Original article

SDS-PAGE analysis of whole cell protein and outer memrbane protein patterns of clinical isolates of *Burkholderia pseudomallei*

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Abstract

Objective: To investigate the banding patterns of whole cell protein (WCP) and outer membrane protein (OMP) of *Burkholderia pseudomallei* (*B. pseudomallei*) in clinical isolates from patients with melioidosis. **Methods**: WCP and OMP of of *B. pseudomallei* in 50 clinical isolates, from 47 patients with melioidosis were prepared and separated by polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gels and stained with Coomassie brilliant blue. The banding patterns were compared by using a laser densitometer and dendrogram. **Results**: There were 6 different banding patterns of WCP and 2 types of OMP. Type 1-5 WCP had 8 common protein bands at 19.0 – 45.0 kDa with identical OMP pattern. The banding patterns of WCP in type 6 were distinct from the others and also its OMP profile. The majority of clinical isolates (37/50, 74%) were in type 1 WCP. Of the remaining isolates, 8 were in type 2, 2 in type 3, and one each was in type 4 to 6. There was no significant association between the WCP typing and the demographic or clinical features of the investigated patients. **Conclusion**: Despite the wide variation of clinical features of melioidosis, the results of this study show that *B. pseudomallei* had a few differences in the WCP and OMP profiles. Therefore typing of WCP and OMP, using SDS-PAGE analysis, could be an alternative method for phenotypic differentiation in clinical isolates of *B. pseudomallei*.

Keywords: SDS-PAGE; *Burkholderia pseudomallei*; Whole cell protein; Outer membrane protein; Melioidosis

INTRODUCTION

Melioidosis, a potentially fatal bacterial infection caused by *Burkholderia pseudomallei* (*B. pseudomallei*), is prevalent in Southeast Asia and Northern Australia, but is occasionally encountered in other countries [1]. In Northeastern Thailand, melioidosis is an important medical problem with an increasing number of cases recognized each year in rice farmers and their families [2]. In Sappasitprasong Hospital, Ubon Ratchatani, Thailand, more than 100 cases of

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culture-proven melioidosis are seen each year [3].

The clinical manifestations of melioidosis vary from asymtomatic sereconversion to fulminant sepsis associated with multiple complications leading to a high mortality[4]. Even after completion of 20 weeks maintenance therapy, relapse occurs in 1/4 of patients with melioidosis[5]. Although many techniques were used to discriminate tying of *B. pseudomallei*, there was no powerful tools for this epidemiological studies. In the present study, Sodium dodecyl sulfate polyacrylamide gel electrophoresis(SDS-PAGE) analysis of whole cell protein (WCP) and outer membrane protein (OMP) of *B. pseudomallei* were investigated.

MATERIALS AND METHODS

Bacterial strains

Fifty clinical isolates of *B. pseudomallei* from 47 patients with culture-proven melioidosis who were admitted to Sappasitprasong Hospital, Ubon Ratchatani, Thailand, were investigated. The bacterial strains were obtained from cultures of the following specimens: blood culture (n = 26), pus (n = 12), urine (n = 5), sputum (n = 3), throat swab (n = 3), and pleural fluid (n = 1). All strains were confirmed to be *B. pseudomallei* by microbiological and biochemical methods as described previously[6]. All isolates were stored in Trypticase soy broth containing 15 % glycerol, at −70℃.

Whole cell protein (WCP) preparation

Clinical isolates were recovered by overnight culture on Columbia agar (Oxoid), at 37℃. Bacterial growth from whole plates were harvested and suspended in 10 mL sterile distilled water. The bacterial suspensions were killed by boiling for 10 mins and then centrifuged at 2 000 × g for 30 mins, at 4℃. Following one wash with sterile distilled water, the cell pellets were resuspended in 1 mL of sterile distilled water, and their protein concentrations were determined using the Lowry method[7]. The WCP suspensions were then stored at −20℃ for subsequent SDS-PAGE analysis, which was performed within 2 months.

Outer membrane protein (OMP) preparation

WCP suspensions were sonicated, on ice, using an ultrasonic homogenizer (B. Braun) at 350 Watts output, for 10 × 1 mins cycles. The debris and unbroken cells were pelleted by centrifugation at 7 000 × g for 15 mins, at 4℃. The supernatant was then centrifuged further at 35 000 × g for 45 mins, at 4℃, and the pellet was resuspended in 10 mL of 1.7 % sodium lauryl sarcosinate in 50 nM Tris pH 7.6 and incubated for 20 mins at room temperature. The extracted OMPs were then pelleted by centrifugation at 35 000 × g for 45 mins at 4℃. The OMPs were resuspended in 100 μL of sterile distilled water, stored at −20℃, and used within 2 months.

