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Prevalence of Melioidosis in the Er-Ren River Basin, Taiwan: Implications for Transmission▽

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An increase in melioidosis cases compared to other areas in Taiwan was observed in the Er-Ren River Basin, southwestern Taiwan, from November 2001 to August 2006. The objective of this study was to determine the association between the level of exposure to *Burkholderia pseudomallei* and the incidence rate of melioidosis and to survey the transmission modes of *B. pseudomallei* in the Er-Ren River Basin. The serosurveillance of melioidosis gave seropositivity rates of 36.6%, 21.6%, and 10.9%, respectively, for residents in regions A, B, and C within the Er-Ren Basin area. Culture and PCR-based detection of *B. pseudomallei* from soil demonstrated that the geographical distribution of this bacterium was confined to a particular site in region B. The distribution of seropositive titers was significantly associated with the incidence rate of melioidosis (120, 68, or 36 incidence cases per 100,000 population in region A, B, or C in 2005), whereas it did not correlate with the geographical distribution of *B. pseudomallei* within the soil. A survey of transmission modes showed that residents with seropositivity were linked to factors such as having confronted flooding and having walked barefoot on soil, which are potential risk factors associated with exposure to *B. pseudomallei*. Our findings indicated that the Er-Ren River Basin in Taiwan has the potential to become a high-prevalence area for melioidosis. This is the first report that documents a high prevalence of melioidosis in an area north of latitude 20°N.

Melioidosis is a fatal infectious disease caused by *Burkholderia pseudomallei*, which is endemic in Southeast Asia and northern Australia (7). Human infection with *B. pseudomallei* usually occurs by inhalation or subcutaneous inoculation and only rarely through ingestion (13). Clinical manifestation includes a variety of symptoms ranging from an unapparent localized chronic infection to a full-blown systemic infection. After the onset of acute septicemia, the mortality rate is about 40% (2). Worldwide, fatal pulmonary melioidosis has been increasing among travelers returning from areas of endemicity (8).

B. pseudomallei is a saprophyte, and it is widely distributed in tropical soil and water but with an uneven distribution (13). The presence of *B. pseudomallei* in soil is associated to some degree with areas having a high incidence of melioidosis (23, 24, 27). Most patients with melioidosis in Thailand are farmers who suffer from heavy exposure to *B. pseudomallei* during agricultural activities (22). Human exposure to *B. pseudomallei* may occur at preschool ages, as serosurveillance in northeast Thailand shows (25). In areas of nonendemicity, the seropositivity rate is relatively low because individuals have little chance to come into contact with the pathogen (5). However, there has been no systematic international study of seroprevalence rates using a consistent, standardized, and quality-controlled serological test. It is thus difficult to make adequate comparisons between countries and regions.

Melioidosis in Taiwan was first reported in 1984 when a traveler was diagnosed as having a pulmonary infection after a drowning incident near Manila, Philippines (18). Since 1994, cases of melioidosis in Taiwan have been steadily increasing and have appeared to be indigenous as these patients had never traveled overseas (3, 16, 19, 20). The clinical manifestations of melioidosis are quite protean, and therefore clinical diagnosis is often difficult. As a result, the true incidence of melioidosis may be higher than is currently recognized (12). Whether this increase in the diagnosis of melioidosis in Taiwan is due to the widespread existence of *B. pseudomallei* or a previous underdiagnosis of cases remains unanswered.

We have previously demonstrated that *B. pseudomallei* can be isolated from cropped soil in southern Taiwan (17) and that city-dwelling individuals have a seropositivity rate of only 5% for melioidosis (5). It appears that specific antibodies against *B. pseudomallei* can still exist in humans even 6 months after exposure (6). The cases of melioidosis that occurred in the Er-Ren River Basin in 2005 represent the highest incidence rate of melioidosis among all regions in Taiwan. Thus, we conducted an epidemiological investigation to determine the seroprevalence rate and geographical distribution of *B. pseudomallei* in the Er-Ren River Basin.

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MATERIALS AND METHODS

Case definition and risk factor analysis. Melioidosis in Taiwan is classified as a notifiable disease, which means that all culture-confirmed cases of melioidosis should be reported to the Centers for Disease Control, Department of Health Taiwan. From 1 November 2001 to 31 August 2006, a total of 133 melioidosis cases were officially documented.

