the local health centre (Figure 3.01; Table 3.01, case KW). At this time, during a general review of melioidosis in PNG, it was noted that melioidosis had been implicated in contributing to a febrile syndrome at Balimo featuring childhood predilection and respiratory involvement refractory to standard treatment resulting in high mortality rates (Reece, 1984). It was given the name by some local healthcare workers the Balimo PUO (pyrexia of unknown origin) syndrome.

A local campaign designed to reveal the extent of melioidosis in the community was established. Appropriate laboratory diagnostic methods were established and clinical awareness was enhanced through local training initiatives. The standard empirical treatment protocols for pneumonia and sepsis at Balimo Health Centre were amended to include melioidosis directed therapy. The program was named *maybe melioidosis* and resulted in confirmation of autochthonous melioidosis in this region with signs and symptoms resembling the melioidosis-implicated Balimo PUO syndrome documented previously (Reece, 1984).

Serology was used to further elucidate the extent of melioidosis in the district. Evidence from Thailand suggests that children in melioidosis hyper-endemic regions demonstrate levels of antibody indicative of sero-conversion up until the age of 4-years (Kanaphun *et al.*, 1993). The extent of antibody production may indicate exposure and successful processing of the organism or latent, subclinical infection.

Currie suggests that high sero-prevalence in hyper-endemic regions may infer protection and help explain the relatively low incidence in hyper-endemic communities where this is a feature compared to areas where it is not (Currie *et al.*, 2000e). Regardless of the clinical relevancy of antibody response in the individual, communities where clinical disease is hyper-endemic often experience the highest sero-prevalence (Ashdown and Guard, 1984). A sero-prevalence study of the community school children, aged between 8 and 13 years, within the Balimo region was undertaken to aid in describing the melioidosis-endemic status of this region compared to others, particularly Thailand and the Top End and north eastern Australia.

The extent and intensity of rainfall has a clear association with melioidosis incidence and disease intensity in hyper-endemic regions (Chaowagul *et al.*, 1989; Merianos *et al.*, 1993; Currie and Jacups, 2003).When seasonal predilection is recognised, this may aid in rapid recognition and clinical diagnosis resulting in expeditious directed antimicrobial therapy.

3.2 Materials and Methods

3.2.1 Study centre

3.2.1.1 Balimo, Western province PNG

Balimo is the demographic capital of the Gogodala language group who number approximately 30,000. Less than 10% live in the urban township and associated periurban villages, with most individuals living as subsistence gardeners, fisherman and hunters scatted throughout approximately 7,000 km^2 of the marine Aramia river flood plain and associated lowland forest. Seasonal flooding feeds lagoon systems which surround villages situated on points of land and islands approximately 30 m above sea level.

Figure 3.02   Map of the Lower Fly region of the Western province. Boxed section
                the approximate boarders of the Gogodala language group (Microsoft
                Encarta Atlas)

Figure 3.03   Balimo urban township

Throughout the 14 villages in the district, individuals live in bush material houses elevated on posts on land sites carved level, many located near lagoons where they use the water for washing, fishing and transport by dugout single hulled canoes. Water for drinking mostly comes from wells, although captured rainwater is also used during the wet season. The average household density is 6 per house and pit toilets are used for waste disposal (data kindly supplied by PNG Census). The Gogodala reflect the importance of their marine environment in strong cultural links with their canoe which is the basis of their clan structure. The social and cultural association with their environment and the geography of their land require constant environmental exposure.

3.2.1.2 Balimo Health Centre

The Unevangelised Field Mission (UFM) established Balimo Health Centre in the 1950's but since 1967 has been the responsibility of the local Evangelical Church of PNG as part of the national Church Health Services. It has the capacity, equivalent to a western hospital, of approximately 80 - 100 beds, with medical surgical, TB/leprosy and maternity wards. The health centre is served by dedicated locally trained nursing staff but relies for medical staff on sporadic expatriate support and occasional Port Moresby based clinical rural rotations. During each phase of the study, the hospital was under the direction of a medical officer from either Australia or PNG and therefore all decisions regarding patient intervention, diagnosis and treatment were made in collaboration with them. The two laboratory staff have basic formal training but have relied mostly on the influence of visiting scientists/technicians over the years and are exceptionally skilled considering the poor access to resources and formal training.

3.2.2   Laboratory diagnosis of melioidosis: culture, isolation and identification

3.2.2.1 Basic bacteriology

Methods of basic bacteriology were established based on standard techniques appropriate for rural laboratories (Cheesbrough, 1987). Brain Heart Infusion broth (Oxoid Australia, Heidelberg, Victoria), 50 ml in 100 ml glass bottles, was used as blood cultures. Two bottles were used per patient and clinical staff were encouraged to place 10 ml of venous blood into each and to repeat three times over a day. Although the ambient temperature was constantly between 25-37°C, a 35°C incubator was used to house the cultures. Human blood (5%) and chocolate agar was made with blood agar base (Oxoid Australia, Heidelberg, Victoria) and with MacConkey and XLD agar provided the solid media. Basic primary and secondary biochemistry tests were used in line with those of Cowan and Steel's Manual (Barrow and Feltham, 1993). Enterobacteriaceae and miscellaneous Gram-negative bacilli identification was augmented by the Microbact 24E (MedVet, Adelaide, South Australia) when necessary.

3.2.2.2 *Burkholderia pseudomallei* selective media

Solid and liquid selective and differential media were prepared as described by Ashdown (Ashdown, 1979c). Sputum samples and swabs from non-sterile sites were pre enriched in selective broth as well as directly plated onto Ashdown agar with 8 mg/l gentamicin (ASH). If the direct culture was negative, the broth was subcultured onto ASH after 48 hr (Appendix 1).

3.2.2.3 Isolate identification

Organisms were presumptively identified as *B. pseudomallei* based on typical colonial appearance, oxidase reaction (positive) and gentamicn resistence inferred from vigorous growth on ASH (Dance *et al.*, 1989c). Each isolate was confirmed with API 20NE (bioMerieux, Baulkham Hills, NSW) or Microbact24E (MedVet,

Adelaide, SA) used as per manufacturer's directions. The isolates were further characterised to confirm their identification and these results are presented in Chapter 5.

3.2.3   Laboratory diagnosis of melioidosis: indirect haemagglutination serology

Serology was used both as a diagnostic aid and as a tool to survey antibody levels in a normal sample of the population. An IHA assay was chosen from all techniques published mostly because of its relatively less reliance on technology.

3.2.3.1 Antigen preparation

Antigen was derived from five strains of *B. pseudomallei* isolated from melioidosis patients presenting in Townsville and was kindly supplied and by Joy Koehler and Bruce Samways from Queensland Health Pathology, The Townsville Hospital. Briefly, purity was checked by inoculating organisms onto blood and MacConkey agar. Identification was carried out using API 20NE (bioMerieux, Baulkham Hills, NSW). Each strain was inoculated into 150 ml of protein free media (Appendix 4) and incubated for 2 weeks at 35°C. The broth cultures were then autoclaved at 121°C for 15 min. Cellular material was deposited by centrifugation and the supernatant was removed and filtered through a 0.45μm pore sized filter (Sarstedt, Inglefarm, SA). Phenol was added to a concentration of 0.5% by volume to preserved the antigen solution. The stock was dispensed into 1 ml aliquots and stored at -70° C.

Optimal dilution was determined by block titration using reactive serum of known IHA titre.

3.2.3.2 IHA test method

Methods described by Ashdown (Ashdown, 1987) were established which were based on optimisation of the IHA for melioidosis diagnostics by Alexander *et al.* (Alexander *et al.*, 1970). Briefly, sheep blood from a nearby captive flock was

collected into a human blood donor pack containing acid citrate dextrose (ACD) (Terumo, North Ryde, NSW) where it could be stored for up to a month at 2-8°C. A portion was washed four times with 0.9% saline (Appendix 1) at pH 7.2 and diluted to a 10% stock solution. As required, 750 µl of the 10% cell suspension was added to 9.25 ml of an optimised dilution of antigen determined previously by block titration using positive serum of a known IHA titre and passively sensitised for 60 min at 37°C. After incubation, the cells were washed with 0.9% saline four times and re-suspended to 10 ml to make a 0.75% cell suspension of sensitised test cells. A 0.75% non-sensitised cell suspension was prepared and used as non-specific agglutination control cells.

A 100 µl volume of serum from each sample was added to 300 µl of 0.9% saline in plastic tubes and inactivated in a 56°C water bath for 30 min. After cooling 100 µl of 10% washed sheep red cells were added and the suspension was incubated at room temperature for 15 min to absorb any non-specific agglutinins. After incubation the suspension was centrifuged and the supernatant was collected and serially diluted in 0.9% saline in series from 1:5 through to 1:80 in wells across a 96 well microtitre plate (Sarstedt, Inglefarm, SA) enabling 14 tests to be screened and two controls to be run on each 96 well plate. The last well of each sample serially diluted series was a 1:5 dilution used for control cells. A titre was the last well in which agglutination was observed and a titre of greater than or equal to 40 was considered significantly reactive based on experience elsewhere and Ashdown's original recommendations (Leelarasamee and Bovornkitti, 1989). This was tested with previously categorised samples. Any reacting samples were further diluted to 1:5120 and re-tested to establish the endpoint titre.

3.2.4 Clinical screening for melioidosis and suspected melioidosis cases

3.2.4.1 Patient selection protocols

A patient selection protocol was established to facilitate samples to be collected and sent to the laboratory. The criteria included:

1. Febrile illness (particular pneumonitis or PUO) refractory to standard treatment, resembling TB;

2. Acute septicaemia, with or without septic arthritis and cutaneous abscesses, resembling *S. aureus* pyomyositis (a condition well known in this region).

3.2.4.2 Clinical screening protocols for melioidosis

Of the patients who attracted clinical suspicion the testing protocol included:

1. Selective culture of sputum (routinely sent for TB investigation);

2. Blood culture on all patients with persistent fever;

3. IHA serology on all patients with persistent fever;

4. Further culture work-up (blood culture, sputum culture - gastric aspirate or throat swab if sputum unavailable -, rectal swab and any pus or wound/abscess that was present) from all patients with a titre of >40;

5. Culture work up on samples from all patients with a persistent fever after antimalarial and/or TB treatment, regardless of IHA result.

3.2.4.3 Case definition

A case definition was established at the health centre based on resources available. Individuals were diagnosed with confirmed melioidosis and documented as such when *B. pseudomallei* was isolated from clinical samples using the above techniques and protocols.

Cases were classified as *suspected* melioidosis when clinical suspicion was high plus when there was evidence of either:

1. Direct familial association during a confirmed outbreak (Table 3.01: MK; SW; SL; GLD; SK; Ki.S);

2. Serological evidence (Table 3.01: SS; LT).

It was the prerogative of the clinician in charge (medical or nursing as the case may have been) to treat *suspected* melioidosis cases with directed melioidosis treatment. Successful treatment outcomes further corroborated the diagnosis.

The cases presented between the years 1981-84 describe an outbreak of "Balimo PUO syndrome" with an association of culture confirmed melioidosis. These data were sourced from an unpublished internal health centre report (Reece, 1984) which was subsequently revealed and included here for the sake of thoroughness.

3.2.5 Rainfall association

Rainfall in Balimo is not recorded. Rainfall data from the closest location was sought and compared to the date of diagnosis of each suspected or confirmed melioidosis case to identify if an association with rainfall could be demonstrate.. Rainfall data from Daru (S 9.0º; E 143.3º) was kindly supplied by Mr Jimmy Gomoga from PNG Bureau of Meteorology. Excel (Microsoft) was used to graph rainfall monthly means 1981 - 2003 against the month melioidosis cases presented at Balimo.

3.2.6 Treatment protocols

Treatment regimens were based on data derived at the time (Sookpranee *et al.*, 1992; Ashdown, 1994) and modified, in consultation with clinical staff of the health centre, according to availability of antibiotics and other local factors.

3.2.6.1 Acute presentation (patient presenting with sepsis)

Initial treatment included a (two) drug combination of IV chloramphenicol or doxycycline or cotrimoxazole at up to twice the standard dose /kg/day for three weeks. If the patient survived the acute episode, oral treatment as below was used as maintenance therapy.

3.2.6.2 Subacute presentation (patient presenting with PUO or localised disease)

Treatment included oral chloramphenicol or doxycycline or cotrimoxazole at up to twice the standard dose /kg/day for three months.

3.2.7 Serology survey

3.2.7.1 Blood collection for sero-prevalence

The community school children were selected as the population cohort to test sero-prevalence. This was based on two factors: data from Thailand indicating that antibody titres were observed in serum samples from children in hyper-endemic regions (Kanaphun *et al.*, 1993); the majority of the patients of either suspected or confirmed melioidosis at Balimo at that time were children. With ethics clearance from the PNG Medical Research Advisory Council and the cooperation of the village communities and local community school authorities, five regional community schools were chosen for the sero-prevalence survey. They all were located within a 10 km radius of the health centre and represented the majority of children of that age

group in the immediate Balimo region. Only when general community agreement was achieved did the sampling take place.

The class roll of each school was used to identify each child and a code was applied to each sample to correspond to each child in each class at each school, e.g. Adiba 5th grade class b child 5th on the list was designated A5b/5. One member of the bleeding team was assigned to one particular class or grade. The children were asked to participate and ticked off as they were bled. At the end of each session the class roll was collected and incorporated into the study data base.

The veneject system (Terumo North Ryde, NSW) was used for venipunctures and 1000 10 ml plain serum tubes with clot activation and gel separation with 21 gauge needles were kindly supplied by the Department of Public Health and Tropical Medicine, James Cook University. The first bleeding was carried out on 28.8.95 and the last on 14.9.95. Due to time constraints it was not possible to conduct the bleeding during the wet season.

The whole blood samples collected from the children were collated and the serum separated the same day after clotting and stored at -20°C in plastic separation tubes (Sarstedt, Inglefarm, SA).

3.2.7.2 Statistical analysis

Sero-prevalence was calculated per school and the positive reactors were further discriminated by age and sex. Binonial distribution confidence limits of 95% were applied to the sero-prevalence data and Chi squared analysis was performed on the data to quantify the significance of any observed sero-prevalence clustering when the data conformed to the limitations of the test, i.e. only when 80% of the expected counts were greater than five and none less than one. When these conditions could not be met the test was not performed.

3.3 Results

3.3.1   Clinical screening for melioidosis and suspected cases

During 1995 - 1998 two active periods of the *maybe melioidosis* campaign were conducted: In January 1994 to January 1995 and March 1998 to July 1998. The screening protocols were able to continue unsupported intermittently into 2000 but ceased soon after as the hospital effectively withdrew most clinical services in response to a lack of national resources. In preparation for the study, community and health centre meetings presented the aims and methods of the study and ongoing clinical meetings outlined melioidosis diagnosis and management issues.

Unfortunately, detailed sampling data from the 1994-5 study was lost with only the results of the culture confirmed cases remaining. Although, during the 1998 initiative details of the numbers and type of samples tested remain. During this time, 245 samples from 170 patient were selectively cultured as per the protocols listed above. Of the total number of samples, 0.5% were ascitic fluid, 0.5% eye swabs, 1% ear swabs, 10% throat swabs, 15% blood cultures, 22% lesions and abscess pus and 51% sputum or gastric aspirates.

The outcome of the screening program is presented in Table 3.01. During the time the program was able to run and including a case diagnosed post mortem in 1983 after the patient was evacuated to Port Moresby from Balimo, nine cases of culture confirmed melioidosis autochthonous to the Balimo region were diagnosed (culture Yes in Figure 3.01) plus a further eight cases of suspected melioidosis (culture NT in Figure 3.01) were identified which fulfilled the criteria. Those patients with familial association are presented (Family Yes in Figure 3.01). The colour matches highlight familial clustering.

Case descriptions are based on medical records or discharge summaries and are included to provide documentation, published in one form, for others to refer. Only

the culture confirmed cases are included along with accompanying description of other cases if details were available and if they were deemed to be relevant.

Table 3.01   Confirmed and suspected melioidosis cases documented in Balimo during 1981-84; 1994-96; 1998; 2000

Patient	Family	Sex	Age	Date	Culture	IHA	Village	Outcome
1981 - 84								
MK	-	F	11	Apr-81	NT	NT	Adiba	well
AW	Yes	M	18	Jan-83	Yes (blood)	NT	Adiba	died
SW	Yes	M	16	Jan-83	NT	NT	Adiba	died
SL	Yes	F	38	Jan-83	NT	NT	Adiba	died
GLD	-	F	11	Nov-83	NT	NT	Kini	died
SK	-	M	12	Apr-84	NT	NT	Adiba	died
1995-96								
KW	-	M	10	Jan-94	Yes (blood)	NT	Adiba	died
ID	-	F	9	Jun-94	Yes(blood)	NT	Kini	died
SS	Yes	M	6	May-94	NT	1024	Adiba	well
GD	-	F	9	Jan-95	Yes (sputum)	>5120	Adiba	well
DS	Yes	F	14	Jan-95	Yes (blood)	NT	Kimama	died
KimS	Yes	M	7	Jan-95	NT	320	Kimama	well
KawS	Yes	M	24	Feb-95	Yes (blood)	NT	Kimama	died
1998; 2000								
NG	Yes	F	4	May-98	Yes (sputum-GA)	40	Balimo	well
TG	Yes	M	6	May-98	Yes (throat)	>5120	Balimo	well
RI	-	M	17	Jul-98	Yes (abscess)	<5	Togowa	well
LT	-	M	13	Apr-00	NT	320	Adiba	died

3.3.2   Descriptions of culture confirmed cases

3.3.2.1 Case AW

An 18-year old male was admitted to the health centre 17.1.83 from Adiba village with fever, dry cough, dyspnea and arthralgia. His chest was clear. Malaria films were reported to be negative. Standard treatment for fever in rural PNG failed to arrest the fever and he was transferred to Port Moresby General Hospital 3.2.83. He died there on the 14.2.83. Diagnosis recorded from post mortem examination was melioidosis septicaemia, pneumonia and septic arthritis.

Case SW was a brother of AW and admitted at the same time with the same signs and symptoms and a more detailed history is available. He weighed 48 kg upon admission (fifth percentile for PNG males) with fever (41°C), pulse rate 90-120/min, respiratory rate 50/min. Chest X-ray revealed right lower lobe consolidation with small effusion noted. Neutropaenia with marked left shift developed during the two weeks of admission. Malaria film and sputum AFB were reported negative. He was treated with standard doses (Anon, 1993) of penicillin, chloramphenicol, tetracycline, isoniazid and streptomicin but died during an attempt to evacuate to Port Moresby 1.2.83.

Case SL was the mother of both AW and SW and admitted to the local sub health centre at Adiba 20.1.83 with fever (39.5°C), dry cough, dyspnea and myalgia. Rhonchi and crepitant rales were heard diffusely throughout the chest. A malaria film was negative but the blood film noted marked neutophilic left shift. Initial treatment with ampicillin failed to arrest the fever. A change to chloramphenicol 500 mg IMI ceased the fever within a day. The patient was discharged on the 1.2.83. During the next day she was readmitted "profoundly ill" and died one month after being transferred to Balimo Health Centre on 2.3.83 with pneumonia.

3.3.2.2 Case KW

In July 1987 a 10-year-old boy was referred to the health centre complaining of fever, cough with pain in both sides of his chest and headache. His chest was clear and fever ceased and he was discharged 9 days later with a diagnosis of bronchitis. On his second admission in April 1992, he presented with a temperature of 37.6°C, abdominal pain and general malaise. Both a blood slide for malaria and a sputum slide for AFB were negative. He was commenced on a standard dose of chloramphenicol and antimalarials and was discharged 8 days later with a diagnosis of PUO.

On 7 January 1994 he presented a third time directly to the health centre with a temperature of 39.9°C, pulse rate of 96/min and a respiration rate of 32/min,

complaining of high fever, nausea and vomiting beginning 5 days earlier. It was noted that he was not eating or drinking well. His chest was clear but he complained of a cough and pain on coughing, no abdominal pain and no masses evident. No improvement was noted 24 hr after antimalarial therapy. On day four his temperature was 40.5°C, a repeat blood slide for malaria was negative and chemotherapy was of no benefit. He developed rigors and vomiting and began complaining of pain in his joints. Blood cultures were taken. During the fifth day the patient was still febrile complaining of pain in all muscles of arms and legs. A discrete lesion was noted on chest x-ray and he was started on IV chloramphenicol, cloxacillin and probenecid.

On day six KW was afebrile but the next day was noted to spike fevers to 38.7 °C. Blood culture results returned "*Enterobacter spp*" sensitive to chloramphenicol. Therapy was continued. It was noted during the next three days that his fever fluctuated and when he became afebrile on day 10 his IV was discontinued and was placed on a standard oral dose of chloramphenicol.

On day 11 and 12 his fever returned to 40.0°C and he was started on gentamicin and short course TB treatment. Repeat blood cultures were taken. In the next three days cutaneous abscesses developed on both arms and left loin, pus was aspirated and he was started on imipenem. He immediately became hypotensive and semi conscious and died 6.00 pm on the 15th day of his third hospital admission.

The initial blood culture isolate, an isolate from the second blood culture and an isolate from pus were later identified as *B. pseudomallei*.

3.3.2.3 Case ID

A 11 year-old girl was referred to the Balimo Heath Centre from a local subhealth centre near Kawiyapo village (approximately 20 km south of Balimo) Upon admission on the 15.6.94 her temperature was 39.8° C , pulse 120/min and respiration 36/min. She had localised swelling of her head and ankle and a groin abscess. The girl and parent denied any family history of TB. She complained of fever, body and

joint pain. A blood film was reported negative for malaria but upon analysis revealed left shift with a total white cell count of 6.1 $\times 10^9$/l. A random blood glucose level was 6.4 mmol/l. A diagnosis of Staphylococcal pyomyositis was made. Despite treatment with chloramphenicol, 1 gm IV QID, penicillin and chloroquine she died on the 26.6.94. Just prior to her death *B. pseudomallei* was isolated from a sample of pus taken from a groin abscess.

3.3.2.4 Case GD

In September 1995, just after the *maybe melioidosis* program was implemented, a 9 year-old girl presented at the health centre after being referred from the Adiba subhealth centre complaining of a persistent fever and general malaise. She was under weight for her age and had to be carried by her father. Her only previous admission was as a 6 month-old with a febrile respiratory illness diagnosed as pertussis. Her blood slide for malaria and sputum AFB were negative and her chest was reported clear on X-ray. She was started on antimalarial treatment and oral chloramphenicol as standard treatment.

Her fever fluctuated for several weeks. Acid fast bacilli were not found in repeat sputum slides and routine sputum culture failed to demonstrate typical pathogens, blood cultures also were negative. Regardless, she was placed on short course TB treatment.

As part of a routine screen of PUO patients melioidosis serology was performed. Her IHA titre was reported to be >5,120. Selective culture of sputum produced a predominant growth of typical *B. pseudomallei.* The organism was subsequently positively identified.

The patient was started on double the standard dose/kg/day of oral chloramphenicol and changed to equivalent doses of cotrimoxizole mid treatment term for a total of 3 months. Her fever responded after 72 hr, she eventually became ambulant and was discharged home well.

3.3.2.5 Case DS

On 21.1.96 an 11 year-old girl was admitted into the Balimo Health Centre from
Kimama village with her younger brother (Case KimS) with a temperature of 40°C,
periorbital swelling, cough, joint and body pain. Her chest had left lower lobe
consolidation on X-ray, she had a total WCC of 9.3×10^9/l with toxic granulation. A
malaria film was negative and no AFB were seen on a smear of sputum. There was a
family history of TB and she was noted to be infected with roundworm at the time of
admission. She was initially treated with aspirin, chloroquine and ampicillin which
failed to arrest the fever. Despite the negative blood film medication was changed to
quinine and fansidar and a diagnosis of chloroquine resistant malaria was made.

Although melioidosis IHA was not available due to lack of sheep red cells, clinical
suspicion of melioidosis resulted in the use of a limited supply of ceftazidime which
halted the fever, but when stocks ran out the fever returned. Medication was changed
to the melioidosis directed drug combination of chloramphenicol, doxycycline and
cotrimoxazole when *B. pseudomallei* was isolated from pus taken from an abscess.
The patient died on 3.3.94 one day after the diagnosis was confirmed.

Case KimS was the 8 year-old brother of DS. His presentation was less acute than his
sister. He was noted to be infested with roundworm. As well as failed malaria
treatment, short course TB treatment was trialed. Melioidosis directed therapy was
instigated as soon as his sister was diagnosed resulting in rapid arrest of the signs of
infection. The organism was not isolated from one set of blood cultures submitted for
culture. A blood sample taken at admission and one taken at the end of his directed
therapy (May 1996) were subsequently tested in IHA with titres of <5 and 320
respectively. This patient has since been admitted with fever in 1997, treated with
chloramphenicol and sent home well.

3.3.2.6 Case KawS

During the admission (12.2.96) of DS and KimS, KawS, the 28-year-old uncle was also admitted with one week history of fever, cough, body pain and rigours since returning to Kimama village from a nearby timber company where he was working. He was 53 kg with a history of asthma and a family history of TB. A blood film was reported to be positive for *Plasmodium falciparum* on 28.2.96. Toxic granulation was also noted with a total WCC of 9.2×10^9/l. A blood urea result was reported to be 17.1 mmol/l. He was given quinine and fansidar which failed to halt signs and symptoms. Clinical suspicion of melioidosis was suspected when the family association with DS and KimS was revealed. Fever ceased after a short trial of ceftaxidime before stocks ran out. The patient was non-compliant with the subsequent change to oral medication. *Burkholderia pseudomallei* was isolated from two blood cultures on the 4.3.96. The patient died 31.3.96.

3.3.2.7 Case NG

During the second search for melioidosis in 1998, on the 19.1.98 a 4 year-old girl was admitted with her mother from Balimo village in the TB ward. Her mother had a positive TB sputum and was on short course treatment. NG was 10 kg (60 percentile for age) with a history of fever and a brother who died 1 year before with "fever". Her temperature was 37.8°C. She complained of a cough, body weakness and diarrhea with occasional blood. A gastric aspirate was negative for AFB. Antimalarials and short course TB treatment failed to arrest the fever.

Late April that year NG was still losing weight with persistent fever regardless of TB treatment and a proactive nutrition diet. Nursing staff recognised NG as meeting the protocol for melioidosis screening. A blood sample was tested for IHA and was reported to have a titre of 40. Culture work-up followed and a pure growth of *B. pseudomallei* was obtained from an early morning gastric aspirate. Fever ceased three days after oral directed therapy was commenced.

3.3.2.8 Case TG

The 7 year-old brother of NG was presented to the health centre by his parents after his sister's diagnosis and treatment. The parents said that TG had been suffering persistent, ongoing mild fever and wondered if he was suffering the same disease. A blood test sample had a titre of >5,120. A throat swab and gastric aspirate was performed and a pure growth of *B. pseudomallei* was demonstrated. TG was commenced on treatment and discharged well.

3.3.2.9 Case RI

RI was a 17 year-old boy from Tagowa village (adjacent to Adiba) in July 1998. Details of his admission were not documented, although it was noted that he had an injury to his foot which resulted in surgical removal of a foreign body. He was admitted with PUO and as his fever was refractory to standard treatment, melioidosis IHA was ordered but repeatedly produced a result of <5. Melioidosis treatment was begun but as the serology test was negative the staff did not continue with treatment after his initial fever subsided. RI returned two months later with a groin abscess. *Burkholderia pseudomallei* was cultured from this site, he was treated successfully.

3.3.3   Association of melioidosis cases with mean monthly rainfall

Although the numbers of confirmed cases is small and the rainfall data from Balimo can only be inferred from the data obtained from Daru, it seems reasonable to conclude that most cases were apparent during the wet season - December - May and therefore melioidosis is likely to experience wet season predilection.
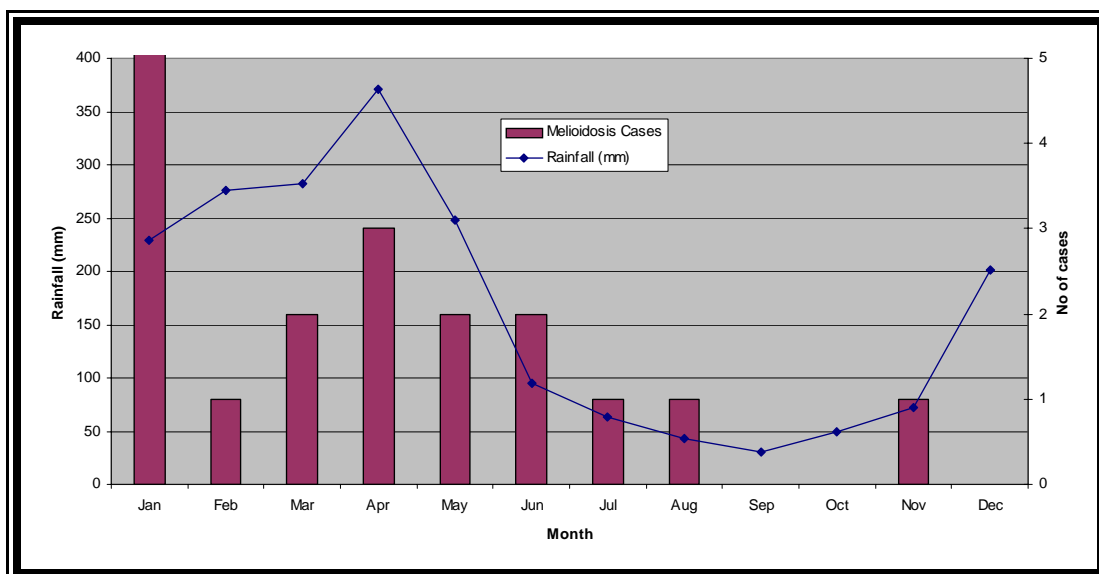
Figure 3.04   Melioidosis cases by month vs. mean annual rainfall at Daru 1981 -
2002

3.3.4   Serological survey

3.3.4.1 Sero-prevalence

A total of 747 children between the ages of eight and 13 years from five community
schools in the Balimo district participated in the study. The sero-prevalence of each
group is presented in Table 3.02. The mean sero-prevalence was 8.1% with a 95%
confidence limit ranging between 6.2 - 10.1%. Buila (the community school for
Balimo Village) 16.9%, Adiba 15.2%, Kimama 4.0% and Saweta 0.7% .

Table 3.02   Sero-prevalence of Balimo District Community School population
using IHA titre of $> / = 40$ as indicative of significant sero-reactive

School	Numbers	Positive	%	95% CL
Adiba	132	20	15.2	9.5 - 22.4
Balimo	178	2	1.1	0.14 - 4.0
Biula	201	34	16.9	12.0 - 22.8
Kimama	101	4	4	1.9 - 9.8
Saweta	135	1	0.7	0.2 - 4.1
Total	747	61	8.2	6.2 - 10.1

Age and sex of the sero-reactive children are presented in Table 3.03. Males were more represented than females with a sero-prevalence peak in the 12 years of age group.

Table 3.03   Sero-prevalence of Balimo District Community School population positive reactors by age and sex

Positive Reactors by Age	%	Positive Reactors by Sex	%
8	16.3	Male	59
9	14.7	Female	41
10	14.7		
11	14.7		
12	24.5		
13	14.7		

3.3.4.2 Statistical analysis

Statistical analysis of sero-prevalence data is presented in Table 4.04. A two by four Chi squared analysis demonstrated significant difference between the sero-prevalence data when all centres were tested ($p = <0.001$). The differences between the sero-prevalence of Adiba and Kimama was significant ($p = <0.01$). A one by one analysis of Kimama vs. Balimo and Kimama vs. Saweta was outside the parameters of the test.

Table 3.04   Statistical analysis of observed sero-clustering between community schools

Chi Squared Analysis	χ^2	df	p value
All Centres	48.69743	4	<0.001
Adiba vs. Balimo	21.01637	1	<0.001
Adiba vs. Saweta	14.90399	1	<0.001
Adiba vs. Kimama	6.957146	1	<0.01
Adiba vs. Buila	0.152872	1	N.S.
Kimama vs. Balimo	Not Valid		
Kimama vs. Saweta	Not Valid		

3.3.4.3 Follow-up of sero-reactive individuals

At the time of the study it was assumed that antibody response may indicate subclinical infection and therefore the sero-reactive children were at risk of reactivation of clinical melioidosis in the future (Charoenwong *et al.*, 1992). A clinic was established to assess the children in the survey whose samples had demonstrated an IHA titre of >/= 80. This was an arbitrary IHA cutoff chosen at the time based on the association of high IHA titres in children and likelihood of infection (Charoenwong *et al.*, 1992).

A chest x-ray was taken and evidence of infection was sought. Swabs from throat, rectum and any lesions were taken and along with a urine sample were broth enriched and cultured as detailed above. In each instance *B. pseudomallei* failed to be isolated. Chest X-rays deteriorated to such an extent during storage in PNG prior to transit to Australia they could not be reported on by a radiologist.

Considering the concern of the community and based on the limited availability of literature at the time, Balimo Health Centre clinical staff considered it clinically prudent to treat all children as if they were sub-clinically infected regardless of culture results. This was a pragmatic decision based on the uncertainty of the health centre's future. This may have been the only chance to intervene in the relentless clinical course of melioidosis. Each child was given a two month course of high dose cotrimoxizole and a new medical record book highlighted "maybe melioidosis". This was designed to encourage clinical staff to consider reactivated melioidosis should the child present with fever refractory to standard treatment in the future. Each child was assessed each week and if compliance to the therapy was affected by side affects the treatment was ceased.

3.4 Discussion

Signs and symptoms of melioidosis are not pathognomic. It is a feature of the natural history of the disease that diagnosis and perceived clinical relevance is dependent upon microbiology that may not be available in regions of likely endemicity. In Thailand from 1955-1966 only three clinical cases of melioidosis had been reported but after improvement in microbiology facilities in rural areas during 1974-1986, 800 cases were reported (Leelarasamee and Bovornkitti, 1989). In tropical regions with access to sophisticated microbiology and medical imaging melioidosis is becoming increasingly recognised as a common cause of sepsis and community acquired pneumonia (Currie *et al.*, 2000d; Dance, 2000d).

Of the 90/1000 childhood deaths in rural PNG, 57% have been attributed to respiratory and infectious disease (Anon, 2000a). Laboratory diagnostics in rural PNG, if available at all, generally consists of microscopy for malaria and AFB and basic haemoglobin estimation. Accordingly, diagnosis of infectious disease is mostly based on clinical experience and treatment is empirical. Papua New Guineans have the lowest number of healthcare workers per capita of any country in the pacific region (Anon, 1998). With postmortem examination being culturally unacceptable in many regions, often the cause of death may not be identified and recorded accurately.

Improving the capacity of the PNG rural laboratory facilities in this study has demonstrated culture confirmed melioidosis outside of Port Moresby for the first time. Of the nine cases, three presented with sepsis, two with pneumonia and one as *Staphylococcus* or tropical pyomyositis. The majority (66%) presented initially with subacute disease diagnosed as PUO with or without respiratory signs. As the majority of cases present as subacute localised melioidosis, early diagnosis of this condition may lead to early treatment which may decrease mortality, as occured with cases GD, NG and TG.

