Antibodies from Patients with Melioidosis Recognize *Burkholderia mallei* but Not *Burkholderia thailandensis* Antigens in the Indirect Hemagglutination Assay

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**The indirect hemagglutination assay routinely used to detect antibodies to *Burkholderia pseudomallei* was
modified to detect cross-reactivity of antibodies to *B. pseudomallei*, *B. mallei*, and *B. thailandensis* antigens. We
demonstrate a lack of cross-reactivity between *B. pseudomallei* and *B. thailandensis* but marked cross-reactivity
between *B. pseudomallei* and *B. mallei*.**

Burkholderia pseudomallei is the causative agent of melioid-osis and a recognized biothreat agent. The organism is present in mud and pooled surface water in Southeast Asia and north-ern Australia, where natural infection is acquired following inoculation or inhalation (11). Culture of *B. pseudomallei* is the gold standard for the diagnosis of melioidosis. However, sero-logical testing is commonly used, particularly in resource-poor regions that lack microbiological culture facilities. Serological testing may also be used as an epidemiological marker of exposure in regions where melioidosis is not known to be endemic and in patients (such as returned travelers or military personnel) from areas where melioidosis is not endemic to investigate suspected exposure to *B. pseudomallei* or *Burkhold-eria mallei* (the causative agent of glanders).

In areas where melioidosis is endemic, high rates of back-ground seropositivity have been noted (4, 8); this limits the specificity of the most commonly used indirect hemagglutina-tion assay (IHA). Seropositivity may be due to prior exposure to *B. pseudomallei* or exposure to the antigenically similar but rarely pathogenic *B. thailandensis* which is present in the en-vironment in Thailand. This may also explain the higher rate of seropositivity seen in Thailand than that seen in Australia, where *B. thailandensis* is not present (4). The aim of this study was to define whether patients with culture-proven melioidosis have antibodies with cross-reactivity to *B. pseudomallei*, *B. thailandensis*, or *B. mallei* antigens presented in a modified IHA.

Patients with melioidosis were prospectively recruited over a 5-month period between June and October 2004 by a study team based at Sappasithiprasong Hospital, Ubon Ratchathani, northeast Thailand. Patients presenting with febrile illnesses were identified by active surveillance of the medical and inten-sive care wards. Culture of *B. pseudomallei* from any sample was considered diagnostic for melioidosis (5). Patients were excluded if they were <14 years of age or declined to partici-pate. Ethical approval was obtained from the Ministry of Pub-lic Health, Royal Government of Thailand.

Pooled antigens were prepared from the following isolates: (i) *B. pseudomallei* clinical isolates 199a and 207a, obtained from patients with melioidosis in northeast Thailand; (ii) *B. thailandensis* isolates E27, E32, and E256, obtained from soil in northeast Thailand; and (iii) *B. mallei* isolates EY2233 (kindly provided by Sumalee Tungpredabkul, Faculty of Science, Ma-hidol University) and ATCC 23344. A loopful of each isolate was inoculated into 5 ml Trypticase soy broth and incubated for 18 h with shaking at 37°C in air, after which 200 μl was spread onto 30 plates of either Columbia agar (for *B. pseudomallei* and *B. thailandensis*) or Columbia agar plus 4% glycerol (for *B. mallei*). Bacteria were harvested after incuba-tion for 72 h at 37°C in air, suspended in 15 ml phosphate-buffered saline, vortexed vigorously, and then autoclaved at 121°C for 15 min. Preparations were centrifuged at 4,000 rpm for 30 min, the supernatants were filtered through a Millipore 0.22-μm filter, phenol was added (0.5%), and the preparation was stored at 4°C until use. An IHA was then performed as previously described (1). Growth of isolates prior to harvest and preparation of antigen represent a departure from the previously reported method in which bacteria are incubated in Rice medium at 37°C in air for 14 days prior to autoclaving, centrifugation, and filtration, as described above. In view of this difference, the *B. pseudomallei* IHA titers were initially compared between the two methods. Overall IHA titers were not significantly different between the two groups. Seven of 117

