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Cloning, expression and purification of outer membrane protein (OmpA) of *Burkholderia pseudomallei* and evaluation of its potential for serodiagnosis of melioidosis

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ABSTRACT

Melioidosis is an emerging infectious disease in India and caused by gram-negative, soil saprophyte bacteria *Burkholderia pseudomallei*. This disease is endemic in Southeast Asia and northern Australia, and sporadic cases of melioidosis are also reported from southern states of India. The present study reports the cloning, expression, and purification of recombinant protein outer membrane protein A (OmpA) of *B. pseudomallei* and its evaluation in indirect enzyme-linked immunosorbent assay (ELISA) format with 87 serum samples collected from Manipal, Karnataka, India. Twenty-three samples from culture confirmed cases (n = 23) of melioidosis, 25 serum samples from patients of other febrile illness and pyrexia of unknown origin (n = 25), and 39 serum samples from healthy blood donors (n = 39) from Kasturba Medical College, Manipal, were tested in this assay format. The assay showed sensitivity of 82.6% and specificity of 93.75%. The recombinant OmpA based indirect ELISA will be a useful tool for serodiagnosis of melioidosis in large scale rapid screening of clinical samples.

1. Introduction

Melioidosis is an infectious disease caused by the bacteria, *Burkholderia pseudomallei*, and is prevalent in subtropical and tropical climates (Dance, 1991). *B. pseudomallei* is a gram-negative, soil bacterium and is classified as a category B biothreat agent by Centre for Disease Control, Tier 1. This disease is endemic mainly in Southeast Asia and northern Australia and affects both human as well as animals (White, 2003). In India, sporadic cases are reported from coastal regions of Maharashtra, Kerala, Karnataka, Tamilnadu, and Pondicherry, and the exact incidence of melioidosis is not known due to misdiagnosis and underreporting (Kanungo et al., 2002). Lack of awareness, low index of suspicion, and inability of rural population to access health services contribute to the paucity of reports from Indian subcontinent. It is also often misdiagnosed with other bacterial infections due to nonavailability of proper diagnostic test assays and kits at the peripheral level health care system.

The clinical manifestations of melioidosis vary with individuals and geographical locations and can mimic a large number of other infections (Meumann et al., 2012; Pagnarith et al., 2010; Yee et al., 1988). In the acute phase of melioidosis, death can occur within 24–48 hours of onset of symptoms, whereas in patients with minimal or negligible symptoms, the disease can progress to a chronic carrier state (Ip et al., 1995; Ngauy et al., 2005). The acute septicemia form can result in multiorgan involvement as often seen in endemic regions.

Epidemiological investigations to study the true prevalence of melioidosis in India require highly accurate diagnostic tools and assays. The diagnosis of melioidosis is intrinsically difficult as clinical presentations vary from patient to patient. The isolation of *B. pseudomallei* by culture from clinical samples remains the most conclusive method for diagnosis of melioidosis. However, culture-based methods are time consuming and may take 5–7 days for diagnosis (Cheng, 2010). Indirect hemagglutination assay (IHA) has been used in endemic areas for diagnosis but has limited utility as a diagnostic test because persistent antibody titers are observed (Cheng et al., 2006). There is a need for development of recombinant protein antigen–based immunoassays for serodiagnosis of melioidosis that is specific and do not cross react with other closely related *Burkholderia* infections. Highly sensitive and specific assays developed with well-characterized recombinant antigens may provide surveillance and prompt diagnosis leading to prompt treatment in endemic areas.

A number of cell-associated antigens have been demonstrated to be immunogenic in patients with melioidosis, including capsular polysaccharide, lipopolysaccharide, and flagellin proteins (Arjcharoen et al., 2007; Chuaygud et al., 2008; Reckseidler-Zenteno et al., 2005). In addition, many putative virulence determinants have also been identified and tested as candidates for use in serodiagnostic assays (Wiersinga et al., 2006). Outer membrane proteins like outer membrane protein A (OmpA) and Omp85 of *B. pseudomallei* are found immunogenic and suggested as potential vaccine candidates (Hara et al., 2009, Su et al.,

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2010). OmpA is not only a potential vaccine candidate but also can be used for serodiagnosis of melioidosis in endemic regions (Allwood et al., 2008).

