# SERODIAGNOSIS OF MELIOIDOSIS IN MALAYSIA

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*ABSTRACT*

***Current diagnosis of melioidosis is based on bacterial culture and/or serology which is becoming increasingly useful. An IgM-ELISA using heat-killed whole cells of Pseudomonas pseudomallei was developed and compared with an indirect haemagglutination technique(IHAT) and an indirect immunofluorescent techique(IFAT). The IgM-ELISA using a P:N ratio of ≥2 had a sensitivity of 91% and a specificity of 96%. All 3 assays were further used in a seroepidemiological survey amongst different groups of patients and healthy individuals. It was found that the IFAT performed better than the IHAT, detecting antibodies to P. pseudomallei in 6% of diabetics, 5% of pyrexics, 8% of pregnant women and 3% of farmers. For the same groups the IgM-ELISA detected antibodies in 1% of pyrexics, 8% of pregnant women and a further 14% of farmers. The IgM-ELISA was found to be sensitive and useful for the serological diagnosis of acute melioidosis.***

*Keywords: serology of melioidosis, IgM-ELISA, indirect immunofluorescent technique(IFAT), indirect haemagglutination technique (IHAT).*

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## INTRODUCTION

Melioidosis is endemic in South East Asia and in Malaysia. The mortality rate, especially in the septicaemic variety, is very high[1] . This is due to several factors such as lack of clinical awareness, delay in identification of the organism by the laboratory, lack of suitable rapid diagnostic techniques and a purely clinical diagnosis of melioidosis is not possible. To compound this, empirical antibiotic therapy seldom includes agents that are active against the causative agent *Pseudomonas pseudomallei.*

Serological diagnosis of melioidosis has been hampered by several factors. These include raised antibody levels in the healthy population in endemic areas[2], the presence of subclinical and asymptomatic infections, poorly standardised antigens and probable cross-reactions with other *Pseudomonas* species. The indirect haemagglutination test(IHAT) was first described by Ileri[3] and subsequently modified and used by several workers[2,4,5], but due to high background titres it was difficult to differentiate current and previous infections using the IHAT. Ashdown[6] developed an indirect immuno-fluorescent test (IFAT) for IgM antibodies and concluded that it appeared to be relevant to the diagnosis of clinical melioidosis. But the IFAT test requires a fluorescence microscope and skill training, both of which are not easily available in developing countries where melioidosis is endemic. The use of enzyme-linked immunosorbent assay (ELISA) has been described by Ashdown and co-workeds[7] for the specific detection of IgM and IgG antibodies. Kunakorn and co-workers[8] have also reported on a direct IgM ELISA and an IgM-capture ELISA.

The need for a simple, sensitive and specific serological test for acute melioidosis is essential for early recognition especially when bacteriological culture takes time and in addition infected sites may be inaccessible to specimen collection. In this study we describe an IgM-capture ELISA technique and the comparison with the IHAT and IFAT using sera from patients with melioidosis and normal healthy individuals. Further to this, a seroepidemiological survey was performed, using sera from healthy diabetics, asymptomatic oil palm estate (FELDA) workers, patients with pyrexia of unknown origin (PUO) and pregnant women.

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## MATERIALS AND METHODS

### Bacterial Strains

Four strains of *P. pseudomallei* obtained from patients admitted at the University Hospital, Kuala Lumpur were used for preparation of antigens. Strain 739499 was isolated from the blood of a 69-year-old male with diabetes mellitus and pulmonary tuberculosis; strain 488026 from the pus of a liver abscess of a 39-year-old male; strain 815602 from urine of a 69-year-old male with benign prostatic hypertrophy, and strain 1-089581 from a chest wall abscess of a 51-year-old male with diabetes mellitius. The strains were isolated on ox blood agar and stored on nutrient agar slopes at room temperature and on beads at -70°C[9].

