Asian Pacific Journal of Allergy and Immunology (1992) 10 : 145-150

Use of Endotoxin Antigens in Enzyme-Linked Immunosorbent Assay for the Diagnosis of *P.pseudomallei* Infections (Melioidosis)

Vimol Petkanjanapong, Pimjai Naigowit, Eiko Kondo and Koomi Kanai

SUMMARY **An enzyme-linked immunosorbent assay (ELISA) with endotoxin preparations of *P.pseudomallei* as antigen was developed for detection of IgG antibodies specific to melioidosis. Forty-seven sera of bacteriologically confirmed melioidosis patients, 55 non-melioidosis sera and 50 sera of healthy blood donors from non-endemic areas were subjected to this assay in comparison with indirect hemagglutination assay (IHA). The data were treated by receiver operating characteristics analysis. The sensitivity, specificity and accuracy in this ELISA were 95.7%, 94.2%, and 94.7%, respectively, with cut-off value of OD = 0.312 at 490 nm. Meanwhile, those in IHA were 81.0%, 91.4%, and 88.1%, respectively, with a cut-off value of $\geqslant$ 1:160. From these results, the ELISA was judged to be more reliable than IHA as the seroassay for diagnosis of melioidosis.**

Melioidosis, an infectious disease caused by *Pseudomonas pseudomallei,* had a wide spectrum of clinical features resembling other acute and chronic parasitic diseases. Definitive diagnosis is possible only by positive specimen culture of *P.pseudomallei.* [1]

On the other hand, efforts have been made by several authors [2,3] to develop a reliable and efficient method for serodiagnosis of *P.pseudomallei* infection. Indirect hemagglutination (IHA), [4] complement fixation assay, [4] indirect immunofluorescent assay (IFA) for IgG [5] and IgM [6] and enzyme-linked immunosorbent assay (ELISA) have been introduced for the diagnosis of melioidosis and for the serosurveillance of inapparent infections. [7,8]

In these serological tests, the employed antigens were preparations from culture filtrates [4,5] or the extract from sonicated cell homogenates. [4] While such preparations were not purified and defined antigens, Ismail *et al* [9,10] used their exotoxin (a 36,000-dalton polypeptide) in ELISA, which has mouse-fatal toxicity, cytotoxicity in cell culture system, and an inhibitory effect on intracellular protein synthesis. This information prompted us to an attempt of ELISA with endotoxin preparations as antigen in melioidosis patients, non-melioidosis patients and healthy blood donors. The data presented here are compared with those of IHA together with receiver operating characteristic (ROC) analysis.

MATERIALS AND METHODS

Serum specimens

Forty-seven blood samples were obtained from bacteriologically confirmed melioidosis patients admitted to Sappasitthiprasong Ubon Hospital as possible seropositive materials to *P.pseudomallei,* and 50 samples were collected from blood donors in nonendemic areas as putative negative sera. Finally, an additional 55 sera were obtained from septicemic patients from whom bacterial species other than *P.pseudomallei* were isolated as causative organisms in Ubon Hospital.

LPS (endotoxin) preparations as antigens

LPS samples were prepared from 4 day-growth of each of the 5 local strains (UB 12, 16, 18, 19, 20) [11]

From the Division of Clinical Pathology, National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Tivanond Road, Nonthaburi, Thailand.