It is purely speculation how the numbers of cases recognised relate to the true prevalence of the disease in this region, but is worth noting that this collection of

cases increases the numbers of reported cases from PNG by over 100%. The lower Western province may represent a new focus of infection in the Australasia/Pacific region linking Asia to the Torres Strait and continental northern Australia.

The duration of the screening program was short and ability to sustain diagnostic protocols within this time was problematic due to resource constraints and the uncertainty of the health centre's future funding sources. Even with the best intentions, positive culture results could have been missed because there was no blood culture media available and serology may have been unable to be performed due to lack of sheep red cells. Power shortages discouraged regular media production and storage of plates. The anecdotal evidence suggests this small collection of culture confirmed and suspected cases, under represents the true prevalence of the disease in this region. To confirm this, a more extensive program would need to be conducted.

Regardless of how this collection of cases reflects the true state of melioidosis in this region, a number of issues are raised in their discovery and description that require further elucidation.

3.4.1   Treatment-refractory febrile disease may not be resistant malaria or TB

In regions of PNG were medical coverage is lacking, diagnosis and treatment is implemented by guidelines provided by manuals of standard treatment (Anon, 1993; Anon, 1997). When treatment protocols fail, emerging antimicrobial resistence of traditional pathogens such as *Plasmodium* spp and *Mycobacterium tuberculosis* is often blamed, although there is no empirical evidence for this assumption. This study has revealed that a treatment-refractory febrile syndrome mimicking TB in some cases, is indeed a previously unrecognised endemic infectious disease. Not withstanding the importance of developing malaria and TB drug resistence, the discovery of culture confirmed melioidosis autochthonous to this region, mostly resulting from the development of laboratory services, raises issues regarding the accuracy of empirical diagnosis elsewhere in PNG and Melanesia which lack basic laboratory support within their health services. It also raises the question of the role of

other non-pathognomic infections for which little is known, particularly arboviruses, leptospirosis and rickettsial disease.

3.4.2 Traditional co-morbidity is not a feature

A particular feature of melioidosis in this region is the general lack of classic predisposing factors commonly associated with acute melioidosis often reported in other endemic regions (Chaowagul *et al.*, 1989; Suputtamongkol *et al.*, 1999; Currie *et al.*, 2000d). Diabetes, liver disease and chronic renal disease are uncommon in Balimo and was not apparent in any of the culture confirmed cases. This may be a function of age of the patient and type of presentation, given that traditional predisposing factors are often not apparent in childhood melioidosis presenting as subacute disease (Lumbiganon and Viengnondha, 1995; Edmond *et al.*, 1998; Currie and Brewster, 2001).

Most Gogodala people live a traditional subsistence lifestyle and most rely on locally gathered food. It is speculation to consider the extent of clinical melioidosis in this region if diets change, as is the case in many villages of Port Moresby, resulting in increase prevalence of diabetes and other traditional co-morbidity factors (Ogle, 2001). All patients were members of a traditional subsistence family, living in rural villages outside the periurban township. This is consistent with the evidence of others which demonstrates that environmental exposure is a important risk factor (Merianos *et al.*, 1993).

Protein and calorie malnutrition is common in village children and particularly apparent in times of drought when fish are scarce and the staple carbohydrate, sago starch, cannot be processed. Of the four cases where data on nutrition as a child was available, three had a percentile of 65% or less at ages between six months and four years. This may contribute to underlying and long lasting cellular immunosuppression (Grunfeld, 2002) that seems apparent in culture confirmed individuals compared to those, in the same family group who are sero-reactive but with no history of clinical melioidosis (Barnes *et al.*, 2004).

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Malaria infection is endemic and has previously been implicated in predisposition to TB (Enwere *et al.*, 1999). Malaria may have been a factor in KawS but was noted in the other patients presented. However, a history of repeated infection cannot be excluded. Two of the cases had evidence of *Ascaris lumbricoides*, with infestation so high, active adult migration was noted per-anus and per-nasopharynx (case DS and KimS). Helminth infection has been shown to induce non-specific cellular immunsuppression (Baird *et al.*, 2002; MacDonald *et al.*, 2002). Lymphatic filariasis prevalence is less than 1% in the immediate region of Balimo (unpublised data) and therefore unlikely to play a role in non-specific immunosupression predisposing melioidosis.

3.4.3   Childhood predilection

Of the 17 cases of either suspected or culture confirmed melioidosis, only four were greater than 18 years of age. The mean age of culture confirmed cases was 12 years (median 10 years) and the mean age of all cases including suspected melioidosis was 13 years (median 11). Although the numbers are low, parotitis which is seen as a feature of childhood melioidosis in Thailand was not a feature in this group (Pongrithsukda *et al.*, 1988). This is consistent with observations made in Australia where it is also rare (Edmond *et al.*, 1998; Edmond *et al.*, 2001; Faa and Holt, 2002).

3.4.4   Regional and familial clustering

Of particular interest in this study is the clinical clustering supported by serology. Of the documented cases of suspected and culture-confirmed cases of melioidosis, eight of the ten come from one particular group of houses within 50 m of each other on Sanebase Point at Adiba village, a village with a high community school sero-prevalence. This clustering represents one extended family group and clan structure. Three individuals are members of an immediate family being a mother and two sons, others being uncles and a nephew. Both NG and TG represent an isolated familial outbreak at Balimo village and cases DS, KimS and KawS represent a familial outbreak on an isolated point of land at Kimama village.

Figure 3.05   Sanebase point, Adiba village

The clinical clustering of cases raises interesting issues of epidemiology and predisposition to infection. Further work needs to be conducted to determine if shared environmental exposure with a proven reservoir or if genetic predisposition, as has been implicated in animal models and in HLA association, play a role in this clustering. Familial association has been demonstrated in the past: presumptive mother to neonate transmission (Abbink *et al.*, 2001); shared familial sero-reactivity (Inglis *et al.*, 1999) associated with culture confirmation; presumptive sexual transmission (McCormick *et al.*, 1975) and presumptive sibling transfer with cystic fibrosis (CF) as a predisposing factor (Holland *et al.*, 2002).

Case NG and TG are siblings with a family history of TB, both presented with subacute melioidosis with respiratory signs at the same time. It is tempting to consider that droplet transfer and naso-pharyngeal colonisation is possible in some environments, mimicking the transmission of TB.

The reservoir of infection remains to be determined. It is interesting to note that meningoencephalitis in children caused by *N. fowleri* has been reported from this region (Tucker and Zerk, 1991). It was speculated at the time that the lagoon system provided the natural reservoir and amoebae were transmitted to the children via the

naso-pharynx as they were washed in the stagnant, warm water. Given that an association between free living amoebae and *B. pseudomallei* has been reported (Inglis *et al.*, 2000b; Inglis *et al.*, 2001; Inglis *et al.*, 2003) it is tempting to consider that there may be an association in this region. Regardless, the washing practices and environmental conditions may equally enable *B. pseudomallei* inoculation either from water as a endogenous reservoir or from water contaminated with soil after times of heavy rain.

Anecdotal evidence in this region has associated the outbreaks of suspected melioidosis, linked to the culture confirmed case AW during 1983 (Table 4.01) with soil excavation during house building on Sanebase point. This may hint at a possible soil based reservoir.

The serological results of the five community schools tested demonstrated significant clustering ($\chi^2 = 49.7$; df = 4; p = <0.001). These results reflect the clinical clustering of outbreaks. The three schools with the highest sero-prevalence, Buila (16.9%), Adiba (15.2) and Kimama (4.0%) are also the three regions where melioidosis outbreaks have been demonstrated. Buila community school is the school that serves Balimo village. This is the first evidence of sero-reactivity to a *B. pseudomallei* immunogen reported from PNG.

Balimo community school is the school that serves the urban township, it and Saweta have similarly low sero-prevalences (Balimo 1.1% and Saweta 0.7%) which are significantly different to Adiba (p = <0.001) and therefore Buila. The children living in the urban township are more likely to use articulated water and wash indoors and rely more on store food rather than gardening, this may decrease their exposure to the environment compared to village children with subsistence farmer parents, living in bush material houses. The low sero-prevalence at Saweta is more curious. The village is located on an island in the main Balimo lagoon system not more that 2 km from Balimo village. There are no apparent differences in geography or demography from other villages where the sero-prevalence is higher. The result may reflect an aberration in random sampling, but clearly requires further elucidation.

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The sero-prevalence at the Kimama village is low compared to Adiba and Buila
(p = <0.01) and given that two culture confirmed cases from Kimama have been
recorded, clinical endemicity does not seem to be reflected in sero-prevalence. This
finding either reflects a deficiency of the assay used in this way (Ashdown, 1981;
Ashdown, 1987) or reflects something unique of this village. Kimama is a village on
the eastern edge of Balimo lagoon approximately 5 km from Balimo village. It is a
large village with many points of land jutting out into the lagoon system. The
outbreak at Kimama resulted from one family group living on the edge of a point of
land reasonably isolated from the rest of the village. This region may represent an
isolated reservoir of *B. pseudomallei* not apparent in other regions throughout the
village and if so may explain the lack of exposure throughout the village. Also, the
sample size was not large therefore the 95% confidence limits of the observed
sero-prevalence ranges from 1.9 - 9.8% (Table 4.02). This range overlaps that of
Adiba, suggesting that regions within Kimama may have sero-prevalence consistent
with that of other regions where clinical evidence has been found. Regardless of these
observations and explanations for the observed clustering, the elucidation of a
reservoir of infection may further clarify the relative risks of acquiring melioidosis
from these regions.

The overall sero-prevalence of this district (6.2 - 10.1%) reflects the sero-prevalence
of the high-risk, well studied hyper-endemic community of the Top End of Northern
Australia (5 - 13%). These regions have a melioidosis incidence of 16.5-34.5/100,000
(Currie *et al.*, 2000e). If population antibody prevalence can be used to predict the
potential for clinical endemicity, Balimo may share a similar clinical burden relative
to these regions that is only sporadically revealed during time of active screening.

Given the poor positive predictive value of the IHA when used in a sample of
disease free individuals from a population with a true prevalence of <5%, in hindsight
treatment of the sero-reactive culture-negative individuals resulting from the
sero-prevalence survey may have been miss-guided (Ashdown *et al.*, 1989;
Thompson and Ashdown, 1989). Indeed individuals with high levels of antibody,
particularly antibody against lipopolysaccaride (LPS), may be less likely to develop

reactivation illness since the antibody response may signify successful processing of the organism and perhaps protection (Charuchaimontri *et al.*, 1999). Regardless, the treatment regimen that was used here was subsequently reported to be used for an asymptomatic yet sero-reactive child with a history of melioidosis in the family (Thummakul *et al.*, 1999).

The follow-up of these individuals in comparison to serology non-reactive individuals and non-treated positive reactors (IHA titres <80), may provide further insights into the role of the antibody response and development of melioidosis.

Regardless of these issues and problems of the IHA test as a sero-diagnostic tool, it proved to be useful in the diagnosis of melioidosis cases, at least in creating a heightened clinical suspicion which encouraged culture investigation. The technology is appropriate for this rural health facility. The sheep flock is 2-3 hours by boat from Balimo but the flock is sustainable. In the brief time the assay was used routinely it aided in the diagnosis of melioidosis thought to be tuberculosis in two cases. The positive result reminded staff to enquire further and seek culture evidence. Although, a negative result in one case prolonged the delay in directed therapy. This highlights the assay's limitations. Although Ashdown found IgM detection with the IFA more useful (Ashdown, 1981), it is unlikely this technology could be used in this setting.

The extent of rainfall has been implicated in the past as a clear factor that predisposes both to the extent and intensity of melioidosis in endemic communities (Merianos *et al.*, 1993; Currie and Jacups, 2003). Although the rainfall statistics reported here are not from Balimo itself but from the provincial capital, the association represented in Figure 3.02 is not perfect, but it is likely that melioidosis incidence follows rainfall, particularly the first rain, after a prolonged dry. It may be reasonable to assume that a break in the dry season may signal increased clinical melioidosis in this community.

The culture and social habits of the village based Gogodala people have changed little since their arrival to this region of PNG. Oral history suggests that people have inhabited this region for over 16 generations. Traditional belief has the Gogodala's

origins in Asia - this is reflected in the derivation of their name: *gogo* - (from the) northwest; *dala* - men. Did migration from regions of melioidosis endemicity import the disease to this region, or did these people stumble across an independent reservoir of infection? Or, of course, is melioidosis equally endemic in similar rural communities but undiscovered? Traditional cultural practices of clan based inter-marriage are still adhered to and may perpetuate particular genetic traits, some of which may predispose susceptibility to particular infectious disease as has been speculated in other ethic groups (Black, 1992). This region, therefore, may provide a unique collection of factors that may further enable the elucidation of epidemiology and predisposition to melioidosis.

Not withstanding the above mentioned epidemiological issues, this study demonstrates the presence of a disease with high case fatality rates, that is not well managed by existing standard treatment. Melioidosis should be considered in the differential diagnosis of febrile illness presenting in individuals from this region. The nationwide significance of melioidosis in PNG is yet to be determined.

CHAPTER 4 - THE RESERVOIR OF INFECTION AND BIOGEOGRAPHY OF MELIOIDOSIS IN PAPUA NEW GUINEA

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4.1 Introduction

Melioidosis is a mostly an environmental disease. Although the organism's ecology is not well elucidated, *B. pseudomallei* seems to be a saprophyte which prefers moist clay type soils and perhaps is an endogenous member of the flora of fresh water (Dance, 2000a). Associations with protozoa and plants have been proposed (Inglis *et al.*, 2001). Regardless of its preferred habitat, it is clear that individuals with a close association with the environment in regions where the organism is endemic are at risk of acquiring the disease (Suputtamongkol *et al.*, 1994a). The disease is more apparent during seasons of heavy rainfall (Currie and Jacups, 2003), this may reflect an increase in the reservoir or more effective transmission from the environment to host during these times. Clearly, when a reservoir of infection is well elucidated, control measures may be investigated.

Burkholderia pseudomallei has never previously been isolated from the environment in PNG. The objective of this series of experiments was to optimise a *B. pseudomallei* soil isolation method based on past techniques and to use the optimised protocol to uncover a reservoir of infection for clinical melioidosis in the Balimo region. This information would confirm endemicity in the region and help elucidate the epidemiology of infection.

The biogeography of microbes, that is the extent or otherwise of geographic boundaries limiting dispersal of microbes, is hotly debated (Cho and Tiedje, 2000; Finlay, 2002; Fenchel, 2003; Whitaker *et al.*, 2003). Given the spatial clustering of clinical melioidosis and the environmental reservoir of *B. pseudomallei*, this disease may provide a model to further this debate. Regardless, analysing associations between melioidosis endemic regions with attributes of climate, geography and lithography with GIS, may enable mapping of regions within PNG, that according to these correlations are conducive to survival and transmission of the organism and therefore potential for clinical melioidosis. If associations could be made and tested, this data would enable more precise targeting of clinical resources that could be used to definitively uncover and manage melioidosis in rural communities.

4.2 Materials and Methods

4.2.1 Selective broth

Although a number of selective broths and methods have been used to isolate
B. pseudomallei from the environment (Ellison *et al.*, 1969; Thomas, 1977; Thomas
and Forbes-Faulkner, 1978/1979; Ashdown and Clark, 1992; Wuthiekanun *et al.*,
1995a), it was decided that the broth based on Ashdown's method (ASHEB) with the
addition of 50 mg/l colistin (Appendix 1) would be used. This was based on reports of
reasonable efficacy, cost and simplicity (Wuthiekanun *et al.*, 1990; Ashdown and
Clark, 1992; Wuthiekanun *et al.*, 1995a). Sample preparation and incubation
conditions were optimised to achieve the highest possible sensitivity with this
method.

4.2.2 Optimisation of soil sample preparation

4.2.2.1 Preparation of *B. pseudomallei* control suspension

Optimal conditions of soil preparation and incubation with high sensitivity was
sought so to ensure confidence in the assay used on field samples. Known
concentrations of NCTC 13178 *B. pseudomallei* in suspension were used for
determining soil isolation sensitivity. Appropriate volumes of the suspension were
then seeded into samples of soil tested previously to be negative for *B. pseudomallei*.
Briefly, a portion of an overnight colony of *B. pseudomallei* on sheep blood agar was
suspended into 2.5 ml of 0.9% saline (Appendix 1) and adjusted to 0.5 McFarlane.
This was assumed to approximate 1×10^8 cfu/ml. A volume of 450 µl of the stock
suspension was serially diluted into 4.5 ml of 0.85% saline down to 10^{-8}. It was
assumed that the suspensions contained 10^8 - 1 cfu/ml respectively. The precise
concentration of the suspensions were subsequently determined by plate count after
10 µl of suspension was subcultured onto ASH agar (Appendix 1).

4.2.2.2 Soil sample preparation

Soil suspension supernatant was prepared by mixing soil with sterile distilled water and letting particulate matter settle. A sample of the supernatant was inoculated into selective broth (Wuthiekanun *et al.*, 1995a). Varying quantities of soil and supernatant inoculum sizes were used to determine if these could alter the sensitivity of the technique (Table 4.01). Presumably, antagonism between soil microflora may be a factor to suppressing *B. pseudomallei* growth. Each method was tested with both 100 µl and 10 ml volumes of inoculum into 10 ml of single and double strength ASHEB respectively.

Table 4.01   Soil preparation methods

Method	Soil (g)	dH$_2$0 (ml)
A	1	40
B	5	40
C	20	40
D	40	40

4.2.2.3 Sensitivity experiments: soil mass and supernatant inoculum volume

Samples were initially prepared in duplicate. For each method (A - D) soil samples were prepared to accommodate 1 ml volumes of each bacterial suspension with known numbers of cfu/ml (10^6 - 1 cfu/ml) in 80 ml sterile plastic containers before the appropriate volume of sterile distilled water was added. These numbers subsequently were calculated to cfu/gm of soil (40 gm of soil seeded). Samples were mixed, let settle and the required volume of supernatant (100 µl or 10 ml) was transferred to appropriately labelled containers containing 10 ml of either single (method A and B) or double strength (method C and D) ASHEB.

Each sample of inoculated broth was incubated at 35°C and 20 µl was subcultured onto ASH and typical colonies were counted after 48 hr incubation. A semi-quantitative method was used to report results:

No colonies = 0; 1 - 10 cfu = 1+; 10 - 50 cfu = 2+; 50 - 100 cfu = 3+; >100 cfu = 4+.

4.2.2.4 Sensitivity experiments: incubation conditions

The preferred method was further optimised by varying incubation conditions. Samples of soil and water slurry were prepared as before and incubated in an orbital shaker overnight at 30°C, mimicking environmental conditions when soil harbouring *B. pseudomallei* is suspended into rain and runoff water. After settling of particulate matter had occurred the preferred volume of supernatant was added to 10 ml of ASHEB in 80 ml sterile plastic containers.

These samples were incubated at 35°C for 48 hr and subcultured onto ASH solid media. The broths were further re-incubated for 3 days (total of 5 days) and re-subcultured. This was done to determine if extended incubation time would increase the specificity of the selective nature of the broth, perhaps through increased selective selection pressure.

During each experiment a non-inoculated sample of distilled water was included in the collection of soil samples being prepared to ensure no carry-over of organism occurred during manipulation. This was repeated when the soil samples of the sites were being analysed.

4.2.2.5 Non-soil sampling

In the houses of families where melioidosis outbreaks had been confirmed, various utensils used to carry and store water were swabbed and swabs were placed in ASHEB broth and incubated for 48 hr and subcultured using the isolation techniques for soil sample slurries.

4.2.3   Isolate identification

Organisms were presumptively identified as *B. pseudomallei* based on typical colonial appearance, oxidase reaction (positive) and gentamicn resistence (Dance *et al.*, 1989c) as described in Chapter 3. Each isolate was confirmed with API 20NE (bioMerieux, Baulkham Hills, NSW) or Microbact 24E (MedVet, Adelaide, SA) used as per manufacturer's directions. Where isolates achieved an adequate clinical suspicion (based on colonial morphology, oxidase and gentamicin reactions) but were not able to be identified positively using the API 20NE, these were classified as "query *B. pseudomallei*". It was subsequently noted that these isolates shared the characteristic of *B. thailandensis* of utilising arabinose (Wuthiekanun *et al.*, 1996; Smith *et al.*, 1997; Brett *et al.*, 1998). For the purposes of this work, the isolates were therefore characterised as either arabinose non-utilisers (ARA-), presumptive virulent *B. pseudomallei* and arabinose utilisers (ARA+), presumptive *B. thailandensis* or related non-virulent species. All isolates were further characterised subsequent to this series of experiments in order to ensure identification and these methods and results are presented in Chapter 5.

4.2.4   Environmental sampling sites

The sites of soil sampling were limited to the regions where clinical melioidosis had been reported, see Chapter 4. This was undertaken to enhance the chance of finding a reservoir which could be further investigated. Sites where logic suggested communities had increased contact with the environment, particularly children, were targeted. They were loosely divided into five categories:

1.   Soil from garden places (GP);
2.   Soil under or near houses (SNH);
3.   Soil from points of land near lagoon (or river for Tapila) frequented by children (PtC);
4.   Soil from mainland regions of village frequented by children (N-PtC);
5.   Soil from walls of water wells or soil adjacent to well (WELLS).

It was not the ambition of this work to obtain a detailed understanding of the extent of the reservoir. Sampling of the Balimo region was undertaken between 31 March 1998 to 29 May 1998. Samples were stored and transported to Townsville for processing as soon as practical.

A "melioidosis non-implicated" control site, Tapila (Tirio) village was chosen approximately 30 km to the south of the Balimo region (Figure 4.01). This region is different in geography, in that the village is adjacent to the Fly river and not associated with swamp land and chosen to provide contrast. The villagers use Balimo health centre as a referral rural hospital and no cases of melioidosis have been recorded or suspected from this village. Soil samples were collected on 5 January 2002. A total of 100 samples were collected.

4.2.5   Soil sampling techniques

A hole approximately 50 cm deep was dug with a spade which had been cleaned of excess soil and disinfected with 70% ethanol between each application. Samples of soil were generally taken at a depth of 30 cm with disposable wooden applicator sticks. Samples were collected into 80 ml sterile, polypropalene containers. Duplicate samples were taken from each site. Global position data was collected at each site so the regions sampled could be plotted and soil type could be inferred from past soil-type mapping exercises.

4.2.6   Biogeography

Associations with various environmental attributes were sought to aid in determining the biogeography of *B. pseudomallei* in this region and therefore provide data on the potential for melioidosis in regions within PNG but outside the scope of this study.

Geographic and climatic data from the PNG Resource Information System (PNGRIS) was kindly supplied by Dr Bryant Allen, Senior Fellow Land Management Group,

Department of Human Geography, Research School of Pacific and Asian Studies, The Australian National University.

The PNG environment was mapped by Resource Mapping Units (RMU) which are determined by unique combinations of altitude, landform, rock type, rainfall and inundation. The actual attributes available for analysis in this study included landform, rock type, slope, relief, altitude, rainfall, seasonality and rainfall deficit, flooding and extent of flooding, vegetation and predominate soil type and are listed in Table 4.09. These attributes of both the Balimo region (considered melioidosis endemic) and Tapila region (considered melioidosis non-endemic) were compared to determine if, by subtraction, unique features consistent with the Balimo region, and therefore, melioidosis endemicity could be inferred.

Latitude and longitude, in the form of decimal degrees of each site sampled was determined by Global Positioning System (GPS) using the Magellan Meridian Gold GPS receiver (Thales Navigation).These points were layered onto resource mapping unit maps and associations with *B. pseudomallei* culture positive soil and the RMU were sought.

4.3   Results

4.3.1   Preliminary optimisation of soil isolation technique

Samples were prepared in duplicate for each criteria mentioned above (Table 4.01). Sensitivity and reproducibility generally increased with the mass of soil prepared and the volume of the supernatant inoculated into broth. Method D achieved a reproducible sensitivity of 400 cfu/ml which, as 40 gm of soil was seeded, equated to 10 cfu/gm of soil (Table 4.02).

Method D (40 gm of soil plus 40 ml of sterile distilled water) was further tested under conditions were the slurry was incubated at 30°C for 24 hr in an orbital shaker before settling. A 10 ml volume of supernatant was inoculated into 10 ml of double strength

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ASHEB and further incubated for 5 days before subculture. Sensitivity was extended to 15 cfu/ml which equated to 0.38 cfu/gm of soil (Table 4.03) . These optimisation conditions were chosen to test the soil samples from the sampled site.

Table 4.02   Initial optimisation of soil isolation technique

CFU / ml	4×10^6	4×10^5	4×10^4	4×10^3	400	40	0
CFU / gm soil	105	104	103	100	10	1	PBS
Method A							
100 µl I	1+	0	0	0	0	0	0
100 µl II	1+	0	0	0	0	0	0
10 ml I	2+	1+	1+	1+	0	0	0
10 ml II	2+	0	0	0	0	0	0
Method B							
100 µl I	1+	0	0	1+	0	0	0
100 µl II	1+	0	1+	1+	0	0	0
10 ml I	1+	1+	0	1+	1+	0	0
10 ml II	2+	1+	1+	1+	1+	0	0
Method C							
100 µl I	2+	2+	0	1+	0	0	0
100 µl II	1+	1+	0	0	1+	0	0
10 ml I	2+	2+	1+	0	0	0	0
10 ml II	2+	2+	1+	1+	0	0	0
Method D							
100 µl I	2+	2+	1+	1+	0	0	0
100 µl II	2+	1+	0	1+	0	0	0
10 ml I	3+	2+	2+	1+	1+	0	0
10 ml II	3+	2+	1+	1+	1+	0	0

Table 4.03   Method D after incubation condition optimisation. Results after five days incubation before subculture. p = pure culture

CFU / ml	1.5×10^6	1.5×10^5	1.5×10^4	1500	150	15	1.5	0.15	PBS
CFU / gm	3.8×10^4	3800	380	38	3.8	0.38	3.8×10^{-2}	3.8×10^{-3}	0
I	4+p	4+p	3+	1+	1+	1+	0	0	0
II	4+p	4+p	4+p	2+	0	1+	0	0	0
II	4+p	4+p	3+	2+	1+	1+	0	0	0

4.3.2   Soil Isolation of *B. pseudomallei* from sample sites

A total of 274 samples were collected from the Balimo region and tested. Of the three villages sampled,144 came from Kimama, 34 from Balimo and 96 from Adiba. Of these 4.0% (11/274) were from garden places (GP), 12.8% were from soil near houses where individuals with culture confirmed melioidosis had been living at the time disease was diagnosed (SNH), 69.7% (191/274) were samples of soil near points of land leading to the lagoon surface where children play and wash (PtC), 5.1% (14/274) were from areas away from the lagoon surface but frequented by children (N-PtC) and 9.5% were from soil near wells or soil from the walls of wells used for washing and drinking (WELLS).

A total of 100 samples were collected from Tapila (Tirio) village. Of the total, 15% were from garden places (GP), 34% from near houses (SNH), 16% from near the bank of the river (PtC) and 35% from regions in the village that are frequented by the local village people (PtC) but are not adjacent to the river. No samples were taken from wells.

Table 4.04   Soil sampling sites and sample types

Village	GP	SNH	PtC	N-PtC	WELL	Total
Balimo region						
Kimama	3	6	119	13	3	**144**
Balimo	0	18	18	1	0	**37**
Adiba	8	11	54	0	23	**96**
Total	**11**	**35**	**191**	**14**	**26**	274
Fly river region						
Tapilia (Tirio)	15	34	16	35	0	**100**

4.3.3   *Burkholderia pseudomallei* isolation (ARA - and ARA +) from villages

Of 274 samples collected from the Balimo region 2.6% (7/274) (95% CI 1.0 - 5.2) were tested positive for ARA - *B. pseudomallei* (Table 4.05). Of the total, five from Kimama village (3.5%, 95% CI 1.1 - 7.9%) and two from Adiba (2.1%, 95% CI 0.3 - 7.3). Culture of the 34 samples collected from Balimo village and 100 samples from Taplia revealed no suspicions isolates, 95% CI 0 - 8.4% and 0 - 3.0% respectively.

Table 4.05   Numbers and percentages of ARA - isolates from each village

Village	Total Samples	No Pos ARA -	% Pos ARA -	95% CI
Balimo region				
Kimama	144	5	**3.5%**	**1.1 - 7.9**
Balimo	34	0	**0**	**0 - 8.4**
Adiba	96	2	**2.1%**	**0.3 - 7.3**
Total	274	7	**2.6%**	**1.0 - 5.2**
Fly river region				
Tapila (Tirio)	100	0	**0**	**0 - 3.0**

A total of 15 ARA+ isolates were isolated from the Balimo region, none from Kimama (95% CI 0 - 2.1%) and four (11.8%, 95% CI 3.3 - 27.5) and 10 (10.4%, 95% CI 5.1 - 18.3) respectively from Balimo and Adiba. A total of five ARA+ isolates were cultured from the 100 samples from Tapila (95% CI 1.6 - 11.3%) (Table 4.06).

Table 4.06   Numbers and percentages of ARA + isolates from each village

Village	Total Samples	No Pos ARA +	% Pos ARA +	95% CI
Balimo Region				
Kimama	144	0	**0**	**0 - 2.1**
Balimo	34	4	**11.8**	**3.3 - 27.5**
Adiba	96	11	**10.4**	**5.1 - 18.3**
Total	274	15	**5.1**	**2.8 - 8.4**
Fly River region				
Tapilia (Tirio)	100	5	**5**	**1.6 - 11.3**

4.3.4   *Burkholderia pseudomallei* isolation (ARA - and ARA +) from sampling sites

Of all samples sites tested across all villages only soil from points of land frequented by children (PtC) tested positive for ARA- isolates. After analysis, a total of 3.7% (95% CI 1.5 - 7.4) of these samples were positive for *B. pseudomallei* (ARA-). These regions are mostly points of land leading to the lagoon and are subject to erosion during heavy rain (Table 4.07).

Table 4.07   ARA - status of isolates relative to sample site in the Balimo region

	Total Samples	ARA -	%ARA -	95% CI
GP	11	0	0	0 - 23.8
SNH	35	0	0	0 - 8.2
PtC	191	7	3.7	1.5 - 7.4
N-PtC	14	0	0	0 - 19.3
Well	26	0	0	0 - 10.9

In contrast to the isolation rates of the ARA- isolates, all regions of the villages produced ARA + isolates (Table 4.08). The highest prevalence was from garden soil (36.4%, 95% CI 10.9 - 69.2) followed by soil near houses (20.0%,95% CI 8.4-36.9), well wall or associated soil (11.5%, 95% CI 2.5 - 30.2) and soil from the regions of mainland village where children play (7.1%, 95% CI 0.18-33.9). The region which demonstrated the lowest prevalence of ARA + isolates was point of land where children play. This region was the only region where ARA- was isolated.

Table 4.08   ARA + status of isolates relative to sample site in the Balimo region

	Total Samples	ARA +	%ARA+	95% CI
GP	11	4	36.4	10.9 - 69.2
SNH	35	7	20.0	8.4 - 36.9
PtC	191	5	2.6	0.8 - 6.0
N-PtC	14	1	7.1	0.18 - 33.9
Well	26	3	11.5	2.5 - 30.2

4.3.5   Non-soil sampling isolation

A total of 13 samples were collected from sites associated with water storage within houses of culture confirmed melioidosis individuals. Of these, four were swabs from buckets used to store water from a house in Kimama village and nine were swabs from similar sites from a house in Balimo village. All were tested negative for either ARA - or ARA + *B. pseudomallei*.

4.3.6   Biogeography

Table 4.09 summaries the environmental characteristics of both regions (Balimo and Tapila) sampled using data obtained from the PNGRIS. Both regions are low attitude with temperatures averaging 30-32°C. The landforms and rock type of the Balimo region is generally more swampy and alluvial compared to the more levee plains and estuarine deposits of Tapila. Balimo is generally wetter and the region has a greater area flooded with a less severe dry season. The soils are similar at both sites, but Balimo is more predominantly hydraquents (Table 5.09). Regardless of soil type, all soils are more likely to be continuously saturated in the Balimo region compared to Tapila. These varying environmental attributes of the Balimo region were categorised as "melioidosis implicated environment"and are graphically represented in Figure 4.01.

