Case cluster shifting and contaminant source as determinants of melioidosis in Taiwan

Dajun Dai1, Yao-Shen Chen2, Pei-Shih Chen3 and Ya-Lei Chen4

1 Department of Geosciences, Georgia State University, Atlanta, GA, USA
2 Division of Infectious Diseases, Kaohsiung Veterans General Hospital, Kaohsiung, Taiwan
3 Department of Public Health, College of Health Sciences, Kaohsiung Medical University, Kaohsiung, Taiwan
4 Department of Biotechnology, National Kaohsiung Normal University, Kaohsiung, Taiwan

Abstract

OBJECTIVES  To assess the geographical distribution of melioidosis contamination sources and the association between the location of melioidosis cases and positive sampling sites for *Burkholderia pseudomallei* in Taiwan.

METHODS  Data on the location of melioidosis cases from 2002 to 2011 were combined with the geographical distribution of *B. pseudomallei* as indicated by the detection of specific flagella gene products measured from 2005 to 2011. Temporal and spatial analyses were used to determine the incidence, cluster shifts and associations between the two datasets.

RESULTS  Melioidosis cases clustered in two ‘hot-spot’ areas with incidence rates that were significantly higher than in neighbouring towns. The incidence rates in the northern area gradually decreased, while the rates in the southern area increased and were temporally associated with the appearance of *B. pseudomallei*-specific flagella genes in water samples.

CONCLUSIONS  Melioidosis hot-spot areas were present in Taiwan. Water contaminated with *B. pseudomallei* serves as a potential transmission vehicle and is correlated with an increase in melioidosis cases; this correlation was stronger than that for *B. pseudomallei*-contaminated soil.

keywords  melioidosis, *Burkholderia pseudomallei*, transmission mode, temporal and spatial analysis

Introduction

Melioidosis, which is caused by the *Burkholderia pseudomallei*, is a potentially fatal infectious disease endemic to tropical areas of south-eastern Asia and northern Australia (Cheng & Currie 2005). The symptoms of melioidosis are variable, ranging from asymptomatic to symptomatic, chronic to acute and local to systemic. Acute melioidosis with sepsis is the most severe form, often resulting in death within a few days of exposure (Wiersinga et al. 2006). The degree of adverse progress of melioidosis generally depends on exposure to large inoculums of *B. pseudomallei* and immune suppression. Diabetes, alcohol abuse and chronic lung or renal diseases are potential risk factors for melioidosis infection (Tamrakar & Haas 2008; Currie et al. 2010). Transmission of melioidosis generally occurs through direct contact with or inhalation of soil or water aerosols contaminated with *B. pseudomallei* (Dance 2000). However, the distribution of *B. pseudomallei* in the environment is restricted by a number of environmental factors, such as pH, water content and UV exposure, as well as by the presence of biological antagonists, such as *B. multivorans* (Inglis & Sagripanti 2006; Palasatien et al. 2008; Lin et al. 2011). Thus, the geographical distribution of melioidosis is usually uneven and is associated with the environmental prevalence of *B. pseudomallei* (Vuddhakul et al. 1999; Su et al. 2007; Chen et al. 2010).

Prior to 2004, melioidosis cases in Taiwan were likely underestimated, as only 25 cases had been reported since 1984, most of which resulted from travel to melioidosis-endemic areas overseas (Hsueh et al. 2001; Chen et al. 2010). Since 2005, however, the number of reported or diagnosed melioidosis cases has increased, and in more than 70% of these cases, the infected individual had never travelled overseas (Su et al. 2007). This increase in the number of reported melioidosis cases might be the result of raised awareness or diagnosis of the disease by physicians in Taiwan since 2005. *B. pseudomallei* isolates harbouring pulsortypes indistinguishable from human isolates have been recovered from the environment, while the rate of bacterial isolation from the environment and melioidosis incidence has risen co-linearly in southern, central and northern Taiwan (Chen et al. 2010). During the typhoon season, the number of melioidosis cases in Taiwan grew in...
the Er-Ren River Basin in 2005 and Kaohsiung city in 2009
(Su et al. 2007, 2011), but clinical experience shows that
melioidosis cases still occur in other areas of Taiwan and
outside the typhoon season.

