

***Burkholderia pseudomallei*-Specific Recombinant Protein and Its Potential in the Diagnosis of Melioidosis**

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Melioidosis, an infectious disease caused by a gram-negative bacillus, *Burkholderia pseudomallei*, is an important public health problem in Southeast Asia and Northern Australia.¹ The bacteria enter the body through wounds or the respiratory tract and disseminate to all systems,² leading to fatality especially in septicemic melioidosis cases. Wide spectra of clinical manifestations are observed in melioidosis ranging from asymptomatic to septicemic infection.^{3,4} Acute septicemic melioidosis is the most severe form of the disease and is responsible for high morbidity and mortality, making melioidosis the most important cause of death in the northeastern part of Thailand. The clinical spectra of melioidosis overlap with those of other infections and thus clinical diagnosis is rather difficult. The diseases to be considered in differential diagnosis with melioidosis are tuberculosis, malaria, typhoid fever, leptospirosis and septicemia caused by other gram-negative bacteria.⁵

Detection of specific anti-

SUMMARY Melioidosis is an important public health problem in Southeast Asia and Northern Australia. This disease is caused by the gram-negative bacilli, *Burkholderia pseudomallei*. Wide spectra of clinical manifestations are observed in melioidosis ranging from asymptomatic to septicemic infection. Although serodiagnostic methods of melioidosis have been improved significantly in recent years, a highly specific diagnostic test that can differentiate asymptomatic seropositive individuals and melioidosis patients remains to be the subject of current investigations. In this study, a *B. pseudomallei*-specific gene, *pBps-1*, expressing a novel 18.7 kDa recombinant protein was selected from genomic libraries of two *B. pseudomallei* virulent isolates by using pooled sera from septicemic melioidosis patients. Nucleotide sequence analysis demonstrated that this gene is unique and does not show substantial similarity with any known genes in the Genbank database. The *Bps-1* recombinant protein was evaluated for its potential in serodiagnosis of melioidosis by Western blot analysis. A high degree of specificity was demonstrated using sera from healthy individuals in the endemic (98.5%) and non-endemic areas (100%), with moderate sensitivity (69.7%) in melioidosis patients. The study demonstrated that this approach can be used to obtain highly specific recombinant antigens such as that described in the present report. A combination of such antigens should provide materials for successful serodiagnosis of melioidosis in the endemic areas.

bodies has been helpful in the diagnosis of melioidosis. However, the most widely used serological test, i.e. indirect hemagglutination assay (IHA) using melioidin as antigen, is known to have poor specificity.⁶ A variety of serological tests for the detection of antibodies to *B. pseudomallei* have been developed and demonstrated good diagnostic poten-

tials. In a recent study by Sirisinha *et al.*,⁷ comparative evaluations of various crude preparations and purified antigens were carried out

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by using the same panel of sera. The preparations included affinity purified 200-kDa antigen, purified lipopolysaccharide (LPS), crude culture filtrate (CCF) containing 30-kDa antigen, and Bps-1 recombinant antigen. However, these antigens showed only moderate degree of sensitivity or specificity. Among them, the Bps-1 recombinant antigen demonstrated highest specificity (91%) with 70% sensitivity in that study.

Specificity of testing is the major problem in the serological diagnosis of melioidosis due to the high background of the antibody in normal population residing in the endemic area. *B. pseudomallei* is present in the soil in the endemic area and the exposure of inhabitants to the organism is high. A serologic assay using suitable antigen which can differentiate asymptomatic from clinical infection is highly needed.

This study describes a selection and characterization of a *B. pseudomallei*-specific gene obtained from screening of two bacterial genomic libraries by using septicemic melioidosis sera. A phenotype of the gene, i.e. recombinant Bps-1 antigenic protein, was shown to be highly specific to the organism.

MATERIALS AND METHODS

Bacterial isolates

Two clinical isolates of *B. pseudomallei*, i.e. K94050 and K96243, were used in this study. The bacteria were incapable to assimilate arabinose and were classified as the Ara- virulent isolates. They were isolated from blood of patients with septicemic melioidosis from Khon Kaen province, north-eastern Thailand in 1994 and 1996, respectively, and kept in 25%

glycerol at -70°C until use. K96243 is a *B. pseudomallei* prototype and has been used in the ongoing International *Burkholderia pseudomallei* Genome Project.

