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Outbreak of multiple clones of imipenem-resistant *Acinetobacter baumannii* isolates expressing OXA-58 carbapenemase in an intensive care unit

S. Pournaras[1], A. Markogiannakis[2], A. Ikonomidis[1], L. Kondyli[3], K. Bethimouti[3],
A. N. Maniatis[1], N. J. Legakis[2] and A. Tsakris[2]*

[1]*Department of Microbiology, Medical School, University of Thessaly, Mezourlo, Larissa, Greece; [2]Department of Microbiology, Medical School, University of Athens, 11527 Athens, Greece; [3]Department of Clinical Microbiology, Red Cross Hospital, Athens, Greece*

Objectives: To investigate the resistance mechanisms and the genetic relationship of imipenem-resistant *Acinetobacter baumannii* isolates recovered in the intensive care unit (ICU) of a tertiary care hospital.

Methods: Imipenem-resistant *A. baumannii* clinical and environmental isolates were collected in the ICU of the Red Cross General Hospital, Athens, Greece between March and October 2002. The isolates were tested by Etest MBL, PCR, RT–PCR and sequencing for carbapenemase-encoding genes, PFGE and synergy experiments using meropenem and the efflux pump inhibitor carbonyl cyanide chlorophenylhydrazone.

Results: During the study period, 15 clinical and two environmental imipenem-resistant (MIC 8 to >128 mg/L) *A. baumannii* isolates were recovered. PFGE showed six different clones that included both clinical and environmental isolates. All 17 isolates were negative by Etest MBL and PCR for genes bla_{IMP}, bla_{VIM}, bla_{SPM}, $bla_{OXA-23-like}$ and $bla_{OXA-24-like}$. Genes $bla_{OXA-51-like}$ and $bla_{OXA-58-like}$ were amplified from 15 and 14 isolates, respectively. Sequencing of $bla_{OXA-51-like}$ amplicons identified bla_{OXA-66} (nine cases) and bla_{OXA-69} (six cases), whereas $bla_{OXA-58-like}$ sequences were classical bla_{OXA-58}. Reverse transcriptase-PCR showed that $bla_{OXA-51-like}$ genes were expressed in 12 and bla_{OXA-58} in 10 isolates; in these isolates, inhibition of OXA enzymes by 200 mM of NaCl reduced carbapenem MICs by up to 4-fold. Overexpression of proton-gradient dependent efflux pumps did not contribute to carbapenem resistance in any isolate. Similarly, although AmpC expression was demonstrated in eight isolates, inhibition of AmpC with cloxacillin did not reduce the MICs of carbapenems significantly.

Conclusions: These findings indicate wide dissemination of OXA-58 carbapenemase, which contributes, at least partially, to the imipenem resistance of unrelated *A. baumannii* isolates in our ICU.

Keywords: oxacillinase, carbapenems, bla_{OXA-51}, bla_{OXA-58}, RT–PCR

Introduction

Carbapenems have a potent activity against acinetobacters and are often used as a last resort for the treatment of infections due to multiresistant *Acinetobacter baumannii* isolates. However, acinetobacters may develop resistance to carbapenems through various combined mechanisms, including AmpC stable derepression, decreased permeability, altered penicillin-binding proteins (PBPs) and, rarely, efflux pump overexpression.[1,2] Moreover, carbapenem resistance among acinetobacters has been sporadically attributed to the production of carbapenemases such as class B IMP-type and VIM-type metallo-β-lactamases and class D OXA-type carbapenemases.[3] Recently, two novel class D oxacillinases with carbapenemase properties, designated OXA-51 and OXA-58, have been described among *A. baumannii* isolates[4,5]; at least six variants of bla_{OXA-51} have been detected in acinetobacters from various regions worldwide.[6]

In our region imipenem-non-susceptible *A. baumannii* are being isolated with increasing frequency from clinical sources.[7] Preliminary susceptibility data in our hospital indicated that

*Corresponding author. Tel: +30-210-746-2140; Fax: +30-210-746-1489; E-mail: atsakris@med.uoa.gr

several infections among patients hospitalized in our intensive care unit (ICU) were caused by imipenem-resistant *A. baumannii* that exhibited cross-resistance to almost all alternative antimicrobials. The similar antimicrobial susceptibility patterns of these isolates prompted an investigation of their genetic relationship and carbapenem resistance mechanisms.

Materials and methods

Bacterial isolates

The study included all imipenem-resistant (MICs of imipenem ≥ 8 mg/L) *A. baumannii* non-repetitive isolates recovered consecutively from clinical infections of separate patients in the general ICU of the Red Cross General Hospital, Athens, Greece, between March and October 2002. We also included imipenem-resistant environmental *A. baumannii* isolates that were recovered during the study period by swabbing the environment and equipment adjacent to the patients as well as by culturing samples from the hands of hospital care workers (HCWs). The isolates were provisionally identified to the genus level by the Vitek 2 automated system (bioMérieux, Marcy l'Étoile, France) and the API 20NE system (bioMérieux), and the identification of *A. baumannii* was performed by a simplified identification scheme.

