Dissemination of imipenem-resistant *Acinetobacter baumannii* strains carrying the IS*Aba1*–*bla*_{OXA-23} genes in a Chinese hospital

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An outbreak of 95 clinical infections with imipenem-resistant *Acinetobacter baumannii* in a Chinese hospital was investigated and the carbapenemase-encoding genes and their relationship with IS*Aba1* of these and a further 16 isolates recovered from the intensive care unit (ICU) environment were analysed. Almost all isolates were resistant to a wide range of antimicrobials; the lowest resistance rates were found for polymyxin E (17.1%), cefoperazone/sulbactam (30.6%) and ampicillin/sulbactam (67.6%). Six pattern types defined by DNA macrorestriction patterns were distinguished among the clinical isolates with dissemination of pattern A (50 isolates) to patients in seven hospital units and pattern B (35 isolates) to eight units; the environmental isolates from ICUs were also of pattern A. All isolates were positive for the *bla*_{OXA-66} and *bla*_{OXA-23} genes. The OXA-23-encoding gene was located 34 bp downstream of IS*Aba1*. No plasmids were detected and conjugal transfer of resistance was not demonstrated. The *bla*_{OXA-23} probe hybridized with 200 and 220 kb *Apal* chromosomal fragments for type patterns A and B, respectively.

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INTRODUCTION

Acinetobacter baumannii is an increasingly important nosocomial pathogen (Bergogne-Bérézin & Towner, 1996), causing a wide range of infections, some serious, in patients in intensive care units (ICUs) (Chastre & Trouillet, 2000). The species can survive for long periods in the hospital environment and is a frequent cause of outbreaks of nosocomial infection, which are often difficult to control (Towner, 2000). Effective treatment is compromised by the high level of resistance to antimicrobials exhibited by hospital strains. Indeed, the emergence of carbapenem resistance in *A. baumannii* has become of global concern, as these β -lactams are often the only active agents against many multiresistant strains (Brown & Amyes, 2006). These agents are being compromised by the emergence of carbapenem-hydrolysing β -lactamases (carbapenemases) of molecular classes B and D (Livermore, 2002). Class B carbapenemases (various IMP-type, VIM-type and SIM-1 metallo- β -lactamases) have been found in *Acinetobacter* spp., but worldwide most *A. baumannii* strains are resistant as a result of the production of OXA-type carbapenemases (Afzal-Shah *et al.*, 2001). Four of the eight known clusters of OXA-type carbapenemases

have been identified in *A. baumannii*, namely OXA-23, OXA-24, OXA-51 and OXA-58 (Brown *et al.*, 2005; Walther-Rasmussen & Hoiby, 2006). The genes encoding OXA-23-like and OXA-51-like enzymes have been found to be linked to IS*Aba1*, with the OXA-58-like encoding gene adjacent to IS*Aba2*, IS*Aba3* and IS*18* (Turton *et al.*, 2006; Poirel & Nordmann, 2006a).

Over a 17-month period, we observed the dissemination of imipenem-resistant *A. baumannii* isolates in clinical infections in several units of a university hospital in China. We describe here the molecular epidemiology of the isolates and the identification of the genes encoding resistance to carbapenems.

METHODS

Bacterial isolates. Ninety-five non-repetitive imipenem-resistant isolates of *A. baumannii* were recovered from clinical infections in hospitalized patients from August 2003 to December 2004 in the First Affiliated Hospital, College of Medicine, Zhejiang University, China. The patients were distributed among ten clinical units, with the majority in the transplant unit (35.6%) and ICU (18.9%) (Table 1). An additional 16 *A. baumannii* isolates were recovered from the environment of the ICU (bed sheets, desks, floors, computer keyboards and mouse, and healthcare workers' hands) in December 2004. All isolates were assigned to the *Acinetobacter calcoaceticus*–*A. baumannii* complex using a Vitek GNI⁺ card (bioMérieux) and species identification was confirmed by sequence analysis of the 16S–23S rRNA gene intergenic spacer region (Chang *et al.*, 2005).

Abbreviation: ICU, intensive care unit.

The GenBank/EMBL/DBJ accession number for the IS*Aba1*–OXA-23 sequence reported in this work is DQ923478.

Table 1. Distribution of DNA pattern types of imipenem-resistant *A. baumannii* in clinical units

ICU, intensive care unit; EICU, emerging ICU; VIP unit, Very Important Person unit; GI unit, gastrointestinal tract unit.