SDS-PAGE analysis

The WCP and OMP suspensions were solubilized in sample buffer (2 g SDS, 20 g sucrose, 1 mL of 2-mercaptoethanol, 0.001 g bromophenol blue in 100 mL of 0.1 M Tris HCl, pH 6.8) at a final concentration of 1 mg/mL and boiled for 5 minutes. Electrophoresis was carried out at constant current of 30 mA using 100 μg WCP per lane, in 10% polyacrylamide gels using Protean IIxi cell (Bio-rad, UK). The gels were stained overnight with Coomassie Brilliant Blue R-250 (0.1%) in methanol:water:acetic acid (3:6:1) and subsequently destained using methanol : water : acetic acid (5:1:5) with shaking for 3-5 hours. The prepared gels were then dried with BioGel Wrap (Bio-rad, UK).

The reproducibility of SDS-PAGE was assessed using randomly selected 10 clinical isolates. Strains of *B. pseudomallei* WCP and OMP were prepared from three batches, grown independently at different times, adhering strickly to the same conditions for all steps in the procedure.

Scanning of gels and computation of similarities

The protein patterns on the dried gels were scanned with a Laser densitometer (Bromma Ultrascan XL, LKB) using a red filter (603 nm).

Similarity between all pairs of traces was expressed as the pearson product-moment correlation coefficient, which was converted to a percentage. A dendrogram was constructed to reflect the similarities between isolates included in the matrix. Strains were clustered by the method of unweighted pair-group with mathematic averages (UPGMA). Computations were carried out on a PC-compatible microcomputer using a program package.

RESULTS

The banding patterns of WCP for each of the 10 clinical control isolates, the patterns of bands obtained on polyacrylamide gels under standard conditions were consistent and reproducible. Of the 50 clinical isolates of *B. pseudomallei*, 6 different banding patterns of WCP were found by visual inspection (Figure 1a). Type 1 to 5 shared common protein bands of the following molecular weight at 19.0, 23.0, 24.5, 26.0, 27.0, 29.0, 38.5 and 45.0 kDa. Of these 8 WCPs, only 4 major protein bands found in type 6 which were different in protein intensity. Type 3 was analysed by a laser densitometer as it had 8 common protein bands without any other additional bands as in other types. Of the 8 common protein bands, the 3 highest protein densities were at 29.0, 26.0 and 38.5 kDa, respectively (Figure 1b).

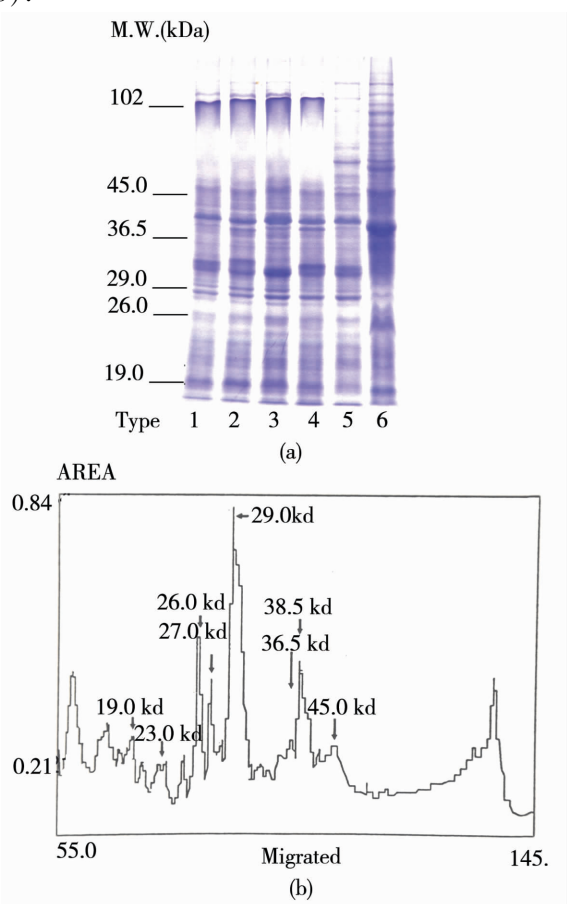

Figure 1 WCP typing of *Burkholderia pseudomallei* by using SDS-PAGE.
a). WCP profiles of 6 different types. b). Densitometric tracing of the electrophoretic WCP pattern of type 3.

