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Retrospective Study on the Diagnostic Value of IgG ELISA, Dot Immunoassay and Indirect Hemagglutination in Septicemic Melioidosis

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SUMMARY **Three serological methods for diagnosis of melioidosis were compared with the culture method currently used as the "gold standard". The diagnostic values of the serological methods were evaluated retrospectively in 306 patients residing in an endemic area. The enzyme-linked immunosorbent assay (ELISA), using affinity purified antigen for detecting specific IgG antibody, showed a slightly higher specificity (86.0%) than the dot immunoassay (DOT) (84.0%) and both were superior to indirect hemagglutination (IHA) (72.0%). The sensitivity of DOT (96.4%) and ELISA (85.7%) were considerably higher than that of IHA (50.0%). The primary benefit of the high negative predictive value of both ELISA (96.4%) and DOT (99.0%) in an area of high prevalence is the ability to rule out most of the non-melioidosis patients.**

Melioidosis is a disease caused by *Burkholderia pseudomallei* and it is endemic in Southeast Asia and Northern Australia. The signs and symptoms of the disease vary considerably from fatal septicemia to latent and chronic localized infection.[1,2] The mortality rate of acute septicemia during the first 48 hours after admission can be high.[3,4] In these patients, the bacteria have disseminated to various organs and are difficult to differentiate from other bacterial infections. Antibiotics given to patients with suspected melioidosis are relatively costly especially considering the potential number of cases in an endemic area. Thus, a rapid and specific diagnosis should be made to enable accurate and successful treatment.[3]

The isolation and identification of the infective organism using the conventional bacterial culture remains the standard protocol. However, this approach is too slow for critically ill patients. Several immunological methods have been developed and used for the detection of either *B. pseudomallei* antibodies or antigens in clinical specimens.[5-12] The detection of antigens in clinical specimens of urine, pus, wound material, sputum and throat swabs has been reported.[7,13] These assays have not been done using blood samples of septicemic cases perhaps because of their low sensitivity and because of interference from other immune complexes (unpublished observation). For the purpose of antibody detection, an indirect hemagglutination (IHA),[9] enzyme-linked immunosorbent assay (ELISA)[5,8,10] and dot immunoassay[5] (DOT) were developed. The antigens used by these methods varied from lipopolysaccharide (LPS) to specific antigens such as 200-kDa surface antigens[14] and a 40-kDa protein,[15] respectively. Although IHAs are still being widely used in endemic areas, numerous false positive and negative results have been documented. We reported the use of a culture filtrated antigen

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in DOT for the detection of *B. pseudomallei* specific antibodies. The sensitivity and specificity of the test were found to be as high as 94.1% and 99.2%, respectively.[5] Rugdech *et al.*[16] purified and characterized a 200-kDa surface antigen by affinity chromatography. This antigen was used in an indirect ELISA for the detection of specific IgG antibody and gave highly satisfactory results.[16]

PCR-based procedures for the detection of *B. pseudomallei* have been described.[17-20] The methods were based on either 23S rRNA[18], 16S rRNA[20], the junction between 16S and 23S rRNA[19] or a specific DNA probe.[21] The performance of these PCR-based procedures on clinical specimens was unsatisfactory,[22] although Haase *et al.*[23] reported a 100% sensitivity of the 16S rRNA primer set. Therefore the conventional immunological methods are still widely used. Moreover, the PCR machine and reagents are quite expensive and the PCR set up still requires experienced technicians making it unsuitable for developing countries. The serological diagnosis is, therefore, of most interest. Sirisinha *et al.*[24] performed a double-blinded multi-center comparative study of various serological methods for the detection of both antigens and antibodies in real clinical settings. None of the serological tests gave satisfactory results when compared with the culture method and all of the tests performed had specificity or sensitivity below the acceptable standard. However, this may have been due to some undefined technical problems in the specimen collection and blood culture identification. We then tried again by choosing IHA, IgG ELISA using affinity-purified antigen and DOT to perform this retrospective (3.5 years) study to evaluate their diagnostic value in 306 sera collected in an actual clinical situation in an endemic area.

