Typing and characterization of carbapenem-resistant Acinetobacter calcoaceticus–baumannii complex in a Chinese hospital

Yun-Song Yu, Qing Yang, Xiao-Wei Xu, Hai-Shen Kong, Gen-Yun Xu and Bu-Yun Zhong

INTRODUCTION

Acinetobacter species can be found in the natural environment, hospital surroundings and on the skin of the human body. It is an important opportunistic nosocomial pathogen and is particularly important in hospital-acquired pneumonia, especially in immunocompromised patients and those with tracheotomy or mechanical ventilation (Forster & Daschner, 1998). Members of the Acinetobacter calcoaceticus–baumannii complex (Acb complex) are the predominant acinetobacter in clinical settings, and isolates are usually multiresistant, complicating therapy. Carbapenems have become the drugs of choice for serious acinetobacter infections in our country and have retained better activity than other antimicrobials. Nevertheless, reports of carbapenem resistance among Acinetobacter species are accumulating steadily (Afzal-Shah & Livermore, 1998). Some early reports described acinetobacters with β-lactamase-independent carbapenem resistance (Clark, 1996; Gehrlein et al., 1991; Urban et al., 1995), but most recent reports describe β-lactamase-mediated resistance. The first known Acinetobacter bauman­nii isolate with a carbapenem-hydrolysing β-lactamase was collected in 1985 in Scotland, and the enzyme was initially designated ARI-1 (Paton et al., 1993), now renamed OXA-23 (Donald et al., 2000). Resistance mediated by IMP- and VIM-type metallo-β-lactamases has been reported subsequently in acinetobacters from Cuba (Perez et al., 1991; Urban et al., 1995), Italy (Cornaglia et al., 1999), Hong Kong (Chu et al., 2001), Japan (Takahashi et al., 2000) and Korea (Yum et al., 2002). However, most carbapenem-resistant acinetobacters have OXA-type β-lactamases with a weak activity against carbapenems; such enzymes have been found in A. bauman­nii isolates from Argentina, Belgium, Kuwait, Scotland, Spain and Singapore (Afzal-Shah & Livermore, 1998; Afzal-Shah et al., 2001; Bou et al., 2000; Donald et al., 2000; Hornstein et al., 1997). Several of these enzymes have been sequenced and are found to form a subgroup among class D β-lactamases, presently comprising the OXA-23, -24, -25, -26, -27 and -40 types (Afzal-Shah et al., 2001; Bou et al., 2000; Donald et al., 2000; Héritier et al., 2003). During the period 2000 to 2002,
the resistance rate of *Acinetobacter* species to imipenem rose from 9 to 18 % in our hospital. It remains unknown whether these carbapenemases are present in these isolates. Thus, we carried out this study to demonstrate the resistance pattern and carbapenemase type prevalent among carbapenem-resistant Acb complex in our hospital.

**METHODS**

**Bacterial isolates.** Forty-five carbapenem-resistant (resistant to both imipenem and meropenem) Acb complex isolates were obtained from the First Affiliated Hospital, College of Medicine, Zhejiang University between October 2000 and September 2002. These isolates from clinical specimens were identified using the VITEK GNI system. The source of these isolates included sputum (*n* = 39), abdominal drainage (*n* = 4), venous line (*n* = 1) and pericardial effusion (*n* = 1).

**Susceptibility testing.** E-test was performed to test the susceptibility of clinical isolates. Twelve antibacterial agents were tested: imipenem, meropenem, ceftazidime, cefotaxime, cefepime, piperacillin/tazobactam, aztreonam, amikacin, ticarcillin/clavulanic acid, cefoperazone/sublactam, ampicillin/sublactam, ciprofloxacin. *Pseudomonas aeruginosa* ATCC 27853 was used as a reference strain for quality control. The data were analysed with WHONET 5 software.

**PFGE.** The procedures were based on the method of Seifert & Gerner-Smidt (1995) with some modification. Pure bacterial cultures were embedded into plugs of low-melting-point agarose after overnight incubation. The plugs were incubated with proteinase K for 48 h at 56 °C and then incubated overnight with 30 µg restriction endonuclease Apal. The digested plugs were loaded into the wells of a 1 % PFGE gel in 0.5 × TBE buffer. Electrophoresis was performed in a CHEF-Mapper XA pulsed-field electrophoresis system for 22 h at 14 V/cm with an electric field of 6 V cm⁻¹ and pulse angle of 120 °. The resultant product was transfected into competent DH5α plasmid DNA was prepared by alkaline lysis, and purified DNA (3–10 ng) was ligated into the pGEM-T Easy vector overnight. The purified DNA (3 µl) was digested with EcoRI digestion and sequenced using an ABI 377 automatic sequencer using the Sanger chain-termination method. The results were compared with data in GenBank.

**Preparation of β-lactamase crude extract and IEF.** β-Lactamase was extracted from 45 isolates of carbapenem-resistant Acb complex and identified using a nitrocefin disc method. Values of *k* was determined according to the instructions of the PhastSystem electrophoresis system (Pharmacia Biotech). The gel was stained with nitrocefin following electrophoresis. In the inhibition assay, the bacteria were first covered with filter paper containing 0.5 mM clavulanic acid or 0.5 mM clavulanic acid for 30 s, followed by nitrocefin stain at the same concentration. Reference standard protein was stained with Coomassie brilliant blue R-250. The pattern was analysed using Curve Expert software 1.3.