For the current study, we made two assumptions: (i)
cases of melioidosis are rarely linked to travel because
>80% of melioidosis patients in Taiwan have a chronic
disease history identical to that reported for endemic
areas (Currie et al. 2010; Hanna et al. 2010) and (ii) all
melioidosis cases are the result of indigenous infection
through direct exposure to neighbouring soil or water
contaminated with B. pseudomallei (Su et al. 2007).
Using these assumptions, we performed temporal and
spatial analyses to determine whether melioidosis cases
clustered in Taiwan, to explore potential sources of
bacterial contamination and to measure the association
between the location of melioidosis cases and the
geographical distribution of B. pseudomallei in the
environment.

Methods
Study cohort and demographics
The melioidosis database was provided by the Taiwan
Centers for Disease Control (CDC), where melioidosis has
been listed in the surveillance system since 2000.
Diagnosis of melioidosis was culture-confirmed for all
patients, who were then registered in the surveillance
system. The addresses of patients (neighbourhood level),
their survival or death after treatment and the suspected
date of their disease exposure were retrieved from the
surveillance system. The suspected date of exposure was
determined by the individuals’ physicians based on each
patient’s history and symptoms. Demographics were
obtained from the annual reports of the Household
Registration Office of each county government. The
average population of each town from 2002 to 2011 was
used in this study.

Environmental sampling
Data on the location of B. pseudomallei in environments
were obtained from two sources: previous studies (Su
et al. 2007; Chen et al. 2010) and the current study. The
sampling period for the previous studies was from 2005
to 2007, while for this study, it was from 2008 to 2011.
For all studies, a similar random sampling strategy as well
as similar sample manipulation and detection methods
was used (see below). Agricultural soil samples from fields
and water from fishing lots, rivers and lakes were
collected within 30 days of rainfall (>25 mm/day)
throughout the summer (June to October) from 632 km²
areas each year. The sampling sites (recorded by global
positioning system) were separated by 2 km² and located
along connecting roads, which allowed transit across the
studied areas. Samples were obtained two or three times
from independent locations within the site during the
study period. If a sampling site was positive for
B. pseudomallei DNA, the same site was sampled again
in the following year and during each subsequent year until
the site returned negative results. Overall, samples were
obtained from 391 sites (water: 188; soil: 203) per year.
Soil samples (100 g) were collected from a depth of
30–60 cm, and water samples (2 l) were collected from a
depth of 100 cm or from the bottom if the water depth
did not exceed 100 cm. All samples were placed into
sterile tubes and sent to the laboratory as quickly as
possible.

Sample manipulation
Two-litre aliquots of each water sample were thoroughly
mixed, passed through a 1-mm screen mesh and filtered
through a cellulose nitrate membrane 45 mm in diameter
(pore size 0.45 μm). The recovered material was then
scraped from the filters and diluted with 1 ml of phos-
phate-buffered saline (PBS, 0.5 ml to be used for DNA
extraction and 0.5 ml for enriched cultures). Soil (15 g) or
concentrated water samples (0.5 ml) were incubated in
50 ml or 5 ml, respectively, of selective Ashdown’s broth
(Ashdown 1979) for 8 h at 37 °C, and subsequently, cell
pellets were harvested.

