SECTION C: UNDERSTANDING THE EPIDEMIOLOGY OF MELIOIDOSIS

The epidemiology of melioidosis has been reviewed in chapters 1 and 2. In this section, the contributions of environmental and bacterial factors to epidemiology are explored further. A geographical information system was created to explore the epidemiology of melioidosis in the Top End; the raw data is depicted visually in Appendix A.

Two outbreaks had been linked to contamination of drinking water supplies in remote communities [1, 2] and potable water in an animal outbreak [3]. It was suggested that the absence or malfunction of chlorinators may have resulted in the persistence of *B. pseudomallei* in these sites. In chapter 12, a study reviewed the impact of chlorinators and drinking water pH on the observed community rates of melioidosis in the Top End region of the Northern Territory. This was reported in the *Transactions of the Royal Society of Tropical Medicine and Hygiene* [4].

In chapter 2, it had been noted that a case cluster was linked to post-cyclonic flooding in a remote community. In chapter 13, a statistical tool was used to identify case clusters in the Top End region; these were found to be linked to extreme weather events (a category 5 tropical cyclone and extensive flooding of the town of Katherine) or a contaminated environmental focus. This manuscript has been submitted to *The International Journal of Epidemiology*.

Previous small studies had suggested that strains of *B. pseudomallei* may differ in their virulence and tissue tropism [5] [6]. In chapters 14 and 15, molecular epidemiological techniques were used to explore this. Using a novel method of analysis, it was determined using pulsed field gel electrophoresis (PFGE) that strains isolated from patients with the same presentation were no more alike than strains isolated from patients with different presentations. This was confirmed using a sequence based typing method, multilocus sequence typing (MLST). In this latter study, it was found that MLST had a similar discriminatory power as PFGE, but conclusions could be drawn regarding the possible global origins and spread of *B.*

pseudomallei. These studies have been published in *Microbes and Infection* and the *Journal of Clinical Microbiology*.

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12. Chlorination and pH of drinking water do not correlate with rates of melioidosis in the Northern Territory, Australia

12.1. Introduction

Although a relationship between monsoonal rains and incident cases of melioidosis is well described, little is known about the environmental determinants of melioidosis. Prompted by two outbreaks of melioidosis with fatalities linked to contamination of the drinking water supply in remote Australian communities [1, 2], the relationship between water pH and the observed rates of melioidosis in the communities across the Top End of the Northern Territory was examined. It was theorized that an acidic environment may provide a selective advantage for this organism, favouring its growth.

Remote communities in the Northern Territory are characterized by geographical isolation. In each community, the small population is generally supplied by a single bore source which provided us with the ideal data to test this hypothesis. Local experience suggests that primary infection is the rule, with reactivation of latent disease occurring in only 3% of cases [3]. Thus, current environmental conditions would be correlated with incident disease rates.

12.2. Methods

Since 1989, a database has been prospectively maintained of patients with culture-positive melioidosis. Patient location has been noted as the address of residence at the time of presentation. We determined rates of melioidosis from the number of cases observed in each remote community over the 13-year period to June, 2002. Population statistics were derived from the mid-interval census in 1996 conducted by the Australian Bureau of Statistics. Previous work had demonstrated no spatial autocorrelation (data not shown) and thus each community was considered independent for statistical analysis.

Water supplies are generally obtained from bores into underground aquifers. Some have continuous chlorination installations; others that do not are chlorinated intermittently in the event of bacteriological failure detected by regular sampling.

Data on routine pH measurements were obtained from the Power and Water Corporation, Darwin.

Rainfall is known to influence the incidence of melioidosis [4]; data were obtained from the Bureau of Meteorology. Statistical tests were performed using Intercooled Stata 7.0. As chlorination may influence the pH of water, these variables were not independent and thus were considered separately. Ethical approval to review clinical data was obtained from the Human Research Ethics Committee of the Territory Health Services and the Menzies School of Health Research.

12.3. Results

Water supply pH data was available for 27 communities and chlorination status for 26 communities. There was no correlation between water supply pH and the crude annual incidence of melioidosis (r= -0.11, p=0.59; Figure 1). Similarly, there was no correlation between presence of a chlorinator and the incidence of melioidosis (r= -0.14, p=0.49; Figure 2). Multiple variable analysis was performed adjusting for the median rainfall; both water supply pH and chlorinators were not correlated with crude annual melioidosis rate (adjusted r^2=0.02, p=0.65 and r^2=0.02, p=0.59 respectively). As expected, the communities with chlorinators had a higher median pH (6.9 vs 5.6, p=0.003).

Figure 12-1: Crude rate of melioidosis and drinking water pH

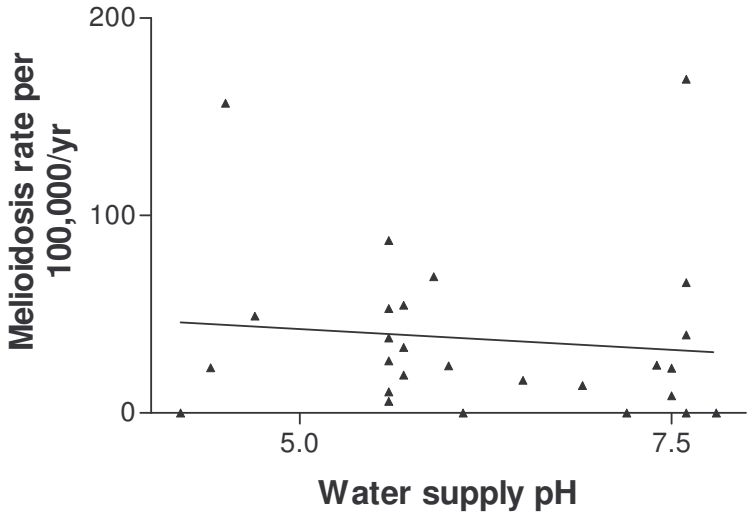
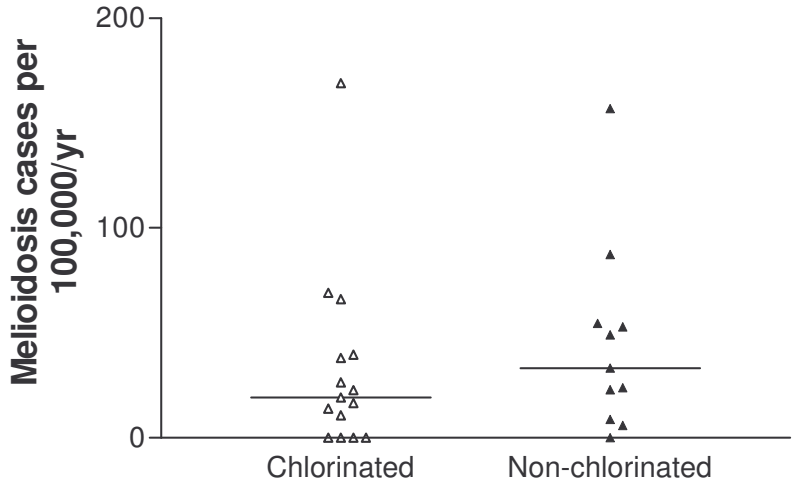


Figure 12-2: Chlorinated water supplies and melioidosis rate



12.4. Discussion

Laboratory studies have suggested that *B. pseudomallei* is tolerant to a wide range of environmental extremes including temperature, acidity [5], and nutrient restriction [6]. An outbreak in a piggery was thought to have been terminated by chlorination of the water supply [7]; *in vitro* studies of the bactericidal ability of chlorine against *B. pseudomallei* have shown conflicting results [8]. *B. pseudomallei* has been shown to have a selective advantage in soils with a low pH *in vitro* [9] and its presence in north-east Thailand has been attributed to the relatively acidic environment of the rice paddy [5, 10].

It has been assumed that the majority of cases of melioidosis arise after inoculation with contaminated mud or surface water, although the contribution of inhalation and ingestion are unknown. In the two outbreaks (15 cases; 7 fatalities), isolates grown from the drinking water supply were indistinguishable from the majority of clinical isolates by molecular techniques, in contrast to the wide genetic diversity seen in the region [1, 2]. These water supplies had relatively low drinking water pH (4.7 and 5.7); one did not have a chlorinator installed and the other had a non-functioning chlorinator.

In the Western Australian outbreak, an aerator was installed in an attempt to correct the acidic water pH, and it was believed that this may have been the site of contamination and growth of *B. pseudomallei* [11] but aerators are only used at one community within the endemic area of the Northern Territory.

A limitation of this study is our inability to control for the prevalence of host factors and behaviours important to acquiring melioidosis in each community. Limited data suggest that there are high rates of risk factors such as diabetes and renal failure in these Aboriginal communities [12].

This study did not demonstrate a relationship between drinking water pH or the use of chlorination installations and melioidosis. Indeed, it was found that communities with a high rate of melioidosis had a wide spectrum of drinking water pH. This study, along with the diversity of isolates outside outbreak situations [2] supports the hypothesis that the majority of cases are related to exposure to surface water and mud. However, contamination of drinking water must be considered in an outbreak situation.

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13. Extreme weather events and environmental contamination are associated with outbreaks of melioidosis in northern Australia

13.1. Introduction

The epidemiology of melioidosis in Australia has been reviewed previously in this thesis. This chapter attempts to define case-clusters of melioidosis and explore associated environmental factors.

The spatial and temporal analysis of melioidosis in northern Australia presents unique problems. The sparse population of the Top End (~150,000 people), encompassing over 500,000 km^2, is irregularly distributed into three urban areas (Darwin, Katherine and Gove), and numerous smaller communities of less than 1000 people (figure 1). Thus, the main determinant of the location of cases is the location of people, and standard geospatial measures such as autocorrelation must be interpreted with caution. In northern Australia, melioidosis is a seasonal disease with 85% of cases occurring in the tropical wet monsoon season [1] during which localized meteorological events, such as flash flooding, may occur. Unlike other areas with endemic disease, rice farming does not occur in the Northern Territory; thus, exposure to mud and surface water is less dependent on seasonal agricultural practices such as rice paddy flooding and planting [2].

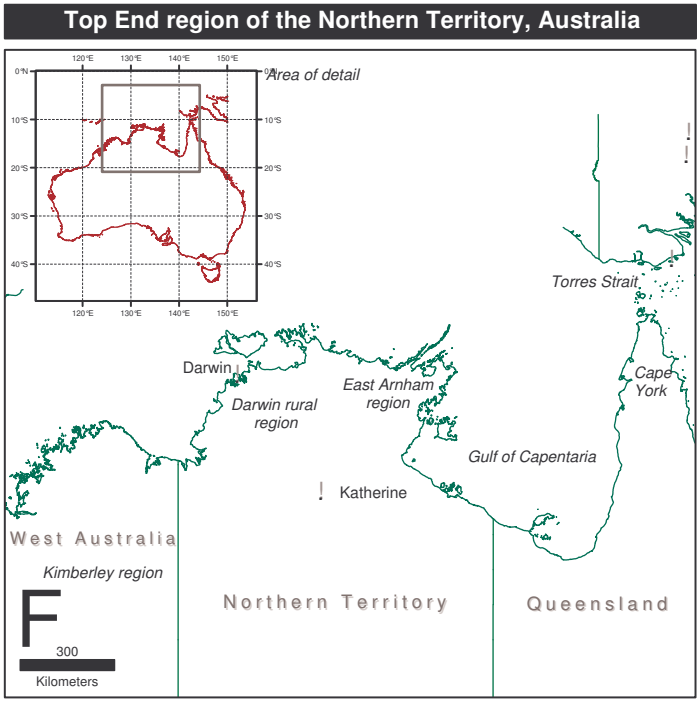
Like many infectious diseases, the incubation period of melioidosis probably depends on the mode and magnitude of inoculation; in the 25% where an exposure is defined, the incubation period is approximately 1 to 21 days (mean 9 days) [3]. However, because of the potential for reactivation from a latent focus, cases presenting up to 29 years after exposure have been documented [4]. The incubation period may be short in situations where exposure to a high bacterial load has been likely, such as near-drownings [5, 6].

