

Dissemination of Class 1, 2 and 3 Integrons among Different Multidrug Resistant Isolates of *Acinetobacter baumannii* in Tehran Hospitals, Iran

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Abstract

A total of 100 non-duplicate *Acinetobacter baumannii* isolates were collected from different hospitals in Tehran and were confirmed as *A. baumannii* by conventional biochemical and API testing. Antimicrobial susceptibility of these isolates was checked by a disk diffusion method in accordance with CLSI guidelines. The isolates were then detected as carrying class 1 and 2 integron gene cassettes by PCR evaluation and then genotyped by REP-PCR. More than 50% (n = 50) of the isolates were multidrug resistant. The results showed that more than 80% of all multidrug resistant *A. baumannii* strains carry a class 1 integron. Distribution of IntI1 and IntI2 among *A. baumannii* isolates was 58% and 14%, respectively. Analysis of a conserved segment of class 1 integron showed a range from 100 bp to 2.5 kb. REP-PCR fingerprinting showed more than 20 genotypes among *A. baumannii* strains. There was no relationship between REP genotypes and the distribution of different classes of integrons. This is a comprehensive study on the distribution of different classes of integrons among *A. baumannii* in Iran. Considering the exact role of integrons in coding drug resistance in bacteria, the findings of this study could help us find antimicrobial resistant mechanisms among *A. baumannii* isolates in Iran.

Key words: *A. baumannii*, hospital isolates in Iran, integron classes

Introduction

Acinetobacter baumannii is an important opportunistic pathogen responsible for a variety of nosocomial infections, including ventilator-associated pneumonia, bacteremia, surgical-site infections, secondary meningitis, and urinary tract infections (von Dolinger *et al.*, 2005; Fontana *et al.*, 2008; Peleg *et al.*, 2008).

Most *A. baumannii* infections are caused by the outbreak strains, which can spread widely and rapidly between patients. Since these strains also exhibit multiple-antibiotic resistance, it has been suggested that epidemic potential among isolates of *A. baumannii* may be linked to the presence of integrons.

Integrons are DNA elements capable of capturing genes by a site-specific recombination mechanism that often carry gene cassettes, containing antibiotic resistance genes (Turton, *et al.*, 2005). Various studies have reported the existence of antibiotic resistance genes located on integrons among *Acinetobacter* spp. (Gallego

and Towner 2001; Navia *et al.*, 2002; Nemeč *et al.*, 2004; Zarrilli *et al.*, 2004).

Several classes of integrons have been described, with class I integrons being the most common and widely distributed among Gram-negative bacteria. Integrons have been found in isolates of *Acinetobacter* spp. from different locations of the world and it has been suggested that multi-resistant isolates of *Acinetobacter* spp. may act as a reservoir of integron-associated antibiotic resistance gene, which could then spread to other pathogens in the hospital environment (Gallego and Towner 2001).

Few studies have hitherto focused on the distribution of antibiotic resistance genes among *Acinetobacter* spp. in Iran (Feizabadi *et al.*, 2008; Taherikalani *et al.*, 2008; Taherikalani *et al.*, 2009; Akbari *et al.*, 2010); however, there is limited information on the detection of different classes of integrons in Iran.

This study aimed to determine the distribution of class 1, 2 and 3 integrons among *A. baumannii* isolates, collected from different clinical specimens in selected

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hospitals, in Tehran and to evaluate any correlations between antibiotic resistance and the carriage of different classes of integrons among *A. baumannii* isolates.

Experimental

Materials and Methods

Bacterial isolates. A total of 100 non-duplicate isolates of *Acinetobacter* spp. were collected from different clinical specimens during 2007–2009. These isolates were confirmed as *A. baumannii* by conventional biochemical testing and 20NE API galleries (BioMerieux, Inc) used in the previous studies (Feizabadi *et al.*, 2008; Taherikalani *et al.*, 2009; Akbari *et al.*, 2010). These studies were carried out in the laboratory of microbiology in Ilam University, Iran. Fifty six percent (n = 56) of the isolates were recovered from wound and trachea. The strains isolated were then stored at -80°C in nutrient broth containing 50% glycerol v/v for further investigation.

Antimicrobial susceptibility testing. Antimicrobial susceptibility testing was performed by disk agar diffusion, according to CLSI guidelines. The applied antimicrobials were as follows: ampicillin-sulbactam (10/10 µg), piperacillin (100 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftioxone (30 µg), cefepime (30 µg), imipenem (10 µg), ciprofloxacin (5 µg), amikacin (30 µg), gentamicin (10 µg) and tetracycline (10 µg). Inoculums of the *A. baumannii* isolates (10⁶ CFU) were swabbed on several Muller-Hinton agar plates, and different disks, impregnated each with different antibiotics, were then placed on these plates. Incubation at 37°C for 24 h was then carried out after which the inhibition zones were read. *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213 and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains.

PCR amplification of integron-associated genes for different integron classes. DNA extraction was carried out using commercial standard kit (Bioner, Republic of Korea) and 4 µl of the suspension were used as the template DNA for PCR.

PCR annealing temperature, primer sequences and the amplicon sizes are listed in Table I (Srinivasan, Rajamohan *et al.*, 2009).

The PCR conditions were as follows: initial denaturation at 95°C for 5 min; 30 cycles with denaturation at 95°C for 30s, annealing at 50°C, 51°C, 52°C and 53°C for 30s for integrons class 1, 2 and 3 and conserved sequence of integron class 1 respectively, and extension at 72°C for 45s followed by final extension at 72°C for 7 min. PCR products were separated by electrophoresis on a 1% agarose gel and were detected by comparison against a 100 bp DNA ladder as a size marker under the visualization of UV light on Geldoc apparatus.

