การเตรียมโปรตีนแอนติเจนจากเชื้อ *Burkholderia pseudomallei* และความเป็นไปได้ในการวินิจฉัยโรคเมลลิออยโดสิส

สุภิญญา พงษ์สังข์, วทม. (จุลชีววิทยา)*
ปัทมา เอกโพธิ์, ปร.ด. (จุลชีววิทยา)*

บทคัดย่อ โปรตีนแอนติเจนที่ถูกปล่อยออกนอกตัวเชื้อ *Burkholderia pseudomallei* ผสมอยู่ในอาหารเลี้ยงเชื้อชนิดเหลวที่เตรียมให้ปราศจากโปรตีน ซึ่งใช้ศึกษาในการทดลองนี้ เรียกว่า culture filtrate แอนติเจนที่เตรียมได้ มีความเข้มข้นของโปรตีน วัดได้ 2 มก/มล เมื่อนำโปรตีนแอนติเจนนี้มาใช้ทดสอบหาปริมาณแอนติบอดีในน้ำเหลืองของคนไข้เมลลิออยโดสิส และในคนปกติที่มีถิ่นพำนักอยู่ในบริเวณชุกชุมของโรค พบว่ามีความไว 80 % และความจำเพาะ 90.6 % จากผลการทดลองแสดงให้เห็นว่าแอนติเจนที่เตรียมได้จากการทดลองนี้ มีความเป็นไปได้ที่จะใช้ในการวินิจฉัยโรคเมลลิออยโดสิส

Abstract **Evaluation the possibility of culture filtrate antigen of *Burkholderia pseudomallei* for diagnosis of melioidosis**

Supinya Pongsunk, M.Sc. (Microbiology)*
Pattama Ekpo, Ph.D. (Microbiology)*

The protein concentration of culture filtrate antigen (CF antigen) of *Burkholderia pseudomallei* prepared in this study was 2 mg/ml. The CF antigen was used for detecting the antibody level of melioidosis patients and normal sera from endemic area. The sensitivity and specificity was found to be 80 % and 90.6 % respectively. The study showed that the CF antigen has a potential used in diagnosis of melioidosis.

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Introduction

Melioidosis is the infection caused by the bacterium *Burkholderia pseudomallei*[1]. *Burkholderia pseudomallei* (formerly called *Pseudomonas pseudomallei*) is endemic in northeastern region of Thailand[2]. The organisms are inhabitants in soil, streams and stagnant water[3]. The clinical manifestation of melioidosis is vary from localized to septicemic infection[4]. Early diagnosis is necessary for proper

* ภาควิชาจุลชีววิทยา คณะแพทยศาสตร์ มหาวิทยาลัยศรีนครินทรวิโรฒ
Department of Microbiology, Faculty of Medicine, Srinakharinwirot University.

management because death may occur within 24 to 48 hours after the onset of infection[5].

Definitive diagnosis of melioidosis are based on bacteriological culture and identification. Because the method is time-consuming (at least 3-4 days) and the result is often too late to be useful, a number of serological tests have been developed to provide rapid and presumptive evidence of the infection[2, 5-8]. However, a number of serological tests currently available are lack of specificity in areas of high melioidosis prevalence. The problem is most likely related to the use of crude antigens which cross-react with other bacteria.

The purposes of present study were to prepare the antigen more specific for *Burkholderia pseudomallei* and to explore its potential used in the diagnosis of melioidosis.

Materials and Methods

Preparation of antigen

Crude culture filtrate CF antigen of *B. pseudomallei* was prepared by culture *B. pseudomallei* in protein-free broth (glycine 1 gram, disodium phosphate 0.25 gram, sodium cholride 0.5 gram and dextrose 0.2 gram made to 100 milliliters by distilled water) incubated at 37°C for 2 weeks. The antigen, which solubilized in supernatant was seperated by centrifugation at 10000 xg for 30 minutes at 4°C, then filtered through 0.45 μm membrane filter. The preparation was concentrated by lyophilized and stored at 4°C. Protein concentration of crude culture filtrate antigen was determined by commercial protein assay and using bovine serum albumin as a standard.