Susceptibility testing

Imipenem and meropenem MICs were determined using an agar dilution method, whereas susceptibility testing against other antimicrobials (amikacin, ciprofloxacin, cefepime, gentamicin, kanamycin, ofloxacin, piperacillin and piperacillin/tazobactam) was performed by disc diffusion. The isolates were also tested by Etest MBL (AB Biodisk, Solna, Sweden) for possible metallo-β-lactamase production. *Pseudomonas aeruginosa* ATCC 27853 was used as control in susceptibility testing and a VIM-type carbapenemase-producing *P. aeruginosa* strain[8] was used as a control for Etest MBL.

Pulsed-field gel electrophoresis

PFGE of *Apa*I-digested genomic DNA of *A. baumannii* isolates was performed with a CHEF-DRIII system (Bio-Rad, Hemel Hempstead, UK).

PCR amplification

PCR testing of the isolates for carbapenemase-encoding genes (bla_{IMP}, bla_{VIM}, bla_{SPM}, $bla_{OXA-23-like}$, $bla_{OXA-24-like}$, $bla_{OXA-58-like}$) was done using consensus primers that were specific for each enzyme group.[5,7–10] The isolates were also screened for the $bla_{OXA-51-like}$ genes using primers that amplify a 353 bp internal fragment (kindly provided by Dr S. Brown, University of Edinburgh, UK), as well as partially degenerate primers (sense: 5′-TGAACATTAAAICACTCTT-3′, anti-sense: 5′-CTATAAAATACCTAATTGTT-3′) that were designed to amplify an 825 bp product in all $bla_{OXA-51-like}$ alleles. Cycling conditions were: an initial denaturation step at 94°C for 5 min, amplification steps of 94°C for 1 min, 54°C for 1 min, 72°C for 45 s for a total of 35 cycles and a final extension at 72°C for 7 min.

RT–PCR and inhibition experiments

RT–PCR was performed for $bla_{OXA-51-like}$ and $bla_{OXA-58-like}$ genes as described previously.[11] RT–PCR was also performed for *ampC* genes[12] to investigate the contribution of AmpC expression to imipenem resistance. To evaluate the expression of *bla* genes, the mRNA of the single copy housekeeping gene, *recA*, was amplified using primers that amplify a 425 bp product. The relative contribution of

OXA-58 enzyme to carbapenem resistance was checked by estimating carbapenem MICs with and without 200 mM of NaCl.[5] Contribution of AmpC β-lactamase was tested by determining carbapenem MICs in agar with and without 200 mg/L of cloxacillin.

Plasmid analysis

Plasmid analysis was performed using an alkaline lysis method. Plasmid and residual chromosomal DNA bands were extracted separately from 0.8% agarose gels using a QIAquick Gel Extraction kit (Qiagen, Hilden, Germany). The respective DNA extracts were subjected to PCR for genes $bla_{OXA-51-like}$ and $bla_{OXA-58-like}$ to check the location of each gene. Plasmid curing experiments used ethidium bromide at the maximum concentration that allowed the growth of isolates (400 mg/L).

Synergy experiments for testing overexpression of efflux pumps

Synergy experiments were performed using imipenem and the efflux pump inhibitor carbonyl cyanide chlorophenylhydrazone (CCCP).[11] Susceptibility testing of imipenem was performed by disc diffusion and agar dilution using Mueller–Hinton agar with and without 12.5 μM CCCP.

Results

During the study period 15 non-repetitive imipenem-resistant *A. baumannii* isolates were recovered from clinical infections of separate patients; eight were from cases of pneumonia, five from bacteraemia and two from urinary tract infections. In addition, two imipenem-resistant *A. baumannii* isolates were recovered from 62 environmental samples; one was recovered from the surface of a patient's bed rails and a second one was recovered from the hands of an HCW. The MICs of imipenem and meropenem for the 17 isolates are shown in Table 1. The isolates were multidrug-resistant exhibiting resistance to all other β-lactams (ampicillin/sulbactam, aztreonam, ceftazidime, cefepime, piperacillin and piperacillin/tazobactam), aminoglycosides (amikacin, gentamicin, netilmicin and tobramycin) and fluoroquinolones (ciprofloxacin and ofloxacin); they were susceptible only to colistin.

PFGE of the clinical and environmental imipenem-resistant isolates showed six distinct genotypes, with two each containing two subtypes (Table 1). The two environmental isolates belonged to the same genotype as two clinical isolates. The infection control team of the hospital implemented restriction of carbapenem usage and strict antiseptic techniques, which included the rigorous use of alcohol-chlorhexidine solutions before and between patient and equipment contact and before leaving the unit.

