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ORIGINAL ARTICLE

Integron-mediated antibiotic multiresistance in *Acinetobacter baumannii* clinical isolates from Spain

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Objective To determine whether non-epidemiologically related, antibiotic-resistant isolates of *Acinetobacter baumannii* from different geographical origins posses common type 1 integrons.

Methods The epidemiologic relationships between seven *A. baumannii* strains recovered from different Spanish hospitals were established by pulsed-field gel electrophoresis, the presence of integrons being determined by PCR and DNA sequencing.

Results Integron analysis showed the presence of four different integrons, containing six different known genes (*aacC1*, *aacA4*, *aadA1*, *aadB*, *oxa21* and *oxa37*) plus an ORF. It was found that the same integron was present in different unrelated strains and that related strains could have different integrons.

Conclusion These results show the potential risk of integron dissemination among different strains of *A. baumannii*.

Keywords Integron, *Acinetobacter baumannii*, resistance

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INTRODUCTION

An important factor that influences the development of multiresistance is the acquisition of mobile genetic elements. Thus, plasmids and transposons carrying resistance genes have been widely described in the scientific literature [1–7]. Integrons have been identified on these mobile elements.

To our knowledge nine different types of integrons have been described up to date, those included in class 1 being by far the most extensively analyzed [8–10].

Class 1 Integrons are composed of three different elements. Two conserved regions: an integrase encoding gene in the 5′ segment (5′CS) and the genes *qacE1*, *sulI* and the *orf5* in the 3′ segment (3′CS). Between these conserved regions a variable region is found in which gene cassettes are inserted [2]. Up to three different gene cassettes are commonly found inserted in one integron. However, exceptionally, a higher number of genes can be found [8]. Furthermore, composed integrons possessing a second 3′CS have also been described. Such structures posses a common ORF (*orf513*) after the first sulfonamide-resistance gene, carrying different resistance genes between this ORF and the second 3′CS [11,12].

Acinetobacter baumannii is the most frequent and clinically important species of the genus *Acinetobacter* [1], usually presenting multiple antibiotic resistance [1,3,13–17]. Different reports identifying integrons as responsible for the presence and acquisition of antibiotic resistance in *A. baumannii* have been published [6,10,18–22]. The presence of type 1 integrons carrying aminoglycoside-resistance genes (*aadB*, *aacA4*, *aacC1*) or β-lactamases encoding genes as *oxa21*, *oxa24* or *oxa37* in *A. baumannii* from Spain have also been previously reported [10,18,22].

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The aim of this study was to investigate the role of type 1 integrons in mediating antibiotic resistance in *A. baumannii*, analyzing whether non-related isolates from different geographical origins possessed common integrons.

MATERIALS AND METHODS

Microorganisms

Seven strains of *A. baumannii* obtained from six different Spanish hospitals (Table 1) were randomly chosen to perform this study. All the strains were characterized as *A. baumannii* according to the criteria of Bouvet and Grimont [23].

Antimicrobial Susceptiblity Testing

Susceptibility to ampicillin, ceftazidime, imipenem, amoxicillin plus clavulanic acid, cotrimoxazole, tetracycline, chloramphenicol, spectinomycin, netilmicin, amikacin, nalidixic acid and ciprofloxacin was determined by the disk diffusion method in accordance with the NCCLS guidelines [24].

Epidemiologic relationships

The genetic relationship of these clinical isolates was established with low-frequency restriction analysis of chromosomal DNA using *Apa*I. Plugs were prepared following the procedure of Gautom [25], and the DNA fragments were separated in a 1% agarose pulsed field gel electrophoresis (PFGE). The electrophoresis conditions were 200 V, 20 h, with pulse times ranging from 5 to 8 seconds. Banding patterns were digitized and stored as TIFF files. Patterns were analyzed using the Lane Manager software (TDI, Madrid, Spain) to calculate Dice coefficients of correlation and to generate a dendrogram by the unweighted pair group method using arithmetic averages (UPGMA) clustering.

Amplification of integrons

PCR amplification of type 1 integrons was done with the set of primers described by Levesque and Roy [27], following previously described conditions and procedures [18]. The amplified products were resolved in 2% agarose gel and stained with ethidium bromide, 0.5 mg/L. The bands were recovered from the gel using the Gene-Clean kit (Bio101, Inc., La Jolla, CA, USA), cloned in a pCRII vector, and transformed into *Escherichia coli* competent cells (Invitrogen BV, Leek, The Netherlands).