Table 4.09   Biogeographical characteristics in RMU of the Balimo and Tapila regions of PNG

Attributes	Balimo Region	Tapila Village
Landform	blocked or drowned valley swamp and associated swampy floodplain	Composite levee plains
Rock type	Aluvial deposits	Estuarine deposits
Slope	Flat	Flat
Relief	Flat - no relief	Flat - no relief
Altitude	<600m; 32-30°C	<600m; 32-30°C
Rainfall	2000 - 2500mm annual	1000 - 1500mm annual
Seasonality	<100 - >200mm; <100mm dominant	<100 - >200mm; <100mm dominant
Rainfall Deficit	Irregular moderate deficit	Regular seasonal severe deficit
Flooding	Near permanent flooding	Near permanent flooding
Flooding extent	>80% of total land area flooded	50 - 80% of total land area flooded
Veg 1	Mixed herbacious swamp vegetation	Large to medium crowned forest; Mixed herbacious swamp vegetation
Veg 2	Swamp grassland	Swamp woodland
Soil 1	Hydaquents - permanently saturated undifferentiated, soft underfoot and mainly fine textured	Fluvaquents - poorly drained, undifferentiated, high (>0.2%) of variable organic C content
Soil 2	Fluvaquents - poorly drained, undifferentiated, high (>0.2%) of variable organic C content	Hydaquents - permanently saturated undifferentiated, soft underfoot and mainly fine textured
Soil 3	Tropohemists - swampy, half decomposed organic soils, often with interbedded mineral layers	
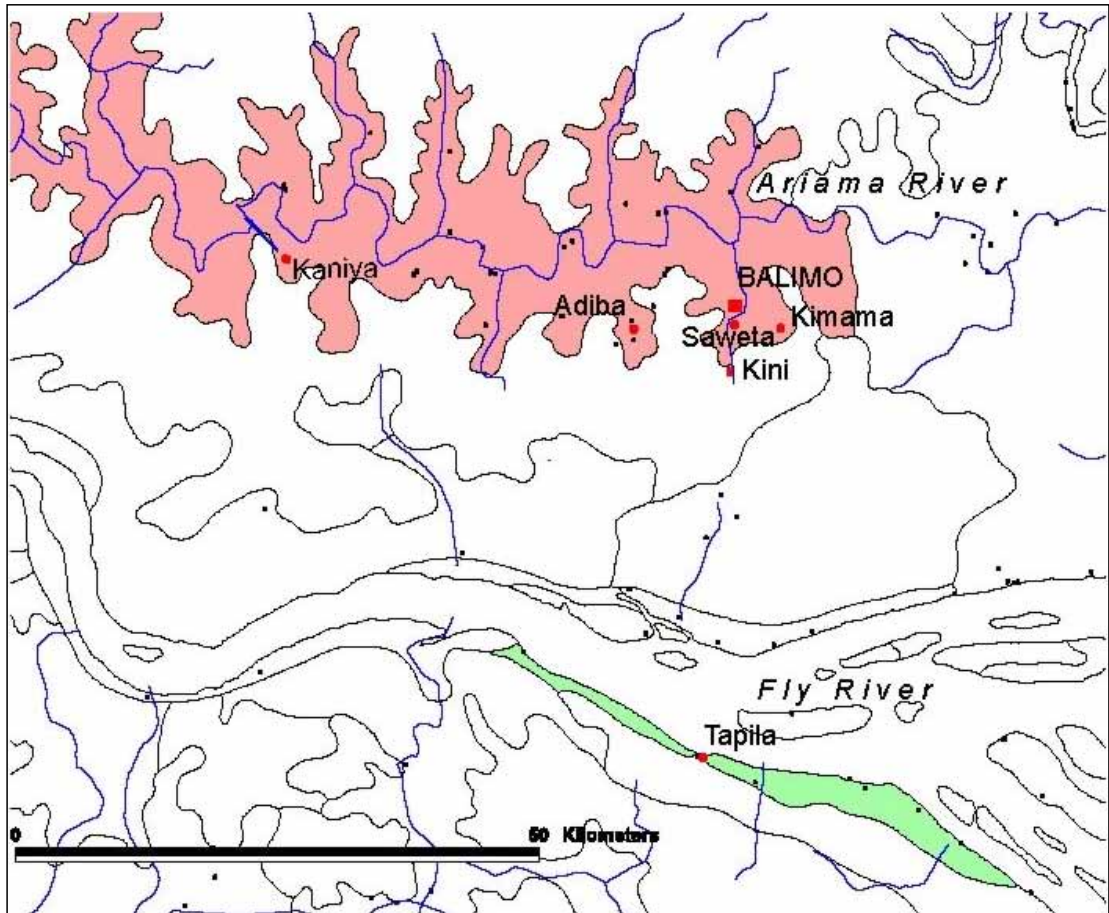
Figure 4.01   Map of RMU of Balimo and Tapila village regions. The "melioidosis
                implicated environment" coloured pink and Tapila village region
                (melioidosis non-implicated) green

Papua New Guinea was mapped in terms of the attributes identified unique to the

Balimo region and including altitude (<600 m) which reflects average ambient

temperature of 30 - 32°C, a temperature which has been shown to be important in

environmental survival of *B. pseudomallei* (Tong *et al.*, 1996). These environmental

attributes were thought to be important indicators of melioidosis biogeography using

the Balimo region as an example of a hyperendemic region in this country. These

attributes included altitude (<600 m), inundation and extent of inundation (80%) plus

the extent of the predominate soil type (hydraquents - poorly drained alluvial

soils).This map is presented as Figure 4.02.

Altitude - <600 m asl
Inundation - near permanent
Inundation extent - 80%
Soils1 - Hydraquents - poorly drained alluvial soils

300   0   300   600 Miles
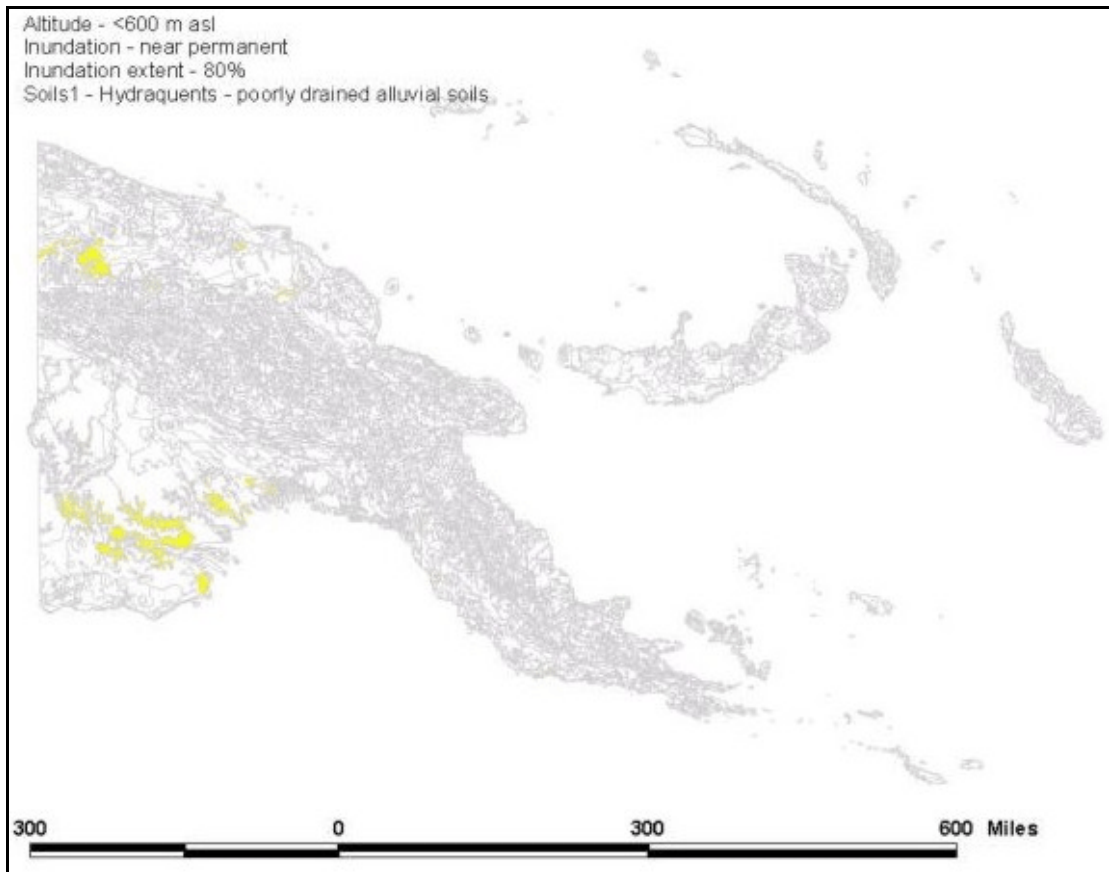
Figure 4.02   National map of PNG showing attributes of altitude, inundation and
extent of inundation and predominant Balimo soil type (hydaquents)

The melioidosis implicated environment is rarely distributed throughout PNG (Figure
4.02). This may reflect the isolated distribution of melioidosis-risk communities
which may explain the rare clinical reports of melioidosis in PNG. The region
highlighted in the west of the East Sepik province (ESP) (further expanded in Figure
4.03) provides an ideal opportunity to further elucidate the biogeographical barriers
of melioidosis which are being speculated to be associated with clinical melioidosis
in this study.
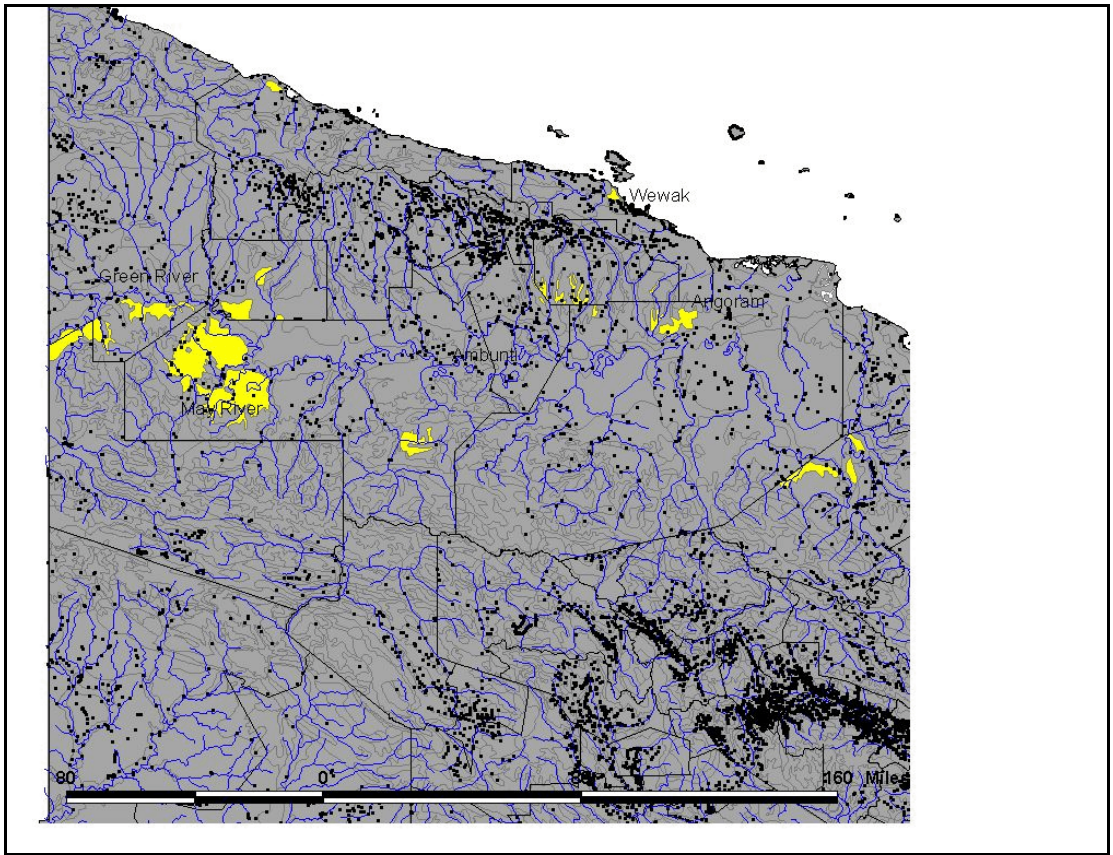
Figure 4.03   Melioidosis implicated environmental region of the ESP

4.4   Discussion

The soil isolation method D optimised with soil mass and incubation condition was able to detect *B. pseudomallei* from soil samples at numbers equating to 0.38 cfu/gm. This is a calculated value based on the ability to recover bacteria successfully to a concentration of 15 cfu/ml spiked into a mix of water containing 40 gm of soil. Assumptions are that exogenous bacteria added into the sample are isolated from the slurry using this technique equally as soil borne *B. pseudomallei* endogenous to the soil samples obtained in the field. Soil bacteria are often associated with complex micro habitats which cultivation-based isolation techniques often misrepresent (Kent and Triplett, 2002).Therefore, the ability to recover exogenously seeded bacteria from the soil slurry probably does not accurately reflect the ease with which bacteria are detected from within this complex microhabitat of *B. pseudomallei* of which little is known (Inglis *et al.*, 2001). Therefore the sensitivity of the assay is likely to be a gross over estimation.

Regardless of the imperfections described above, the optimisation experiments were undertaken to obtain some sense of confidence in the assay and assurance that a negative result was not simply due to poor *in vitro* sensitivity. The extended incubation period and agitation which proved to increase *in vitro* sensitively may also enhance the availability of endogenous organism from a microhabitat, be it clay microspheres or vacuoles within protozoa. A genomic detection method, although previously undertaken (Brook *et al.*, 1997) and generally recommended for this work (Kent and Triplett, 2002) was not considered because of the need to obtain viable organism from the samples for further phenotypic and molecular epidemiological analysis.

The quantitation of *B. pseudomallei* numbers within positive samples have been corelated with regions in terms of incidence of infection: high numbers have been from regions samples from regions with high incidence (Smith *et al.*, 1995b).Quantitation of positive samples within this region may provide more information regarding risk of acquiring melioidosis. The lack of technical accuracy

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inherent in the most probable number technique (MPN) could be clarified with the application of real time quantitative PCR with proven specific primers (Filion *et al.*, 2003). This assay along with sensitive cultivation techniques may help to demonstrate the relevance and indeed presence of uncultivatable strains (Inglis *et al.*, 2001). Regardless of what technique is used, sensitivity results which can be used to accurately determine the utility of the assay in demonstrating *B. pseudomallei* from the environment will always be complicated by the lack of complete assurance that detecting the presence of organism seeded into samples equates to the detection of organism locked away in soil microhabitats with unknown characteristics.

Regardless of the true sensitivity of the method used, *B. pseudomallei* from soil sampled from regions associated with clinical outbreaks in PNG has been demonstrated for the first time. The overall prevalence of soil-borne ARA non-utilising *B. pseudomallei* from the environment within the Balimo region associated with clinical melioidosis isolates was 2.6% (95% CI 1.0 - 5.2).When compared to other melioidosis endemic regions, these isolation rates are at the lower end of those achieved in regions of Thailand were melioidosis is considered hyperendemic (4.4% - 20.4%) (Vuddhakul *et al.*, 1999) but higher than those rates reported from North Queensland (1.7%) (Thomas, 1977). It should be noted that most of the soil samples taken from the Thailand study were taken from cultivated rice fields which have previously found to be particularly rich reservoirs of *B. pseudomallei* (Dance, 1991b). The process of rice cultivation presumably enhances growth rates (and therefore isolation rates) through aeration and transmission by contaminated farming equipment (Liesack *et al.*, 2000). Therefore the results may not reflect isolation rates of uncultivated regions and therefore true endogenous habitat, as do to some extent, the PNG samples.

Of the three villages that had been associated with outbreaks: Adiba (08° 03.929S; 142° 52.552E) , Kimama (08° 03.058S; 143° 00.042E) and Balimo (08° 03.058S; 143° 00.042E), analysis of soil samples from Adiba (2.1%, 95% CI 0.3 - 7.3) and Kimama (3.5%, 95% CI 1.1 - 7.5) demonstrated *B. pseudomallei* from regions within the villages where children frequent. The fact that the analysis of samples from

Balimo village failed to demonstrate evidence of *B. pseudomallei* may be related to sampling error or inherent error in isolation methods based on cultivation as described previously. The sample size required to reveal between 2% and 5% positive within a population of infinite size with 95% confidence is 59 - 149 (Cannon and Roe, 1982). Although none of the 34 samples analysed from the Balimo village demonstrated *B. pseudomallei*, this is still consistent with the prevalence achieved from the other villages (2% - 5%). Further sampling in this region would elucidate the true extent of the reservoir.

Of the six regions within the two villages tested which had reported confirmed melioidosis outbreaks only one region was consistently culture positive. Further sampling of the environment surrounding the villages would eliminate sample biases associated with this observation. Regardless, the analysis of the 191 samples collected from regions were children play close to points of land leading to the lagoon (PtC) demonstrated the organism in 3.7% (95% CI 1.5 - 7.4) of the samples. This soil at these regions rarely drys, is mostly clay based and is subject to seasonal flooding. This observation is consistent with past reports which demonstrated the importance of soil water content for *B. pseudomallei* survival in the environment (Thomas, 1977; Thomas *et al.*, 1979; Tong *et al.*, 1996). Also, the extent of inundation appears to be a critical difference between the environment of Balimo relative to most other regions in PNG. Further analysis of physio-chemical attributes of this soil and indeed analysis of the soil profile in these regions may help define the microhabitat more fully.

It is interesting to speculate that if cultivation of the environment to the extent of intensive rice farming undertaken in Thailand was undertaken in this region of PNG (as it is in the Morobe province), how this might alter the environment in favour of more enhanced growth of *B. pseudomallei* and therefore the heightened risk of acquiring melioidosis. In regions of PNG where rice cultivation is being considered, where the status of melioidosis is not known, it may be prudent to undertake soil prevalence studies.

These regions (points of land) often lead into the lagoon. The soil profile is exposed through season rainfall and through excavation when houses are being prepared to be built. At times of steady rain (late wet season and early dry season) these points of land are often grassed over, this may protect individuals from being exposed to soil harbouring *B. pseudomallei*. At the end of the dry season, at the time of sudden heavy rain, the points of land are usual bare. Soil from the various profiles that are exposed is washed into the lagoon system where children play and wash (Figure 4.04).

Figure 4.04   Digi point Kimama village, soil on this incline harbours
               B. pseudomallei

Children often have minor abrasions and other lesions on extremities, the contamination of these lesions may encourage infection. Also, the vigorous playing in the muddy waters at the base of these runoffs may encourage pernasal inoculation as seems to be the case when children are washed in these waters which also presumably harbour free living amoebae (Tucker and Zerk, 1991). These factors may help explain modes of transmission *per se* but also the perceived childhood predilection in particular (see Chapter 3).

Molecular typing (Lew and Desmarchelier, 1993; Trakulsomboon *et al.*, 1994; Currie *et al.*, 2001) of the isolates matched from the clinical outbreaks and associated environmental sources needs to be undertaken to further substantiate this hypothesis of transmission (see Chapter 6).

The ratio of ARA non-utilising / ARA utilising isolates from the environment corelates with the incidence of disease in the community (Trakulsomboon *et al.*, 1999; Vuddhakul *et al.*, 1999). That is, the higher the ratio the higher the incidence of infection. The regions where melioidosis is endemic in Thailand range from a ratio of ARA- / ARA+ of between 0.4 - 1.7. The ratio in this study is 0.5, the lower end of what is expected in Thailand.

It is interesting to speculate on the relationship between the different species of *Burkholderia* to one another in the environment. Perhaps antagonism between the ARA+ against the ARA - prevents colonisation of the immediate environment by the ARA - to the extent that critical numbers are not achieved to sustain sufficient exposure to produce hyperendemic disease in the community. It is interesting to note that the regions within villages that harbour *B. pseudomallei* (PtC) are also regions which least harbour the ARA+ species. This may be an aberration of sampling error but perhaps is the manifestation of microbial antagonism between species.

Not withstanding the difficulties in sustaining a laboratory service capable of providing melioidosis diagnosis capacity within an under resourced rural health service, melioidosis does seem generally spatial in the Balimo region. The role of ARA+ in modulating infection potential is speculative (Dance, 2000a). Work on the immuno-relatedness and bacteriocin properties of the indigenous ARA + relative to indigenous ARA- isolates may elucidate this further.

Soil was the only environmental sample cultured mostly because water filtration equipment was not available at Balimo and water in the volumes required for accurate analysis could not transported to Australia easily (as apposed to small aliquots of soil). This is an obvious limitation. Water has been proposed to be an endogenous

source of *B. pseudomallei* associated with clinical outbreaks (Inglis *et al.*, 2000a; Currie *et al.*, 2001). Well water used for drinking and bathing would be an obvious source for investigation, given the soil contamination possibilities. The fact that no *B. pseudomallei* was found near or as a part of existing well soil profiles may be a sampling error aberration. Only 26 samples were analysed and none demonstrated the organism. Regardless, the true positive rate is unlikely to be >10% (Cannon and Roe, 1982).

Nitrogen fixing capacity of various *Burkholderia* have recently been reported (Estrada-De Los Santos *et al.*, 2001). Given the potential for members of the genera to share mutualistic relationships with plants, an audit of native, particularly leguminous plants, may reveal a reservoir. Introduced rice-like grass covers the surface of the lagoon system most of the wet season.

The biogeography of bacteria, Archaea and microscopic eukaryotes is contentious (Fenchel, 2003). Microbes have been generally considered not to be bound by geographic barriers as are macro fauna. This is mostly due their size, abundance and the extent of their metabolic adaptiveness (Finlay, 2002). The widely accepted theory that the preferred environment merely selects out populations of organisms which are ubiquitously dispersed has been recently challenged for the hyperthermophilic Archaea (Whitaker *et al.*, 2003). Presumably these organisms cannot survive dispersion outside their extreme ecological requirements. This seems unlikely to be the case for the *Burkholderia* given their relatively non-fastidious nutritional requirements, although true endemicity (not associated with mere dispersal) has been reported in the *Pseudomonas* genus (Cho and Tiedje, 2000).

The spatial clustering of clinical melioidosis prevalence is widely reported and is consistent with an associated reservoir (Ashdown and Guard, 1984; Chaowagul *et al.*, 1989; Li and He, 1992; Currie *et al.*, 1994; Suputtamongkol *et al.*, 1994a; Heng *et al.*, 1998; Inglis *et al.*, 1999; Vuddhakul *et al.*, 1999; Dance, 2000c; Currie *et al.*, 2001). Not withstanding the critical role of the laboratory to identify melioidosis, these reports seem to reflect a genuine spatial distribution which may be due to ecological

factors not yet fully elucidated. This region of PNG with environmental and demographic stability, may be a region where these ecological attributes are able to be studied.

The "melioidosis implicated environment" as described by the RMU provided by the PNGRIS demonstrated some differences between a melioidosis implication region (Balimo) and melioidosis non-implicated (Tapila) (Table 4.09). The differences mainly are associated with the extent of innundation. Past reports of the habitat of *B. pseudomallei* have implicated water either in soil, stored or artificially articulated as probable habitats (Thomas, 1977; Thomas *et al.*, 1979; Pruekprasert and Jitsurong, 1991; Inglis *et al.*, 1998b; Inglis *et al.*, 1999; Inglis *et al.*, 2000a). Flooded rice fields certainly provide a habitat for a vast array of microbes (Liesack *et al.*, 2000). Regardless, these aquatic environments presumably require contamination from a permanent habitat and therefore associated with a more complex soil, plant or animal microhabitat.

The national PNG map produced which plot "melioidosis implicated environment" attributes are the result of selecting attributes which are more likely associated with Balimo than Tapila (Figures 4.01 and 4.02). These data provides the opportunity to test the hypothesis that the environmental factors determined here have a relationship with soil prevalence of *B. pseudomallei* and therefore potential for acquisition from the environment and thus clinical melioidosis in the communities which reside in and use these environments. This can be undertaken by establishing screening programs in contrasting regions and perhaps through sero-epidemiology (Srikitjakarn *et al.*, 2002).

Regardless of the accuracy of the perceived correlation of the environmental attributes plotted and potential for mapping clinical melioidosis, this information may at least provide a tool for further defining this relationship. It will allow the first layer of data which may help provide a model for remote sensing detection of melioidosis in PNG, techniques which have been used for other infectious diseases, particularly

those with well defined environmental requirements (Odoi *et al.*, 2004; Tum *et al.*, 2004).

It is uncertain how the two regions presented here (Balimo and Tapila) reflect true melioidosis "endemic vs. non-endemic" environments. Sero-epidemiology, further clinical and environmental screening may help to further classify Tapila's endemicity status and thus provide a more accurate comparison with Balimo. Quantitative assays of determining prevalence of organism in the environment with high sensitivity which do not rely on cultivation (Filion *et al.*, 2003) might be useful. At least though, the data presented provides subtractive comparison on which speculation of ecology can be further investigated. The lack of knowledge regarding the ecology of *B. pseudomallei* limits this application and misleading results could be produced if hypotheses are not tested with accurate clinical investigations. Not withstanding this problem, remote GIS application supported by research on the ecology of *B. pseudomallei* in determining at-risk communities, would be particularly useful in PNG where logistics of establishing screening programs randomly would waste stretched healthcare resources.

CHAPTER 5 - THE CHARACTERISATION OF *BURKHOLDERIA PSEUDOMALLEI* AND *BURKHOLDERIA PSEUDOMALLEI*-LIKE ISOLATES DERIVED FROM PAPUA NEW GUINEA
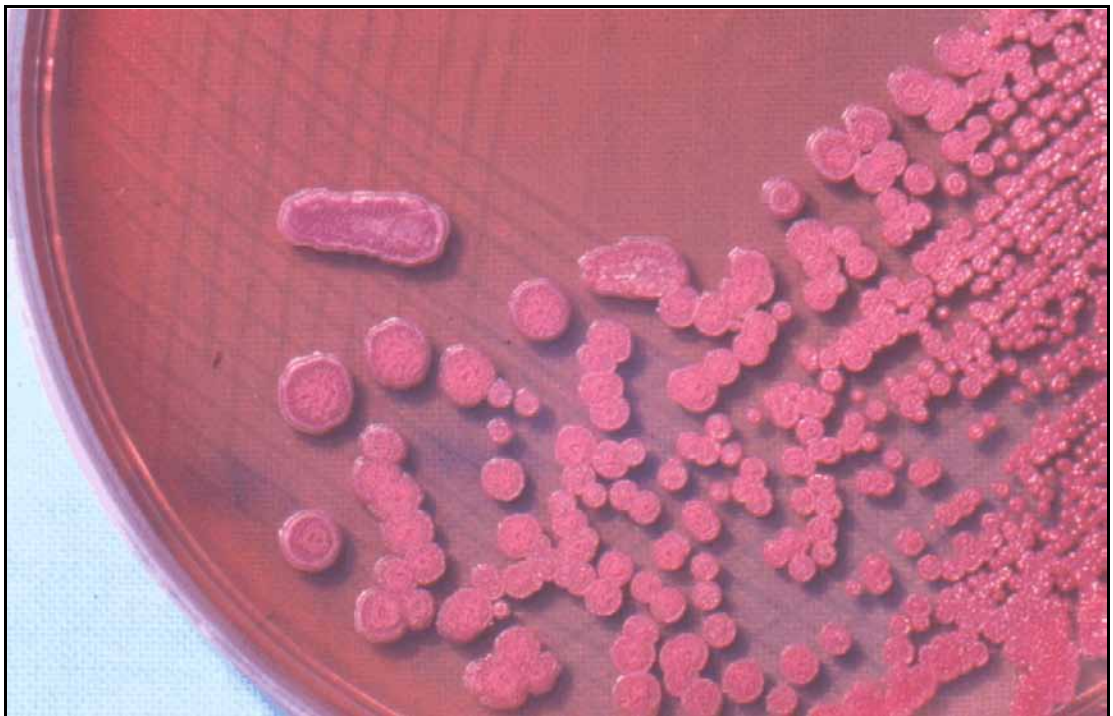
5.1 Introduction

Burkholderia pseudomallei isolates derived from PNG from either clinical samples or the environment were identified with a panel of simple phenotypic tests based on previous descriptions (Ashdown, 1979b; Dance *et al.*, 1989c) (Chapter 3 and Chapter 4). Further definitive phenotypic and genotypic characterisation was required for three purposes:

1. Confirm the identification of the isolates implicated in the study;
2. Further categorise the arabinose assimilating isolates isolated from the environment;
3. Provide an identification bench mark by which this simple panel of tests could be compared and assessed.

Isolating the organism from a clinical sample is the diagnostic gold standard for melioidosis (Kanaphun *et al.*, 1993). The cultivation of *B. pseudomallei* from clinical sites is relatively trouble free particularly with the use of selective and differential primary agar used by experienced bacteriologists (Ashdown, 1979c; Howard and Inglis, 2003). Heightened clinical suspicion in endemic regions and use of these media with identification systems that are standard in most laboratories usually provide a correct identification. However, in regions where the disease is non-endemic but imported, or in regions without a sophisticated laboratory service or clinical staff familiar with melioidosis, standard methods may fail (Inglis *et al.*, 1998a; Lowe *et al.*, 2002; Koh *et al.*, 2003).

The recent characterisation of an initially described biotype of *B. pseudomallei* (Wuthiekanun *et al.*, 1996) which was found to be avirulent in animal models, has generated much interest. The organism has been described as *B. thailandensis* (Brett *et al.*, 1998). It has been implicated in immune cross reaction with *B. pseudomallei* in melioidosis endemic regions, which as well as confusing sero-epidemiology results, may also modulate infection with *B. pseudomallei* (Parry *et al.*, 1999; Vuddhakul *et al.*, 1999; Currie *et al.*, 2000e). The prevalence of *B. pseudomallei* in the

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environment, compared to *B. thailandensis*, seems to corelate to the prevalence of clinical melioidosis. Also, the organism has been used in the unravelling of virulence mechanisms of *B. pseudomallei* through subtractive hydridisation and other techniques (Chaiyaroj *et al.*, 1999; Brown and Beacham, 2000; Brown *et al.*, 2000; DeShazer *et al.*, 2001; Reckseidler *et al.*, 2001; Rainbow *et al.*, 2002; Woods *et al.*, 2002; Parry *et al.*, 1999; Vuddhakul *et al.*, 1999). Clearly, further characterisation and study of the PNG derived arabinose utilising *Burkholderia spp* isolate may contribute to these endeavours.

5.2   Materials and Methods

5.2.1   Comparison of in-field identification panel and confirmation tests

All viable isolates from the clinical (Chapter 3) and environmental (Chapter 4) screening studies transported to and stored at Townsville (School of Biomedical Sciences, James Cook University), were included. Isolates were originally classified in the field as *B. pseudomallei* based on the outcome of simple phenotypic tests including, colonial morphology on ASH and presumptive gentamicin resistance, cellular morphology (Gram-negative, small bipolar rods) and oxidase reaction, as previously described (Chapter 3) As the ASHEB was prepared with 50 mg/l colistin, presumptive colistin resistance of the environmental isolates was also assumed. The outcome of these tests and thus initial field identification was compared to more detailed phenotypic and genotypic testing.

5.2.2   In-field identification criteria

5.2.2.1 Colonial morphology, gentamicin and colistin resistance

Primary selective and differential agar of Ashdown was used with 8 mg/l gentamicin (Ashdown, 1979c). Mauve coloured colonies (combination of crystal violet plus neutral red) were considered suspicious as was any colony demonstrating typical rugose colonial morphology. When the presence of the typical odour (as determined

through routine manipulation of agar plates) suggested *B. pseudomallei*, suspicion was heightened.

5.2.2.2 Oxidase reaction

The oxidase test was undertaken as per method of Kovac and presented in Appendix 1. *Pseudomonas aeruginosa* (ATCC 27853) was used as a positive control and *Escherichia coli* (ATCC 25922) was used as a negative control.

5.2.3   Confirmation tests

5.2.3.1 API 20NE

Commercial phenotypic identification profiles were determined by the API 20NE (bioMerieux, Baukham Hills, NSW) as used as per manufacturer's instructions. Profile numbers were analysis by ApiLab Plus version 3.3.3 (bioMerieux, Baukham Hills, NSW).

5.2.3.2 Single carbohydrate utilisation agar

Both L-arabinose and glucose single carbohydrate agar were prepared following the methods of Wuthiekanun *et al.* (Wuthiekanun *et al.*, 1996). Briefly, a 2% carbohydrate agar was prepared with a minimal salt solution (Appendix 1). The agar and minimal salt solution was dissolved and sterilised. The carbohydrate was filter sterilised through a 0.22 μm pore filter (Sarstedt, Inglefarm, SA) and added to the agar when it had cooled to 56°C. A 0.5 MacFarland suspension of each organism to be tested was prepared from a signal colony grown on 48 hr incubated ASH. The agar plates were spotted with 3μl of the suspension. All plates were incubated in air at 35°C and reported after overnight incubation. Growth was compared to relevant controls for interpretation (see Figure 5.02 for a representation). The glucose plates were also used as a control for the viability of the suspension. *Burkholderia thailandensis* was kindly supplied by Dr Robert Norton of QHPSS, The Townsville

Hospital and used to control for arabinose assimilation. *Burkholderia pseudomallei* NCTC 13178, was used as a negative control.

5.2.3.3 Amoxicillin + clavulanate susceptibility

All isolates were tested for *in vitro* susceptibility to amoxicillin + clavulanate (Augmentin) with the disk diffusion method using a 30 µg disc (Oxoid Australia, Heidelberg, Vic). This was undertaken to determine if *in vitro* sensitive and resistance patterns could be used for diagnostic purposes. *Burkholderia pseudomallei* has been reported as susceptible to Augmentin and *B. cepacia* seems inherently resistant (Suputtamongkol *et al.*, 1991; Suputtamongkol *et al.*, 1994b; Hoban *et al.*, 2003). This pattern, if reproduced *in vitro* may aid developing countries differentiate *B. pseudomallei* from *B. cepacia.*

5.2.3.4 Polymerase chain reaction method

Polymerase chain reaction method protocols which amplified different specific products of *B. pseudomallei* (Winstanley and Hart, 2000) and both *B. pseudomallei* and *B. thailandensis* (Dharakul *et al.*, 1999b) were used to confirm the phenotypic identification. The multiplex PCR described by Dharakul *et al.* discriminates between *B. pseudomallei* (405bp and a 243bp product) and *B. thailandensis* (243bp product only). The type III secretion gene-based PCR of Winstanley and Hart discriminates only *B. pseudomallei* (548 kb product).The published primers were used but further optimisation was conducted and included DNA extraction, annealing temperature and magnesium concentration. Further, a specificity study was carried out on a selection of organisms either related clinically or taxonomically to *B. pseudomallei* (Table 5.05).

5.2.3.5 DNA extraction methods

Two methods of DNA extraction were tested: one a full standard bacterial DNA extraction, using phenol chloroform, hexadecyltrimethylammonium bromide (CTAB)

and ethanol precipitation (Ausubel *et al.*, 1995). The other was based on the method applied by Dharakul *et al* (Dharakul *et al.*, 1996) to blood samples and bacterial colonies and used with the multiplex PCR to differentiate arabinose positive and negative isolates (Dharakul *et al.*, 1999b).

Extraction methods were based on the protocols of Ausubel *et al.* (Ausubel *et al.*, 1995).The full extraction method involved taking a single colony grown on ASH overnight and resuspended in digestion buffer (Appendix 2) and allowed to digest over 1-2 hr at 56°C with intermittent agitation. When the suspension had cleared, digestion was complete. Polysaccharides were removed by adding 80 µl of 5 M NaCl and mixing, followed by 0.7 M NaCl/10% CTAB and mixing. This mixture was then incubated for 10 min at 65°C. Precipitated polysaccharides were removed by the addition of an equal volume of chloroform:isoamyl alcohol (24:1) with vigorous mixing so an emulsion formed and then the phases were separated by centrifugation at 12,000 *g* for 5 min. The upper aqueous layer was removed and then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) in the same way as the chloroform extraction of polysaccharides. The phenol/chloroform extraction was then followed with another chloroform extraction.

The nucleic acids (NA) were then precipitated with 0.6 ml of isopropanol alcohol by incubating for 10 min on ice and then centrifuging for 30 min at 12,000 *g* at 4°C. The pellet of NA was then washed twice with 70% ethanol by adding 1 ml of ethanol and then vortexing before centrifuging at 12,000 *g* for 5 min each. Ethanol was removed from the pellet with an extended Pasteur pipette. The pellet was then allowed to air dry at room temperature for 10 min and then redissolved in 45 µl of 10 mM TE buffer (Appendix 2).

The quick extraction method consisted of taking a colony from ASH and resuspended in digestion buffer (Appendix 2). The suspension was then incubated for 2 hr at 56°C and then the proteinase K (PK) was inactivated at 95°C for 10 min.

5.3 Results

Clinical screening and environmental sampling for *B. pseudomallei* during the study enabled the viable collection and subsequent storage of 10 clinical isolates, from six patients, (Chapter 4) and 30 isolates that were derived from the environment (Chapter 5).Where multiple isolates from the same clinical case are presented, they differed in either sample type origin or time of isolation or represent a distinct and reproducible colonial morphotype which resulted from the culture of one clinical sample. Of the 30 isolates derived from the environment, seven demonstrated the characteristics of typical *B. pseudomallei* (TBps) and 23 were identified as suspected *B. pseudomallei* (SBps). Of the 23 SBps isolates, 15 subsequently were identified as *B. thailandensis* by PCR. Whereas eight failed to be identified although they shared similar phenotypic characteristics to the other *Burkholderia spp*. Of this eight, five isolates were those detected from samples taken at Tapila.

