

SEROPREVALENCE OF *BURKHOLDERIA PSEUDOMALLEI* IN EAST TIMORESE REFUGEES: IMPLICATIONS FOR HEALTHCARE IN EAST TIMOR

PK Armstrong^{1,2}, NM Anstey^{3,4}, PM Kelly^{1,2,3}, BJ Currie^{3,4}, N Martins^{3,4}, P Dasari^{3,4} and V Krause¹

¹Center for Disease Control, Casuarina; ²Communicable Diseases Branch, NSW Health, North Sydney; ³Infectious Diseases Division, Menzies School of Health Research, Casuarina, Northern Territory; ⁴ Charles Darwin University, Darwin, Northern Territory, Australia

Abstract. Melioidosis is a disease with protean clinical manifestations caused by the bacterium *Burkholderia pseudomallei*. It is endemic in countries surrounding the newly independent East Timor, but has yet to be isolated or demonstrated serologically in that country. One illness that can be clinically indistinguishable from melioidosis is pulmonary tuberculosis, a condition with a very high prevalence in East Timor. We used an indirect hemagglutination test (IHA) to measure antibodies to *B. pseudomallei* in 407 East Timorese evacuated to Darwin, Australia, in September 1999. Assuming a positive IHA titer as $\geq 1:40$, the overall seroprevalence rate was 17.0%, in keeping with other seroprevalence studies from the region. The IHA titres ranged up to 1:320. After adjusting for age, females were 2.5 times more likely to be seropositive than males ($p=0.0001$). There was an inverse relationship between seropositivity and age. This study shows that exposure to *B. pseudomallei* occurs in East Timor melioidosis is also likely to occur. Due to the lack of laboratory facilities at present, it may be some time before a laboratory-confirmed case proves that melioidosis occurs. In the meantime, clinicians in East Timor should include melioidosis in the differential diagnosis of the many conditions that it may mimic.

INTRODUCTION

Burkholderia pseudomallei is the causative organism for melioidosis, a disease of humans and some domestic and wild animals that has protean clinical manifestations. Melioidosis occurs in the tropics, predominantly within the latitudes of 20° North and South of the equator. East Timor (estimated population 780,000 in May, 2000) (Anonymous, 2000) is a newly independent country located within the eastern part of the Indonesian archipelago, 9° South of the equator. Although presently no seroepidemiological studies suggest, or clinical or laboratory reports confirm, the existence of melioidosis in East Timor, the known epidemiology of the disease would support its presence there. To thrive in nature, *B. pseudomallei* requires relatively high temperatures, high humidity, and abundant rainfall, con-

ditions that exist in East Timor (Thomas *et al*, 1979). Melioidosis is known to occur in the neighboring countries of (northern) Australia, Papua New Guinea, Singapore and Malaysia (Dance, 1991).

It is important to determine whether or not melioidosis occurs in East Timor so it may be considered in the differential diagnosis of the many illnesses it may mimic. Without evidence that melioidosis exists there, consideration of the disease is unlikely to occur. Melioidosis presents in many guises, but in East Timor an important disease entity for it to be distinguished from is pulmonary tuberculosis, a disease which can clinically be impossible to differentiate from chronic pulmonary melioidosis. Tuberculosis is a major public health problem in East Timor, with a point prevalence (estimated from the same cohort of East Timorese as in this study) of 542/100,000 for smear positive cases and 2,060/100,000 for culture positive cases (Kelly *et al*, 2002).

It is important to differentiate the epidemiology of the clinical disease *melioidosis*, from

Correspondence: PK Armstrong, Communicable disease Branch, NSW Health, LMB 961, North Sydney; NSW, Australia 2059.

Tel: 61-2-9391-9207; Fax: 61-2-9391-9189

E-mail: paarm@doh.health.nsw.gov.au

subclinical infection with *B. pseudomallei*. Case series and case-control studies supply data for the former, whereas seroepidemiological methods aid in describing the latter. A significant proportion of the population of a country or region may be seropositive yet no clinical cases may be recorded there (Sanford, 1995). Because the geographical distribution of melioidosis includes mainly developing countries, this apparent paradox may be due to an inability to isolate the organism because of resource constraints, rather than absence of the disease.