A dose-response relationship between rainfall and the incidence of melioidosis has long been suspected. In this study, outbreaks, or cases clustered in time and location, were defined and explore associated environmental factors.

13.2. Methods

The Top End of the Northern Territory (between latitudes 10°S and 20°S) is regarded as endemic for melioidosis (Figure 13-1). Since 1989 a database has been maintained containing the demographic and clinical details of all cases of melioidosis in the Northern Territory. An analysis of cases between 1 January, 1990 and 31 October, 2002 was performed.

Figure 13-1: Top End region of the Northern Territory



The most likely place of exposure to *B. pseudomallei* was regarded as the geographical location in which the patient resided at the time of admission. Latitude and longitude were determined from the Gazette of Australia. Population statistics were determined from the mid-interval point, the 1996 Census (Australian Bureau of Statistics). Patients with first presentations of acute (symptoms <2 months) cases of culture-confirmed melioidosis were considered. Cyclone tracks, pressures and wind strength were drawn from the Bureau of Meteorology archive of significant weather events and from an archival web site (http://australiasevereweather.com/cyclones/index.html). Cyclones are

conventionally classified by maximum wind speed which may not necessarily correlate with rainfall. Table 13-1 details tropical cyclones in the Top End region.

With permission from affected communities, epidemiological investigations were conducted after suspected outbreaks occurred. Detailed histories were taken from individual patients seeking to determine exposure to potential environmental pathogens. Sampling from soil, water and other possible sources of exposure were performed.

Table 13-1: Categories of cyclones and a list of cyclones in the Top End region between 1990 and 2002.

Category	Definition	Cyclones in Top End region*
Category 1	Strongest gust <125 km/h (Gales; central pressure >985)	TC Laurence (Dec 10-12, 1990, no landfall – WA border) TC 0838 (Jan 27-28, 1996 – Qld border) TC Rachel (Jan 2-10, 1997 – WA border, coast islands, Darwin region) TC Phil (Dec 26, 1996 – Jan 3, 1997 – passed over Top End region) TC Steve (Feb 27 – Mar 11, 2000 – passed inland over Top End region) TC Winsome (Feb 10-11, 2001, Qld border)
Category 2	Strongest gust 125-169 km/h (Destructive winds; central pressure 985-790kPa)	TC Mark (Jan 7-10, 1992 – E Arnhem region) TC Ethyl (Mar 8-13, 1996 – Qld border) TC Sid (Dec 24-29, 1997 – north coast Darwin and E Arnhem region) TC Les (Jan 22 – Feb 1, 1998 – passed over Top End region)
Category 3	Strongest gust 170-224 km/h (Very destructive winds; central pressure	TC Warren (Mar 4-6, 1995, Qld border)

	970-945 kPa)	
Category 4	Strongest gust 225-279 km/h (Very destructive winds; central pressure 920-945 kPa)	TC Neville (April 7-13, 1992, coastal islands, Darwin region)
Category 5	Strongest gust >280 km/h (Very destructive winds; central pressure <920kPa)	TC Thelma (Nov 30 – Dec 11, 1998 – coastal islands, Darwin region)

*TC: Tropical Cyclone; Qld: Queensland; WA; West Australia.

We then examined the clinical characteristics of outbreak patients and compared these to patients not involved in outbreaks. Pulsed field gel electrophoresis was performed as previously described [7] with strain types defined at the level of "closely related" [8].

The scan statistic determines the probability that incidence of disease within a defined time-space window is the same as the incidence outside the window, adjusted for population and based on the Poisson distribution. Clusters were defined as cases within a 150 km radius and within 21 days; a Monte Carlo simulation with 999 simulations at 0.05 probability level was used to test significance. Clusters were also detected if they occurred within 21 days in all areas. Weather events affect a variable area, so to explore the possibility that incubation may be shorter in outbreak situations, a sensitivity analysis was performed altering the radius window between 100 to 300 km and the time period between 7 to 28 days. Although it may be argued that such outbreaks result in statistical overdispersion and therefore the negative binomial distribution may more accurately model the rates of disease than the Poisson distribution, the relatively small number of cases attributable to outbreaks would argue against this. This method of analysis, developed by Kulldorf for the National Cancer Institute, was initially designed to determine "hot spots" of cancer

incidence [9] but has been used to investigate clustering of cases of Creutzfeldt Jacob disease, West Nile fever and listeriosis, as well as chronic diseases [10-12].

Analysis and statistical calculations for clusters were performed using SaTScan 3.0 (Statistical Research and Applications Branch, National Cancer Institute, Bethesda, MD, United States). Fisher's exact test compared proportions using Intercooled Stata 7.0 for Windows (STATA Corporation, College Station, Texas, USA).

Ethical approval for this study was obtained from the Human Research Ethics Committee of the Department of Health and Community Services and the Menzies School of Health Research. As many remote communities are characterized by small numbers of people with a cultural connection to the area, specific care was taken not to disclose identifying information for remote Aboriginal communities in this paper pursuant with the conditions of our submission.

13.3. Results

During the period 1 January 1990 and 31 October 2002, there were 274 cases of culture-confirmed melioidosis presenting with acute symptoms. In addition, 50 patients were excluded due to presentations with reactivated or chronic disease, 5 due to residency outside the endemic area and 27 due to serological diagnoses only. Thirty communities recorded cases of melioidosis from the 41 census areas (total population of 143,633) defined in this study. The annual rate of acute melioidosis was 14.9 cases per 100,000 population.

Five clusters were identified by the primary analysis parameters (scanning window of 21 days and 150 km radius) involving 22 patients. Sensitivity analysis revealed a further cluster of 2 patients using a narrow scan window (7 days, 100km) and a further 3 patients in a wider scan window (28 days, 300km) giving a total of 27 patients. Table 13-2 details these clusters.

Table 13-2: Outbreaks identified and probable explanatory events

Cluster	Location	Cases/population	Relative risk (p-value)	Period and area of scan window	Associated event
1	Coastal communities A	6 cases / 1734 people	425 (p=0.001)	8 - 27 Dec 1998; 38 km radius	TC Thelma
2	All areas*	16 cases / 143633 people	9.8 (p=0.001)	8 Dec 1998 – 9 Jan 1999	TC Thelma
3	Katherine	6 cases / 7046 people	97 (p=0.001)	3 – 20 Feb 1998	Extensive flooding following TC Les
4	Coastal community B	3 cases / 2059 people	95 (p=0.003)	4 – 8 Mar 1994; Single community	Contamination of drinking water supply
5	Katherine district community C	2 cases / 273 people	9000 (p=0.002)	23 – 24 Jan 2001; Single community	Contaminated detergent

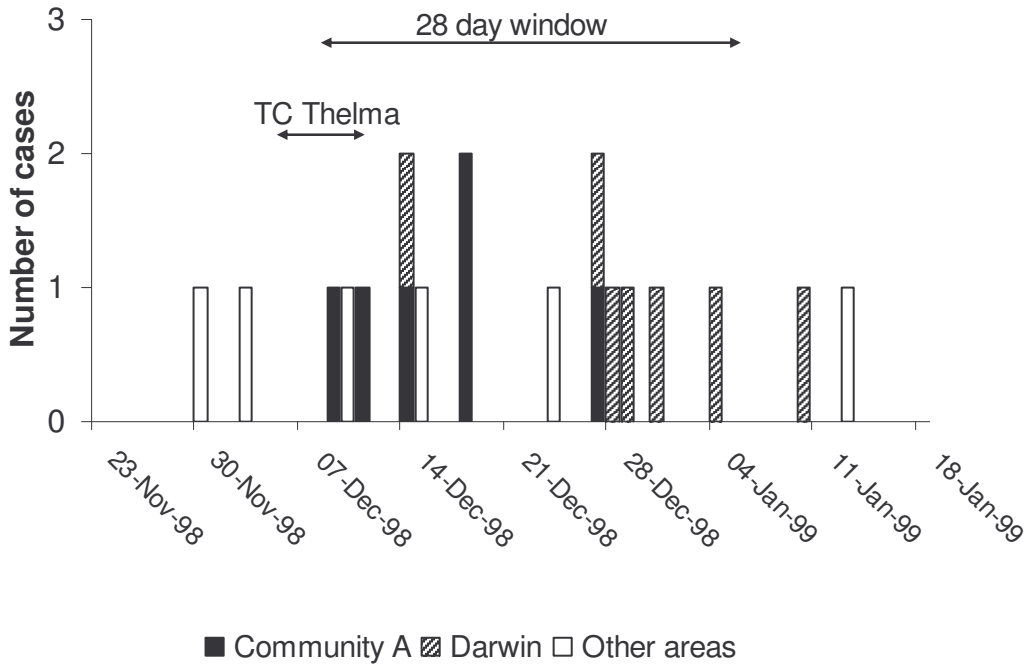
*Incorporates patients in cluster 1

13.3.1. Clusters associated with weather events

During the study period, 13 cyclones were recorded in the Top End area of the Northern Territory (Table 13-1). Of these, two were linked with the identified clusters. The first tropical cyclone (TC) Thelma developed in the Timor Sea on December 6, 1998 and progressed into a severe tropical cyclone of category 5 (estimated central pressure 925 hPa, maximum estimated gusts of up to 320 km/h). Destructive winds and extreme rainfall were experienced in the Darwin regional and adjacent coastal communities on December 10, 1998. Areas of Darwin recorded their highest rainfall totals for over 25 years, with one station in the Darwin region recording 1022mm of rain in 24 hours. TC Thelma moved away from the coast to the west and weakened the following day. This extreme weather event was linked to clusters 1 and 2. Patients included in cluster 1 were from one coastal community in the Darwin region. Cluster 2 incorporated all six patients from cluster 1 and a further

six patients from the Darwin region. The incidence of cases associated with TC Thelma is illustrated in Figure 13-2. This compares to the corresponding 28 day period the previous year, where only 4 cases of acute melioidosis presented (3 from Darwin, 1 from another community).

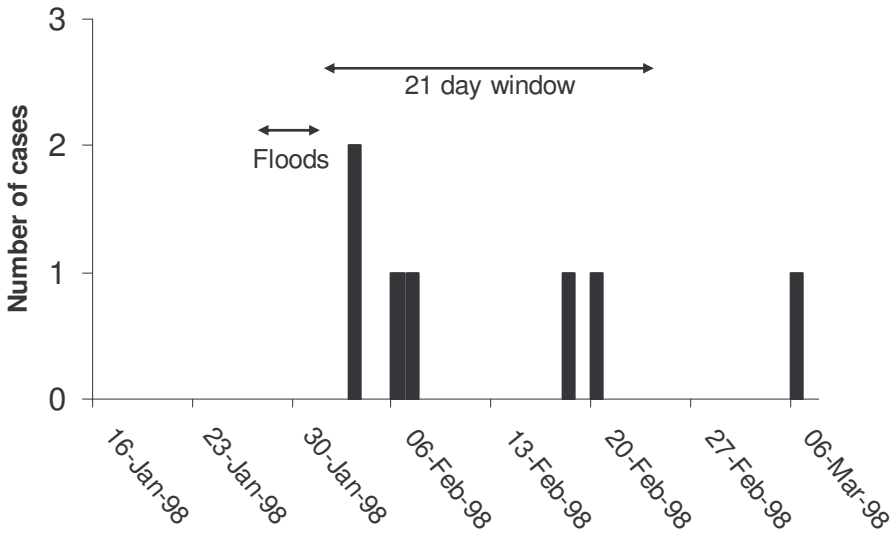
Figure 13-2: Incident cases of melioidosis in relation to TC Thelma, December 1998 (clusters 1 and 2)



The second TC in the time period, Les, formed in the Gulf of Carpentaria to the east of the Top End in mid January, 2000. Although not associated with strong winds (category 2), it was associated with heavy rainfall (300-400mm), especially in the catchment area of the Katherine and Upper Roper rivers. For 48 hours prior to January 27, 2000, this was the highest total recorded in the region and caused extensive flooding of the Daly and Katherine rivers. At the town of Katherine, the water level was above the flood level of 16m from January 26 to January 31, 2000 and peaked at 20.4m on January 27, 2000, the highest recorded level. Inundation of the central business district led to the evacuation of the area and was associated with severe disruption to communications and extensive property damage in Katherine. This event was linked to the 6 cases in cluster 3 (Figure 13-3). This compares with

the corresponding 28-day period in the previous year, where no cases of melioidosis presented from the Katherine region.