Correspondence : Koomi Kanai

of *P.pseudomallei* in 1.5 l of Muller-Hinton medium. These strains were identified by colony morphology, biochemical tests, pH-activity pattern of acid phosphatase, fatty acid profiles by gas-liquid chromatography [11] and indirect imunofluorescence microscopy (Kurata *et al,* in preparation). After heating at 120° C for 30 minutes, the bacterial mass was harvested from the culture and washed with water by centrifugation at 10,000 rpm for 30 minutes. The sedimented cell mass was combined and suspended in 20 ml of water and treated by sonication to make lysate. LPS was then extracted from this lysate by the phenol-water method of Westphal and Jann. [12] Briefly stated, the lysate diluted with 50 ml of water was kept at 65°C and added to 70 ml of 90% phenol. The mixture was stirred vigorously at 65° C. Thereafter, it was cooled to 10° C and centrifuged at 5,000 rpm for 15 minutes to obtain the water layer I. The phenol layer was extracted again as before by adding 70 ml of water. The water layer II here obtained was combined with the water layer I and subjected to dialysis for 4 days. The dialysed water extract was centrifuged at 3,000 rpm for 20 minutes to remove the materials unsolved. The supernatant was then centrifuged at 100,000 × g for 60 minutes. The resulting sediment was washed 5 times by such centrifugation with 10 ml of water. The final sediment was suspended in small amount of water and lyophilized.

ELISA

An indirect ELISA was employed. Preliminarily, the optimal conditions for the assay were determined by checkerboard titration concerning the concentrations of antigen, serum, and enzyme conjugates. The 96 flat-bottomed wells of ELISA plates (Nunc Micro Well Plates, Denmark) were coated with 100 μl of 5 μg LPS per ml of PBS (pH 7.4) and left to stand overnight at 4° C. The contents of the wells were removed by washing out, and the plates were blocked by the addition of 180 μl of 1% bovine serum albumin in PBS pH 7.4 to each well. After incubation in a moist chamber at 37° C for 90 minutes, the plates were washed three times with PBS pH 7.4 containing 0.05% Tween 20 (PBS-Tween and dried by blotting.

A 100 μl volume of each test sample and control serum, diluted 1:100 with PBS-Tween, was added to individual wells and the plates were incubated at 37° C for 90 minutes. After the plates were washed three times with PBS-Tween, 100 μl of horseradish peroxidase-labeled rabbit antibody to human IgG antibody (Dakopatts AS, Copenhagen, Denmark) diluted 1:1,000 in PBS-Tween was added to each well, and the plates were incubated at 37° C for 90 minutes. The plates were washed three more times with PBS-Tween and 150 μl of o-phenylenediamine (OPD) substrate was added to each well. The substrate was prepared by dissolving 4 mg o-phenylenediamine (Sigma) in 10 ml of phosphate-citrate buffer, pH 5.0, and 40 μl of 3% H_2O_2. The plates were incubated in the dark at room temperature, and the reaction was stopped after 5 minutes by the addition of 50 μl of 2 N H_2SO_4 to each well. The optical density (OD) of developed yellow-orange color was measured spectrophotometrically by the Microplate Autoreader EL 309, (Bio-Tek instruments) at 490 nm.

Two standard serum samples were employed; a negative control and a positive control, the former being determined by the IHA test, IgG-IFA, and IgM-IFA, and the latter being a pool of serum specimens obtained from culture-positive patients with high titers of IHA and IgG-IFA.

IHA

IHA test was performed in a microtiter system using *P.pseudomallei* culture filtrate (melioidin) as antigen. The antigen was prepared from bacterial culture of glycine broth incubated at 37° C for 14 days. The culture was killed at 121°C for 15 minutes and centrifuged at 4,000 rpm for 1 hour. The supernatant was then passed through the membrane filter of 0.45 μm pore-size. Saline-washed sheep erythrocytes were sensitized by incubating them with the antigen at 37° C for 1 hour, and excess antigen was removed by washing 3 times with isotonic saline. Serum specimens were inactivated at 56° C for 30 minutes and adsorbed with a saline-washed nonsensitized sheep erythrocytes (5 per cent) at room temperature for 30 minutes before testing. Serial twofold dilutions) (1:10 to 1:10,240) were made from the inactivated serum specimens in PBS, and each dilution was incubated with the sensitized erythrocytes for 2 hours at room temperature. The endpoint titer was defined as the highest dilution in which hemagglutination occurred.