Construction of *B. pseudomallei* genomic libraries

B. pseudomallei were grown overnight at 37°C in 100 ml BHI broth (Gibco BRL, Paisley, UK). Genomic DNA from *B. pseudomallei* were prepared by using the proteinase K, SDS and CTAB/NaCl solution method.⁸ The DNA was size-fractionated after partial digestion with *EcoRI* (New England Biolabs, Hertfordshire, UK). The DNA size of 2-10 kb was selected by low melting point agarose electrophoresis (Gibco BRL, Paisley, UK). Genomic libraries were constructed in a λ ZAP II bacteriophage (Stratagene, Cambridge, UK). The 2-10 kb size-fractionated fragments of bacterial genomic DNA were ligated to *EcoRI*-digested dephosphorylated λ ZAP II DNA using T4 DNA ligase (New England Biolabs). The recombinant DNA was packaged into a lambda phage using an *in vitro* packaging system (Stratagene, Cambridge, UK) and plated on the *Escherichia coli* strain XL1Blue-MRF' cells.

Screening and characterization of the recombinant clones expressing the antigenic genes

The recombinant clones expressing the antigenic proteins were screened from the genomic libraries by detection with pooled sera from melioidosis patients. The pooled melioidosis sera were thoroughly absorbed with *E. coli* whole cell lysate to remove antibodies to *E. coli* which were used as the host cells for library expres-

sion.⁹ Positive clones were analyzed by SDS-PAGE and stained with Coomassie brilliant blue and compared with the whole cell lysate of *E. coli*. The presence of the recombinant proteins was confirmed by Western blotting using the *E. coli* absorbed melioidosis sera.

The size of the inserted DNA fragment was determined by digestion with *HindIII* and *XbaI* (New England Biolabs). The nucleotide sequences of antigenic genes were obtained by automated nucleotide sequence analysis using BigDye terminator sequencing chemistry. The sequences were compared with the sequences deposited in the Genbank database (National Center for Biotechnology Information, Bethesda, Maryland, USA).

Western blot analysis of the recombinant Bps-1

The recombinant protein antigen was evaluated with a panel of human sera as followed. Seventy-six serum samples were obtained from bacteriologically confirmed cases of melioidosis, 46 of which had septicemic melioidosis and 30 had localized melioidosis. One hundred and thirty-two negative-control serum samples were taken from healthy blood donors from Khon Kaen province which is an endemic area of melioidosis and 100 serum samples were taken from healthy blood donors from Bangkok which is a non-endemic area for this disease.

In addition, 75 serum samples from patients with clinical suspicion of melioidosis, without *B. pseudomallei* isolation from clinical specimens; were tested; 5 of which had isolated gram-positive bacteria, 21 patients had isolated other gram-

negative bacteria, and 49 had no bacteria isolated.

Western blotting was performed after electrophoresis as follows: the protein was blotted onto a nitrocellulose membrane (0.45 µm pore size; Schleicher & Schuell, Dassel Germany). The recombinant protein on the nitrocellulose membrane was then incubated with melioidosis sera diluted at 1:2,000 for 1 hour and stained with alkaline phosphatase-conjugated goat anti-human immunoglobulins (Kirkegaard and Perry Laboratories, Maryland, USA) at a dilution of 1:2,000 for 1 hour. Alkaline phosphatase substrate, i.e. nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma, MO, USA) was then added.

RESULTS

Protein expression from recombinant clones in λZAP II libraries were screened by enzyme immunoassay. A recombinant clone from the K94050 library and a recombinant clone from the K96243 library, which were very strongly positive by enzyme immunoassay, were selected. Subsequent analysis showed that these two clones from different libraries were identical. They had identical nucleotide sequences and encoded for the same protein. A recombinant clone from *B. pseudomallei* isolate K94050 library was selected for further study and was named *pBps-1*. After SDS-PAGE analysis, the recombinant protein of 18.7 kDa in size (named Bps-1) was detected and was confirmed to be antigenic by Western blotting (Figs. 1 and 2).

The immunoreactivity of the recombinant Bps-1 protein was evaluated in melioidosis patients (n = 76), and in healthy blood