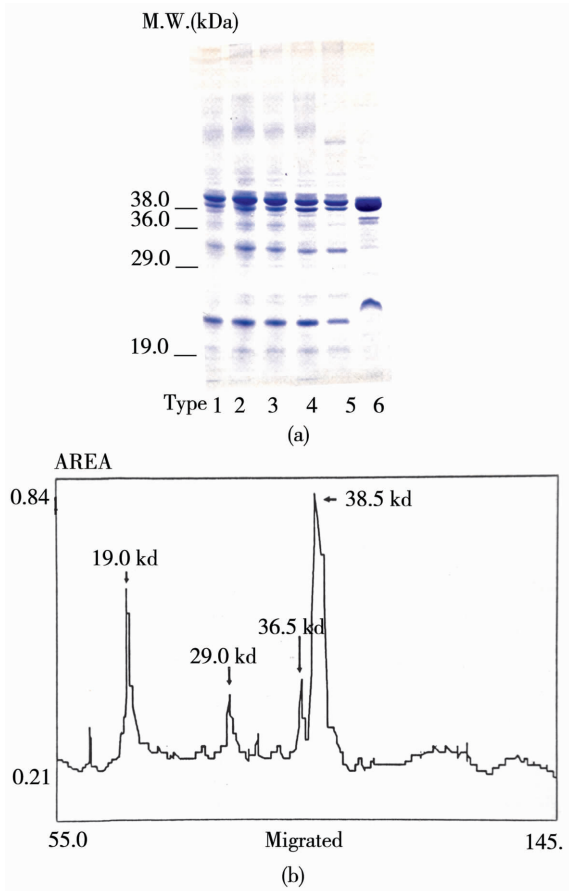

Figure 2 OMP typing of *Burkholderia pseudomallei* by using SDS-PAGE.
a). OMP profiles of 2 different types. b). Densitometric tracing of the electrophoretic OMP pattern of type 2.

Six of the 50 strains representing the 6 types of WCP, were used to determine OMP profiles by SDS-PAGE. Among the 6 strains, only 2 different OMP patterns occurred. Type 1-5 WCPs had identical OMP patterns (type A) whereas type 6 WCP revealed a unique OMP pattern (type B) (Figure 2a). Type A OMP consisted of 4 major bands at 19.0, 29.0, 36.5 and 38.5 kDa as shown in Figure 2b. Bands at 22.0, 35.0 and 38.0 kDa are seen in type B OMP pattern.

The similarity analysis of WCP profiles was based on discremination of 8 protein bands between 14 kDa and 31 kDa. There were 6 basic clusters defined with 10 % different limits (Figure 3). Types 1-5 were closely similar (75 % – 85 %). Type 6 showed only 24 % similarity to other types by using this programme. Visual inspection, laser densitome-

try and the computer-generated similarity all gave comparable results i. e. 6 WCP types. Types 1-4 contained 102.5 kDa band which was predominantly glycoprotein, as demonstrated by PAS staining (data not shown). Type 6 was clearly distinct from the other types in having a pattern of protein bands between 22.5 and 78.0 kDa, however this type consisted of a single strain only. Type 5 could also be distinguished visually in the high molecular weight range of bands (50 – 100 kDa) but this type also consisted of only a single strain. The majority of clinical isolates (37/50; 74 %) were in type 1 WCP. Of the remaining isolates, 8 were in type 2, 2 in type 3, and each was in type 4 to type 6. The WCP patterns of isolates with different colonial appearances (e. g. mucoid, dry and wrinkled, or smooth) and from different sources of isolations were not distinguished by this method (data not shown).

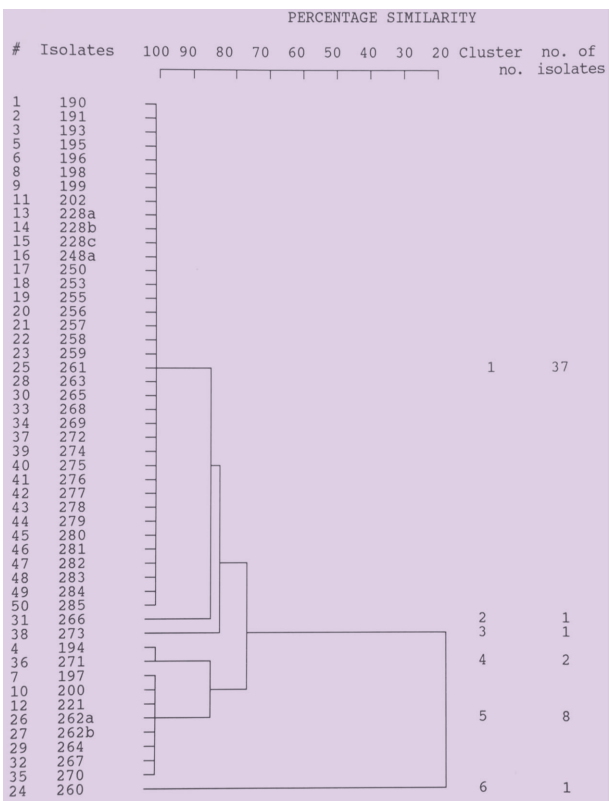

Figure 3 Numerical analysis of WCP electrophoresis of 50 different isolates of Burkholderia pseudomallei using the correlation coefficient and clustered using the unweighted pair-group method using average (UPGMA).