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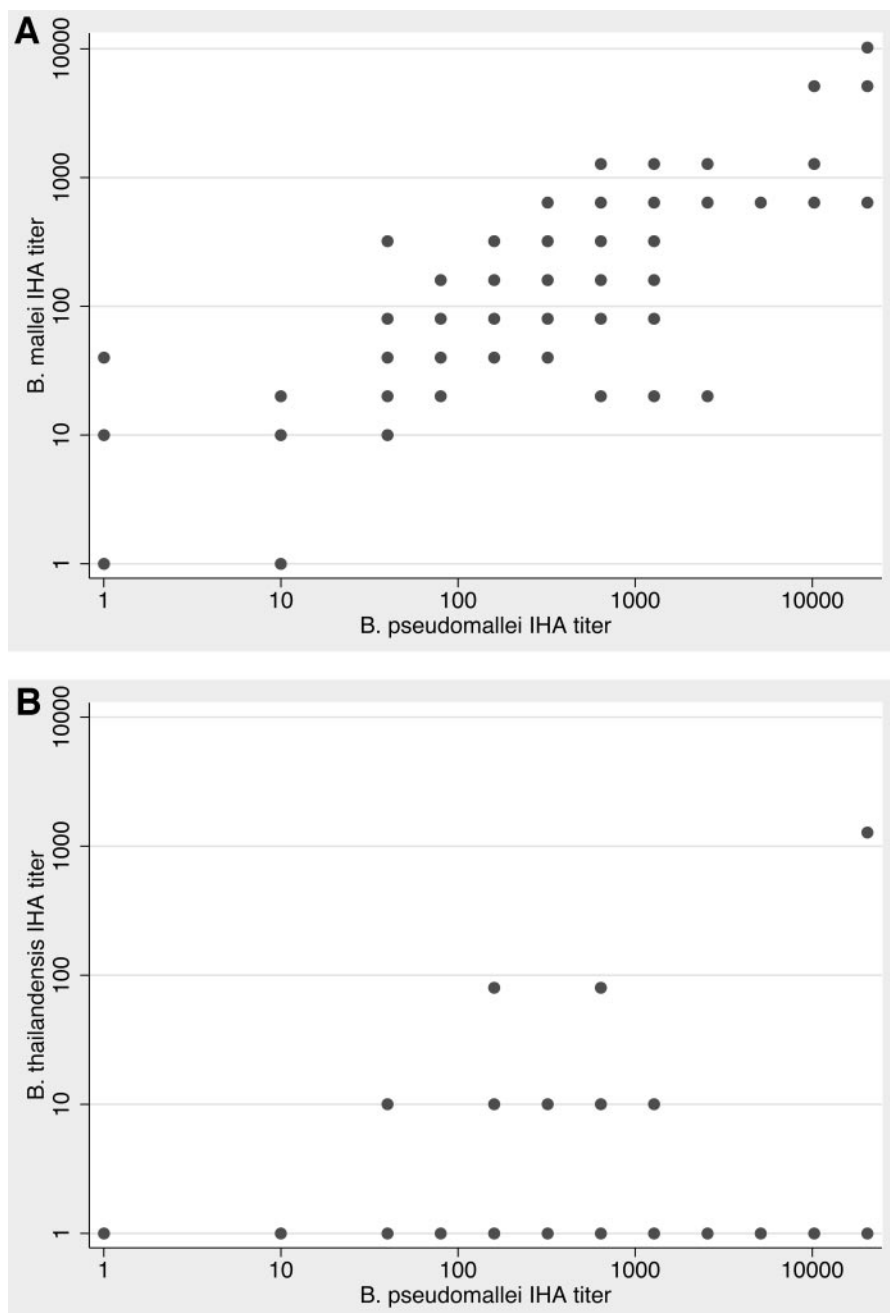
FIG. 1. IHA titers of (A) *B. mallei* and (B) *B. thailandensis* versus *B. pseudomallei* in patients with melioidosis. Each dot represents one or more patients.

sera (5.9%) gave IHA titers that were ≥2 dilutions different between the methods (five samples were 2 dilutions different, and two samples had a 3-dilution difference); of these titers, three were higher using the conventional method, and four were higher using the modified method. We propose that growth of bacteria on agar plates prior to antigen preparation for IHA is a reasonable alterative to prolonged growth in broth culture.

