COMPARISON OF THE POLYMERASE CHAIN REACTION AND SEROLOGIC TESTS FOR DIAGNOSIS OF SEPTICEMIC MELIOIDOSIS

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Abstract. For diagnosis of melioidosis, we compared polymerase chain reaction (PCR)-based DNA detection and three serologic methods with the culture method currently used as gold standard. The diagnostic values of the serologic methods were evaluated in 130 patients. All these patients resided in an endemic area. An enzyme-linked immunosorbent assay (ELISA) gave slightly higher specificity (86.2%) than a dot immunoassay (DOT) (85.3%), but was superior to an indirect hemagglutination assay (IHA) (79.8%). The sensitivities of the DOT (85.7%) and ELISA (71.4%) were considerably higher than that of IHA (61.9%). However, the PCR was the most sensitive (95.2%) and specific (91.7%). Nevertheless, DOT and ELISA are more practical for local hospitals. With the high negative predictive value of both the ELISA (94.0%) and DOT (96.9%) in a high prevalence area, clearly these methods can rule out most of the non-melioidosis patients.

INTRODUCTION

Melioidosis, an important infectious disease caused by Burkholderia pseudomallei, is known to be endemic in parts of Southeast Asia and northern Australia. The signs and symptoms of this disease resemble those of many other bacterial infections. In septicemic melioidosis, the mortality rate during the first 48 hr after admission can be as high as 80–90%. These septicemic patients show dissemination of the bacteria to various organs. Therefore, a rapid and sensitive diagnosis is needed since treatment with appropriate antibiotics can reduce the mortality by half. Currently, the isolation and identification of the causative agent by the conventional bacterial culture and biochemical tests remains the gold standard for definitive diagnosis. However, misidentification of B. pseudomallei using the API 20NE test has been reported. Antibody detection, an indirect hemagglutination assay (IHA), an enzyme-linked immunosorbent assay (ELISA), and a dot immunoassay (DOT) have been developed.

The PCR amplification of B. pseudomallei DNA by the polymerase chain reaction (PCR) is very sensitive and relies on a present infection. However, a more recent report by Haase and others using a 16S rRNA primer set showed that it performed poorly in an actual clinical setting.

This work is the first report on a comparison between the PCR, serological and culture methods for the diagnosis of melioidosis using samples collected in an actual clinical situation in an endemic area.

MATERIALS AND METHODS

Collection and preparation of clinical specimens. The study was performed with 130 patients admitted to Srinarind Hospital during the period from January, 1997 through August, 1998 with suspected bacterial sepsis. Their sera were collected on the first day of admission and used to detect the bacterial DNA and specific antibodies by PCR, IHA, ELISA, and DOT respectively. For the PCR, 1 ml of heparinized blood was centrifuged (12,000 × g for 5 min). The red blood cells were lysed by the addition of 1 ml of sterile distilled water, vortexed, and centrifuged at 12,000 × g for 5 min. The pellet was washed twice with TE buffer (10 mM Tris-HCl, 10 mM EDTA, pH 8.0), resuspended in 10 μl of TE, and boiled for 10 min. Prior to the PCR, the samples were centrifuged at 12,000 × g for 5 sec and 5 μl of the supernatant was used for amplification. The serum samples obtained were kept frozen at −20°C and subjected to testing by the ELISA, DOT, and IHA.

Culture for B. pseudomallei. The blood samples were subjected to routine hemoculture using an automatic culture system (Bactec 9240, Becton Dickinson and Company, Sparks, MD) and conventional biochemical tests.