Figure 13-3: Incident cases of melioidosis in relation to Katherine floods in January, 2000 (cluster 3)



13.3.2. Clusters associated with environmental contamination

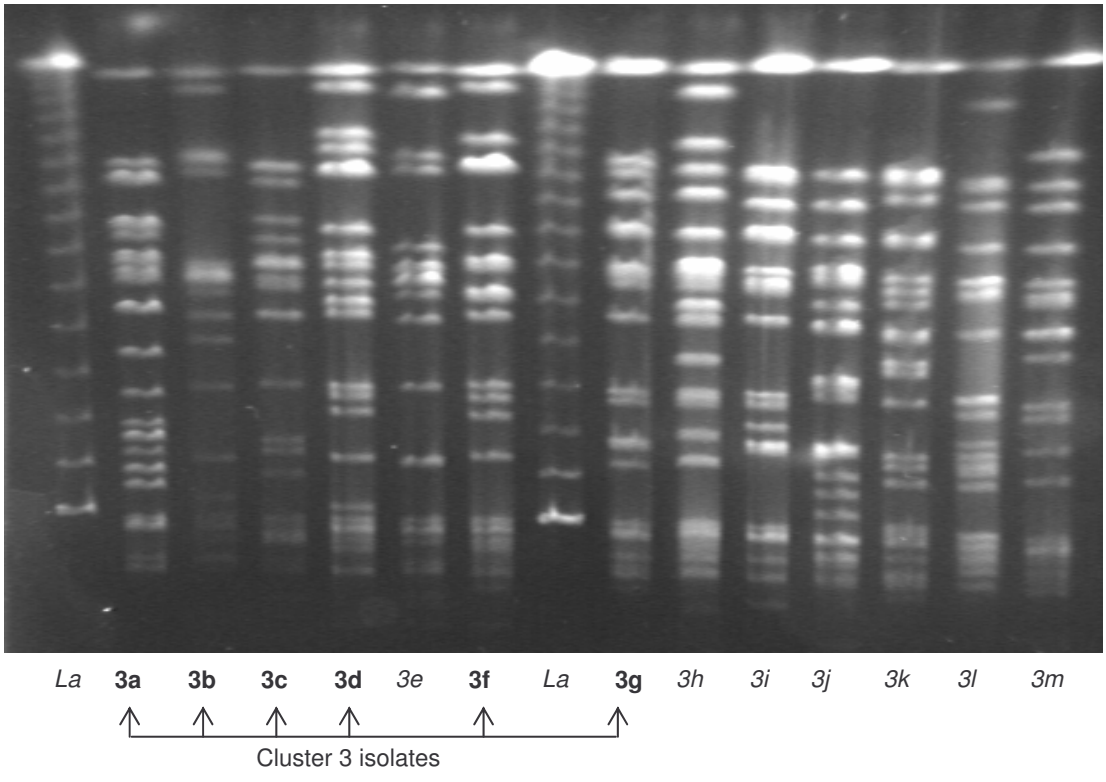
Two clusters were associated with environmental contamination. Cluster 4 was part of a longer outbreak that involved 9 patients over 28 months that has previously been reported [13]. Finally, the two patients in cluster 5, from a small community of 273, were exposed at a garage to a common detergent container from which *B. pseudomallei* was recovered [14].

Of the 27 patients involved in outbreaks, the mortality was 11%, the proportion with severe sepsis was 15%, pneumonia was seen in 52% and genitourinary disease was seen in 15%. The proportion of patients with a history of diabetes was 30%. These proportions were not statistically different to patients not involved in outbreaks (mortality 16.2%, severe sepsis 25%, pneumonia 50%, genitourinary disease 13%, diabetes 38%, Fisher's exact: all $p>0.05$)

13.3.3. Pulsed field gel electrophoresis

PFGE was performed from three patients in cluster 3 and all six patients in cluster 1, linked to severe weather events. Two of six isolates from patients in cluster 1 were identical. Two isolates from patients in cluster 3 were possibly related but others were unrelated (Figure 13-4). In contrast, clusters 4 and 5, linked to environmental contamination, have previously been shown to be mostly clonal [13, 14].

Figure 13-4: PFGE gel for isolates in cluster 3: Isolates 3a, 3b, 3c, 3d, 3f and 3g were from patients involved in cluster 3; other isolates were taken from patients in the same geographic area outside the outbreak period. La denotes lambda ladder (ProMega Markers)



13.4. Discussion

Epidemiologists have traditionally analyzed clustering in the single dimension of time, rather than geographical space. Geographical information systems (GIS) provide a powerful but underutilized tool to explore patterns of disease. A previous GIS investigation restricted to cases in the Darwin urban area revealed an association between higher incidence of melioidosis and deep mottled yellow earths

[15], which was also supported by earlier sampling studies from north Queensland [16]. Conversely, saline mud and clays were associated with a lower incidence of melioidosis [15]. These associations were consistent when validated in the outer Darwin region and in the Top End region. The unique population distribution of the Northern Territory, together with the seasonality of melioidosis precluded traditional geospatial analyses, such as weights of evidence, Bayesian analysis and spatial correlation.

This analysis seeks to detect unusual clusters of cases. The background rate of "usual" disease is defined and simulated using the Poisson distribution for the geographic area during a time period 'window'. Observed cases within each window are compared to expected cases, and a relative risk with its associated probability calculated. Where it has been suggested that a large window between 10-50% of the population of interest is defined, a much smaller geographic area was chosen to reflect the relatively localized influence of meteorological conditions and the broad study area. Similarly, the temporal window used, 21 days, covers the incubation period of acute melioidosis and sought to detect relatively short-lived meteorological phenomena. Sensitivity analysis allowed for variations in the incubation period potentially associated with high or low inoculum and non-point source outbreaks of up to a week in duration, occurring over a wider area.

In this study, five such unusual clusters during a 12 year period were defined involving 10% of the cases of acute melioidosis seen during this time. Although the environmental determinants of the background rate of melioidosis may differ from that seen in an outbreak situation, case-clusters may still provide valuable clues as to factors that may be important.

What were the unusual events surrounding these clusters? A link was observed between extreme weather events and three of the outbreaks of melioidosis identified by this method. For the residents of the Top End, TC Thelma and the Katherine floods undoubtedly represent the most catastrophic weather events during the study period; both caused extensive property damage and required evacuation of inhabitants from the worst-affected areas. A recent review of all Australian cases also noted an association between a cluster of five cases from an isolated

Queensland community and flooding following a TC in January, 2002 [17]. Although tropical cyclones are an annual occurrence in the region, apart from TC Tracy in 1974 which destroyed Darwin city, most have not been associated with widespread destruction to communities in recent decades.

The relative contribution of each possible mode of acquisition of melioidosis is yet to be defined, whether inhalation, inoculation or ingestion. Significant differences between the pattern of disease during outbreaks and those at other times have not been found, although, an association between monsoonal rainfall and severity of illness and the incidence of pneumonia has been previously noted, suggesting a possible shift from inoculation to inhalation during periods of heavy wind and rain [18].

Microenvironmental conditions during the monsoonal wet may be similar to the larger scale environmental conditions seen during extreme weather events. Alternatively, cases during the dry season are more likely to be chronic or reactivated disease, having been acquired during a previous wet season.

Other associations also resulting in clusters of melioidosis cases were noted in relation to environmental contamination, of which two were detected in this study. It was determined that one of these outbreaks was part of a larger case-cluster of nine cases occurring over a 28 month period. This outbreak has previously been reported and was found to be linked, both epidemiologically and by molecular typing, to contamination of an unchlorinated drinking water supply [13]. A second outbreak in a small community was linked epidemiologically to contaminated detergent used in a garage. In this outbreak multiple strain types were isolated from the detergent but none was found to match the two clonal clinical isolates [14]. The observation that these outbreaks have not reoccurred since the removal of the putative source vindicates our approach to the investigation of such case clusters.

The clonality of outbreaks linked to environmental contamination reflects the acquisition of a strain established in a particular environmental niche from which exposure occurs. However the isolates from extreme weather events were generally not clonal, suggesting that widespread weather events which favour the acquisition

of *B. pseudomallei* are responsible for these outbreaks rather than exposure to a particular strain.

There are several limitations to our study. Host parameters were not considered, where risk factors such as diabetes, excessive alcohol intake and renal disease are known to be important determinants of disease [1, 19]. An advantage of this method of analysis is that if it could be assumed that the underlying population and risk factor profile of the denominator population remains static, these factors would be controlled for in the analysis. While this may seem a significant assumption, census data from 1990 and 2002 does reveal only small changes to the total population.

Ascertainment of all cases linked to weather events is not possible due to the variation in incubation period for melioidosis which can be up to many years after exposure. Previous work suggests an incubation period of up to 21 days in the majority of cases [3], with shorter incubation associated with situations of exposure to high inoculates. It is known that an additional four patients present at the Katherine floods presented up to three months after the event; these were not included in the cluster as they were outside of the 21-28 day window used in this study.

In addition, meteorological phenomena are notoriously difficult events to quantify; not only within the constructs of time and space, but also in their detail – rainfall of varying amount and rate, mean and peak wind speed and hydrological measures of flooding. Because of this complexity and the paucity of such data from remote communities, an analysis using disease incidence was performed, but the exact meteorological determinants of disease and their probable interaction with soil types are yet to be defined. One intriguing observation suggests such an interaction; few cases in the area of the Daly River south of Darwin have been observed; this area is noted for saline mud and clays found to be associated with lower rates of melioidosis. Extensive flooding of this river in February 1998 and again in March 2000 was not associated with outbreaks of melioidosis.

It is concluded that outbreaks, or time-space clusters of cases of melioidosis, are associated with extreme wind and rainfall conditions or with environmental

contamination. Prospective use of this method may highlight times and areas where intensive environmental sampling may have a higher yield if bacterial recovery is to help elucidate the molecular epidemiology of this environmental pathogen. This work also suggests that public awareness and prevention campaigns might help prevent such outbreaks if commenced during such weather events, although outbreaks only contribute a minority of cases to the total public health burden of melioidosis. Ongoing work is aimed at elucidating factors responsible for the background rate of disease, such as water contamination, soil type and patterns of rainfall as well as factors determining the different manifestations of melioidosis.