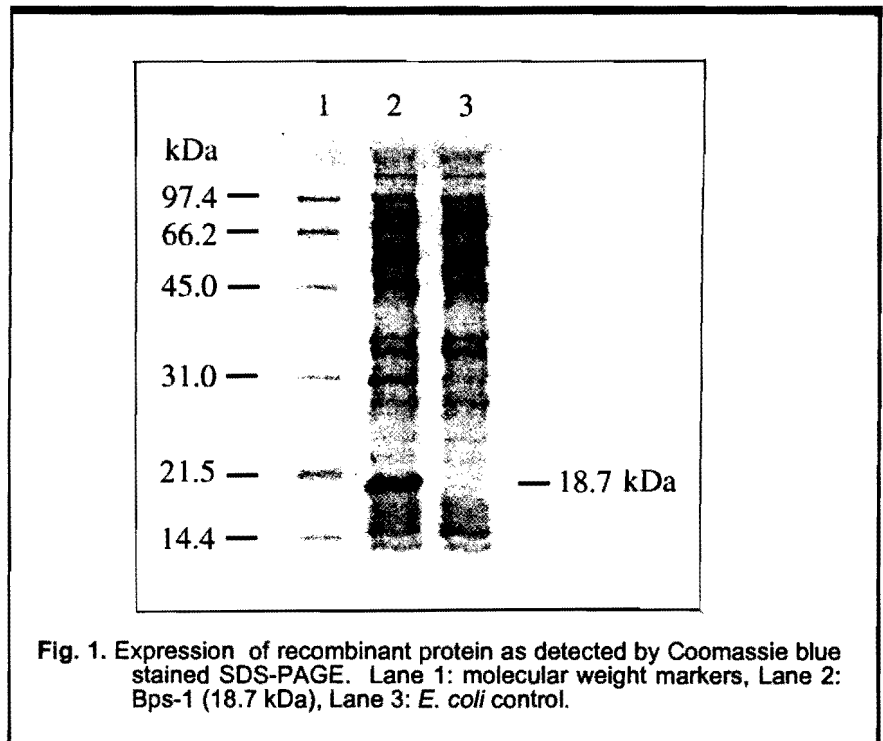


Fig. 1. Expression of recombinant protein as detected by Coomassie blue stained SDS-PAGE. Lane 1: molecular weight markers, Lane 2: Bps-1 (18.7 kDa), Lane 3: *E. coli* control.

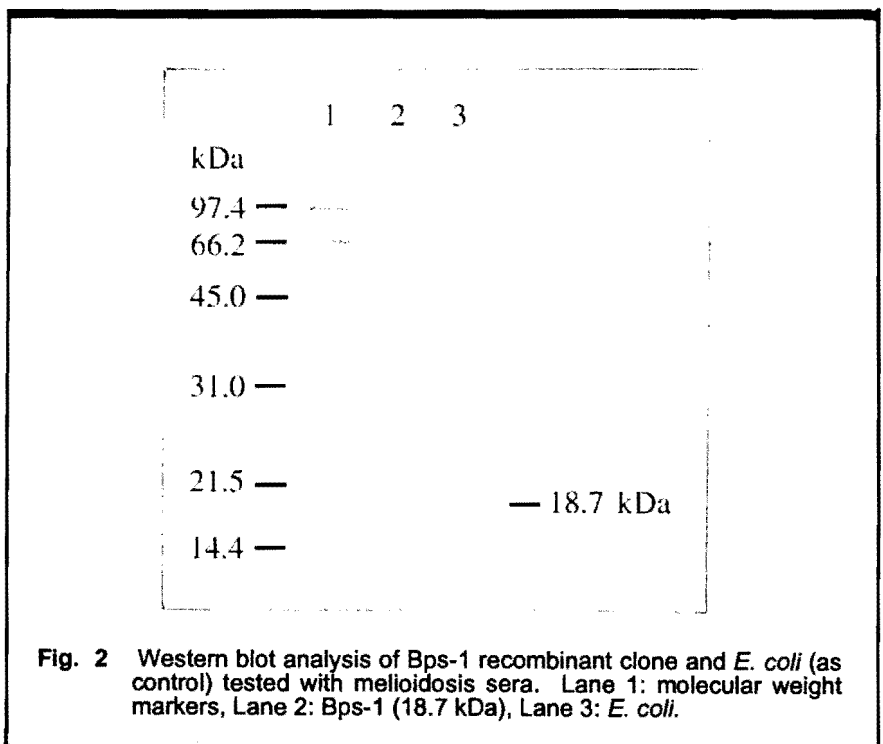


Fig. 2. Western blot analysis of Bps-1 recombinant clone and *E. coli* (as control) tested with melioidosis sera. Lane 1: molecular weight markers, Lane 2: Bps-1 (18.7 kDa), Lane 3: *E. coli*.

donors in both the endemic and non-endemic areas (n = 232). The 18.7 kDa recombinant *B. pseudomallei* protein was summarized in Table 1. The result of the Western blot analysis for detecting antibodies against the

Table 1 Western blot analysis for the detection of antibody to Bps-1 recombinant protein in sera of melioidosis patients, healthy blood donors and non-melioidosis patients

Group	No. of serum samples	No. of sera reactive to Bps-1 recombinant protein (%)
Melioidosis	76	53 (69.7)
Septicemic	46	32
Localized	30	21
Healthy blood donors	232	2 (0.9)
From endemic area	132	2
From non-endemic area	100	0
Other infections	75	9 (12.0)
Gram-positive bacteria were isolated	5	0
Other gram-negative bacteria were isolated	21	4
No bacterial growth	49	5

The sensitivity of Western blot using Bps-1 recombinant protein antigen was 69.7% (53/76) and the specificity was 99.1% (230/232). The antibody to Bps-1 recombinant protein was present in only 1.5% (2/132) of healthy blood donors in the melioidosis endemic area and none (0/100) of healthy blood donors in the non-endemic area.