Statistical methods

The indices of sensitivity, specificity, and accuracy at various ELISA and IHA titers were calculated as follows: For sensitivity, $a/(a+c)\times 100$, for specificity, $d/(b+d)\times 100$, for accuracy, $(a+d)/(a+b+c+d)\times 100$, where a is the number of true positive samples, b is the number of false positive samples, c is the number of false negative samples, and d is the number of true negative samples. Cut-off level for IHA was set at 1:160 on the basis of published data and our own data (Pimjai *et al,* in preparation). The cut-off point for ELISA was set at OD = 0.320 in view of the distribution pattern of reaction intensity (Fig. 1). To give a further statistical rationale to these settings, the ROC curve was constructed by ploting the true positive rate (sensitivity) against the false positive rate (1-specificity). The overall accuracy of a test can be evaluated as the area under the ROC curve; the larger the area, the better the test. [13]

RESULTS

The distribution of ELISA antibody titers in three groups of examinees is shown in Fig. 1 in the form of a scatter diagram. The distribution pattern is rather clear-cut separating the melioidosis group from the groups of other diseases and blood donors. The horizontal line set at the critical level (OD = 0.312) of antibody in the melioidosis group excludes almost all subjects of the other 2 groups below the level, leaving only 3 and 2 above the line, respectively. Meanwhile, the titer distribution within the patient group is rather wide in the range upto OD 2.5 at the highest.

IHA titers exhibited a distribution pattern as shown in Fig. 2, where the cut-off value was set at $\geqslant$ 1:160 with 81% sensitivity, 91.4% specificity, and 88.1% accuracy. Nine out of 47 melioidosis sera showed the low titers below the cut-off value while out of 55 other infectious sera and 3 out of 50 blood donor sera had higher IHA titers than cut-off value.

The results are shown in Table 1. The table demonstrates that the cut-off level at OD = 0.312 gives the most acceptable indications for the above three items when taken together, namely 95.7% sensitivity, 94.2% specificity and 94.7% accuracy. In the lower level of cut-off point the sensitivity was increased upto 100%, but the specificity was decreased to 59%.

