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 DH5α 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 cefazidime disc (30 µg) or a cefotaxime disc (30 µg) 30 mm away from a disc containing amoxycillin/clavulenate (60/10 µg), 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 µg ml⁻¹) plus ceftazidime (50 µg 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 conjugation 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 µg 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 A. baumannii isolates, E. coli DH5α 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 µg 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 DH5α, 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 3·2 × 10⁻⁸ (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-lactama alone and in combination with the β-lactamase inhibitor clavulanic acid

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>A. baumannii</th>
<th>Cured A. baumannii</th>
<th>E. coli DH5α</th>
<th>E. coli transconjugant</th>
<th>Cured E. coli transconjugant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin+SBT</td>
<td>&lt;8</td>
<td>&lt;8</td>
<td>0·125</td>
<td>&lt;0·5</td>
<td>&lt;0·5</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>&gt;512</td>
<td>0·5</td>
<td>0·5</td>
<td>&gt;512</td>
<td>0·5</td>
</tr>
<tr>
<td>Amoxicillin+CLA</td>
<td>&lt;16</td>
<td>0·5</td>
<td>0·125</td>
<td>&lt;16</td>
<td>0·125</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>&gt;512</td>
<td>&gt;0·125</td>
<td>&gt;0·5</td>
<td>&gt;512</td>
<td>&gt;0·125</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>&gt;512</td>
<td>0·5</td>
<td>0·125</td>
<td>256–512</td>
<td>0·125</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>&gt;256</td>
<td>0·5</td>
<td>0·125</td>
<td>256</td>
<td>0·125</td>
</tr>
<tr>
<td>Cefotaxime+CLA</td>
<td>&lt;0·5</td>
<td>0·5</td>
<td>0·125</td>
<td>&lt;0·5</td>
<td>0·125–0·5</td>
</tr>
<tr>
<td>Cefuroxime</td>
<td>&gt;512</td>
<td>0·5</td>
<td>0·5</td>
<td>&gt;512</td>
<td>0·5</td>
</tr>
<tr>
<td>Cefotaxime+CLA</td>
<td>&gt;256</td>
<td>0·125–0·5</td>
<td>0·125–0·5</td>
<td>&gt;256</td>
<td>0·5</td>
</tr>
</tbody>
</table>

Recent, 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 DH5α 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 DH5α 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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