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Evolution of multidrug-resistant *Acinetobacter baumannii* isolates obtained from elderly patients with respiratory tract infections

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***Objectives*: To study the evolution between 1999 and 2002 and mechanisms of antibiotic resistance in a multidrug-resistant *Acinetobacter baumannii* clone predominant in isolates from elderly patients with respiratory tract infections.**

***Methods*: Susceptibility to antimicrobials was determined using an agar dilution method. Bacterial clones were identified by PCR-fingerprinting and PFGE with *Apa*I. Carbapenemases were detected by phenotypic tests; by PCR with primers specific for *bla* $_{OXA-40}$, *bla*$_{IMP}$, *bla*$_{VIM-1}$ and *bla*$_{VIM-2}$; and by hybridization with DNA probes. Class 1 integrons were detected using PCR.**

***Results*: In 1999 isolates were grouped into two main genotypes: clone I (33%) and clone II (55%). These were also detected in 2002 with a different distribution: clone I (69%), clone II (22%). Resistance to amikacin, meropenem and imipenem increased significantly in clone I over this time, whereas clone II was not affected. In 2002, the incidence of *bla*$_{OXA-40}$ rose to 91% in clone I isolates with some also harbouring *bla*$_{VIM-2}$ and *bla*$_{IMP}$ genes. Different class 1 integrons were detected ranging in size from 550 to 1200 bp. No relationship was found between carbapenemases and class 1 integrons.**

***Conclusions*: In elderly patients, a single clone became predominant among *A. baumannii* isolates, coinciding with an increase in antibiotic resistance rates. The majority of isolates harboured the *bla*$_{OXA-40}$ carbapenemase gene and some of them also harboured *bla*$_{VIM-2}$ and *bla*$_{IMP}$ genes. The presence of class 1 integrons also increased over time.**

Keywords: *A. baumannii*, carbapenemases, OXA-40, resistance, integrons

Introduction

In recent years, *Acinetobacter baumannii* has emerged as an important pathogen, responsible for serious infections and nosocomial outbreaks which particularly affect patients who are already critically ill with underlying diseases.[1] *Acinetobacter* spp. can complicate persistent respiratory infections, causing significant health problems in the elderly, with high rates of mortality.[2]

The emergence and rapid spread of multidrug-resistant isolates causing nosocomial infections are of great concern.[1] Carbapenem antibiotics are considered the agents of choice to treat serious infections caused by *A. baumannii*, but progressive antimicrobial resistance has made treatment very difficult. Although many mechanisms are involved, carbapenemases are the major effectors, with increasing concern regarding their spread.[3] The most important clinically significant carbapenemases in *A. baumannii* are class D (OXA) types, of which four families have been described.[4] In the Iberian Peninsula, outbreaks of an OXA-40-producing clone have been identified, mainly in hospitals from Northern Spain and Portugal.[5]

Elderly patients have a significant incidence of chronic obstructive pulmonary disease and nosocomial pneumonia with a high mortality rate, yet few studies have focused on the evolution over time of the *A. baumannii* isolates from these patients. The purpose of this study was to analyse the clonal diversity, the evolution and mechanisms of resistance among *A. baumannii* isolates from elderly patients with respiratory tract infections.

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Materials and methods

The study included 60 and 49 *A. baumannii* isolates obtained from patients attending a hospital in Osakidetza (Bilbao, Northern Spain) during the years 1999 and 2002, respectively. Isolates represented 50% of the total number of *A. baumannii* collected at the Microbiology Service. Patients included in this study showed potential risk factors for *Acinetobacter* infections including old age (>65 years), underlying diseases and lower respiratory tract illness (Table 1). All isolates were recovered from sputum and had been previously identified to the species level by tDNA fingerprinting.

The susceptibility of isolates to cefotaxime, ceftazidime, imipenem, meropenem, amikacin and gentamicin was determined using an agar dilution method according to the guidelines of the CLSI. *Pseudomonas aeruginosa* ATCC 27853 was used as a control strain.