We report in this study the cloning, expression, purification, and evaluation of outer membrane protein (OmpA) of *B. pseudomallei* for the serodiagnosis of melioidosis. The 675-bp *ompA* gene was cloned and expressed in pET-SUMO bacterial expression system in expression host *Escherichia coli* strain BL21 (DE3). The SUMO fusion protein was purified by single-step purification and yielded 96 mg of pure protein per liter of shake culture. An indirect enzyme-linked immunosorbent assay (ELISA) was confirmed and standardized with recombinant OmpA (rOmpA) protein followed by evaluation with a panel of 87 clinical samples. The sensitivity, specificity, and usefulness of this assay for clinical diagnosis of melioidosis in India are described. This simple, cost-effective serological assay that is sensitive and specific in nature will be useful in serosurveillance in areas where the actual incidence of this disease is unknown.

2. Materials and methods

2.1. Bacterial strains and cloning vector

B. pseudomallei strain NCTC1688 was obtained from National Collection of Type Cultures, UK, and cultured using Brain Heart Infusion (BHI) broth and agar under Bio Safety Laboratory Level 3 (BSL3) containment (Himedia chemicals, Mumbai, India). The expression vector pET-SUMO used in the study was procured from Invitrogen, CA, USA. The maintenance host *E. coli* strain DH5α and expression host *E. coli* strain BL21 (DE3) were obtained from Sigma chemicals (St. Louis, MO, USA) and routinely cultured and maintained in Luria broth (Difco, Detroit, USA). The antibiotic selection was performed by supplementing kamamycin (50 μg/mL) (Sigma) in broth and agar as a resistance gene for kanamycin present in pET-SUMO vector.

2.2. Serum samples

A total of 87 serum samples collected from Kasturba Medical College (Manipal, Karnataka, India) in year 2012 were stored at −80 °C and further tested via indirect ELISA (iELISA). The serum samples were grouped into 3 categories for evaluation by iELISA. Group I included 23 culture-confirmed melioidosis serum samples, group II include 25 serum samples collected from patients with other febrile illnesses (malaria, typhoid, and pyrexia of unknown origin), and group III include 39 serum samples collected from healthy blood donors.

2.3. Cloning and expression of ompA in pET-SUMO vector

B. pseudomallei strain NCTC 1688 was cultured in BHI broth, and DNA was extracted from an overnight grown fresh culture by conventional Phenol-Chloroform DNA extraction method. DNA purity was checked via agarose gel electrophoresis. The extracted DNA was used as template to amplify the *ompA* gene of *B. pseudomallei*. Primers used to amplify this gene were forward primer 5′ATGAATAAACTTTCAAAGCTC3′ and reverse primer 5′TTACTGCGCCGGAACGGT3′. The position of the primer corresponds to 3039644 to 3040318 position of the *ompA* gene in chromosome 1 of *B. pseudomallei* strain K96243 (accession no. NC_006350.1). PCR conditions were standardized by performing $MgCl_2$ gradient PCR and temperature gradient PCR. The amplification protocol for the 675-bp amplified product was initial denaturation of 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 50 s, annealing at 56 °C for 50 s, extension at 72 °C for 1 min, followed by final elongation at 72 °C for 10 min. The pET-SUMO expression vector is a linearized vector that follows a Thymine Adenine (TA) cloning strategy. The amplified PCR product was gel purified using a Qiagen gel extraction kit (Qiagen, Hilden, Germany); yield was checked via agarose gel electrophoresis. The purified PCR product was then ligated into the pET-SUMO expression vector and transformed in BL21 (DE3) strain of *E. coli* as per standard protocol. Positive clones were selected on Luria Bertini (LB) agar plates containing kanamycin (50 μg/mL), and PCR using forward and reverse primers to OmpA was used to detect the presence of recombinant plasmid. Orientation of the insert was confirmed by performing PCR with SUMO vector's forward 5′ AGATTCTTGTACGACGGTATTAG3′ and reverse 5′TTACTGCGCCGGAAC GGT3′ sequencing primers. Clones with the correct orientation were further confirmed by sequencing analysis before expression of recombinant protein. In order to express the OmpA, overnight grown culture of a positive clone was inoculated in 10 mL of LB broth containing kanamycin in 1:20 ratio (500 μL initial culture in 10-mL broth) and incubated at 37 °C with shaking at 180 rpm. The expression profile of recombinant protein was studied at hourly intervals from 0 to 5 hours by inducing with increasing Isopropyl β-D-1-thiogalactopyranoside (IPTG) concentrations of 0.25, 0.5, 1.0, 1.25, 1.5, 2.0, and 3.0 mmol/L. The un-induced and induced cultures were collected at different time intervals and lysed in 2× sample lysis buffer and then analyzed on 15% SDS PAGE gel as described by Laemmli (1970). In order to determine the solubility of protein, pelleted bacterial cultures were treated with lysis buffer containing lysozyme for 30 min at 4 °C, sonicated, and centrifuged at 8000 rpm (or 7600 × g) for 30 min. The supernatant and cell pellet were then analyzed by Sodium Dodecyl Sulphate Polyacylamide Gel Electrophoresis (SDS-PAGE) that included a molecular mass marker (cat. #SM1811; MBI Fermantas, Hanover, MD, USA).