In addition, Bps-1 specific antibody was present in 12% (9/75) of patients suspected of having melioidosis but had other or no bacteria isolated, resulting in 88% specificity of the test. The antibody was detected in none (0/5) of the patients who had isolated gram-positive bacteria and in 4/21 of those who had isolated gram-negative bacteria. Of note, three of the four were highly positive against the affinity purified antigen and two were also highly positive by IHA (titer 1:1,280, 1:2,560), suggesting that they actually had melioidosis.

The antibody was also detected in 5/49 patients in whom no causative organisms were iden-

tified, two of which were also highly positive by ELISA against the affinity purified antigen and by IHA.

DISCUSSION

A variety of serological tests for the detection of antibodies of *B. pseudomallei* have been developed and demonstrated good potentials in the diagnosis of melioidosis. However, the specificity of the tests is the major problem in the serological diagnosis of melioidosis due to the high background of the antibody in normal population in the endemic area. The appropriate antigen to be used in serological assays that can differentiate asymptomatic versus clinical infection is urgently needed. In a recent study by Sirisinha *et al.*⁷ various crude preparations and purified antigens were evaluated by using the same panel of sera. Although none of the tests could achieve the target of both 90% specificity and sensitivity, some preparations of antigens, including affinity purified (200 kDa), purified LPS, CCF (30 kDa), and Bps-1

recombinant antigen showed a moderate degree of either sensitivity or specificity. All three antigens, except Bps-1 recombinant antigen, were derived from the *B. pseudomallei* culture. Being classified as an organism requiring a Biosafety Level 3 containment facility, mass culture of living *B. pseudomallei* for the production of antigens for immunodiagnostic purposes would not be a method of choice. It is clear that preparation of antigenic protein from a recombinant clone would be a safer approach.

The recombinant clone, *pBps-1*, was selected from the genomic libraries of two different isolates of *B. pseudomallei* by using melioidosis sera. The analysis of this clone by comparing it with known nucleotide sequences in the Genbank database demonstrated that it encoded a highly specific antigen of *B. pseudomallei* that did not match with the sequences of any bacteria in the Genbank database at the nucleotide sequence as well as at the amino acid sequence levels. This data provided an additional confirmation on the degree of

specificity since it is more difficult to extensively test with the sera from those infected with many other bacteria.

The recombinant Bps-1 protein was shown to be highly specific with 98.5% and 100% specificity in healthy blood donors in the endemic and non-endemic areas, respectively. The cross-reactivity of Bps-1 recombinant protein antigen which was evaluated in non-melioidosis patients was initially at 12%. By using the same panel of non-melioidosis sera, the cross-reactivity seen in Bps-1 recombinant protein was better than that reported of the immunoaffinity-purified antigen (17.5%) and by IHA (27.8%).⁶

The antibody to Bps-1 recombinant protein antigen was found in 4 patients with other gram-negative bacterial infections and 5 patients with no bacterial growth. Three of the 4 patients with another gram-negative bacterial infection and 3 of the 5 patients with no bacterial growth also had antibodies to melioidosis as detected by IHA and ELISA,⁶ suggesting that these individuals were seropositive for melioidosis. By excluding those

that may actually have had *B. pseudomallei* infection, the Bps-1 antibody would be present in only 4% (4/75) of these negative-control patients, resulting in 96% specificity. The cross-reactivity was decreased to only 4%.

Moreover, the Bps-1 recombinant protein antigen was recognized by only 1.5% of healthy individuals in the endemic area, and not recognized by that from a non-endemic area. This finding indicated that the Bps-1 recombinant protein antigen was highly specific to *B. pseudomallei*.

The Bps-1 recombinant protein antigen, therefore, has good diagnostic potential as a specific antigen for detecting antibody for melioidosis. Although the sensitivity of the Western blot test using Bps-1 protein was not satisfactory at present, improvement of the sensitivity might be achieved by using more sensitive format of the test such as immunochromatography.

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