USE OF CULTURE-FILTRATED ANTIGEN IN AN ELISA AND A DOT IMMUNOASSAY FOR THE DIAGNOSIS OF MELIOIDOSIS

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Abstract. An enzyme-linked immunosorbent assay (ELISA) and a dot immunoassay with culture-filtrated antigen were developed for detection of *Burkholderia pseudomallei* specific antibodies in melioidosis patients. Sixty-eight sera of bacteriologically confirmed melioidosis patients, 45 sera of other bacterial infected patients and 80 sera of healthy blood donors from endemic area were investigated. The samples were subjected to those assays in comparison with indirect hemagglutination (IHA). The sensitivity, specificity, positive and negative predictive values in this dot immunoassay were 94.1%, 99.2%, 98.5% and 96.9%, respectively, with cut-off dilution at 1:4,000, whereas those in ELISA were 92.6%, 96.8%, 94.0% and 96.0%, respectively, with cut-off value of OD = 0.47 at 490 nm. Meanwhile, those in IHA were 64.7%, 93.6%, 84.6%, 83.0% respectively, with a cut-off value of ≥ 1:80. The results in this study demonstrated that the dot immunoassay was more reliable and rapid than ELISA as the serological test for diagnosis of melioidosis.

INTRODUCTION

Melioidosis is an infectious disease caused by *Burkholderia (Pseudomonas) pseudomallei*. The disease is endemic in Southeast Asia and Australia (Ashdown and Guard, 1984; Dance, 1990; Leelarasamee and Bovornkitti, 1989; Patamasucon *et al*, 1982). Recently, increasing numbers of cases have been reported from Korea, Hong Kong, Bangladesh, India, Iran, Turkey, England, France, Africa, the former USSR and the United States (Dance, 1990; Jenkins *et al*, 1990; Leelarasamee and Bovornkitti, 1989; Ranghavan *et al*, 1991; So *et al*, 1984; Struelens *et al*, 1988). Infection is usually acquired from contamination of soil and water through pre-existing skin abrasions or by inhalation of infectious dust particles. The disease is often associated with underlying illness of immunocompromised hosts (Tanphaichitra, 1989). The clinical features of this disease are protean and ranges from subclinical to acute septicemic forms (Chaowagul *et al*, 1989; Dance, 1991; Sanford and Moore, 1971; Thompson and Ashdown, 1989; White and Dance, 1988; Wilson *et al*, 1987). In acute septicemic melioidosis, the mortality rate is 80-90%, with death occurring 24-48 hours after onset (Chaowagul *et al*, 1989). Rapid and specific diagnosis is thus critical. Definite diagnosis is dependent on isolation and identification of the causative agent from clinical specimens. However, the bacteriological method is time consuming and may provide results too late for effective therapy. A variety of serological tests have been used for the detection of antibodies to *B. pseu-domallei* such as agglutination, indirect hemagglu-tination (IHA), complement fixation (CF), immuno-fluorescence (IFA), enzyme-linked immunosorbent assay (ELISA) and gold blot (Alexander *et al*, 1970; Anuntagool *et al*, 1993; Appassakij *et al*, 1990; Ashdown, 1987; Ashdown *et al*, 1989; Kunakorn *et al*, 1990, 1991; Nigg, 1973; Petkanjanapong *et al*, 1992). Alternatively, other diagnostic tests for detection of *B. pseudomallei* in samples using either polyclonal antibodies or specific DNA probe were also developed (Sermswan *et al*, 1994; Wongratana-cheewin *et al*, 1990, 1993). Most of the former methods still have problems associated with inter-pretation of the results and some occasionally false-positive reactions, whereas the application of the later methods in patient specimens remains to be in-vestigated.

In past studies using antibody detection for diagnosis of melioidosis, the employed antigens were prepared from crude extract or culture-filtrated of which their complexity had not been characterized. In the present study, the culture-filtrated antigen prepared in synthetic medium which was shown to give the highest specificity for *B. pseudomallei* (Wongratanacheewin *et al*, 1993) was used to develop

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an ELISA and a dot immunoassay. The results presented are compared between ELISA and dot immunoassay and their applications to routine diagnosis is described.

