Iron regulated outer membrane proteins (IROMPs) as potential targets against carbapenem-resistant *Acinetobacter* spp. isolated from a Medical Centre in Malaysia

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**Background & objectives:** Carbapenem-resistant *Acinetobacter* spp. have gained increasing significance as opportunistic pathogens in hospitalized patients. Carbapenem resistance is often associated with the loss and/or decrease in outer membrane proteins (OMP) and overexpression of multidrug efflux systems. However, carbapenem-hydrolysing β-lactamases of Ambler Class B (metallo-enzymes) and Ambler Class D (oxacillinases) have also been detected in *Acinetobacter* spp. In this study we have investigated the role of the iron regulated outer membrane protein (IROMPs) and the loss of a 29-kDa OMP in carbapenem resistance of *Acinetobacter calcoaceticus*.

**Methods:** Carbapenem resistant clinical isolates (n=39) of *Acinetobacter baumannii / calcoaceticus* were used. Identification of *Acinetobacter* spp. at species level was done by amplified ribosomal DNA restriction analysis (ARDRA). MIC was evaluated using agar dilution method according to CLSI standards. Presence of outer membrane proteins were determined by SDS-PAGE. A representative strain of *A. calcoaceticus*, S26 with the loss of 29-kDa OMP was selected for further analysis as strain S26 had unique resistance mechanism, that is, the presence of IMP-4 metallo-β-lactamases. IROMPs were expressed under iron deficit conditions. Bands corresponding to IROMPs were excised from SDS-PAGE and used to immunize rabbits for the production of polyclonal antibodies. The antibodies raised against IROMPs were detected by an in-house ELISA and then used for bactericidal activity against carbapenem resistant *A. baumannii / calcoaceticus*.

**Results:** All isolates were resistant to all antibiotics including imipenem and meropenem and had loss of a 29-kDa OMP. The polyclonal antibodies showed bactericidal effect against the organism tested and it specifically killed the bacteria grown in iron deficit medium.

**Interpretation & conclusions:** In this study, a 29-kDa OMP has been identified to be the major outer membrane protein in *A. baumannii / calcoaceticus* and loss of this porin and overexpression of IROMPs have contributed to carbapenem resistance. Polyclonal antibodies raised against IROMPs may have a role in antimicrobial therapy in these isolates.

**Key words** *Acinetobacter* spp. - imipenem - iron regulated outer membrane protein (IROMPs) - meropenem - multiresistant - outer membrane protein (OMP) - SDS-PAGE
Acinetobacter baumannii / calcoaceticus are oxidase-negative, non-motile bacteria which appear as Gram-negative coccobacilli. These bacteria are widely found in nature, mostly in water and soil. This opportunistic pathogen has also been isolated from the normal flora of human skin, respiratory tract, gastrointestinal tract and various other sites in patients, especially in immunocompromised patients. A. baumannii / calcoaceticus is often associated with hospital-acquired infections such as blood stream infections, meningitis, ventilator-associated pneumonia, wound, and urinary tract infections. It has been reported worldwide and is now becoming one of the most difficult healthcare-associated infections to control and treat. The treatment of A. baumannii / calcoaceticus infections is becoming a serious problem due to multiple acquired resistances following indiscriminate use of antibiotics and often carbapenems remain as the only alternative drug for the treatment of Acinetobacter spp. infection.

The common carbapenem resistance mechanisms in A. baumannii / calcoaceticus include acquisition of carbapenemases, β-lactamases which are capable of hydrolyzing carbapenems, reduced affinity of penicillin binding proteins, and low permeability of outer membrane proteins. However, a few studies have shown the importance of the outer membrane protein in carbapenem resistant A. baumannii / calcoaceticus isolates. Almost all the previously reported studies have shown the role of OMP towards carbapenem resistant A. baumannii / calcoaceticus involving one mechanism.

Iron is one of the most important nutrients required by all forms of living organisms including microorganisms. However, the availability of iron is poor in the environment as most of it is found complexed with transferrin, lactoferrin, ferritin, haem, and haemoglobin. In order to survive in a human host, bacteria form siderophores called iron-regulated outer membrane proteins (iROMPs). It has been reported that monoclonal antibodies raised against iROMPs of A. baumannii are bactericidal. Here, in this study, we have incorporated another important factor to further analyze our findings to investigate the survival of carbapenem resistant A. calcoaceticus with the loss of 29-kDa outer membrane protein in iron deficit conditions.