Polymerase chain reaction. The PCR amplification of B. pseudomallei DNA was performed and interpreted as described previously. The PCRs were performed using a DNA thermal cycles (2400; Perkin-Elmer, Norwalk, CT). The reactions were carried out in a 50-μl volume containing PCR buffer (20 mM Tris-HCl, pH 8.4, 4 mM MgCl2, and 50 mM KCl), 50 μM of each dNTP, 1 μM of each primer (LPS1; 5'-CTTCTCA-GATTGCTGCAAACCC-3' and LPS2; 5'-CGGATGACA-CTCGAATCCACCG-3'), 5 μl of sample, and 1 unit of Taq
DNA polymerase. The template DNA was initially denatured at 94°C for 5 min. The amplification procedure was composed of 40 cycles at 94°C for 30 sec, 75°C for 30 sec, and 72°C for 45 sec. Amplified products were analyzed by electrophoresis on a 2% agarose gel and were visualized by staining with ethidium bromide. The positive product was confirmed by hybridization with a Digoxigenin-11-dUTP (Roche Diagnostics, Germany) labeled 23LPS \( B. \) pseudomallei specific DNA probe.21 The samples that showed negative results were tested again in another PCR system. The primers used were IGF1 (5’-GGGCAGGCAGCATTGGATTGG-3’) and IGF2 (5’-AGA-GTGTGTTGGGTGGGAGGG-3’), which amplify a 165-base pair sequence in human chromosome 12.22

**Serology for \( B. \) pseudomallei.** Three serologic tests were evaluated with the sera from suspected septicemic patients. These included an ELISA, DOT, and IHA that have been described in detail in previous reports.

**Enzyme-linked immunosorbent assay.** An indirect ELISA was performed to detect specific IgG antibodies to \( B. \) pseudomallei using immunoaffinity-purified antigen.11 Sera samples diluted 1:2,000 in phosphate-buffered saline with 1% bovine serum albumin and 0.05% Tween 20 were added to the microtiter plates (Immunoplate II; Nunc, Roskilde, Denmark) coated with 0.5 \( \mu \)g/ml of the antigen and incubated for 1 hr at 30°C. The plates were then washed with saline containing 0.05% Tween 20 and a 1:1,000 dilution of horseradish peroxidase-conjugated rabbit anti-human IgG (\( \gamma \) chain specific; Dakopatts, Copenhagen, Denmark), was added. The color was developed by using o-phenylenediamine (0.1 mg/ml) as substrate. Samples were incubated room temperature for 40 min. The reaction was stopped with 5 N \( \text{H}_2\text{SO}_4 \) and the absorbance value at a wavelength of 490 nm was determined. A cut-off optical density value > 0.170 represented the mean + 3 SD of the values obtained from healthy individuals in the endemic area in the northern part of Thailand.

**Dot immunoassay.** Fifty microliters (2.5 \( \mu \)g/ml) of \( B. \) pseudomallei antigen9 was applied to a nitrocellulose membrane with a 96-well filtration manifold (Gibco-BRL, Gaithersburg, MD) under an adjusted vacuum. The membrane was then washed with phosphate-buffered saline, pH 7.2 (PBS), blocked with 2% skim milk in 20 mM Tris buffer saline, pH 7.5 (TBS), and 50 \( \mu \)l of 1:4,000 diluted serum samples in 1% skim milk-TBS were added in duplicate. The samples were incubated at room temperature for 60 min. The samples were washed with PBS and horseradish peroxidase-conjugated rabbit antibodies to human IgG, IgA, and IgM, (\( \kappa \) and \( \lambda \) chain specific; Dakopatts) diluted 1:4,000 in 1% skim milk-TBS were added and incubated for 60 min at room temperature. The color was developed by the addition of 35 mg of 4-chloro-1-naphthol (Sigma, St. Louis, MO) in 7 ml of absolute methanol mixed with 20 \( \mu \)l of 30% \( \text{H}_2\text{O}_2 \) in 63 ml of TBS. A positive signal was indicated by the development of a purple dot.

**Indirect hemagglutination assay.** The IHA test was performed using \( B. \) pseudomallei culture filtrate (meliodin)\(^\text{9}\) as antigen. Serum specimens were incubated 56°C for 30 min and adsorbed with saline-washed non-sensitized sheep erythrocytes at room temperature for 30 min before testing. Each of two-fold diluted serum specimens (1:10–1:10,240) was incubated with the sensitized erythrocytes for 2 hr at 37°C. The cut-off level was set at 1:160.