**Chromosomal DNA homology.** PFGE patterns showed that the 45 isolates of Acb complex were classified into two genotypes, type A and B. Type A was dominant (*n* = 44), with four subtypes A1 (*n* = 10), A2 (*n* = 32), A3 (*n* = 1) and A4 (*n* = 1). Subtype A1 was the dominant subtype in our hospital from October 2000 to May 2001, being found in the ICU (*n* = 6), liver transplantation unit (*n* = 2) and urology (*n* = 1). From June 2001, subtype
A2 became the prevalent subtype in the hospital (Fig. 1). An outbreak caused by this subtype was documented during the period from May to September 2002. This subtype was isolated from 21 patients (21/33), most from the ICU (n = 17), others from the respiratory department (n = 1), liver transplantation unit (n = 1), thoracic surgery wards (n = 1) and geriatric wards (n = 1). The only type B isolate (isolate 16) was from haematology (see supplementary data online).

### Carbapenemase produced by Acb complex

The three-dimensional test confirmed that 43 of 45 isolates produced an imipenem-hydrolysing β-lactamase. This enzyme was not inhibited by clavulanic acid or cloxacillin. The metalloenzyme screening test indicated that this enzyme was not inhibited by 2-mercaptoethanesulfonic acid. All isolates tested were negative for OXA-24 by PCR amplification. Only one isolate was positive for OXA-23 (isolate 16). The amplified band was approx. 1000 bp. Cloning and sequencing confirmed that the sequence of the PCR product was the same as published OXA-23 gene sequence. IEF analysis showed that 42 isolates had two bands, of different pIs (6.40 and 7.01). Only isolate 16 had bands of 6.64 and 7.17 on IEF analysis. All bands were not inhibited by clavulanic acid or cloxacillin.

### DISCUSSION

Carbapenem antibiotics have the most extended spectrum of antibacterial activity among all β-lactams. They are stable to extended-spectrum β-lactamases (ESBLs) and AmpC produced by Gram-negative organisms. However, carbapenem resistance is emerging and increasing in clinical isolates, especially in *P. aeruginosa* and *A. baumannii*, as such antibiotics are increasingly used in clinical practice.

Our study suggested that imipenem-resistant Acb complex was also highly resistant to meropenem. All the isolates tested were multiresistant. The most active agents against these resistant isolates were cefoperazone/sulbactam and ampicillin/sulbactam, with susceptibility rates of 63.0% and 43.5%, respectively. This may be due to the unique activity of sulbactam against *Acinetobacter* species. Sulbactam acts synergistically with cephalosporins in the treatment of infections caused by such isolates. These results are consistent with previous reports from other countries (Levin et al., 2003). Most isolates of Acb complex were intermediate resistant to ceftazidime, cefotaxime and cefepime, and highly resistant to amikacin, aztreonam, piperacillin/tazobactam and ticarcillin/clavulanic acid. PFGE patterns indicated that the prevalence of carbapenem-resistant Acb complex in our hospital was high.

### Table 1. MIC of 12 antibiotics against Acb complex

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (mg l⁻¹)</th>
<th>Susceptibility (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>MIC₅₀</td>
</tr>
<tr>
<td>Cefoperazone/sulbactam</td>
<td>2–256</td>
<td>8</td>
</tr>
<tr>
<td>Ampicillin/sulbactam</td>
<td>4–¡256</td>
<td>12</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>8–¡256</td>
<td>16</td>
</tr>
<tr>
<td>Cefepime</td>
<td>8–¡256</td>
<td>16</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0–125–¡32</td>
<td>32</td>
</tr>
<tr>
<td>Amikacin</td>
<td>4–¡256</td>
<td>256</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>32–¡256</td>
<td>256</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>16–¡256</td>
<td>256</td>
</tr>
<tr>
<td>Ticarcillin/clavulanic acid</td>
<td>¡256</td>
<td>256</td>
</tr>
<tr>
<td>Imipenem</td>
<td>¡32</td>
<td>32</td>
</tr>
<tr>
<td>Meropenem</td>
<td>¡32</td>
<td>32</td>
</tr>
</tbody>
</table>

Fig. 1. PFGE patterns of selected imipenem-resistant isolates. The PFGE pattern is identical for lanes 1 and 3–10. These isolates were from the same clone and belonged to the prevalent subtype A1. Lanes 11–14 had the same pattern and were defined as subtype A2. Lane 2 was defined as subtype A3. M, λ DNA ladder marker.
hospital was due to an epidemic isolate. Subtype A1 was the dominant isolate before May 2001. Subtype A2 was prevalent after June 2001, and an outbreak due to A2 developed from May to September 2002. Subtype A2 was isolated from 21 patients. Therefore, measures should be taken to control the spread of this epidemic isolate.

Carbapenem resistance may be mediated by one of four mechanisms: enzymic inactivation by β-lactamase, loss of outer-membrane porin, alteration of penicillin-binding protein and specific drug efflux pumps (Nakae et al., 1999). In recent years, the number of reports of acquired carbapenemase in common pathogens such as P. aeruginosa, A. baumannii and Enterobacteriaceae has increased (Nordmann & Poirel, 2002). Outbreaks caused by these ESBLs-producing isolates make the situation worse. Few effective agents are available for these infections, which have a high mortality rate. Major carbapenemases found in Acinetobacter species were metalloenzyme and OXA-type enzymes. A preliminary study on the carbapenemases revealed that all resistant isolates isolated in our hospital produced an imipenem-hydrolysing carbapenemase. However, OXA-23 was detected in only one isolate. As reported previously (Donald et al., 2000), the pI of this enzyme was 6.64. Metalloenzymes were not found in any of these isolates. These results suggest that carbapenem resistance of Acb complex in our hospital was not mediated by a metalloenzyme. There may be another unknown carbapenemase that cannot be inhibited by clavulanic acid, cloxacinil or 2-mercaptoopropanoic acid.

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REFERENCES