There were 66,103 residents in 32 villages surrounding the Er-Ren River Basin in 2005. Between February 2006 and April 2006, 624 serum samples were collected from residents by convenience sampling. All blood specimens were collected by a staff nurse following an Institutional Review Board procedure established by the Centers for Disease Control, Department of Health Taiwan. The numbers of samples were predetermined to be at a ratio of 1:100 among residents in each village and to cover the total population in the Er-Ren River Basin. The 624 residents who provided their blood samples also filled out a questionnaire at the same time. This questionnaire covered topics such as demographics, family history, housing conditions, the environment, life or work routines, and travel history. Only significant or interesting variables were selected and are mentioned in this study.

Serodiagnosis. The serum samples were tested for melioidosis antibodies using an indirect enzyme-linked immunosorbent assay (6). Briefly, 96-well polystyrene microtiter plates were coated with *B. pseudomallei* flagellin (0.5 μg/ml) in coating buffer (50 mM carbonate/bicarbonate buffer [pH 9.6]) at 4°C overnight. The plates were then blocked for 2 h using 100 μl of bovine serum albumin (1 mg/ml; GIBCO, Grand Island, NY). Next, they were washed three times with saline-Tween solution (0.9% [wt/vol] NaCl and 0.05% [vol/vol] Tween 20 in phosphate-buffered saline [PBS]). The wells were incubated at 37°C for 1 h with twofold serial dilutions of the sera in PBS and then washed with saline-Tween solution and incubated with diluted (1:1,000) anti-human immunoglobulin G conjugated with peroxidase (Zymed, South San Francisco, CA) at 37°C for 1 h. Finally, the wells were washed again with PBS three times, and 100-μl volumes of 1-Step Turbo tetramethylbenzidine enzyme-linked immunosorbent assay substrate (Pierce) were added to each well. The optical density at 450 nm of the wells was determined using a microplate reader (Anthos 2010). When the average of the optical density readings of the test sample was greater than that of the negative controls plus 2 standard deviations, the test sample was considered to be positive for the specific antibody. The highest dilution of the tested sample that still gave a positive result was considered the endpoint titer and is listed in the results.

Soil sampling. Soil samples were collected from various cropped fields that were located on both sides of the main Er-Ren River and its branches. The sampling sites were separated by between 0.5 km and 1 km and stretched from Kuan-Yin Village to Wan-Fu Village. The area surveyed was about 130 km^2, 0.37% of the total area of Taiwan. In the Er-Ren Basin, the rainfall season is from May to September and can reach up to 1,200 mm/month during July. In this study, the sampling time was from October 2005 to December 2005, which is the dry season in this area (average rainfall is <20 mm/month). In total, 311 sampling sites were examined (ca. 2 sites/km^2). Each site was sampled from three separate holes at the same time. Approximately 100 g of soil sample was obtained at a depth of 30 to 60 cm from the bottom of each hole and placed into a sterile tube.

PCR detection. The genomic DNA of bacteria present in the soil was isolated using a soil genomic DNA extraction kit (GeneMark, Taiwan) and purified using another kit (IsoQuick; ORCA Research, Inc.). Two primer sets (16SrRNA gene, forward, 5′-CGGCAGCGCGGGCTTCGG-3′; reverse, 5′-TGTGGCTGGTCG TCCTCTC-3′ and 5′-CACTCCGGGTATTAGCCAGA-3′; flagellum gene, forward, 5′-CTGTCGTCGACGGCCGTG-3′; reverse, 5′-ATTGTTGACCGTCG CGAG-3′) were used to amplify species-specific amplicons (243 and 405 bp for the 16S RNA gene and 267 bp for the flagella gene) (14, 17). The PCR mixture consisted of 1 pg genomic DNA, 0.5 μmol of each primer, 250 μmol/liter deoxynucleotide triphosphate (dNTP), 1× PCR buffer, and 1 U *Taq* polymerase with a final volume of 50 μl. The PCR profile consisted of 40 cycles of 1 min at 94°C, 30 s at 60°C, and 1 min at 72°C, with a final extension step of 10 min at 72°C. The products were visualized by 1.5% agarose electrophoresis. When amplicons of both the 16S RNA gene and the flagellum gene were observed, the sample was considered to be positive for *B. pseudomallei* (17).

Enrichment, culture, and identification of *B. pseudomallei*. Soil samples (15 g) were placed individually into 50 ml of Ashdown's broth (26) in a 250-ml flask. The flask was shaken vigorously for 5 min and then incubated at 150 rpm/min and 42°C for 2 days. The cultures were repeatedly streaked onto Ashdown's medium at 0, 1, and 2 days. The plates were incubated at 37°C for 2 to 6 days to allow the dry, wrinkled, violet-to-purple colonies typical of *B. pseudomallei* to grow. These typical colonies were stored in Luria-Bertani broth containing 15% glycerol at −80°C for further identification.