Finally, this data suggests an intriguing hypothesis. There has been increasing interest in the potential effects of global climate change on the incidence of diseases in humans, with the observation that increasing extreme weather events may be linked to the apparent increasing intensification of the El Niño Southern Oscillation since the 1980s. If the incidence of melioidosis is linked to extreme weather events, predictions of long-term climate change may have significant implications for the future incidence of this disease, similar to that postulated with cholera, tick-borne encephalitis and malaria [20, 21].

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14. *Burkholderia pseudomallei* strain type, based on pulsed field gel electrophoresis, does not determine disease presentation in melioidosis

14.1. Introduction

Host risk factors, including diabetes, alcohol abuse and renal failure, have been well defined and demonstrated to correlate with outcome. However, less is known about bacterial strain variation in determining the severity and pattern of disease. Previous work has hinted at differential virulence and tropism in *B. pseudomallei*, but conclusions have been difficult to draw from relatively small sample sizes [1, 2]. The presence of differential strain tropism and virulence would have significant implications for the identification of virulence factors, for biodefense studies and vaccine strategies.

Using clinical data collected over the past 13 years, strain tropism and differential virulence was explored in isolates of *B. pseudomallei* in the tropical Top End of the Northern Territory, Australia based on strains defined by pulsed field gel electrophoresis.

14.2. Methods

Since 1989, a collection of *B. pseudomallei* isolates has been maintained, encompassing isolates from all human cases seen in the Northern Territory. A sample of isolates, representing the full range of clinical and geographical diversity, was typed by pulsed-field gel electrophoresis (PFGE). A single isolate from each patient was considered to preserve the assumption of independence of observations. Five distinct clinical presentations were of particular interest; severe sepsis (based on the presence of sepsis and markers of end-organ dysfunction [3]), pneumonia, genitourinary infection, skin/soft tissue infection and central nervous system infection. The proportions of patients with pneumonia, genitourinary infection, skin and soft tissue infection and severe sepsis were similar to previously reported series of patients with melioidosis [4]. All isolates from patients with central nervous system infection, a less common but unique manifestation of melioidosis were typed [5], to allow us to draw conclusions from these strains. First, primary presentations of disease were considered, recognizing that haematogenous dissemination to other

sites is seen in 8% of patients presenting with pneumonia [4]. A secondary analysis was performed by all sites (both primary and other sites) of infection in each patient.

PFGE was performed using *SpeI* as published elsewhere [6]. In summary, pure colonies were grown overnight in Todd-Hewitt broth. A resuspension was mixed with a 2% low melting point agarose and pipetted into PFGE plug molds. Plugs were lysed overnight at 50°C in a proteinase K-containing lysis buffer, rinsed with TE buffer and stored at 4°C. Plugs were digested overnight with *SpeI*. PFGE was performed using the CHEF-III DR system using concatemerized lambda phage DNA as a standard. PFGE gels were stained with ethidium bromide and digitally photographed using a Gel Doc 1000 system (Biorad Laboratories).

DNA macrorestriction patterns were analyzed for similarity using the Dice coefficient. A dendrogram was formed from the unweighted pair group matching band average (UPGMA). Clusters were then distinguished using the Tenover criteria "closely related" (1 - 3 band difference) or identical (no band differences)[7]. The diversity of isolates defined by these methods was assessed using Simpson's index of diversity *D*, where for *s* strain types, p_i represents the proportion of the total number of isolates represented by the i^{th} cluster [8].

$$D = 1 - \sum_{i=1}^{s} p_i^2$$

Simpson's index represents the probability that any two isolates chosen at random from this sample would not be expected to match. The variance of this estimate of the true diversity λ in a sample size *n* is given by [9]:

$$\sigma^2 = \frac{4}{n}[\sum_{i=1}^{s} p_i^3 - (\sum_{i=1}^{s} p_i^2)^2]$$

The similarity matrix formed using the Dice coefficient of size (n × (n-1))/2 was analyzed. This matrix details the similarity coefficient of each pair of isolates. Pairs of isolates could either be disease concordant (for example, both isolates from patients with pneumonia) or discordant (for example, one isolate from a patient with pneumonia and one from a patient without pneumonia). The similarity coefficients from concordant isolates were classed as "within group" and those from discordant

isolates as “between group”. It was surmised that if there was a tendency of strains to cluster on the basis of disease presentation, then the similarity coefficients within each group would be higher than those between groups.

Analysis of electrophoretic patterns was performed using Bionumerics (Applied Maths, Belgium). Band optimization and tolerances were the minima required to match lambda ladder standards; these were found to be 0.4% optimization and 0.6% position tolerance. Statistical tests were performed using Intercooled Stata 7.0 for Windows (College Station, Texas), except for Simpson’s index which was calculated by hand. The Epanechinkov kernel density estimation was used to graphically illustrate the distribution of similarity coefficients and the Mann Whitney two-sample (Wilcoxon ranksum) test to compare the equality of the coefficients thus obtained. Clinical significance was based both on the results of statistical tests and a visual comparison of the distribution of similarity coefficients, particularly at higher values. Ethical approval for this study was obtained from the Human Research Ethics Committee of the Department of Health and Community Services and the Menzies School of Health Research.

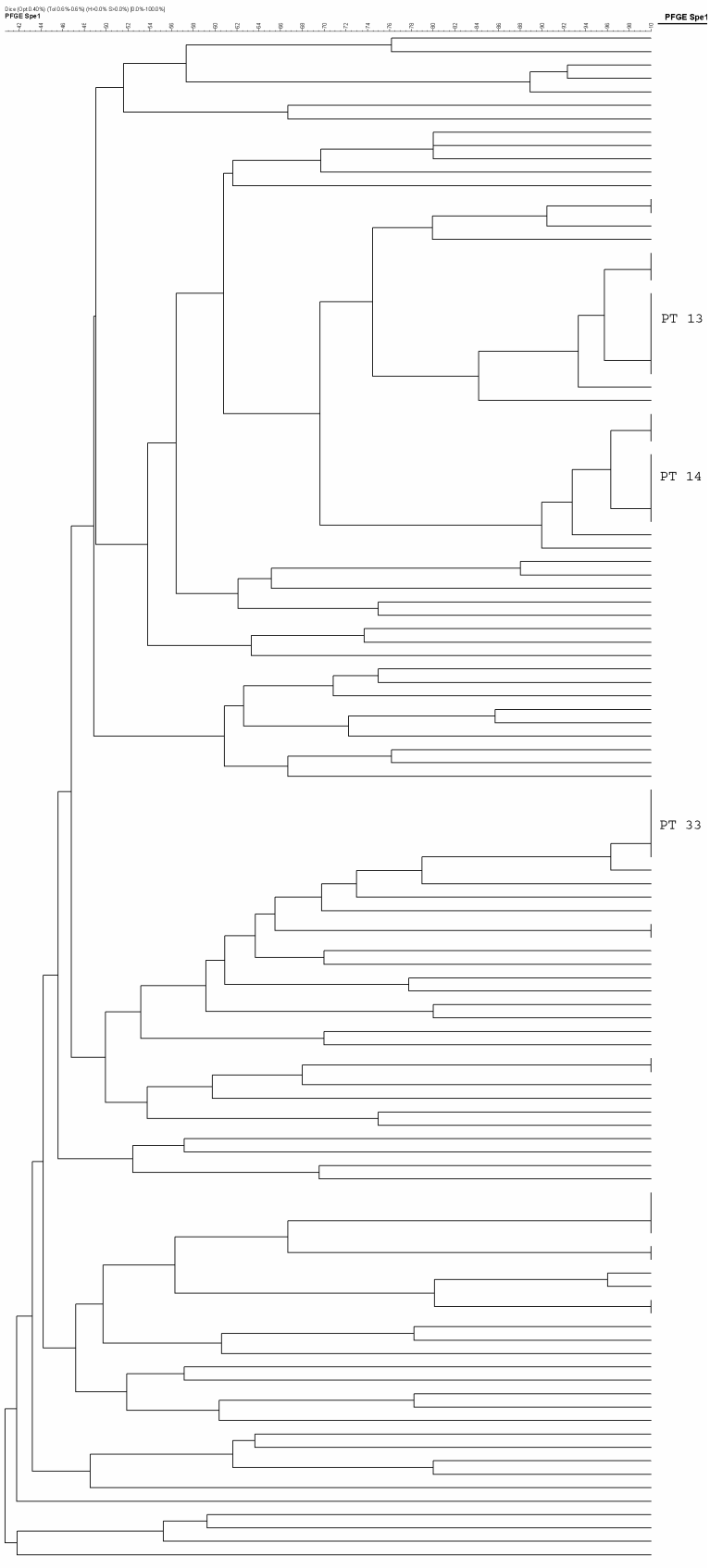
14.3. Results

Isolates from 114 patients were considered of which pneumonia was the primary diagnosis in 47, genitourinary infection in 24, central nervous system involvement in 11 and skin or soft tissue infections in 12. Additionally, 4 patients with pneumonia had secondary involvement of the genitourinary tract, three patients with pneumonia had secondary infection of skin and/or soft tissue and one patient with central nervous system infection had secondary genitourinary infection. Twenty nine patients had severe sepsis, including 19 patients with pneumonia, 5 patients with genitourinary infection and 1 patient with CNS involvement. Twenty six isolates were from patients in the Darwin urban region.

14.3.1. Diversity of isolates and analysis by PFGE strain type.

Based on a cluster definition of closely related or identical, there were 71 PFGE strain types (PT) defined. The relationship between the UPGMA dendrogram and PFGE strain types is depicted in figure 1. Several strain types of multiple isolates were identified, the largest of these contained 12 isolates (PT 13 and 14) and 8 isolates (PT 33). These strain types were widely distributed over time (PT 13: 10 years; PT 14: 11 years; PT 33: 7 years).

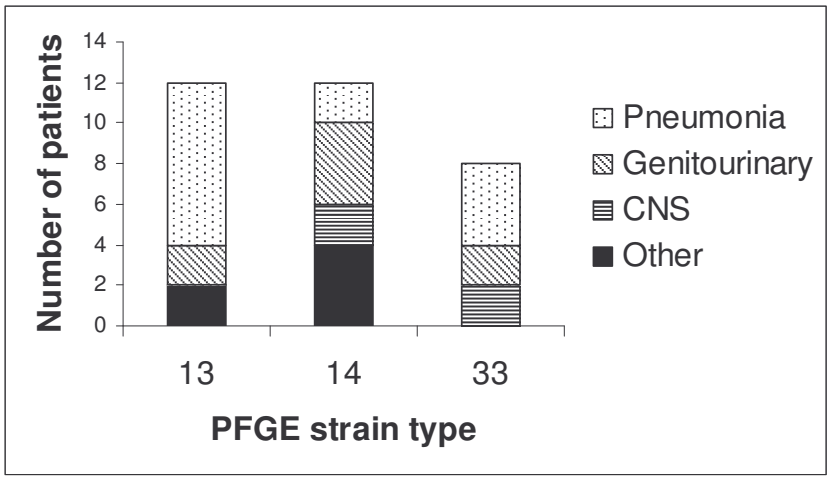
Figure 14-1: Dendrogram based on UPGMA and Dice coefficient from SpeI-*restricted B. pseudomallei isolate DNA*



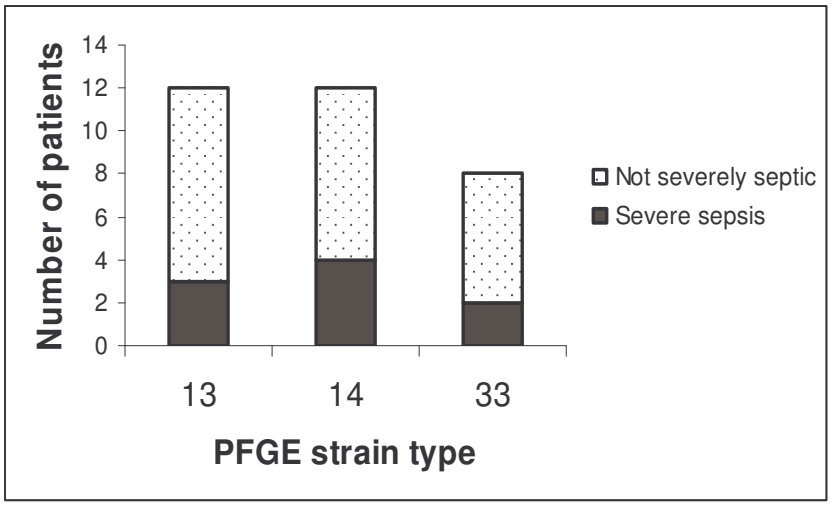
For this group of 114 isolates, Simpson's index was calculated at 0.91 (95% CI: 0.88, 0.93). There were no significant differences between the site of disease on presentation (figure 2a) nor the presence of severe sepsis (figure 2b) between the three strain types containing more than five isolates. These three strain types incorporated isolates derived from patients both in the Darwin urban region and from rural communities elsewhere in the Top End region of the Northern Territory.