### Preparation of antigens

A 100 ul of a preculture of each of the strains prepared in 3 ml nutrient broth containing 1% yeast extract (NB+1%YE) incubated at 37°C for 3 hours, was inoculated into 10ml NB+1%YE. Following overnight incubation at 37°C, the broth cultures were centrifuged at 10,000 x g for 15 minutes, the pellets resuspended in 8% formalin in phosphate buffered saline (PBS) and treated for 48 hours to render the organisms non-viable. The formalised cells were centrifuged further at 10,000 x g for 15 minutes and the pellets resuspended in 250 ul of PBS. Pellets of all 4 bacterial strains were pooled, washed three times with sterile PBS and finally resuspended in 1.0ml of sterile PBS prior to storage at 4°C. The protein concentration of the prepared antigen was determined as 6.4mg/ml by a modified Lowry's method[10].

### Sera

A total of 378 sera were used in this study; 11 samples were

from eleven patients with culture-positive melioidosis and were collected immediately after culture was positive; 90 were from healthy blood donors collected at random from the community, 75 from patients with a clinical diagnosis of pyrexia of unknown origin (PUO), 50 from asymptomatic patients with a history of diabetes mellitus, 72 from pregnant women visiting the antenatal clinic, and 80 from healthy oil palm estate (FELDA) workers. The samples were not matched for race, age or sex. Positive control serum used was from a Thai patient with confirmed melioidosis and the negative control serum was from a newborn baby.

**Modified IgM-capture ELISA**

The assay of Bundo et al[11] and Lam et al[12] was modified to detect the presence of IgM antibodies to *P. pseudomallei* infections. To each well of a 96-well microtitre plate, 200 ul (0.125 ug/ml) of mouse anti-human IgM (μ-chain specific) (Dako-Patt or Sigma Chemicals) in 0.05M carbonate-bicarbonate buffer (pH 9.6) was added and incubated at 4°C overnight and then washed three times with PBS (pH 7.5) containing 0.05% (vol/vol) Tween 20 (Sigma Chemicals). The coated plates were stored immediately at 4°C until further use. To each well 200 μl of 5% (wt/vol) bovine serum albumin (BSA) (Sigma Chemicals) in PBS was added and incubated at 37°C for 0.5 hour to block non-specific binding sites. Following three washes with PBS-Tween 20, 100 μl of test serum diluted 1:500 in PBS-Tween was added to duplicate wells. Positive and negative sera were similarly added and the mixture incubated at 37°C for 1 hour. Following washing, 100 μl of heat-killed *P. pseudomallei* (equivalent to 20ug/ml protein), rabbit anti-*P. pseudomallei* immunoglobulin (1:1000) and peroxidase-labelled anti-rabbit immunoglobulins (1:100) (Dako-Patt) diluted in PBS-Tween 20 were added sequentially and incubated at 37°C for 1 hour at each stage. The wells were washed with PBS-Tween 20 between each stage. Freshly prepared orthophenylene diamine substrate solution was added and incubated for 20 minutes at room temperature in the dark after which the reaction was stopped by the addition of 20 μl of 8N $H_2SO_4$. The optical density (OD) at 492 nm was measured using an ELISA reader (Multiscan MR600, Dynatech). The OD values of the test sera were divided by the OD value to give a negative control. The result was expressed as a positive to negative ratio (P:N)[13].

**Indirect haemagglutination assay (IHAT)**

The assay was performed as described by Khupulsup and Petchclai[5]. Sensitised sheep erythrocytes were prepared using 250μg/ml of antigen to an equal volume of 1% sheep red blood cells. Briefly, the test sera were inactivated at 56°C for 30 minutes, then diluted 1:10 with 5% sheep erythrocytes and incubated at room temperature for 30 minutes to adsorb heterophile antibody. The adsorbed serum was serially diluted two-fold using 50 μl amounts in a microtiter plate starting from 1:10 using 0.15M PBS containing 0.5% BSA. 50 ul of sensitised sheep erythrocytes was added to each well with the exception of one well in which unsensitised sheep erythrocytes in diluent was added as control for non-specific haemagglutination. Following incubation at room temperature for 1 hour, the plates were examined for haemagglutination. Positive and negative control sera were included for each plate. An IHAT titre of ≥1:80 was considered positive as recommended by Khupulsup and Petchclai[5].