**Material & Methods**

*Bacterial strains and susceptibility testing:* Isolates of carbapenem-resistant *Acinetobacter* spp. (n=39) were obtained from patients who were hospitalized in University Malaya Medical Centre, Kuala Lumpur, Malaysia, from August 2003 until March 2004. This included one representative strain of *A. calcoaceticus* S26 which had a unique resistance mechanism, i.e., the presence of IMP-4 metallo-β-lactamases. The strains were obtained from invasive and non invasive sites which include blood, tracheal secretion, sputum, throat swab, peritoneal fluid, wound, bronchial lavage, and urine. Besides that, these strains were also selected based on their resistance to carbapenems, imipenem and meropenem. API20NE System (bioMérieux SA, Marcy-l’Etoile, France) and growth at 44°C was performed to identify and differentiate between A. baumannii and A. calcoaceticus. Further species level identification was done by amplified ribosomal DNA restriction analysis (ARDRA) as described by Johannes *et al*. Briefly, 1500 bp fragment of the 16S rRNA gene was amplified using the universal primers - forward 5’-GGCTCAGATTGAACGCTGCGGC-3’ and reverse 5’-TACCTGTTACGACTCTC-3’. The PCR reaction mixture of the final volume of 50 µl contained 20 µM deoxynucleotide triphosphate, 1x reaction buffer, 1.5 mM MgCl₂, 2.5 U Taq DNA polymerase (Fermentas, Lithuania, USA) and approximately 1 µl of template DNA. The thermocycle protocol used was: an initial denaturation step at 94°C for 5 min, followed by 25 cycles of denaturation at 94°C for 45 sec annealing at 58°C for 45 sec, elongation at 72°C for 3 min, followed by a final extension step at 72°C for 7 min. The restriction enzymes *AluI*, *CfoI*, *MboI*, *RsaI* and *MspI* (Gibco BRL, Paisley, UK) were used in separate reactions to digest the amplimers, according to the manufactures’ instructions. Restriction fragments were resolved in 2 per cent agarose gel and the profiles were compared with those reported earlier.

Minimum inhibitory concentrations (MICs) for the *A. baumannii / calcoaceticus* isolates were determined and interpreted according to Clinical Laboratory Standard Institute (CLSI, 2005). The antibiotics used in this study include imipenem, meropenem, ceftazidime, cefotaxime, gentamicin, amikacin, ciprofloxacin and ampicillin.

*Outer membrane protein extraction:* Outer membrane proteins were prepared based on the modified method of Matsuyama *et al*. Briefly, *A. calcoaceticus* was grown on 50 ml of iron rich medium and on iron-deficit medium to early stationary growth phase. Cells were pelleted by centrifugation for 10,000 X g for 20 min at 4°C, washed twice with 10 mM phosphate buffer, pH...
The cells were then resuspended in 20 ml of the same buffer before being subjected to cell disruption by sonication for 30s at 30 KHz output with 30 sec intervals. Cell debris was removed by centrifugation at 10,000 X g for 20 min at 4°C. The supernatant was transferred to an ultra tube and the outer membrane fraction was pelleted by ultra centrifugation at 100,000 X g for 35 min at 4°C. The pellet was then resuspended in 20 ml of 10 mM phosphate buffer, pH 7.2 containing 2 per cent (v/v) Triton X-100. The suspension was incubated at 37°C for 20 min. The Triton X-100 insoluble fraction was recovered by ultracentrifugation at 100,000 X g for 35 min at 4°C. The pellet was then washed once with 10 ml phosphate buffer, pH 7.2 and recovered by centrifugation at 100,000 X g for 35 min at 4°C. The protein pellet was resuspended in 100 µl of Laemmli buffer (Sigma-Aldrich, St. Louis, USA) and stored at -20°C till use. The concentration of protein was determined using Bradford method and the protein samples were analyzed via SDS-PAGE.