Clonal relatedness of the isolates was investigated by RAPD–PCR-fingerprinting with the primers M13 (5′-GAGGGTGGCGGTTCT-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′)[6] and by PFGE with the *Apa*I enzyme.[7]

Digital images of the gels were analysed using the *Molecular Analyst/Macintosh Fingerprinting* program (Image Analysis System, Bio-Rad Laboratories), which identifies the positions and intensities of the bands in each lane of a gel and then calculates a similarity coefficient (S_{AB}) for every pair of strains. Those isolates with an S_{AB} value of >0.72 were clustered together.

Phenotypic detection of carbapenemase activity was carried out following the modified Hodge test, and metallo-β-lactamases were detected using the double-disc synergy test (DDST).[8] Control strains were *E. coli* ATCC 25922 (Hodge test), *A. baumannii* SM28 (OXA-40), *P. aeruginosa* 3/P/10586 (VIM-1), *P. aeruginosa* P4824 (VIM-2) and *P. aeruginosa* A327 (IMP-1).

Detection of bla_{OXA-40}, bla_{VIM-1}, bla_{VIM-2} and bla_{IMP} genes was carried out using PCR with primers P2 5′-TTCCCCTAACATGAATTTGT-3′, P1 5′-GTACTAATCAAAGTTGTGAA-3′; IMP-F 5′-CTACCGCAGCAGAGTCTTTG-3′, IMP-R 5′-AACCGATTTTGCCTTACCAT-3′; VIM-Diar 5′-AGGTGGGCCATTCAGCCAGA-3′, VIM-1upv 5′-GTCGCAAGTCCGTTAGCCCAT-3′ and VIM-2upv 5′-GATTCTAGCGGTGAGTATCCG-3′, following the conditions described in the corresponding references.[9] The sizes of the fragments were 1023, 610, 583 and 587 bp, respectively.

To detect the presence of class 1 integrons, PCR was performed with primers 5′CS (5′-GGCATCCAAGCAGCAAG-3′) and 3′CS (5′-AAAGCAGACTTGACCTGA-3′). To identify the presence of carbapenemase genes within the integron, PCRs were developed combining 5′CS with the corresponding OXA-40, VIM and IMP reverse primers under the same conditions as those for amplification of individual genes.[6,9]

For hybridization experiments, gels of PCR amplicons or total DNA were transferred to a nylon membrane and hybridized with the corresponding carbapenemase probes made from PCR-generated fragments and labelled with digoxigenin-dUTP. Detection of hybrids was carried out using an anti-digoxigenin antibody following the manufacturer's instructions (Roche Diagnostics).

Table 1. Distribution of clones and clinical data of the corresponding patients

Year	Genotype	Isolates	Patients	Sex (M/F)	Age (range)	Diagnosis		
						COPD	PN	CB
1999	I	20	19	18/1	77 (65–91)	14	2	3
	II	33	25	21/4	79 (65–103)	15	4	6
	others	7	5	3/2	77 (70–83)	2	1	2
2002	I	34	18	16/2	78 (69–87)	4	2	12
	II	11	3	2/1	71 (65–74)	1	0	2
	others	4	4	3/1	77 (72–85)	1	0	3

COPD, chronic obstructive pulmonary disease; PN, pneumonia; CB, chronic bronchiectasis.

Results and discussion

Distinct genotypes were recognized in 1999 but the majority of the isolates were grouped into two main genotypes: clone I (33%) and clone II (55%). These two major clones were also detected in 2002, but the proportions differed: clone I accounted for 69%, whereas clone II accounted for 22% of the isolates. Patients with several isolates in both years maintained the same clone throughout. Studies carried out in the same hospital including all patients from 1999 identified up to 21 different genotypes,[6] but only clones I and II have been isolated in elderly patients, meaning that these clones are endemic in these patients.

From 1999 to 2002 there were important increases in resistance to aminoglycosides and carbapenems in isolates of clone I while clone II maintained the same level of resistance.