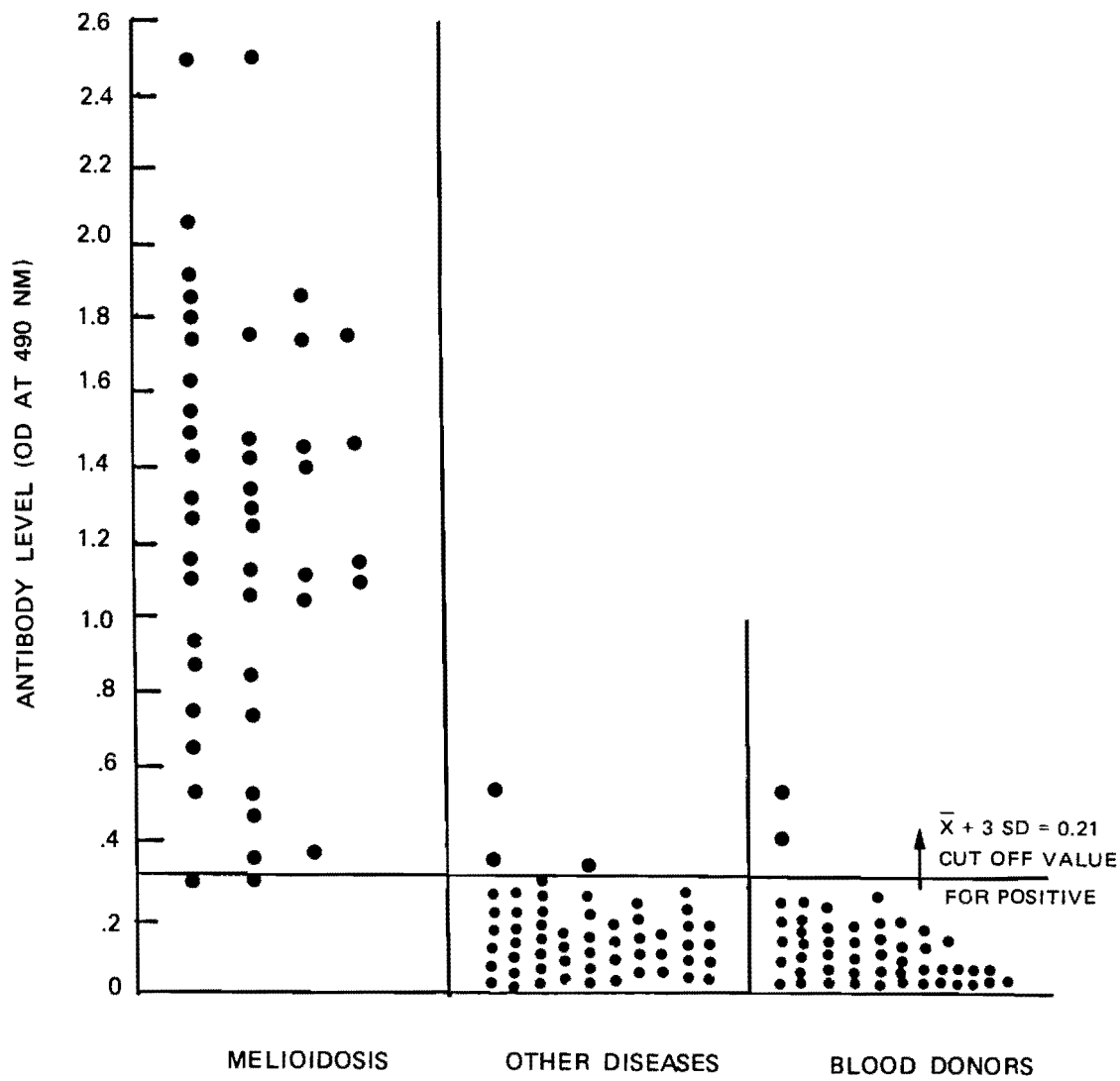

Fig. 1 Scattered diagram of ELISA antibody titers to *P. pseudomallei* in three groups of sera.