Figure 14-2: Distribution of major strain types by (a) site of infection and (b) severity of infection.

(a)



(b)



14.3.2. Analysis by Dice coefficient

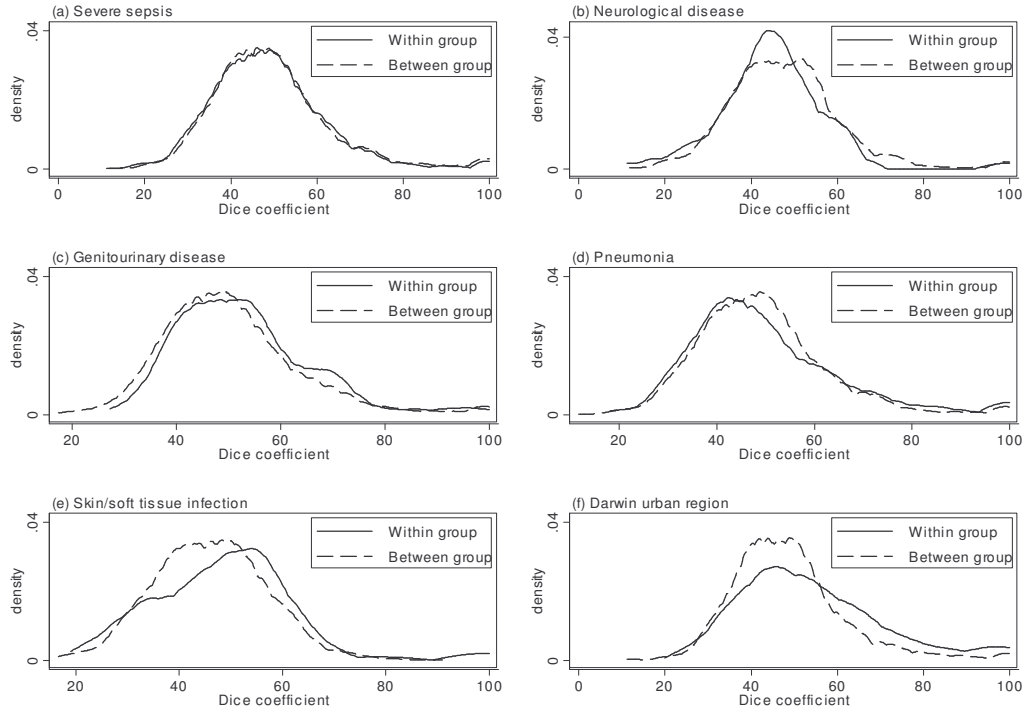
When considering all 6,441 isolate pairs, the median Dice coefficient was 47.62 (IQR: 40.0, 55.6). There were no significant differences in median Dice coefficient when comparing within group coefficients and between group coefficients for severely septic patients, central nervous system disease, pneumonia or skin and soft tissue disease ($p>0.05$). Median Dice coefficients were marginally higher when comparing within group coefficients and between group coefficients for patients with genitourinary disease ($p=0.003$) and significantly higher for patients from the Darwin urban region ($p<0.001$). Summary statistics of within-group and between group comparisons are detailed in table 1 and illustrated graphically in figure 3.

Analysis by the presence of any site (primary or secondary) demonstrated statistical significance for within group and between group coefficients for genitourinary infection (within group median Dice coefficient 50.0, IQR: 43.5, 57.2; between group median 47.6, IQR 40.0, 56.0, $P<0.001$). Analysis by other clinical presentations did not result in significant changes to median Dice coefficients or significance (data not shown)

Table 14-1: Dice coefficients for within group and between group comparisons (median, interquartile range and number of isolate pairs)

	Median Dice coefficient for within-group pairs	Dice coefficient for between group pairs	Significance
Severe sepsis	47.6 (41.7, 56.0), n=406	47.6 (40.0, 55.6), n=2465	P=0.15
Neurological disease	45.6 (38.1, 45.5), n=55	47.6 (40.0, 54.6), n=1133	P=0.13
Genitourinary disease	51.1 (43.5, 58.8), n=276	50.0 (41.7, 57.2), n=2436	P=0.003
Pneumonia	47.1 (40.0, 57.2), n=1081	47.6 (40.0, 55.6), n=3149	P=0.55
Skin and soft tissue infection	50.0 (40, 57.2), n=66	45.5 (38.1, 52.6), n=1224	P=0.11
Darwin urban region	50.0 (41.7, 63.6), n=325	47.6 (40.0, 54.6), n=2288	P<0.001

Figure 14-3: Distribution of Dice coefficients for within group (solid line) and between group isolates (dashed line) for (a) severe sepsis, (b) neurological disease, (c) genitourinary disease, (d) pneumonia, (e) skin/soft tissue infection (f) Darwin urban region. Within group comparisons are between isolates derived from patients with the same presentation/locality and between group comparisons are between isolates from patients with the presentation and those without the presentation



14.3.3. Discussion

This method provides a simple conceptual framework to test the degree of clustering based on DNA typing systems which may be simply expressed as "Are isolates from patients with the same disease presentation more similar than isolates from different disease presentations?" Although there was some statistical association in this study when considering presentation, differences in the measures of similarities between groups were not large, suggesting there are no strain differences between isolates causing different disease presentations.

Primary diagnoses were used in the principal analysis as subsequent seeding of other sites is well recognized [4, 10]. In the secondary analysis, the within group comparison demonstrated some clustering; this may be interpreted as an equivocal finding which needs further exploration by other methods. In addition, the higher similarity within isolates from the Darwin region compared to isolate pairs from Darwin and outside Darwin suggests that there is some geographical localization of strain types.

Although it is recognized that host and environmental factors are important in determining the severity and pattern of this protean disease, less work has been done to determine the contribution of bacterial factors. A major advance in understanding came with the discovery that Thai arabinose-assimilating (ara^+) environmental isolates were relatively avirulent. [11]. This variant, subsequently shown to represent a different species (*B. thailandensis*) has not been found in Australia to date. However, Ulett et al showed that virulence of 42 ara^- isolates, measured by a LD_{50} for Balb/c mice, varied between 10 and $>10^6$ CFU [2]. Of the three PFGE clone types defined in this study, two clones (containing 4 and 6 isolates respectively) showed LD_{50} that were similar, suggesting some strain variation in virulence.

In addition, one study demonstrated some differential clustering between human, animal and environmental isolates [12], and another study described ribotypes that were not found in humans [13]. Similarly, recent multilocus sequence typing suggests that *B. mallei* may be a subspecies of *B. pseudomallei* that exhibits tropism for animals [14]. However, it has also been demonstrated that a single clone of *B. pseudomallei* has been isolated from local soil, animals and a human [15].

Small studies of isolates from humans with clinical disease have suggested possible bacterial tropism. In one study, Pitt found virulence correlated with ribotype, with three ribotypes associated with fatalities and one of these with relapses [16]. A second study from Queensland using multi-locus enzyme electrophoresis (MLEE) and randomly amplified polymorphic DNA (RAPD) typing demonstrated that clusters corresponded with neurological and pneumonic disease (n=9) and soft tissue abscesses (n=9) [1].

Of particular interest were possible correlations with neurological disease and genitourinary disease. These presentations appear much more common in Australia compared to reports from other countries [17] and regional variation in strain types with tropism for these sites is a possible factor. The lack of clear clustering among strains from patients with these presentations suggests that as-yet-undefined behavioural differences resulting in a unique mode of acquisition, or other host susceptibility factors may be more important in determining these clinical manifestations. This work also supports recent findings defining an association between the severity and pattern of disease and the intensity of prior rainfall [18], and a report that a single clone may result in a variety of disease manifestations, including neurological melioidosis [19].

There are several limitations to this method of analysis. PFGE is an imprecise measure of genetic relatedness as band changes depend on genetic change at restriction enzyme cleavage sites. Thus, there is not a linear relationship between band similarities on pulsed-field gel electrophoresis and genetic relatedness. Matching bands may also not contain homologous genetic material and there is poor resolution of fragments of similar size [20]. Similarly, if recombination of restriction enzyme sites or genetic material represented by whole bands is prominent, genetically diverse isolates may appear more closely linked. Criteria have been developed for determining the degree of genetic difference when considering isolates from an outbreak situation [7], but have not been formally developed for population-based investigations.

The typing of isolates by sequence-based methods such as MLST provides direct evidence of genetic relatedness. Macrorestriction analysis of *Staphylococcus aureus* has demonstrated a correlation between band-based similarity values and clonal clusters defined by MLST [21] as well as temporal and geographical correlation [22]. Similarly, correlation between relatedness defined by PFGE and MLST has also been noted for *Bartonella henselae* [23]. For *B. pseudomallei*, Godoy et al demonstrated a close correlation between clusters distinguished by MLST and PFGE, supporting the validity of PFGE for this organism [14].

This study also addresses the background diversity of isolates found in the endemic Top End of the Northern Territory, important in considering the validity of PFGE as a tool for epidemiological studies [24]. Simpson's index of diversity [8] represents the chance that any two isolates selected at random would not match when using this typing method and thus reflects the sensitivity of the analysis. Based on a Tenover definition of closely-related or identical, a large diversity of isolates was found, reinforcing the discriminatory ability of PFGE when applied to micro-epidemiological investigations of *B. pseudomallei* outbreaks.

Although PFGE typing may be an imperfect tool for measuring genetic relatedness, it demonstrates good resolving power for *B. pseudomallei* in this context at the "closely related" level. Previous work has demonstrated that PFGE typing may correlate with virulence in an *in vivo* mouse model. Despite the large apparent variation in virulence of *B. pseudomallei* in the mouse model [2], this work suggests that the variation in strain type may not be as important as host co-morbidities, the route of acquisition and the size of inoculum in determining the clinical manifestations of melioidosis.

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15. Isolates of *Burkholderia pseudomallei* from northern Australia are distinct on multilocus sequence typing but are not correlated with clinical presentation

15.1. Introduction

Multilocus sequence typing (MLST) is a genetic typing method based on sequence variation in seven housekeeping genes. The slow rate of change in these alleles and the lack of ambiguity in assigning alleles make this an ideal method to compare strains within and between laboratories. MLST has been used to define relationships between bacterial clones and track global spread and antibiotic resistance in *Neisseria meningitidis, Streptococcus pneumoniae, Streptococcus pyogenes, Staphylococcus aureus* and other organisms. In addition, it has allowed the definition of the dominant mechanism of genetic change in these bacteria [1-3]. For *B. pseudomallei*, a MLST method has recently been described and has noted the overlap between *B. mallei* and *B. pseudomallei*, and the clear resolution of these isolates from those of the less virulent *B. thailandensis* [4].

