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Demonstration of an outer membrane protein that is antigenically specific for *Acinetobacter baumannii*

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Abstract

Acinetobacter baumannii is an emerging nosocomial pathogen that is resistant to many types of antibiotics, and hence, a fast, sensitive, specific, and economical test for its rapid diagnosis is needed. Development of such a test requires a specific antigen, and outer membrane proteins (OMPs) are the prime candidates. The goal of this study was to find a specific OMP of *A. baumannii* and demonstrate the presence of specific IgM, IgA, and IgG against the candidate protein in human serum. OMPs of *A. baumannii* ATCC 19606 and 16 other clinical isolates of *A. baumannii* were extracted from an overnight culture grown at 37 °C. Protein profiles were obtained using sodium dodecyl sulfate polyacrylamide gel electrophoresis, and Western blot analysis was performed to detect the presence of IgM, IgA, and IgG against the OMP in host serum. An antigenic 34.4-kDa OMP was uniquely recognized by IgM, IgA, and IgG from patients with *A. baumannii* infection, and it did not cross-react with sera from patients with other types of infection. The band was also found in the other 16 *A. baumannii* isolates. This 34.4-kDa OMP is a prime candidate for development of a diagnostic test for the presence of *A. baumannii*.

Keywords: *Acinetobacter baumannii*; Nosocomial infection; Diagnostic biomarker; Outer membrane protein

1. Introduction

Nosocomial or hospital-acquired infections caused by *Acinetobacter baumannii* constitute one of the major causes of morbidity and mortality in hospitals, especially among patients staying in intensive care units (ICUs) (Playford et al., 2007) and in immunosuppressed patients (Levin et al., 2003; Wisplinghoff et al., 2007). Controlling and treating the infection and eliminating its spread are difficult because of the bacterium's prolonged environmental survival and its resistance to a variety of antimicrobial agents (Bergogne-Berezin and Towner, 1996; Bernards et al., 2004; Koulenti and Rello, 2006). A prevalence survey conducted under the auspices of the World Health Organization (WHO) in 55 hospitals of 14 countries representing 4 WHO regions (Europe, Eastern Mediterranean, Southeast Asia, and Western Pacific) showed an average of 8.7% of hospital patients had nosocomial infections (WHO/CDS/CSR/EPH/2002.12). The WHO also calculated that at any given time, over 1.4 million people worldwide suffer from infectious complications acquired in hospitals. In Malaysia, the prevalence of nosocomial infections is underestimated and the incidence rate is unknown at the country level. In Malaysia, nosocomial infections caused by *A. baumannii* will continue to prevail because of the bacterium's increased propensity to acquire resistance to multiple antibiotics, the increased numbers of vulnerable patients, and the failure of staff to comply with infection control procedures (Deris et al., 2009).

Acinetobacter spp. cause a variety of diseases ranging from pneumonia to serious blood or wound infections, and the symptoms vary depending on the disease (Bergogne-Berezin and Towner, 1996). *Acinetobacter* can spread to susceptible people via person-to-person contact, contact with contaminated surfaces, or exposure in the environment. It poses very little risk to healthy people, but people with weakened immune systems, such as those with chronic lung disease or other chronic diseases, those with previous antimicrobial drug exposures, patients on a ventilator, those with a prolonged hospital stay, or

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those who have open wounds, may be more susceptible to infections with *A. baumannii* (Lee et al., 2004).

There are many species of *Acinetobacter* and all can cause human disease. However, *A. baumannii* accounts for almost 80% of reported *Acinetobacter* infections (Bergogne-Berezin and Towner, 1996) and is an increasingly important cause of nosocomial infection, particularly in ICUs. Outbreaks of *Acinetobacter* infections typically occur in ICUs or settings in which very ill patients are admitted. Recently, a drug-resistant *A. baumannii* was responsible for an outbreak of bacteremia in more than 240 American troops in Iraq (Centers for Disease Control and Prevention, 2004; Davis et al., 2005; Scott et al., 2007).

Although *A. baumannii* has been recognized as an emerging nosocomial pathogen and is highly resistant to many types of antibiotics, the presence of this organism still is detected using conventional culture methods and its identification is by biochemical means. This conventional method is time consuming and may take at least 2–5 days to produce results. Late detection of the bacteria in patients causes problems in disease control and can result in severe complications in patient care and management. A new, fast, sensitive, specific, and economical test is needed for the rapid detection of *A. baumannii* infections. Development of such a test would require finding specific antigenic proteins of *A. baumannii*, and outer membrane proteins (OMPs) are prime candidates for recognition by host antibodies. This study was conducted to demonstrate the presence of an antigenic and specific OMP(s) in *A. baumannii* and to identify the presence of specific immunoglobulins in patient sera against the candidate protein(s). This protein(s) then can be utilized as a potential biomarker in the development of a diagnostic test against *A. baumannii*.