Soil samples (1 g), water concentrates (0.5 ml) and cell
pellets were separately mixed with incubation buffer
(GeneMark, Taipei, Taiwan) for further DNA extraction.
The samples were shaken for 30 min at 55 °C and
centrifuged, and DNA was extracted using a genomic DNA
extraction kit for soil samples (GeneMark) or a standard
purification kit for water samples and pellets (IsoQuick,
ORCA Research Inc., Bothel, WA, USA). The specific
primers for the fliC flagellum gene were used to amplify
species-specific fliC amplicons using PCR (267 bp) (Su et al.
2007). This PCR can distinguish B. thailandensis, B. cepa-
cia complex and Pseudomonas sp. from B. pseudomallei
with as few as 0–10 cells per reaction (Chen et al. 2002;
Kao et al. 2003). As controls, the universal V3 region of
16S RNA (197 bp) was used (Sheffield et al. 1989). If the
PCR results were negative based on the control primer
amplification, 1 ml of CaCO₂-saturated water was added
to the soil samples before DNA extraction. A positive
sampling site was defined as a sampling site that was
positive for fliC at least once using either enriched or non-
enriched preparations during the sampling period.
Statistics

The statistics used total population and density, melioidosis cases and the cadastral divisions for Taiwan. A GIS (geographic information system) program, ArcGIS 10 (Environment System Research Institute; ESRI Inc., Redlands, CA, USA) was used for the spatial analysis. Cases were aggregated at the township level and analysed using the local Moran’s I:

\[
I_i = \frac{x_i - \bar{X}}{s_i} = \frac{1}{n-1} \sum_{j=1,j\neq i}^{n} w_{ij}(x_i - \bar{X})
\]

where \(n\) equals the total number of towns in the study area. Statistical testing for local Moran’s I significance levels was performed by means of randomisation (Crichton et al. 2007). In addition, the Poisson model for spatial scan statistics at SatScan (http://www.satscan.org) was used to detect where cases were significantly clustered compared to the underlying at-risk population residing in those areas.

Ripley’s K function tests the clustering of case locations at multiple different distances:

\[
L(d) = \frac{A}{\pi n(n-1)} \sum_{i=1}^{n} \sum_{j=1,j\neq i}^{n} k(i,j)
\]

where, \(d\) is the distance and \(n\) is the total number of cases. \(A\) refers to the total area derived from the minimum bounding rectangle of the cases, and \(k(i,j)\) is a weight value. Simulated outer boundary values were chosen to correct for underestimates near the edges.

The mean centre of the melioidosis case distribution was calculated by averaging the \(x\) - and \(y\)-coordinates of all new melioidosis cases that occurred in the hot-spot areas within 1 year. The distribution of melioidosis cases, namely whether the neighbouring water or soil was contaminated with \(B.\ pseudomallei\), was determined using Fisher’s exact test (where \(P < 0.05\) was defined as significant). The association between melioidosis incidence and the positive detection of \(B.\ pseudomallei\)-specific DNA in the environmental samples was determined using linear regression.

Table 1 Summary of melioidosis incidence in Taiwan from 2002 to 2011

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of Cases</th>
<th>Incidence (per 100 000)</th>
<th>95% CI *</th>
</tr>
</thead>
<tbody>
<tr>
<td>2002</td>
<td>2</td>
<td>0.009</td>
<td>0.001–0.032</td>
</tr>
<tr>
<td>2003</td>
<td>5</td>
<td>0.022</td>
<td>0.007–0.051</td>
</tr>
<tr>
<td>2004</td>
<td>13</td>
<td>0.057</td>
<td>0.03–0.098</td>
</tr>
<tr>
<td>2005</td>
<td>76</td>
<td>0.332</td>
<td>0.262–0.426</td>
</tr>
<tr>
<td>2006</td>
<td>31</td>
<td>0.135</td>
<td>0.092–0.192</td>
</tr>
<tr>
<td>2007</td>
<td>17</td>
<td>0.074</td>
<td>0.043–0.118</td>
</tr>
<tr>
<td>2008</td>
<td>44</td>
<td>0.19</td>
<td>0.138–0.255</td>
</tr>
<tr>
<td>2009</td>
<td>45</td>
<td>0.194</td>
<td>0.142–0.26</td>
</tr>
<tr>
<td>2010</td>
<td>44</td>
<td>0.19</td>
<td>0.138–0.255</td>
</tr>
<tr>
<td>2011</td>
<td>45</td>
<td>0.193</td>
<td>0.141–0.259</td>
</tr>
</tbody>
</table>

*CI, confidence interval.