MATERIALS AND METHODS

Serum specimens

Sera from 68 melioidosis patients admitted to Srinagarind Hospital, Faculty of Medicine, Khon Kaen University were collected for positive samples. Diagnosis was confirmed by isolation of *B. pseudomallei* from their clinical specimens. Eighty control samples were obtained from blood donors at The Center Blood Bank, Srinagarind Hospital. Forty-five sera from individual septicemic patients with other bacterial infections *ie Staphylococcus aureus* (11 cases), *Salmonella* spp (3 cases), *Escherichia coli* (8 cases), *Enterobacter* spp (4 cases), *Streptococcus* spp (7 cases), *Klebsiella pneumoniae* (6 cases), *Proteus mirabilis* (1 cases), *Pseudomonas aeruginosa* (3 cases), *P. stutzeri* (1 case) and *Mycobacterium tuberculosis* (1 case) were also included for evaluation of the specificity of the tests.

Antigen preparation

The *B. pseudomallei* isolated from a patient with disseminated septicemic melioidosis was used for antigen preparation. The culture-filtrated antigen was prepared in Modified Proskauer and Beck medium (MPB) as described by Wongratanacheewin *et al* (1990). Briefly, the bacteria were cultured in MPB medium for 3 weeks at 37°C. The culture-filtrated antigens were collected by centrifugation at 12,800*g* 4°C for 20 minutes (Beckman, USA) and filtered through a 0.45 μm membrane. The antigens obtained were then dialyzed thoroughly against three changes of PBS and kept frozen at-20°C until used. This antigen was used for development of ELISA and dot immunoassay.

Indirect hemagglutination (IHA)

IHA test was performed in a microtiter system using *B. pseudomallei* culture-filtrated (melioidin) (Ashdown, 1987) as antigen for detection of antibodies. The antigen was prepared by growing bacteria in glycine broth at 37°C for 14 days. After killing the bacteria at 121°C for 15 minutes, the supernatant was collected after centrifuged at 4,000 rpm for 1 hour and filtered 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, thereafter they were washed three times with saline solution to remove excess antigens. Serum specimens were incubated at 56°C for 30 minutes and adsorbed with 5% saline-washed non-sensitized sheep erythrocytes at room temperature for 30 minutes before testing. Each of two-fold diluted serum specimens (1:10 to 1:10,240) was incubated with the sensitized erythrocytes for 2 hours at 37°C. The end-point titer was defined as the highest dilution in which hemagglutination occurred. Cut-off level was set at 1:80 on the basis of our routine hospital use.

Enzyme-linked immunosorbent assay (ELISA)

An indirect ELISA of Engvall and Perlman (1971) was performed for the detection of *B. pseudomallei* specific antibodies. Optimal conditions for antigen, serum and enzyme conjugates were predetermined by a checkerboard titration.

The 96 flat-bottomed wells of EIA plates (Costar, MA, USA) were coated with 100 μl of antigen at 5 μg/ml in carbonate buffer pH 9.6 and incubated overnight at 4°C. The plates were then washed three times with 0.05% (vol/vol) Tween-20 (Sigma, MO, USA) in 0.15 M phosphate buffer saline, pH 7.4 (PBS-Tween). Each time the washing fluid was left in the wells for 3 minutes. The plates were dried and blocked with 5% skim milk in PBS for 60 minutes at 37°C and then similarly washed prior to adding the samples. Serum samples were diluted serially (3-fold) from 1:500 in PBS-Tween containing 2% skim milk and 100 μl of each dilutions were added. In the sample that gave a titer lower than 1:500, the dilution will be repeated by starting from 1:50. The plates were incubated at 37°C for 60 minutes. After the plates were washed 3 times with PBS-Tween, 100 μl of horseradish peroxidase-conjugated rabbit antibody to human IgG, IgA, IgM, kappa and lambda (Dakopatts AS, Copenhagen, Denmark) diluted 1:8,000 in 2% skim milk PBS-Tween was added to each well. The plates were then incubated at 37°C for 60 minutes. After washing for 3 times, 100 μl of ortho-phenylenediamine (OPD) substrate was added to each well. The substrate was prepared by dissolv-