5.3.1 Simple screening: initial "in-field" identification

Details of the clinical isolates' origins are presented in Table 5.01 and isolates derived from the environment are presented in Table 5.02 Isolates were classified as either TBps or SBps based on outcomes of the simple panel of tests described. Colonial morphology was a major determining factor and descriptions were based on past reports (Nigg *et al.*, 1956; Ashdown, 1979c; Wuthiekanun *et al.*, 1990; Walsh and Wuthiekanun, 1996). Isolates judged to be TBps consistently produced the classic colonial morphology of being sightly irregular in edge, rugose (increasingly so after extended incubation) and mauve in colour - or at least a combination of violet and red. Growth after 24 hr was obvious but not until 48 hr did these characteristics become conclusive (Figure 5.01). The earthy odour, although no longer an accepted identification criteria, was also obvious during routine manipulation. All clinical isolates which met the colonial morphological criteria met all other requirements of the identification criteria, including cellular morphology (Gram-negative bacilli), oxidase (positive), gentamicin resistance (assumed if growth on ASH) and vigorous growth on ASH after 48 hr. Of the 30 isolates derived from the environment, seven

met the requirements of TBps and the remaining 23 were classified as SBps (Table 5.03).

 The identification criteria for the SBps was mostly subjective but was made when all criteria could be met, but when colonial morphology was slightly more mucoid and glossy rather than rugose or crinkled (Figure 5.02) - this feature was consistent and did not revert after subculture or extended incubation (Dance *et al.*, 1989c).

Figure 5.01   TBps   Figure 5.02   SBps

Colonial variants were identified when particular characteristics appeared consistent upon repeat sub culture. This included C6, a small colony variant from case NG (Chapter 4) and C9 and C10 colour variants from case RI (Chapter 4). These were chosen for further investigation due to past reports of variants demonstrating clinically significant phenotypic divergence (Kishimoto and Eveland, 1975; Haussler *et al.*, 1999).

Table 5.01   Clinical isolates and identification based on simple on-field tests

Lab No	Case	Nature of Sample	Village	Initial ID
C1	GD	Sputum	Adiba	T.Bps
C2	Kawaki S	Blood	Kimama	T.Bps
C3	Kawaki S	Blood	Kimama	T.Bps
C4	DS	Pus Swab, abscess	Kimama	T.Bps
C5 (large)	NG (large)	Sputum	Balimo	T.Bps
C6 (small)	NG (small)	Sputum	Balimo	T.Bps
C7	TG	Throat Swab	Balimo	T.Bps
C8	TG	Gastric Aspirate	Balimo	T.Bps
C9 (red)	RI (red)	Pus Swab	Togowa	T.Bps
C10 (blue)	RI (blue)	Pus Swab	Togowa	T.Bps
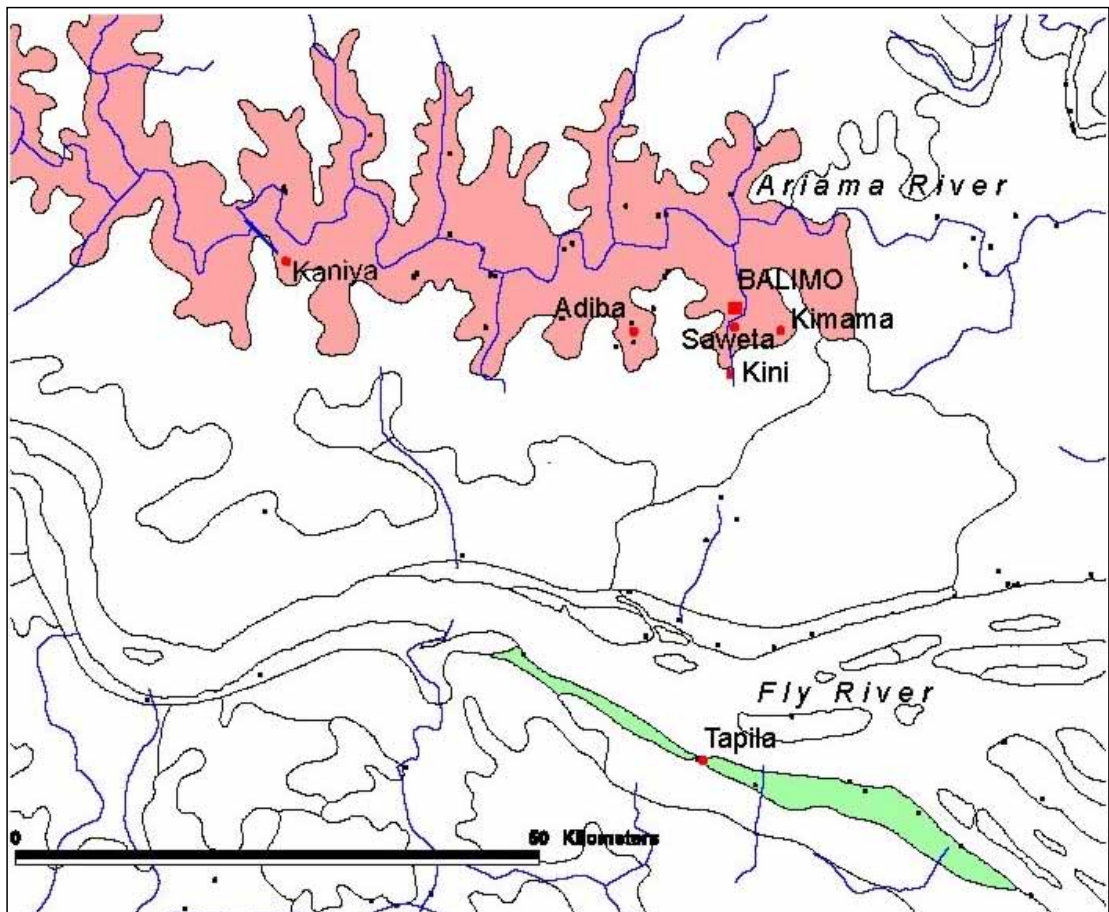
Figure 5.03   Location of sites within the study centre

Table 5.02   Environmental isolates and identification based on simple on-field tests

Lab No	Sample	Village	Initial ID
A67	Red clay	Adiba/Sanebase	TBps
A78	Brown top soil	Adiba/Sanebase	TBps
K33	Brown clay mix	Kimama	TBps
K41	Grey sand loose	Kimama	TBps
K93	Brown loose	Kimama	TBps
K113	Brown clay mix	Kimama	TBps
K141	Red clay mix	Kimama	TBps
E1	Clay	Balimo	SBps
E2	Clay	Balimo	SBps
E3	Clay	Balimo	SBps
E4	Clay	Balimo	SBps
A4	Sandy clay	Adiba/Sanebase	SBps
A9	Garden/clay 30cm	Adiba/Sanebase	SBps
A16	clay wall	Adiba/Sanebase	SBps
A19	Clay wall	Adiba/Sanebase	SBps
A20	Clay wall	Adiba/Sanebase	SBps
A21	Inside s/p garden	Adiba/Sanebase	SBps
A22	Inside s/p garden	Adiba/Sanebase	SBps
A23	Layered clay	Adiba/Sanebase	SBps
A24	Layered clay	Adiba/Sanebase	SBps
A30	Coloured clay	Adiba/Sanebase	SBps
A36	Clay	Adiba/Sanebase	SBps
A37	Clay	Adiba/Sanebase	SBps
K5	Clay/from above	Kimama	SBps
K31	Clay mix	Kimama	SBps
F10	Tilled surface soil	Tapila	SBps
F13	Tilled Surface Soil.	Tapila	SBps
F63	Surface.	Tapila	SBps
F78	Surface.	Tapila	SBps
F8	Surfaces 2.5-10cm	Tapila	SBps

5.3.2 Confirmation tests

5.3.2.1 API 20NE and arabinose assimilation

The API 20NE (bioMerieux, Baulkham Hills, NSW) identification was consistently more accurate when read after 48 hr. Of the 17 isolates identified as TBps with the simple tests scheme all achieved an identification probability with this system of between 98.3 - 99.9% (Table 5.03 and 5.04). These results represent four analytical profile numbers: 1156576 (99.9%, 6/17); 1556576 (99.8%, 7/17); 1556577 (99.8%, 3/17); 1556574 (98.3%, 1/17).

The analysis of isolates identified as SBps resulted in mixed analytical profiles, none of which suggested the isolates were *B. pseudomallei*. The most common identity was *B. cepacia* and occasionally *Pseudomonas fluoresence* (Table 5.04), although on only six occasions a probability of correct identification of >90% was achieved, demonstrating that the organisms are unlikely to be represented in the system's database.

Arabinose utilisation using single carbohydate utilisation and the API correlated perfectly with the TBps (all negative) and SBps (all positive) (Table 5.03). The viability of all isolate suspensions was confirmed with the use of glucose single carbohydrate agar, spots were cultured on this media in parallel with the arabinose single carbohydrate agar and all grew (Table 5.03). Representative plates of arabinose utilisation are included (Figure 5.04).

Table 5.03   Clinical isolates API20NE and arabinose assimilation

Lab No			API20NE	Profile No	API Interpretation	
	ARA	Gluc	24 hr	48 hr	24 hr	48 hr
C1	-	+	1156576	1156576	*B.ps 99.9%*	*B.ps 99.9%*
C2	-	+	1556576	1556576	*B.ps 99.8%*	*B.ps 99.8%*
C3	-	+	1156576	1156576	*B.ps 99.9%*	*B.ps 99.9%*
C4	-	+	1154574	1556576	*Ps.aerug 99.5%*	*B.ps 99.8%*
C5	-	+	1156576	1156576	*B.ps 99.9%*	*B.ps 99.9%*
C6	-	+	1054576	1156576	*Ps.aerug 59.1%*	*B.ps 99.9%*
C7	-	+	1356576	1156576	*B.ps 98.4%*	*B.ps 99.9%*
C8	-	+	1156576	1556577	*B.ps 99.9%*	*B.ps 99.8%*
C9	-	+	1156576	1556577	*B.ps 99.9%*	*B.ps 99.8%*
C10	-	+	1156576	1556577	*B.ps 99.9%*	*B.ps 99.8%*
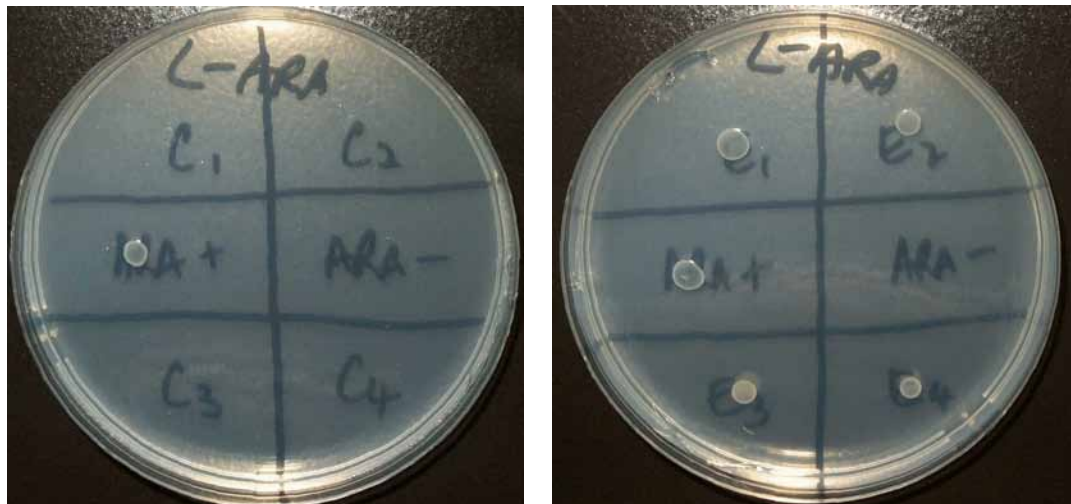
Figure 5.04   Arabinose and assimilation tests C1-C4, neg and E1-E4, pos

Table 5.04   Environmental isolates API20NE and arabinose assimilation

Lab No	ARA	Glu	24 hr	48 hr	24 hr	48 hr
A67	-	+	1156576	1556576	*B.ps 99.9%*	*B.ps 99.8%*
A78	-	+	1056576	1556576	*B.ps 81.7%*	*B.ps 99.8%*
K33	-	+	1156574	1556574	*B.ps 92.5%*	*B.ps 98.3%*
K41	-	+	1156576	1556576	*B.ps 99.9%*	*B.ps 99.8%*
K93	-	+	1156576	1556576	*B.ps 99.9%*	*B.ps 99.8%*
K113	-	+	1156576	1556576	*B.ps 99.9%*	*B.ps 99.8%*
K141	-	+	1056576	1156576	*B.ps 81.7%*	*B.ps 99.9%*
E1	+	+	1357574	1557577	*Ps. fluor <70%*	*B.cep 99.9%*
E2	+	+	1057574	1557575	*Ps. fluor 71.7%*	*Ps. fluor 84.3%*
E3	+	+	1257574	1557577	*Ps. fluor <70%*	*B.cep 74.6%*
E4	+	+	1057574	1557577	*Ps. fluor 71.7%*	*B.cep 74.6%*
A4	+	+	1457777	1757777	*B.cep 99.9%*	*B.cep <70%*
A9	+	+	1757577	1757777	*B.cep 93.1%*	*B.cep <70%*
A16	+	+	1456577	1557777	*B.cep 77.5%*	*B. cep 93.1%*
A19	+	+	1056577	1157777	*B.ps 81.7%*	*B.cep 66.7%*
A20	+	+	1257577	1757777	*B.cep 98.9%*	*B.cep <70%*
A21	+	+	1257577	1757777	*B.cep 98.9%*	*B.cep <70%*
A22	+	+	1057576	1657776	*B.cep 98.8%*	*B.cep <70%*
A23	+	+	1657577	1757777	*B.cep 99.9%*	*B.cep <70%*
A24	+	+	1656577	1757777	*B.cep 97.3%*	*B.cep <70%*
A30	+	+	1656577	1757777	*B.cep 97.3%*	*B.cep <70%*
A36	+	+	1756577	1757777	*B.ps 98.4%*	*B.cep <70%*
A37	+	+	1654577	1757777	*Ps.aerug 70.2%*	*B.cep <70%*
K5	+	+	1654557	1757577	*Ps.aerug <70%*	*B.cep 93.1%*
K31	+	+	1754577	1757777	*Ps.aerug 91.4%*	*B.cep <70%*
F10	+	+	46777	57777	*B. cep 99.0%*	*B.cep 99.7%*
F13	+	+	1066777	1067777	*B. cep 94.9%*	*B.cep 99.8%*
F63	+	+	56777	157777	*B. cep 99.6%*	*B.cep 56.6%*
F78	+	+	46777	57777	*B. cep 99.0%*	*B. cep 99.7%*
F8	+	+	56577	57777	*B. cep 95.1%*	*B. cep 99.7%*
NCTC 13178						
B. pseudomallei	-	+		1157574		*B. ps 92.5%*
B. thailandensis	+	+		1057554		*Ps. fluor 99.8%*
ATCC 27853						
Ps. aeruginosa	-	'+		1354575		*Ps. aerug 99.9%*

5.3.2.2 Polymerase chain reaction

The PCR conditions for both the 16S RNA gene and TTS gene protocols were optimised for annealing temperature and magnesium concentration using a gradient thermal cycler. Temperatures in the gradient used ranged from 50°C to 70°C. The optimum was 60°C for both protocols. A magnesium concentration of 2 mM was chosen for the 16S rRNA PCR as it resulted in two bands of equal intensity for the arabinose negative controls, while a concentration of 1.5 mM resulted in optimum intensity while maximising specificity for the TTS PCR.

The method of quick DNA extraction failed to produce DNA of reproducibly high quality so the full extraction method was chosen.

A collection of bacteria, mostly wild isolates, from the organism collection stored at the School of Biomedical Science, James Cook University, was selected for the specificity trial. The list and results are presented in Table 5.05. The 16S PCR analysis of several bacteria, using the optimised conditions, resulted in non-specific PCR products, while the TTS PCR was entirely specific for TBps. In most cases the non-specific 16S RNA products were clearly distinguished from the 243 and 405 bp size products of the *B. pseudomallei* isolates. However, analysis of the *P. fluorescens* isolate demonstrated a band only slightly smaller than the 243 bp product. Reducing the magnesium concentration to 1.5 mM eliminated all non-specific products except the *P. fluorescens* but this concentration also reduced the intensity of the specific 405 bp product. Therefore, it was decided that 2 mM magnesium should be used for unknown PNG isolates and unusual bands of similar size sequenced if required.

Table 5.05   Results of PCR systems specificity study

Microorganism	16S 243 bp	16S 405 bp	Other 16S product	TTS 548 bp
Proteus vulgaris	-	-	-	-
Enterococcush faecalis	-	-	-	-
Serratia marcescens	-	-	>500	-
Enterobacter aerogenes	-	-	-	-
Klebsiella pnemoniae	-	-	-	-
Acinetobacter spp			300	-
Pseudomonas fluorescens	-	-	240*	-
Burkholderia cepacia	-	-	-	-
Aeromonas hydrophilia	-	-	-	-
Streptococcus epui	-	-	-	-
Vibrio cholera (non 01)	-	-	-	-
E. coli ATCC 25972	-	-	-	-
Burkholderia thailandensis	-	+	-	-
Pseudomonas aeruginosa	-	-	180 / 300	-
Staphylococcus aureus	-	-	-	-
Rhodococcus spp	-	-	-	-
Streptococcus agalacticae	-	-	-	-
Bacillus spp	-	-	-	-
Clostridium perfringens	-	-	-	-
Xanthomonas spp	-	-	-	-
Streptococcus pyogenes	-	-	-	-
NCTC 13178	+	+	-	+

* lower intensity that the 243bp band

The analysis of all isolates identified as TBps resulted in the PCR products consistent with *B. pseudomallei* (Table 5.06). None of the 30 isolates identified as SBps after analysis with either PCR protocols demonstrated products consistent with *B. pseudomallei*. Of these isolates, 15 demonstrated products consistent with *B. thailandensis* and seven demonstrated either no products or products inconsistent with either *B. pseudomallei* or *B. thailandensis*. Figure 5.05 and Figure 5.06 are representative of the results and the product size interpretation. Isolates LD39 and K38 are included on the gels but are not included in the study because, although they garnered suspicion initially, they failed to produce consistent growth and colonial morphological characteristics on ASH and were therefore excluded as not fulfilling the criteria of the simple panel of tests. The isolates obtained from Tapila were

analysed at a different time and do not feature on the gels presented here. Results from the subsequent run are included in Table 5.05.

5.3.2.3 Amoxicillin + clavulanate susceptibility

Burkholderia pseudomallei is has not been validated for use with the NCCLS disk diffusion system (NCCLS, 2004). Regardless, all clinical isolates (TBps) except C10 produced a measurable zone of inhibition similar in dimension to that expected from a "sensitive", e.g. acceptable range for *E. coli* ATCC 25922 (19 - 25 mm). Interestingly, isolate C10 is a colonial variant of C9, isolated from case RI. All environmentally derived TBps produced a susceptible range (based on the *E.coli* criterion) , whereas all SBps except F10 produced zone diameters less than 19 mm, the analysis of most SBps isolates demonstrating no measurable zone (Table 5.06).

Table 5.06   Clinical isolates PCR and AMC30 *in vitro* susceptibility results

Lab No	AMC30 zone diam (mm)	16S 405 bp	16S 243 bp	TTS 548 bp
C1	30	+	+	+
C2	23	+	+	+
C3	25	+	+	+
C4	24	+	+	+
C5	20	+	+	+
C6	20	+	+	+
C7	22	+	+	+
C8	26	+	+	+
C9	26	+	+	+
C10	0	+	+	+

Table 5.07　　　Environmental isolates PCR and AMC30 *in vitro* susceptibility results

Lab No	AMC30 zone diam (mm)	16S 405 bp	16S 243 bp	TTS 548 bp
A67	24	+	+	+
A78	24	+	+	+
K33	24	+	+	+
K41	20	+	+	+
K93	25	+	+	+
K113	26	+	+	+
K141	25	+	+	+
E1	11	-	+	-
E2	11	-	+	-
E3	9	-	+	-
E4	12	-	+	-
A4	0	-	+	-
A9	0	-	+	-
A16	0	-	+	-
A19	0	-	+	-
A20	0	-	+	-
A21	0	-	+	-
A22	0	-	+	-
A23	0	-	+	-
A24	0	-	+	-
A30	0	-	+	-
A36	0	-	+	-
A37	0	-	-	-
K5	0	-	-	-
K31	0	-	-	-
F10	35	-	-	-
F13	0	-	-	-
F63	0	-	-	-
F78	0	-	-	-
F8	0	-	-	-
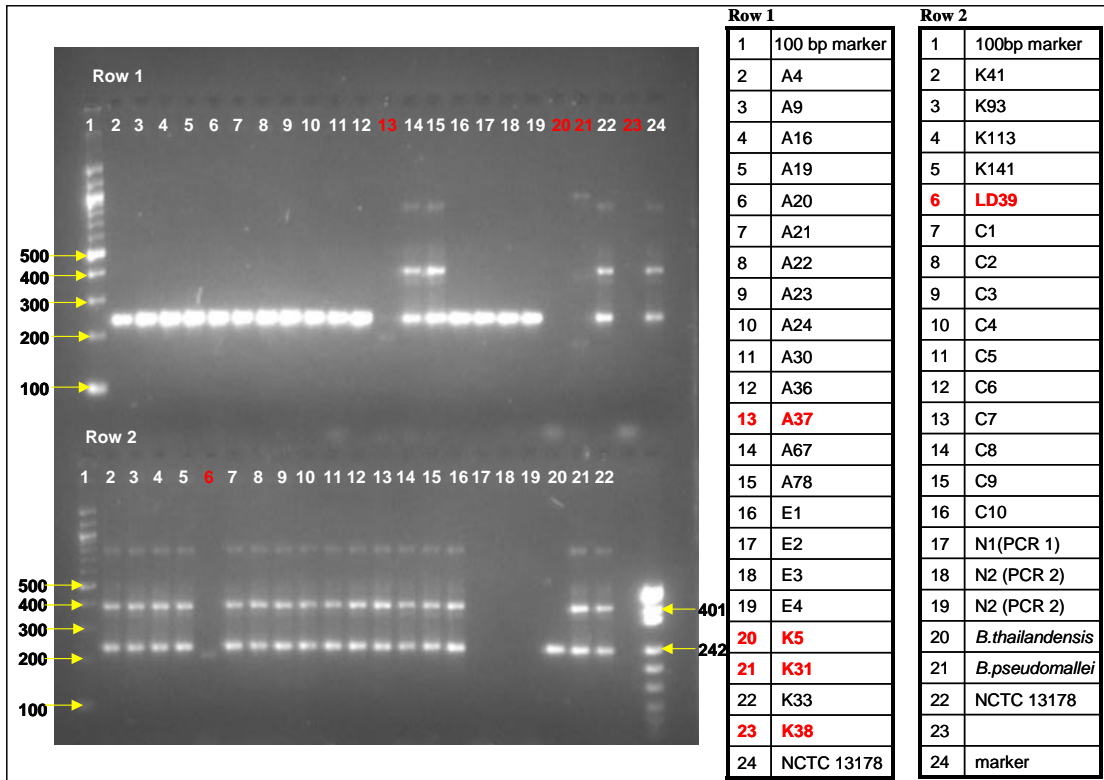
Figure 5.05   16S PCR representative gel

Row 1			Row 2	
1	100 bp marker		1	100bp marker
2	A4		2	K41
3	A9		3	K93
4	A16		4	K113
5	A19		5	K141
6	A20		6	LD39
7	A21		7	C1
8	A22		8	C2
9	A23		9	C3
10	A24		10	C4
11	A30		11	C5
12	A36		12	C6
13	A37		13	C7
14	A67		14	C8
15	A78		15	C9
16	E1		16	C10
17	E2		17	N1(PCR 1)
18	E3		18	N2 (PCR 2)
19	E4		19	N2 (PCR 2)
20	K5		20	*B.thailandensis*
21	K31		21	*B.pseudomallei*
22	K33		22	NCTC 13178
23	K38		23	
24	NCTC 13178		24	marker
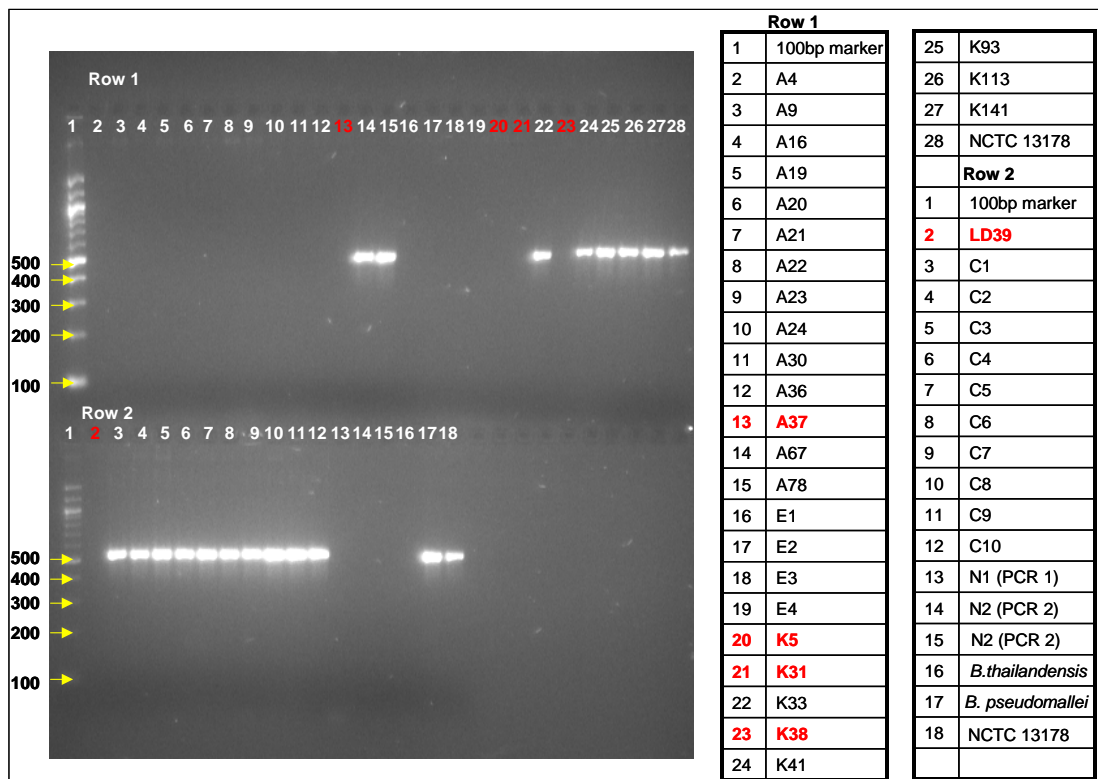
Figure 5.06   TTS PCR representative gel

Row 1				
1	100bp marker		25	K93
2	A4		26	K113
3	A9		27	K141
4	A16		28	NCTC 13178
5	A19			Row 2
6	A20		1	100bp marker
7	A21		2	LD39
8	A22		3	C1
9	A23		4	C2
10	A24		5	C3
11	A30		6	C4
12	A36		7	C5
13	A37		8	C6
14	A67		9	C7
15	A78		10	C8
16	E1		11	C9
17	E2		12	C10
18	E3		13	N1 (PCR 1)
19	E4		14	N2 (PCR 2)
20	K5		15	N2 (PCR 2)
21	K31		16	*B.thailandensis*
22	K33		17	*B. pseudomallei*
23	K38		18	NCTC 13178
24	K41			

5.4   Discussion

Melioidosis in PNG appears to be a disease of rural communities. The number of cases uncovered in the Balimo region of the Western province exceeds the total number of reported cases from elsewhere in PNG (Currie, 1993). To enhance the sustainability of local diagnostic regimens in rural health services, it is imperative that cost effective technologies are used. To this end, in the provision of a sustainable melioidosis diagnostic centre within a rural community hospital in PNG it was decided to establish a simple set of diagnostic tests that could be maintained with minimal external input (Dance *et al.*, 1989c). This was tested against phenotypic and genotypic standards of *B. pseudomallei* identification and proved to be reliable in identifying *B. pseudomallei* from clinical samples and also from the environment. This clearly demonstrates that successful diagnosis of melioidosis in resource poor communities is possible without access to reference laboratories. It also highlights the association between a skilled laboratory workforce and positive patient outcomes, for without laboratory evidence, melioidosis may be mis-diagnosed.

All TBps isolates derived either from clinical samples or the environment were confirmed to be *B. pseudomallei* using the phenotypic criteria of the API20NE and genotypic criteria of two specific PCR systems. Although the number of isolates is small, this work corroborates studies of others working in developing world environments where simple identification tests are used confidently to identify *B. pseudomallei* (Dance *et al.*, 1989c; Wuthiekanun *et al.*, 1996). The application of these simple identification criteria against organisms derived from the environment further confirmed the ability of these criteria, used by the staff, to recognise subtle characteristics that differentiate *B. pseudomallei* from other organisms in this region. No isolates initially identified as SBps were identified as *B. pseudomallei* using confirmatory tests. Further, 15 of the 23 SBps isolates were confirmed as *B. thailandensis* based on the PCR products of the 16SrRNA PCR (Dharakul *et al.*, 1999b). Isolates with these characteristics have previously never been reported from PNG and have not been isolated from Australia to date. The remaining eight of the

ARA+ isolates that were not identified by the PCR systems used, remain to be characterised fully.

Although there have been reports that mucoid strains of *B. pseudomallei* may go unrecognised (Howard and Inglis, 2003), in this small study the characteristics of a slightly mucoid, less rugose colonial appearance appears to have differentiated the PNG *B. thailandensis* from *B. pseudomallei*.

Of the total 23 SBps isolates, upon analysis eight failed to demonstrate PCR products that defined the organisms as either *B. pseudomallei* or *B. thailandensis*. During the analysis of these eight, five isolates achieved acceptable API20NE identification probability (>90%) for *B. cepacia* (K5, F10, F13,F78 and F8). However, the analysis of two isolates confirmed as *B. thailandensis* using PCR (E1 and A16) produced API outcomes similar to *B. cepacia*. This therefore suggests the API 20NE database may not be sufficiently developed to speciate non-clinically relevant *Burkholderia* spp. Given this commercial phenotypic identification system is for clinical use, it is reasonable that users should expect this limitation.

Overall, the API analytical profile numbers remained reasonably consistent with past reports (Dance *et al.*, 1989c), although an inability to utilise citrate seemed a notable exception. This could be explained by poor readability and/or, as in this case, when the strip is incubated at >25°C (Dance *et al.*, 1989c; Inglis *et al.*, 1998a). Regardless, the system was reliable when used as per manufacturer's instructions and could be used to confirm isolate identity in this setting if required. However, the perfect correlation of API identification outcomes and those achieved from the simple panel of tests, indicates that it is not necessary to rely on this expensive method for routine identification in rural settings.

The National Committee for Clinical Laboratory Standards (NCCLS) performance standards does not list zone diameter interpretive standards for the disk diffusion method of antimicrobial sensitivity testing of *B. pseudomallei* (NCCLS, 2004). As this is the preferred method for susceptibility testing in developing countries, it is

tempting to use it regardless of calibration to a MIC but this application may pose problems (Lumbiganon *et al.*, 2000). Regardless, the assay may be used as a diagnostic tool. The *in vitro* susceptibility of isolates against Augmentin proved a useful identification criterion which was able to further corroborate the simple diagnostic tests, particularly when *B. cepacia* is suspected. Upon analysis, all but one of TBps demonstrated zone diameters of >19 mm and all SBps confirmed by PCR to be *B. thailandensis* demonstrated a zone diameter of <19 mm. Most (11/15) produced no zone. Interestingly, the clinical isolate that when tested demonstrated *in vitro* resistance is a colonial variant of C9. This is consistent with past reports of antimicrobial resistance related to colonial morphology (Dance *et al.*, 1989c; Haussler *et al.*, 1999). In this case though, colour rather than size was the differentiating feature. The other colonial variants based on size (C5 and C6) did not demonstrate any differences with any tests used. Further antimicrobial susceptibility testing may be required.

Of the remaining eight isolates which were unclassified by PCR, only one produced a zone diameter of >19mm (F10) when tested against Augmentin. Interestingly, this isolate was tested 99.7% *B. cepacia* by the API20NE. Given *B. cepacia* is inherently resistant to Augmentin, this result may highlight inadequacies of the API20NE system in identifying the *Burkholderia*.

The PCR systems used in this study are already outdated, most notably in relation to template preparation where commercial DNA extraction kits are now routine, negating the complexities of CTAB extraction methods. But also in relation to the amount sequence data is available on the genome of *B. pseudomallei*. Since the primers of these assays were designed, sequencing of the *B. pseudomallei* genome has been completed by collaborators at the Sanger Centre (Holden *et al.*, 2004). With this detail and other reforms, more sophisticated and robust PCR systems can be developed which may aid in isolate identification and characterisation with sustainable clinical application (Yang and Rothman, 2004). Clearly real time PCR may have an application not only in a clinical setting but also as a tool for quantifying organism numbers from environmental samples (Filion *et al.*, 2003). It may be some

time before this technology will be applied to melioidosis diagnostics in rural regions where the disease is mostly endemic.

Regardless, the PCR systems in this study proved to be useful in confirming the identification of isolates thought to be *B. pseudomallei*. It provided a genotypic benchmark to which phenotypic identification systems could be compared. As correlation with identification using the simple algorithm was perfect, the PCR adds nothing to the routine identification of isolates in this setting and certainly does not warrant the costs and training required for implementation, although further optimisation for clinical use may have merit.

Further phylogenetic analysis to define the relatedness between Southeast Asian derived *B. thailandensis* (and indeed other *Burkholderia* spp) and this PNG strain will determine if a new species classification is warranted. If so, further study of this organism and its interaction with *B. pseudomallei* in this environment and in animal hosts could help expand knowledge of both *B. pseudomallei* ecology and pathogenesis (Reckseidler *et al.*, 2001; Kent and Triplett, 2002). Preliminary studies on virulence of the PNG *B. thailandensis* strains have demonstrated they are avirulent in a mouse model (Ulett *et al.*, 2001).

The role that *B. thailandensis* plays in mediating *B. pseudomallei* survival in an environmental habitat and/or infection in hosts remains unclear. It is clear that in regions where both *B. thailandensis* and *B. pseudomallei* are endemic the regions with higher ratio of *B. pseudomallei* are regions with the highest melioidosis endemicity (Trakulsomboon *et al.*, 1999; Vuddhakul *et al.*, 1999). Both organisms share common epitopes (Wuthiekanun *et al.*, 1996) and exposure to *B. thailandensis* may explain a high degree of protective cross-reactive immunity where melioidosis is endemic (Currie *et al.*, 2000e). However, the inability of *B. thailandensis* to establish infection sufficient to cause disease may preclude a detectable and indeed protective immune response regardless of cross-reactivity.