MIC_{90} values of imipenem, meropenem and amikacin rose significantly in 2002 for clone I compared with the values in 1999, while the values for other antibiotics were similar in both years (Table 2). Resistance rates among clone II isolates did not differ from 1999 to 2002 (100% to cefotaxime and ceftazidime,

Table 2. MIC values and level of resistance among *A. baumannii* isolates belonging to clone I

Antibiotic	Year 1999				Year 2002			
	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)	% resistance	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)	% resistance
Cefotaxime	64	128	8–>128	84	>128	>128	32–>128	88
Ceftazidime	32	>128	2–>128	73	>128	>128	2–>128	77
Imipenem	1	128	<1–>128	43	64	128	<1–>128	66
Meropenem	2	32	<1–>128	21	>128	>128	<1–>128	77
Amikacin	32	64	0.25–128	21	128	>128	8–>128	83
Gentamicin	>128	>128	0.03–>128	79	>128	>128	4–>128	77

70% to imipenem, 80% to meropenem, 60% to amikacin and 70% to gentamicin).

Since clone I became predominant at the same time that resistance rates increased, the main interest of this study was to analyse the mechanisms involved: carbapenemases and class 1 integrons, both of which are frequently related to aminoglycoside inactivating enzymes.

In 1999, seven isolates of clone I were Hodge test-positive and two of them carried the $bla_{\text{OXA-40}}$ gene. In 2002, 22 isolates were Hodge test-positive and PCR-positive for the $bla_{\text{OXA-40}}$ gene. Metallo-β-lactamase activity by the DDST was not detected, but gene amplification showed three PCR-positive isolates for $bla_{\text{VIM-2}}$ and one for bla_{IMP}. This is the first time that metallo-β-lactamase genes have been detected in our hospital.

All positive results were obtained in imipenem-resistant isolates and only one susceptible strain was positive for $bla_{\text{OXA-40}}$. The percentage of imipenem-resistant isolates PCR-positive for the $bla_{\text{OXA-40}}$ gene was 22% in 1999 and 91% in 2002.

All imipenem-resistant isolates belonging to clone II from both years were positive in phenotypic carbapenemase tests and carried the $bla_{\text{OXA-40}}$ gene, which could suggest that this gene spread from one clone to the other. Two clone II isolates were PCR-positive for $bla_{\text{VIM-2}}$.

The relatedness of isolates of both clones harbouring or lacking genes for OXA or metallo-carbapenemases was confirmed by PFGE.

During the past few years, the spread of antibiotic resistance genes has been associated with the presence of class 1 integrons, the most common and prevalent in clinical isolates.[6] In 1999 all isolates of clone I harboured 760 bp integrons, which carried genes for aminoglycoside-modifying enzymes.[6] In 2002, 50% of clone I isolates carried not only these integrons, but also combinations of other 1200 and 550 bp structures. Although other mechanisms cannot be discarded, we infer that these elements contributed to the increase in resistance to aminoglycosides in the isolates analysed in the study. In 1999, isolates of clone II harboured unique 550 bp integron structures, but another integron structure of 1200 bp was also present in 2002. Our results are in agreement with those of other authors, who have reported a high incidence of class 1 integrons in nosocomial isolates in Spain and in other countries.[10]

Although the presence of the $bla_{\text{OXA-40}}$ gene in different clones suggested the presence of a mobile element, PCRs with 5′CS/3′carbapenemase primers and DNA hybridization showed that there was no relationship between $bla_{\text{OXA-40}}$ and class 1 integrons.

Resistance to carbapenems is a worrying global phenomenon, since they are the drugs of choice in serious *Acinetobacter* infections. There are few studies of carbapenemases in our country, and they seem more common in other European countries. It is important to note that the same *A. baumannii* clone producing the OXA-40 enzyme has been identified in several hospitals.[5]

In conclusion, our results demonstrate that in elderly patients the majority of *A. baumannii* isolates belong to clone I, which has became predominant due to the increase of resistance over time. This clone harbours several integrons and produces an OXA-40-type carbapenemase. This fact emphasizes the need for a greater and rigorous control in these patients to prevent the dissemination of multiresistant isolates in the hospital environment, which would cause a serious public health problem.

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Transparency declarations

None to declare.

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