The environmental isolates of *B. pseudomallei* were confirmed by biochemical tests and their molecular characteristics. The biochemical tests were performed using an ID32 GN profile (API System; bioMérieux, France). The molecular characteristics were evaluated according to the presence of the specific amplicons for the 16S RNA and flagellum genes (see above).

Statistical evaluation. Statistical analyses were carried out using the χ^2 test (Epi Info, version 5.01b, 1991) and the χ^2 exact test (StatXact, version 2.05, 1991). The significance of differences between two groups was defined as $P < 0.05$. A binomial distribution was used to construct a 95% confidence interval for the incidence of melioidosis.

RESULTS

From November 2001 to August 2006, 54.1% (72/133) of melioidosis cases in Taiwan occurred in the Er-Ren River Basin in southwestern Taiwan (Fig. 1). Detailed information on the incidence rate of melioidosis in Taiwan, the Er-Ren River Basin, and the various regions within the Er-Ren River Basin are listed in Table 1. The Er-Ren River Basin had an incidence of 70/100,000 in 2005, the highest incidence rate compared in Taiwan. We set out to determine if residents in Er-Ren River Basin were exposed to *B. pseudomallei*. Therefore, 624 serum samples were collected from residents to detect the presence of specific antiflagellin antibodies. The Er-Ren River Basin can be divided into three regions based on the seropositivity rate, namely high (A [36.6%]), medium (B [21.6%]), and low (C [10.9%]) (Table 2). The seropositivity rate in the Er-Ren River Basin was associated with the incidence of melioidosis as follows: 120/100,000 in region A, 68/100,000 in region B, and 36/100,000 in region C (Table 1). If each region was subdivided into several sites (A1 and A2; B1, B2, and B3; and C1, C2, and C3) based on the presence of geographical barriers such as river segmenting or discontinuous locations (Fig. 1), then the association between seropositivity and incidence rate was not sustained for each intradivision (Table 1). Sites B3 and C3 exhibited seropositivity rates among the residents of 23.8% and 9.2%, respectively (Table 2), although these sites did not report melioidosis cases in 2005.

To determine if the geographical distribution of *B. pseudomallei* was linked to seropositivity in residents in the Er-Ren River Basin, bacterial isolation combined with PCR-based detection were used to detect the presence of *B. pseudomallei* in soil specimens. The results indicated that the geographical distribution of culturable *B. pseudomallei* was confined to site B3, where there was a 26.4% positive bacterial isolation rate for *B. pseudomallei* (Table 2). Nevertheless, the presence of *B. pseudomallei* was detected over an extensive region using the PCR-based technique. There were positivity rates of 3.1% to 6.7%, 2.6% to 33.0%, and 0% to 5.6% in regions A, B, and C, respectively (Table 2). However, the actual incidence of melioidosis (Table 1) and the seropositivity rate for *B. pseudomallei* (Table 2) did not correlate with the presence of *B. pseudomallei* in soil from the Er-Ren River Basin in these areas.

To address the possible transmission modes of *B. pseudomallei* in the Er-Ren River Basin, a survey was conducted to evaluate the daily routine of the residents. Variables including sex, age, travel history, and occupation were first excluded since these variables did not show statistical significance. Being barefoot was a significant factor (29.9%; $P < 0.05$), as reported