In the previous chapter, an exploration of differential virulence and strain tropism was performed using PFGE. However, this analysis was limited by the uncharacterized nature of the genetic change tracked using this method. In this study, the possibility of geographical localization and variation in strain tropism and virulence in *B. pseudomallei* was performed using MLST and compared results of MLST with pulsed-field gel electrophoresis (PFGE) typing.

15.2. Methods

Since 1989, a collection of *B. pseudomallei* isolates has been maintained, encompassing isolates from all human cases seen in the Northern Territory of Australia. Isolates had been stored at -70°C in Todd Hewitt broth (Oxoid Australia, Melbourne, Australia) with 20% glycerol. A sample of 87 isolates, representing the full range of clinical and geographical diversity, was typed by multilocus sequence typing (MLST). A single isolate from each patient was selected to preserve the assumption of independence of observations. Clinical classification of human cases

was as previously described [5]; severe sepsis was defined according to standard criteria [6]. Geographical location was taken from the address at which the patient resided at the estimated time of infection.

To examine the resolving power of MLST, results of MLST were compared with PFGE on a subset of 17 isolates. This subset included two groups, with five and eight isolates respectively, where isolates were not linked epidemiologically but had identical PFGE banding patterns. A third cluster was derived from a small remote community with four cases over an 11 month period, but where isolates were not all related on PFGE typing.

Bacteria were cultured on chocolate agar (Oxoid Australia), subcultured in Todd Hewitt broth and DNA extracted using a DNeasy Tissue Kit (Qiagen, Hilden, Germany). MLST was performed at Imperial College London as recently reported [4]. The alleles at each of the seven loci were assigned by comparing the sequences to those at the *B. pseudomallei* MLST website (http://b*pseudomallei*.mlst.net/). Novel sequences were assigned new allele numbers and were deposited in the MLST allele database. The allele numbers at each locus provide the allelic profile of each strain and each distinct allelic profile is assigned as a sequence type (designated by the prefix ST). A list of currently described sequence types is detailed at the MLST website; the 47 novel sequence types from this study have been submitted to this database.

Isolates from northern Australia were compared to other isolates in the MLST database. Many of the isolates in the MLST database were from imported infections in non-endemic countries and these were removed from the comparator group. Other isolates from Australia were also removed since it was unknown whether these were from imported infections or those acquired in endemic areas of Australia. The comparator groups included 62 sequence types where the country of origin was known; a subset of this group included the 25 sequence types recovered from countries of south east Asia.

The relatedness among isolates was displayed as a dendrogram by the unweighted pair group method with arithmetic averages (UPGMA) method using the matrix of

pair-wise differences between the allelic profiles of the isolates [7]. The sequences of the seven loci from the isolates characterized here and from the comparator group from the *B. pseudomallei* MLST database, were joined in-frame to produce a concatenated sequence of 3,399-bp and a minimum evolution tree was constructed by means of the Kimura 2-parameter method for estimated pairwise genetic distances, using the MEGA 2.1 program (Kumar et al, Molecular Evolutionary Genetics Analysis software, Arizona State University, Tempe, Arizona, USA). The significance of the nodes on the tree was evaluated using the bootstrap technique with 1000 re-samplings from the dataset.

PFGE was performed at the Menzies School of Health Research as previously described using *SpeI* [8]; DNA macrorestriction patterns were analyzed for similarity using the Dice coefficient. A dendrogram was formed using the UPGMA method. Band similarities were assessed using the Tenover criteria "closely related" (one to three band difference) or identical (no band differences) [9].

Ethical approval for this study was obtained from the Human Research Ethics Committee of the Department of Health and Community Services and the Menzies School of Health Research.

15.3. Results

Eighty seven isolates were typed by MLST. There were 48 sequence types defined in this study, of which only one (ST36, where the isolate already in the MLST database was from elsewhere in Australia) had been described previously. Figure 15-2 shows the clustering of the sequence types on a UPGMA tree obtained using differences in the allelic profiles and their relatedness to the 62 sequence types in the MLST database from other endemic areas.

The majority of sequence types from the Northern Territory differed from all other sequence types at more than one locus (linkage distance of > 0.14); four pairs of sequence types differed at only one of the seven loci (single locus variants) and one group of three sequence types were also closely related, with two sequence types being different single locus variants of the third sequence type. Although none of the

isolates was identical in sequence type to isolates from other countries in the MLST database, in a few cases there were closely related strains in the database. For example, ST110 was a single locus variant of ST23, previously sourced from both Australia and Thailand, and ST105 was a single locus variant of ST18, sourced from Kenya.

15.3.1. Geographical distribution of sequence types

Genetic diversity was evident at each of the three geographical regions of the tropical Top End of the Northern Territory. In the Darwin region, incorporating the surrounding rural areas, nine sequence types were seen in 13 isolates. In the Katherine rural region, five sequence types were demonstrated in five isolates, and in the East Arnhem region, 16 sequence types were seen in 21 isolates.

Since the sequence types of isolates from northern Australia were distinct from that described from other endemic regions a further analysis explored whether these isolates clustered apart from the 25 sequence types of south east Asia. On a dendrogram constructed using the concatenated sequences, the Australian isolates in this study were appeared clustered together and were distinct from those from countries of south east Asia (Figure 15-3). However, isolates of *B. pseudomallei* are very uniform at house-keeping loci [4] and the nodes on the minimum evolution tree therefore had very poor bootstrap support.

15.3.2. Strain tropism and virulence

There was no evidence of strain tropism or differential virulence. Presentations with severe sepsis (n=14) were caused by strains of 13 different sequence types. Skin and soft tissue infections (n=5) were caused by strains of five different sequence types, melioidosis encephalomyelitis (n=9) was caused by eight different sequence types and prostatic infections (n=11) were caused by eight different sequence types. Individual sequence types were associated with multiple sites of infection, such as ST109 which was associated with prostatic and neurological presentations. There was also no evidence of associations between disease presentation and the clustering of isolates on the minimum evolution tree obtained using the concatenated sequences (data not shown).

15.3.3. Comparison with PFGE strain types

All isolates from one PFGE clone (clone 1; n=8) were of the same sequence type (ST109). Isolates from another clone defined on PFGE typing (clone 2; n=5) as identical were assigned to two sequence types (ST132 and ST133) but these were very closely related as they differed at only one locus. Both these clusters contained isolates from patients that were not epidemiologically linked and which were recovered over periods of eight years (Table 15-1). A third group of isolates from a remote community over an 11-month period showed concordance of PFGE and MLST results; two of the four isolates were indistinguishable by PFGE and had identical sequence types. The other two isolates had unique genotypes by both PFGE and MLST (Table 15-2 and Figure 15-1).

Figure 15-1: Comparison of PFGE and MLST for isolates not epidemiologically linked but clonal on PFGE (PFGE clone 1 and PFGE clone 2) and a case cluster from a remote community.

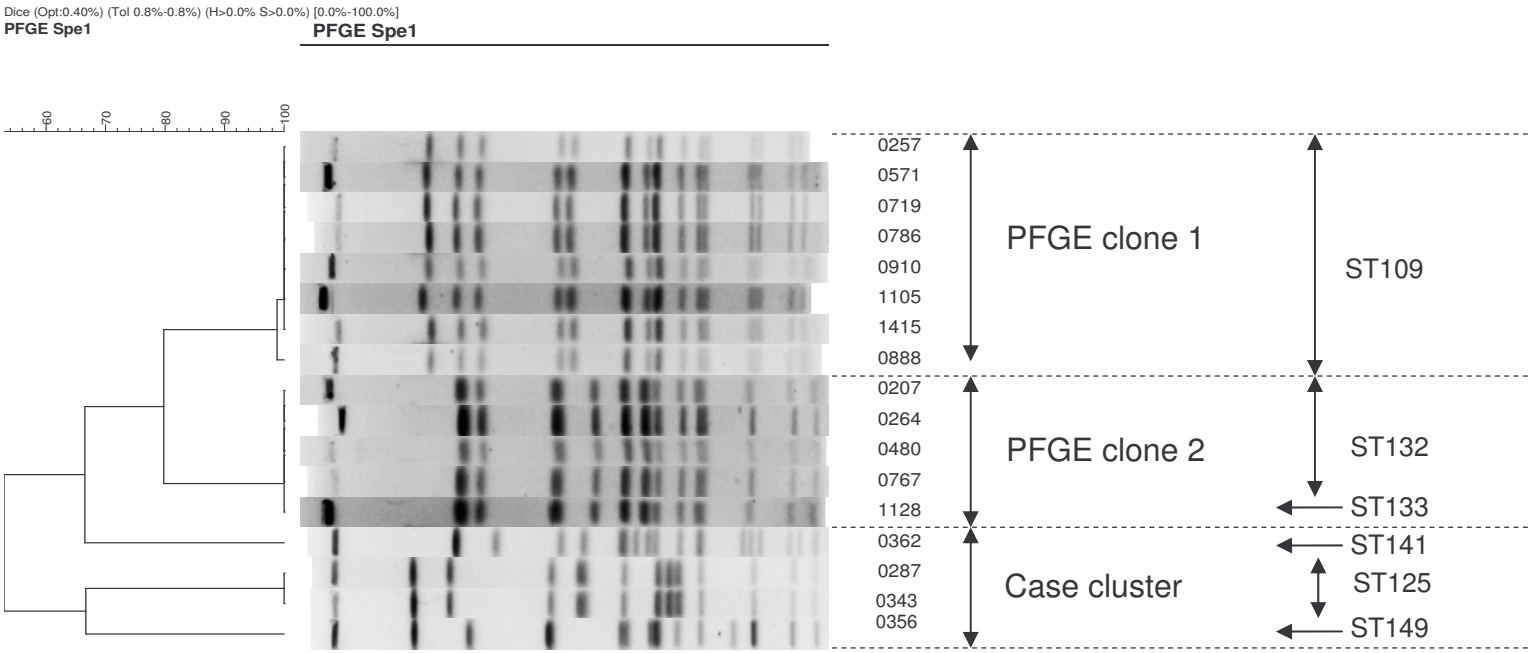


Table 15-1:Isolates not epidemiologically linked but clonal on PFGE: comparison with MLST

Year	**Isolate number**	**PFGE clone**	**ST**	***ace***	***gltB***	***gmhD***	***lepA***	***lipA***	***narK***	***ndh***
1994	257	1	109	1	2	13	4	1	19	1
1997	571	1	109	1	2	13	4	1	19	1
1998	719	1	109	1	2	13	4	1	19	1
1999	786	1	109	1	2	13	4	1	19	1
1999	888	1	109	1	2	13	4	1	19	1
2000	910	1	109	1	2	13	4	1	19	1
2000	1105	1	109	1	2	13	4	1	19	1
2002	1415	1	109	1	2	13	4	1	19	1
1993	207	2	132	1	16	13	4	6	21	1
1994	264	2	132	1	16	13	4	6	21	1
1997	480	2	132	1	16	13	4	6	21	1
1999	767	2	132	1	16	13	4	6	21	1
2001	1128	2	133	1	16	13	4	15	21	1

ace: acetyl coenzyme A reductase; *gltB:* glutamate synthase; *gmhD:* ADP glycerol-mannoheptose epimerase; *lepA:* GTP-binding elongation factor; *lipA:* lipoic acid synthetase; *nark:* nitrite extrusion protein; *ndh* NADH dehydrogenase.

