การประเมินชุดทดสอบเพื่อตรวจวินิจฉัยโรคเมลิโอติส
แบบรวดเร็วโดยใช้ crude culture filtrate แอนดีเจน
ของเชื้อ Burkholderia pseudomallei

อุเทน รุ้งพานิชย์¹  สุกิจณ์ยา พงษ์สัจจ์² และ ปั๋ทนภา เอกโพธิ์³

¹ ภาควิชาวิทยาภูมิภูมิ คณะแพทยศาสตร์ศิริราชพยาบาล มหาวิทยาลัยมหิดล
² ภาควิชานุเคราะห์ คณะแพทยศาสตร์ มหาวิทยาลัยอัสสัมชัญ

บทคัดย่อ

โรคเมลิโอติส เป็นโรคดื้อที่มีสาเหตุมาจากเชื้อแบคทีเรีย Burkholderia pseudomallei
ซึ่งเป็นโรคดื้อดื้อที่เป็นสาเหตุสูงสุดของภาวะเจ็บป่วยและเสียชีวิตในประเทศไทย
ประเภทต่าง ๆ ในแอน
เชื้อสร้างความเสี่ยงได้ และอาจทำให้จำเป็นต้องตรวจทดสอบเพื่อ
ต้องปฏิบัติการตรวจการหายเกินข้อต่อส่งต่อ
ซึ่งต้องมีการร่วมวิจัย
ให้การประเมินชุดทดสอบแบบรวดเร็วสำหรับวินิจฉัยโรคเมลิโอติสที่ได้พัฒนาขึ้นใหม่ โดยใช้หลักการ indirect enzyme linked-immunosorbent assay (ELISA) และ latex agglutination (LA) โดย
ใช้ crude culture filtrate แอนดีเจน เพื่อตรวจสอบแอนิเดนท์ที่มักจะดื้อ B. pseudomallei ใน
ชิ้นของผู้ป่วย โดยได้ทำการทดสอบด้วยยี่ชีวิตตัวละ 187 ตัวอย่าง จำแนกออกเป็นตัวอย่างซึ่งมาจาก
ผู้ป่วยโรคเมลิโอติส ระยะแรกพบ จำนวน 82 ตัวอย่าง ตัวอย่างซึ่งมาจากผู้ป่วยโรคดื้อ
แบบที่เรียกชื้อต่อม ๆ ระยะเดียวกัน จำนวน 51 ตัวอย่าง และตัวอย่างซึ่งมาจากเอดอกท์ที่มีสุขภาพดี
ซึ่งอาจต้องอยู่ในบริเวณที่มีภาวะระบาดของโรค จำนวน 54 ตัวอย่าง จากการบริเวณที่เก็บกล่องเฉพาะสิ่ง
เชื้อซึ่งเป็นวิธีการมาตรฐานวัดความ indirect ELISA มีความไว (sensitivity) 82.93% ความถูกต้อง
(specificity) 88.57%, positive predictive value 85.00% และ negative predictive value 86.92% สำหรับวิธี latex agglutination มีความไว 82.93%, ความถูกต้อง 70.48%, positive
predictive value 68.69% และ negative predictive value 84.09% จากการศึกษาพบว่า การ
ตรวจตนเองดื้นั้นที่จำเป็นต้องใช้ B. pseudomallei โดยใช้ crude culture filtrate แอนดีเจน
สามารถพัฒนาเป็นชุดทดสอบแบบรวดเร็วสำหรับวินิจฉัยโรคเมลิโอติสในพื้นที่มีการระบาดของโรคได้
Evaluation of Crude Culture Filtrate Antigen from *Burkholderia pseudomallei* for Rapid Diagnosis of Melioidosis

Utane Rungpanich¹, Supinya Pongsunk² and Pattama Ekpo¹

¹Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.
²Department of Microbiology, Faculty of Medicine, Srinakharinwirot University, Prasanmitr Campus, Sukhumvit 23, Bangkok, Thailand.

Abstract

Melioidosis is an infection caused by *Burkholderia pseudomallei*. The disease is an important cause of morbidity and mortality in Thailand, other countries in Southeast Asia, and northern Australia. The current method for the diagnosis of melioidosis is based on bacteriological culture. However, the method is time-consuming. In this study, we evaluated the use of an indirect enzyme-linked-immunosorbent assay (ELISA) and latex agglutination (LA) using crude culture filtrate antigen for detecting specific *Burkholderia pseudomallei* antibodies in patients’ sera. A total of 187 sera were tested. These samples comprised of 82 acute phase sera from patients with melioidosis, 51 acute phase sera from those with other bacterial infections, and 54 from normal healthy individually living in an endemic area. By using bacterial culture as the gold standard, it was found that, the sensitivity, specificity, positive predictive value and negative predictive value of the indirect ELISA were 82.93%, 88.57%, 85.00% and 86.92%, respectively. For the LA, these values were 82.93%, 70.48%, 68.69% and 84.09%, respectively. Thus, it can be concluded that the specific antibody detection by using the crude filtrate antigen is useful for the rapid diagnosis of melioidosis in an endemic area.