Perhaps more likely is that the presence of *B. thailandensis* in the environment antagonises *B. pseudomallei*. This has been known to be a feature of the proteobacteria and particularly *Pseudomonas* spp and *Burkholderia* spp of the gamma group and has been exploited in the agricultural industry (Kent and Triplett, 2002; Parret and De Mot, 2002; Xie *et al.*, 2003). If antagonism exists, it may do so to such an extent that numbers of *B. pseudomallei* sufficient to cause disease are prevented from maintaining a permanent, clinically relevant habitat.

The discovery of bacteriocins produced by clinically avirulent *Burkholderia* spp against *B. pseudomallei* with or without association with phage, may elucidate not only novel antimicrobial therapy for clinical use but also bioremediation (Cain *et al.*, 2000; Raaijmakers *et al.*, 2002; Bano and Musarrat, 2003) of land implicated with clinical melioidosis. Although problematic (van Veen *et al.*, 1997), environments with proven clinical melioidosis association could have their reservoir of infection reduced, through either probiotic antagonism or through direct bacteriocin application.

Regardless of this potential focus of future research, the simple tests highlighted here to characterise *B. pseudomallei* from implicated clinical samples and from the environment may be used in this setting with confidence.

CHAPTER 6 - THE MOLECULAR EPIDEMIOLOGY OF MELIOIDOSIS IN THE BALIMO REGION OF PAPUA NEW GUINEA
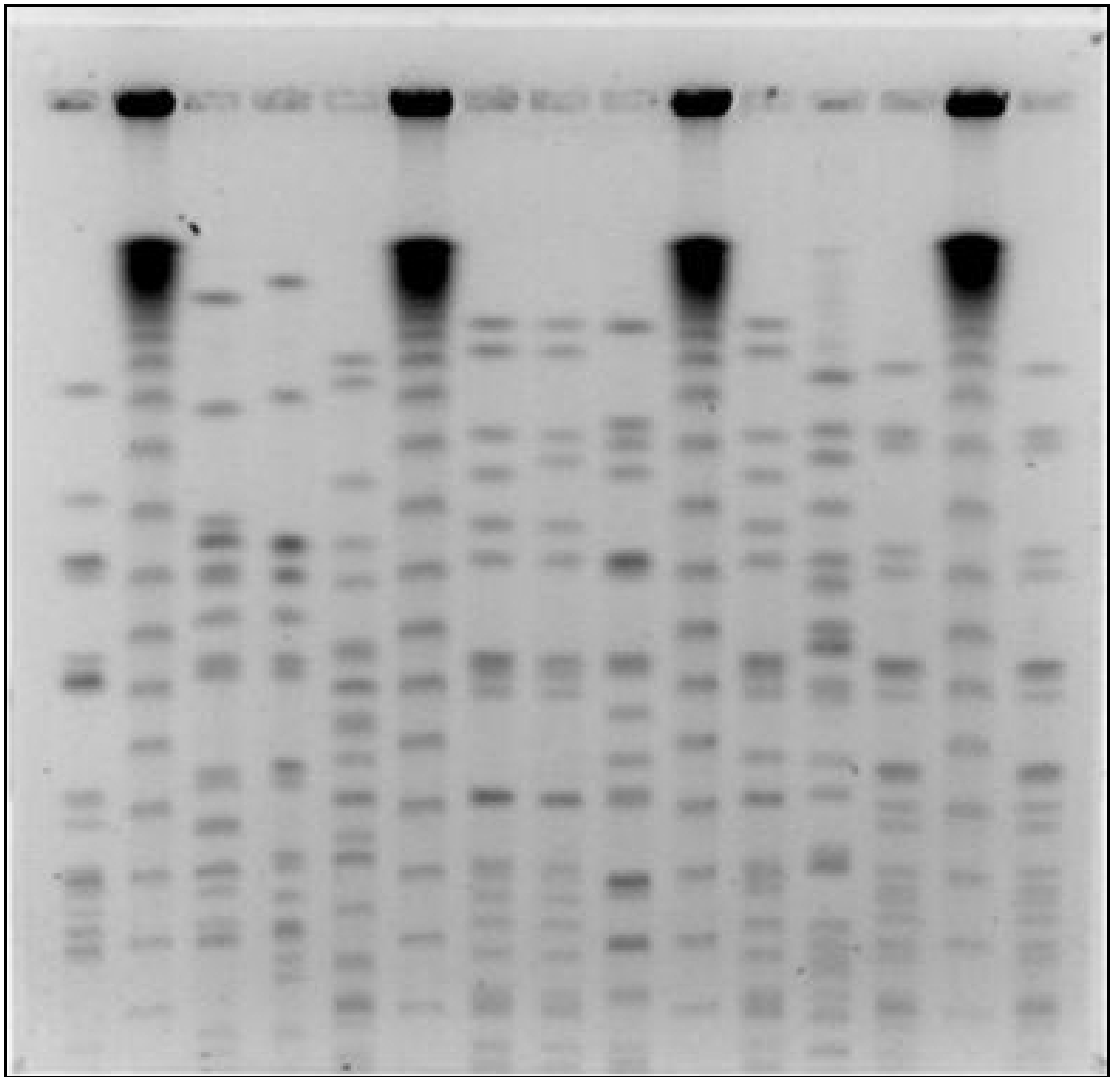
6.1   Introduction

Although melioidosis has been recognised for over 90 years, precise modes of transmission, other than isolated examples of iatrogenic transmission, remain mostly speculative (Markovitz, 1993). Compared to other sources of sepsis, a source of initial infection resulting in acute melioidosis is rarely discovered (Chaowagul *et al.*, 1989). Given *B. pseudomallei* is recognised as a saprophyte, comparison of genetic relatedness between clinical and environmental isolates aids in the quest for establishing a mode of transmission by identifying a source of infection (Currie *et al.*, 1994; Inglis *et al.*, 1999; Currie *et al.*, 2001). Supporting this, descriptive epidemiology often implicates environmental exposure with melioidosis (Merianos *et al.*, 1993; Suputtamongkol *et al.*, 1999).

Isolated cases of human to human and animal to human transmission have been reported, but most have not been substantiated by culture confirmation and molecular epidemiology and result from either unique issues of co-morbidity or social behaviour (McCormick *et al.*, 1975; Kunakorn *et al.*, 1991; Idris *et al.*, 1998; Abbink *et al.*, 2001; Holland *et al.*, 2002) which are uncommon in the general population.

Given this accumulated evidence, there is general agreement that subcutaneous inoculation, inhalation and perhaps ingestion from an environmental source are considered likely modes of transmission (Leelarasamee and Bovornkitti, 1989; Dance, 1991a; White, 2003).

An environmental reservoir of infection for melioidosis in the Balimo region of PNG has been demonstrated (Chapter 4). These isolates and those isolated from individuals have been characterised and confirmed to be *B. pseudomallei* (Chapter 5). Within this group of 17 isolates there exists three clinical/environmental epidemiological matches from three regions. All environmental strains of *B. pseudomallei* from these three regions have been isolated from areas frequented by the individuals and their families. These regions being either from points of land where the houses are built (as was the case at Digi and Sanebase points) or adjacent to the lagoon that individuals

either use for recreation, washing or as a thoroughfare from one point of land to another (Teleme).

Other isolates in the collection include sibling matches, colonial variants from a single sample and unrelated clinical cases.

 Through study of the genetic relatedness of the isolates from these cases and their environmental or clinical match, the mode of transmission of melioidosis may be elucidated. This knowledge linked to a proven reservoir, may enable the establishment of disease control strategies that may be used to reduce the risk of melioidosis in this region (Inglis *et al.*, 2000a; Na-ngam *et al.*, 2004).

6.2   Materials and Methods

6.2.1   Isolates and locations

All isolates implicated in the study as either *B. pseudomallei* or *B. thailandensis* were included. Of the *B. pseudomallei* isolates three clinical-environmental matches existed and are represented as colour matches in Table 6.01. The letter "C" used as a prefix denotes clinical isolates, the prefix "A" or "K" denotes an environmentally derived isolate from either Adiba or Kimama respectively. The isolates highlighted red are implicated with case GD at Sanebase pt (08° 03.927′S; 142° 52.549′E) and include C1, A67 and A78. The isolates highlighted blue are implicated with case KawS, and include C2, C3, K33 and K41 from Teleme, Kimama village (08°03.187′S; 143° 00.072′E). The isolates highlighted green are implicated with case DS, and include C4, K113 and K141 and are from Digi point, Kimama village (08° 03.058′ S; 143° 00.42′ E). Isolates C5 and C6 are variants from case NG from Balimo village (08° 02.493′S; 142° 57.484′E) and these match with sibling, case TG whose isolates C7 and C8 represent isolates cultured from different sites (throat and gastric aspirate respectively). Finally, isolates C9 and C10 are derived from case RI who resides at Togowa village, approximately 5 km south of Adiba village on the same lagoon system (GPS data not available).

Table 6.01   Isolates of *B. pseudomallei* implicated in the study

Lab No	Nature of Sample	Case/Location	Village
C1	Sputum	GD	Adiba
C2	Blood	Kawaki S	Kimama
C3	Blood	Kawaki S	Kimama
C4	Pus Swab, abscess	DS	Kimama
C5	Sputum	NG	Balimo
C6	Sputum	NG	Balimo
C7	Throat Swab	TG	Balimo
C8	Gastric Aspirate	TG	Balimo
C9	Pus Swab	RI	Togowa
C10	Pus Swab	RI	Togowa
A67	red clay	Sanebase Village	Adiba
A78	brown top soil	Sanebase Village	Adiba
K33	brown clay mix	Teleme	Kimama
K41	gray sand loose	Teleme	Kimama
K93	brown loose	Gawa Dama Pt	Kimama
K113	browm/mix	Digi	Kimama
K141	red/mix	Digi	Kimama

Table 6.02   PNG *B. thailandensis* isolates included for molecular typing

Lab No	Nature of Sample	Case/Location	Village
E1	clay - canoe place	Mission pt	Biula
E2	clay - canoe place	Mission pt	Biula
E3	clay - canoe place	Mission pt	Biula
E4	clay - canoe place	Mission pt	Biula
A4	Sandy clay	Washing well 1	Adiba/Sanebase
A9	garden/clay 30cm	Siniwa house	Adiba/Sanebase
A16	clay wall	Wiyawa house	Adiba/Sanebase
A19	clay wall	Wiyawa house	Adiba/Sanebase
A20	clay wall	Wiyawa house	Adiba/Sanebase
A21	inside s/p garden	Wiyawa house	Adiba/Sanebase
A22	inside s/p garden	Wiyawa house	Adiba/Sanebase
A23	layered clay	Wiyawa house	Adiba/Sanebase
A24	layered clay	Wiyawa house	Adiba/Sanebase
A30	coloured clay	Public wash	Adiba/Sanebase
A36	clay	Public drink	Adiba/Sanebase

6.2.2 DNA macro restriction analysis

DNA macro restriction analysis using pulse field gel electrophoresis of all PNG isolates was conducted in the laboratory of Prof. Bart Currie, Menzies School of Health Research, Darwin NT. Technical support was kindly provided by Mark Mayo and the gels presented here were kindly prepared by Daniel Gal according to published methodology (Gal *et al.*, 2004). Briefly, an overnight colony grown on chocolate agar was transferred to 20 ml of Todd Hewitt broth (Oxoid Australia, North Ryde, NSW) and incubated overnight with agitation. The suspension was centrifuged and washed and subsequently resuspended in 10 ml of PET IV (Appendix 3). To 1 ml of resuspended suspension 1 ml of molten 2% low melting point agarose (Appendix 3) was added and vortexed. Moulds (BioRad, Regents Park, NSW) were filled and left at RT to cool before being refrigerated at 4°C to solidify for 30 min.

Cell walls were lysed to reveal total genomic DNA after blocks were placed in 2 ml of lysis buffer (Appendix 3), with the addition of 1 mg/ml, of Proteinase K (PK). After overnight incubation at 37°C the blocks were washed several times in TE buffer (Appendix 3) and resuspend in this buffer and stored at 4°C until dialysis.

Degraded protein was cleared through dialysis in restriction enzyme buffer (Promega, Annandale, NSW) for 30 min. Buffer was changed and blocks were incubated with 10 U of *SpeI* (Promega, Annandale, NSW) overnight at 37°C to digest genomic DNA.

A 1.2% agarose gel in 100 ml of tris base, boric acid and EDTA (TBE) of appropriate size was made with a 15 well comb. Blocks were placed centrally in the wells ensuring no air between gel and the leading edge. A lambda 50 kb - 1 Mb ladder (Promega, Annandale, NSW) was added in appropriate wells. Each well was then filled with low melting point agarose. The CHEF-DR III (BioRad, Regents Park, NSW) was loaded with 2 l of 0.5× TBE and 100 µM of thiurea and precooled to 14°C for 30 min. The PFGE conditions included switching times: initial 10-30 sec for 5.8 hr; second 30 - 40 sec for 15.6; final 40 - 90 sec for 5.4 hr. Temperature was set at 14°C, time 24 hr, V/cm 6.0, and angle 120°.

Bands were stained with 0.5 µg/ml ethidium bromide in 400 ml TBE buffer for 1 hr. The gels were destained in fresh 0.5× TBE buffer for 30 min. Where required, the band divergence was assessed using pairwise comparison and differences analysed as per methods of Tenovor *et al.* (Tenover *et al.*, 1995).

6.3   Results

Of the 17 confirmed *B. pseudomallei* isolates implicated in the study, including the
three clinical / environmental matches, all shared the same macro restriction pattern
regardless of location and source of isolate (Figure 6.01 and Figure 6.02) . This type
consists of 17 bands ranging from approximately 50 - 1000 kb. Band 4
(approximately 460kb) and band 6 (approximately 350kb) are faint and are not clear
in some lanes, this seems to be a function of gel staining rather than a point mutation
resulting in the creation of a new restriction site as there is no corresponding smaller
oligonucleotide that should be present if this is the case (Tenover *et al.*, 1995). These
isolates can therefore be characterised as indistinguishable as per the categorisation of
Tenover *et al* (Tenover *et al.*, 1995) and therefore typically represent the same strain.
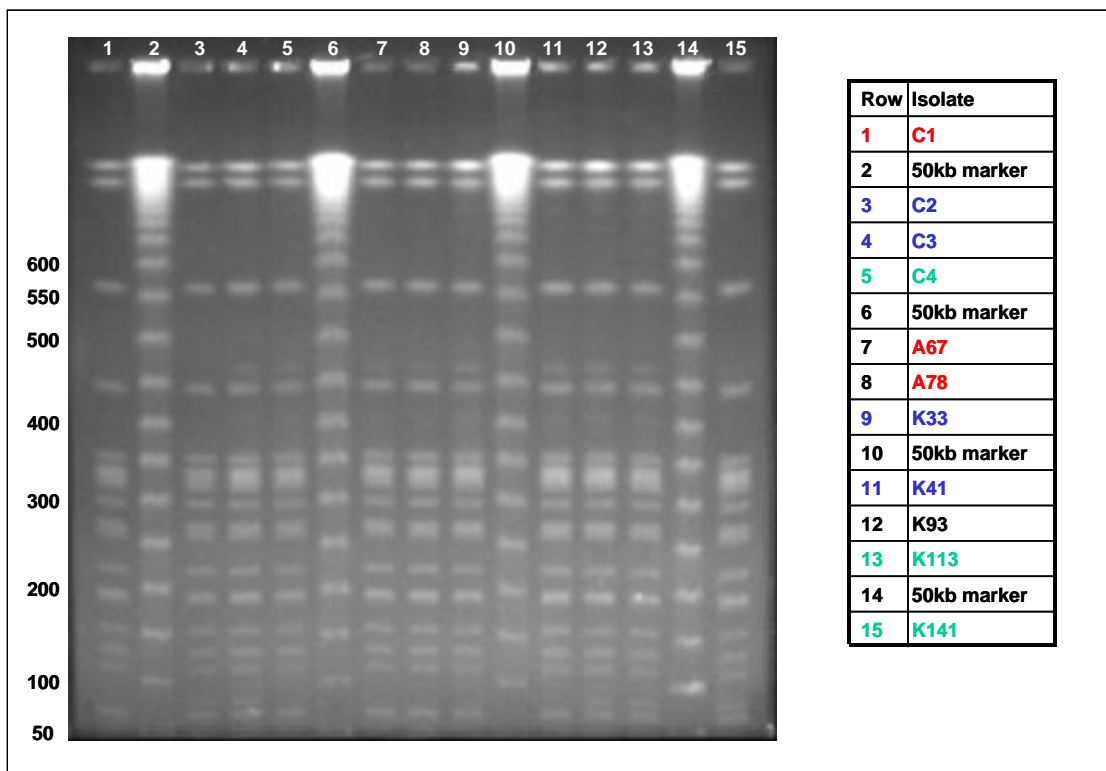
Figure 6.01   *SpeI* digest PFGE patterns of three epidemiologically associated
               B. pseudomallei isolate groups (red Adiba, blue Teleme Kimama
               village, green Digi pt Kimama village)

Row	Isolate
1	A37
2	50kb marker
3	C5
4	C6
5	C7
6	50kb marker
7	C8
8	C9
9	C10
10	50kb marker
11	E1
12	E2
13	E3
14	50kb marker
15	E4
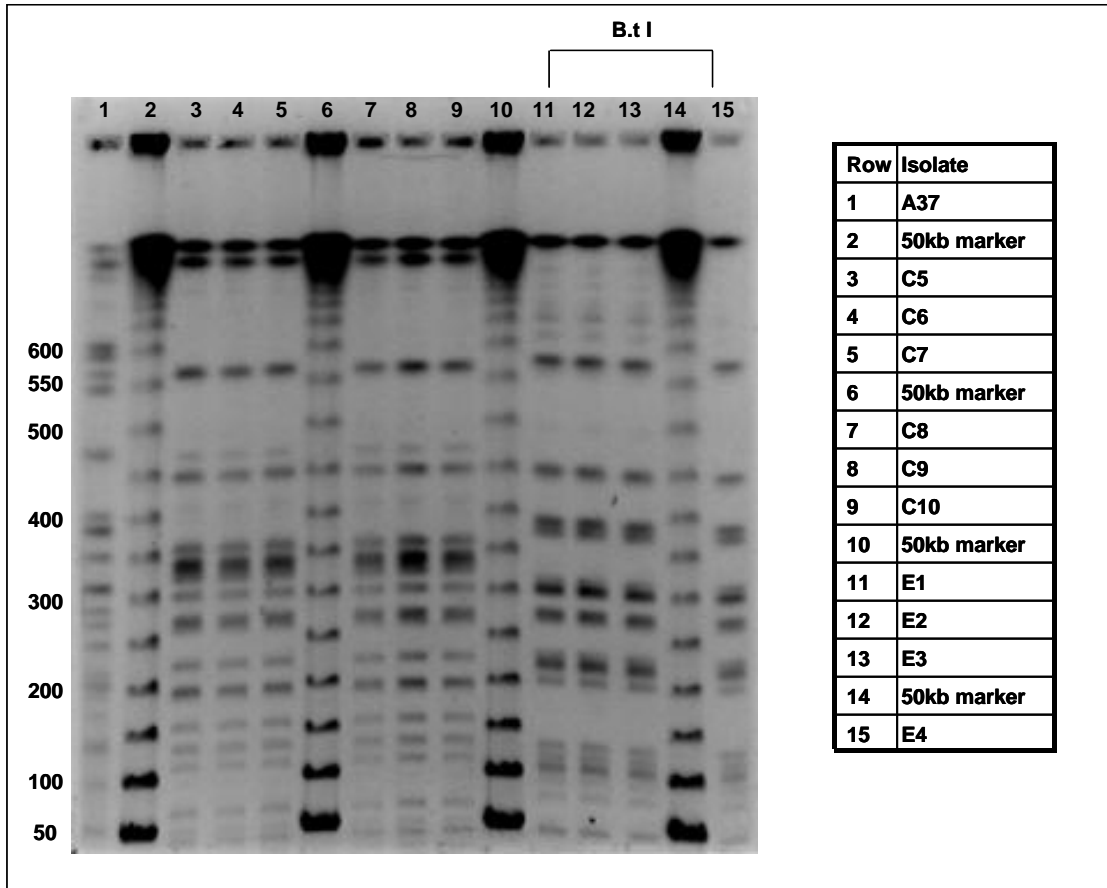
Figure 6.02   *SpeI* digest PFGE patterns demonstrating all clinical derived
B. pseudomallei (C prefix) with the same genotype as
epidemiologically unrelated clinical and environmental isolates shown
in Figure 6.01. Included in rows 11 - 15 are PNG *B. thailandesis*
genotype 1. Isolate A37 (row 1) uncharacterised (see chapter 5)

In contrast, upon macro restriction analysis of the 15 isolates that shared the

characteristics of *B. thailandensis*, 10 genotypes were demonstrated (Figure 6.02 and

Figure 6.03). The four isolates from Buila (E1 - E4) shared the sample type (I) , two

groups of two from Adiba shared two separate types (II and VI), but all others were

separate, with varying degrees of similarity.

143

Row	Isolate
1	A4
2	50kb marker
3	A9
4	A16
5	A19
6	50kb marker
7	A20
8	A21
9	A22
10	50kb marker
11	A23
12	A24
13	A30
14	50kb marker
15	A36
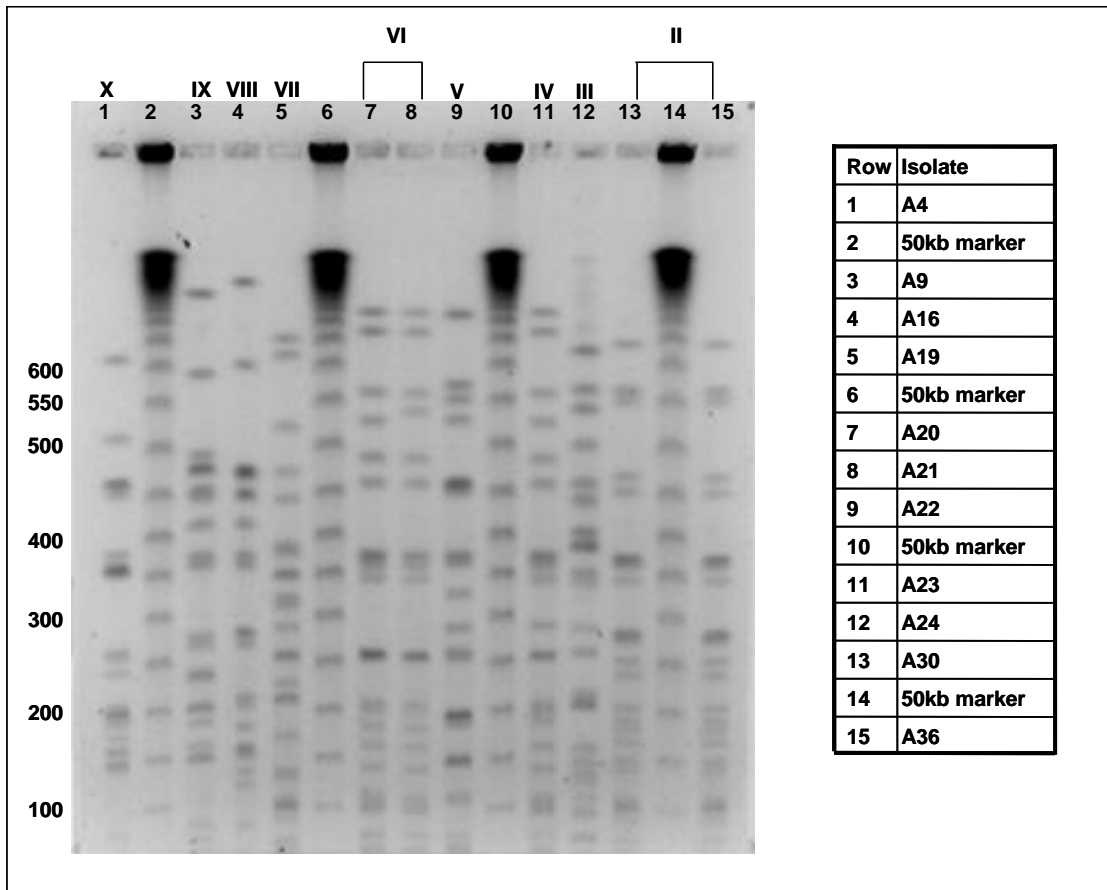
Figure 6.03   *SpeI* digest patterns of PNG *B. thailandensis* genotypes II - X

Also, the analysis of isolate A37 (Figure 6.02), an uncharacterised suspected *Burkholderia* spp, demonstrated a different pattern than any *B. thailandensis* or *B. pseudomallei* genotype.

6.4 Discussion

The demonstrated clonality between epidemiological matches of clinical and environmental isolates supports the general hypothesis that the organism responsible for melioidosis in this region resides in the soil which is closely associated with the lagoon system. Although, the general lack of genetic diversity across unrelated isolates raises other issues (see later).

Fundamentally, it is reasonable to assume that when houses are built at these sites, either during their construction or as a result of subsequent human activity in the immediate area, the organism is transmitted to a host and that if the host is susceptible to infection, melioidosis results. Although the actual mode of transmission still remains speculative, the fact that 80% of the melioidosis cases in Balimo are children with no apparent co-morbidity or predisposition (Chapter 3) suggests that something unique to children or children's behaviour might be involved.

Figure 6.04   House building at Sanebase pt Adiba village

Certainly two issues are striking and might be involved: children are more likely to wash (bathe) in groups, in the muddy waters of the lagoon adjacent to points of land found to be reservoirs of *B. pseudomallei*. Adults are more likely, in this community, to bathe alone in private places or wash on land with water fetched from wells. This washing activity of children mostly is robust play, and is likely to enable transmission of water borne organisms (either endogenous in origin or exogenous through contamination of rainfall runoff from adjacent land) through either pernasal or subcutaneous inoculation through preexisting lesions (see later) (Figure 6.05). Adults watch this activity in dry houses with amusement.

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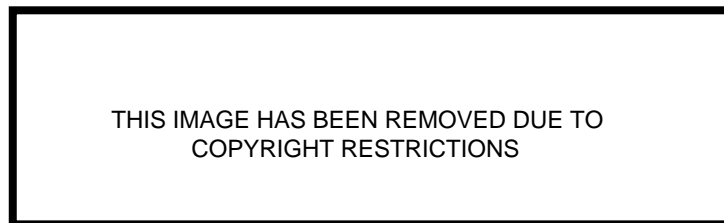
Figure 6.05   Children washing at Sanebase pt Adiba village

The other factor which becomes apparent observing children in this region (compared to adults) is the extent of undressed superficial lesions on children. Infected cuts and abrasions, are less apparent (undressed at least) in adults. This further substantiates the hypothesis that subcutaneous inoculation through preexisting lesions may play a

role. Although no individual diagnosed with melioidosis in this study had a history of *B. pseudomallei* infected wound.

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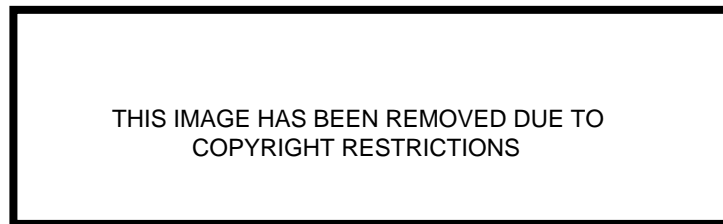
Figure 6.06   Children at Digi pt Kimama village

The role water plays in transmission or as a endogenous habitat *per se* remains speculative. Clearly, studies of water and the rhizosphere, phylosphere and gemnosphere of aquatic plants would demonstrate this more clearly. Regardless of the true endogenous reservoir, the extent and movement of water in this region as a vehicle of *B. pseudomallei* transmission throughout the region must be a factor. Similar to air currents transmitting fungal spores (Fenchel, 2003), water will disperse and lead *B. pseudomallei* to a environment where it may more permanently reside due to its encounter with favourable attributes. Clearly though, the sites selected for analysis here, are biased toward the clinically associated regions. The extent of the reservoir and therefore the ability to infer modes of transmission through molecular epidemiology, is limited to the extent of sampling in the region.

Regardless, Adiba and Kimama are villages on separate lagoon systems joined by the Aramia river approximately 10 km apart. The river, which seasonally floods, fills and partially empties these lagoon systems. Human movement is mostly though canoe travel and occasionally motorised dingy. Clearly the extent of dispersion potential is enormous. Hydrodynamic analysis of water movement with more detailed soil isolation data may help elucidate this by tracking currents from proven reservoirs to other regions and testing for *B. pseudomallei* prevalence and association.

Regardless of these observations, the general lack of genetic diversity of *B. pseudomallei* across regions highlighted through this analysis raises other broad issues:

1. Do the results reflect a failure of this typing technique to demonstrate genetic divergence in this group of bacteria?
2. Does this perceived clonality across regions and time reflect attributes of *B. pseudomallei* ecology and survival unique to the environment in this region?
3. Was melioidosis relatively recently imported to this region and does infection of a susceptible human host provide a cycle of selection and maintenance of a genetically non-diverse but virulent *Burkholderia* spp in the environment?

The lack of genetic diversity demonstrated with this assay is in contrast to past reports where this technique has been used to confirm genetic diversity within genotypes produced by other techniques (Trakulsomboon *et al.*, 1994; Vadivelu *et al.*, 1997). Indeed across other applications, PFGE macro restriction genotypes have proven rarely heterogenous by other typing techniques (Anderson *et al.*, 1991; Tenover *et al.*, 1994; Tenover *et al.*, 1995; Godoy *et al.*, 2003). Pulsed field gel electrophoresis has been used in past studies of melioidosis epidemiology to implicate clonality and epidemiological relatedness (Trakulsomboon *et al.*, 1994; Vadivelu *et al.*, 1998; Inglis *et al.*, 1999; Currie *et al.*, 2001).Multiple genotypes have been demonstrated among unrelated strains. Although, it has been highlighted that single PFGE genotypes occasionally are demonstrated among collection of isolates with no

apparent epidemiological association (Vadivelu *et al.*, 1997). This perhaps highlights the broad limitations of molecular typing in categorising clonality when it is applied outside the assumptions for use, rather than a failure of the technique *per se* (Tenover *et al.*, 1995).

The rare restriction enzyme *SpeI*, is not widely used to produce PFGE digest patterns of *B. pseudomallei*, but it is recommended for this genus (Tenover *et al.*, 1995). Also, *SpeI* analysis of *B. pseudomallei* isolates from a single site has demonstrated both multiple genotypes and clinical and environmental clones (Gal *et al.*, 2004). Further, the fact that *SpeI* analysis of the 15 PNG *B. thailandensis* isolates demonstrated 10 genotypes suggests that the principle of its use in this setting is sound for this bacterial genus.

Further substantiating this observation, the *B. pseudomallei* isolates that represent the single PFGE *SpeI* genotype have been subjected to *Xbal* macro restriction digestion analysis and RAPD PCR using five arbitrary primers. These techniques have not been able to discriminate this single genotype further (Appendix 5). Although isolate K93, an environmental isolate not directly related to either epidemiological associated group, did upon RAPD analysis demonstrate some divergence. This may suggest that RAPD molecular typing may represent a more sensitive tool to further confirm PFGE demonstrated macro restriction genotypes.

The utility of molecular typing to demonstrate epidemiological associations between isolates, is a balance of readability of gels and the extent of the genome represented by the patterns (Anderson *et al.*, 1991; Lew and Desmarchelier, 1993; Sexton *et al.*, 1993; Trakulsomboon *et al.*, 1994; Haase *et al.*, 1995a; Tenover *et al.*, 1995). Given that size of the *B. pseudomallei* genome is over 7 million bp and that the *SpeI* restriction site is 6 bases in size and that <20 bands are produced from the digest, the pattern only represents a very small amount of comparative genomic data. Indeed even the use of DNA-DNA hybridisation or sequence data as the gold standard for genomic comparison is problematic in that clones, if subjected to different environmental stress, may achieve divergent genetic recombinations over a relatively

short generational time (Storz and Hengge-Aronis, 2000). Clearly the variables of molecular typing are considerable and the tool, in this context, as apposed to an application for identifying the isolated source of a nosocomial outbreak from a restricted selection of isolates, for example (Tenover *et al.*, 1994; Shopsin and Kreiswirth, 2001; de Andrade *et al.*, 2003; Trindade *et al.*, 2003), supports a general hypothesis rather than provides forensic style evidence. This perhaps explains conflicting interpretations when various techniques have been used to infer phenotypic association with genotypes (Norton *et al.*, 1998; Ulett *et al.*, 2001).

The adaptive evolution of prokaryotes is different than that of sexual beings, particularly in relation to the low rates of recombination and the extent and mobility of extra-chromosomal elements (Levin and Bergstrom, 2000), but the fundamental drivers of genetic diversity is still a function of the length of evolutionary history and the extent of selection pressure. Given that microbial replication even during stationary phase with only the single stressor of starvation can result in genetic divergence of the progeny of a parent strain (Korona, 1996; Papadopoulos *et al.*, 1999), it seems implausible that clones of *B. pseudomallei* in the environment and those infecting individuals, are not exposed to selection pressure which may result in the selection of more favourable genetic recombination of some form (Storz and Hengge-Aronis, 2000). Presumably subtle movements occur as is demonstrated in developing *in vivo* resistance to antimicrobials - suggested to be plasmid or at least mobile transposon mediated (Dance *et al.*, 1988), but these are undetected with the restriction enzyme digest used for macro restriction genotyping or random arbitrary primers. It is also unlikely that the environment in this region implicated with melioidosis is so idyllic that sufficient selection pressure for more fit mutants to be selected doesn't happen. Although, clonal stability over 25 years has been demonstrated even in temperate climes, presumably environments less ideal that those of the tropics (Currie *et al.*, 1994).

The presumption that *B. pseudomallei* is endogenous to this region may be unfounded. Perhaps melioidosis was relatively recently imported through animals. Past missionary activity did establish sheep and cattle production, some sourced from

melioidosis endemic regions of Australia. This activity may have imported *B. pseudomallei* to the region where it established an isolated niche. Similar circumstances exist where clonal stability is a feature (Currie *et al.*, 1994). A study on the phyogenetics of isolates implicated in this study, perhaps using multilocus sequencing of house keeping genes (Godoy *et al.*, 2003), comparing isolates from Port Moresby, the Torres Strait and mainland Queensland, may shed light on the origins and movement of *B. pseudomallei* within this region.