The Etest MBL was negative in all 17 imipenem-resistant isolates. The isolates were also negative for bla_{IMP}, bla_{VIM}, bla_{SPM}, $bla_{OXA-23-like}$ and $bla_{OXA-24-like}$ genes. The $bla_{OXA-51-like}$ and $bla_{OXA-58-like}$ genes were amplified in 15 and 14 isolates, respectively. Sequencing of $bla_{OXA-51-like}$ amplicons identified bla_{OXA-66} (nine cases) and bla_{OXA-69} (six cases), while all $bla_{OXA-58-like}$ alleles encoded classical OXA-58 enzyme.

Plasmids were analysed in six isolates (one each of PFGE types I, IIa, IIb, IIIa, IIIb and VI) that harboured both $bla_{OXA-51-like}$ and bla_{OXA-58} genes; three isolates carried single plasmids of ~24–70 MDa; one isolate had two plasmids of ~45 and 3 MDa; two isolates had no detectable plasmids. The large plasmids and the chromosomal band of each isolate were extracted from the

Table 1. PFGE types, MICs (mg/L), synergy test results and PCR results for the 17 carbapenem-resistant *A. baumannii* isolates

Strain	Site of isolation	PFGE type	MIC of IPM	MIC of MEM	Cloxacillin (200 mg/L) MIC of IPM	NaCl (200 mM) MIC of IPM	NaCl (200 mM) MIC of MEM	PCR results bla_{ampC}	PCR results $bla_{oxa\text{-}51}$	PCR results $bla_{OXA\text{-}58}$	RT–PCR results bla_{ampC}	RT–PCR results $bla_{OXA\text{-}51}$	RT–PCR results $bla_{OXA\text{-}58}$
72	Blood	I	64	8	64	32	4	+	+	+	–	+	+
111	Hands of an HCW	I	64	8	64	32	4	+	+	+	–	+	+
155	Patient's bed	I	64	8	64	32	4	+	+	+	–	+	+
189	Bronchial	I	8	2	8	4	1	+	–	+	+	–	+
132	Bronchial	IIa	16	2	16	8	1	+	+	–	+	+	–
182	Blood	IIa	16	2	16	8	1	+	+	+	–	+	+
144	Blood	IIb	64	16	64	64	4	+	+	+	+	+	+
157	Bronchial	IIb	64	16	64	32	4	+	+	+	–	+	+
176	Bronchial	IIIa	32	4	16	8	1	+	+	+	+	+	+
223	Bronchial	IIIa	8	2	8	4	1	–	–	+	–	–	+
231	Blood	IIIa	>128	>128	>128	128	128	+	+	+	+	+	–
201	Urine	IIIb	16	16	16	16	16	+	+	+	+	–	–
229	Blood	IIIb	16	16	16	16	16	+	+	+	–	–	–
242	Bronchial	IIIb	32	8	16	32	8	+	+	+	+	–	–
208	Bronchial	IV	16	4	16	16	4	–	+	–	–	+	–
255	Urine	V	>128	>128	>128	128	128	+	+	–	+	+	–
263	Bronchial	VI	8	2	8	8	1	+	+	+	–	+	+

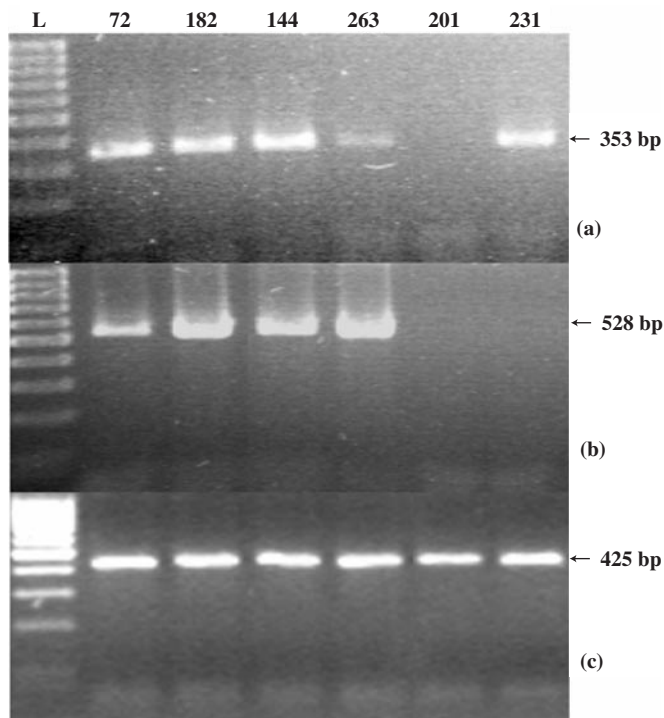HCW, hospital care worker; IPM, imipenem; MEM, meropenem.