**Indirect Immunofluorescent antibody technique (IFAT)**

The assay was performed as described by Ashdown et al[6]. Briefly, the antigen was air dried and heat fixed onto teflon coated slides. Each test serum was then serially diluted two-fold in PBS at dilutions of 1:10 to 1:160 and allowed to incubate with the antigen at 37°C for 30 minutes in a moist box. Following three washes with PBS (pH 7.4), fluorescein-labelled antihuman immunoglobulin (MFOI, Wellcome Research Laboratories) was added, allowed to incubate for a further 10 minutes, washed three times with PBS, then dried and mounted with buffered glycerol. Positive and negative sera were included for each test. Fluorescence was read in degrees of 3+ to negative using a Zeiss large universal microscope with an epifluorescence condenser III RS. Fluorescence observed at a dilution of ≥1:40 was considered positive for the presence of antibodies to *P. pseudomallei*[6].

**RESULTS AND COMMENTS**

The IgM-ELISA was developed to detect IgM antibodies to P. *pseudomallei*. The positive:negative (P:N) cut off was based on the OD values obtained from serum samples of 11 patients with laboratory confirmed diagnosis of melioidosis and sera from 90 healthy blood donors from the community. Using the relative operative cut-off (ROC) analyses[14], at a P:N cut-off value of >1, the sensitivity was 91% (10/11) and specificity was 76%, at P:N cut off value of >2, sensitivity remained 91% whereas specificity improved to 96%; and at P:N cut-off >3, the sensitivity reduced to 64% (7/11) and specificity increased to 100% (Table I). Thus the best P:N cut-off value as determined by this analysis was ≥2 with a sensitivity of 91% and specificity of 96%.

The indirect haemagglutination assay (IHAT)[5] and indirect immunofluorescent antibody technique (IFAT)[6] detected both IgM and IgG antibodies. The cut-off values used were as previously determined in their respective

**Table 1 - Relative operative cut-off value analysis for the IgM-ELISA**

| P:N cut-off value | No. of specimens | | % | % |
|---|---|---|---|---|
| | True positive | False positive | sensitivity[a] | specificity[b] |
| >0.5 | 11 | 89 | 100 | 1 |
| >1 | 10 | 22 | 91 | 76 |
| >2 | 10 | 4 | 91 | 96 |
| >3 | 7 | 0 | 64 | 100 |
| >4 | 7 | 0 | 64 | 100 |
| >5 | 6 | 0 | 55 | 100 |
| >6 | 5 | 0 | 46 | 100 |
| >7 | 4 | 0 | 36 | 100 |

a : Positive cases: 11 melioidosis cases
b : Negative cases: 90 healthy blood donor:

**Table II - Detection of antibodies to *P. pseudomallei* using different cut-off values**

| Study groups | Total No. | IHAT | | | IFAT | | |
|---|---|---|---|---|---|---|---|
| | | 1:20 | 1:40 | 1:80 | 1:20 | 1:40 | ≥1:80 |
| melioidosis | 11 | 7 | 5 | 5 | 10 | 10 | 8 |
| healthy blood donors | 90 | 0 | 0 | 0 | 0 | 0 | 0 |
| pyrexics | 75 | 1 | 0 | 0 | 14 | 4 | 0 |
| pregnant women | 72 | 0 | 0 | 0 | 26 | 6 | 2 |
| diabetics | 50 | 4 | 2 | 0 | 13 | 3 | 0 |
| farmers | 80 | 6 | 6 | 2 | 2 | 2 | 2 |

**Table III - Comparison of three assays to detect antibodies to *P. pseudomallei* amongst control groups of sera.**

| Assays (cut-off value) | | | Control sera | |
|---|---|---|---|---|
| IgM-ELISA (P:N≥2) | IHAT(≥1:80) | IFAT(≥1:40) | Melioidosis | Healthy blood donors |
| + | + | + | 5 | 0 |
| + | + | - | 0 | 0 |
| + | - | - | 0 | 4 |
| + | - | + | 5 | 0 |
| - | + | - | 0 | 0 |
| - | + | + | 0 | 0 |
| - | - | + | 1 | 0 |
| - | - | - | 0 | 86 |
| | | Total no. of sera | 11 | 90 |