**SDS-PAGE:** SDS-PAGE analysis was performed with the mini-protein II system (8.2 by 7.2 by 0.75 cm; BioRad Laboratories, USA). The stacking gel and separating gel contained 4 and 12 per cent acrylamide respectively. A total of 25 µg of protein of each sample was denatured by boiling for 10 min and cooled immediately prior to loading. Electrophoresis was carried out at constant voltage at 100 V for 90 min. The protein gels were visualized by staining with Coomassie blue R (Sigma, St. Louis, USA). The apparent molecular weights of the proteins detected were estimated by comparison with a broad-range protein standard obtained from New England Biolabs, United Kingdom. One well served as a negative control which contained only sample dilution buffer and loading dye.

**Extraction of IROMPs:** IROMPs were characterized according to a modified method of Goel and Kapil. Briefly, *Acinetobacter calcoaceticus* were grown in iron depleted chemically defined medium (CDMFe<sup>-</sup>) and in iron rich (CDMFe<sup>+</sup>) medium. Medium without iron (CDMFe<sup>-</sup>) contained less than 0.04 mM Fe<sup>3+</sup>, and for medium with iron (CDMFe<sup>+</sup>), FeSO<sub>4</sub> was added to give a concentration of 60 mM. Overnight cultures in CDMFe<sup>-</sup> and CDMFe<sup>+</sup> were allowed to grow at 37°C with aeration (150 rpm) until late log phase at optical density of (OD<sub>600</sub>) of 0.4. The cultures were then subjected to OMPs extraction according to the modified method of Matsuyama et al. IROMPs extracted from the SDS-PAGE gel were used as antigen to immunize rabbits.

**Preparation of polyclonal sera:** To raise polyclonal antibodies in rabbit, a first dose of IROMP (300 µg/ml) was given intraperitoneally (ip) mixed with 250 µl of complete Freund’s adjuvant (CFA) followed by three doses (300 µg/ml each) in Freund’s incomplete adjuvant at intervals of one week. A final booster was given in normal saline, intraperitoneally, just four days before sacrifice. The experiments reported herein were conducted according to the principles in the Guidelines for the Care and Use of Laboratory Animals. An ELISA was carried out to test the reactivity of the polyclonal antibodies from rabbit sera with the IROMP (10 µg/ml). Briefly, 96-well microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with antigen diluted in phosphate buffer saline (PBS). The plates were washed four times by dispensing 250 µl PBS-T (PBS + 0.05% Tween) to each well. Then, the plates were blocked with 1 per cent BSA in PBS for 1 h at 37°C, and washed four times with PBS-T. One hundred microlitres of rabbit sera dilute 2-fold serially was added to the wells. The plate was incubated for 1 h at room temperature and washed four times with PBS-T. Goat anti-rabbit IgG horseradish peroxidase (Incstar Corporation, Stilwater Minnesota, USA) diluted at 1:2,000 was added to the wells. After 1 h of incubation at room temperature, the plates were washed four times with PBS-T and the last wash was with PBS only before the addition of the 2, 2’-azinobis (3-ethylbenzthiazoline sulphonic acid) (ABTS) substrate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, Meryland, USA). Optical densities at 410 nm (OD<sub>410</sub>) were measured after 15 min of incubation at room temperature.

**Bactericidal activity:** Total viable count was performed to demonstrate bactericidal activity of polyclonal antibodies raised against IROMPs according to the modified method described by Poolman et al. First, a standard growth curve was plotted and used as control. Briefly, 1 ml of known amount of *A. calcoaceticus* culture approximately 10<sup>8</sup> cfu/ml was inoculated into CDMFe<sup>-</sup> medium and CDMFe<sup>+</sup> medium. The cultures were vortexed well and incubated at 37°C with shaking at 250 rpm. Every half an hour, 10 µl of the culture suspensions were diluted in 90 µl of Luria broth in a test tube and followed by a 10-fold serial dilution until to 10<sup>6</sup> cfu/ml. Each serially diluted culture suspensions about 100 µl each was spread onto Mueller-Hinton agar plates. The plates were incubated at 37°C overnight. Total viable count was done between 18 and 24 h. This procedure was repeated every half hour until the bacterial growth reached late log phase. Following
this, total viable count method was performed on polyclonal antibodies to obtain bactericidal activity. In this experiment, polyclonal antibodies with 2 different dilution factors were used to obtain different concentration of antisera; 1:5 and 1:10. The appropriate concentrations of polyclonal antibodies were added to the *A. calcoaceticus* culture medium at early stationary phase. Finally, the growth curve graphs were plotted and the data obtained were analyzed.