Sera from 117 patients with culture-confirmed melioidosis were evaluated. Patient ages ranged from 15 to 82 years (median, 48.5 years; interquartile range [IQR], 38 to 57); 56 pa-

tients (48%) were female. The majority of patients were rice farmers (79%), and 70% had diabetes mellitus. Infection was associated with septic shock in 22 patients (19%), and the overall in-hospital mortality was 28%.

The *B. pseudomallei* titers ranged from 0 to 1:20,480 (median, 1:320; IQR, 1:80 to 1:1,280), and those for *B. mallei* ranged from 0 to 1:10,240 (median, 1:160; IQR, 1:40 to 1:640). There was significant cross-reactivity between the two assays (Spearman's $\rho = 0.78$, $P < 0.0001$ [Fig. 1]). The IHA titers were identical between the IHAs using *B. pseudomallei* or *B. mallei* antigens in 46 (39%) of the 117 cases. The titer was

greater with the *B. pseudomallei* IHA than with the *B. mallei* IHA in 57 cases and less in 14 cases. Antibodies recognizing *B. thailandensis* were not detected in 98 (84%) samples. Of the remainder, 14 samples had a titer of 1:10, one had a titer of 1:20, three had a titer of 1:80, and one had a titer of 1:1,280 (the *B. pseudomallei* titer in this case was 1:20,480). There was no significant cross-reactivity between the *B. thailandensis* assay and those based on *B. pseudomallei* (Spearman's $\rho = 0.17$, $P = 0.06$) and *B. mallei* antigens (Spearman's $\rho = 0.09$, $P = 0.35$); the borderline P value in the case of *B. pseudomallei* was entirely due to the single high *B. thailandensis* case mentioned above.

The finding of cross-reactivity of *B. mallei* antigens by antibodies raised against *B. pseudomallei* is consistent with previous studies demonstrating antigenic relatedness between the two species (2). In addition, there is increasing evidence of a close phylogenetic relationship based on similarities in the genome sequence and multilocus sequence typing (6, 7, 9). It is unlikely that our patient group had been exposed to *B. mallei* previously, as human glanders is not recognized to occur in Thailand.

However, our patient population is likely to be repeatedly exposed to both *B. thailandensis* and *B. pseudomallei*, which are both present in soil in northeast Thailand (10). We found that there was little detectable cross-reactivity between *B. thailandensis* and *B. pseudomallei* antigens by the IHA. The generally absent or very low titers to *B. thailandensis* in our patient population indicate that environmental exposure to this organism is not very immunogenic or that these antibodies are not recognized by the IHA; in this way, *B. thailandensis* differs from *B. pseudomallei*, for which background seropositivity is a major problem in areas of endemicity. This study suggests that exposure of individuals to *B. thailandensis* is unlikely to interfere with the results from the *B. pseudomallei* IHA.

Some antigens, such as lipopolysaccharide, are known to be conserved between these *Burkholderia* species (3), while others are immunologically unique (12). A limitation of the IHA is that the antigens generated are poorly characterized. It is generally assumed that the use of multiple strains would result in a broad representation of antigens, but cell-free supernatant is likely to be depleted of many of the major cell surface-associated antigens.

The explanation for the different rates of seropositivity between Australia and Thailand remains unclear. It is unlikely that the intensity of exposure, maintaining seropositivity in Southeast Asian patients, is responsible, as the rates of seropositivity were higher in immigrants to Australia from Southeast Asia (4). However, it is possible that intense exposure earlier in life may result in a prolonged seropositivity. Differences in immunological responses to *B. pseudomallei* in Asian and Australian patients have not been described, but variations in Th1/Th2 responses may be important. Finally, the IHA is a poorly standardized test with different isolates (typically local clinical strains) used in Australia and Thailand; the effect of these differences has not been defined.

Future publication of the *B. thailandensis* genome sequence will shed new light on the similarities and degree of homology between these saprophytic *Burkholderia* species. We conclude that the *B. pseudomallei* IHA is unlikely to be confounded by antibodies to *B. thailandensis*, but that this assay is unable to distinguish between infection by *B. pseudomallei* and infection by *B. mallei*.

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