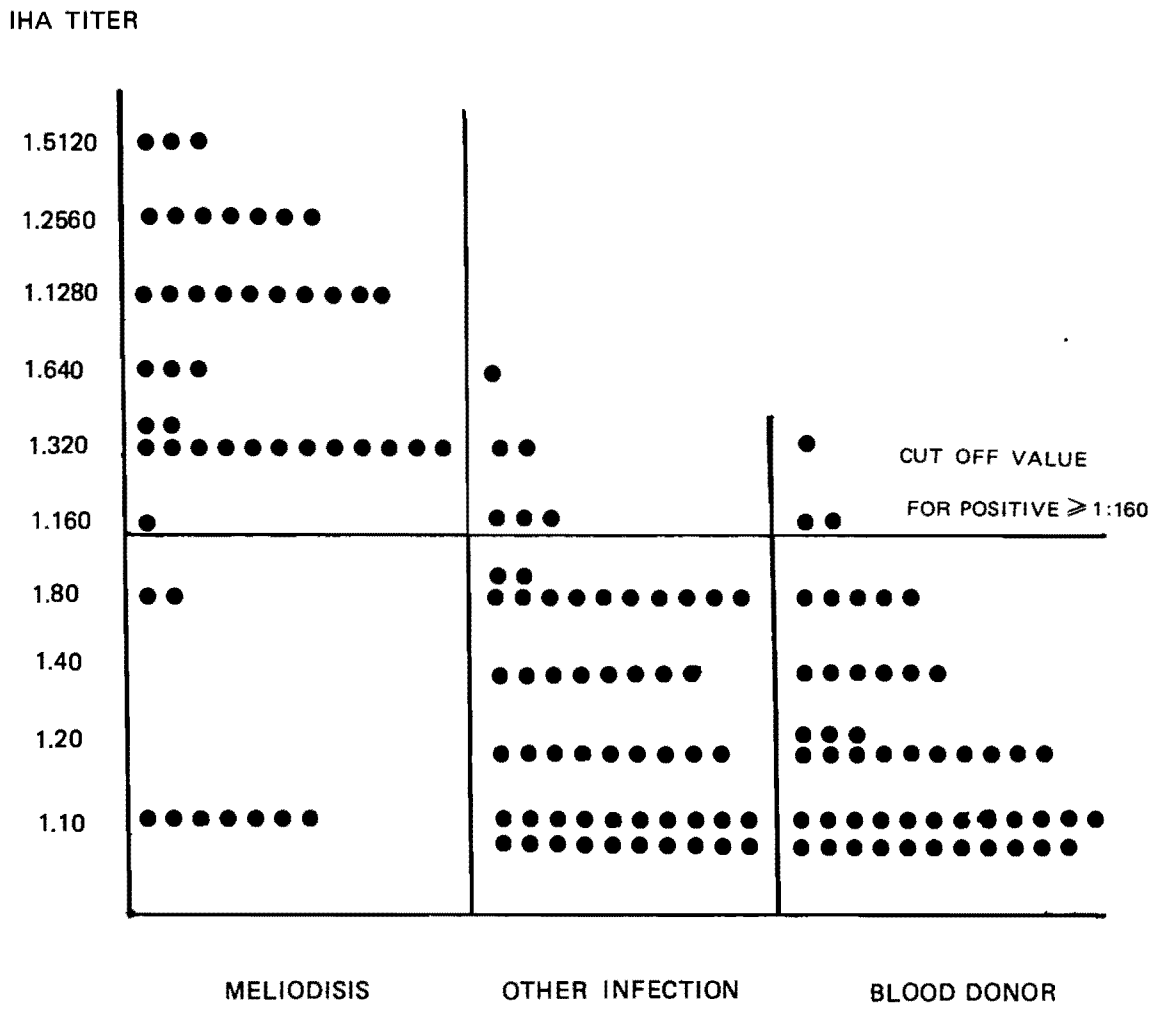

Fig. 2 Scattered diagram of IHA antibody titers to *P. pseudomallei* in three groups of sera.

Table 1. ROC analysis of ELISA

Cut–off value for positive at 490 nm	Sensitivity (%)	Specificity (%)	Accurcy (%)	False positive
$\bar{x}$ + SD (0.157)	100.0	59.0	71.7	41.0
$\bar{x}$ + 2SD (0.234)	100.0	86.6	90.7	13.4
$\bar{x}$ + 3SD (0.312)	95.7	94.2	94.7	5.8
$\bar{x}$ + 4SD (0.390)	91.4	96.1	94.7	3.9

Table 2 shows comparison of efficiency by ROC analysis between ELISA and IHA. In IHA, the cut-off level was set at 1:160 according to the experiences of the previous authors including ourselves (Pimjai *et al*, in preparation). In each case, ELISA was better than IHA, especially in sensitivity and accuracy. This was confirmed graphically by ROC curves in Fig. 3, where the curve of ELISA runs above that of IHA.