DNA Sequencing procedures

Plasmid DNA, with the cloned integrons, was extracted and directly sequenced with the Thermosequenase Dye Terminator Sequencing kit (Amersham, Cleveland, OH, USA) in an automatic DNA sequencer (377; Applied Biosystems,

Table 1 Source and epidemiologic relationships between the strains

Strain/Year	Hospital/City	Drug Resistances	PFGE type	Integron type
87/1988	HCP/Barcelona	Cm, Amp, Caz, A/C	B	1700/*aadB, oxa-21*
74I/1997	HLP/Madrid	Tc, Cm, Sxt, Spt, Net, Ak, Amp, Caz, A/C, Nal, Cip	D	550/*aacC1* 2100/*aacA4, ORF, oxa37*
875/1995	HVR/Sevilla	Tc, Cm, Sxt, Spt, Net, Ak, Amp, Caz, Imp, A/C, Nal, Cip	C	550/*aacC1* 750/*aadB*
6R/1997	HDO/Madrid	Tc, Cm, Sxt, Spt, Net, Ak, Amp, Caz, Imp, A/C, Nal, Cip	A	550/*aacC1* 750/*aadB* 1700/*aadB, oxa21*
F_{14}/1990	HVH/Barcelona	Tc, Cm, Sxt, Spt, Ak, Amp, Caz, A/C, Nal, Cip	A	>2Kb/*aacA4, oxa21, aadA1*
709R/1997	HSJ/Reus	Tc, Cm, Sxt, Spt, Net, Ak, Amp, Caz, A/C, Nal, Cip	A	550/*aacC1* 750/*aadB*
203/1997	H.LP/Madrid	Cm, Spt, Net, Ak, Amp, Caz, A/C, Nal, Cip	B	750/*aadB*

Tc, Tetracycline; Cm, Chloramphenicol; Sxt, Cotrimoxazole; Spt, Spectinomycin; Net, Netilmycin; Ak, Amikacin; Amp, Ampicillin.
Caz, Ceftazidime; Imp, Imipenem; A/C, Amoxicillin plus clavulanic acid; Nal, Nalidixic acid, Cip, Ciprofloxacin.
HCP, Hospital Clínic i Provincial; HDO, Hospital Doce de Octubre; HLP, Hospital La Princesa; HSJ, Hospital Sant Joan; HVH, Hospital Vall d'Hebró; HVR, Hospital Virgen del Rocio.

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Foster City, CA, USA). The sequencing strategy included an initial sequencing with the primers originally used to amplify the integrons and the posterior designing of novel primers to move downstream into the more central gene cassettes.

RESULTS

All seven *A. baumannii* isolates were resistant to chloramphenicol, ampicillin, amikacin, ceftazidime and amoxicillin plus clavulanic acid. Only isolate 87 showed susceptibility to nalidixic acid, ciprofloxacin, tetracycline, cotrimoxazole, spectinomycin, Finally isolates 87 and F14 both showed susceptibility to netilmicin (Table 1).

The application of the Dice coefficient to the results obtained by low-frequency restriction analysis of chromosomal DNA and PFGE showed the presence of four different profiles, which were arbitrarily named A to D. The criteria used to define clonal relations among strains was based on a difference of less than three bands, corresponding in the dendrogram to 85% similarity (Figure 1). Group A was composed of three strains (6R, F_{14} and 709R) group B included two strains (87 and 203) while the remaining groups were all of single isolates.

PCR amplification with integron-specific primers (Figure 2) resulted in the amplification of two common bands of circa 550 bp and 750 bp respectively in 5 out of 7 strains. Upon sequencing, the smallest band was found to be an integron carrying an *aacC1* gene, while the larger one carried a single *aadB* gene. Two unrelated strains (87 and 6R) carried an integron with a molecular size of 1700 bp containing two genes: an *aadB* and an *oxa21*. The two largest integrons, with a 2.1 kb and >2.1 kb were found in isolates 74I and F_{14} respectively. The 2.1 kb integron contained an *aacA4* and an *oxa37*, plus a putative unknown protein encoding region with a longer sequence than AJ251519. The largest one with >2.1 kb contained three genes.an *oxa21*, *aadA1* and an *aacA4* (Table 1).