Table 15-2:Isolates epidemiologically linked but polyclonal on PFGE: comparison with MLST

Date	**Isolate number**	**PFGE clone**	**ST**	***ace***	***gltB***	***gmhD***	***lepA***	***lipA***	***narK***	***ndh***
Feb 1995	362	3	141	4	16	3	4	1	9	6
March 1994	287	4	125	1	14	20	1	15	9	1
Oct 1994	343	4	125	1	14	20	1	15	9	1
Jan 1995	356	5	149	11	2	14	2	1	6	1

ace: acetyl coenzyme A reductase; *gltB:* glutamate synthase; *gmhD:* ADP glycerol-mannoheptose epimerase; *lepA:* GTP-binding elongation factor; *lipA:* lipoic acid synthetase; *nark:* nitrite extrusion protein; *ndh* NADH dehydrogenase.

15.4. Discussion

Previous work on *B. pseudomallei* has defined its genetic relationship to *B. mallei* and *B. thailandensis* and identified three clones responsible for an outbreak in Hong Kong [4]. In this study, a broad diversity of strains was seen in isolates from the endemic region of Northern Territory of Australia and no clinical correlates of strain types were evident.

The northern Australian isolates examined here appear to be distinct from those described from south east Asia. No sequence type from isolates in this study has been demonstrated in other countries and only two previously identified sequence types in the MLST database have been noted from Australia as well as other countries (ST23, found in Australia and Thailand and ST60, found in Australia and Fiji). However, it is unclear whether the Australian isolates of ST23 and ST60 in the MLST database were acquired in endemic northern Australia or were imported infections from other countries to the non-endemic temperate regions of Australia. With a few exceptions, Australian isolates appeared to cluster on the minimum evolution tree distinct from those recovered in south east Asia.

The minimum evolution tree attempts to define the relatedness between all strains, but the validity of these relationships depends on the relative impact of recombination and point mutation to the divergence between strains, which is presently unknown. Furthermore, *B. pseudomallei* is genetically quite uniform at housekeeping loci [4]; the concatenated sequences from many of the isolates differ at only one or a small number of nucleotide sites. Therefore, nodes on the minimum evolution tree had poor bootstrap support. Further studies with larger numbers of isolates are required to explore the distinctiveness of the genotypes recovered in different endemic areas although the lack of sequence diversity may make it difficult to obtain robust population genetic inferences about the extent and nature of geographic substructure.

What then can be concluded about the origins of *B. pseudomallei* in Australia? Australia has been isolated geographically for many millions of years. However, during that time, exchange of flora and fauna is well recognized through various transient land links with south east Asia. The spread of *B. pseudomallei* can result from contact with humans or animals. Previous outbreaks in non-endemic countries have implicated imported animals from endemic countries [10, 11]. Clusters in temperate Australia have also been attributed to animals brought in from the tropical north [12, 13].

Despite its recent description in Australia, the genetic and geographical diversity found does not support theories that melioidosis was introduced by servicemen returning to Australia following the Second World War [14]. Prior to European contact, human contact between south east Asia traders and indigenous Australians may have resulted in its introduction to or from northern Australia; however, the distinct nature of Australian isolates does not support repeated exchange of *B. pseudomallei* strains between these regions. Alternatively, *B. pseudomallei* is a soil saprophyte and intercontinental wind dispersal has been noted for a wide variety of organisms [15, 16]. However, it is likely that this mechanism of dispersal is restricted to fungi and spore-forming bacteria; although *B. pseudomallei* can survive in waterless soil for 30 days, it is sensitive to ultraviolet light exposure and thus long-range dispersal seems unlikely [17].

It is therefore hypothesized that the diverse but distinct phylogeny of strains in both south east Asia and Australia, including the lack of *B. thailandensis* and *B. mallei* in Australia, may reflect geographical isolation over a longer period of time. It is possible that *B. pseudomallei* originated in Australia or south east Asia and was propagated though animal migration during the Miocene period around 15 million years ago, when south east Asia was linked to the Australia-New Guinea continent though a land bridge. Movements of flora and fauna along this route has been implicated in the palaeogeography of plants, including rice (*Oryza sativa*) [18, 19], and animals including Australia's earliest endemic rodents [20, 21].

Other sequence types are common amongst various south east Asian countries; this may suggest that migration of humans and animals may have resulted in their

subsequent dissemination and shared phylogenies. The widespread distribution of ST40 (*B. mallei*) is likely to represent later successful adaptation and restriction to a particular niche (a mobile equine host) and subsequent dissemination though trading routes though the Middle East to Europe, Africa and South America. Occasional reports of melioidosis or environmental isolates of *B. pseudomallei* from Africa and South America may reflect even more ancient origins of *B. pseudomallei* or may relate to more recent importation from the major endemic regions.

Strain tropism from geographically localized sequence types may have been an explanation for the distinct clinical syndromes seen in Australia, in particular the more frequent neurological and prostatic disease compared to other endemic countries [5, 22] and the near absence of parotid disease. Previous studies of small numbers of isolates have suggested that strains may have demonstrated tissue tropism; one study, using multilocus enzyme electrophoresis and random amplified polymorphic DNA analysis (RAPD), defined one cluster associated with pneumonia (n=9) and another associated with neurological or soft tissue infection [23]. Another study using ribotyping suggested certain ribotypes correlated with fatal outcomes and others with relapse [24]. Furthermore, considerable and consistent differential virulence of *B. pseudomallei* strains has been demonstrated in a mouse model [25]. However, in this study using a more precise and unambiguous method of strain characterisation and of determining the genetic relatedness between isolates, no evidence was found of strain tropism or differential virulence in isolates from northern Australia.

As a tool for epidemiological investigation, MLST appears to provide a similar level of discrimination to PFGE whether based on *XbaI* [4] or *SpeI* restriction. Isolates of two clones defined by PFGE derived from isolates that were not epidemiologically linked were closely-related or identical on MLST. Conversely, isolates from a case cluster that was polyclonal on PFGE were also polyclonal on MLST. Although MLST provides results that are comparable between laboratories, PFGE typing is quicker and cheaper in our context to define the molecular epidemiology of specific case clusters. Precise assignment of the genotype can then be made by characterising one isolate of each cluster by MLST.

It is concluded that, in contrast to previous studies, there is no evidence to support strain tropism of *B. pseudomallei*; differences in the mode of acquisition, inoculating dose and host factors are more likely to result in differences in presentations and outcomes of melioidosis. Within the Top End of the Northern Territory, *B. pseudomallei* strains are distributed widely throughout remote locations. These strains are distinct from those found in other endemic areas in south east Asia, possibly reflecting Australia's geographic isolation. For local epidemiological investigations, PFGE typing and MLST provide similar results.

Figure 15-2: Relatedness among isolates was displayed as a dendrogram by the UPGMA method using the matrix of pair-wise differences between the allelic profiles of the isolates. Clusters and arrows indicate Australian isolates from this study. Number of Australian isolates from this study indicated in parentheses; single unless otherwise indicated

(Figure on following page).

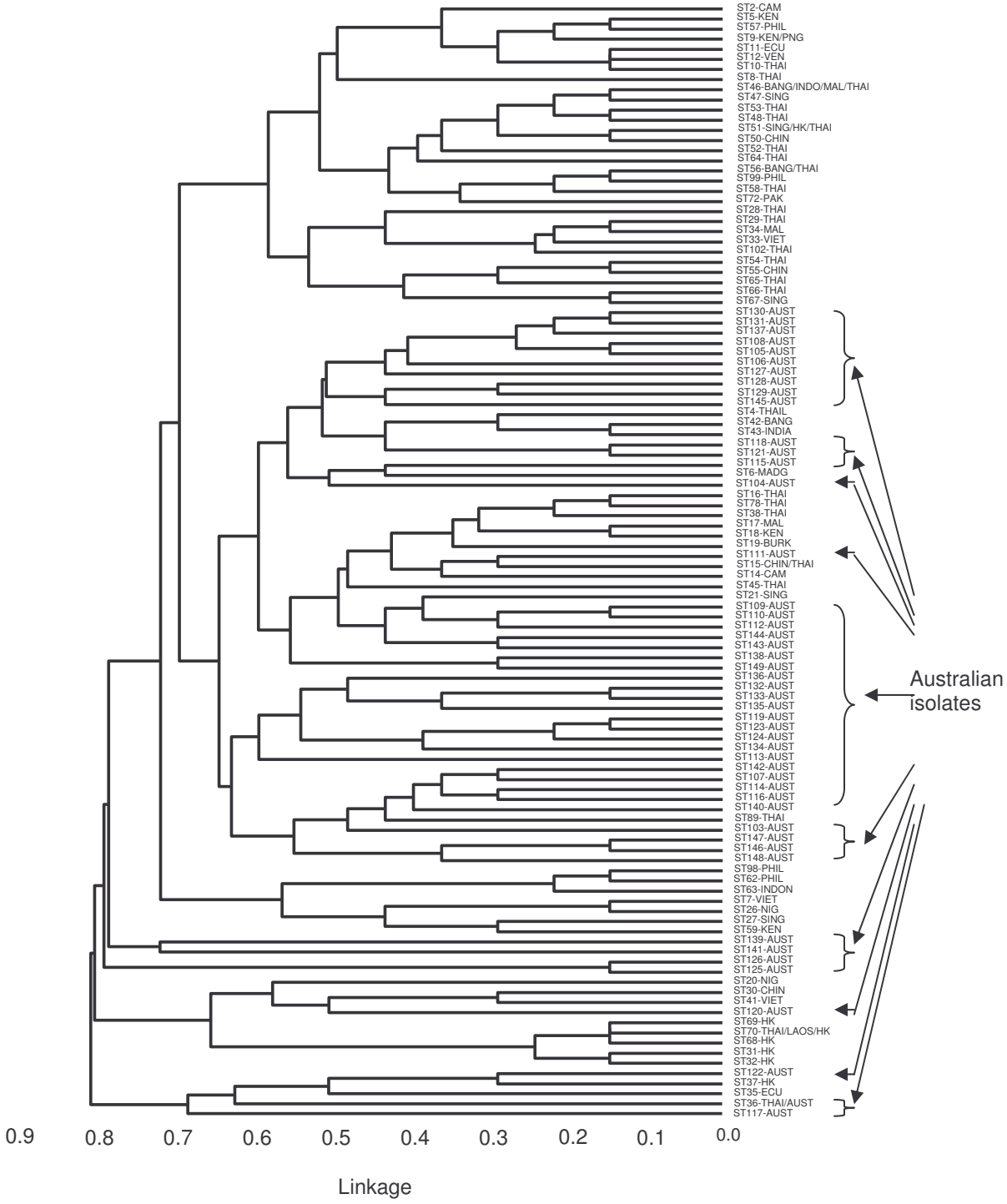
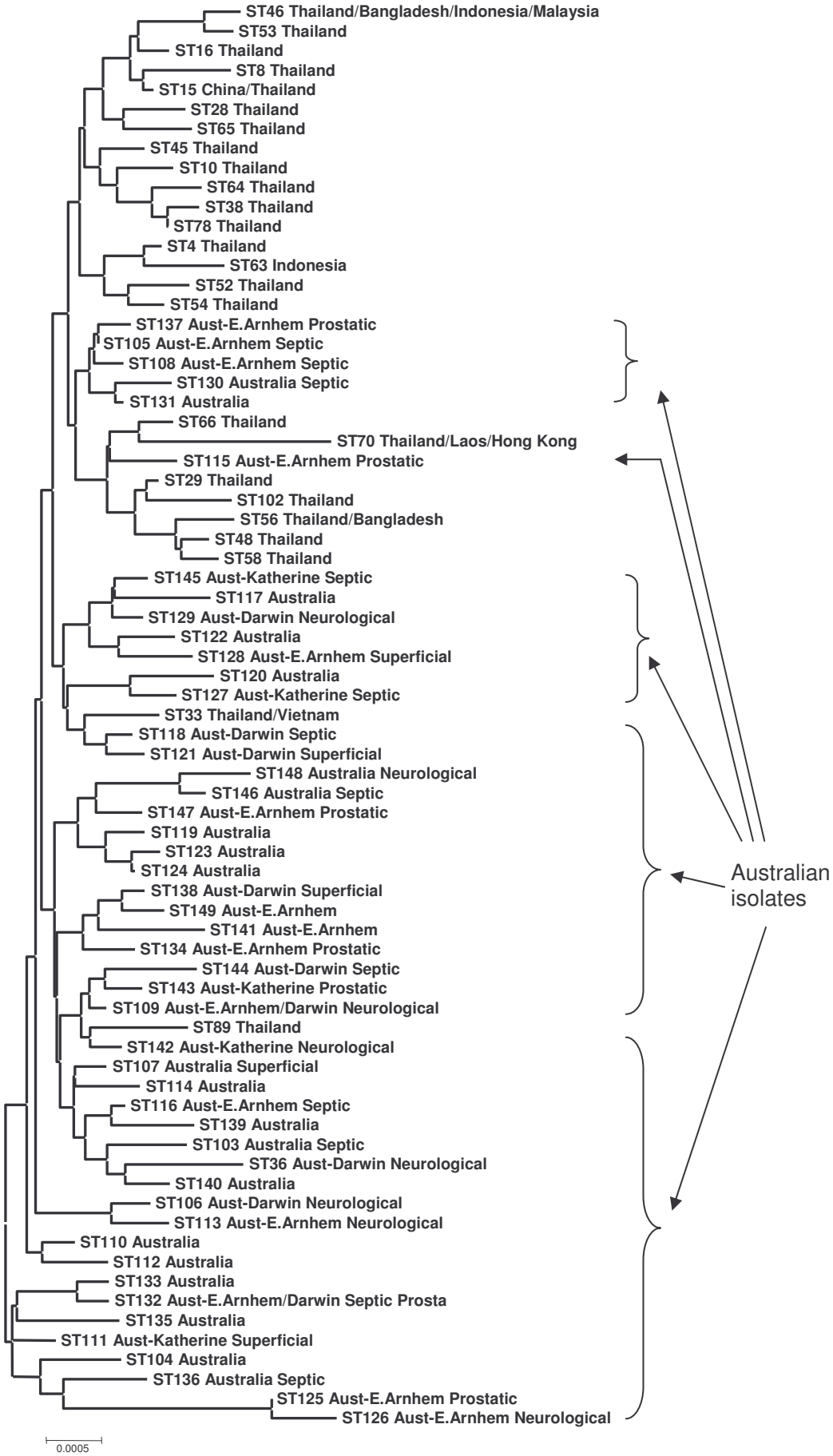