Keywords: Melioidosis, *Burkholderia pseudomallei*, crude culture filtrate antigen, indirect ELISA, latex agglutination

Correspondence: Pattama Ekpo, Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand. Tel.: 66-2418-0569 Fax: 66-2418-1636 E-mail: sipep@mahidol.ac.th
**Introduction**

Melioidosis is an infection caused by *Burkholderia pseudomallei*. The endemic areas are countries in Southeast Asia including Thailand, and northern Australia (Chaowagul et al, 1989). The disease may manifest itself in acute, subacute, and chronic forms (Howe et al, 1971). In the northeastern part of Thailand, the acute form is an important cause of morbidity and mortality. Early diagnosis and appropriate antibiotic treatment are very helpful for reducing the mortality rate of this disease (White et al, 1989). The clinical symptoms of melioidosis cannot be differentiated from septicemia caused by other organisms. The definitive diagnosis of this disease is based on bacteriological isolation and biochemical identification. However, the method is time-consuming, taking 2–6 days, and by the time the result is obtained, it is often too late to be useful. A number of serological tests have been developed, based on various antigen-antibody system, to provide rapid and presumptive evidence of the infection (Ananttagool et al, 1993; Appassakij et al, 1990; Asdown et al, 1998; Dharakul et al, 1997; Desakonn et al, 1994; Ismail et al, 1987; Kunakorn et al, 1990; Leelarasamee et al, 1989; Petkanjanapong et al, 1992; Sermswan et al, 2000; Sirisinha et al, 2000; Wongratanacheewin et al, 1990; Wongratanacheewin et al, 1995). However, the tests which are currently available lack of either specificity or sensitivity. In this study, we have evaluated an indirect ELISA and latex agglutination (LA) methods, using the crude culture filtrate (CCF) antigen prepared from *B. pseudomallei*, for the rapid diagnosis of melioidosis in an endemic area.

**Materials and Methods**

**Bacteria**

The *Burkholderia pseudomallei* strains used in this study were isolated from blood specimens obtained from patients with bacteriologically proven disease admitted to Sappasitprasong Hospital, Ubon Ratchathani and Khonkaen Regional Hospital, Khonkaen, Thailand.

**Preparation of antigen**

Crude culture filtrate (CCF) antigen was prepared from 10 strains of *B. pseudomallei* isolated from blood samples (Pongsunk et al, 1999). The bacterium was grown for 2 weeks in a liter of protein-free broth containing 133.21 mM glycine, 17.61 mM disodium phosphate, 85.56 mM sodium chloride and 11.10 mM dextrose, at 37°C with agitation. The culture was centrifuged for 30 min at 10,000 × g, and the supernatant was filtered through a 0.45 µm pore filter sterilizing unit. The supernatant was then filtered in an ultrafiltration unit (Amicon, USA) fitted with a 10-kDa cutoff membrane. The concentrated supernatant was dialyzed against phosphate buffered saline (PBS), pH 7.4.

The protein concentration of the CCF antigens was determined by using a commercial protein assay kit (BioRad Laboratories,
USA). The antigen was kept at -20°C in small aliquots until used.

Clinical specimens

One hundred and eighty seven sera were obtained from patients, which the diagnosis was based on the results of blood culture and biochemical tests, and blood donors at Sappasitprasong Hospital, Ubon Ratchani and Khonkaen Regional Hospital, Khonkaen, which are in the northeastern part of Thailand. The specimens were divided into 3 groups: 82 and 51 acute phase sera were the specimens obtained from patients with culture proven melioidosis and from those with other bacterial infections, respectively, and 54 normal sera. Of 51 sera from patients with other bacterial infections, 37 sera were from patients with Gram-negative bacterial infections, i.e. Acinetobacter spp., Aeromonas spp., B. cepacia, E. coli, Enterobacter spp., Klebsiella spp., Proteus spp., Pseudomonas spp., and Salmonella spp., and 14 sera were from those with Gram-positive infections, i.e. Bacillus spp., Staphylococcus spp., and Streptococcus spp.

