

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

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This study was designed to investigate the prevalence of carbapenem-resistant *Acinetobacter calcoaceticus*–*baumannii* complex (*Acb* complex) and to type carbapenemases. The relatedness of 45 isolates of carbapenem-resistant *Acb* complex collected from a clinical setting was analysed by PFGE. The carbapenemases produced by these isolates were typed by IEF, a three-dimensional test, 2-mercaptopropanoic acid inhibition assay, PCR and DNA cloning and sequencing. Results showed that all 45 isolates were resistant to multiple antibiotics including meropenem. The resistance rates to cefoperazone/sulbactam and ampicillin/sulbactam were 2.2 and 6.5 %, respectively. About 71.7–78.3 % of these isolates were intermediately resistant to cefepime, ceftazidime and cefotaxime. Forty-five isolates were classified into type A (98 %) and B (2 %) based on their PFGE patterns. Most of type A isolates were from the ICU. Type A was the dominant isolate, including subtypes A1 (22 %), A2 (71 %), A3 (2 %) and A4 (2 %). Only one isolate, from the haematology department, belonged to type B. Forty-three isolates (96 %) were positive for carbapenemase. One isolate had two bands by IEF, the pIs of which were 6.64 and 7.17. The band with the pI of 6.64 was OXA-23. The other 42 isolates produced two bands with pIs of 6.40 and 7.01 which could not be inhibited by clavulanic acid, cloxacillin or 2-mercaptopropanoic acid. It can be concluded that the prevalent carbapenem-resistant *Acb* complex isolates from this hospital all had similar β -lactamase patterns.

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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 car-

bapenem 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.*, 1996), 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,

Abbreviation: *Acb* complex, *Acinetobacter calcoaceticus*–*baumannii* complex.

A dendrogram of the PFGE results is available in JMM Online.

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/sulbactam, ampicillin/sulbactam, 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 *Apa*I. 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 °C, with an electric field of 6 V cm⁻¹ and pulse angle of 120°, and the pulse time increased from 5 to 20 s. A λ DNA ladder was used as molecular mass marker. Resultant bands were stained with ethidium bromide and observed under UV light. The interpreting criteria were described by Tenover *et al.* (1995). The relationships between all isolates were analysed using the SPSS software package and presented as a dendrogram.

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 pIs were 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 cloxacillin 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.

Three-dimensional test to determine imipenem-hydrolysing ability. A colony of *Escherichia coli* ATCC 25922 strain was suspended to approximately 10⁸ c.f.u. ml⁻¹ in Mueller–Hinton (MH) broth and spread on MH plates with a cotton swab. An imipenem disc (10 µg; Oxoid) was put on the centre of the plate. Four slots of 1 × 15 mm perpendicular to the edge of the disc were cut out. The slots were 5 mm from the edge of the imipenem disc. The following samples were added to the slots: 40 µl crude enzyme extract, crude enzyme extract plus 2 mM clavulanic acid (36 µl crude enzyme extract plus 4 µl clavulanic acid), crude enzyme extract plus 2 mM cloxacillin (36 µl crude enzyme extract plus 4 µl cloxacillin) and 40 µl PBS (negative control). After incubation at 35 °C for 24 h, arrow-like lawns of bacterial growth around the slot in the direction of the imipenem disc indicated that the enzyme could hydrolyse imipenem. The growth pattern on the plate also revealed

whether or not the enzyme was inhibited by clavulanic acid or cloxacillin.

Metalloenzyme assay. A colony of each acinetobacter isolate was suspended to 10⁸ c.f.u. ml⁻¹ with MH broth and spread on an MH plate. An imipenem disc (10 µg) was put on the centre of the plate. A blank disc (without antibiotic) was put 2.5 cm away from the edge of the imipenem disc and 2 µl 2-mercaptopropanoic acid stock solution was added. The plate was incubated overnight at 35 °C. Enlargement of the imipenem-inhibition zone near the 2-mercaptopropanoic acid disc indicated production of a metalloenzyme (Arakawa *et al.*, 2000). A positive control (IMP-4-producing *A. baumannii*) was kindly provided by Prince of Wales Hospital, Chinese University of Hong Kong.