Clinical specimens

Serum specimens were collected from 20 patients with hemoculture positive for *B. pseudomallei* and 32 normal sera from endemic area (Khon Kaen Province).

Antibody production

Ten micrograms of crude culture filtrate of *B.pseudomallei* in 100 μl of normal saline solution (NSS) mixed with an equal volume of complete Freund's adjuvant were injected intraperitoneally into mice. Booster by intraveneous injection with the same dose of antigen in NSS. One week later serum samples were collected and stored at -20°C until required for used.

Screening anti-CF antigen antibody of *B. pseudomallei*

Indirect enzyme linked immunosorbent assay (Indirect ELISA) employing crude culture filtrate antigen of *B.pseudomallei* coated microelisa plate was used. Two micrograms per millilitre of CF antigen were diluted in 0.05 M Carbonate buffer (pH 9.6) at 4°C overnight. After washing the plate with 0.05 % V/V Tween 20 in NSS (Tween saline), blocked with 1 % BSA-Tween saline at 37 °C for 1 hour then washed. Patient's sera or normal sera at dilution of 1:200 (in 1 % BSA-Tween saline) was added to the plate and incubated at 37 °C for 1 hour. The plate was washed again and biotinilated anti human immunoglobulin at dilution of 1:40000 was added and incubated at 37 °C for 1 hour, then washed. The 1:40000 avidin conjugated with horseradish peroxidase was added. The plate was incubated at 37 °C for 1 hour and washed. Then the substrate TMB was added and the absorbance value was read at 450 nm. Each sample was performed in duplicate.

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli[9]. The antigen was subjected to SDS-PAGE in 10 % acrylamide gel and 3.5 % stacking gel. After electrophoresis the gel was stained with Coomassie blue for detection of protein.

Results

Characterization of *B. pseudomallei* culture filtrate antigen

The culture filtrate of *B. pseudomallei* was prepared from 500 millilitres of protein-free culture broth. After lyophilization, the CF antigen was resuspended with distilled water to protein concentration of 2 mg/ml. A total volume of this preparation was 2 millilitres. Then, the characteristic pattern of CF antigen was performed by running in SDS-PAGE and stained with Coomassie blue. The results showed only 3 faint bands (too faint to be photographed). Even the little amount of protein antigen presented in the preparation but the immunoreactivity of the antigen with specific antibody raised in mice was strong enough for further study.

Evaluation of the diagnostic potential of *B. pseudomallei* culture filtrate antigen

Mouse polyclonal antiserum raised against CF antigen was used as a positive control in the test system (used anti-mouse immunoglobulin as secondary antibody instead of antihuman immunoglobulin in materials and methods). The results of indirect ELISA are shown in Figure 1. The scattergram presented in fig. 1 shows that cutoff limit at OD 0.2 a large majority of 20 specimens of patients with septicemic melioidosis were positive. On the other hand, only 3 from 32 samples had OD over cutoff value. The sensitivity of the test system was 80 % and the specificity was 90.6 %.