Indirect ELISA for antibody detection

CCF antigen at a concentration of 3 µg/ml in 0.05 M carbonate buffer, pH 9.6 was coated on the 96-well microtiter plate at 4°C overnight. After that, the wells were washed with normal saline solution containing 0.05% Tween (NSST). Then, 100 µl of NSST containing 2% BSA were added to each well and the plate was incubated at 37°C for 1 hour. After incubation, the plate was washed again. One hundred microlitre of patients’ sera at a dilution of 1:200 in 1% BSA- NSST were added to each well in the plate and incubated at 37°C for 1 hour. After washing, 100 µl of biotinilated anti-human IgG (Dakopatts, Denmark) at a dilution of 1:1,000 were added and incubated at 37°C for 1 hour. After washing, 100 µl of 1:1,000 streptavidin conjugated with alkaline phosphatase (Dakopatts) in 1% BSA-NSST were added. The plate was incubated at 37°C for 1 hour and washed again. Finally, the substrate, p-nitrophenylphosphate (BioRad Laboratories), was added and the absorbance value was read at 405 nm. Each sample was assayed in duplicate. Positive and negative reference specimens were always included in each experiment. Direct conjugate control well, in which the buffer was used instead of the specimen, was also included in every plate for providing the data of the extent of non-specific binding to the plate.

Latex agglutination (LA) for antibody detection

The CCF antigen sensitized latex particle was prepared by adsorption 250 µg of CCF antigen onto 500 µl of 0.793 µm sulfonated latex bead (Interfacial Dynamics Corporation, Portland, Oregon, USA) in 0.1 M borate buffer pH 8.5 for overnight. After washing with the borate buffer, the latex beads were blocked with 1% BSA in the borate
buffer for 30 minutes, washed again, and resuspended to 0.5% in storage buffer (0.02 M phosphate buffer, pH 7.4, 0.15 M NaCl, 1% BSA, 5% glycerol, and 0.1% NaN₃). The LA was performed on a glass slide using an equal volume (50 µl) of the 1:4 diluted serum and the latex reagent. The slide was rotated for at least 2 minutes. The result was read visually. Positive and negative reference specimens were always tested in each experiment.

**SDS-PAGE**

The protein profile of CCF antigen was examined by SDS-PAGE in a minigel apparatus (Bio-Rad Laboratories). A 3.5% stacking and a 12% separating acrylamide gel were used. A sample was solubilized under denaturing condition by using sample buffer (0.0625 M Tri-HCl pH 6.8, 1% SDS, 10% glyceral, 5% 2-mercapterethanol and bromphenol blue) and heated in a boiling water bath for 5 min before being loaded onto the gel. The electrophoresis was performed at a constant current of 170 mA, until the bromphenol blue reach the bottom edge of the gel. After that, the separated polypeptide bands were visualized by staining with Coomassie blue R-250.

**Statistical analysis**

Sensitivity, specificity, positive predictive value and negative predictive value of the ELISA and LA test were calculated as follows:

\[
\text{Sensitivity} = \frac{a}{a+b} \\
\text{Specificity} = \frac{d}{c+d} \\
\text{Positive predictive value} = \frac{a}{a+c} \\
\text{Negative predictive value} = \frac{d}{b+d}
\]

Where 

- \(a\) = number of positive ELISA or LA test in specimens positive for \(B. \text{pseudomallei}\) as identified by blood culture and biochemical tests
- \(b\) = number of negative ELISA or LA test in specimens positive for \(B. \text{pseudomallei}\) as identified by blood culture and biochemical tests
- \(c\) = number of positive ELISA or LA tests in negative control specimens
- \(d\) = number of negative ELISA or LA tests in negative control specimens

**Results**

**Characterization of the CCF antigen**

The CCF antigen prepared from 10 blood culture isolates of \(B. \text{pseudomallei}\) was pooled. The protein yield of the antigen prepared from a liter of bacterial culture medium was 1 mg. The SDS-PAGE analysis of the CCF antigen revealed one major polypeptide band with the molecular weight of 30-kDa (Fig. 1). In addition, several minor bands were also revealed but were not shown up clearly in the Fig. 1.
Evaluation of the diagnostic potential of the CCF antigen

Pooled positive and pooled negative sera obtained from patients with melioidosis, and sera from normal healthy individuals were used in establishment of the indirect ELISA and LA. The established ELISA was used for the assay of 82 sera obtained from patients with melioidosis, 51 sera from those with other bacterial infections and 54 sera normal controls. The results are shown in Figure 2. It can be seen that, by using a cutoff level for positive result at 0.4 A<sub>405</sub>, a false positive results were obtained with sera from patients infected with Acinetobacter spp. (1/2), Enterobacter spp. (1/2), E. coli (4/20), Klebsiella spp. (2/8), Salmonella group B (1/1) and Staphylococcus spp. (2/6). Of the 54 normal sera and 82 melioidosis patients’ sera, 1 false positive and 14 false negative results were also observed, respectively. Thus, using blood culture as the gold standard and the cutoff level for positive results of ELISA at 0.4 A<sub>405</sub>, the sensitivity, specificity, positive predicative value and negative predictive value of the test were 82.93%, 88.57%, 85.00%, and 86.92%, respectively. For the LA using 1:4 diluted serum, there was a cross reactivity with sera obtained from patients infected with Acinetobacter spp. (2/2), Bacillus sp. (1/1), Enterobacter spp. (1/2), E. coli (11/20), Klebsiella spp. (2/8), Salmonella group B (1/1), Staphylococcus spp. (3/6), Streptococcus group A (2/4) and Sterptococcus pneumoniae (1/2). Seven of 54 normal sera and 14 of 82 melioidosis patients’ sera also gave positive