Using the same laboratory methodology used in the present study, seroprevalence estimates for exposure to *B. pseudomallei* in healthy blood donors from endemic countries in South-east Asia were 26.5% (Malaysia) and from 4-47% (Thailand). The upper estimates of the Thai studies may have been biased by cross reactivity with antibodies to avirulent *B. pseudomallei*-like organisms (recently renamed *Burkholderia thailandensis*) found in some areas of that country (Trakulsomboon, *et al*, 1999). These figures compare with seropositivity rates in non-endemic areas of Australia and USA of between 0 and 1% (Strauss *et al*, 1969; Ashdown *et al*, 1989).

There is scant information in the published literature regarding the relationship of age and gender with seroprevalence. However, it is known that clinical melioidosis is more likely to occur in older age groups, (Suputtamongkol *et al*, 1994) and in males rather than females (Guard *et al*, 1984; Kanaphun *et al*, 1993; Suputtamongkol *et al*, 1994; Faa and Holt, 2002). Within an endemic country, seroprevalence rates vary widely according to the region within the country (Strauss *et al*, 1969; Werner *et al*, 1998).

Sera collected from a cohort of East Timorese people were analysed retrospectively to calculate the seroprevalence rate of exposure to *B. pseudomallei* and thereby provide indirect evidence on whether or not melioidosis occurs in East Timor.

MATERIALS AND METHODS

Subjects

Following the referendum for independence in East Timor in 1999, more than 2000 people sought refuge at the United Nations compound

in the capital Dili. From this group, 1,863 were evacuated in 2 groups to Darwin, Australia, over a 4-day period in mid-September 1999 (Evans *et al*, 2000). At least one blood sample was collected in EDTA from 603 refugees. Each of the first group (Group 1) of 347 refugees had blood drawn on arrival in Darwin to establish the prevalence of malaria in that group. The remaining 256 samples were obtained from members of the second group (Group 2, 1,516 individuals) who attended a temporary "Chest/Fever Clinic", if they were found to be febrile, have an abnormal chest X ray suggestive of past or current pulmonary tuberculosis, or were pregnant. All plasma samples were stored at -70°C until tested in June 2002. Testing for antibodies to *B. pseudomallei* was carried out in 8 separate batches over a 3-week period. Each batch was randomly selected from the pool of remaining samples (initially 603), and comprised between 28 and 118 samples. In total, serum samples from 407 individuals were tested.

Laboratory methods

Indirect hemagglutination (IHA) was the test method used, based on that developed by Ileri in 1965 and later modified by Strauss *et al*, (1969). The amount of antibody was quantified by observing the maximum serum dilution that resulted in hemagglutination. The serological test used was not thought to cross-react with other pathogens apart from the closely related *B. thailandensis*, an organism that has only been isolated from certain areas of Thailand (Trakulsomboon *et al*, 1999), and *Legionella pneumophila* (Klein, 1980).

The antigen used in the IHA test was polysaccharide derived from a sample of 5 strains of *B. pseudomallei* chosen for their geographical diversity in the Northern Territory, Australia. The optimum dilution for each new batch of antigen was determined by a block titration method. Three control sera of known titer, and validated at an external reference laboratory, were tested with each sample batch; a negative control, a low positive control, and a high positive control. Sheep erythrocytes, the antigen carrier, were suspended and sensitized by adding the *B. pseudomallei* antigen and incubating the mixture at 37°C for 1 hour. The plasma speci-

mens to be tested were first heated to 56°C to inactivate complement, after which non-sensitized sheep erythrocytes were added to adsorb any non-specific hemagglutinins. After centrifugation, the supernatants were transferred to a round-bottomed microtiter plate and serially diluted 2-fold, commencing at 1:10 and finishing with 1:10,240. After two hours incubation at room temperature, the well erythrocyte patterns were manually read with the aid of a microtiter test reading mirror. A test result was interpreted as negative if hemagglutination was observed at a titer of <1:20, as borderline if the titer was 1:20, and positive if it was \geq 1:40. For the purposes of our study, a subject was considered seropositive if the titer was \geq 1:40, in keeping with previously published seroepidemiological studies.