The differences between the genetic divergence of the *B. pseudomallei* and PNG *B. thailandensis* suggest that this typing technique is sound for the molecular epidemiology of organisms of this genus. Furthermore, this phenomenon may highlight the importance of selection pressure from either the human susceptible host and/or single celled eukaryotes or plants in this region (Inglis *et al.*, 2000b; Inglis *et al.*, 2001) in selecting out a single genotype of *Burkholderia* spp from the environment with the fitness to adapt to an intracellular lifestyle. This virulent genoytpe may be cycled back into the environment and maintained through continued exposure and release, as has been speculated elsewhere (Ketterer *et al.*, 1975; Dance, 1991a; Currie *et al.*, 1994). As virulence, and therefore an ability to adopt an intracellular habitat, appears not to be a feature of the PNG *B. thailandensis* strains (Ulett *et al.*, 2001), clonal selection of this species in this way is not a feature, therefore resulting in increased genetic diversity.

Faecal carriage of *B. pseudomallei* has been demonstrated in humans with melioidosis (Wuthiekanun *et al.*, 1990). It is interesting to speculate that the use of pit toilets might be associated with the maintenance of *B. pseudomallei* in environments within regions where all other epidemiological factors converge. Perhaps a prospective study of faecal carriage of infected individuals and environmental sampling adjacent to pit toilets may elucidate this further.

Genetic diversity of intracellular pathogens has also been associated with the degree of the immune response by the host when challenged (Grenfell *et al.*, 2004). It seems that the degree of immunity a host may demonstrate may maintain a single genotype,

either through a lack of selection pressure (no effective immunity) or through a lack of time to develop an evolutionary history (overwhelming immunity). Perhaps hosts in this region represent either of these scales rather than intermediate immunity where both immune selection and adaptation collide, producing divergence.

Regardless of this speculation, the purpose of undertaking this study was to gather evidence on modes of transmission so disease control strategies could be developed. Although it may not be the only means with which *B. pseudomallei* is transmitted, it seems that individuals living in houses close to points of land are at risk and that children are more prone to exposure through robust play in the environment; behaviour peculiar to children. Percutaneous and perhaps pernasal inoculation results. Clearly, basic primary health care protecting lesions from environmental exposure will be useful. More contentious is the quarantining of points of land implicated in melioidosis. Some families affected by melioidosis have already relocated into the body of the villages that seem more safe, clearly more extensive work needs to be carried out to determine that these regions are a lesser risk.

The use of alkalising agents to reduce a local reservoir may have merit (Na-ngam *et al.*, 2004). More interesting is the use of bacteriocins or phage produced by other members in the family Pseudomonadaceae, as has been the case in controlling agricultural pests (Cain *et al.*, 2000; Adhikari *et al.*, 2001; Parret and De Mot, 2002; Xie *et al.*, 2003). Analysis of any antagonistic affects between the PNG *B. thailandensis* and *B. pseudomallei* may help clarify the potential of this option.

CHAPTER 7 - SERO-EPIDEMIOLOGY OF MELIOIDOSIS IN PAPUA NEW GUINEA
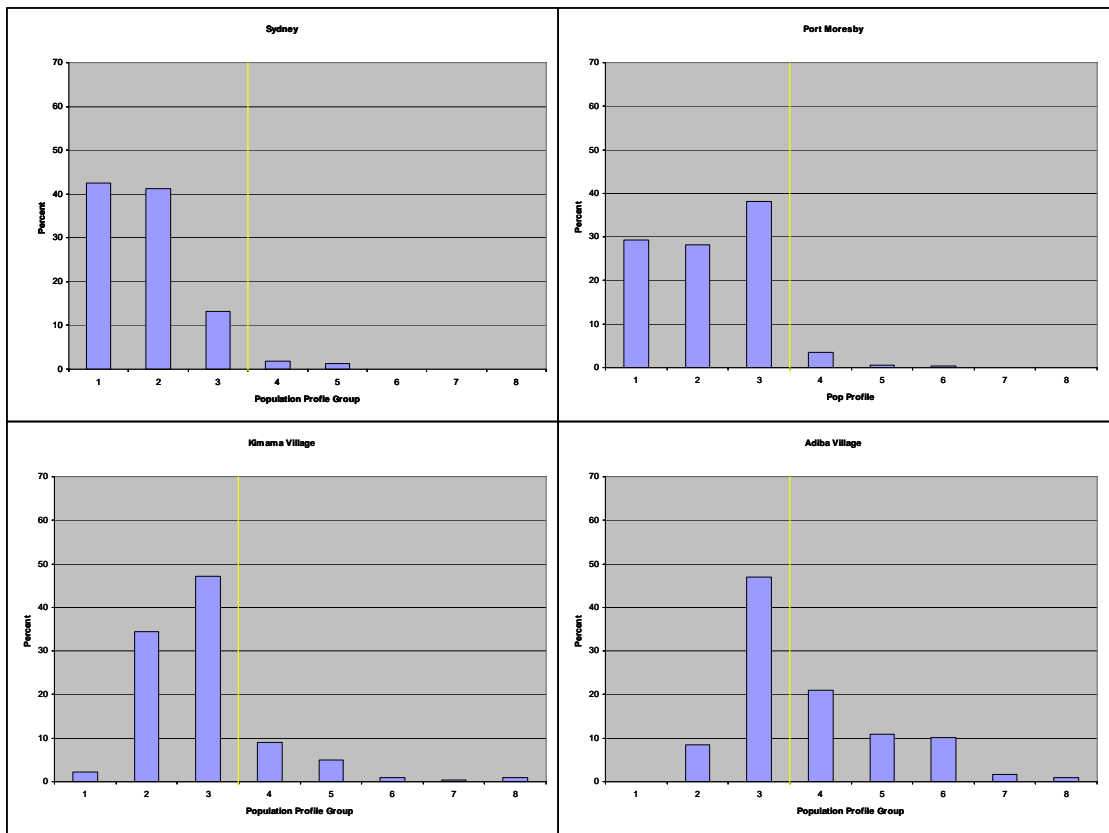
7.1   Introduction

Autochthonous melioidosis has been demonstrated in the Balimo region of the Western province of PNG but a logical question which follows is: to what extent does melioidosis contribute to the national infectious disease burden? Clearly, as PNG has the lowest number of health care workers per capita of any country in the Pacific region (Anon, 2000b), the assumption that melioidosis is under-recognised in Balimo mostly because of a lack of facilities and trained human resources (Dance, 1991a), can equally be applied throughout the other rural provinces.

Given the apparent clinical and environmental spatial clustering of melioidosis, establishing clinical melioidosis screening programs within health programs in underdeveloped communities in an ad hoc manner, that is to say without any clinical suspicion, is unlikely to be sustainable and perhaps is an inappropriate use of scarce health resources. Studies on the sero-epidemiology of melioidosis in PNG was undertaken that could provide an insight into the extent of melioidosis throughout the Western province and from selected regions elsewhere in PNG.

If in the analysis of samples taken from individuals from regions with varying degrees of clinical endemicity, sero-reactivity was found to be dependent on region, then samples taken from individuals living in communities with unknown clinical endemicity could be analysed and then be compared. Assumptions on the extent of clinical endemicity could be inferred and be subsequently tested with clinical screening programs.

In an attempt to validate this approach, different methods of data presentation were used and assessed for statistical soundness in comparing regions. Particular note was taken as to how the methods of analysis could be applied in rural PNG with the least analytical imprecision.

7.2 Materials and Methods

7.2.1 ELISA assay preparation and validation

An indirect IgG ELISA was designed based on the description of Ashdown *et al* (Ashdown *et al.*, 1989) but using antigen derived from *B. pseudomallei* isolated from a PNG patient. The assay was optimised in terms of antigen concentration and conjugate dilution and then validated for use against samples from melioidosis culture confirmed patients (kindly supplied by Dr Robert Norton, QHPSS The Townsville Hospital) and disease control samples from a non-endemic region (kindly suppled by Mr Noel Person, PaLMS, Royal North Shore Hospital, Sydney).

7.2.1.1 Antigen preparation

Of the clinical isolates in the collection, four (C1 - C4) were chosen to prepare antigen. Each isolate was grown on TSA (Appendix 1) and growth was harvested into 20 ml of sterilised distilled water. The bacteria were heat killed at 85°C for 15 min. The suspensions were then sonicated (Biosonik III untrasonicator, 20Kc/sec) with two bursts of 20 min with the suspensions on ice. Approximately 1 ml of 0.1 mm diameter glass beads (Biospec Products, Bartlesville) was added to aid in cell disruption. The sonicate was centrifuged at 10,000 *g* and a portion of the supernatant passed through a 0.22 µm syringe filter (Sarstedt, Inglefarm, SA). Both filtered and non-filtered fractions were aliquoted and stored separately at -70°C before use.

7.2.1.2 ELISA optimisation

Each fraction (unfiltered and filtered) of the sonicates prepared from each isolate was tested for immunoreactivity against serum previously determined to be reactive and a sample previously tested as non-reactive. The antigen preparation that achieved the largest positive / negative ratio was selected for further optimisation. Briefly, antigen was serially diluted in coating buffer (TropBio, Townsville, QLD) across the rows of a 96 well plate (Sarstedt, Inglefarm, SA) to a total volume in each well of 100 µl. The

plates were incubated overnight in a humidified environment. After incubation the contents of the plates were discarded. Plates were washed with ELISA wash buffer (TropBio, Townsville, QLD) and serum, diluted 1:100 in ELISA buffer (TropBio, Townsville, QLD) was added in 50 µl volumes. After a 60 min incubation at RT, the plate contents were discarded and the plates were washed three times in washing buffer (Tropbio, Townsville, QLD). A 50 µl volume of peroxidase-conjugated goat anti-human IgG (Jackson ImmunoResearch, West Grove, PA) diluted 1/8000 in ELISA buffer, was added and plates were incubated for 60 min at RT. After incubation plate contents were discarded and the plate washed four times in ELISA washing buffer. After washing, 100 µl of a one pack peroxidase substrate solution (ABTS, Biomediq, Doncaster, VIC) was added and colour was left to develop for 60 min. The plates were read using dual wavelength at 414 and 492 nm to limit non-specific absorbance. This basic configuration was used to find a high and low positive reactor in the serum bank. A total of 88 samples were tested and the most positive and negative was chosen and used to further optimise the assay in terms of antigen concentration and conjugate dilution through checkerboard titration.

7.2.1.3 Standard curve

A collection of standards was prepared which could be used to internally control the assay and establish boundaries for population profiles (see later). Briefly, a sample of the most reactive serum was subjected to 11 two-fold serial dilutions in a sample of the most non-reactive serum. This resulted in 11 samples representing the most positive (undiluted reactive serum, sample 1) to the most negative (1/1,024 diluted sample, sample 11). The samples were analysed and results graphed and a sigmoidal association resulted. Linerisation of the model was performed by eliminating selected samples represent the 1/2,1/64,1/256 and 1/512 dilution. This resulted in seven standard samples which represented a linear representation of absorbance readings expected from the use of this assay. These standards were aliquoted and stored at -70°C and analysed in duplicate for each subsequent run. Standards and a conjugate control were run in duplicate.

7.2.1.4 Validation and establishment of a cutoff

The assay was not intended to be used as a diagnostic test. However, to allow the calculation of the relative sero-prevalence for each region sensitivity and specificity were established. Firstly, a cutoff was established based a method outlined by Kurstak (Kurstak, 1985) and modified to ensure between-run comparison was possible. Briefly, 119 serum samples from individuals presenting with an infectious disease but not melioidosis from a non-endemic region were analysed for mean absorbance and a standard deviation was calculated. From this data, an absorbance value two standard deviations plus the mean was calculated which represented the reactivity cutoff within 95% confidence limits. Along with these samples, seven control samples described above were also analysed in duplicate. The standard which when analysed represented the closed absorbance to the cutoff value was considered the "cutoff standard". Standard sample number 3 was determined to be the cutoff standard, all samples when analysed demonstrated absorbance readings greater than those that resulted from the analysis of sample 3 were considered reactive.

Sensitivity, specificity and positive and negative predictive values were determined through the analysis of 164 serum samples from individuals presenting with an infectious disease but not melioidosis and 102 serum samples from individuals with culture confirmed melioidosis, respectively. All samples were analysed and subjected to the reactivity cutoff established as described above. Validation was calculated by subjecting data to classic two by two table analysis as described by Gerstman (Gerstman, 1998) (Table 7.03).

7.2.2   Study centres and sampling techniques

Study centres were chosen that represented regions of known melioidosis prevalence and regions where melioidosis was unknown were selected to be tested. Sero-reactivity resulting from the analysis of samples taken from individuals could be further analysed and tested to be dependent on region and melioidosis prevalence. The study centres chosen are listed in Table 7.01. Regions include, urban temperate

Australia (Sydney), urban settlements of the national capital district of PNG (Port Moresby), rural central province (Woitape), periurban East New Britain province (Rabaul), rural coastal and inland East Sepik province (Angorum and Ambunti), northern Western province (Rumginae) Mid northern Western province (Kiunga), mid Western province (Lake Murray), Balimo region (Balimo village, Saweta village, Kimama village, Buila station, Adiba and Kaniya), southern Fly river village (Tapila) and coastal Western province (Daru).

The regions chosen to represent varying known degrees of melioidosis prevalence were Sydney (considered no risk, no autochthonous melioidosis reported), Port Moresby (considered slight risk, endemic but rare, sporadic cases reported only), Kimama (considered moderate risk, endemic, not widespread, three isolated cases within one family outbreak in one centre of village, implicated environmental reservoir demonstrated) and Adiba (considered high risk, multiple cases confirmed and more suspected, implicated environmental reservoir demonstrated). If sero-reactivity could be shown to be dependent on region and with these regions representing varying degrees of melioidosis prevalence, samples could be taken from individuals within regions of unknown melioidosis prevalence and the sero-reactivity could be measured. Results could be compared with the sero-reactivity data from melioidosis prevalent regions and clinical prevalence could be inferred.

Venous blood samples from individuals were taken by the methods previously described (Chapter 3). From the different regions of PNG samples were collected from healthy volunteers. Samples from Sydney were provided by Mr Noel Person from the serum bank at the microbiology department PaLMS, Royal North Shore Hospital Sydney and represent sera from individuals being investigated for an infectious disease found subsequently not be melioidosis. The work in PNG was performed in accordance with the ethics requirements of the PNG Medical Research Advisory Committee under the approval reference number MRAC 02.25. Informed consent was obtained prior to any sampling initiative.

Table 7.01   Study centres, samples sizes, locations and melioidosis status

Region	Village / City	n	Lat	Long	Melioidosis Status
Australia	Sydney	159	34º0'S	151º0'E	Remote risk of imported disease
Central Province	Woitape	59	8º33'S	147º15'E	Unknown
	Port Moresby	293	9º27'S	147º11'E	Sporadic
East Sepik	Ambunti	141	4º13'S	142º49'E	Unknown
	Angorum	161	4º4'S	144º4'E	
East New Britian	Nonga	110	4º10'S	152º9'E	Unknown
Western province					
North	Rumginae	217	5º54'S	141º16'E	Unknown
	Kiunga	83	6º7'S	141º18'E	
Central	Lake Murray	75	6º47'S	141º25'E	Unknown
South	Daru	62	9º4'S	143º11'E	Unknown
	Tapila (Tirio)	161	8º26'S	143º1'E	Soil isolation negative, clinical status unknown
Balimo region					
Balimo lagoon based villages	Balimo village	208	8º3'S	142º56'E	2 cases, no soil isolates
	Kimama	223	8º1'S	142º58'E	3 cases, soil isolate from implicated site
	Buila (periurban station)	59	8º2'S	142º57'S	Periurban station village
	Saweta (Waligi)	47	8º4'S	142º58'E	No cases
Aramia river based village					
	Kaniya (Mumuni)	211	8º0'S	142º37'E	Unknown
Adiba lagoon					
	Adiba	119	8º4'S	142º52'E	multiple cases, soil isolates

7.2.3   Presentation of data and methods of region comparison

7.2.3.1 Positive rate and true prevalence

Relative sero-reactivity of regions was graphically presented as percent positive rate (numbers of samples tested reactive divided by total numbers multiplied by 100) with 95% confidence limits and true prevalence based on definition and calculation of

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Rogan and Gladen (Rogan and Gladen, 1978) (positive rate + sensitivity - 1 / sensitivity + specificity - 1) which, because it includes validation of the assay in the determination of prevalence, is a more accurate measure of sero-prevalence. Subjective assumptions could be made regarding the similarity of sero-reactivity of regions based on comparing true prevalence values and using positive rate 95% confidence limits as a guide to epidemiological relatedness.

7.2.3.2 Statistical analysis of variance

Various statistical analyses were applied using SPSS for windows, version 11 (SPSS Inc, Chicago, IL). Attempts were made to determine if sero-reactivity (as measured by raw absorbance readings) was dependent on region. Firstly, absorbance data for each region was tested for normality using Q plot analysis and found to be not normally distributed even when log transformed, therefore non-parametric statistical methods of comparison were sought which might be applied so an analysis of variance could be applied to data. The Kuskal-Wallis test, non-parametric one way ANOVA was used to test whether several independent samples taken from independent regions were from the same population and the Mann-Whitney test was used when two groups were compared.

7.2.3.3 Population profile histograms

Variation of between-run conditions, such as incubation temperature and reagent lot, may alter the relative median absorbance readings resulting from the analysis of samples. This will limit the use of the assay as a epidemiological tool in the comparison of sero-reactivity between regions as variations of the rank of absorbance readings would result which would not be associated with relative sero-reactivity. This would particularly be the case if the assay was to be used in the field where stability of incubation conditions could not be guaranteed. A method of semi-quantitative sero-prevalence analysis was therefore sought to infer clinical prevalence which would not be limited by this variation.

To graphically represent subtle changes in the rank of absorbance readings which resulted from the analysis of samples taken from individuals within regions (without detailed statistical analysis), a population profile histogram was established for each region. Briefly, raw absorbance readings resulting from the analysis of samples from each region were ranked within ranges established as a result of the analysis of standards which represent known degrees of sero-reactivity, as per Table 7.02. Upon analysis, the percentage of samples from individuals within the population with absorbance readings within each rank was calculated and graphed as a histogram demonstrating eight population profile groups demonstrating low - high IgG levels. The resultant histogram therefore expresses the distribution of IgG antibody within the samples tested and therefore the population.

Histograms were prepared from samples of populations with degrees of known prevalence (and therefore know risk of acquiring melioidosis). This was undertaken so regions where prevalence data (and melioidosis status) is being sought but unknown could be sampled and analysed and compared to histograms from regions of known prevalence and clinical prevalence inferred.

Table 7.02   Derivation of population profile groups

Standard Curve Sample	Original Titre	STD ranges	Population Profile Group
1	1	<7	8 - most reactive
2	4	6 to 7	7
3	8	5 to 6	6
4	16	4 to 5	5
5	32	3 to 4	4
6	128	2 to 3	3
7	1024	1 to 2	2
		>1	1 - least reactive

7.3   Results

7.3.1   ELISA validation

A total of 266 samples were analysed to validate the assay. Of this total, 102 were from melioidosis culture-confirmed individuals and 164 were from individuals being investigated for an infectious disease but were not suffering from melioidosis. Of the culture confirmed group, 80 samples were reactive using the cutoff criteria described above, this resulted in a sensitivity of 78.4%. Of the 164 disease control group, four were reactive, this resulted in a specificity of 97.6%. The positive and negative predictive values were 95.2% and 87.9% respectively (Table 7.03).

Table 7.03   ELISA validation

ELISA	Culture		
	Positive	**Negative**	**Totals**
Reactive	80	4	84
Non-reactive	22	160	182
Totals	102	164	266
Sensitivity	78.4%		
Specificity	97.6%		
Positive Predictive value	95.2%		
Negative Predictive value	87.9%		

7.3.2   Positive rate and true sero-prevalence

The true sero-prevalence of regions tested ranged from 0% (Kiunga) to 55.4% (Adiba) (Table 7.02) and can be broadly divided into those centres with a true prevalence of <10% (Sydney, Woitape, Nonga, Port Moresby, Ambunti, Angorum, Lake Murray, Kiunga and Rumginae) and those centres with a true prevalence of >10% (Daru, Tapila, Balimo Village, Kimama, Buila Station, Kaniya and Adiba). Clearly those centres with the closest proximity to the Aramia river floodplain (Balimo District) had the highest true sero-prevalence. Regions which when sampled and tested resulted in a true prevalence of <2.6 were outside the sensitivity of the assay (sensitivity of 97.4%) and considered invalid and represent a limitation of assessing regions with low

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prevalence. The positive rate overestimated sero-prevalence when true prevalence was <10% and overestimated sero-prevalence when true prevalence was >10%.

Table 7.04   Sero-prevalence of all regions. Regions highlighted in red are regions chosen to represent known varying degrees of clinical prevalence

Location	n	Positive Rate	95% CI	True Prevalence
Sydney	159	3.1	1 - 7.7	0.8
Woitape	59	6.8	0.2 - 16.5	5.6
Rabaul	110	2.7	0.6 - 7.8	0.3
Port Moresby	293	4.1	2.1 - 7.0	2.1
Ambunti	141	3.5	0.1 - 8.1	1.4
Angorum	161	3.1	0.1 - 7.1	0.8
Lake Murray	75	4	0.8 - 11.3	2
Kiunga	83	1.2	0 - 6.5	0
Rumginae	217	6.5	3.6 - 10.6	5.2
Daru	62	12.9	5.7 - 23.9	13.7
Tapila	161	16.1	10.8 - 22.8	18
Balimo Village	208	31.7	25.5 - 38.5	38.5
Kimama	223	16.1	11.6 - 21.6	18
Buila Station	59	18.6	9.7 - 30.9	21.3
Saweta	47	10.6	3.5 - 23.1	10.7
Kaniya	211	26.1	20.1 - 32.5	31
Adiba	119	44.5	35.4 - 53.9	55.4

7.3.3   Statistical analysis of variance

For all regions sampled and tested, the resultant sero-reactivity was shown to be dependent on region (Kruskal Wallis test; H = 968.1; 16 df; p = <0.001). Two groups emerge when true sero-prevalence becomes greater than positive rate, those that share a true sero-prevalence of <10% (Figure 7.01) and those that share a true sero-prevalence of >10% (Figure 7.02). Although for each group sero-reactivity is also dependent on region, within each group are clusters which represent the same population in terms of sero-reactivity. For example, sero-reactivity of samples tested from Woitape (TP 5.6%), Rabaul (TP 0.3%) and Port Moresby (TP 2.1%) are not significantly different (p = 0.514). Ambunti (TP 1.8%) and Angorum (TP 0.8%)

(p = 0.760) and Lake Murray (TP 2%) and Kiunga (TP 0%) (p = 0.662) respectively
are also not significantly different to one another.
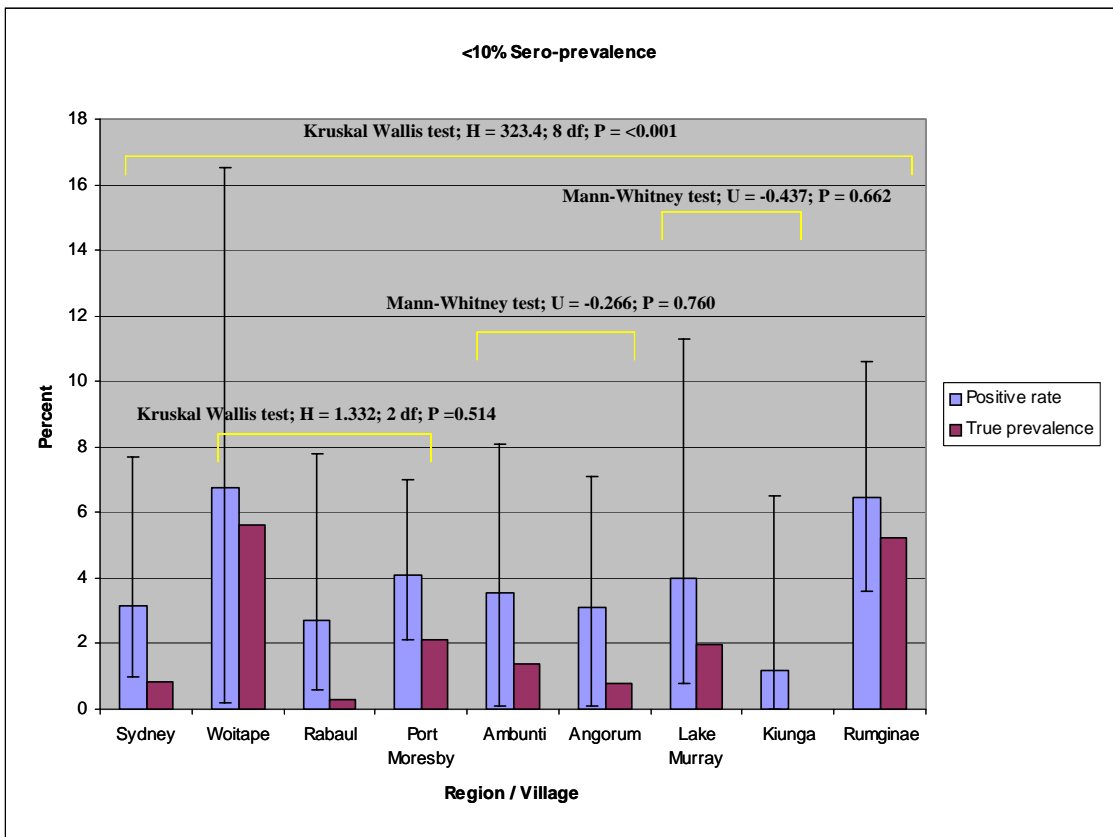
Figure 7.01   Regions demonstrating true prevalence of <10%

Within the group of regions which share a true prevalence of >10%, greater
heterogenicity is demonstrated (Figure 7.02). All of these regions are from either the
Aramia flood plain or southern Western province.
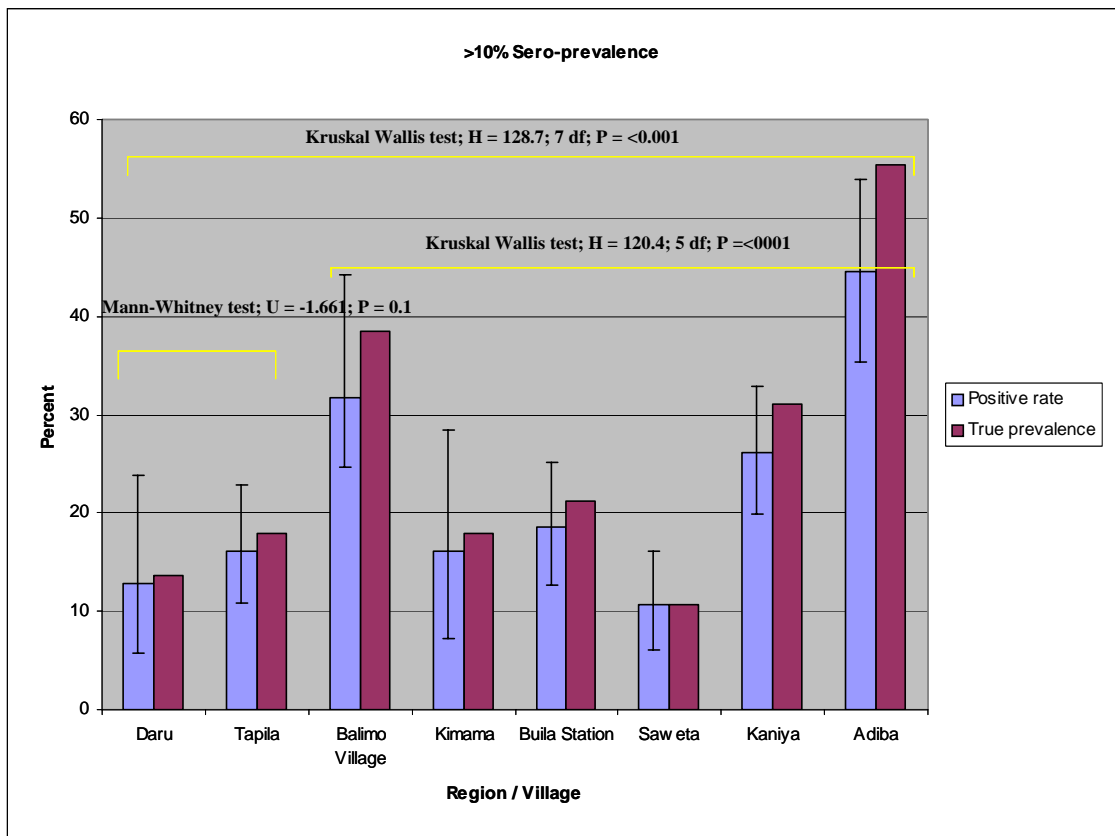
Figure 7.02   Regions demonstrating true prevalence >10%

Further, when regions was stratified according to melioidosis prevalence, sero-reactivity was found to be dependent on regions which represent varying degrees of melioidosis prevalence (Figure 7.03) (H = 337.2, 3 df, p = <0.001).

Of the four communities tested in terms of sero-reactivity Kimama and Adiba villages, both are clearly different to each other and to Sydney and Port Moresby (Figure 7.01). However, the confidence limit ranges of Sydney and Port Moresby suggest these regions are epidemiologically related in terms of melioidosis sero-prevalence. Further analysis of the raw absorbance data however demonstrates that Sydney and Port Moresby are also independent regions in term of sero-reactivity (U = 10249.9; p = <0.001). Therefore the divergence in the clinical prevalence of melioidosis of these regions can be demonstrated in differences in sero-reactivity.

Sero-prevalence of regions with known melioidosis prevalence

Kruskal Wallis test; H = 337.2; 3 df; P = <0.001

Mann-Whitney test; U = 10249.9; P = <0.001

Positive rate
True prevalence

Percent

Sydney n = 159   Port Moresby n = 293   Kimama n = 223   Adiba n = 119

Region and sample size
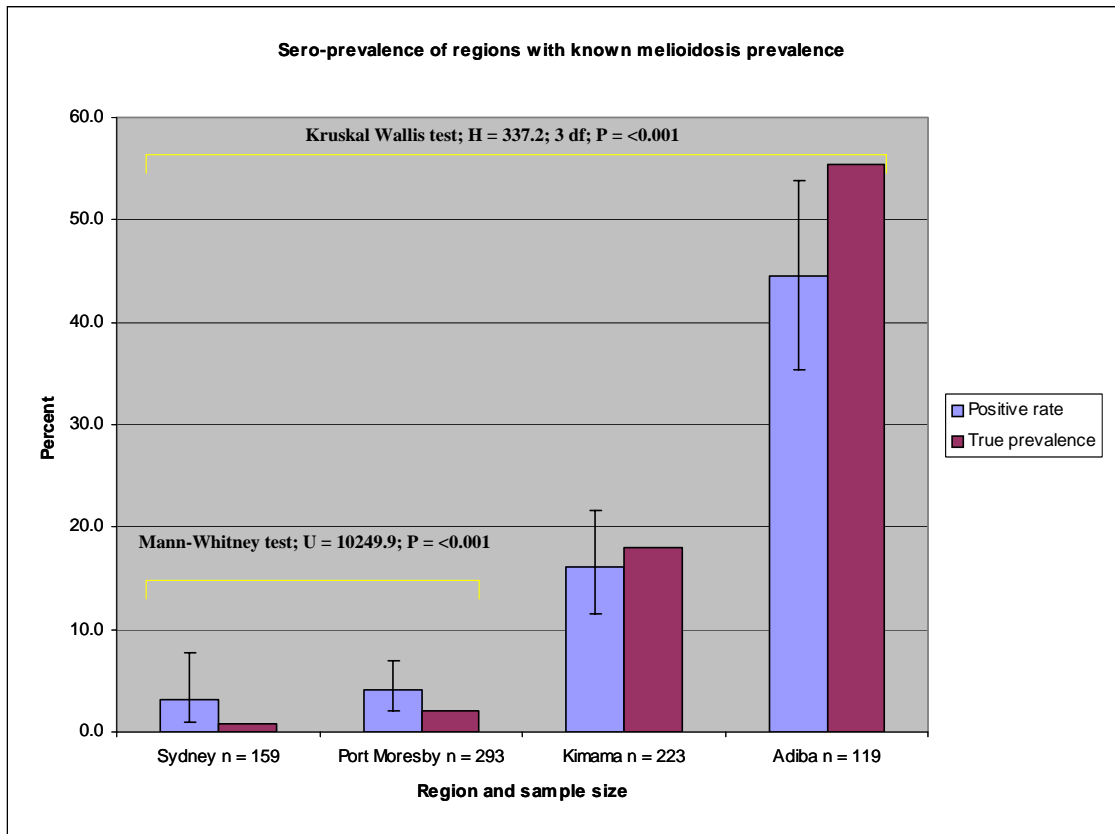
Figure 7.03   Comparison of the sero-prevalence between regions of varying degrees of melioidosis prevalence

Using sero-reactivity expressed as positive rate and true prevalence, it can be demonstrated that within the Aramia flood plain region of the Western province exists the highest true prevalences, all greater than 10%. This high true sero-prevalence corelates with the prevalence of clinical disease, which seems more apparent in this region of PNG compared to others. Although, given that the prevalence of clinical melioidosis has not been thoroughly studied in communities outside this region, this assumption remains to be tested.

Adiba has by far the highest true prevalence and it appears from the 95% confidence intervals that the sero-reactivity demonstrated from the analysis of samples taken from individuals here is unprecedented in the population's extent of sero-reactivity. This seems to corelate with the clinical experience, where nine of the 17, either confirmed or suspected, cases of melioidosis have been reported (Chapter 3).

7.3.4 Population profile histograms

Sero-reactivity can be further described by the use of population profile histograms. The population profile histograms for the know melioidosis prevalent localities of Sydney, Port Moresby, Kimama and Adiba are shown in Figure 7.04. The shapes of the histograms reflect a shift of IgG in the community from a skew to the left (most sero-non reactive region, Sydney) to a skew to the right (most sero-reactive region, Adiba). The sero-reactivity histograms are significantly different to each other (as determined above when raw absorbance rank was analysed) and therefore reflect the varying prevalence of melioidosis in these communities. Further, as the analysis of internal standards determine the boundaries within which the absorbance readings of samples are placed to create the histograms, this method enables between-run comparison possible, regardless of variation of median absorbance readings which may vary between each run due to variables such as incubation temperature and reagent lot.

To semi-quantify methods of comparison (as apposed to simply proposing similarities by comparing the the shape of the histogram visually) a simple set of criteria was chosen to represent the histograms based on movements of the population profile groups, shaping the histogram, which reflect movements of IgG within the population. These were related to clinical prevalence of the region based on literature available and are presented in Table 7.03 and Figure 7.04.