**Table IV - Prevalence of antibodies to *P. pseudomallei* in the different study groups.**

| Study groups | Total No. | IHAT No.(%) | IFAT No.(%) | IgM-ELISA No.(%) |
|---|---|---|---|---|
| melioidosis | 11 | 5(46) | 10(91) | 10(91) |
| healthy blood donors | 90 | 0 | 0 | 4(3) |
| pyrexics | 75 | 0 | 4(5) | 1(1) |
| pregnant women | 72 | 0 | 6(8) | 6(8) |
| diabetics | 50 | 0 | 3(6) | 0 |
| farmers | 80 | 2(3) | 2(3) | 11(14) |

published reports. The IHAT using a cut-off value of ≥1:80 detected only 45%(5/11) of melioidosis sera as positive and 100% (90/90) of normal sera as negative (Table II). On the other hand, using a cut-off value of 1:40[14] did not alter the sensitivity or specificity values. Analysis of the results obtained by the IFAT indicated that at a cut-off value of 1:80, 73%(8/11) of melioidosis sera were detected as positive whereas all normal sera remained negative. At a cut-off value of 1:40[6] the IFAT detected 91% (10/11) of melioidosis sera as positive and all normal sera as negative. If the cut-off value was further decreased to 1:20, the same results were observed. In addition, a large proportion of melioidosis samples (8/11) had cut-off values of ≥1:80.

Using the cut-off values as previously mentioned, a comparison was made of ability of the 3 assays to detect *P. pseudomallei* antibodies. All 3 assays agreed on the detection of 45% (5/11) of melioidosis sera as positive and 85% (86/90) of the normal sera as negative. (Table III) In addition, the IgM-ELISA and IFAT detected a further 45% (5/11) melioidosis sera as positive, giving a concordance rate of 95% (96/101) between the two assays. Although Kunakorn et al[8] reported that the IHAT was the most sensitive assay, in our hands this assay performed less optimally than either the IFAT or IgM-ELISA.

Further to the assay evaluation, a seroepidemiological survey was carried out using all three assays, on the prevalence of antibody levels to *P. pseudomallei* amongst different groups of patients and healthy individuals (Table IV). The groups included patients with a diagnosis of diabetes mellitus and pyrexia of unknown origin (PUO), pregnant women and farmers. Amongst the 3 assays, using the IHAT cut-off value at ≥1:80, antibodies were detected amongst 3% of farmers. Amongst the PUO and diabetic groups the IFAT (≥1:40) detected antibodies in 5% (4/75) and 6% (3/50) of patients respectively who were negative by IHAT and IgM-ELISA (P:N≥2). However, the IgM-ELISA detected an additional sample in the PUO group as positive ie 1% (1/75). In samples from pregnant women, 80% (6/72) were identified as positive for antibodies by both the IFAT and IgM-ELISA. Four of the six samples were detected by both assays and the remaining 2 samples were different for each of the assays (data not shown). Amongst the sera from farmers, antibodies were detected in 3% (2/80) of samples by IHAT, in 3% (2/80) of samples by IFAT and 14% (11/80) of samples by IgM-ELISA. On further inspection of the data (not shown) all 3 assays agreed on the detection of antibodies in the 2 samples. However the IgM-ELISA detected an additional 9 samples as positive.

Further to the report by Kunakorn et al[8] on the performance of the IHAT, if the cut-off value for the IHAT was reduced to 1:40, no change on the sensitivity value occurred amongst our control groups. However amongst the diabetics and farmers, an additional 4% (2/50) and 5% (4/80) of samples respectively would have been classified as positive (Table II).