Ethical approval

Ethical approval for the study was obtained from the Top End Human Research Ethics Committee at the Menzies School of Health Research in Darwin, Northern Territory, Australia. Consent to test the de-identified plasma was given by the Head of the Division of Health Services of the United Nations Transitional Authority for East Timor.

Statistical analysis

Two outcome factors are reported: 1) proportion who are seropositive (IHA titer \geq 1:40), and 2) geometric mean titers. The influence of age and gender as explanatory variables of seropositivity was calculated. Age was examined as an ordinal categorical variable (15-year age groups). A subgroup analysis of children (age

<15 years) was carried out (3-year age groups).

Data were entered into an Access database (Microsoft Corporation, USA) and statistical analysis was performed using STATA[™] software (version 7.0, Stata Corporation, College Station, Texas, USA). The chi-square test was used to compare proportions. Adjusted relative risks for various age groups and for gender were computed using the Mantel-Haenszel method. For comparisons where the underlying assumption of normality was violated, the Wilcoxon rank-sum test was used. A p-value <0.05 was chosen as the level of significance for all tests; 95% confidence intervals are reported.

RESULTS

The age and gender breakdown of the total population of refugees (n=1,863) was similar to that of East Timor as a whole: 51.5% were males, 52% were less than 20 years and 15% under 5 years. The mean age was 21.1 years (range 0 to 96 years). Other demographic data, such as region of origin and occupation, were unknown, but the Group 1 (n=347) study population was more likely to be weighted towards those with an urban origin. The baseline characteristics of the study subjects by age and gender are shown in Table 1. Overall, 58.2% of the study sample were males, although this proportion varied according to age group, with a smaller proportion in the lower age groups. The mean age was 28.8 years; this differed by gender [males-31.1 years, females-25.5 years (p<0.001 for difference)].

Table 1
Baseline characteristics of study subjects and rates of seropositivity to *B. pseudomallei* (IHA antibody titer >1:40), by 15 year age groups.

Age (years)	Mean age (years)	No. of cases (%)	Percentage males	Seroprevalence (%)		
				Male (n=237)	Female (n=170)	Overall (n=407)
<15	9.4	68 (16.7)	39.7	18.5	34.2	27.9
15-29	23.8	157 (38.6)	54.8	8.1	30.1	18.5
30-44	35.4	138 (33.9)	68.8	9.5	18.6	12.3
>45	54.6	44 (10.8)	65.9	6.9	13.3	9.3
Overall	28.8	407 (100)	58.2	9.7	27.1	17.0

Table 2
Seropositivity rates to *B. pseudomallei* in children (<15 years) in 3-year age groups.

Age group (years)	Number of children	Percentage seropositive ^a
0-2	4	25.0
3-5	9	22.2
6-8	18	38.9
9-11	17	35.3
12-14	20	15.0
Overall	68	27.9

^ap=0.38 (for trend)

Table 3
Range of IHA antibody titers to *B. pseudomallei*.

Titer	Test interpretation	Number of cases (n=407)	Percentage of cases
<1:20	Negative	275	68.6
1:20	Borderline	46	15.5
1:40	Positive	35	8.6
1:80	Positive	19	4.7
1:160	Positive	12	3.0
1:320	Positive	3	0.7

Seroprevalence rates

The overall seroprevalence rate (IHA titer $\geq 1:40$) was 17.0% (95%CI 13.4-21.0%) (Table 1). There was an inverse relationship between the crude seroprevalence rates and increasing age group (p=0.002 for trend). When those subjects less than 15 years old were analysed separately (in 3-year age groups), no unidirectional trends in rates of seropositivity were found (Table 2). The association between increasing age and seropositivity was weaker after adjusting for gender (p=0.04 and 0.05 for the 30-44 and ≥ 45 years age groups, respectively, compared to the referent (<15 years) age group. Those in the youngest age group were more than 2.5 times as likely to be seropositive than the oldest age group.