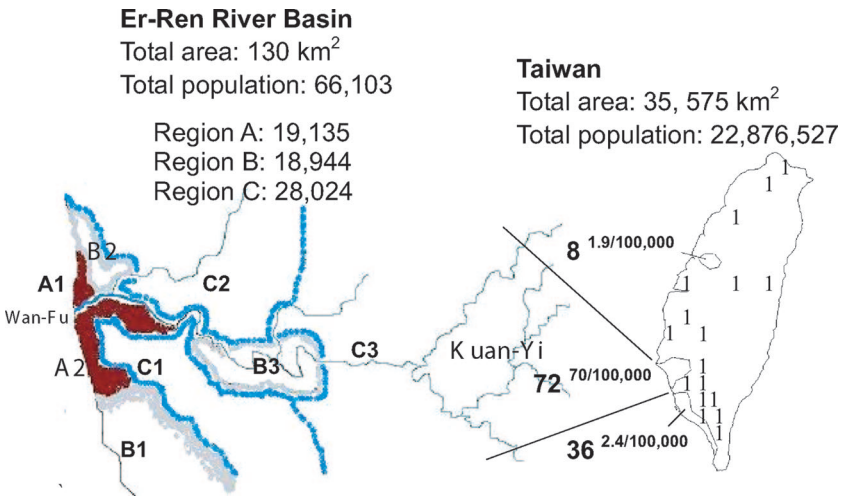

FIG. 1. Map of case distribution and divisions for the melioidosis serosurveillance. The melioidosis cases in Taiwan (2001 to 2006) are indicated on the right-hand side. The numbering indicates the numbers of cases in specific areas. The incidence of melioidosis in clusters of cases in 2005 is listed to the upper right of the indicated number of cases. The surrounding areas of the Er-Ren River Basin are enlarged in the map on the left. The letters A, B, and C indicate the regions with distinct seropositive rates for melioidosis or different isolation rates for *B. pseudomallei* (see text). Region A was subdivided into sites A1 and A2 because the two sites are separated by the river and the agriculture involves dramatically different crops.

from region A (Table 3), where there was a 36.6% seropositivity rate for residents (Table 2). In addition, experiencing flooding within the last 6 months was also a significant factor reported from region A (32.8%; $P < 0.05$) and B (34.2%; $P < 0.05$) (Table 3). Only 13.4% of residents complained of this flooding in region C, where there was only a 10.9% seropositivity rate for residents. It seems that walking barefoot and flooding are important factors that might result in a high risk of exposure to *B. pseudomallei* in region A. The same questionnaire was given to 30 patients who had contracted melioidosis in the last 6 months, with a response rate of 43% (13/30). The results showed that 38.5% were farmers and 46.2% were living near fields, but only 7.7% had walked barefoot on the soil. It is interesting that 38.5% of respondents had experienced a flood within the past 6 months. It appears that water contact is the most important factor contributing to infection with *B. pseudomallei* among residents in the Er-Ren River Basin.

DISCUSSION

Melioidosis in Taiwan has been recognized as an emerging disease (16). However, its prevalence has not yet been fully evaluated despite a substantial increase in sporadic cases over recent years (3, 16, 19, 20). Data from 2001 to 2006 show that cases of melioidosis are not evenly distributed; 54.1% of cases were localized to the Er-Ren River Basin in southwestern Taiwan. We have confirmed that residents in the various regions within Er-Ren River Basin exhibited 10.9% to 36.6% seropositivity for melioidosis, which is significantly higher than the 2.5% to 5% that has been reported among Taiwanese in

TABLE 1. Summary of the annual incidence of melioidosis in Taiwan in 2005

Location	Incidence rate/100,000 population (95% CI)[a]
Taiwan	0.3 (0.25–00.40)
Er-Ren River Basin	70 (49.49–89.69)
Region and subdivision	
A	120 (71.10–169.29)
A1	60 (7.44–60.12)
A2	166 (89.59–166.39)
B	68 (31.33–105.91)
B1	73 (1.49–73.33)
B2	124 (43.14–124.30)
B3	ND
C	36 (13.57–57.80)
C1	55 (7.25–55.25)
C2	52 (51.99–213.49)
C3	ND

[a] Data are shown as the incidence rate per 100,000 population, with 95% confidence intervals in parentheses. ND, no confirmed case found.

TABLE 2. Summary of the seroprevalence and geographical distribution of *B. pseudomallei* in the Er-Ren River Basin

Region and subdivision	Seroprevalence[a]		Geographical distribution		
	Sampling size (*n*)	% Seropositive	No. of detection sites	% Isolation	% PCR positive
A	183	36.6			
A1		37.1	30	0.0	6.7
A2		35.6	32	0.0	3.1
B	176	21.6			
B1		23.1	42	0.0	11.9
B2		19.5	38	0.0	2.6
B3		23.8	91	26.4	33.0
C	265	10.9			
C1		12.5	24	0.0	0.0
C2		11.1	18	0.0	5.6
C3		9.2	36	0.0	0.0

[a] Significance ($P < 0.05$), A > B > C.