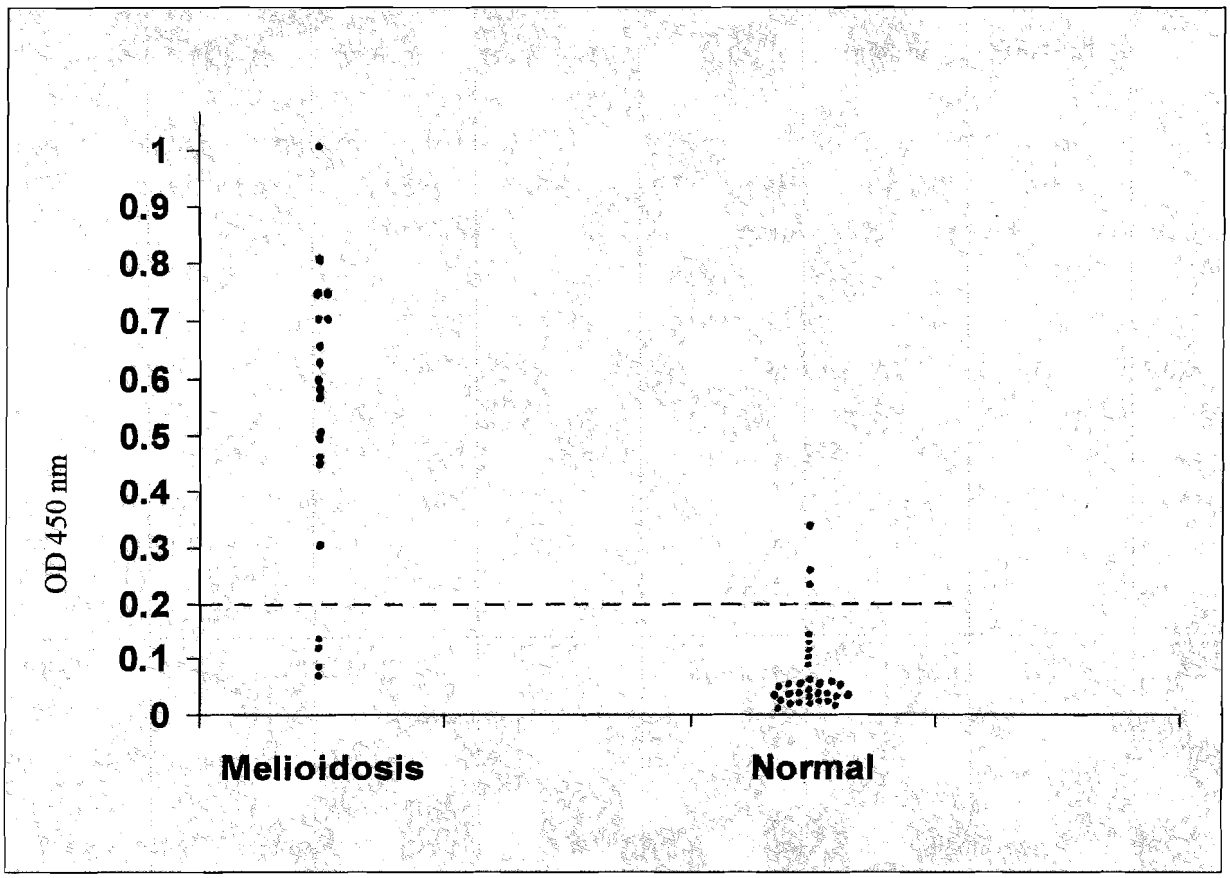

Figure 1 : Scattergram of ELISA. Sera were collected from patients with septicemic melioidosis (20 samples) and normal sera from endemic area (32 samples). Cutoff value is OD 0.2 OD 450 nm ; optical density at 450 nm.

Discussion

The expectation of culture filtrate antigen which shed from the organisms during culture will composed of less complex mixture than crude extract antigen. Thus, this antigen may be more specific. The results presented in this study showed that CF antigen was specific to *B. pseudomallei*, since the preliminary study showed a satisfactory specificity.

Even the CF antigen has a potential used as an antigen for detection of specific antibody in patient's sera, for diagnosis of melioidosis but bacterium are an hazardous agent (the mode of transmission is by inhalation). The specific component purified by the recombinant DNA technology will be safer than the culture methods and a large quantity of the specific antigen can be purified more easily without variation from lot to lot. Thus, the specific protein component will be purified by using the combination of the MAbs and recombinant DNA technology should be explored for the further study.

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