Accuracy of Enzyme-Linked Immunosorbent Assay Using Crude and Purified Antigens for Serodiagnosis of Melioidosis

Narisara Chantratita,1,* Vanaporn Wuthiekanun,1 Aunchalee Thanwisai,1 Direk Limmathurotsakul,1 Allen C. Cheng,2 Wirongrong Chierakul,1 Nicholas P. J. Day,1,2 and Sharon J. Peacock1,3

Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand1; Menzies School of Health Research, Charles Darwin University, Darwin, Northern Territory, Australia2; and Center for Clinical Vaccinology and Tropical Medicine, Nuffield Department of Clinical Medicine, University of Oxford, Churchill Hospital, Oxford, United Kingdom3

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Five enzyme-linked immunosorbent assays developed to detect antibodies to different Burkholderia pseudomallei antigen preparations were evaluated as diagnostic tests for melioidosis in northeast Thailand. The highest diagnostic indices were observed for an affinity-purified antigen (sensitivity, 82%; specificity, 72%) and crude B. pseudomallei antigen (sensitivity, 81%; specificity, 70%), an improvement over the indirect hemagglutination assay (sensitivity, 73%; specificity, 64%).

Burkholderia pseudomallei is the causative agent of melioidosis, a disease of public health importance in Southeast Asia and northern Australia (4, 22). This bacterium is present in the environment in areas of endemicity where natural infection is acquired following inoculation or inhalation (4, 22). Clinical manifestations of melioidosis are broad ranging and are often difficult to differentiate from other infections, but accurate diagnosis is important, since melioidosis requires prolonged antimicrobial therapy of ≥20 weeks to achieve cure (4, 24). Although culture of B. pseudomallei represents the diagnostic gold standard, rural hospitals in Asia may lack culture facilities but often have access to serological tests. The most widely used serological test for the diagnosis of melioidosis is the indirect hemagglutination assay (IHA) (5, 6, 8, 13, 14). However, this assay has a low diagnostic accuracy in settings of endemicity, since rates of seropositivity in the general population are high (12, 23); this is most likely the result of repeated environmental exposure to B. pseudomallei or possibly the highly related but nonviralent Burkholderia thailandensis. Many previous attempts have been made to improve the accuracy of serology tests for melioidosis using a range of antigen preparations in an enzyme-linked immunosorbent assay (ELISA) format (7, 10, 15, 17, 19, 20). Some studies have suggested that immunoglobulin G (IgG) assays using whole-cell antigens are more specific for acute infection (7, 10, 20). Studies using purified antigens such as lipopolysaccharide (LPS) or exopolysaccharide (EPS) have also been conducted (10, 15, 17, 18), but these have generally been evaluated in small sample collections and have demonstrated varying sensitivities and specificities. The aim of this study was to compare the diagnostic utilities of ELISAs using crude and purified B. pseudomallei antigens (LPS, EPS, and affinity-purified antigen) in a single highly defined population of patients with suspected melioidosis in northeast Thailand. We also assessed the relationship between antibody responses to different B. pseudomallei antigen preparations and the extent of cross-reactivity between B. pseudomallei and B. thailandensis antigens.

Serum samples were obtained upon admission from unselected adult patients presenting with suspected melioidosis to Sappasithiprasong Hospital, Ubon Ratchathani, northeast Thailand, between June and October 2004. Patients with suspected melioidosis were identified by active ward surveillance, and multiple samples were taken for culture. Isolation of B. pseudomallei from any sample was considered diagnostic. Of the 322 patients clinically suspected to have melioidosis, 120 had culture-confirmed infection, and the remainder (n = 202) acted as controls. Ethical approval was obtained from the Ethics Subcommittee, Faculty of Tropical Medicine, Mahidol University.

Crude antigens were prepared from aqueous extracts of B. pseudomallei K96243 and B. thailandensis E32 (isolated from soil in northeast Thailand) grown on Trypticase soy agar at 37°C for 2 days. Bacteria from 10 Trypticase soy agar plates were harvested into 30 ml of phosphate-buffered saline (PBS), pH 7.2, and heat killed at 80°C for 1 h. Bacterial LPS and EPS were extracted using the hot-phenol chloroform method, as previously described (17). EPS alone was extracted from the LPS-defective mutant B. pseudomallei SR1015 (16), and EPS alone was extracted simultaneously from B. pseudomallei K96243. LPS alone was extracted from the EPS-defective mutant B. pseudomallei SRM 117 (both mutants kindly provided by Donald Woods, University of Calgary, Canada) (9). The monoclonal antibody 4B11 to B. pseudomallei EPS (3) was used to prepare affinity-purified antigen, as previously described (17). All suspensions were sterile tested, quantitated on the basis of carbohydrate content using the orcinol-sulfuric acid method (21), and stored at −20°C prior to use.