DISCUSSION

SDS-PAGE analysis of cellular proteins is often valuable in microbial classification and typing. Many kinds of proteins in bacterial cells have been used for these purposes, including cytoplasmic protein, OMP, cell envelope protein and WCP. WCP is a whole bacterial cell extract containing outer and inner membrane and cytoplasmic proteins. Electrophoresis of WCP preparations has been used as an epidemiological tool in typing many different bacterial species[8-11]. One of the problems with gel electrophoresis is reproducibility of results. Bacterial culture conditions, sample preparation, storage conditions and the physical conditions during gel electrophoresis may all cause variation in the results[12]. In our study electrophoretic protein patterns were reproducible, although the sharpness of individual bands was reduced after prolonged storage of WCP at –20℃ (>2 months).

This study has shown 6 types of WCP patterns and 2 types of OMP patterns among 50 clinical isolates of *B. pseudomallei* from patients in northeastern Thailand. Type 5 and 6 were significantly different from the remainder. Type 5 was isolated from pus in diabetic labourer patient with a Psoas abscess. Type 6, was isolated from the urine or diabetic patient with septicaemia and a knee abscess. This latter patient was a merchant and died after 3 day hospitalization. There was no association between WCP types and different colonial appearances or disease severity (clinical features, organ involvement, complications) or geographic distribution or antibiotic susceptibility. Gotoh *et al.*[13] using sucrose density gradient centrifugation followed by SDS-PAGE found five major protein bands in the outer membrane fractions but there was no difference in the OMP pattern in 12 strains of *B. pseudomallei*. One of the major protein bands had a Mr of 38.0 kDa which served as a porin, also found in this study. Sexton *et al.*[14] found protease enzyme at a molecular weight of 36.0 kDa which may be a significant pathogenic determinant in infections caused by *B. pseudomallei*. This finding is corresponding to this study in the presence of banding in OMP.

Early studies of the antigenic constitution of *B. pseudomallei identified four distinct antigens*, *including a heat-stable O-antigen*[15]. Dodin and Fournier[16] found 2 serotypes, I and II against O-antigen. The serotypes were associated with geographic origin. Lipopolysaccharide (LPS) is a highly conserved antigen in *B. pseudomallei*[17]. This LPS was not detected in other Pseudomonas species except in *P. mallei*. Most tested isolates in this study were contained in glycoprotein which was not immunogenic substance (data not shown).

Ribotyping is a technique that is increasing used as an epidemiological typing method, however it is relatively time consuming, and requires radioisotopes[18]. Sexton *et al*.[14] found only 10 ribotypes from 84 clinical and environmental isolates of *B. pseudomallei* from Thailand. Lew and Demarcheliер[19] have found 22 ribotypes among 100 isolates from human, animal, and environmental sources, although the largest ribotype group contained 29 isolates. Further ribotyping experiments yield 12-44 distinct patterns depending on each cutting enzyme[20,21] None of these types could be assigned to a single animal species, a geographic region or clinical course[21,22]. A possible solution for the problem of standardization in ribotyping technique should be done. The DNA probe specific for *B. pseudomallei* DNA has been developed[23]. The probe was applied to total DNA digests from 60 clinical isolates of *B. pseudomallei*, but only 8 different types could be distinguished. It was proposed that this probe may be used for the application in epidemiological studies of *B. pseudomallei* as ribotyping. The current gold standard, pulse-field gel electrophoresis (PFGE) has been employed successfully in epidemiological studies[21,24-26]. Pitt *et al*.[21] found 226 PFGE profile types within 350 *B. pseudomallei*. However, this procedure is labour intensive, taking long time, and expensive like a multilocus sequence typing technique (MLST). Godoy *et al*.[27] identified 71 sequence types in 147 *B. pseudomallei* isolates. These MLST results are comparable with PFGE. Another variable number of tandem repeats technique may be a new promising tool for typing *B. pseudomallei* strains in endemic areas as the method is obviously cheaper and easier. Therefore, the WCP and OMP analysis as described in this study is simple and reproducible for epidemiological study but may lack the necessary discriminatory power to differentiate the clinical isolates of *B. pseudomallei*.

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