The rate was higher for females overall, and in all age groups, and the association with age

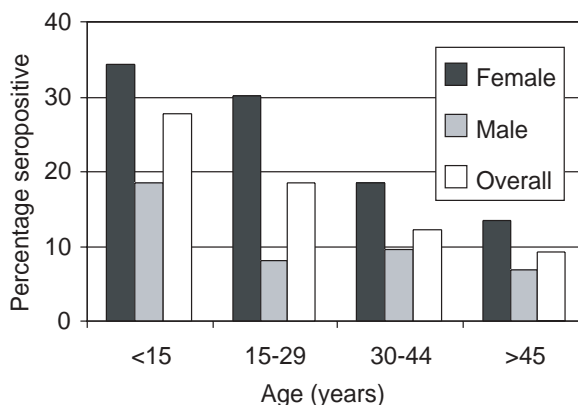


Fig 1–Graph showing relationship between seropositivity rate to *B. pseudomallei* by age group and gender.

was stronger in females (Fig 1). Gender remained significantly associated with seroprevalence after adjusting for age (p=0.0001). Overall, females were 2.5 times more likely to be seropositive than males (data not shown).

Geometric mean titers (GMT)

There was a narrow range of positive titers, with no titer exceeding 1:320 (Table 3). The overall GMT was 1:15. Considering only those subjects who had a positive titer ($\geq 1:40$), there were no significant differences in GMTs between males or females, or consistent trends with age.

DISCUSSION

The overall seroprevalence rate of 17.0% in our sample concurs with rates reported elsewhere in the region (Khupulsup and Petchclai, 1986; Appassakij *et al*, 1990; Wongratanacheewin *et al*, 1995; Norazah *et al*, 1996) as was the spread of titer results (Khupulsup and Petchclai, 1986; Norazah *et al*, 1996). This suggests that *B. pseudomallei* is, as expected, endemic in East Timor. Locally acquired clinical cases of melioidosis, with laboratory confirmation, need to be documented before it can be definitively stated that the disease occurs in that country. It is likely that the lack of case reports thus far are because the illness has not been suspected or microbiological methods have not been available.

Unlike the findings of clinical case series of melioidosis, we found a higher seroprevalence in

females compared to males. We do not have demographic data, such as occupational, social and health data, to further explore the reasons for this finding, and cannot exclude selection bias. Anecdotal reports suggests that the bulk of the physical activity in rural areas rests with females, and the resulting increased contact with soil and ground-water may explain the difference in seroprevalance rates along gender lines. Previously published seroepidemiological studies have failed to report on gender as an epidemiological factor of interest, although in reports of clinical cases of melioidosis, males normally predominate. However, in a seroepidemiological survey of 109 adult Aboriginal adults from an East Arnhem Land community in the Northern Territory of Australia, where 75% of clinical cases of melioidosis are known to occur in males (Currie *et al*, 2000a), a higher rate of seropositivity was also found in females (17% compared to 7%, $p=0.12$) (Anstey and Currie, unpublished data). Why there would be a higher seropositivity rate in females, yet a higher rate of clinical disease in males, is unclear. Although there is currently no evidence for natural immunity to *B. pseudomallei* from the environment, it is conceivable that if more females are exposed and are possibly "immune" from a younger age, then there would be a smaller pool of susceptible adult females for disease development and more "at risk" males of older ages to manifest clinical symptoms.