Table 7.05   Locality risk profile criteria

Locality Risk Profile	Representative Region	Risk of acquiring melioidosis
Population profile group 3 < 1 and 2	Sydney	Low
Population profile group 3 > 1 and 2	Port Moresby	Slight
Population profile group 4 < 1	Kimama	Moderate
Population profile group 4 < 2	Adiba	High
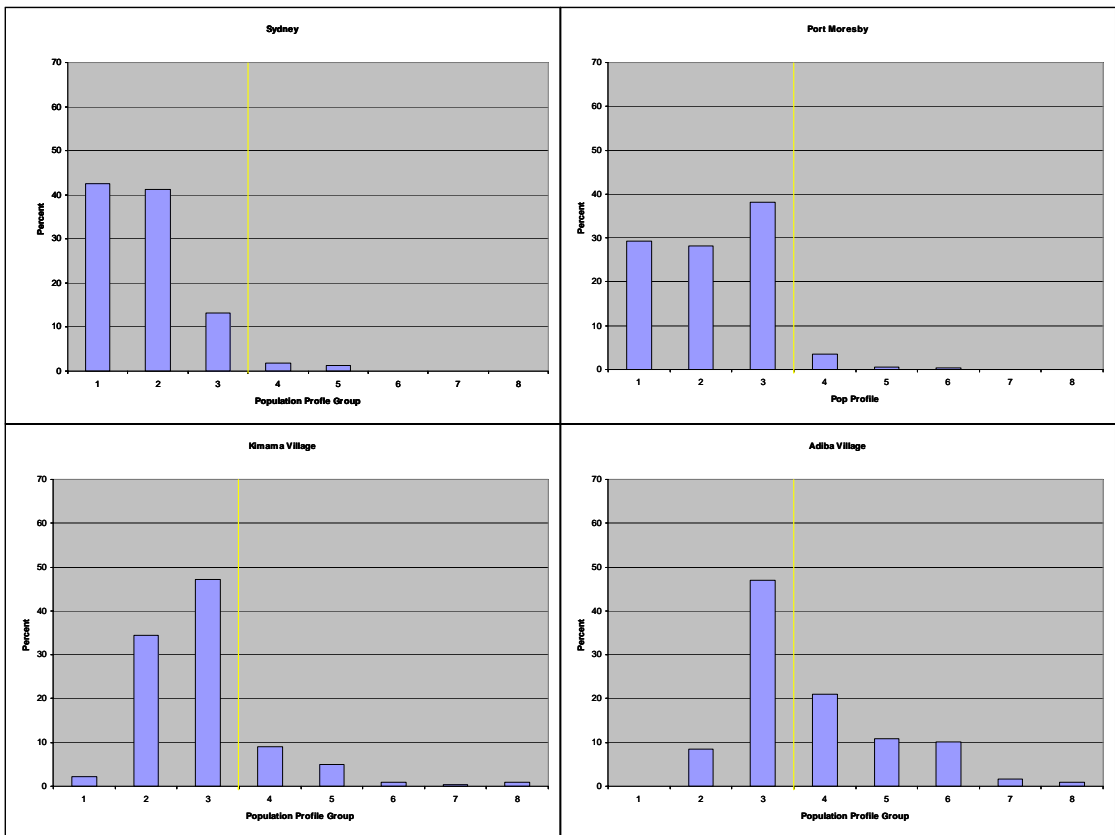
Figure 7.04   Shift of sero-reactivity from low prevalence regions through to higher absorbance readings

The histograms of localities of unknown prevalences that have resulted from the analysis of samples from these regions are presented in Figures 7.04 - 7.07.

Regions which share locality risk profile similar to Sydney, are those regions geographically distant to the Balimo region: East New Britain, East Sepik and Central province (Figure 7.05 and 7.09). The regions which share a locality risk group similar to Port Moresby which represent a slight risk, include Rumginae, Kiunga and Lake Murray. These regions are located in the mid and northern regions of the Western province (Figure 7.06 and 7.09). The regions that share a locality risk similar to Kimama (moderate) include villages within the Balimo district with known melioidosis endemicity and neighbouring regions in the south of the province including Daru, Tapila, Saweta and Buila (Figure 7.07 and 7.09). Regions which share the high risk locality region with Adiba include Balimo village, a village with known endemicity and Kaniya, a rural village on the Aramia river to the west of Adiba and Balimo (Figure 7.08 and 7.10).

Table 7.06   Locality risk group analysis of unknown melioidosis prevalence regions

Group	Risk Locality	Associated regions
1	Sydney (No risk)	Ambunti, Angorum Woitape, Rabaul
2	Port Moresby (Slight risk)	Kiunga, Lake Murray Rumginae
3	Kimama (Moderate risk)	Daru, Tapila Kimama,Saweta Buila
4	Adiba (High risk)	Kaniya, Balimo
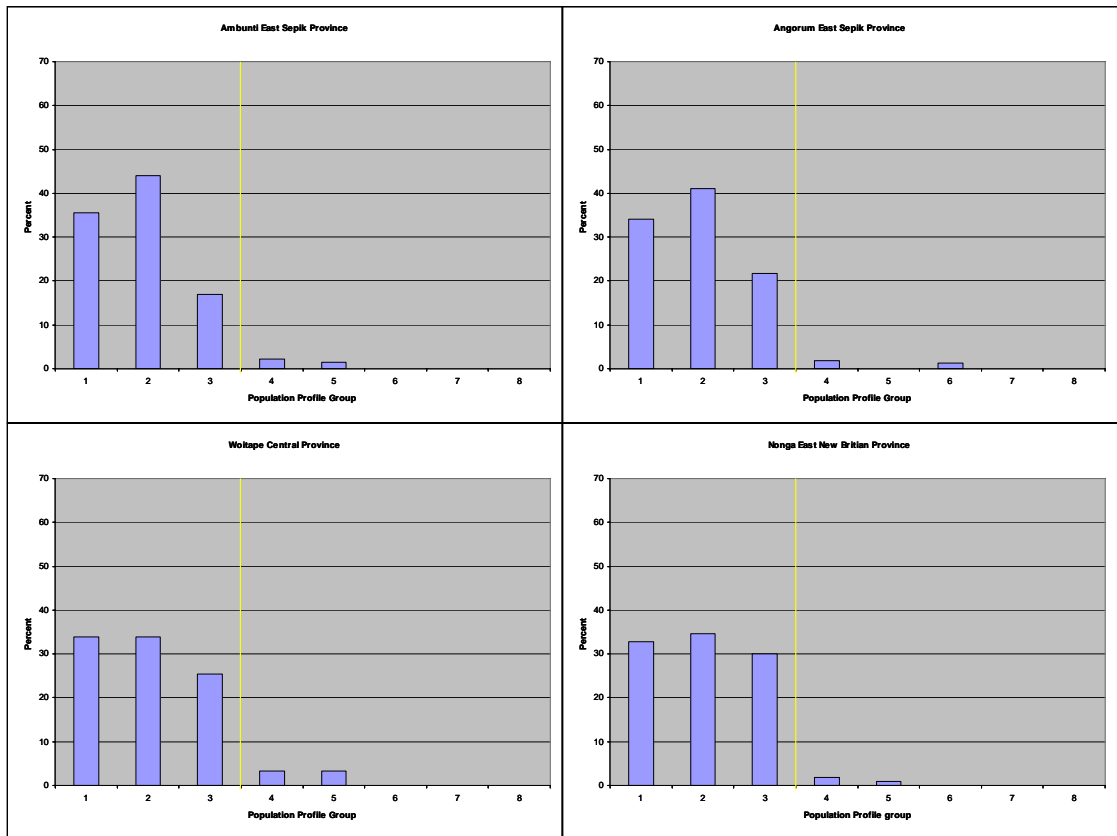
Figure 7.05   Histograms representing locality risk-group 1

When the sero-epidemiology data is overlayed onto the biogeography map, it can be seen that a correlation exists between the high risk groups and those regions which share melioidosis-implicated biogeographical attributes (Figure 7.09 and Figure 7.10).
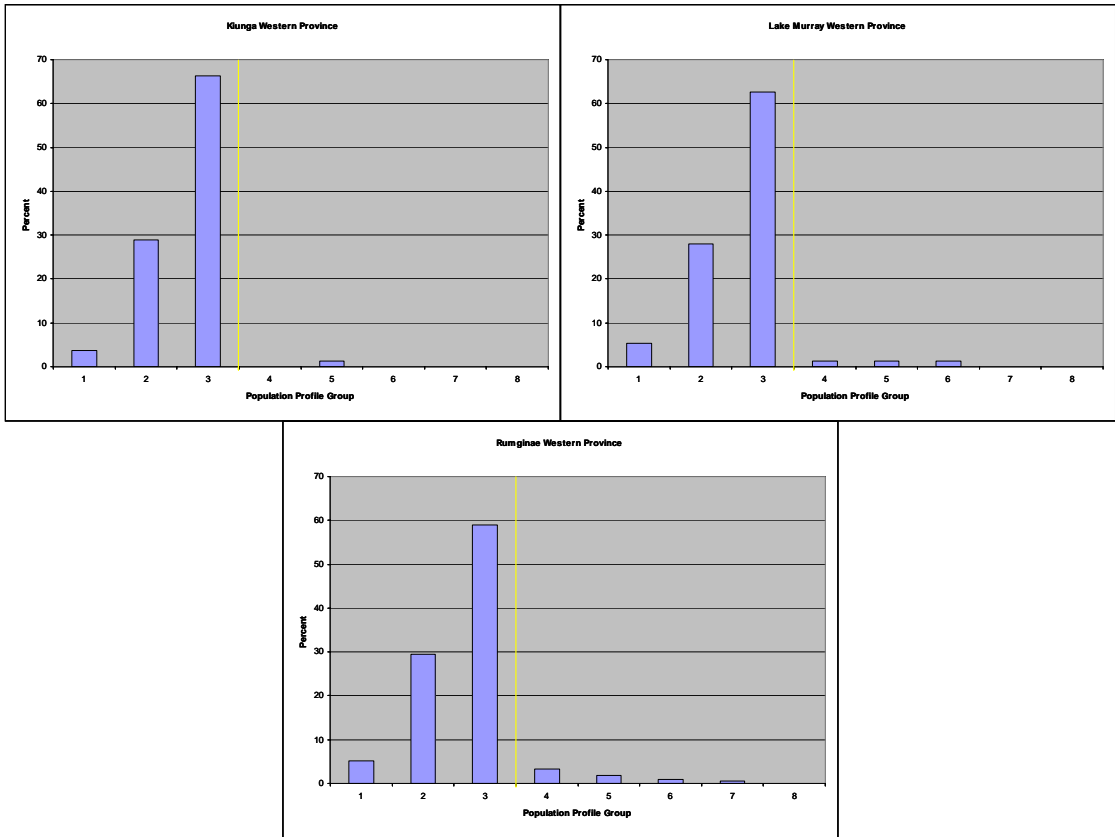
Figure 7.06   Histograms representing locality risk-group 2
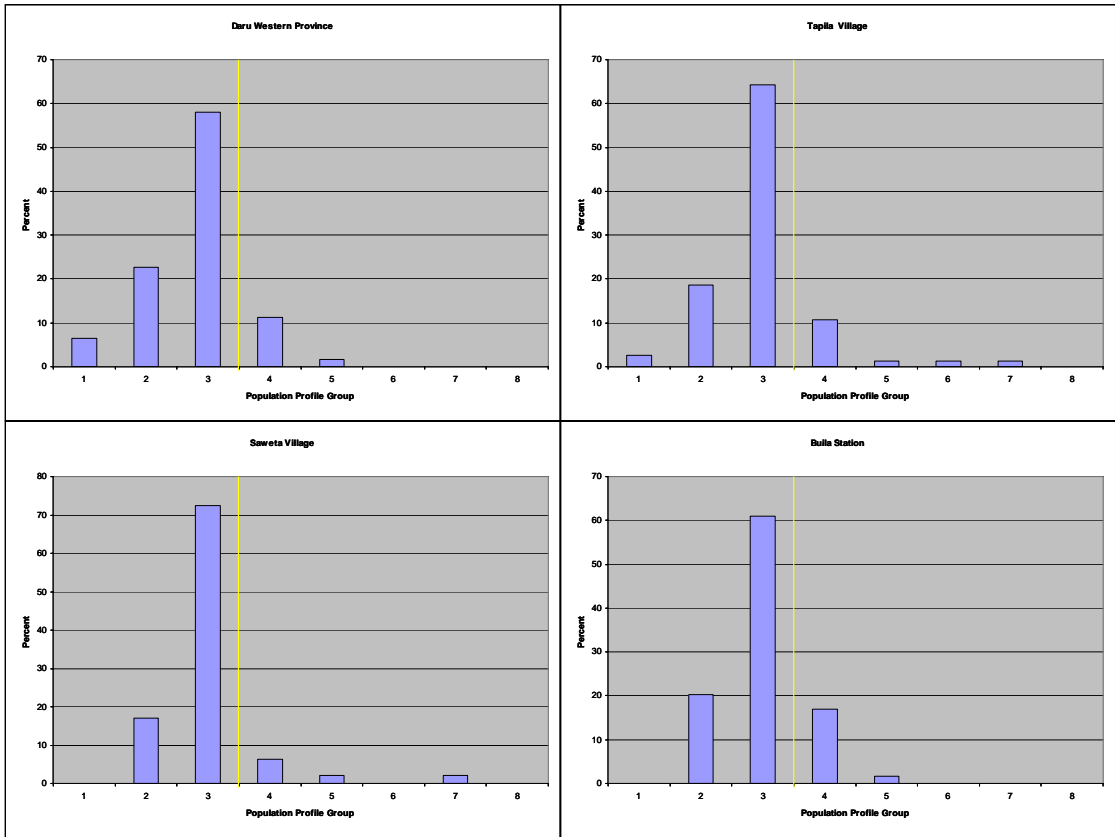
Figure 7.07   Histograms representing locality risk-group 3
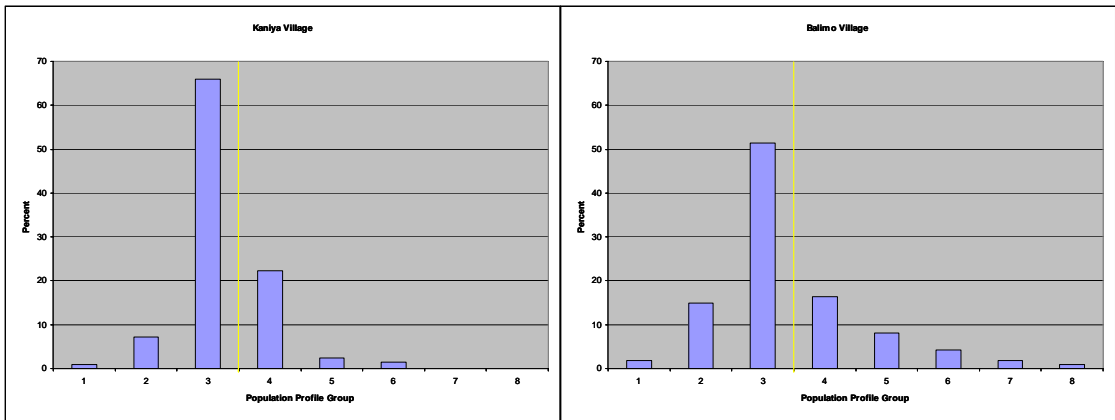
Figure 7.08   Histograms representing locality risk-group 4
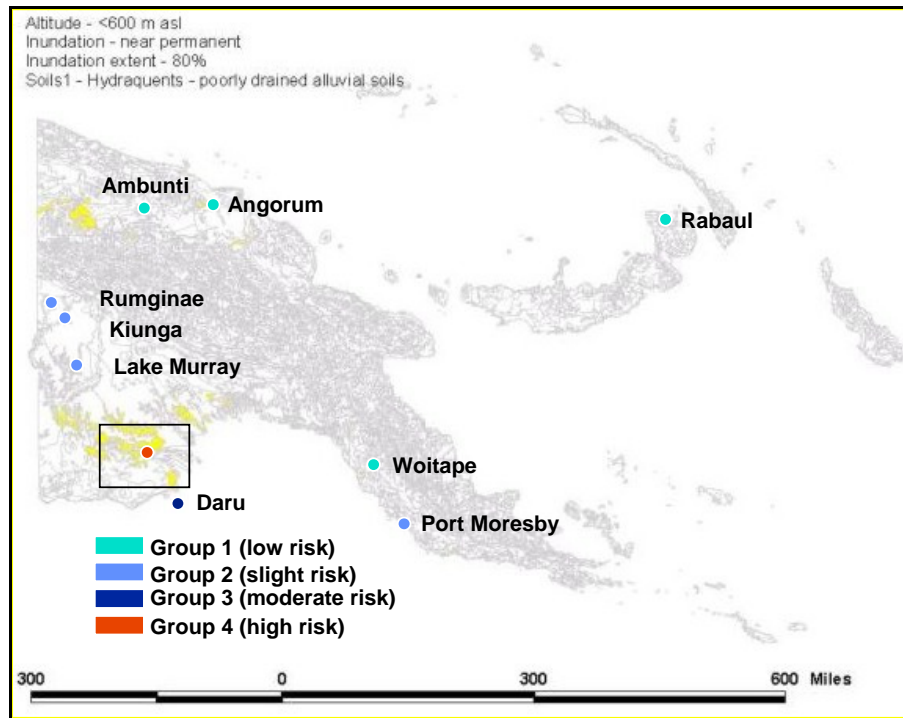
Figure 7.09   National Sero-epidemiology. Locality risk groups. (See detail of boxed
                region in Figure 7.10)
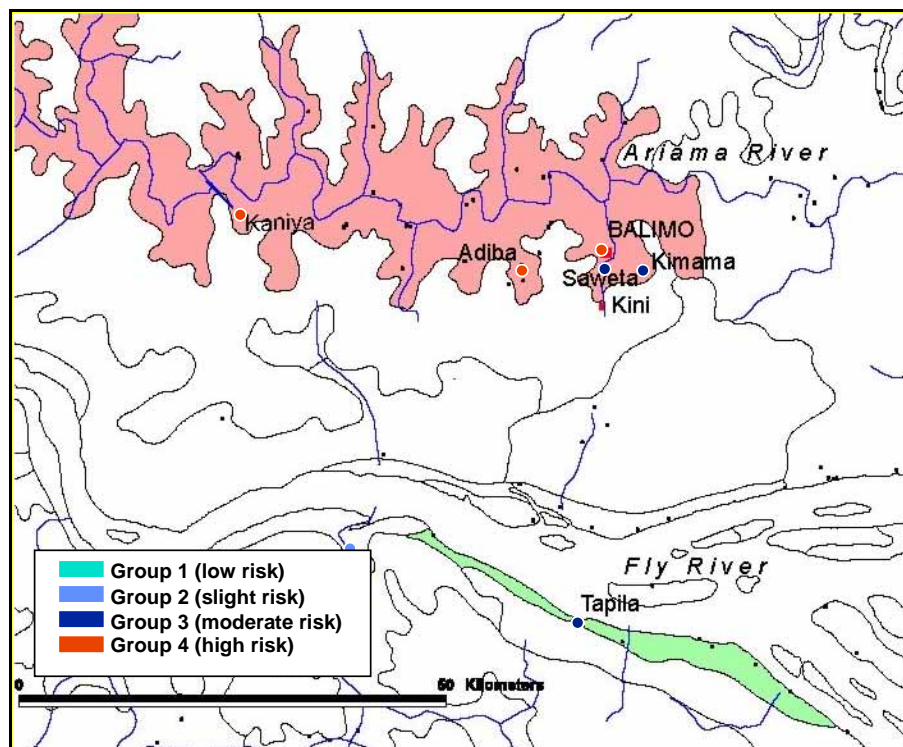
Figure 7.10   Aramia river region. Population profile risk groups (Boxed region of
                Figure 7.09). Pink represents environmental attributes of Balimo.
                Green represents environmental attributes of Tapila

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7.4   Discussion

Individuals tested within the population of the Aramia flood plain region of the Balimo region have the highest sero-reactivity. Not only does sero-reactivity appear to corelate to clinical risk of acquiring melioidosis (Chapter 3), but the data is further corroborated when the population profile risk groups were overlayed on the biogeographical map (Chapter 4).

Studies conducted elsewhere demonstrate an association between the prevalence of *B. pseudomallei* in the soil, the risk or acquiring melioidosis through exposure to this reservoir and an underlying high sero-prevalence (Kanaphun *et al.*, 1993; Wuthiekanun *et al.*, 1995a; Vuddhakul *et al.*, 1999). The sero-reactivity in animals seems to corroborate these clinical findings (Srikitjakarn *et al.*, 2002). It also seems possible that levels of IgG decrease in individuals after successful treatment (Vasu *et al.*, 2003) reflecting a decrease in the challenge by the organism to the host immune system.

It therefore seems reasonable to conclude that the presence of IgG detected in an individual with an assay of appropriate validation can be the result of either infection, subclinical or otherwise, or exposure to the organism which may or may not result in infection, subclinical or otherwise. Either way, it is therefore reasonable to conclude that the presence of high levels of IgG in individuals within a population represents high level exposure rates which may lead to high prevalence within the population. This would be the result of either a uniquely large reservoir of infection in this region or particular behaviour which predisposes these individuals to exposure to a shared reservoir. Clearly also, the manifestation of disease in individuals within these regions is also dependent on the extent of host risk factors (Chaowagul *et al.*, 1989; Currie *et al.*, 2000d).

The use of serology for the diagnosis of melioidosis in individuals is problematic. This is mostly due to either poor validation (Cuzzubbo *et al.*, 2000) or when it is used in regions were true prevalence is not high enough to achieve reasonable positive predictive values (Rogan and Gladen, 1978). Therefore, the diagnosis of subclinical

infection based on sero-reactivity alone does, in terms of the individual, becomes unfounded in terms of reactivation illness (Ashdown and Guard, 1984; Thompson and Ashdown, 1989). This situation reflects the poor positive predictive value of the assay when used on serum collected from a clinically healthy individual within a population where the true clinical prevalence is <1%.

Alternatively, the use of sero-epidemiology requires simply that an association can be made with population sero-reactivity, as measured in an appropriate sample of individuals, and regions, which reflect differences in clinical prevalence of disease.

The non-parametric statistical analysis of variance that compares rank of absorbance demonstrated that for all regions sampled and tested sero-reactivity was dependent on region (Kruskal Wallis test; H = 968.1; 16 df; p = <0.001). Furthermore, when regions with known varying degrees of clinical endemicity are tested, sero-reactivity was also dependent on region (Kruskal Wallis test; H = 337.2 3 df; p = <0.001). Even when positive rates seem epidemiologically similar, as with Sydney and Port Moresby, they too were statistically significantly different (Mann-Whitney test; U = 10249.9; p = <0.001) (Figure 7.03). This highlights some points in the presentation of sero-reactive data used to infer epidemiological features.

Clearly the reporting of "sero-prevalence" without calculating true prevalence (Rogan and Gladen, 1978) can over or under estimate the true sero-prevalence. Also, simply comparing positive rates, without including 95% confidence limits can give false associations when attempting to infer shared prevalence. Further, a true representation is best achieved by comparing raw absorbance readings regardless of the cut off, as this best describes movement of IgG in the collection of samples of individuals which reflects exposure and therefore clinical risk of acquiring melioidosis in the population.

Using these statistics may aid in grouping regions with shared absorbance rank, and if within this group there resides regions of known clinical endemicity, the potential for clinical disease can be inferred in regions of unknown prevalence. A limitation of this technique is the possibility of increasing type 1 error each time an analysis is

performed resulting in false conclusions (Gerstman, 1998). But also, the selective comparisons made may bias the data towards a preferred outcome.

The use of population profile histograms enables a simple and graphical representation of the distribution of titres in a sample of a population which can be used to compare regions and therefore infer potential for clinical disease. The advantage of this semi-quantitative method is that the population profile groups are determined by absorbance boundaries set by running internal standards. Thus, any artefactual variation between runs resulting from altered incubation conditions, perhaps resulting from running the assay in different field conditions, will be controlled. Therefore, the results between regions can be compared with confidence that any variation in absorbance between regions is the result of differences in sero-reactivity in samples from the relative populations.

Histogram shape resulting from the analysis of samples from regions of known clinical endemicity can be defined (Table 7.05). Individuals from regions of unknown melioidosis endemicity can be sampled and tested and can be be grouped according to these definitions (Table 7.06). Assumptions can therefore be made regarding risk of acquiring melioidosis and these can be tested with screening programs. Clearly, the definitions of risk localities is relatively arbitrary and only through further detailed clinical screening would this association be verified.

Although these findings suggest the Aramia flood plain region of the Balimo district is substantially more melioidosis-endemic than other regions in PNG, further study would need to be undertaken in contrasting regions to further corroborate these results.

CHAPTER 8 - MELIOIDOSIS IN PORT MORESBY: A PROSPECTIVE STUDY

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8.1   Introduction

Past evidence of melioidosis endemicity in PNG has been based on reports from Port Moresby, the urban capital (Egerton, 1963a; Egerton, 1963b; Rampling, 1964; Rowlands and Curtis, 1965; De Buse *et al.*, 1975; Lee and Naraqi, 1980; Barnes *et al.*, 1991; Currie, 1993). Clearly these reports have been sporadic and rare. The sero-epidemiology and biogeography analysis undertaken earlier (Chapters 4 and 7) would seem to corroborate this observation.

Not withstanding this, the difficulty of diagnosing melioidosis within a TB endemic region has been highlighted in the past (Puthucheary *et al.*, 1992) and it is noted with interest that a number of melioidosis cases in Balimo have been associated with a TB diagnosis (Chapter 3).

A clinical screening program was established at Port Moresby, both in the clinical microbiology laboratory and the Central Public Health laboratory, to determine if melioidosis is apparent in Port Moresby, particularly the TB patient cohort. This was undertaken for two reasons:

1.   To test the hypothesis that melioidosis is present in undiagnosed TB patients throughout PNG (using the urban melting pot of Port Moresby as the sample) and contributes to treatment-refractory TB;

2.   To determine, in a specific prospective study, the true clinical incidence of melioidosis in Port Moresby and use this data to corroborate or otherwise the national sero-epidemiological and biogeographical investigation.

8.2   Materials and Methods

8.2.1   The study centres

Two laboratories were chosen to establish the screening programs: Port Moresby General Hospital Pathology, Microbiology department (POMGHP) and the Central

Public Health Laboratory (CPHL). The POMGHP study was conducted between 6 October 2000 to 1 June 2001 and the CPHL study was conducted between 11 February 2002 to 5 August 2002. Procedures and protocols were discussed with relevant staff and directors and managers of the laboratories and clinical staff. Initial training was provided, then the study was allowed to run its course without any further intervention. Although, as the selective culture procedures of the CPHL project departed significantly from routine duties, a local Medical Laboratory Assistant (MLA) was specifically placed in the laboratory to undertake this work.

8.2.2 Samples and procedures

The study was laboratory-based to eliminate clinical acumen as a bias. All screening procedures have been previously described (Chapter 3) and are based on selective cultivation of *B. pseudomallei* described by Ashdown (Ashdown, 1979c). Identification protocols have also been previously described and are based on the simple test algorithm of Dance *et al.* (Dance *et al.*, 1989c) which have been demonstrated to provide adequate accuracy (Chapter 5).

The sample types chosen for analysis at the POMGHP included all sputum, joint and body fluids, abscess pus and blood cultures. In the CPHL all sputum samples sent for TB investigation were selectively cultured for *B. pseudomallei* prior to decontamination. The samples were initially placed in Ashdown selective broth (Appendix 1) as per enrichment methods used in earlier studies which were found to increase recovery of respiratory samples (Wuthiekanun *et al.*, 1990; Wuthiekanun *et al.*, 2001). After 48 hr incubation, the broth was subcultured onto ASH and incubated for up to 3 days. Samples directly cultured onto ASH (all samples processed in the POMGHP) were checked daily over a 72 hr incubation period for typical colonies.

8.3   Results

8.3.1   Port Moresby General Hospital, Pathology Laboratory project

Over the study period a total of 267 body fluid samples were screened and 2,285 blood cultures. Of the body fluid samples received for analysis, 57% were sputum, 13% pleural fluid, 11% peritoneal or other abdominal fluid, 7% knee or other joint aspirate, 3% undefined aspirates, 2% pericardial aspirates and <1% each of various organ fine needle aspirates, including liver and kidney. None of these samples demonstrated *B. pseudomallei* upon selective culture.

Of the 2,285 blood culture samples analysed, two yielded *B. pseudomallei*, one patient of whom the blood culture was collected and tested positive also had a urine sample submitted which was also culture positive. In total, 0.09% (95% CI 0.01 - 0.32) of all blood cultures submitted for analysis yielded *B. pseudomallei*.

Table 8.01   Number and type of samples selectively cultured for *B. pseudomallei* at the POMGH Pathology 6.10.00 - 1.6.01

Port Moresby General Hospital Pathology Laboratory Project				
Sample Type	**Number Tested**	**Positive for** *B. pseudomallei*	**Percent Positive**	**95% Confidence Limits**
Tracheal	8	0	0	0 - 31.2
Sputum	153	0	0	0 - 1.9
Abscess pus	4	0	0	0 - 52.7
Pleural effusion	35	0	0	0 - 8.2
Pericardial aspirate	4	0	0	0 - 52.7
Organ FNA	4	0	0	0 - 52.7
Knee and other joints	19	0	0	0 - 14.6
Peritoneal asp	36	0	0	0 - 8.0
Unspecified asp	12	0	0	0 - 22.1
Blood	2285	2	0.09	0.01 - 0.32

Given that there are on average approximately 2,567 blood cultures submitted for analysis to the clinical microbiology laboratory at Port Moresby (data kindly supplied by Clement Manesuia, Microbiology department, PMGH), approximately 0 to 8 cases of melioidosis each year would be expected.

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8.3.2 Central Public Health Laboratory project

Over the study period a total of 1309 samples of sputum from 529 patients were selectively cultured for *B. pseudomallei*. During this time one sample, the second submitted by the patient, demonstrated *B. pseudomallei* when selectively cultured. Given there are approximately 1,899 new cases of TB diagnosed each year in POM without microbiological evidence (Anon, 2000a) and given, in this study, 0.24% (95% CI 0.01 - 1.3) of all patients tested TB slide negative were positive for melioidosis, it can be expected that between less than one and up to 24 cases of treatment-refractory slide negative TB may be undiagnosed melioidosis (Table 8.02).

Table 8.02   Number of sputum samples and patients selectively cultured for
B. pseudomallei, including data on TB in Port Moresby (Anon, 2000a)

Category	Numbers / Percent	95% Confidence Limits
Total number of samples	1309	
Total number of patients	529	
Number patients TB slide pos	112	
Number patients TB slide negative	417	
Number positive melioidosis cases	1	
Percent patients in TB slide neg cohort Positive for melioidosis	0.24	0.01 - 1.3
Yearly average newly detected TB cases slide negative in POM*	1899	
Predicted annual number of melioidosis cases in TB slide negative cohort in POM	4.6	0.19 - 24.6

Although at the time of diagnosis the relevant clinician was notified, of the three individuals whose samples demonstrated *B. pseudomallei* (Table 8.03), only one case history was able to be recovered retrospectively (MG) and will be detailed below. The clinic records of Case WM were able to be found and the patient was traced to Kila Kila settlement (a urban settlement frequented by Gulf and Western province expatriates) but an extensive search of this settlement failed to locate the patient or his relatives (uncertainty of his correct name exacerbated this difficultly). It is assumed that the patient was visiting POM from the Gulf province and was a relatively recent visitor to POM. Speculation from local clinic staff and those that knew of him suggest he was sent back home to die as a result of TB treatment failure. No details of case HB

are known apart from the details received on the pathology sample and are listed in Table 8.03.

Table 8.03   Case details from POM

POM Cases			
Date	Case	Isolate No	Sex / Age
7.5.01	HB (blood culture)	POM1	M/50
7.5.01	HB (urine)	POM2	
1.6.01	MG	No isolate*	M/50+
11.3.02	WM (sputum)	POM3	M/48

* isolate inadvertently discarded

Case MG was a male adult above 50 years of age who had been living in Morata (outskirts of POM) since early 1970s. His origins were the Mumeng subdistrict Morobe province, southwest of Lae. He worked as a labourer for a local Waigani general practitioner where his duties included maintenance of the facilities. He developed gardens at and near his residential address.

He is described as being a regularly sick individual with diabetes mellitus (DM) and was admitted to POMGH 1.6.01 with fever and a persistent ulcer on his left foot. He had a left leg abscess which was incised and drained 27.5.01. His pulse was 82/min and blood pressure 180/100. He had hepatomegaly and general abdomen distention. Ultrasound of his abdomen however was normal. He was pale and confused and uncooperative. He was admitted and precribed chloramphenicol IV QID with a diagnosis of septic pyomyositis secondary to DM. Initial pathology demonstrated an Hb of 8.8 g/dl with MCV 80 fl, total WBC 12.5×10^6/l and random blood glucose of 8.8 mmol/l but which fluctuated between 2.1 - 12.3 mmol/l during his admission.

His fever persisted despite chloramphenicol and antimalarial treatment. Liver function tests revealed albumin 23, ALP 430, ALT 74 and AST 188. He become afebrile on day 5 and remained afebrile until day 10 until the abscess on his right leg, which had developed since his admission, was incised and drained. He become febrile on day 11 and died later that evening.

Cases HB and MG of the POMGHP study appeared during a year of above average rainfall just after the average peak in March (Figure 9.01).

Monthly Rainfall Port Moresby
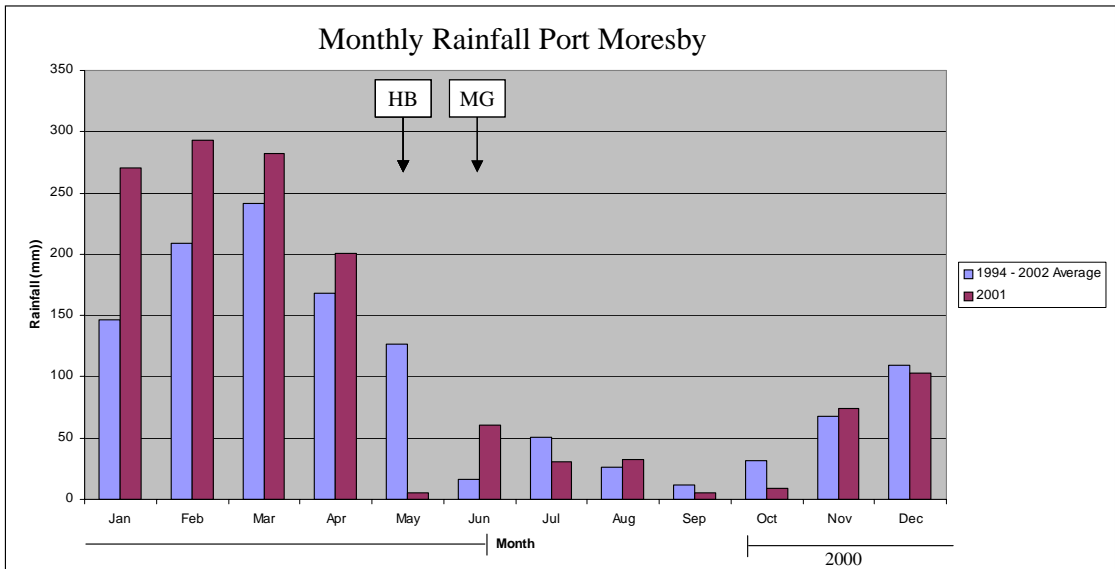
Figure 8.01   Monthly rainfall Port Moresby, yearly average (1994 - 2002) and 2001.Scale below X axis indicates POMGHP study time frame and arrows indicate presentation of case

In contrast case WM was discovered during a year of below average rainfall, although during the wettest month (Figure 8.02).