ing 10 mg o-phenylenediamine (Sigma) in 100 ml of 0.1 M citrate phosphate buffer, pH 5.0, and 10 µl of 30% H_2O_2. The plates were incubated in the dark at room temperature, and the reaction was stopped after 10 minutes by the addition 100 µl of 4 N H_2SO_4 to each well. The optical density was read at 490 nm in an ELISA reader (Dynatech Instruments Inc, CA, USA).

The levels of specific antibody in the serum were determined either by reading the OD values at a dilution of 1:4,500 (This dilution gave the best discrimination between pooled positive and pooled negative samples) or by the multiple dilution end-point estimation method as described by Svennerholm *et al* (1984). Antibody titers were then calculated from the end-point giving an absorbance of 0.5 unit above the background. A positive reference serum was also included in every plate to correct for day-to-day and plate-to-plate variations that might be present.

Dot immunoassay

A 50 µl of 2.5 µg/ml *B. pseudomallei* antigen was applied to an nitrocellulose membrane (Hoefer Scientific Instrument, CA, USA) with a 96-well filtration manifold (Gibco BRL, MD, USA) under an adjustable vacuum. The antigen was allowed to filter through the membrane for 30 minutes. The membrane was washed 3 times with PBS. Blocking was performed with 2% skim milk in 20 mM Tris buffer saline, pH 7.5 (TBS) overnight at 4°C to prevent nonspecific binding. After washing 3 times, the membrane can either be kept at -20°C until used. Fifty µl of samples diluted at 1:4,000, 1:8,000, 1:16,000, 1:32,000 in 1% skim milk-TBS were added duplicately in the wells. The samples were incubated at room temperature for 60 minutes. The membrane was again washed 3 times, and the horseradish peroxidase-conjugated rabbit antibody to human IgG, IgA, IgM, kappa and lambda (Dakopatts AS) diluted 1:4,000 in 1% skim milk-TBS was applied and incubated for another 60 minutes. The membrane was then washed and the color was developed by addition of chromogenic substance solution containing 35 mg of 4-chloro-1-naphthol (Sigma) in 7 ml of absolute methanol mixed with 20 µl of 30% H_2O_2 in 63 ml of TBS. The positive signal was indicated by the deposition of a purple dot. For standardization, each time an assay was performed, both positive and negative controls were included.

Sensitivity, specificity accuracy and predictive values

Sensitivity for the detection of specific antibodies to *B. pseudomallei* antigens was calculated from the number of serologically positive cases among patients with bacteriologically proven melioidosis. The criteria used for positive result were either (1) ELISA titer was equal to or above the arithmetic mean + 99% confidence limit of normal controls; or (2) the optical density of ELISA at a dilution of 1:4,500 was equal to or above the arithmetic mean + 4 SD normal controls; or (3) the purple dot of dot immunoassay as observed by naked eye.

The indices of specificity and accurary of the assays system was calculated as follows: 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, d is the number of true negative samples.

RESULTS

The IHA results in diagnosis of melioidosis were shown in Table 1. When the cut-off value was set at ≥ 1:80, 24 out of 68 melioidosis sera showed the low titers below the cut-off while 5 out of 45 other infectious sera and 3 out of 80 blood donor sera had higher IHA titers than cut-off values.

Table 1

Comparison of IHA, ELISA and dot immunoassay for the detection of antibodies to *B. pseudomallei* in various source of specimens.

Source of specimens	Numbers of positive/total tested				
	IHA[a]	ELISA		Dot immunoassay[b]	
		Titer[c]	OD[d]	1:4,000	1:8,000
Melioidosis	44/68	63/68	63/68	64/68	56/68
Other diseases	5/45	10/45	4/45	1/45	1/45
Normal	3/80	0/80	0/80	0/80	0/80

[a]Positive result is specimens giving IHA titer equal to or above 1 : 80

[b]Positive result is specimens giving the purple dot (as observed by naked eye) at certain dilutions.

