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Identification of immunogenic proteins from *Burkholderia cepacia* secretome using proteomic analysis

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ABSTRACT

Burkholderia cepacia is an opportunistic human pathogen associated with lung infections. Secretory proteins of *B. cepacia* are known to be involved in virulence and may mediate important host–pathogen interactions. In the present study, secretory proteins isolated from *B. cepacia* culture supernatant were separated using two-dimensional gel electrophoresis, followed by Western blot analysis to identify the immunogenic proteins. Mice antibodies raised to *B. cepacia* inactivated whole bacteria, outer membrane protein and culture filtrate antigen detected 74, 104 and 32 immunogenic proteins, respectively. Eighteen of these immunogenic proteins which reacted with all three antibodies were identified and might be potential molecules as a diagnostic marker or a putative candidate vaccine against *B. cepacia* infections.

1. Introduction

Burkholderia cepacia is a Gram-negative, non-sporulating motile bacillus found in a wide variety of terrestrial and aquatic habitats [1]. It is an invasive pathogen and therefore capable of adhering and colonising prior to entering cells. The bacterium is also known to produce secretory enzymes such as protease [2], lipase [3] and phosphatase [4], which are virulence factors that damage host extracellular membrane to promote invasion. It is also known to produce haemolysin, which induces lysis of erythrocytes and host cell death [5], as well as unique cable pilus that is involved in adhesion and colonisation of the respiratory tract [5]. *B. cepacia* has emerged as an important opportunistic human pathogen associated with life-threatening pulmonary infections that affect immunocompromised individuals [1]. It causes respiratory infections in cystic fibrosis patients associated with acute necrotising pneumonia, and also other infections such as septicemia or bacteremia which are often lethal.

Secretory proteins play important roles in the pathogenesis of many bacterial infections. They represent the inter-phase of the bacterium–host interaction [6]. Secretory proteins of intracellular pathogens are exposed to the host immune system and are therefore the primary antigen targets of host immune response [7]. Numerous novel secretory proteins such as those produced by *Pseudomonas aeruginosa* [8], *Staphylococcus aureus* [9] and *Helicobacter pylori* [10] have been previously identified by subjecting bacterial culture supernatants to two-dimensional gel electrophoresis (2-DE) and mass spectrometry analyses.

Identifying immunogens are important for development of diagnostics, vaccines and passive immunotherapies. The comprehensive profiling of bacterial immunogenic proteins is now made possible due to the recent progress in genomic and proteomic technologies [11]. Identification of the immunogenic proteins will be a further step towards the understanding of the humoral immune response during *B. cepacia* infections. The aim of the present study was to identify and characterise common immunogenic proteins of *B. cepacia* secretome by a combination of 2-DE and Western blot assay using polyclonal antisera raised in mice using different antigen preparations of *B. cepacia*.

2. Materials and methods

2.1. Bacterial strain, growth and culture condition

Clinically isolated *B. cepacia,* CQK strain was obtained from a non-cystic fibrosis patient from the University Malaya Medical Centre. The strain was cultured on nutrient agar at 37 °C, overnight and fresh colonies were resuspended in 10 ml Luria Bertani (LB) (Difco, USA) broth and mixed under vigorous agitation condition at 37 °C (OD_{600nm} of 0.5–0.8). The bacteria were recovered by

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centrifugation with fresh LB and used to inoculate a second liquid culture to obtain an OD_{600nm} of 0.01. Liquid culture (50 μl) was inoculated into 500 ml fresh LB medium and was grown to stationary-phase. The quantification of isocitrate dehydrogenase (ICD) (an intracellular enzyme) in stationary-phase culture supernatant was estimated by colorimetric method according to Anderson et al. [12].

2.2. Precipitation of secreted proteins

Supernatant was obtained from the stationary-phase bacterial culture that was centrifuged for 40 min and at 20,000 × *g* and 4 °C. The supernatant was then filtered through a 0.22 μm pore size membrane filter to remove residual bacteria. The supernatant was then precipitated by prechilled 25% (w/v) trichloroacetic acid (TCA) [10]. The mixture was incubated on ice for 2 h and centrifuged for 30 min at 40,000 × *g* at 4 °C. Pellets were dissolved in cold acetone and washed twice before air dried.

2.3. Protein sample preparation

The protein pellets were solubilised in lysis buffer (8 M urea, 4% CHAPS, and 2% Pharmalyte pH 4–7). Protein amount was estimated using bovine serum albumin as a standard [13]. The resulting protein extract was either stored at −80 °C or immediately used for 2-DE.