2. Methods

2.1. Collection and culture of bacterial strains

Sixteen clinical strains of *A. baumannii* used in this study were obtained from the Department of Medical Microbiology & Parasitology, School of Medical Sciences, Universiti Sains Malaysia, Malaysia. These clinical isolates were obtained from septicemic patients admitted at the Universiti Sains Malaysia Hospital from June 2005 to October 2006. A reference, *A. baumannii* ATCC 19606, was obtained from the Belgian Coordinated Collection of Microorganisms, Belgium. This reference strain was the standard organism used when performing protein profiling in this study. Serum was collected from patients confirmed by culture to have *A. baumannii*.

A. baumannii ATCC 19606 and the 16 clinical isolates were maintained in nutrient slants agar and trypticase soy broth with 20% glycerol. Working cultures were prepared by inoculating one single isolated colony in 10 mL of nutrient broth, which was then incubated overnight at 37 °C with shaking at 200 rpm in an orbital shaker (Forma Orbital Shaker, Model-420, Gyeonggi-Do, Korea). The purity of each culture was determined by inoculating it on blood agar. For experimental purposes, the organism was grown in nutrient broth at 37 °C for 18 h. Both *A. baumannii* ATCC 19606 and the clinical isolates were identified using the API 20NE system (BioMerieux, Marcy l'Etoile, France) according to the manufacturer's instructions.

2.2. OMP and inner membrane protein preparations

Bacteria were grown in nutrient broth. OMPs were extracted using a previously described method (Ismail et al., 1991). Briefly, bacteria were grown in 2 L of nutrient broth and incubated in a shaker (Forma Orbital Shaker) at 37°C at 200 rpm for 18 h until late log phase was reached. Cells were harvested by centrifugation at 15 900 × *g* for 30 min and resuspended in 8 mL of 0.01 mol/L HEPES buffer (pH 7.4). This suspension was then mixed with 8 μL of 10 mmol/L DNAse, 8 μL of 10 mmol/L RNAse, 8 μL 10 mmol/L of lysozyme, and 800 μL of 100 mmol/L phenylmethylsulfonyl fluoride. Bacterial cells were disrupted by vortexing with glass beads (0.2 mm in diameter) for 1.5 h, with 1 min alternate on ice until 95% lysis was achieved. Cell lysis was monitored using the Gram stain method. The cell lysate obtained was aspirated and the glass beads were washed with 0.01 mol/L HEPES buffer until the washings were clear. Unbroken cells were removed by centrifugation using a high-speed refrigerated centrifuge (Kubota, Model 6930, Tokyo, Japan) at 7800 × *g*, 4 °C for 15 min. The supernatant was then centrifuged with an ultracentrifuge (Hitachi, Model CP 80MX, Ibaraki-Pref, Japan) at 145,100×*g* at 4 °C for 1 h (using rotor type P40 ST) to obtain cell envelopes. The cytoplasmic membrane (inner membrane) was detached from the outer membrane by adding 0.01 mol/L HEPES containing 4% Triton X-100 (Bio-Rad, Hercules, CA). The mixture was incubated at room temperature for 10 min. The insoluble OMPs were pelleted using the ultracentrifuge at 181 800×*g* at 4 °C for 1 h (using rotor type P55 ST2). The pellet was resuspended with 4 mL of 30 mmol/L Tris–HCl, pH 8.0. The supernatant, which consisted of the inner membrane, was stored at –20°C for further use to determine the presence or location of the specific protein in it.

2.3. Determination of protein concentration

Protein concentration was determined using the Bio-Rad protein assay kit. Briefly, 1 mL of Bio-Rad protein assay stock solution was mixed with 4 mL of Phosphate Buffered Saline (PBS), pH 7.4 to make the working solution. In a microtiter plate, 140 μL of the solution was pipetted into each well and 10 μL of protein sample or protein standard (Bovine Serum Albumin standards of 50 to 500 μg) was added. The solution was mixed thoroughly and absorbance was measured at λ595 nm using the ELISA reader (Multiskan EX Thermo Labsystem, Helsinki, Finland). Relative measurement of protein concentration was determined by comparing the protein absorbance to