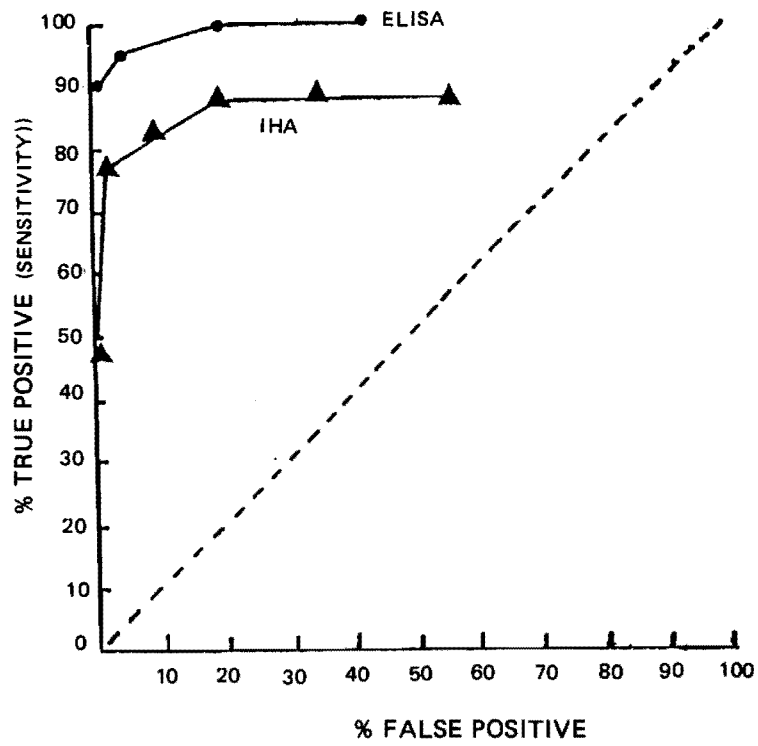

Fig. 3 ROC curves of ELISA and IHA.

DISCUSSION

The IgG-ELISA introduced here employed an endotoxin preparation extracted from *P.pseudomallei* cells as antigen. The results were satisfactory, giving high values for sensitivity and specificity, 95.7% and 94.2%, respectively. These values are higher in sensitivity and lower in specificity than those reported by Ashdown *et al*[7] in their IgG-ELISA. These authors used a cell-sonicate preparation as antigen. The higher specificity value in their results may

Table 2. Comparison by ROC analysis between IHA and ELISA for melioidosis diagnosis

		IHA (cut–off value : ⩾ 1:160) in		ELISA (cut–off value : ⩽ OD 0.312) in	
		Patients	Blood donors & other diseases	Patients	Blood donors & other diseases
Total tests		47	105	47	105
Positive		38	9	45	6
Negative		9	96	2	99
ROC analysis (%)	Sensitivity	81.0		95.7	
	Specificity	91.4		94.2	
	Predicitive value of positive	81.0		88.2	
	Predicitive value of negative	91.4		98.0	
	False positive	8.6		5.8	
	False negative	19.0		4.3	
	Accuracy	88.1		94.7	

be due to the employment of blood donors of nonendemic areas as the normal sera.

Kunakorn *et al*[8] evaluated IgM-ELISA compared with IHA, using the same samples of test (melioidosis) and control sera. The *P.pseudomallei* antigen was a soluble sonicated antigen for ELISA and protein-free broth culture filtrate for IHA. The IgM-ELISA gave 87.5% sensitivity and 92.2% specificity at 1:500 serum dilution and a positive to negative ratio (P/N) cut off > 4, while those for IHA were 88% and 97.4%, respectively. They recommended that the IgM-ELISA and IHA should be used in combination for serodiagnosis of melioidosis.

On the other hand, our IgG-ELISA revealed a superior efficiency in every item of ROC analysis than IHA, with the cut-off point set at 1:160. This was confirmed when ROC curves were compared between these two assays. In Fig. 3 the curve of ELISA runs above that of IHA, thus indicating that the former is the more efficient test to separate positive and negative reactions.

It is not clear at this moment to what extent the use of endotoxin (LPS) has contributed to the successful results of our IgG-ELISA. Though the immunogenic exotoxins have been demonstrated in the culture filtrate of *P.pseudomallei,* we have little information about the endotoxins of this species of bacteria. Only the endotoxin property of *P. pseudomallei* has been reported in and has been described as mouse body weight decreasing activity.[14,15] Meanwhile, the serotyping of *P. pseudomallei* by O-antigenicity is not yet developed, and generally it has been thought that there was only one O-serotype. Endotoxin is a molecular structure on the bacterial surface and the polysaccharide moiety may play an important role in immunogenicity. Further study is needed to make endotoxin-ELISA a routinely applicable serology in usual clinical laboratories.

Tattawasart *et al*[16] developed an ELISA for the detection of *P.pseudomallei* antigens using plates coated with IgG antibodies. However, this is still in an experimental level, as is the exotoxin ELISA. DNA probe technology will be introduced sooner or later for detection of the bacterium. What is the most satisfactory method for rapid and reliable diagnosis of melioidosis remains problem for future exploration.