## DISCUSSION

Serodiagnosis of acute melioidosis has been focused mainly on the detection of IgM antibodies. Kunakorn et al[8] developed an indirect ELISA and an IgM-ELISA and these assays were evaluated using 16 melioidosis positive sera and 153 normal sera. The sensitivities for the indirect ELISA and IgM-capture ELISA were 88% and 75% respectively and the specificities were 92.2% and 91.5% respectively.

In the present study the IgM-ELISA for detection of IgM antibodies based on 11 melioidosis culture positive sera had a sensitivity of 91% and a specificity of 96%. It was found that one sample was negative by both the ELISA and IFAT. Retrospectively, it was found that this serum sample came from a highly immunocompromised patient. Similarly, the false positive results may be due to a subclinical infection or cross-reactions with antibodies due to other *Pseudomonas* infections or presence of IgM antibodies to *Legionella*[7]. During the optimisation of the IgM-ELISA, interference due to rheumatoid factor was tested and found to be absent. The IHAT which detects total antibodies did not perform as well (sensitivity 45%; specificity 100%) even if the cut-off value was lowered from ≥1:80 to≥1:40 (Table II). This may have been due to the sensitisation of sheep erythrocytes being less than optimal. In comparison, the IFAT which also detects total antibodies performed better with a sensitivity of 91% and specificity of 100%, using a cut-off value of ≥1:40. Thus in our experience the IgM-ELISA performed optimally for the detection of active melioidosis and the IFAT may be used in combination to detect IgG in chronic melioidosis and also as a confirmatory test. The ELISA can be developed in kit form which ensures optimally standardised, quality controlled reagents and the results can be read visually. However, with the IFAT, a fluorescence microscope is essential, which may not be available in endemic areas. In addition, the interpretation of results is subjective and quality of antigen must be maintained by frequent subculture of *P. pseudomallei*. The small number of positive sera is a reflection of the difficulty in collecting samples as 37% of our septicaemic melioidosis patients died within 48 hours of admission[1], before culture results were available.

In the prevalence study it was found that four of the 75 samples (5%) from PUO sera were positive by IFAT,

suggesting the possibility of melioidosis but active infection was ruled out as all were negative by the IgM-ELISA. Once again, the presence of IgG due to slowly progressive infection or cross-reaction with related species of *Pseudomonas* is a possibility. In addition, one further sample was positive by the IgM-ELISA indicating the possibility of a current infection or cross-reaction. Amongst the antenatal sera, five of 72 samples (7%) were positive by both IFAT and IgM-ELISA indicating the presence of both IgG and IgM antibodies. The IFAT detected one further sample indicating the possibility of IgG only and the IgM-ELISA detected one more sample indicating the presence of IgM only respectively. However, none of the seven patients had overt signs of infection. Pregnancy has been described as one of the predisposing underlying conditions[15] for *P. pseudomallei* infection but the effect of immunological changes accompanying pregnancy on the serodiagnosis of melioidosis is unclear. However, as a specific antigen was used in the assay and only 7 of 72 samples (10%) were positive, these results suggest a current or progressive infection or a cross-reaction with other *Pseudomonas* infections.

Amongst sera from 50 diabetic patients, none was positive by the IgM-ELISA, indicating no active disease but 3 (6%) were positive by IFAT, suggesting the presence of IgG in a slowly progressive or chronic infection. Diabetes mellitus is a well recognised risk factor[1] and it could be postulated that the increased level of blood sugar enables glycocalyx formation by the organism to evade phagocytosis or enhance extracellular survival leading to latent or persistent infection[16].

Farmers and outdoor workers form another well recognised occupational risk group as soil is the natural habitat of *P. pseudomallei*: 14% (11/80) of samples were positive by the IgM-ELISA and only 3% (2/80) were positive by all these assays (Table IV). The results indicate probable past subclinical infection and possible recent or current mild infection which may have been disregarded.

The spectrum of melioidosis is a continuum of various stages and the immunological status of these stages has yet to be clearly defined, and the gold standard for the serological diagnosis of melioidosis also has yet to be established, especially to discriminate between active and chronic infections. New diagnostic methods and perhaps serial or sequential immunological studies are urgently needed.


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