Figure 1. RT–PCR products showing expression of the bla_{OXA-51} (a), bla_{OXA-58} (b) and *recA* (c) genes of *A. baumannii* isolates representative of each PFGE type that carry both bla_{OXA-51} and bla_{OXA-58}. Lane L, 100 bp DNA ladder. Sizes of the amplified fragments are indicated by arrows. The index numbers of the isolates are those listed in Table 1.

gel and used as templates in PCR for $bla_{OXA-51-like}$ and bla_{OXA-58}. Results suggested that plasmids carried bla_{OXA-58} in isolates of type I, IIIa, IIIb and VI, and $bla_{OXA-51-like}$ in type I and IIIb. Consistent with this, $bla_{OXA-51-like}$ was cured from the isolates of type I and IIIb, and bla_{OXA-58} from types I, IIIa, IIIb and VI, indicating that in types I and IIIb the same plasmid might harbour both genes.

RT-PCR showed that $bla_{OXA-51-like}$ genes were expressed in 12 isolates, and bla_{OXA-58} in 10 isolates (Figure 1); in these isolates, inhibition of oxacillinases with 200 mM of NaCl reduced carbapenem MICs by up to 4-fold (Table 1). Interestingly, three isolates that expressed neither $bla_{OXA-51-like}$ nor bla_{OXA-58} showed no reduction in carbapenem MICs in the presence of 200 mM of NaCl (Table 1). AmpC expression was demonstrated by RT–PCR in eight isolates, but inhibition of AmpC with cloxacillin did not reduce carbapenem MICs significantly (Table 1). None of the isolates exhibited significant synergy between imipenem and CCCP, indicating that overexpression of proton-gradient dependent efflux pumps did not contribute to the imipenem resistance. Presence or absence of OXA-58 did not affect patient mortality (data not shown).

Discussion

Several clones of imipenem-resistant *A. baumannii* producing OXA-58-carbapenem-hydrolysing oxacillinase and naturally occurring OXA-51-type oxacillinases have emerged in a general ICU of a Greek tertiary hospital. This spread could have been facilitated by the extensive use of carbapenems for treating the multidrug-resistant Gram-negative bacteria that are commonly isolated in our unit. In addition, the detection of a common clone among environmental and clinical imipenem-resistant *A. baumannii* indicates that environmental contamination contributes to the difficulty in restricting spread of this organism in our hospital. Surveillance cultures and strict antiseptic techniques possibly reduced the further spread of these bacteria.

Our bla_{OXA-58}-bearing acinetobacters had alleles identical to that described in Toulouse[5] and other southern European regions,[13] showing wide geographical spread of this gene. Also, our bla_{OXA-51} alleles resembled those described previously among *A. baumannii* from different regions (Spain, Hong Kong, Singapore and Turkey).[6] It has been recently shown that $bla_{OXA-51-like}$ alleles are very poor carbapenemases and naturally present in almost all *A. baumannii* isolates, regardless of their susceptibility or resistance to carbapenems.[14] The negative amplification of this gene in two isolates of the present study might be due to point mutations, even though our partially degenerate primers amplify all known $bla_{OXA-51-like}$ alleles.

In accordance with previous findings,[13] bla_{OXA-58} was plasmid-mediated in many of our isolates. Héritier *et al.*[1] demonstrated that OXA-58 exhibits weak carbapenemase activity and plays a role in carbapenem resistance in *A. baumannii*, particularly when bla_{OXA-58} is highly expressed. These authors also suggested that overexpression of efflux pumps might contribute to higher levels of carbapenem resistance, but proton-gradient dependent efflux pumps appeared not to contribute to the carbapenem resistance of our isolates. Although bla_{OXA-58} was present and expressed by most of the isolates, NaCl reduced carbapenem MICs no more than 4-fold, with imipenem MICs remaining in the resistant category. Similarly, inhibition of AmpC by cloxacillin did not affect MICs of carbapenems significantly. This suggests that much of the resistance in our isolates may depend on impermeability or other combined mechanisms that additionally affected resistance to other antibiotics such as expanded-spectrum cephalosporinases.[2] This is also supported by the finding that two isolates with the highest carbapenem MICs (>128 mg/L) did not express bla_{OXA-58}, but only $bla_{OXA-51-like}$ gene. Further studies are needed to determine whether imipenem-resistant *A. baumannii* expressing OXA-58 carbapenemase exist in other Greek hospitals and to evaluate the dissemination of these and other class D carbapenemases among acinetobacters with variable carbapenem MICs.

Transparency declarations

We have no conflicts to declare.

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