Results

Melioidosis incidence and distribution

A total of 322 melioidosis cases were reported to the Taiwan CDC from 2002 to 2011. The annual incidence of melioidosis peaked (0.332/100 000 persons) in 2005 and remained relatively high (ca. 0.19/100 000) from 2008 to 2011 (Table 1). Analysing the annual case distribution geographically revealed that 57.5% (185/322) of melioidosis cases appeared in south-western Taiwan, including three counties in the north and five counties in the south (>10 cases/town on average) (Figure 1a). Of these cases, 22.7% (42/185) were fatal and also clustered to specific areas (Figure 1b). When incidence rates were measured at the town level, the two highest rates occurred in rural towns: Jiading in the north and Tsygun in the south (Figure 1c). Using local Moran’s I, the northern high-incidence area included four towns (Er-Ren River Basin) and the southern high-incidence area three towns (Zoynan Region). These rates were significantly higher than the mean rate of melioidosis incidence calculated by neighbourhood (Figure 1d). When SatScan was used to perform the evaluation, again, the cases in the Er-Ren River Basin and Zoynan Region formed a statistically significant cluster (\(P < 0.01\)) (Figure 1d). However, the data interpretation for the cluster of the three towns in the south tip of Taiwan needs caution because their populations (population density was 16–228 people/km², compared to 2042 people/km² for Jiading and 3268 people/km² for Tsygun) are small and tend to have large spatial variation (Mu & Wang 2008).

Melioidosis cluster analysis

To determine the clustering of melioidosis cases, Ripley’s K function was used to analyse the distribution of all
Figure 1. The distribution of melioidosis in Taiwan. All melioidosis cases (a) or deaths (b) were mapped. The incidence of melioidosis cases is shown based on the population average from 2002 to 2011 (c). Based on the local Moran’s I, the incidence rates in certain areas (dark colour) were significantly higher than those in neighbouring areas (light colour) at the town level (d). The dotted lines indicate the clustering by the SatScan analysis.
melioidosis cases (or fatal cases) in high-incidence areas. Our results indicate that there was a cluster of both cases (Figure 2a) and deaths (Figure 2b) in the Er-Ren River Basin and the Zoynan Region. Comparison of the L(d) values for all cases revealed that melioidosis cases were clearly clustered within a 7.5 km area according to the greatest distance observed compared to the expected data (Figure 2a). The significant distance for deaths to be considered a cluster was selected to be 2.5 km in the Er-Ren River Basin and 5.5 km in the Zoynan Region (Figure 2b).

Contaminated sources
To explore the possible environmental sources of B. pseudomallei contamination, different sampling sites (water: 188 and soil: 203) were tested for specific B. pseudomallei PCR products (fliC gene amplicons) (Figure 3). Many melioidosis cases were located in the rural areas surrounding fishing lots or city lakes (Figure 3). From 2005 to 2011, approximately 13.5% (25/188) of water sampling sites and 25.9% (53/203) of soil sampling sites were positive for B. pseudomallei. It was assumed that each positive sampling site was a potential source of contamination with B. pseudomallei, and some patients were linked to more than one contaminated source because of the heterogeneous distribution of B. pseudomallei in the environment. A total of 163 melioidosis cases were identified for patients who lived near a contaminated water source, according to the 7.5 km distance derived by Ripley’s K function for case clustering. This distribution was significantly higher than that for cases reported by patients who lived near contaminated soil (Table 2). If deaths were assumed to result from exposure to large numbers of B. pseudomallei from neighbouring environments, then 145 cases involving deaths in the Er-Ren River Basin or Zoynan Region were located within 2.5 or 5.5 km, respectively, of the distances determined by Ripley’s K function for clustering. The number of deaths near water sites contaminated with B. pseudomallei was higher than that of deaths near contaminated soil (Table 2).