DNA type	Transplant unit	ICU	EICU	Kidney unit	Medical dept	Respiratory unit	VIP unit	Neurology unit	GI unit	Vascular unit	Total
A	23	16	2	4	2		2			1	50
B	10	2	7	6	7	1	1		1		35
C			1	1	2	1		1			6
D						2					2
E				1							1
F	1										1
Total	34	18	10	12	11	4	3	1	1	1	95

Susceptibility testing. The MICs of nine antimicrobial agents for all isolates were determined by the agar dilution technique on Mueller–Hinton agar plates containing serially twofold-diluted agents following the Clinical and Laboratory Standards Institute guidelines (CLSI, 2006). The agents tested were imipenem (MerckKGaA), meropenem (Sumitomo Pharmaceuticals), piperacillin (Sigma), ceftazidime (Sigma), cefepime (Sino-American Shanghai Squibb Pharmaceuticals), aztreonam (Sigma), amikacin (Sigma), gentamicin (Sigma) and ciprofloxacin (Bayer). The activity of six other agents, ampicillin/sulbactam, cefoperazone/sulbactam, piperacillin/tazobactam, polymyxin E, minocycline and trimethoprim/sulfamethoxazole, was determined by Etest (AB Biodisk). *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls. Results were interpreted according to published recommendations (CLSI, 2006).

PFGE. DNA was prepared from isolates and cleaved with 40 U *ApaI* (Sangon) as described previously (Gouby *et al.*, 1992). Electrophoresis was performed on a 1% agarose gel (Sangon) in 0.5 M Tris/borate/EDTA buffer on a CHEF-Mapper XA PFGE system (Bio-Rad) for 22 h at 14 °C, with run conditions of 6 V cm⁻¹, a pulse angle of 120° and pulse times from 5 to 20 s. A λ DNA ladder (Amersham Biosciences) was used as molecular mass marker and bands were stained with ethidium bromide (0.5 mg l⁻¹) and photographed under UV light. Band profiles were interpreted by the criteria of Tenover *et al.* (1995).

Isolation and characterization of β -lactamases. Crude β -lactamase extracts from bacterial suspensions were prepared by sonication (Yu *et al.*, 2004) and pIs were determined by electrophoresis in a PhastSystem (Amersham Pharmacia Biotech) following staining with nitrocefin (Oxoid). Reference standard proteins were stained with Coomassie brilliant blue R-250. An OXA-23-producing *A. baumannii* isolate was used as a positive control (Yu *et al.*, 2004). Patterns were analysed with CURVE EXPERT software version 1.3.

Detection of metallo- β -lactamases. An imipenem/EDTA double disc synergy test was performed according to Lee *et al.* (2003). IMP-4-producing *A. baumannii* (Chu *et al.*, 2001) and VIM-2-producing *P. aeruginosa* (Yu *et al.*, 2006) were used as positive controls.

PCR amplification of the carbapenemase-encoding gene. The PCR system and parameters used have been described previously (Jeon *et al.*, 2005; Lee *et al.*, 2005; Woodford *et al.*, 2006). The primers used to amplify the IMP-like, VIM-like, SIM-1, *bla*_{OXA-23}-like, *bla*_{OXA-24}-like, *bla*_{OXA-58}-like and *bla*_{OXA-51}-like genes are listed in Table 2. PCR mapping experiments using combinations of the IS*Aba1* primers and the *bla*_{OXA-23}-like and *bla*_{OXA-51}-like reverse primers were performed to detect the IS*Aba1*/*bla*_{OXA-23}-like, IS*Aba1*/*bla*_{OXA-51}-like and *bla*_{OXA-51}-like/IS*Aba1* sequences.

Sequencing of PCR products. Purified PCR products were cloned into the pGEM-T Easy vector (Promega) according to the manufacturer's instructions. The resultant product was transformed into competent *E. coli* DH5 α cells and selected on MacConkey agar supplemented with 50 μ g ampicillin ml⁻¹. Plasmid DNA was prepared with the aid of a plasmid extraction kit (Sangon), digested with *EcoRI* (Sangon) and sequenced with an ABI 3730 automatic sequencer using the Sanger chain-termination method. Sequences were analysed using the BLAST network service of the National Center for Biotechnology Information.

Conjugation and plasmid extraction. Conjugation experiments were performed as described previously (Yan *et al.*, 2000), with rifampicin-resistant *E. coli* 600 as the recipient. Mueller–Hinton plates supplemented with rifampicin (512 μ g ml⁻¹) and imipenem (2 μ g ml⁻¹) were used to select for transconjugants. A Qiagen Plasmid Midi kit was used to extract plasmids of imipenem-resistant *A. baumannii* strains.