MATERIALS AND METHODS

Collection and preparation of clinical specimens

The study was performed with specimens from patients with suspected bacterial sepsis (not only melioidosis) admitted to Srinagarind Hospital, Khon Kaen, Thailand, between June, 1993 and December, 1996. Sera were collected routinely for laboratory diagnosis and left over specimens were then kept in the serum bank. Excluded from the study were sera from which a complete and conclusive diagnosis was not available. Samples that were collected after the day of admission were also excluded. Altogether 306 serum samples fit these criteria and were used to retrospectively detect specific antibodies against *B. pseudomallei* by IHA, IgG ELISA and DOT.

Culture for *B. pseudomallei*

The blood culture data were obtained from the hospital's routine hemoculture laboratory which used an automatic culture system (Bactec 9240, Sparks, Md, USA) and conventional biochemical tests.

Enzyme-linked immunosorbent assay (ELISA) for detecting specific IgG antibody using affinity-purified antigen

Indirect ELISA was performed to detect the specific IgG antibodies to *B. pseudomallei* using immunoaffinity-purified antigen.[8] The microtiter plates (Immunoplate II, Nunc, Roskilde, Denmark) were coated with 0.5 μg/ml of the antigen and incubated for 1 hour at 30°C. Serum samples, diluted to 1:2,000 in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA) and 0.05% Tween 20, were added to the microtiter plates. After the plates were washed with saline containing 0.05% Tween 20, a 1:1,000 dilution of horseradish peroxidase-conjugated rabbit anti-human IgG (γ-chain specific; Dakopatts, Copenhagen, Denmark) was added. The color was developed by using o-phenylenediamine (0.1 mg/ml) as substrate. Incubation was at room temperature for 40 minutes, the reaction was stopped with 5 N H_2SO_4, and the absorbance value at a wavelength of 490 nm was determined. A cut-off optical density (OD) value of > 0.170 represented the mean + 3 SD of the values obtained from healthy individuals in the endemic area of northeastern Thailand.

Dot immunoassay (DOT)

Fifty microliters of 2.5 μg/ml *B. pseudomallei* 40 kDa culture-filtrated protein antigen[5] was applied onto a nitrocellulose membrane using a 96-well filtration manifold (Gibco BRL, MD) under an optimally adjusted vacuum. After washing, 2% skim milk in 20 mM Tris buffered saline, pH 7.5 (TBS) was used for blocking and then 50 μl of 1:4,000 diluted patient serum samples in 1% skim milk-TBS were added in duplicates. The samples were incubated at room temperature for 60 minutes. After washing, the horseradish peroxidase-conjugated rabbit antibody to human IgG, IgA, IgM, kappa and lambda (Dakopatts) diluted 1:4,000 in 1% skim milk-TBS, was applied and incubated for another 60 minutes. Color was developed by addition of 35 mg of 4-chloro-1-naph-

Table 1 Serological results when compared with the culture method

Culture results	Number of positives by		
	IgG ELISA	DOT	IHA
B. pseudomallei (n = 56)	48	54	28
Other microorganisms (n = 62)	11	13	20
No growth (n = 188)	24	27	50

Table 2 Serological and culture results of 62 patients infected with other microorganisms in the study

Hemoculture results	Number of patients	Number of positives by		
		IgG ELISA	DOT	IHA
Gram-positives				
Staphylococcus aureus	24	4	7	10
Streptococcus pneumoniae	1	1	1	1
Streptococcus (groups B and D)	2	1	1	1
Bacillus spp.	6	2	2	4
Gram-negatives				
Escherichia coli	3	1	1	1
Klebsiella pneumoniae	2	0	0	0
Klebsiella spp.	1	0	0	0
Pseudomonas aeruginosa	3	0	0	0
Pseudomonas fluorescense	1	0	0	0
Pseudomonas spp.	1	0	0	0
Acinetobacter sorbia	1	0	0	0
Acinetobacter hydrophilia	1	0	0	0
Acinetobacter anitratus	3	1	0	0
Vibrio parahaemolyticus	1	0	0	0
Salmonella (group D)	6	0	0	0
Other: Yeast	3	0	0	0
Mix infections	3	1	1	3
Total	62	11	13	20

Table 3 Sensitivity, specificity and positive and negative predictive values of IgG ELISA, DOT and IHA in the study

Tests	Sensitivity (%)	Specificity (%)	Predictive values (%)	
			Positive	Negative
IgG ELISA	85.7	86.0	57.8	96.4
DOT	96.4	84.0	57.4	99.0
IHA	50.0	72.0	28.6	86.5

thol (Sigma, Mo, USA) in 7 ml of absolute methanol mixed with 20 µl of 30% H_2O_2 in 63 ml of TBS. A positive result was indicated by the deposition of a purple dot.