2.4. Two-dimensional gel electrophoresis

A total of 400 μg of protein sample was added with the rehydration buffer (8 M urea, 2% CHAPS, 0.002% bromophenol blue) and applied onto IPG strips (pH 4–7, 13 cm). In-gel rehydration was performed for 18 h at room temperature before the strips were subjected to isoelectric focusing. Proteins were focused using the IPGphor system (GE Healthcare, Uppsala, Sweden) as described by Görg et al. [14]. The strips were transferred onto 12% SDS-PAGE [15] for the second-dimension which was carried out in a vertical gel electrophoresis system (GE Healthcare, Uppsala, Sweden). After electrophoresis, the gel was subjected to either staining or electroblotting.

2.5. Image analysis

The protein spots were visualised by staining with Coomassie Brilliant Blue (CBB) G-250 [16]. Three biological growth experiments were performed to increase the reproducibility of the results. The gels were scanned and analysed with an Image Scanner and Image Master™ 2D Platinum version 5.0 (GE Healthcare, San Francisco, CA, USA).

2.6. Antibody production

Culture filtrate antigen (CFA), outer membrane proteins (OMP) antigen and inactivated whole bacteria proteins (WBP) were prepared using the methods described by Chenthamarakshan et al. [17], Molloy et al. [18] and Whitlock et al. [19], respectively. Six weeks old pathogen free male BALB/c mice [Animal Ethics: MP/08/02/2005/JSU (M)] were immunised via subcutaneous once a week for 4 weeks with respective antigens. Presence of antibodies against bacteria antigen was evaluated by an enzyme-linked immunosorbent assay (ELISA); with *B. cepacia* WBP, OMP and CFA as coating antigen, respectively. Sera that elicited high anti-antigen proteins bacterial antigen titres were used to probe Western blots. Unimmunised mice sera were used as negative control for ELISA.

2.7. Western blot analysis

Replicate 2-DE gels were transferred onto nitrocellulose membrane in a semi-dry condition using transfer apparatus (Amersham Biosciences, Uppsala, Sweden) for 2 h at room temperature in Tris–Glycine transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) [20]. After the proteins have been transferred, the membranes were blocked with 3% gelatine for 1 h and washed with phosphate buffered saline (PBS) containing 0.05% Tween (PBST) twice with 15 min interval. The membranes were then incubated overnight at 4 °C with mouse polyclonal anti-*B. cepacia* WBP, OMP and CFA at a dilution of 1:1000, respectively. Unimmunised mice sera with a dilution of 1:1000 were used as negative control immunoblot. The membranes were agitated at room temperature for 30 min before washed three times using PBST, followed by incubation in alkaline phosphatase-conjugate goat anti-mouse IgG secondary antibody (Calbiochem, California, USA) with a 1:5000 dilution. After the final washing step, Western Blue Stabiliser Alkaline Phosphatase substrate (Promega, Madison, USA) was subjected onto the membranes for visualisation.

2.8. Mass spectrometry

Selected protein spots were excised from the CBB G-250 stained 2-DE gels of the *B. cepacia* secreted proteins and digested with solution of sequencing-grade modified trypsin (Promega, Madison, USA). The peptides released from the gel were sent to Biomolecular Research Facility, University of Newcastle, Australia for further analysis by matrix-assisted laser desorption/ionisation-time-of-flight mass spectrometry (MALDI-TOF MS).

2.9. Protein identification

The spectra obtained from mass spectrometry data were submitted for database searching using MASCOT as the search engine (Matrix Science, London, UK). The subsequent search settings were used: carboxymidomethylation of cystein was fixed modification and oxidation of methionine was selected as variable modification; maximum number of missed cleavages: 1; peptide tolerance: 100 ppm. All searches were BLAST analysed with a non-redundant NCBI library (http://ncbi.nlm.nih.gov) database comprising annotated proteins of *B. cepacia* complex. To study the predicted mode of secretion, cellular localisation and protein domains of the proteins, the available bioinformatics databases were used (www.cds.dtu.dk).

3. Results

3.1. 2-DE profile of B. cepacia secretome

B. cepacia secretory proteins were extracted from the supernatant of stationary-phase culture in LB medium in order to limit the contamination of medium-derived polypeptides, were profiled (Fig. 1). Approximately, a total of 265 highly resolved protein spots were detected on the 2-DE gel stained with CBB G-250 [15] using the Image Master™ 2D Platinum version 5.0 software (GE Healthcare, Sweden). In order to exclude the possibility that these proteins are contaminants of bacterial lysis, two consecutive washing steps was included before inoculation and ICD enzyme assay was preformed and low levels of activities were detected.

As a starting point, linear IPG strip in the p*I* range of pH 3–10 was used to profile the *B. cepacia* secretome (data not shown). As a large number of protein spots were observed in the range of pH 4–7, linear IPG strip in this range was used for better separation of proteins. Therefore, acidic (p*I* < 4) and basic (p*I* > 7) proteins were