Southern blot hybridization analysis of the OXA-23 gene. For Southern blot hybridization, plasmids and *ApaI*-digested PFGE genomic DNA were transferred from electrophoresis gels to nylon membranes (Bio-Rad) and hybridized with [α -³²P]dCTP (DuPont)-labelled OXA-23 gene fragments. After washing, the membrane was compressed with a storage phosphor screen (Kodak, Japan) for 48 h and then scanned for hybridization reactions.

RESULTS

A total of 1018 *A. baumannii* isolates were recovered from various specimens in 2003, and 17.6% were resistant to imipenem. Specimens from which *A. baumannii* was isolated comprised respiratory secretions (53.7%), blood (20%), urine (10.5%), sterile fluids and catheter tips (8.4%), and wounds (7.4%).

Antibiotic susceptibility

All of the imipenem-resistant isolates of *A. baumannii* exhibited high resistance rates (>95%) to the penicillins, cephamycins, extended-spectrum cephalosporins, carbapenems, monobactams, quinolones, minocycline and aminoglycosides. The resistance rates for cefoperazone/sulbactam and ampicillin/sulbactam were 30.6 and 67.6%, respectively, and the most active agent was polymyxin E with 17% resistance.

Table 2. Primers used in this study

Primer	Sequence (5'→3')	Target	Reference
OXA-23-likeF	GATGTGTCATAGTATTCGTCGT	<i>bla</i> _{OXA-23} -like	Jeon <i>et al.</i> (2005)
OXA-23-likeR	TCACAACAACATAAAAGCACTGT		
OXA-24-likeF	ATGAAAAAATTTATACTTCCTATATTCAGC	<i>bla</i> _{OXA-24} -like	Jeon <i>et al.</i> (2005)
OXA-24-likeR	TTAAATGATTCCAAGATTTTCTAGC		
OXA-51-likeF	TAATGCTTTGATCGGCCTTG	<i>bla</i> _{OXA-51} -like	Woodford <i>et al.</i> (2006)
OXA-51-likeR	TGGATTGCACTTCATCTTGG		
OXA-58-likeF	AAGTATTGGGGCTTGTGCTG	<i>bla</i> _{OXA-58} -like	Woodford <i>et al.</i> (2006)
OXA-58-likeR	CCCCTCTGCGCTCTACATAC		
IS <i>Aba</i> 1F1	AACGATTGCGAGCATC	IS <i>Aba</i> 1/ <i>bla</i> _{OXA-23} -like	This study
OXA-23-likeR1	GTCAACCAGCCCACTT		
IS <i>Aba</i> 1F2	AGGCTATAAAGCGTTGA	IS <i>Aba</i> 1/ <i>bla</i> _{OXA-51} -like	This study
OXA-51-likeR2	CTTCTGTGGTGGTTGC		
OXA-51-likeF3	ATGAACATTAACACTCTTACTTA	<i>bla</i> _{OXA-51} -like/ IS <i>Aba</i> 1	This study
IS <i>Aba</i> 1R3	CACATTCACAATACGGTCTTTAC		
IMP-likeF	CATGGTTTGGTGGTTCTTGT	<i>bla</i> _{IMP} -like	Jeon <i>et al.</i> (2005)
IMP-likeR	ATAATTTGGCFFACTTTGGC		
VIM-likeF	ATTGGTCTATTTGACCGCGTC	<i>bla</i> _{VIM} -like	Jeon <i>et al.</i> (2005)
VIM-likeR	TGCTACTCAACGACTGAGCG		
SIM-1F	TACAAGGGATTGCGCATCG	<i>bla</i> _{SIM-1}	Lee <i>et al.</i> (2003)
SIM-1R	TAATGGCCTGTTCCCATGTG		

Molecular typing

The 95 clinical isolates were grouped into six clonal patterns by PFGE. The two predominant patterns, A represented by 50 isolates and B by 35 isolates, exhibited slight variation (between one and three band differences) within each profile, but this was insufficient to distinguish subtypes confidently within the profile. Pattern C was represented by six isolates, pattern D by two isolates and patterns E and F by single strains (Fig. 1 and Table 1). The first isolates of pattern A were from the sputum of a patient in the ICU in August 2003 and this type first appeared in the transplant unit in October 2003; the ICU environmental isolates were also of pattern A. Clonal pattern B first appeared in an ICU patient in October 2003 and pattern C in the 'emerging' ICU ward in December 2003. The remaining types were isolated in the following 6 months.