2.4. Purification of rOmpA under denaturing conditions

The recombinant protein was purified using Ni-NTA affinity column chromatography, and small scale purification was done using a Qiagen Ni-NTA spin column under denaturing conditions using the protocol as specified by the manufacturer (Qiagen, Hilden, Germany), and then bulk purification was carried out by affinity column chromatography using Ni-NTA Superflow purchased from Qiagen (Hochuli, 1990). In order to purify rOmpA protein, induced bacterial culture was pelleted, washed with 1× Phosphate Buffered Saline (PBS), and dissolved in 8-mL lysis buffer (8 mol/L urea, 100 mmol/L NaH_2PO_4, 10 mmol/L Tris–Cl, 20 mmol/L β-mercaptoethanol, 1% Triton X-100, 1 mmol/L phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride (PMSF), pH 8.0) and sonicated for 10 min with a pulse interval of 5 s and continuously stirred at room temperature for 1 hour at 180 rpm. The lysed cell suspension was centrifuged at 10,000 rpm (or 12,000 × g) for 30 min at room temperature, and supernatant was collected. The Ni-NTA agarose and cleared lysate were mixed in 1:3 ratio for 1 hour to allow the 6× His tagged protein to bind with Ni^{+2} in the column. The agarose lysate mix was packed in a 15-mL column, and unbound proteins in the column were washed 3 times using wash buffer (8 mol/L urea, 100 mmol/L NaH_2PO_4, 10 mmol/L Tris–Cl, 1% Triton X-100, 10% glycerol, pH 6.3). The rOmpA protein was eluted from the column using elution buffer (8 mol/L urea, 100 mmol/L NaH_2PO_4, 10 mmol/L Tris–Cl, pH 4.5) in 1-mL fractions and analyzed on 15% SDS-PAGE using molecular mass marker (cat. #SM1811; MBI Fermentas). The pooled fractions of purified protein were dialysed overnight in 2 L of dialyzing buffer containing 50 mmol/L sodium phosphate buffer and 300 mmol/L NaCl and concentrated using an Amicon ultra centrifugal filter device (Millipore Pvt. Ltd, Bangalore, India). The concentration of purified recombinant protein was estimated by Lowry method using 1 mg/mL Bovine Serum Albumin (BSA) as standard (Lowry et al., 1951).

2.5. Characterization of rOmpA

2.5.1. Western blot analysis

The purified rOmpA protein was characterized by Western blot analysis according to procedure described by Towbin et al. (1979). The protein was transferred electrophoretically from the 15% polyacrylamide gel to a nitrocellulose membrane (Pall Corporation, New York,