DISCUSSION

Many different genes responsible for antibiotic resistance have been found in integrons, among which those coding for different aminoglycoside-modifying enzymes, β-lactamases or dihydrofolate reductases can be found [2,8,18,19,27]. Moreover, some different ORF with unknown functions have been described [8,20,27,28]. In this study, five different integrons have been found, all of them presented at least one gene encoding an aminoglycoside-modifying enzyme. The *aacC1* and the *aadA1* genes were only found in one type of integron each, whereas *aadB* and *aacA4* genes

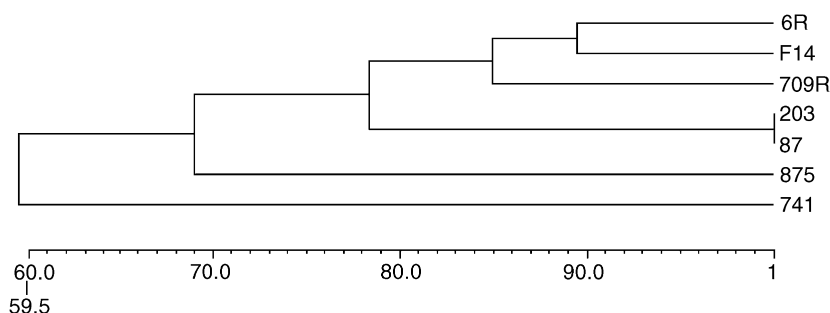

Figure 1 Dendrogram of the clonal relationship of the selected strains.

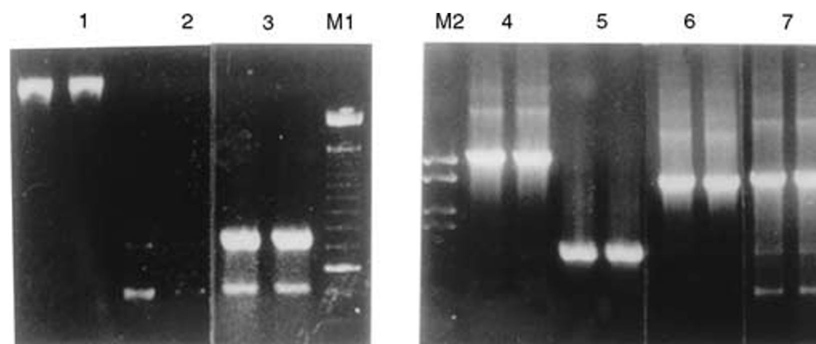

Figure 2 PCR amplification of integrons. PCR amplification of integrons. Lane M Molecular weight marker (100 bp ladder, GIBCO BRL, Gaithersburg, MD), lane 1, strain 875, lane 2, strain 6R; lane 3, strain 87; lane 4, strain 709; lane 5, strain 74I; lane 6, strain 203; lane 7, strain F_{14}.

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were each located in two different sized integrons. As for the β-lactamases, an *oxa21* gene was found in two different integrons, while an *oxa37* gene was located in one. This high prevalence of aminoglycoside-modifying enzymes in *A. baumannii* integrons is in accordance with Bissonnette and Roy [8], who described *aadA1* and *aadA2* genes as being the most frequently found cassettes in multiresistance integrons. Furthermore, these genes have been previously described in *A. baumannii* clinical isolates from other European countries, suggesting wide dissemination [19,20]. In a similar way, the most frequently encoded β-lactamase genes in these genetic elements include the OXA type [7,8,18,19]. Once more these results are in agreement with what has been published regarding *A. baumannii* integrons, among which a high prevalence of both aminoglycoside-modifying enzymes and β-lactamases have been found [10,18–20].

Our results point out the possibility that unrelated strains, with a different geographical origin, acquire the same integron, as shown with the common integrons of circa 550 bp (present in strains belonging to all the different PFGE-types) and 750 bp (from strains belonging to the PFGE-types A, B, and C) as well as the integron carrying an *aadB* and an *oxa21* of circa 1700 bp present in strains of type A and B. Interestingly, these results also show that related strains may possess unrelated integrons. Thus, the two isolates belonging to type B (strains 87 and 203) had a different integron profiles. Similarly, isolate F_{14} had an integron >2 kb that was absent from the other isolates of type A.

The integrons found in our strains carried genes that were identical or closely related to others that have been previously found in integrons from other organisms such as *Pseudomonas aeruginosa* [5,20,27,29]. Both *A. baumannii* and *P. aeruginosa* are important nosocomial pathogens living in environments with high antibiotic pressure such as intensive care units, and thus frequently showing multiresistance. The close similarity between the integrons present in *P. aeruginosa* and *A. baumannii*, as for example that found in isolate 74I, suggest the transfer of genetic elements between these two microorganisms, which could be plasmid-mediated as has previously been observed in *Salmonella* [4]. Further studies are required to elucidate the process by which microorganisms evolve towards multirresistance.

In conclusion, our results show that integrons can play an important role in the acquisition of multiresistance in *A. baumannii*, especially resistance to β-lactam and aminoglycoside antibiotics, and suggest the potential transfer of genetic material between *A. baumannii* and *P. aeruginosa*.

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