ST2-CAM
ST5-KEN
ST57-PHIL
ST9-KEN/PNG
ST11-ECU
ST12-VEN
ST10-THAI
ST8-THAI
ST46-BANG/INDO/MAL/THAI
ST47-SING
ST53-THAI
ST48-THAI
ST51-SING/HK/THAI
ST50-CHIN
ST52-THAI
ST64-THAI
ST56-BANG/THAI
ST99-PHIL
ST58-THAI
ST72-PAK
ST28-THAI
ST29-THAI
ST34-MAL
ST33-VIET
ST102-THAI
ST54-THAI
ST55-CHIN
ST65-THAI
ST66-THAI
ST67-SING
ST130-AUST
ST131-AUST
ST137-AUST
ST108-AUST
ST105-AUST
ST106-AUST
ST127-AUST
ST128-AUST
ST129-AUST
ST145-AUST
ST4-THAIL
ST42-BANG
ST43-INDIA
ST118-AUST
ST121-AUST
ST115-AUST
ST6-MADG
ST104-AUST
ST16-THAI
ST78-THAI
ST38-THAI
ST17-MAL
ST18-KEN
ST19-BURK
ST111-AUST
ST15-CHIN/THAI
ST14-CAM
ST45-THAI
ST21-SING
ST109-AUST
ST110-AUST
ST112-AUST
ST144-AUST
ST143-AUST
ST138-AUST
ST149-AUST
ST136-AUST
ST132-AUST
ST133-AUST
ST135-AUST
ST119-AUST
ST123-AUST
ST124-AUST
ST134-AUST
ST113-AUST
ST142-AUST
ST107-AUST
ST114-AUST
ST116-AUST
ST140-AUST
ST89-THAI
ST103-AUST
ST147-AUST
ST146-AUST
ST148-AUST
ST98-PHIL
ST62-PHIL
ST63-INDON
ST7-VIET
ST26-NIG
ST27-SING
ST59-KEN
ST139-AUST
ST141-AUST
ST126-AUST
ST125-AUST
ST20-NIG
ST30-CHIN
ST41-VIET
ST120-AUST
ST69-HK
ST70-THAI/LAOS/HK
ST68-HK
ST31-HK
ST32-HK
ST122-AUST
ST37-HK
ST35-ECU
ST36-THAI/AUST
ST117-AUST
Australian isolates
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Linkage

Figure 15-3: Tree constructed from the concatenated sequence of the seven MLST loci from B. pseudomallei isolates illustrating distribution of Australian sequence types (clusters indicated by brackets and unclustered strains by arrows) and those from other endemic areas.

(Figure on following page)

Nodes in the minimum evolution tree are poorly supported by bootstrap resamplings, reflecting low sequence diversity.


ST46 Thailand/Bangladesh/Indonesia/Malaysia
ST53 Thailand
ST16 Thailand
ST8 Thailand
ST15 China/Thailand
ST28 Thailand
ST65 Thailand
ST45 Thailand
ST10 Thailand
ST64 Thailand
ST38 Thailand
ST78 Thailand
ST4 Thailand
ST63 Indonesia
ST52 Thailand
ST54 Thailand
ST137 Aust-E.Arnhem Prostatic
ST105 Aust-E.Arnhem Septic
ST108 Aust-E.Arnhem Septic
ST130 Australia Septic
ST131 Australia
ST66 Thailand
ST70 Thailand/Laos/Hong Kong
ST115 Aust-E.Arnhem Prostatic
ST29 Thailand
ST102 Thailand
ST56 Thailand/Bangladesh
ST48 Thailand
ST58 Thailand
ST145 Aust-Katherine Septic
ST117 Australia
ST129 Aust-Darwin Neurological
ST122 Australia
ST128 Aust-E.Arnhem Superficial
ST120 Australia
ST127 Aust-Katherine Septic
ST33 Thailand/Vietnam
ST118 Aust-Darwin Septic
ST121 Aust-Darwin Superficial
ST148 Australia Neurological
ST146 Australia Septic
ST147 Aust-E.Arnhem Prostatic
ST119 Australia
ST123 Australia
ST124 Australia
ST138 Aust-Darwin Superficial
ST149 Aust-E.Arnhem
ST141 Aust-E.Arnhem
ST134 Aust-E.Arnhem Prostatic
ST144 Aust-Darwin Septic
ST143 Aust-Katherine Prostatic
ST109 Aust-E.Arnhem/Darwin Neurological
ST89 Thailand
ST142 Aust-Katherine Neurological
ST107 Australia Superficial
ST114 Australia
ST116 Aust-E.Arnhem Septic
ST139 Australia
ST103 Australia Septic
ST36 Aust-Darwin Neurological
ST140 Australia
ST106 Aust-Darwin Neurological
ST113 Aust-E.Arnhem Neurological
ST110 Australia
ST112 Australia
ST133 Australia
ST132 Aust-E.Arnhem/Darwin Septic Prosta
ST135 Australia
ST111 Aust-Katherine Superficial
ST104 Australia
ST136 Australia Septic
ST125 Aust-E.Arnhem Prostatic
ST126 Aust-E.Arnhem Neurological
0.0005
Australian isolates

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SECTION D: CONCLUSIONS AND FUTURE DIRECTIONS

Karl Popper argues that science is a process of falsification. In this thesis, a number of hypotheses were generated and some tested.

Does G-CSF benefit patients with severe melioidosis? The experience in Darwin does suggest this, at least to the degree that a trial is not possible there. Although confounders may explain some the benefits, it is unlikely to account fully for the dramatic change in mortality demonstrated for this specific disease. Evidence could not be drawn from *in vitro* models or other clinical trials.

The benefits of G-CSF are being tested in the ongoing trial in Thailand. However, it is clear that the generalizability of results from this may be limited to similar resource-constrained settings. A trial is also underway in Darwin examining the use of G-CSF in patients with non-melioidosis sepsis. Future studies that are planned in Thailand will have the same limitations but are obviously of great relevance to the majority of patients with melioidosis in a similar context. Protocols have been drafted for a comparison of meropenem with ceftazidime at multiple centres in northeastern Thailand.

One approach to reduce mortality in patients with melioidosis in Thailand is by the use of rapid diagnostic tests. The currently available method used in Ubon Ratchathani is immunofluorescence; however, the sensitivity of this test is moderate, its use is limited to patients from whom a direct specimen is available and to centres where specialized microscopy is available. Other rapid tests to date have proved disappointing or have remained untested in the field; none is currently available commercially.

The wider issue, however, in considering potential interventions for patients with melioidosis is the management of patients with severe sepsis generally. The clinical syndromes of severe sepsis and septic shock defined a group at a highest risk of death from melioidosis; this readily identifiable group forms the best target group to test future interventions.

Although the mortality of melioidosis is higher than those of other causes of sepsis, non-antibiotic management is similar. Future trials could test sustainable interventions in severely septic patients of any cause. Such interventions could be adapted from those successfully implemented in intensive care units in developed countries; it would be important to assess their efficacy and cost-effectiveness in resource-constrained situations. These might include studies of goal-directed resuscitation guided by non-invasive parameters, of different modalities of renal replacement therapy or of strategies to rapidly correct metabolic derangements such as hyperglycaemia and acidosis.

What are some of the determinants of the epidemiology of melioidosis? It had been suggested by previous work that water chlorination may reduce the risk of exposure to *B. pseudomallei*; this was not reflected in the overall Top End epidemiology of melioidosis. Conversely, suggestions that post-cyclonic flooding were associated with a case cluster in Queensland were borne out in an analysis of case-clusters in the Top End. This, and the associations with foci of environmental contamination, has implications for future outbreak investigations and prevention strategies.

Ongoing work is underway creating risk maps of Ubon Ratchathani province in Thailand; in the future this could be used to explore the impact of other environmental variables such as soil type and drainage on the epidemiology of melioidosis. More importantly, a correlation of melioidosis risk and agricultural practices could provide an evidence base for prevention measures.

Are bacterial factors responsible for the variations in the severity of disease and clinical pattern? This was explored using two molecular typing techniques. Where previous work had suggested that strains may vary in virulence and tissue tropism, this was not seen in the work described in this thesis using both typing techniques. However, geographical localization of strains was seen, strongest at a global level allowing conclusions to be drawn on the possible routes of spread in the endemic regions of south east Asia and northern Australia. Work is currently underway to determine if this phylogenetic signal is present within Australian isolates to establish local patterns of spread.

These findings suggest, however, that host factors and the variation in the mode and magnitude of acquisition are more important factors than bacterial strain variation in determining the pattern of disease. Humans are obviously more complex organisms to study, but work by other groups into the immunology of this disease will contribute to unraveling this mystery and defining adaptive responses that may be used in a potential vaccine.