Pilot studies were performed to define the optimal concentration of serum and antigen using pooled positive and pooled control sera (5 patients with culture-proven melioidosis and 5...
TABLE 1. Test characteristics of five *B. pseudomallei* ELISA and IHA in 322 patients with suspected melioidosis

| Assay                        | Cutoff | Sensitivity (%) | Specificity (%) | PPV (%) | NPV (%) | % Correctly classified | LR + | LR − | AUROCC |
|------------------------------|--------|-----------------|-----------------|---------|---------|------------------------|------|------|--------|
| Affinity-purified antigen    | 0.386  | 82 (74, 88)     | 72 (66, 78)     | 64 (56, 71) | 87 (81, 92) | 76 | 3.0 | 0.25 | 0.80 (0.75, 0.85) |
| Crude *B. pseudomallei*      | 0.482  | 81 (73, 87)     | 70 (63, 77)     | 62 (54, 69) | 86 (80, 91) | 74 | 2.7 | 0.27 | 0.80 (0.74, 0.84) |
| EPS + LPS                    | 0.434  | 77 (68, 84)     | 74 (75, 86)     | 64 (55, 72) | 84 (78, 89) | 75 | 3.0 | 0.31 | 0.79 (0.74, 0.84) |
| LPS                          | 0.683  | 66 (57, 74)     | 81 (75, 86)     | 67 (58, 75) | 80 (74, 85) | 75 | 3.4 | 0.42 | 0.80 (0.74, 0.85) |
| EPS                          | 0.133  | 64 (55, 75)     | 74 (68, 80)     | 60 (51, 68) | 78 (71, 83) | 71 | 2.5 | 0.48 | 0.72 (0.66, 0.77) |
| IHA                          | 1:160  | 73 (64, 80)     | 64 (57, 71)     | 55 (47, 63) | 80 (73, 86) | 67 | 2.0 | 0.43 | 0.75 (0.69, 0.80) |

* Values in parentheses represent 95% CIs. Sensitivity, specificity, LR +, and LR − were calculated at the cutoff OD.

healthy volunteers who were IHA negative, respectively) at a dilution range of 1:1,000 to 1:16,000. The six antigens were diluted in 0.05 M carbonate buffer, pH 9.6, to achieve a range of concentrations. Each antigen was added in duplicate to a 96-well ELISA plate in a fixed volume of 100 μl per well and maintained overnight at 4°C. Plates were then washed three times with normal saline containing 0.05% Tween 20. After a further washing step, 100 μl of sera diluted in PBS with 1% bovine serum albumin and 0.05% Tween 20 were added to wells in duplicate and incubated at room temperature for 1 h. After washing of the plates as described above, a 1:2,000 dilution of horseradish peroxidase-conjugated rabbit anti-human IgG (Dako, Copenhagen, Denmark) was added, and the mixture was incubated at room temperature for 1 h. After the plates were washed six times, the enzymatic activity was determined using a substrate solution containing O-phenylenediamine (0.67 mg/ml) and hydrogen peroxide (1.5%), using incubation at room temperature for 30 min. The reaction was stopped with 50 μl of H₂SO₄, and the absorbance value (optical density [OD]) was determined at a wavelength 492 nm. The optimal concentrations for each antigen were as follows: LPS, 100 ng/ml; EPS, 500 ng/ml; EPS plus LPS, 100 ng/ml; 4B11 affinity-purified antigen, 30 ng/ml; crude *B. pseudomallei*, 250 ng/ml; and crude *B. thailandensis*, 250 ng/ml. The optimal concentration of sera was 1:4,000 for all ELISAs. The 322 individual sera were tested in duplicate using these optimized values. The IHA was performed as previously described (1), using pooled antigens from clinical isolates 199a and 207a of *B. pseudomallei* cultured from patients with melioidosis in northeast Thailand. The diagnostic cutoff used was 1:160. The highest positivity rates were observed for the affinity-purified antigen and crude *B. pseudomallei* antigen IHA. AUROCC were statistically equivalent for the affinity-purified antigen, crude *B. pseudomallei* antigen, EPS plus LPS, and LPS IHA (P = 0.76).