[c]Positive result is specimens giving titer equal to or above the geometric mean titer with 99% confidence limits of the normal controls.

[d]Positive result is specimens giving absorbance equal to or above the mean absorbance with 4SD of the normal controls.

The distribution of ELISA antibodies in three groups of samples expressed as either antibody titers or absorbances are shown in Fig 1. The scattergram of ELISA absorbances is more clear-cut when separating the melioidosis group from the control groups than that of ELISA titers. However, using either of the cut-off limits in both diagrams, a large majority (except 5 cases) of the melioidosis sera were positive (Table 1). When sera from other bacterial diseases are concerned, ELISA absorbance can exclude most subjects below the level, leaving only 4 above the cut-off line (Fig 1 and Table 1). This makes the expression of ELISA as an absorbance more advantage than as a titer.

Result from the dot immunoassay of the representative 3 groups of sera clearly demonstrated that only the melioidosis sera gave strong signal at dilution of 1:4,000 (data not shown). Those purple signals of the melioidosis sera were easily observed by the naked eye whereas those of sera from other bacterial infections or healthy controls provided negative signals. The detection limit of the dot immunoassay is showed in Table 1. It is found that the optimal sera dilution for the dot immunoassay was 1:4,000, giving the highest positive cases (64 from 68) in the melioidosis group.

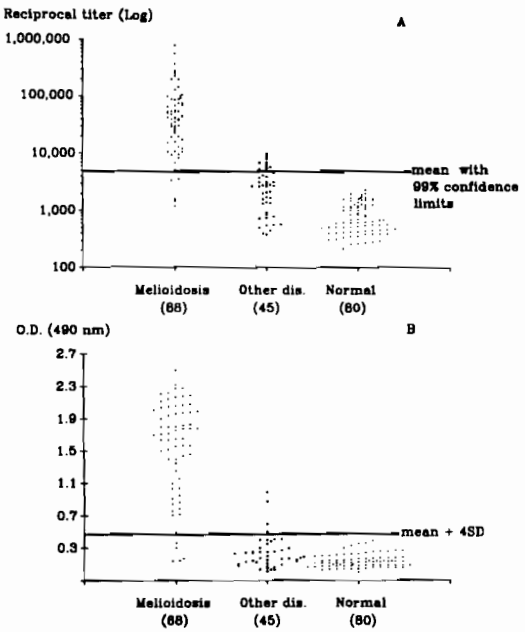

Fig 1–Scattered diagram of ELISA antibody titers (A) and ELISA absorbances (B) to *B. pseudomallei* in three groups of sera. The cut-off value for positive were either mean with 99% confidence limits or mean+ 4SD. Numbers in parenthesis were number of sera tested.

The sensitivity, specificity, accuracy and predictive values are shown in Table 2. In IHA the cut-off value was set at 1:80 with 64.7% sensitivity, 93.6% specificity and 83.4% accuracy. The sensitivity and specificity of ELISA were determined at both end-point value and absorbance. Results from Tables 1 and 2 demontrate that ELISA absorbance gives more acceptable indications, namely 92.6% sensitivity, 96.8% specificity, 95.3% accuracy, 94.0% positive and 96.0% negative predictive values, than that of ELISA titer. On the other hand, the dot immunoassay, using sera dilution of 1:4,000, clearly shows the most satisfactory result with 94.1% sensitivity, 99.2% specificity, 97.4% accuracy, 98.5% positive and 96.9% negative predictive values. From the results above, ELISA was better than IHA and dot immunoassay was better than ELISA, especially in sensitivity, specificity and accuracy.