PCR amplification of OXA gene. A heating and boiling method was used to prepare the templates for PCR amplification. Primers were designed according to Afzal-Shah *et al.* (2001). Primers used in this amplification were: OXA-23: P1, 5'-GATGTGTCATAGTATTCGTCG-3'; P2, 5'-TCACAACAATAAAAGCACTG-3'; OXA-24: P3, 5'-GTACTAATCAAAGTTGTGAA-3'; P4, 5'-TTCCCCTAACATGAATTGT-3'. The PCR system (50 µl) was composed of 1× PCR buffer, 2 mM MgCl₂, 200 µM dNTPs, 500 µM primers, 1.6 U *Taq* enzyme and 10–100 ng DNA template. Parameters for PCR were pre-denaturation at 94 °C for 5 min, followed by 30 cycles of 94 °C for 25 s, 50 °C for 45 s and 72 °C for 90 s, followed by a final extension at 72 °C for 10 min.

Purification, cloning and sequencing of PCR product. PCR products were purified using a commercial DNA purification kit. The purified DNA (3 µl) was ligated into the pGEM-T Easy vector overnight at 4 °C. The resultant product was transfected into competent *E. coli* DH5α, and inoculated on a MacConkey plate supplemented with 50 µg ampicillin ml⁻¹. White colonies were picked and inoculated into LB medium containing ampicillin. Flasks were incubated overnight at 37 °C with shaking. Plasmid DNA was prepared by alkaline lysis, identified by *Eco*RI digestion and sequenced with an ABI 377 automatic sequencer using the Sanger chain-termination method. The results were compared with data in GenBank.

RESULTS

Antibiotic susceptibility of *Acb* complex

MICs of 12 antibiotics against *Acb* complex are presented in Table 1. All 45 isolates of *Acb* complex were resistant to both imipenem and meropenem. The resistance rates to cefoperazone/sulbactam and ampicillin/sulbactam were the lowest, at 2.2 and 6.5 %, respectively. Approx. 19.6, 23.9 and 15.2 % of these isolates were resistant to ceftazidime, cefotaxime and cefepime, respectively, and 71.7–78.3 % were intermediately resistant. *Acb* complex isolates tested were highly resistant to piperacillin/tazobactam, ticarcillin/clavulanic acid, ciprofloxacin, aztreonam and amikacin, with resistance rates ranging from 93.5 to 100 %.

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

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

Antibiotic	MIC (mg l ⁻¹)			Susceptibility (%)		
	Range	MIC ₅₀	MIC ₉₀	Resistant	Intermediate	Susceptible
Cefoperazone/sulbactam	2–256	8	24	2.2	34.8	63.0
Ampicillin/sulbactam	4–≥256	12	64	6.5	50.0	43.5
Ceftazidime	8–≥256	16	64	19.6	71.7	8.7
Cefepime	8–≥256	16	48	15.2	78.3	6.5
Cefotaxime	8–≥256	32	96	23.9	73.9	2.2
Ciprofloxacin	0.125–≥32	32	32	95.7	0	4.3
Amikacin	4–≥256	256	256	93.5	4.3	2.2
Aztreonam	32–≥256	256	256	100	0	0
Piperacillin/tazobactam	16–≥256	256	256	97.8	0	2.2
Ticarcillin/clavulanic acid	≥256	256	256	100	0	0
Imipenem	≥32	32	32	100	0	0
Meropenem	≥32	32	32	100	0	0

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 en-

zyme was not inhibited by clavulanic acid or cloxacillin. The metalloenzyme screening test indicated that this enzyme was not inhibited by 2-mercaptopropanoic 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 intermediately 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

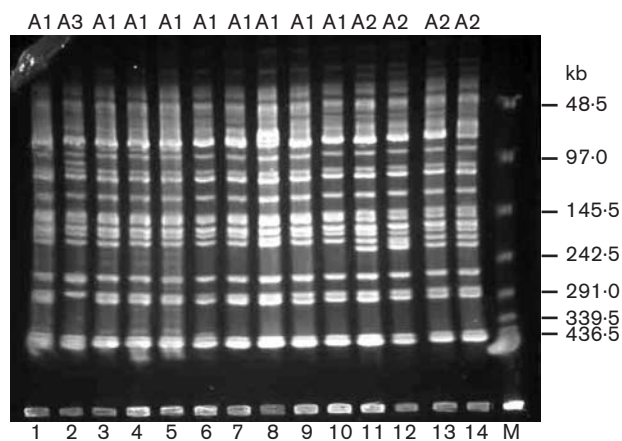


Fig. 1. PFGE patterns of selected imipenem-resistant isolates. The PFGE pattern is identical for lanes 1 and 3–10. These isolates were from the same clone and belonged to the prevalent subtype A1. Lanes 11–14 had the same pattern and were defined as subtype A2. Lane 2 was defined as subtype A3. M, λ 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, cloxacillin or 2-mercaptothiopropanoic acid.

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