Fig. 1  SDS- PAGE profiles of the crude culture filtrate (CCF) of B. pseudomallei
Fig. 2 The use of the indirect ELISA for antibody detection. The three groups are: sera from melioidosis patients; other bacterial infections; and healthy volunteers from the endemic area.
and negative results, respectively. Thus, with the same gold standard, the sensitivity, specificity, positive predictive value and negative predictive value of the LA test were 82.93%, 70.48%, 68.69%, and 84.09%, respectively.

Discussion

The septicemic form of melioidosis which is the most severe form of the disease was found as 60% of all patients with this disease in the northeast of Thailand (Chaowagul W et al, 1989). Rapid diagnosis and appropriate antibiotic treatment are needed to save life of these patients. A number of serological tests for specific antibody to *B. pseudomallei* have been developed (Appassakij et al, 1990; Ashdown et al, 1998; Dharakul et al, 1997; Mathai et al, 2003; Phung et al, 1995; Sermswan et al, 2000; Sirisinha et al, 2000; Wongratanacheewin et al, 1995). Whole cell or crude antigen (Appassakij et al, 1990; Ashdown et al, 1998; Dharakul et al, 1997; Mathai et al, 2003; Sermswan et al, 2000; Sirisinha et al, 2000; Wongratanacheewin et al, 1995) and partially or purified antigen were used in these tests (Dharakul et al, 1997; Phung et al, 1995; Sermswan et al, 2000; Sirisinha et al, 2000).

The use of whole cell or crude antigen is problematic in areas of endemicity, particularly in Thailand, where rates of background seropositivity may be up to 30 to 47% (Khupulsup et al, 1986). The high antibody level in the healthy population in the endemic area, due to subclinical exposure to *B. thailandensis* or *B. pseudomallei* early in life (Anuntagool et al, 1998; Kanaphun et al, 1993), interferes with the interpretation of results (Leelarasamee et al, 1989; Naigowit et al, 1989). Furthermore, the strains used to formulate the antigen are not standardized and there is heterogeneity of LPS, a major component in the whole cell or crude antigen, in difference strains (Anuntagool et al, 2000). The antibodies against atypical LPS in patient’s serum may not react with the LPS in the antigen prepared from the wild type strain (Anuntagool et al, 2000).

Efforts have been made to refine the antigen targets, including refinement of LPS (Sirisinha et al, 2000), glycolipid (Phung et al, 1995), and, 19.5-, 30- (the CCF antigen), 40-, 200- kDa proteins (Dharakul et al, 1997; Sermswan et al, 2000; Sirisinha et al, 2000). The LPS, and, 19.5-, 30-, 40-, 200- kDa proteins antigens were evaluated using the same assay, ELISA, to detect the specific IgG in the acute phase sera obtained from patients with culture proven melioidosis and from those with other bacterial infections (Sirisinha et al, 2000). The sensitivity and specificity of these tests were various from 74 to 82% and 75 to 80%, respectively. The sensitivity and specificity of these tests appeared to be more than the IHA using whole cell antigen. Among these antigens, the 30- kDa protein or CCF antigen
was the best antigen that gave the highest sensitivity (82%) and specificity (80%).

In this study we compared an indirect ELISA and LA using CCF antigen for the detection of the specific antibody in the same group of specimens. It was found that CCF antigen can be used successfully as a diagnostic reagent both in ELISA and LA method. Although, the ELISA showed higher specificity than LA test (88.57% vs. 70.48%), the LA is very simple, cheap, and there are no requirements for any complicated reagents or instruments.

Thus, the CCF antigen has a potential for being a valuable antigen for the diagnosis of melioidosis in an endemic area. However, the improvement of antigen preparation method such as the use of combination of recombinant DNA technology and monoclonal antibody specific to 30-kDa protein (Pongsunk et al, 1999) will be very helpful for the preparation of the purified specific protein antigen and reducing the risk of infection during the antigen preparation and also overcome the disadvantage of using conventional method which gave loss yield of protein.

**Acknowledgements**

We thank Mrs. Nuanchan Piyasangthong, Khonkaen Regional Hospital, Khonkaen, and Miss Nittaya Thirawattanasuk, Sappasitprasong Hospital, Ubon Ratchathani, Thailand, for providing bacterial strains and serum samples.

**References**