To determine whether water was the major contamination source for melioidosis acquisition, the association between the percentages of B. pseudomallei-positive water samples and the annual incidence of melioidosis in the north (Er-Ren River Basin) and the south (Zoynan Region) was analysed. The results indicate that the number of positive water samples collected from the north gradually fell year after year but that the number of positive samples rose in the south (Figure 4a). The mean centre of case distribution also shifted from north to south (Figure 4b). The incidence rate reached 56/100 000 in 2005 in the north but dropped after 2005 (Figure 4c), while in the south, the rate was low in 2005 but rose to 20/100 000 in 2011 (Figure 4d). The increase or decrease in the annual incidence of melioidosis was associated with changes in the percentages of water samples that tested positive for B. pseudomallei, both in the south ($r^2 = 0.7001$) and the north ($r^2 = 0.4162$). Positive soil samples showed an identical pattern of change from north to south (Figure 4a); the correlation efficiency (incidence rate against positive rate) was $r^2 = 0.4656$ in the north and $r^2 = 0.0006$ in the south.

Discussion
Clinical experience indicates that the number of cases of melioidosis in Taiwan has substantially increased in recent
years, but the melioidosis incidence rate was relatively low (ca. 0.02–0.19/100 000) compared to northern Australia (7.98–21.3/100 000) and Thailand (5.4–24.2/100 000), where melioidosis is endemic (Currie et al. 2004; Limmathurotsakul et al. 2010). In this study, we found that melioidosis cases were clustered in specific geographical

Figure 3. Case distribution, sampling sites, terrains and land usage in hot-spot areas.
Melioidosis results from exposure to widely distributed environments contaminated with *B. pseudomallei* (Dance 2000). Despite the fact that *B. pseudomallei* can be isolated from soil or water, it is difficult to determine the true density of the organism in the environment because of its uneven and non-homogenous distribution in soil, with colony counts ranging from 10 to 1200 cfu (Wuthiekanun et al. 2005; Kaestli et al. 2007). Moreover, UV exposure and dryness limit the extensive distribution of this bacterium (Chen et al. 2003; Inglis & Sagripanti 2006; Palasatien et al. 2008), while various strains of the widely distributed soil and water microbes *B. multivorans* and *B. cenocepacia* antagonise the growth of *B. pseudomallei* (Lin et al. 2011). The isolation rates for *B. pseudomallei* are therefore as low as 27–28% per rice field, even when the sampling sites were as close together as possible and when the fixed-interval sampling strategy was used (Wuthiekanun et al. 2009; Limmathurotsakul et al. 2010). The rate of false negatives is relatively high if the soil or water was sampled using a random sampling strategy because some particular areas harbouring *B. pseudomallei* might not be covered (Limmathurotsakul et al. 2010). However, for large-scale investigations tackling many topographical barriers, irregular terrain and different land usages, random sampling is still commonly used to investigate *B. pseudomallei* in the environment and remains the strategy for demonstrating the association between the presence of *B. pseudomallei* in the environment and the high incidence of melioidosis in endemic areas (Vuddhakul et al. 1999; Chen et al. 2010; Rattanavong et al. 2011). PCR-based techniques can improve the positive detection of *B. pseudomallei* in the environment (Kaestli et al. 2007); however, in this study, approximately 13.5% and 25.9% of positive water and soil samples, respectively, were detected using random sampling. Melioidosis cases were clustered in certain towns, indicating that higher exposure to *B. pseudomallei* could be occurring in those areas where *B. pseudomallei* can be easily detected in soil or water.

