Plasmid-borne extended-spectrum β -lactamase in a clinical isolate of *Acinetobacter baumannii*

Acinetobacter baumannii has emerged over the last decade as a significant opportunistic pathogen. Although it is generally associated with benign colonization of hospitalized patients, it is responsible for about 10 % of nosocomial infection in intensive care unit (ICU) patients, causing a wide range of infections such as bacteraemia, nosocomial pneumonia, urinary tract infection, secondary meningitis and burn and wound infections (Bergogne-Berezin, 2001; Joshi, 1998; Levin *et al.*, 2003; Poirel *et al.*, 1999). In *Acinetobacter*-associated nosocomial infection, the major problem encountered by ICU clinicians relates to the readily transferable antimicrobial resistance expressed by this organism (Bergogne-Berezin, 2001; Joshi, 1998). In addition to intrinsic resistance, *A. baumannii* has the ability to acquire resistance to many major classes of antibiotics including newer β -lactams (Perilli *et al.*, 1996). The presence of resistance plasmids (R-plasmids) is a significant feature of this organism, and plasmid profiling has been proposed as a method of epidemiological typing for *Acinetobacter* (Joshi, 1998; Perilli *et al.*, 1996). Although *A. baumannii* colonizes hospitalized patients, approximately 30 % of isolates are associated with frank infection in ICU patients and, in this setting, tend to demonstrate variable susceptibility profiles (Dy *et al.*, 1999). Nevertheless, despite the rising clinical importance of *A. baumannii* compared with other nosocomial pathogens in developing countries, this organism has been widely overlooked. In this study, five isolates of *A. baumannii* isolated in 1998 from exudates of post-surgical wound sepsis from our teaching hospital were examined. The isolates were found to harbour transferable extended-spectrum β -lactamase (ESBL)-encoding resistance determinants on a 66·1 kb conjugative plasmid.

The isolates were identified (Von Graevenitz, 1995) and biotyped according to the method of Bouvet & Grimont (1987). *Escherichia coli* DH5 α was used as the

recipient strain for bacterial conjugation experiments. The size of the test plasmid was estimated using *E. coli* NCTC 50192 carrying plasmids of 154, 66·1, 37·6 and 7·7 kb.

Antimicrobial susceptibility testing of clinical isolates of *A. baumannii*, *E. coli* transconjugants and their plasmid-cured derivatives was first performed by the disc-diffusion technique (Hi-Media). The MIC of the isolates was determined according to NCCLS guidelines (NCCLS, 1997). *E. coli* ATCC 25922 was included as a control strain for susceptibility testing. The antibiotics tested are listed in Table 1. ESBL production was confirmed by using a double-disc synergy (DDS) test, which was performed by placing a ceftazidime disc (30 μ g) or a cefotaxime disc (30 μ g) 30 mm away from a disc containing amoxycillin/clavulanate (60/10 μ g), as described previously (Jarlier *et al.*, 1988). ESBL production was considered positive when an enhanced zone of inhibition was visible between the β -lactam- and β -lactamase inhibitor-containing disks.

Plasmid DNA was isolated by the alkaline lysis method, separated by electrophoresis on 0·7 % (w/v) agarose gels using TBE buffer and stained with ethidium bromide as described by Sambrook *et al.* (1989).

Bacterial conjugation experiments were performed by the membrane filter mating technique (Willets, 1988). An initial donor-to-recipient ratio of 1 : 20 was used for mating. Mueller–Hinton agar plates containing rifampicin (300 μ g ml⁻¹) plus ceftazidime (50 μ g ml⁻¹) were used for selection of transconjugants. For each conjugation experiment, the plasmid profiles and antibiograms of five randomly selected transconjugants were analysed for the presence of plasmid DNA and co-transfer of resistance determinants. The transconjugation frequency was calculated as the number of transconjugants per donor viable cell counts. Curing of plasmid-encoded antibiotic-resistance determinants from both the donor and transconjugant

was carried out using ethidium bromide (10–512 μ g ml⁻¹) as described previously (Stanisich, 1988) and the plasmid profiles of derivatives were analysed at various concentrations of curing agent during growth to determine optimal plasmid curing. The growth cycles were repeated five times to ensure plasmid curing and antibiograms and MICs were determined each time to correlate curing of plasmid with resistance determinants in cured derivatives. All experiments were repeated at least three times and were highly reproducible.

During analysis, all five isolates of *A. baumannii* were identified as biotype 9 and were found to express resistance to routine β -lactams tested in our laboratory. Table 1 shows MIC values of various β -lactams in the *A. baumannii* isolates, *E. coli* DH5 α transconjugants and their plasmid-cured derivatives. The isolates were positive for production of ESBL activity when tested by a positive DDS test with ceftazidime or cefotaxime (Table 1). The plasmid profiles appeared to be similar for all isolates, with plasmid sizes estimated to be approximately 66·1, 18·1 and 5·2 kb (Fig. 1). Curing of plasmids by ethidium bromide at a subinhibitory concentration of 512 μ g ml⁻¹ was highly efficient. The disappearance of antibiotic resistance to expanded-spectrum cephalosporins with the concurrent loss of all three plasmids from all *A. baumannii* isolates suggested that the ESBL determinants were plasmid-borne.