**RESULTS**

The main objective of the study was to compare the performance of a PCR and three serologic methods in a real clinical setting using conventional culture as the gold standard. One hundred thirty patients with suspected septicemia admitted to Srinagarind Hospital, from January 1997 through August 1998 were included. Of these, 21 were found to be culture positive for \( B. \) pseudomallei, 70 were culture negative, and 39 were culture positive for other microorganisms (Table 1). In the 21 culture-positive patients, the PCR showed 20 positive results while the ELISA, DOT, and IHA showed 15, 18, and 13 positive results, respectively. When the 39 samples from patients infected by other microorganisms were analyzed, the ELISA and PCR showed only two positive results while the DOT and IHA showed three and seven positive results, respectively (Table 1). The false-positive results of PCR were samples from patients with \( \text{Acinetobacter anitratus} \) (1 of 2) and \( \text{Candida} \) sp. (1 of 2) whereas most of the false positive in ELISA and DOT were from gram positive infections (2 of 2 in ELISA) and (2 of 3 in DOT) respectively (data not shown). The specificity, sensitivity, and positive and negative predictive values of these tests as summarized in Table 2, demonstrated that PCR is the most sensitive (95.2%) and specific (91.7%) test for diagnosis of melioidosis.

**DISCUSSION**

Melioidosis is an important public health problem in Southeast Asia and northern Australia. In an acute septicaemic infection, the mortality rate in untreated cases can be as high as 80–90%, with the majority of deaths occurring 24–48 hr after onset. A delay in diagnosis may lead to this high

**TABLE 1**

<table>
<thead>
<tr>
<th>Culture results (n = 130)</th>
<th>Number positive by</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR ELISA DOT IHA</td>
<td></td>
</tr>
<tr>
<td>( B. ) pseudomallei (n = 21)</td>
<td>20 15 18 13</td>
</tr>
<tr>
<td>Other microorganisms (n = 39)</td>
<td>2 2 3 7</td>
</tr>
<tr>
<td>Negative (n = 70)</td>
<td>2 13 15</td>
</tr>
</tbody>
</table>

* PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay; DOT = dot immunoassay; IHA = indirect hemagglutination assay. \( B. \) = \( \text{Burkholderia} \).
† The differences in positivity between the PCR and the ELISA (\( P = 0.28 \)) and the PCR and the DOT (\( P = 0.42 \)) were not statistically significant (by \( t \) test).

**TABLE 2**

<table>
<thead>
<tr>
<th>Tests</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>Predictive values (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR</td>
<td>95.2</td>
<td>91.7</td>
<td>69.0</td>
</tr>
<tr>
<td>ELISA</td>
<td>71.4</td>
<td>86.2</td>
<td>50.0</td>
</tr>
<tr>
<td>DOT</td>
<td>85.7</td>
<td>85.3</td>
<td>52.9</td>
</tr>
<tr>
<td>IHA</td>
<td>61.9</td>
<td>79.8</td>
<td>37.1</td>
</tr>
</tbody>
</table>

* For definitions of abbreviations, see Table 1.
mortality rate. Rapid and specific diagnosis is thus extremely critical for the management of patients. Definitive diagnosis is dependent on the isolation and identification of the causative agent from clinical specimens. Several serological methods for the detection of the antibody specific to B. pseudomallei have been developed.\(^\text{[8,9,11,13,14]}\) However, the detection of antibody is of limited use in endemic areas since the majority of the population already may have been exposed to the organism.\(^\text{[15]}\) Although methods for antigen detection in urine, sputum, pleural fluid, and pus have been developed,\(^\text{[10,18,19]}\) none of them were used with serum samples. This might be due to the low sensitivity and unsuccessful use of the method in the patients’ sera (Wongratanacheewin, S., and others, unpublished data). Several PCR-based procedures for the detection of B. pseudomallei have been described,\(^\text{[2,25–27]}\) and one method, based on a specific DNA probe, was found to give the highest specificity.\(^\text{[28]}\) To determine the most reliable and practical method for the diagnosis of melioidosis in an endemic area in an actual clinical situation, the PCR, IgG ELISA, DOT, and IHA were selected for this comparative study.