**Table 2** Summary of the distribution of melioidosis cases neighboring water or soil

<table>
<thead>
<tr>
<th>Distance*</th>
<th>Origins contaminated with <em>Burkholderia pseudomallei</em></th>
<th>Fisher’s exact test**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>Soil</td>
</tr>
<tr>
<td>7.5 km</td>
<td>163</td>
<td>74</td>
</tr>
<tr>
<td>2.5 km</td>
<td>145</td>
<td>18</td>
</tr>
<tr>
<td>In Er-Ren River Basin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.5 km</td>
<td>In Zoynan Region</td>
<td></td>
</tr>
</tbody>
</table>

*The distance based on Ripley’s K function analysis

**Presence or absence of melioidosis cases in water vs. in soil

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**Figure 4.** Rates of positive PCR testing, mean centres and incidence of *Burkholderia pseudomallei* in hot-spot areas. Water (○, ○) or soil (■, □) samples were collected from northern (black) or southern (white) areas from 2005 to 2011 (a). The mean centre of the case distribution area per year was mapped (b). The shifts of melioidosis incidence rate in the north (c) and the south (d) are shown.
Spatial analysis revealed that in northern Australia, melioidosis is particularly endemic in Townsville, North Queensland, where one set of melioidosis cases was clustered into the area of the old course of a major waterway (Corkeron et al. 2010). In Northern Territory of Australia, all B. pseudomallei-positive environmental sites (n = 104) were permanently waterlogged, waterlogged during the wet season or irrigated (Kaestli et al. 2007). Water as a transmission vehicle for B. pseudomallei has been suggested when melioidosis case clusters are associated with the water supply (Currie et al. 2001; Draper et al. 2010). In south-east Asia, the occupational risk of melioidosis is correlated with a farmer’s exposure to contaminated soils through agricultural activity; however, approximately 86% of those diagnosed with melioidosis also lived in the vicinity of a river (Cheng & Currie 2005; Rattanavong et al. 2011). Indeed, many cases of melioidosis in our study were found in areas near water (fishing lots, river or lakes). If water is contaminated with B. pseudomallei, it serves as a vehicle to spread the melioidosis.

It is very difficult to determine the broadest distance of melioidosis case clusters from each contamination source. Ripley’s K function provides a tool for measuring the possible distance that significantly associates with a case cluster (Corkeron et al. 2010). Using a broad line for the distance that was generated by Ripley’s K function analysis, melioidosis cases appear to result more frequently from exposure to water than to soil contaminated with B. pseudomallei. In previous studies, we have found that the distribution of B. pseudomallei in Taiwan, unlike in endemic areas, is usually confined to specific areas (Chen et al. 2010; Lin et al. 2011). In this study, we found a high density of B. pseudomallei present upstream of the Er-Ren River, as indicated by the positive detection of specific DNA each year of the study. However, further investigation is required to determine whether B. pseudomallei persisted in the water or came from remote soil harbouring the bacteria, such as the high-density upstream area detected for the river.

Although the transmission modes of melioidosis are not fully understood, person-to-person transmission is rare (Dance 2000). The appearance of fixed contamination sources in the environment or the existence of certain vectors or vehicles for pathogen transmission lends strength to the assumptions made in spatial and temporal measurements (Kitron & Kazmierczak 1997; Corkeron et al. 2010; Ditsuwon et al. 2011). Environmental sampling has been widely used to assess whether B. pseudomallei is present in an effort to identify the geographical distribution of the organism and the related risk of infection to humans and livestock. Determining the relationship between the geographical distribution of melioidosis cases and sites of positive sampling for B. pseudomallei provides insight into the transmission modes of B. pseudomallei in high-risk areas for melioidosis incidence in Taiwan.

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Corresponding Author Ya-Lei Chen, Department of Biotechnology, National Kaohsiung Normal University, Kaohsiung, Taiwan. Tel.: 886 7 6051366, Fax: 886 7 6151353, Email: dan1001@ms31.hinet.net