**Results & Discussion**

All the 39 isolates were identified as *A. baumannii-calcoaceticus* complex using the API20NE system. Growing at 44°C showed that 36 were identified as *A. baumannii* and 3 were *A. calcoaceticus*. The species were further confirmed by amplified ribosomal DNA restriction analysis (Fig. 1). All isolates were multi-resistant to all the antimicrobials tested including imipenem and meropenem (Table). In a previous study we have shown that Class 1 integrons were detected in 31 of 39 isolates\(^8\). Two of the isolates harboured an integron-borne *blaIMP*-4 metallo-β-lactamase, 1 of which was located on a 36-kb plasmid.

The OMP profile via SDS-PAGE showed that all the carbapenem resistant *A. baumannii / calcoaceticus* isolates had a missing OMP band at about 29-kDa (marked by the red arrow) as shown in Fig. 2 suggesting that loss of a 29-kDa OMP is one of the leading factors to carbapenem resistance among these *A. baumannii / calcoaceticus* isolates.

The S26 isolate was used as representative of carbapenem resistant isolate with the loss of 29-kDa OMP and its survival when grown in iron deficit medium, was investigated. S26 was seen to overexpress bands corresponding to IROMPs in comparison to the isolate which was grown in the presence of iron in the medium (Fig. 3).

The polyclonal antibodies raised against IROMPs in rabbits showed bactericidal activity against the organism tested and specifically killed the bacteria grown in iron deficit medium in a dose dependant manner (Fig. 4). Five-fold dilution of the polyclonal antibody reduced the growth of *A. calcoaceticus* from 10^6^ to 10^4^ cfu/ml from the time point of 3 to 3.5 h up to 7 h suggesting that the polyclonal antibodies raised against IROMPs are bactericidal. However, in the presence of iron, the bactericidal effect of the polyclonal antibodies was only observed up to the time point of 1.5 to 2 h (Fig. 5) after which the organism recovered from the bactericidal effect and started to grow well.

<table>
<thead>
<tr>
<th>β-lactams</th>
<th>MIC (µg/ml) for isolates (Σn=39)</th>
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<tr>
<td></td>
<td>Animal study (n=1) S26</td>
</tr>
<tr>
<td>Imipenem</td>
<td>32</td>
</tr>
<tr>
<td>Meropenem</td>
<td>128</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>256</td>
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<td>Cefotaxime</td>
<td>512</td>
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**Table.** MICs of β-lactams for S26 (representative strain used for animal study) and other 38 isolates

![Fig. 1. RE Digestion of 16S rDNA using *AluI*, *CfoI* and *MboI* enzymes for species level identification.](image-url)
Understanding the contribution of OMPs to antibiotic resistance in *A. baumannii* has been a challenge. Unfortunately, it is difficult to accurately compare the loss of OMPs as there is variability in the number of observed OMPs\(^{10}\). Reduced expression of many different OMPs have been reported in various studies\(^{9,11,12,13,16}\).

Recently, a 43-kDa protein in *A. baumannii* was identified as a homologue of OprD; a well-studied OMP which is frequently associated with imipenem resistance in *P. aeruginosa*\(^3\). We demonstrated a dual role for the OMP in this study. As suggested earlier\(^20\), blocking the iron uptake pathways of the bacteria might reduce the uptake of iron, thus inhibiting the growth of the bacterium. Therefore, this could be a good target for antimicrobial therapy as antibodies produced against IROMPs may have a protective role in the course of infection in human host.

In conclusion, our study showed that the dual mechanisms of outer membrane protein are important factors leading to carbapenem resistance mechanisms among *A. baumannii/calcoaceticus* isolates. There are however, other important factors such as carbapenemases, oxacillinases, mobile genetic elements, and efflux pump which may contribute to carbapenem resistance in *A. baumannii/calcoaceticus* and act via one or multi-mechanisms. Hence, understanding carbapenem resistance mechanisms might be crucial for the development of novel therapeutic strategies.

References


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