To further evaluate the five ELISAs, we examined seropositivity in individuals resident in areas of high and low melioidosis endemicity (northeast and southern Thailand, respectively). Serum was obtained from 300 children resident in northeast Thailand who presented to Sappasithiprasong Hospital between 2004 and 2005 for reasons other than melioidosis (age range, 1 to 14 years; median, 7.5 years, interquartile range, 4 to 11 years) and 113 adults without melioidosis presenting to Takuapa General Hospital, Phangnga, southern Thailand, between 2004 and 2005 (age range, 19 to 91 years; median, 37 years; interquartile range, 29 to 47 years). Figure 1 shows the proportion positive for each of the assays using the diagnostic ELISA cutoffs given in Table 1 and an IHA cutoff of ≥1:160. The highest positivity rates were observed for EPS and IHA; this is consistent with the lower diagnostic indices for these assays in the patient population with suspected melioidosis. These data provide further evidence for the presence and extent of seropositivity to *B. pseudomallei* in Thailand, a phenomenon that has a major effect on serodiagnostic test specificity.

Serum from the 322 patients with suspected melioidosis was tested using the crude *B. thailandensis* antigen ELISA. Diagnostic indices (for the diagnosis of melioidosis) were calculated for the *B. thailandensis* antigen ELISA using a cutoff of 0.441...
and were as follows (95% confidence intervals [CIs] are indicated in parentheses): sensitivity, 78% (69 to 85%); specificity, 74% (67 to 80%); PPV, 64% (55 to 71%); NPV, 85% (78 to 90%); correct classification, 75%; LR +, 3.0; LR −, 0.31; AUROC, 0.80 (0.75 to 0.85).

The relationship between antibody responses to different antigen preparations was examined for the 322 sera from patients with suspected melioidosis. Table 2 shows the correlation between data for the five *B. pseudomallei* ELISAs, the *B. thailandensis* ELISA, and the IHA. There was high correlation between crude *B. pseudomallei* antigen, affinity-purified antigen, and both EPS plus LPS and LPS, but a lower correlation was observed between crude *B. pseudomallei* antigen and EPS. There was a high correlation between affinity-purified antigen and both EPS plus LPS and LPS alone but a lower correlation between affinity-purified antigen and EPS alone. This suggests that the affinity-purified antigen, which was prepared using a monoclonal antibody to EPS, is likely to be contaminated with EPS antigen. The IHA correlated poorly with all five ELISAs, suggesting that alternative bacterial antigens are presented by the IHA. The *B. thailandensis* ELISA correlated most closely with *B. pseudomallei* ELISAs containing LPS; this is consistent with previous reports of antigenic similarity between LPS of the two species (2).

We conclude that the IgG *B. pseudomallei* ELISA represents an improvement over IHA. The affinity-purified antigen and crude *B. pseudomallei* antigen ELISA had the greater diagnostic performance; the crude antigen ELISA has the added advantage that antigen is simple to prepare. However, the goal remains to improve the accuracy of serological tests for the diagnosis of melioidosis. Devising an assay that has a clinically useful degree of predictive accuracy in areas of high endemicity where antibody levels are high in the healthy population represents a major challenge. The utility of alternative purified bacterial targets and the simultaneous use of multiple targets using immunoassay technology require investigation.

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### Table 2. Correlation between data from five *B. pseudomallei* ELISAs, the IHA, and an ELISA using crude *B. thailandensis* tested using sera from 322 patients presenting with suspected melioidosis

| Assay                   | Crude *B. pseudomallei* | Affinity-purified antigen | EPS + LPS | LPS | EPS | IHA | Crude *B. thailandensis* |
|-------------------------|-------------------------|----------------------------|-----------|-----|-----|-----|----------------------------|
| Crude *B. pseudomallei* | 1.0000                  |                            |           |     |     |     |                            |
| Affinity-purified antigen | 0.9596                  | 1.0000                     |           |     |     |     |                            |
| EPS + LPS               | 0.9461                  | 0.9553                     | 1.0000    |     |     |     |                            |
| LPS                    | 0.9115                  | 0.9256                     | 0.9384    | 1.0000 |     |     |                            |
| EPS                    | 0.7033                  | 0.6864                     | 0.6819    | 0.5302 | 1.0000 |     |                            |
| IHA                    | 0.6001                  | 0.5889                     | 0.5750    | 0.5377 | 0.4886 | 1.0000 |                            |
| Crude *B. thailandensis* | 0.8881                  | 0.9079                     | 0.8960    | 0.9410 | 0.5131 | 0.5229 | 1.0000                     |

* Results are correlation coefficients.
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