TABLE 3. Summary of the questionnaire results

Variable	Characteristics of survey responders[a]			
	Region (responders)			Melioidosis ($n = 13$)[b]
	A ($n = 67$)	B ($n = 38$)	C ($n = 29$)	
Sex				
No. male	32	13	14	8
No. female	35	25	15	5
Age				
21–40 yr	2	3	1	0
41–60 yr	23	11	10	0
>60 yr	42	24	18	13
Lifestyle				
% Had been in area of endemicity[c]	29.9	26.3	27.6	NA[d]
% Farmers	20.9	21.1	20.7	38.5
% Working or living near fields	49.2*	21.1	48.3*	46.2
% Frequently worked in nearby fields	34.3*	23.7	37.9*	NA
% Often barefoot on soil	29.9*	15.8	17.2	7.7
% Had been flooded within last 6 mo	32.8*	34.2*	13.4	38.5

[a] *, $P < 0.05$.
[b] Due to the limited number of valid questionnaires, statistical analysis was not performed.
[c] Areas of endemicity were defined to include Southeast Asia and northern Australia.
[d] NA, over 50% of responders did not answer.

general (5). Moreover, environmental samples that yielded positive PCR or culture results demonstrated the presence of *B. pseudomallei* in the cropped soil in the Er-Ren River Basin. Taken together, the Er-Ren River Basin in Taiwan should be described as a high-risk region for the occurrence of a number of melioidosis cases.

Early classification of *B. pseudomallei* environmental isolates divided them into pathogenic (arabinose nonassimilating) and nonpathogenic (arabinose assimilating) strains. However, nonpathogenic strains are now classified as *Burkholderia thailandensis* (1) and are easily distinguished from the pathogenic strains based on specific 16S RNA gene and flagellum gene amplicons (14, 21). Using the presence of these specific amplicons as detected by a PCR-based technique, it was demonstrated that *B. pseudomallei* existed in a wide range of soil samples. However, it was sometimes not possible to enrich these PCR-positive soil specimens to allow isolation of *B. pseudomallei* in this study. This may be because a cross-reaction of PCR occurred between *B. pseudomallei* and other unidentified organisms. In addition, the presence of nonviable or unculturable *B. pseudomallei* could also result in negative results for the bacterial cultures. Since we have previously demonstrated *B. pseudomallei* is capable of survival and growth in soil media mimicking the Taiwan environment for 6 months (4), a very low level of bacteria or the presence of unculturable bacteria might be the origin of the infection risk. This risk would occur when conditions that restrict the growth of *B. pseudomallei* are removed and the organism becomes more prevalent.

B. pseudomallei inhabits soil or water in tropical areas and in particular is found between latitudes 20°N and 20°S (7). Its geographical distribution is uneven. For example, the isolation rate was 37% in Pattalung but only 3% in Trang, neighboring provinces in southern Thailand (15). In this study, the isolation rate from site B3 was high at 26.4%. Beyond this site, the distribution of *B. pseudomallei* was low, although the presence of the bacteria was occasionally detected by PCR. This indicates that site B3 is a potential risk area that may be involved in spreading this bacterium across the Er-Ren River Basin. This is the first reported area with such a high isolation rate for *B. pseudomallei* north of latitude 20°N.

In this study, the order of the seropositive titers of the various regions correlated with the degree of the incidence rate of melioidosis, but there was no direct correlation with the presence of viable *B. pseudomallei* at specific sites within the Er-Ren River Basin. Since the geographical distribution of *B. pseudomallei* is usually uneven, the transmission of the infectious organism causing melioidosis from one site to another has been proposed to occur through vectors such as floodwater or wind (10). In one particular instance, during an outbreak of melioidosis in Australia, it was found that the disease was spread by a water conduit (11). Based on our data, 32.8% or 42.3% of residents shared common experiences of flooding in regions A or B. The seropositivity and incidence rates for melioidosis in both regions were significantly higher than in region C. It is possible that residents were infected by the bacterium through floodwater. Alternatively, there could have been direct contact with propagating unculturable bacteria that had rapidly proliferated under suitable growth conditions.

The annual incidence of melioidosis in the Er-Ren River Basin in 2005 was 70/100,000, which is higher than those of areas where melioidosis is endemic: for example, 16.5/100,000 in the top end of the Northern Territory, Australia, and 4.4/100,000 in Ubon Ratchathani Province in northeast Thailand (7). Indeed, the presence of *B. pseudomallei* in soil and a high seropositivity rate in residents were clearly evident for the Er-Ren River Basin. Physicians who practice in this area should be aware of melioidosis when patients present with an unknown fever or community-acquired pneumonia. Special attention should be given to seropositive individuals because *B. pseudomallei* may persist in humans for a long time and can easily reactivate, which can lead to patient relapse, especially among immunocompromised individuals, in whom the disease can be fatal (7, 9).

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