During mating experiments with *E. coli* DH5 α , conjugational transfer of the ESBL-encoding determinant found to be present on a 66·1 kb plasmid from the donor strain of *A. baumannii* occurred at a frequency of $\sim 3\cdot2 \times 10^{-4}$ (transconjugants : donor). All transconjugants obtained from *A. baumannii* demonstrated the same plasmid profile, usually similar to that of the donor plasmid profile of 66·1 kb (Fig. 1, lane 2). No significant differences were noted among the patterns of β -lactam susceptibility of all transconjugants obtained from donor *A. baumannii* isolates (Table 1). Resistance to

Table 1. MICs of β -lactams alone and in combination with the β -lactamase inhibitor clavulanic acid

MICs are given in $\mu\text{g ml}^{-1}$. CLA, Clavulanic acid; SBT, sulbactam.

Antibiotic	<i>A. baumannii</i>	Cured <i>A. baumannii</i>	<i>E. coli</i> DH5 α	<i>E. coli</i> transconjugant	Cured <i>E. coli</i> transconjugant
Ampicillin+SBT	<8	<8	0-125	<0.5	<0.5
Amoxycillin	>512	0.5	0.5	>512	0.5
Amoxycillin+CLA	<16	0.5	0-125	<16	0-125
Cefazolin	>512	>0.125	>0.5	>512	>0.125
Cefoperazone	>512	0.5	0-125	256–512	0-125
Ceftazidime	>256	0.5	0-125	256	0-125
Ceftazidime+CLA	<0.5	0.5	0-125	<0.5	0.125–0.5
Cefuroxime	>512	0.5	0.5	>512	0.5
Cefotaxime	>256	0.125–0.5	0.125–0.5	>256	0.5
Cefotaxime+CLA	<0.5	0-125	0-125	<0.5	0.5

Fig. 1. Plasmid DNA profiles after agarose gel electrophoresis. Lanes: 1, reference plasmids isolated from *E. coli* MCTC 50192; 2, *E. coli* DH5 α transconjugant; 3, *A. baumannii* clinical isolate. Molecular sizes are in kb.

cefazolin, cefoperazone, cefotaxime and ceftazidime was always co-transferred with plasmid DNA, suggesting that the resistance determinants were carried by the same 66.1 kb conjugative plasmid.

Recently, multiple-antibiotic-resistant *A. baumannii* have been widely reported from ICU (Bergogne-Berezin & Towner, 1996). In the authors' own hospital, the isolates of *A. baumannii* reported were predominantly resistant to newer β -lactams (Joshi, 1998; Joshi *et al.*, 2003). β -Lactam resistance has been reported in many nosocomial pathogens, including *Acinetobacter* spp.

(Mulvey *et al.*, 2003), but plasmid-borne transferable ESBL-producing *Acinetobacter* has not been reported to date. PER-1-type ESBL-producing *Acinetobacter* spp. have been described (Vahaboglu *et al.*, 2001), but no plasmid-mediated transferability was found. The results of this study confirm the presence of ESBL-producing isolates of *A. baumannii* isolated from ICU. The similar antibiograms, biotypes, specimen types and plasmid profiles of the isolates are highly indicative of clonal spread. A plasmid of 66.1 kb was readily transferred into *E. coli* DH5 α by filter mating with concurrent transfer of ESBL determinants. This demonstrated that the ESBL was plasmid-encoded and could be readily spread among other organisms by mere conjugation. The plasmid-borne status of these ESBL determinants was further confirmed by plasmid-curing experiments of donor and *E. coli* transconjugants and antimicrobial susceptibility and DDS results. We did not characterize the ESBL in detail, but focused only on the transferable nature of ESBL genes carried on a conjugative plasmid. The transconjugation experiments in *E. coli* DH5 α also support the hypothesis that plasmid-borne ESBL genes can be readily disseminated to other nosocomial pathogens. The ESBL activity was inhibited by the β -lactamase inhibitor clavulanic acid. A significant decrease in MIC of cefotaxime and ceftazidime also confirmed the involvement of ESBL production. Possible spread of resistance markers via conjugative plasmid transfer has been reported (Bergogne-Berezin & Towner, 1996; Joshi, 1998), but transferable ESBL production has not been reported so far in *Acinetobacter* spp. Third-generation cephalosporins are widely used in our ICU, but fourth-

generation cephalosporins are not readily available. The selective pressure of third-generation β -lactams would be high and is perhaps responsible for the repeated selection of mutants of resistance determinants (Bergogne-Berezin, 2001).

It is known that ESBL-mediated resistance may increase the basal level of naturally occurring multi-drug resistance among other nosocomial pathogens by dissemination and integration of R-plasmids. Therefore, it is advisable to include *A. baumannii* in screening programmes for ESBL-producing Gram-negative aerobes. Future studies on characterization of the ESBL and the epidemiology of the source of the plasmid in this study are in progress.

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