Carbapenemase of *A. baumannii*

All isolates were negative by the imipenem/EDTA double disc synergy test. Isoelectric focusing analysis revealed the presence of a single band of pI 6.64, which is identical to the reported value for OXA-23 (Donald *et al.*, 2000). All isolates were positive by PCR for *bla*_{OXA-23}-like and *bla*_{OXA-51}-like genes, and cloning and sequencing confirmed that the PCR products were 100% identical to the OXA-23 and OXA-66 genes (GenBank accession nos AY795964 and AY949204). All isolates were positive for IS*Aba*1/*bla*_{OXA-23}-like but negative for IS*Aba*1/*bla*_{OXA-51}-like and *bla*_{OXA-51}-like/IS*Aba*1 genes (Fig. 2). No IMP-like, VIM-like, SIM-1, OXA-58-like or OXA-24-like genes were detected.

Repeated attempts to transfer the OXA-23-encoding gene by conjugation failed and no plasmids were detected or hybridized with the *bla*_{OXA-23} probe. However, chromosomal

Fig. 1. *Apal*-digested PFGE patterns of selected imipenem-resistant *A. baumannii* strains. Strains 58, 97, 19, and 109 are pattern A; strains 135 and 134 are pattern B; strain 108 is pattern C; strain 131 is pattern D; and strain 129 is imipenem susceptible. M, λ DNA ladder marker.

Fig. 2. PCR map and sequencing result. All imipenem-resistant *A. baumannii* strains were positive for the *bla*_{OXA-23} and *bla*_{OXA-66} genes; *ISAbal* is upstream of the *bla*_{OXA-23} gene.

DNA fragments digested with *Apal* showed two hybridization patterns represented by 200 and 220 kb bands for PFGE patterns A and B, respectively (Fig. 3), which is indicative of a chromosomal location for the *bla*_{OXA-23} gene of *A. baumannii* in our hospital.

DISCUSSION

Carbapenems have the most extended antimicrobial spectrum of antibacterial activity among all of the β -lactams. However, carbapenem resistance is emerging and increasing in clinical isolates, especially in *P. aeruginosa* and *A. baumannii*. A limited number of antimicrobial agents maintain high activity against OXA-23-producing *A. baumannii*, including polymyxin, sulbactam and minocycline (Dalla Costa *et al.*, 2003). Sulbactam has a direct antimicrobial effect against *A. baumannii* and also acts synergistically with ampicillin against this organism (Levin *et al.*, 2003). Nebulized polymyxin E is increasingly being

used for the treatment of respiratory infections caused by multiresistant *A. baumannii* due to the high concentrations that can be achieved in the lung, and is sometimes combined with intravenous administration, despite concerns of nephrotoxicity previously reported in the literature (Li *et al.*, 2005; Lolans *et al.*, 2006).

For all of the patients studied, imipenem-resistant strains of *A. baumannii* were all recovered at least 72 h after initial hospitalization. This strongly suggested nosocomial acquisition of the organism and was supported by the widespread environmental contamination found for the predominant strain type in the ICU. This is a very common feature of hospital outbreaks due to multiresistant opportunist Gram-negative organisms such as acinetobacters and augmented, no doubt, by the increased resistance to desiccation observed with epidemic strains of *A. baumannii* (Jawad *et al.*, 1998), which promotes their persistence in the hospital ward environment. It is therefore important to monitor and control the spread of such strains by the use of molecular epidemiological tools to inform infection control measures.

In recent years, OXA-23-producing *A. baumannii* strains have been reported around the world (Poirel & Nordmann, 2006b), whilst *bla*_{OXA-66} belonging to the OXA-51/69 cluster was recently found to be the intrinsic β -lactamase gene in *A. baumannii* isolates from Greece (Ikonomidis *et al.*, 2006). In this study, *bla*_{OXA-23} and *bla*_{OXA-66} genes were detected in all isolates and *bla*_{OXA-23} was located chromosomally. *ISAbal* occurred upstream of the *bla*_{OXA-23} gene. This is consistent with the finding of Turton *et al.* (2006), who concluded that *ISAbal* may have an important role in the expression of OXA-23. It is noteworthy that these characteristics were shared by a number of PFGE-defined genotypes in this study and this might suggest that the *ISAbal/bla*_{OXA-23} unit is mobile.

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Fig. 3. Southern blot hybridization analysis (right panel) of *Apal*-digested chromosomal DNA fragments (left panel) from imipenem-resistant *A. baumannii* strains of PFGE pattern types A and B. Location of the *bla*_{OXA-23} gene on DNA fragments of ~220 kb in type A and ~200 kb in type B.

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