IgG ELISA, DOT, and IHA were evaluated in 130 patients with suspected septicemia. The detection of the specific IgG antibody by ELISA was the most specific while the DOT was found to be the most sensitive test. The slightly higher sensitivity of the DOT may be related to the fact that it detected total antibody (IgG, IgA, and IgM), whereas the ELISA and IHA detected either IgG or IgM, respectively. However, the different antigens used in these methods may also influence the test results. The antigen used in the ELISA was shown to be a high molecular weight (200 kD) exopolysaccharide,\(^\text{[27]}\) whereas in the DOT and IHA, the antigens were a protein and carbohydrate, respectively.\(^\text{[8,13]}\) The ELISA and DOT were also used to detect the antibodies in 23 patients with localized melioidosis. The result showed that the ELISA and DOT had the same sensitivity (19 of 23 = 82.6%) (data not shown). This data suggested that patients with localized melioidosis develop specific antibodies in sera that could be detected by these methods. In the case of the standard IHA, the present results showed lower sensitivity and higher specificity than those previously reported.\(^\text{[8]}\) This might be due to the use of 1:160 cut-off titer instead of 1:80. Evaluation of these serologic tests in the present study clearly shows that the IHA is definitely inferior to the other two serologic tests and should not be used in the diagnosis of melioidosis. This is important because the IHA is currently used in most diagnostic laboratories.

The PCR was found to be more sensitive and specific when compared with the serologic tests. The positive diagnosis of serologic methods in other microbial-infected cases (two by the ELISA and three by the DOT) (Table 1) may result from the antibody that persisted from previous infections because the purified antigen used in the ELISA had never been demonstrated to have cross-reacted with other bacteria and the PCR results were negative in this study. Since the criterion for positive results in the study was the presence of B. pseudomallei in blood samples, five patients with positive cultures for swab, sputum, or urine specimens could therefore be identified as negative (Wongratanacheewin S and others, unpublished data). This may explain the slightly lower specificity of the serologic tests. Moreover, there were three culture-negative patients who were positive by all serologic tests and the PCR. These patients were clinically diagnosed as having melioidosis since they responded well to the antibiotic regimens used for the treatment of melioidosis. This observation confirmed the previous report that patients with melioidosis may have a circulating B. pseudomallei in the blood below the threshold value of the culture method.\(^\text{[29]}\)

A recent report by Haase and others\(^\text{[20]}\) using a 16S rRNA primer set for a nested PCR for buffy coat specimens showed 10 PCR-positive cases among 30 non-melioidosis patients. This result led them to conclude that the PCR still presents difficulties in the diagnosis of melioidosis. The nested PCR is not ideal for clinical application due to the high propensity of contamination from carryover products of the first to the second round and the increased time and complexity of the method. Our PCR system showed only 8.3% (9 of 109) false-positive results. The PCR diagnosis using only one pair of primers as presented in this study is therefore a better system for a rapid diagnosis of septicemic melioidosis. There are a number of advantages of PCR diagnosis when compared with the serologic tests and the culture method. The PCR diagnosis is more rapid and sensitive than the latter. Nevertheless, it should be kept in mind that the presence of circulating B. pseudomallei in healthy people has been reported (Sirisantha J and others, unpublished data). A positive result in the PCR can also be related to the persistent presence of viable and/or nonviable bacteria in the macrophages of latent infected persons who are not clinically diagnosed as being infected with B. pseudomallei. This may result in negative hemocultures and positive results in the PCR when the bacteria cells were detected by template preparation techniques. Moreover, the apparatus used in the PCR is currently available only in the regional hospital and some provincial hospitals in the endemic area of northeastern Thailand. Also, the time needed for transportation of specimens from small rural health center to the regional hospital must be considered.

In summary, the results obtained in this study indicate the usefulness and some advantages of the PCR versus serologic tests in the diagnosis of septicemic melioidosis. Because of the difficulties in an actual clinical application of the PCR, the serologic tests (IgG ELISA and DOT) still can be used as effectively as conventional methods, especially in a developing country. With the high negative predictive value of both the ELISA (94.0%) and the DOT (96.9%) in an area of high disease prevalence, clearly these methods can rule out most of the non-melioidosis patients. Very recently, we have reported on the use of monoclonal antibody–based latex agglutination for the detection of B. pseudomallei in hemocultures of community-acquired septicemic patients. A combined use of culture and immunologic methods can thus reduce the 2–7 days commonly used for the culture method to less than 30 hr.\(^\text{[29]}\)

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PCR AND SEROLOGIC TESTS FOR DIAGNOSIS OF MELIOIDOSIS

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