DISCUSSION

The results presented in this study clearly demonstrated that the dot immunoassay with culture-filtrated antigen gave the highest values for sensitivity (94.1%) and specificity (99.2%). The culture-filtrated antigen used in this study was already characterized and found to have a major *B. pseudomallei* specific antigen with M_r of 40 kDa (Wongratanacheewin *et al*, 1993). The results from immunoblotting showed that this culture-filtrated antigen contained less complicated SDS-PAGE profile than crude antigens (Wongratanacheewin *et al*, 1993). Sera from individuals melioidosis were proved to be reacted consistently and specifically with that of 40-kDa. These all results indicated that the culture-filtrated antigen have a good potential to be used in immunodiagnosis of melioidosis. It is not clear at this moment that our culture-filtrated antigen used in this study contained any exotoxin that played a role in pathogenesis or disease progression, although our preliminary data demonstrated that this antigen had an inhibitory effect on human lymphoproliferation (data not shown). Ismail and colleagues (1987) developed a sensitive and specific enzyme immunoassay for detection of antibody to *B. pseudomallei* exotoxin in mice. Although such antibody was found only in 54.4% Malaysian military personnel

Table 2

Sensitivity, specificity, accuracy, false positive false negative, and predictive values of the IHA, ELISA and dot immunoassay.

Methods	Sensitivity (%)	Specificity (%)	Accuracy (%)	False positive (%)	False negative (%)	Predictive values (%)	
						Positive	Negative
IHA	64.7	93.6	83.4	6.4	35.3	84.6	83.0
ELISA (Titer)	92.6	92.0	92.2	8.0	7.4	86.3	95.8
ELISA (OD)	92.6	96.8	95.3	3.2	7.4	94.0	96.0
Dot immunoassay (1:4,000)	94.1	99.2	97.4	0.8	5.9	98.5	96.9
Dot immunoassay (1:8,000)	82.4	99.2	93.3	0.8	17.6	98.2	91.2

(Embi *et al*, 1992), the evidence of the anti-exotoxin antibodies in the diagnosis of melioidosis remains to be investigated. Since our serum series were obtained from patients resided in the endemic area, it is possible that some patients might have been exposed to previous subclinical *B. pseudomallei* infections and that the current episode represent a reinfection. The use of specific IgM antibody as a diagnostic parameter does not have an advantage over those of specific IgG antibody. However, this IgM antibody might be useful in the case of patients with first infection or those residing in the non-endemic areas. therefore, our methods were developed to detect *B. pseudomallei* specific immunoglobulins (IgG, IgA and IgM) whereas those reported by other investigators detect either IgG or IgM (Anuntagool *et al*, 1993; Ashdown *et al*, 1989; Kunakorn *et al*, 1990). This could answer the question why the sensitivity of our ELISA and dot immunoassay are higher than those reported by Ashdown *et al* (1989) and Anuntagool *et al* (1993) for their IgG-ELISA. Although these authors used cell-sonicate antigens, their higher ELISA specificity value may be due to the employment of sera from unrelated disorders in non-melioidosis specimens and from non-endemic areas in the control group.

Kunakorn and associates (Kunakorn *et al*, 1990) evaluated the used of protein-free culture-filtrated in IHA. The sensitivity and specificity were 88% and 97.4%, respectively. The antigen used in their IHA is a polysaccharide component though to be thymus independent and capable of activating B-cells to produce only IgM antibody (Ashdown, 1987). Kunakorn *et al* (1991) demonstrated the combination of IgM gold blot and protein A gold blot with crude sonicated antigens in serodiagnosis of melioidosis. Their results in specificity (94.3%) were lower than our methods. Moreover, their methods were more expensive to perform. Recently, Petkanjanapong *et al* (1992) developed IgG-ELISA using endotoxin as antigen. Their IgG-ELISA gave comparable sensitivity with our dot immunoassay, nevertheless our dot immunoassay gave greater specific and accuracy. Since the antigen dot nitrocellulose in the dot immunoassay can be kept at -20°C, it makes this assay more rapid with results easily obtained by the naked eye within 3 hours. Moreover, the technique is very simple and can be performed even with 1 specimen or a large quantity of specimens at a time. The rapidity and simplicity of our dot immunoassay make it possible to use in septicemic cases routinely or in epidemiological stydies and even during emergencies in hospitals lacking special equipment.

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