ACKNOWLEDGEMENTS

We thank Miss Nuanchawee Wetprasit and Mr. Wanchai Maneeboonyoung for their technical cooperation. Thanks are also expressed to Dr Monkol Kunakorn for his advises concerning ROC analysis, and to Mrs Suwanna Navacharoen for her secretarial assistance in preparation of the manuscript. A part of the budget for this study was supplied from Japan International Cooperation Agency (JICA).

REFERENCES

1. Leelarasamee A, Punyagupta S. Melioidosis. Epidemiology with Thai experience. p. 238-64. In S. Punyagupta; T. Sirisanthara and Stapatayavong, B, eds, Melioidosis 1989; Bangkok Medical Publisher, Bangkok.
2. Kanai K, Dejsirilert S. *Pseudomonas pseudomallei* and melioidosis, with special reference to the status in Thailand. Jpn J Med Sci Biol 1989; 41 : 123-57.
3. Nigg C. Serological studies on subclinical melioidosis. J Immunol 1963; 91 : 18-28.
4. Alexander AD, Huxsoll DL, Warner AR Jr, Shepler V, Dorsey A. Serological diagnosis of human melioidosis with indirect hemagglutination and complement fixation tests. Appl Microbiol 1990; 20 : 825-33.
5. Ashdown LR. Demonstration of human antibodies to *Pseudomonas pseudomallei* by indirect fluorescent antibody staining. Pathology 1981a; 13 : 597-601.
6. Ashdown LR. Relationship and significance of specific immunoglobulin M antibody response in clinical and subclinical melioidosis. J Clin Microbiol 1981b; 14 : 361-4.
7. Ashdown LR, Johnson RW, Koehler JM, *et al.* Enzyme-linked immunosorbent assay for the diagnosis of clinical and subclinical melioidosis. J Infect Dis 1989; 160 : 253-60.
8. Kunakorn M, Boonma P, Khupulsup K, *et al.* Enzyme-linked immunosorbent assay for immunoglobulin M specific antibody for the diagnosis of melioidosis. J Clin Microbiol 1990; 28 : 1249-53.
9. Ismail G, Embi MN, Omar O, *et al.* A competitive immunosorbent assay for detection of *Pseudomonas pseudomallei* exotoxin. J Med Microbiol 1987; 23 : 351-7.
10. Ismail G, Mohamed R, Rohana S, *et al.* Antibody to *Pseudomanas pseudomallei* exotion in sheep exposed to natural infection. Veterinary Microbiol 1991; 27 : 277-82.
11. Kondo E, Neigowit P, Petkanchanapong W, *et al.* Acid phosphatase activity and fatty acid pattern for identification of fresh clinical isolates of *Pseudomonas pseudomallei.* Jpn J Med Sci Biol 1991; 44 : 195-211.
12. Westphal O, Jann K. Bacterial lipopolysaccharide extract with phenol-water and further application of the procedure. Methods Carbohydr Chem 1965; 5 : 83.
13. Weinstein MC, Fineberg HV. Clinical Decision Analysis. W.B. Saunders, Philadelphia, 1980.
14. Rapaport FT, Miller JW, Ruch J. Endotoxic properties of *Pseudomonas pseudomallei* Arch Pathol 1961; 71 : 429-36.
15. Iwasa S. Petkanchanapong V, Naigowit P, *et al.* Endotoxic property of *Pseudomonas pseudomallei* as detected by the body-weight decreasing reaction in mice. A comparison with *P.cepacia* and *P.aeruginosa.* Jpn J Med Sci Biol 1992; 45 : 33-47.
16. Tattawasart U, Wongratanacheewin S, Wongwajana S, *et al.* Enzyme-linked immunosorbent assay for the detection of *Pseudomonas pseudomallei* culture filtrate antigen. Proc 2nd Western Pacific Congr Infect Dis Chemother 1990; Jomtien-Pattaya, Thailand, Dec. 11-4.