Monthly Rainfall Port Moresby
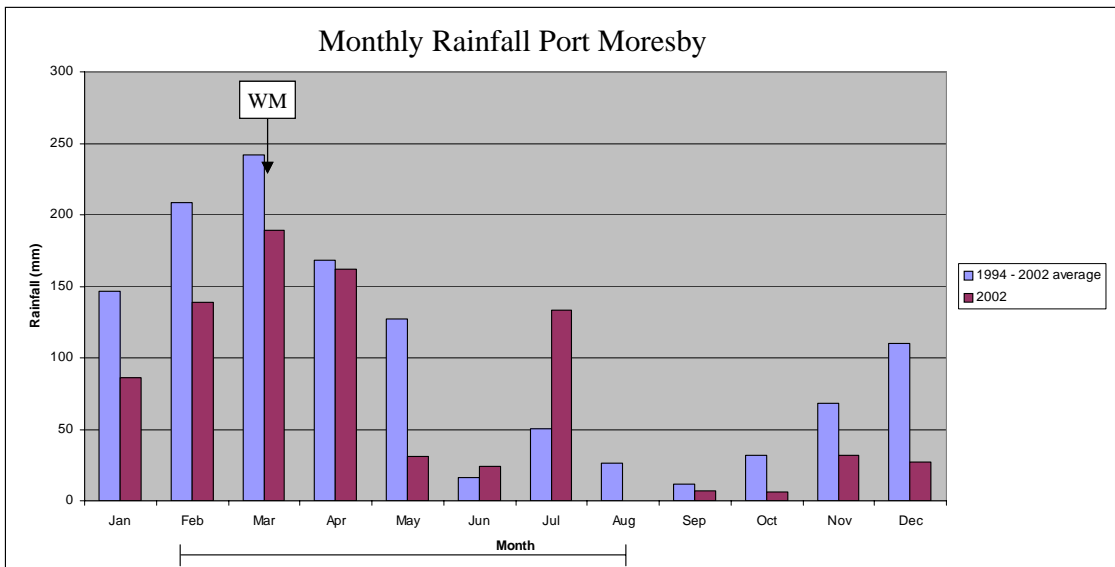
Figure 8.02   Monthly rainfall Port Moresby, yearly average (1994 - 2002) and 2002. Scale below X axis indicates CPHL study time frame and arrows indicate presentation of case

8.4   Discussion

This study detected three cases of melioidosis in the POM region over a period of approximately 14 months. A study period where samples from 3081 patients were cultured. If this is an accurate representation of the prevalence of melioidosis, clearly this evidence supports past studies which have concluded that melioidosis is rare in Port Moresby, particularly when compared to Northern Australia (Rowlands and Curtis, 1965; De Buse *et al.*, 1975; Lee and Naraqi, 1980; Barnes *et al.*, 1991; Currie, 1993). Also, these results corroborate the sero-epidemiolology (Chapter 7) and biogeography (Chapter 4) data that Port Moresby is a low melioidosis- risk region.

Although, if history is a guide, these cases would not have been identified without the specific melioidosis selective culture and identification protocol intervention provided by the study. Also, the study does demonstrate that melioidosis is mis-diagnosed as slide-negative TB and raises the issue of melioidosis in treatment-refractory, slide-negative TB in PNG. This case would not have been demonstrated without the specific intervention of this study. Further, within the 95% confidence limits of this analysis, it is possible that the incidence of melioidosis in Port Moresby is similar to Townsville (Norton, 2003). Although the numbers may be small, perhaps melioidosis investigation and empirical treatment needs to be considered in patients presenting with slide-negative, treatment-refractory TB in PNG.

Given the strong wet season predilection seen elsewhere in the region (Currie and Jacups, 2003), it is reasonable to speculate that the extent of rainfall is also a factor in Port Moresby. Cases HB and MG were diagnosed during a year of above average annual rainfall at the end of the wet season (Figure 8.01). Repeating the study with a commercial blood culture system and ensuring adequate blood volume is cultured, may increase isolation rates (Hall *et al.*, 1976). Also, as soft tissue lesions were not included for selective culture in the study, there is a chance that melioidosis ulcers were not identified.

The case WM presented with the DM and chronic liver disease, two of the co morbidity factors for melioidosis frequently encountered in endemic regions

throughout the world (Suputtamongkol *et al.*, 1999; Currie *et al.*, 2000d). It is interesting to speculate how the increasing burden of diabetes in PNG (Ogle, 2001) will select out the seemingly isolated reservoir of *B. pseudomallei* from the environment in POM, manifesting in an increased incidence of melioidosis in this region. Regardless, melioidosis should be considered in patients presenting with sepsis secondary to diabetes in PNG so appropriate antimicrobial therapy is considered, particularly during and just after the wettest months of the year (Currie and Jacups, 2003).

Past reports of animal melioidosis in POM have implicated a single farm as a source of infection, resulting in both bovine and porcine infection (Egerton, 1963a; Rampling, 1964). It was noted that cattle have been imported from North Queensland. It is interesting to speculate that animal imported melioidosis was the source of a stable clone of *B. pseudomallei* that has persisted over time, resulting in isolated outbreaks as is the case in southern Western Australia and elsewhere (Ketterer *et al.*, 1975; Ketterer *et al.*, 1986; Golledge *et al.*, 1992; Currie *et al.*, 2000e). Clearly, molecular epidemiology and phylogenetic analysis of isolates derived from POM, Balimo, the Torres Strait and mainland Queensland, is required to further elucidate the origins and movement of melioidosis in PNG and throughout the region.

CHAPTER 9 - FINAL DISCUSSION

The primary objective of this study was to demonstrate the extent to which melioidosis contributes to the disease burden in the Balimo district of PNG. To that extent autochthonous melioidosis has been demonstrated in the Balimo district of PNG and diagnosis and treatment protocols have been established which have decreased case fatality rates. Autochthonous melioidosis seems to explain the fatal Balimo PUO syndrome previously documented (Reece, 1984). The fact that this study has participated in saving the lives of at least six children within this rural community is its greatest contribution, particularly as the study's context was laboratory capacity building and not primarily a clinical initiative. It again demonstrates the importance of laboratory capacity in the diagnosis of melioidosis where clinical awareness is lacking.

The epidemiology of melioidosis in this region predisposes children because they are more likely than adults to acquire the organism through contact with the reservoir of infection, which seems to be the points of land leading to the lagoon where they wash. Their robust play, with or without exposed lesions in these regions will enable primary infection. In terms of disease manifestation, an inability to mount an adequate CMI response may be involved (Barnes *et al.*, 2004) as might the co-morbidity of parasite co-infection or malnutrition. Regardless, it could be expected that if the traditional co-morbidity factors of diabetes and liver and renal disease were to become apparent in this region, the incidence of melioidosis would increase and adult infection would be more common.

The sero-epidemiology data supports the clinical findings and speculation that the Sanebase point of Adiba village is a hot spot for autochthonous melioidosis within the district, although further and more extensive sampling may expand and redefine this further. Regardless, this village may provide a model for further analysis of the environmental microbiology of melioidosis.

Of particular interest is the significance of the narrow genetic diversity of *B. pseudomallei* in the region (Chapter 6). This indeed may represent a short evolutionary history or simply an inability of the techniques used to discriminate differences between isolates. But also, perhaps *B. pseudomallei* has selected out a

preferred niche including susceptible hosts and a means with which to cycle back into the environmental through the use of pit toilets. Further sampling and molecular epidemiology will elucidate this further.

Analysis of the environment using the PNGRIS suggests that the Balimo region possesses unique attributes within PNG (Chapter 4). Whether this represents biogeographic boundaries for the environmental reservoir for melioidosis remains to be tested. Clearly both the geographical and clinical association with the Torres Strait (Faa and Holt, 2002) remains to be elucidated and would provide a interesting biogeographical study (Cho and Tiedje, 2000; Fenchel, 2003). It is speculation at this stage to associate a shared primordial continental relationship in ancient Gondwanaland to endemic melioidosis in southwestern PNG and southeastern Australia. Perhaps also involved is the enormous extent of the Fly river delta runoff into the Gulf of Papua and the Torres Strait in the dispersal of *B. pseudomallei* from a primordial reservoir at Balimo throughout the region.

Although it seems possible that alterations in the treatment protocols for sepsis and pneumonia may assist in arresting the high cases fatality rates of melioidosis in the region (Chapter 3) (Anon, 1993; Anon, 1997), prevention remains problematic. Perhaps an interesting approach would be to investigate the relationships between the diversity of PNG-derived *B. thailandensis* organisms recovered from this region and their interactions with the virulent *B. pseudomallei*. Early observations suggest there is antagonism at play (J. Eliman, School of Biomedical Science, JCU unpublished data) and although problematic may be used as either bioremediation or other forms of bacteriocin mediated control (van Veen *et al.*, 1997; Cain *et al.*, 2000; Adhikari *et al.*, 2001; Kent and Triplett, 2002; Raaijmakers *et al.*, 2002; Bano and Musarrat, 2003).

As the sero-epidemiological data corelates with both the clinical and biogeographical data gathered to date, this supports its use as a tool to identify melioidosis at-risk communities. It may be used in the future to aid in further defining the extent of melioidosis prevalence in the region and in providing evidence to support the establishment of screening programs, particularly where health resources are scarce.

Finally, although the prevalence of melioidosis in Port Moresby does seem genuinely low, given the differences of health care quality and availability between PNG and Australia are enormous, it seems inappropriate to suggest, based on past, mostly expatriate influence that melioidosis prevalence is lower in PNG than it is in northern Australia. Clearly a failure to investigate melioidosis in slide negative, treatment-refractory TB patients in PNG, due to both a lack of clinical awareness and laboratory capacity, will artefactually decrease melioidosis incidence in PNG. These deficiencies are endemic and widespread throughout PNG. It is likely that the true prevalence of melioidosis in Port Moresby is similar to that experience in north southeastern Australia. Equally, it is inappropriate to suggest that all communities in PNG share similar at-risk status. The work underlies apparent regional clustering that is common to melioidosis endemic regions world wide. Searching further with the tools established in this study may further determine the extent of the iceberg that is undiagnosed melioidosis in this region. This may lead to the early diagnosis of melioidosis and subsequently lead to decreased fatality rates of pneumonia and sepsis.

APPENDICES

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APPENDIX 1

BACTERLOGICAL MEDIA AND REAGENTS

Brain Heart Infusion Broth (Blood Culture Broth)

Brain heart Infusion Broth base (Micro diagnostics, New Farm, QLD)	37.0 g
Distilled water	1,000 ml

Add and dissolve base in small amount of distilled water with gentle heat. Top up to 1,000 ml. Add appropriate volume to appropriate sized glass bottles and autoclave at 121°C for 15 min.

5% Sheep Blood Agar

Blood Agar Base (Oxoid Australia, Heidelberg, Victoria)	40.0 g
Distilled water	1,000 ml

Mix and dissolve agar base. autoclave at 121°C for 15 min and cool to 56°C. To 1000 ml add 50 ml of citrate anticoagulated sheep blood. Cool and pour.

5% Chocolate Blood Agar

Blood Agar Base (Oxoid Australia, Heidelberg, Victoria)	40.0 g
Distilled water	1,000 ml

Mix and dissolve agar base. Autoclave at 121°C for 15 min and cool to 56°C. To 1,000 ml add 50 ml of citrate anticoagulated sheep blood. Gently heat until red cells are lysed. Cool and pour.

MacConkey Agar

MacConkey Agar (Oxoid Australia, Heidelberg, Victoria)	51.5 g
Distilled water	1,000 ml

Mix, dissolve, autoclave at 121°C for 15 min.. Cool and pour.

1% Aqueous Neutral Red

Neutral red (Sigma-Aldrich, Castle Hill, NSW)	1 g
Distilled water	100 ml

Mix and dissolve.

0.1% Crystal Violet

Crystal violet (Sigma-Aldrich, Castle Hill, NSW)	0.1 g
Distilled water	100 ml

Mix and dissolve.

Ashdown's Selective Agar (8mg/L gentamicin)

Tryptone (Oxoid Australia, Heidelberg, Victoria)	12.0 g
Technical Agar #3 (Oxoid Australia, Heidelberg, Victoria)	12 g
1% aqueous neutral red	4 ml
0.1% crystal violet	4 ml
Glycerol (Sigma-Aldrich, Castle Hill, NSW)	32 ml
Distilled water	800 ml
Gentamicin sulphate (50 mg/ml injectable, Troy Labs, Smithfield, NSW)	128 µl

Dissolve tryptone and agar in distilled water. Add neutral red crystal violet and glycerol. Autoclave at 121°C for 15 min.. Cool, add gentamicin aseptically and pour.

Ashdown's Selective Broth (environmental broth, 50 mg/l colistin)

Tryptone (Oxoid Australia, Heidelberg, Victoria)	15 g
0.1% crystal violet	5 ml
Distilled water	995 ml
Glycerol (Sigma-Aldrich, Castle Hill, NSW)	40 ml
Colistin Sulfomethate Sodium (150 mg/2 ml, Pfizer, West Ryde, NSW)	528 µl

Dissolve tryptone in distilled water. Add crystal violet and glycerol. Autoclave at 121°C for 15 min.. Cool, add colistin aseptically. Transfer 20 ml volumes aseptically to sterile Macartney bottles.

Ashdown's Selective Broth (clinical broth, 5 mg/l colistin)

Tryptone (Oxoid Australia, Heidelberg, Victoria)	15 g
0.1% crystal violet	5 ml
Distilled water	995 ml
Glycerol (Sigma-Aldrich, Castle Hill, NSW)	40 ml
Colistin Sulfomethate Sodium (150 mg/2 ml, Pfizer, West Ryde, NSW)	53 µl

Dissolve tryptone in distilled water. Add crystal violet and glycerol. Autoclave at 121°C for 15 min.. Cool, add colistin aseptically. Transfer 20 ml volumes aseptically to sterile Macartney bottles.

Minimal Salt Solution

Ammonum Chloride (Sigma-Aldrich, Castle Hill, NSW)	20 g
Ammonium nitrate (Sigma-Aldrich, Castle Hill, NSW)	4 g
Anhydrous sodium sulphate (Sigma-Aldrich, Castle Hill, NSW)	8 g
Dipotassium hydrogen orthophosphate (Sigma-Aldrich, Castle Hill, NSW)	12 g
Potassium dihydrogen orthophosphate (Sigma-Aldrich, Castle Hill, NSW)	4 g
Magnesium sulphate (Sigma-Aldrich, Castle Hill, NSW)	0.4 g
Distilled water	1,000 ml

10% L-arabinose

L-arabinose (Sigma-Aldrich, Castle Hill, NSW)	10 g
Distilled water	100 ml

Add carbohydrate and heat gently to dissolve.

10% Glucose

Glucose (Sigma-Aldrich, Castle Hill, NSW)	10 g
Distilled water	100 ml

Add carbohydrate and heat gently to dissolve.

2% Agar

Technical Agar #3 (Oxoid Australia, Heidelberg, Victoria)	2 g
Distilled water	100 ml

Add and dissolve agar. Autoclave at 121°C for 15 min.

L-Arabinose Single Carbohydate Agar

Minimal salt solution	25 ml
2% agar	75 ml
10% L-arabinose	2 ml

Each solution was prepared separately before mixing. The 2% agar solution was autoclaved. The minimal salt solution and carbohydrate was filter sterilised.

Glucose Single Carbohydrate Agar

Minimal salt solution	25 ml
2% agar	75 ml
10% Glucose	2 ml

Each solution was prepared separately before mixing. The 2% agar solution was autoclaved. The minimal salt solution and carbohydrate was filter sterilised.

Tryptone Soya Agar

Tryptone Soya Agar (Oxoid Australia, Heidelberg, Victoria)	40 g
Distilled water	1,000 ml

Mix and dissolve. Autoclave at 121°C for 15 min. Cool and pour.

0.9% Normal Saline

NaCl (Sigma-Aldrich, Castle Hill, NSW)	9 g
Distilled water	1,000 ml

Oxidase Reagent

Tetramethl-*p*phenlenediamine dihydrochloride (Sigma-Aldrich, Castle Hill, NSW)	0.1 g
Distilled water	10 ml

Dissolve reagent in distilled water. Cover vessel with foil. Discard when discoloured or when quality control fails.

APPENDIX 2

PCR REAGENTS PROTOCOLS

TE (Tris-HCL / EDTA) Buffer

Tris-HCL (pH 7.8) (Crown Scientific, Garbutt, QLD)	10 mM
EDTA (Sigma-Aldrich, Castle Hill, NSW)	5 mM

Digestion Buffer

TE buffer	576 µl
Proteinase K (Progen Biosciences, Darra, QLD)	200 µg/ml
Tween 20 (Sigma-Aldrich, Castle Hill, NSW)	0.5% (w/v)

PCR 10× Reaction Buffer

Tris-HCL (pH 8.8)	670 mM
$(NH_4)_2SO_4$ (Sigma-Aldrich, Castle Hill, NSW)	166 mM
Triton X-100 (Bioscientific, Gymea, NSW	4.5% (w/v)
Gelatin (Crown Scientific, Garbutt, QLD)	2 mg/ml

TTS PCR Protocol

Reagent	Volume (μl)	Final Concentration
Template DNA	2.5*	50 - 100 ng
DNTP (5 nM)	1	200 μM
Taq (5 U/μl)	0.1	1 U/25 μl
Buffer (10×)	2.5	1×
MgCl$_2$ (25 μM)	2.0	2.0 mM
BpTTSR (25 μM) 5'-GAGGACGGTTTGGGACGAA	0.5	0.5 μM
BpTTSF (25 μM) 5-'GTTCAATCTGCTCTTTCCGTT	0.5	0.5 μM
Double distilled water	16.4	
Total volume	25	

16S Multiplex PCR Protocol

Reagent	Volume (μl)	Final concentration
Template DNA	2.5*	50-100 ng
DNTP (5 mM)	1	200 μM
Taq (5.5 U/μl)	0.1	1 U/25 μl
Buffer (10×)	2.5	1×
MgCl$_2$ (25 mM)	1.5	1.5 mM
16S-42L (25 μM) 5'-CGGCAGCTCGGGCTTCGG	0.5	0.5 μM
16S-266R (25 μM) 5'-TGTGGCTGGTCGTCCTCTC	0.5	0.5 μM
16S-427R (25 μM) 5'-CACTCCGGGTATTAGCCAGA	0.5	0.5 μM
Double distilled water	16.4	
Total volume	25	

* final volume of template is DNA concentration dependent

Thermal Cycling TTS and 16S PCR

1. Initial denaturation: 94°C 4 min

2. Denaturation: 94°C 50 sec

3. Anneal: 60°C 50 sec

4. Extension: 72°C 1 min

5. Repeat steps 2-4 35 times

6. Final extension: 72°C 10 min

7. Soak cycle: 4°C

APPENDIX 3

PULSED FIELD GEL ELECTROPHORESIS BUFFERS AND REAGENTS

PET IV

Tris-HCL (pH 8.0)	10 μM
NaCl (Sigma-Aldrich, Castle Hill, NSW)	20 μM
EDTA (Sigma-Aldrich, Castle Hill, NSW)	100 μM

Lysis Buffer

Tris-HCL (pH 8.0)	10 μM
EDTA (Sigma-Aldrich, Castle Hill, NSW)	100 μM
Brij-58 (Bioscientific, Gymea, NSW)	0.5% (v/v)
Sodium deoxycholate (Sigma-Aldrich, Castle Hill, NSW)	0.2% (w/v)
Sodium lauryl sarcosinate (N-lauroylsarcosine)	0.5% (w/v)
(Sigma-Aldrich, Castle Hill, NSW)	

TBE (10×)

Tris base (Crown Scientific, Garbutt, Qld)	216 g
Boric acid (Sigma-Aldrich, Castle Hill, NSW)	110 g
EDTA (0.5 M)	80 ml

Make up to 2 l with distilled water. Filter (0.22 μm) to prevent precipitation. Store in separate 0.5 l Schott bottles.

Low melting point agarose (50 ml)

Nusieve GTG agarose (BioWhittaker Molecular Application) (DKSH Australia Pty. Ltd. Hallam, VIC)	0.75 g
SeaKem Gold agarose (BioWhittaker Molecular Application) (DKSH Australia Pty. Ltd. Hallam, VIC)	0.25 g
PET IV buffer	50 ml

APPENDIX 4

IHA ANTIGEN PREPARATION REAGENTS

IHA protein free media

Asparagine (Sigma-Aldrich, Castle Hill, NSW)	3.5 g
KH_2PO_4 (Sigma-Aldrich, Castle Hill, NSW)	1.0 g
Ammonium citrate (Sigma-Aldrich, Castle Hill, NSW)	1.0 g
$MgSO_4$ (Sigma-Aldrich, Castle Hill, NSW)	0.5 g
Ferric ammonium citrate (Sigma-Aldrich, Castle Hill, NSW)	1.0 g
Sodium citrate (Sigma-Aldrich, Castle Hill, NSW)	1.0 g
Dextrose (Sigma-Aldrich, Castle Hill, NSW)	5.0 g
Glycerol (Sigma-Aldrich, Castle Hill, NSW)	35 ml
Distilled water (up to)	500 ml

APPENDIX 5

PRELIMINARY RESULTS OF FURTHER MOLECULAR TYPING OF *BURKHOLDERIA PSEUDOMALLEI* DERIVED FROM PNG

DNA Macro restriction analysis using *XbaI* digests does not discriminate isolates within a single *Spe1* digest genotype

(Rai, G., Lazzaroni, S. and Warner, J.M.. 2004 in preparation for publication)

The following images represent *XbaI* digest macro restriction analysis of the epidemiologically implicated *B. pseudomallei* isolates described in Chapter 6. *XbaI* digest does not enable discrimination between *SpeI* digest macro restriction analysis of these isolates.

These are interim results demonstrating suboptimal gels. Upon repair of equipment the procedure will be repeated to obtain optimal results.

Lane	Isolate
1	MWM
2	C1
3	C2
4	C3
5	C4
6	MWM
7	A67
8	A78
9	K33
10	K141
11	MWM

Lane	Sample
1	MWM
2	C5
3	C6
4	C7
5	C8
6	MWM
7	K41
8	K93
9	K113
10	C9
11	C10
12	MWM
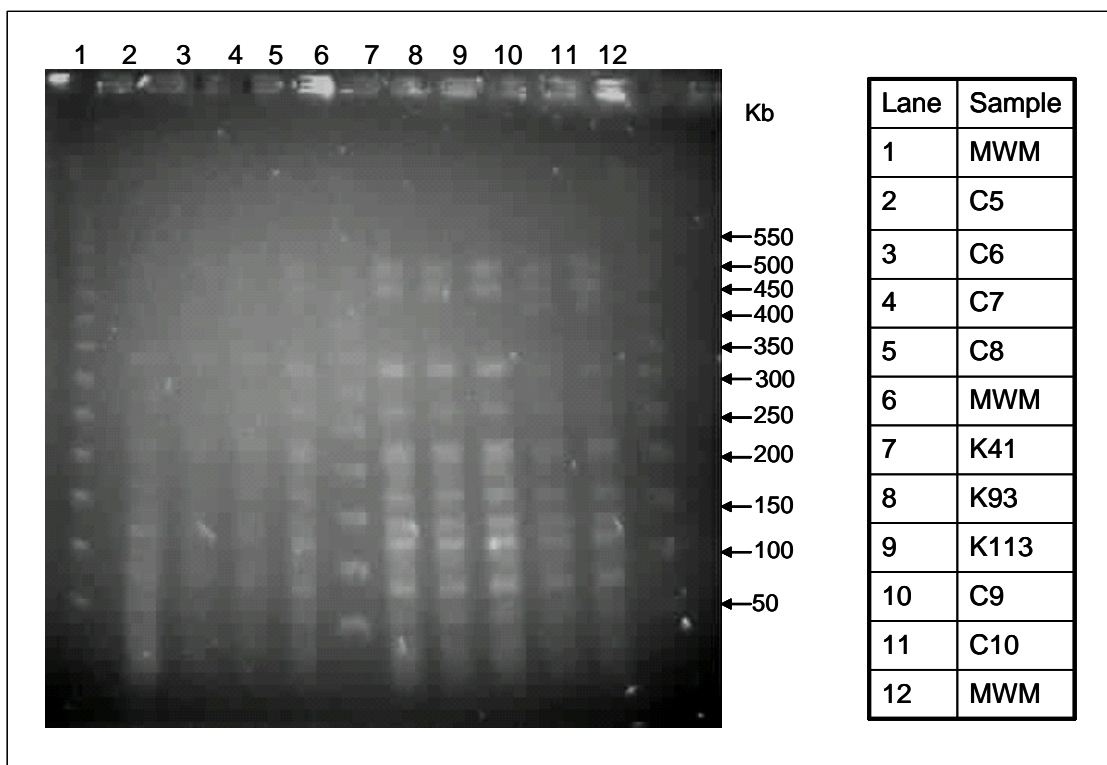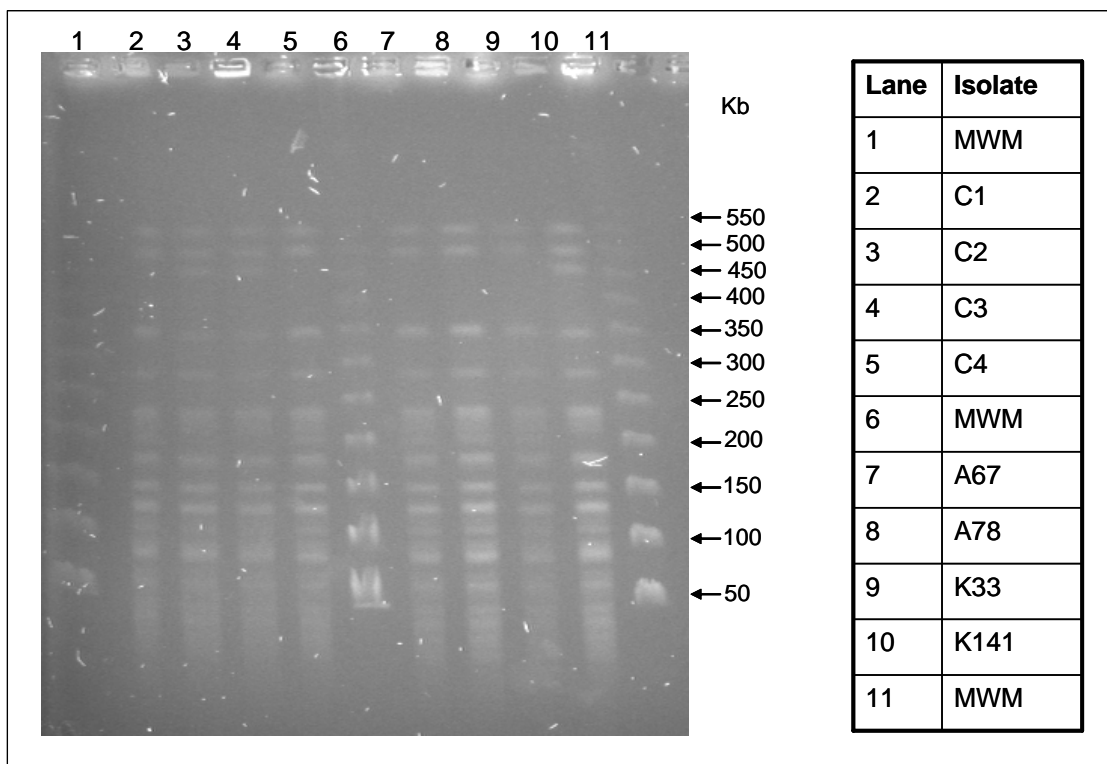
RAPD PCR using five arbitrary primers does not discriminate isolates within a single macro restriction genotype demonstrated by *Xbal* and *Spe1* digests using PFGE.

(Rai,G., Govan, B. and Warner, J.M. 2004 in preparation for publication)

The following images represent RAPD PCR analysis of the PNG derived *B. pseudomallei* isolates which SpeI digest macro restriction analysis failed to discriminate (chapter 6). The results demonstrate that RAPD PCR is unable to further discriminate the epidemiologically associated collection of isolates. After analysis, the only isolate to demonstrated divergence was K93 which is an environmental isolate unrelated to this group.

Primer	Sequence (5′to 3′)
A (208)	ACG GCC GAC C
B (228)	GCT GGG CCG A
C (272)	AGC GGG CCA A
D (277)	AGG AAG GTG C
E (287)	CGA ACG GCG G

Primer A

Lane	Sample
1	MWM
2	C1
3	C2
4	C3
5	C4
6	A67
7	A78
8	K33
9	K41
10	K93
11	K113
12	K141
13	Control
14	MWM

Primer B

Lane	Sample
1	MWM
2	C1
3	C2
4	C3
5	C4
6	A67
7	A78
8	K33
9	K41
10	K93
11	K113
12	K141
13	Control
14	MWM

Primer C

Lane	Sample
1	MWM
2	C1
3	C2
4	C3
5	C4
6	A67
7	A78
8	K33
9	K41
10	K93
11	K113
12	K141
13	Control
14	MWM

Primer D

Lane	Sample
1	MWM
2	C1
3	C2
4	C3
5	C4
6	A67
7	A78
8	K33
9	K41
10	K93
11	K113
12	K141
13	Control
14	MWM

Primer E

Lane	Sample
1	C1
2	C2
3	C3
4	C4
5	A67
6	A78
7	K33
8	K41
9	K93
10	K113
11	K141
12	Control
13	MWM

Primer C

Lane	Sample
1	MWM
2	C1
3	C2
4	C3
5	C4
6	A67
7	A78
8	K33
9	K41
10	K93
11	K113
12	K141
13	Control
14	MWM

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Ahmed, K., Enciso, H.D., Masaki, H., Tao, M., Omori, A., Tharavichikul, P. and Nagatake, T. (1999) Attachment of *Burkholderia 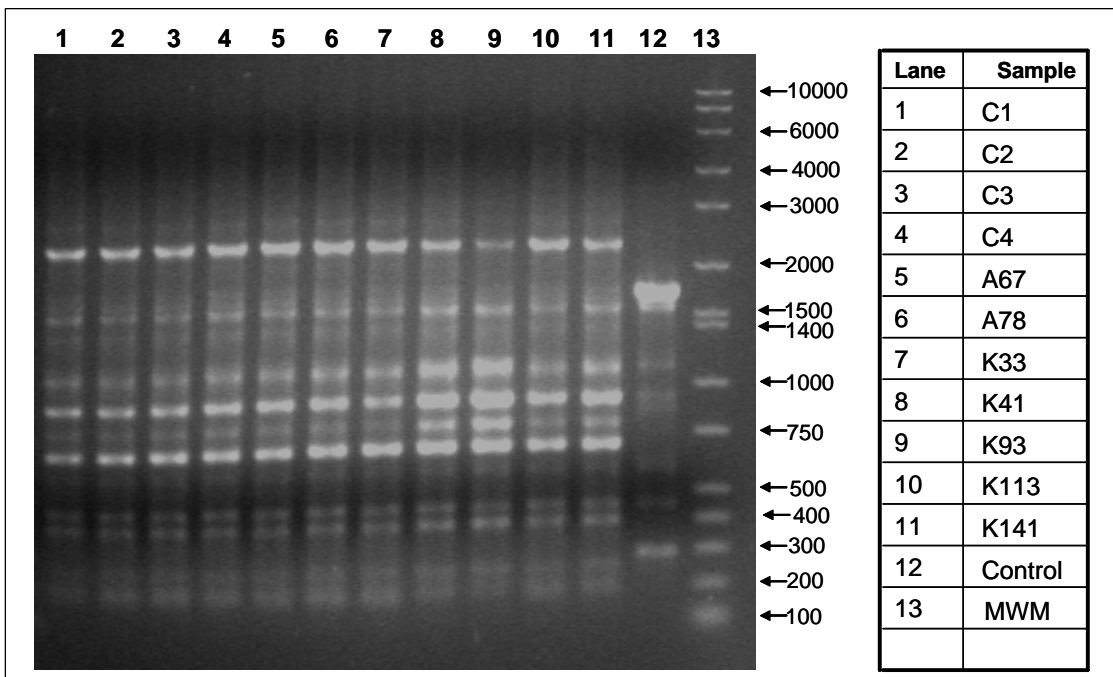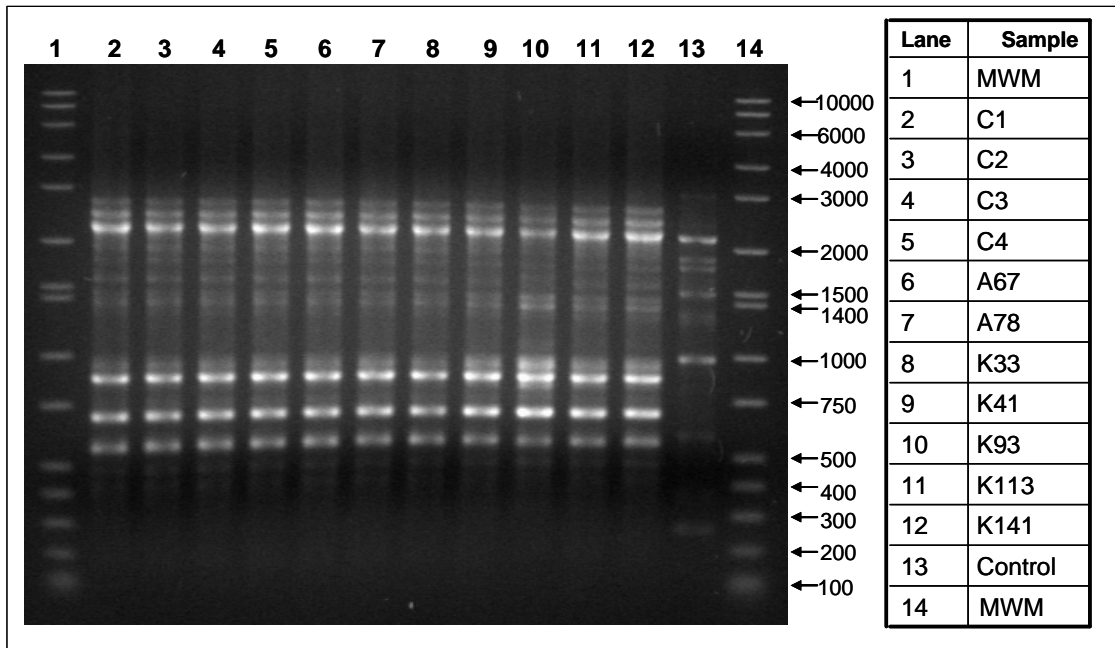pseudomallei* to pharyngeal epithelial cells: a highly pathogenic bacteria with low attachment ability. *Am J Trop Med Hyg* **60**: 90-3

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