The relationship between age and seropositivity to *B. pseudomallei* is poorly understood. The finding of an inverse relationship with age was unexpected and the reasons for it are speculative. Children may have high rates of exposure to the organism, and natural waning of antibody levels may occur with age, concomitant with decreased exposure in adulthood. An alternative explanation is that the younger refugees may have come from backgrounds where a higher rate of seroprevalence would be expected, such as rural inhabitants who are more likely to be exposed to soil and/or surface water. However, this is less likely, as most of those less than 15 years old were accompanied by their parents, who would have had a similar exposure risk. Although the numbers in the youngest age group in our study were small, we did not find

an increasing seroprevalence rate with age in children as was found in a Thai study (Kanaphun *et al*, 1993).

A limitation of this study is possible selection bias in the choice of study subjects. Although the refugee group had a similar age and sex profile to East Timor as a whole, it is likely that the study subjects were not entirely representative of the whole population. More than half the individuals who had blood samples drawn were from the first of two groups of refugees, which were largely made up of East Timorese employees of the United Nations and their families. This group was therefore likely to be weighted towards an urban origin and a higher socio-economic status (SES). The second group of refugees comprised individuals from rural and urban areas and were more likely to be representative. However, because rural dwellers would be more likely to have exposure to the organism due to their closer contact with the soil, the weighting towards urban dwellers with higher SES would be expected to lead to a *lower* seroprevalence estimate than the one we found. Refugees who had blood samples taken, thereby becoming study subjects, may not have been representative of the larger group. Blood samples were taken from all individuals of the first refugee group but samples from the second refugee group were only taken if they were referred to the "Chest/Fever clinic". It is unknown whether this group would be at a higher or lower risk of exposure to *B. pseudomallei* than the group as a whole. Although these limitations may have an effect on the magnitude of the seroprevalence estimate, they do not compromise the important finding of this study, that exposure to *B. pseudomallei* occurs in East Timor, and are unlikely to explain the age and gender findings.

Assuming the seroprevalence found in this study equates with the existence of clinical melioidosis, what impact will this knowledge have on the health-care of East Timor? Microbiological laboratory capacity is limited and the number of skilled health-care workers is low. Melioidosis can be a difficult disease to diagnose with certainty, even with sophisticated diagnostic resources. With this in mind, diagnosis would need

to be made on clinical grounds and would probably occur late in the course of a particular illness, after failure of conventional treatment. If a person presented with acute, fulminant melioidosis, effective treatment would remain difficult due to the scarcity and expense of appropriate antibiotics. However, the chronic forms of the disease would be more amenable to treatment, owing to the lower cost of ambulatory antibiotic treatment that would normally be required. The indolent nature of this form of the illness would allow a trial of treatment without the necessity of microbiological confirmation. The most important group that would fall into this category would be those sputum-smear negative pulmonary tuberculosis cases that fail conventional anti-tuberculous treatment. Other chronic forms of melioidosis that would be amenable to such treatment would include suppurative infections of the skin and liver.

To put the potential importance of melioidosis in perspective, it should be remembered that morbidity and mortality due to this illness is likely to be small compared to other important communicable diseases in East Timor. If the incidence of melioidosis were equal to that of the Top End of the Northern Territory for example (16.5 per 100,000/year) (Currie *et al*, 2000b), it would be expected that approximately 130 new cases a year would occur, compared to over 100,000 cases of malaria, 1,800 new cases of tuberculosis and 1,300 cases of measles (Anonymous, 2000). Melioidosis will therefore remain a low health priority for some time. Notwithstanding this, the information provided by this study will be useful when, at some stage in the future, more resources are able to be devoted to the diagnosis and treatment of melioidosis.

In conclusion, this seroprevalence study has demonstrated that exposure to *B. pseudomallei* occurs in East Timor and therefore that melioidosis also probably occurs. Because of lack of laboratory capacity at present, it may be some time before microbiologically proven disease is reported. Realistically, it is the chronic forms of the illness that would be amenable to treatment in East Timor, especially chronic pulmonary melioidosis. In the meantime, melioidosis should be included in the differential diagnosis of the

many clinical entities that it can mimic. Attempts to confirm culture positive cases in East Timor should target individuals with these conditions.

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