Indirect hemagglutination (IHA)

IHA test was performed using culture-filtrated *B. pseudomallei* (melioidin)[9] as the antigen. 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 the two-fold diluted serum specimens (1:10 - 1:10,240) was incubated with the sensitized erythrocytes for 2 hours at 37°C. The cut-off level was set at 1:160.

RESULTS

Positive hemoculture for *B. pseudomallei* were found in 56 out of 306 bacterial sepsis serum specimens obtained from the serum bank, 188 were culture negative and 62 were positive for other microorganisms (Table 1). Of the 56 culture proven samples, only 2 were found to be negative by DOT, whereas 8 and 28 were negative by IgG ELISA and IHA, respectively. IgG ELISA gave 35 false positive results in the rest of the 250 control patients while DOT and IHA gave 40 and 70 false positives, respectively. Of the 62 patients with other infections, IgG ELISA also gave the best specificity (11/62) whereas DOT and IHA gave 13 and 20 false positives, respectively (Table 1). Approximately 72 to 85% of the false positives by IgG ELISA and DOT were samples from patients with gram positive bacterial infections with more than half being *Staphylococcus aureus*. (8/11 in IgG ELISA and 11/13 in DOT) (Table 2). The comparative results

demonstrated that DOT is the most sensitive (96.4%) while IgG ELISA gave the best specificity (86.0%). IHA is far behind in both specificity and sensitivity (Table 3).

DISCUSSION

Melioidosis is an important public health problem in Southeast Asia and Northern Australia. The mortality rate in an acute septicemic infection of inappropriately treated cases can be as high as 80 to 90%. The clinical signs and symptoms of septicemic melioidosis cannot be differentiated from similar conditions caused by other organisms. Rapid and specific laboratory diagnosis is thus extremely critical for the management of patients. In this study, the detection of the specific IgG antibody by ELISA was the most specific while the DOT was found to be the most sensitive (Table 3). The higher sensitivity of the DOT was due to the detection of all isotypes of the antibodies while ELISA detected only IgG antibodies. The different antigens used by these methods may have influenced our findings. The antigen used in the IgG ELISA was the affinity-purified 200-kDa exopolysaccharide[14] whereas in the DOT and IHA, we used a 40-kDa protein[15] and carbohydrate, respectively. The data obtained from the 62 patients whose hemocultures were positive for other microorganisms confirmed the greater specificity for IgG by ELISA. The positive results of IgG ELISA and DOT in patients with gram-positive bacterial infections are most likely to be true positives caused by previous exposure to *B. pseudomallei* as these samples were positive by more than one serological test. However, the possibility of a mixed infection in which the gram-positive organisms had overgrown the *B. pseudomallei* cannot be ruled out. The sensitivity and specificity of IgG ELISA (85.7% and 86.0%) and the sensitivity of the DOT (96.4%) were not very different from previous reports[5,16] (81% and 90% of IgG ELISA and 94.1% of DOT). Nevertheless, the specificity of DOT was much lower. This may reflect the real clinical situation used in this study compared to previous reports[5]. A few sera in which all serological tests gave positive results produced a negative culture. In these cases, the number of bacterial cells in the patients' blood may have been too low for culture identification. In the case of IHA, the present results showed lower sensitivity and higher specificity than previously reported. This might be due to the use of a 1:160 cut-off titer instead of a 1:80.

In summary, the results obtained in this study indicate the usefulness of IgG ELISA and DOT over the IHA in the diagnosis of melioidosis. The high negative predictive value of both IgG ELISA (96.4%) and DOT (99%) can be used to rule out nearly all cases of non-melioidosis patients among bacterial sepsis cases in the endemic areas.

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