REP-PCR Finger-printing. DNA extraction was carried out by DNA extraction kit (Bioner, Republic of Korea) and 4 µl of the extract was used as the template DNA. The primer pair REP1, 5'-IIIGCGCCGICATCAGGC-3' and REP2, 5'-ACGTCTTATCAGGCCTAC-3' were used to amplify putative REP-like elements in the genomic bacterial chromosomes (Bou *et al.*, 2000). Amplification reaction was performed in a final volume of 25 µl. Each reaction mixture contained 2.5 µl of 10X PCR buffer, 1.25 U Taq DNA polymerase (Fermentas, UK), and 0.8 µl of mixed dNTPs (Fermentas, UK), 1.5 µl of 25 Mm MgCl₂, 1 µl of 10 pmol primers and 50 ng of bacterial DNA. Amplification reaction was carried out by thermal cycler (Ependorff, Germany) with an initial denaturation at 94°C for 10min, followed by 30 cycle of denaturation at 94°C for 1 min, annealing at 45°C for 1 min, and extension at 72°C for 1 min, followed by final extension at 72°C for 16 min. Aliquots of each sample were subjected to electrophoresis in 1.2% agarose gels. Amplified products were detected by Geldoc apparatus after staining with ethidium bromide (50 mg/L) and the created photographs were then analysed visually and with the TotalLab TL120 software.

Results

The most effective antimicrobial agents against *A. baumannii* isolates were as follows: gentamicin 55% (n=55), imipenem 47% (n=47), ampicillin-sulbactam, ami-

Table I
Primers used in PCR amplification of integron classes 1 to 3

Target gene	Forward	Reverse	Annealing Temperature (°C)	Size of amplicon (bp)
<i>intI 1</i>	5'-ACATGTGATGGCGACGCACGA-3'	5'-ATTTCTGTCTGGCTGGCGA-3'	50	300
<i>intI 2</i>	5'-CACGGATATGCGACAAAAAGGT-3'	5'-GTAGCAAACGAGTGACGAAATG-3'	51	962
<i>intI 3</i>	5'-GCCTCCGGCAGCGACTTTCAG-3'	5'-ACGGATCTGCCAACCTGACT-3'	52	1041
Conserved Segment of <i>IntI 1</i>	(5'-CS) 5'-GGCATCCAAGCAGCAAG-3'	(3'-CS) 5'-AAAGCAGACTTGACCTGA-3'	53	Variable

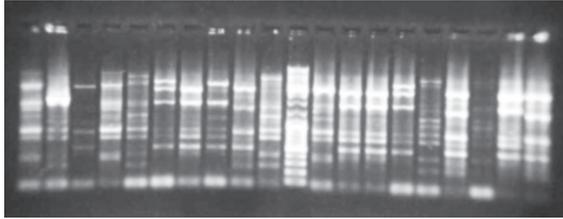


Fig. 1. Variable amplicon size of conserved segments of integron class 1.

Lane 1 (negative control: DDW); Lanes 3 and 8 (clinical positive sample); Lanes 2 and 4-7 and 9-11 and 13-21 (clinical negative samples).

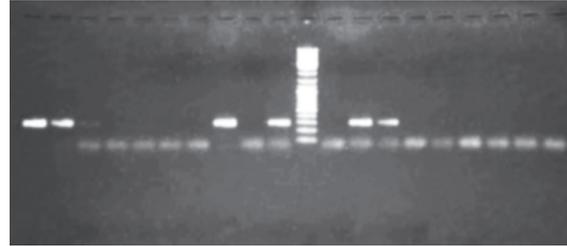


Fig. 3. PCR of class 2 integron among clinical *A. baumannii* isolates. Lanes 1-2, 8, 9, 13-14 (clinical positive isolates), Lane 11 (100 bp DNA size marker); Lanes 3-7, 9, 12, 15-20 (clinical negative isolates).

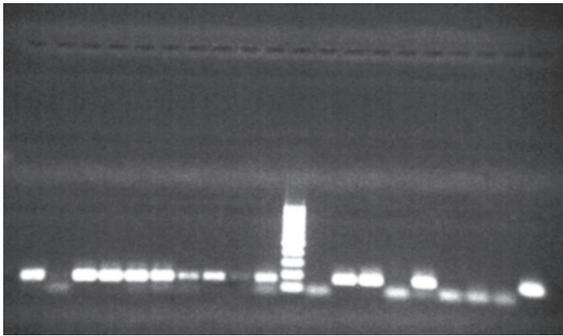


Fig. 2. PCR of class 1 integron among clinical *A. baumannii* isolates.

Lanes 1-2, 4-5, 7-8, 10 (clinical positive isolates), Lane 11 (100 bp DNA size marker), Lanes 12-20 (clinical positive isolates).

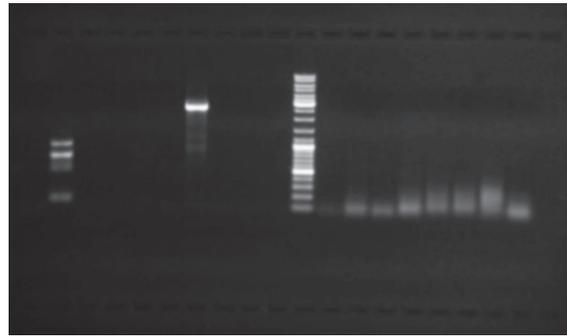


Fig. 4. REP PCR. Pattern of genomic DNA from 19 clinical *A. baumannii* isolates.

Lanes 1-10, 12-20 (clinical isolates of *A. baumannii*), Lane 11 (DNA Ladder 100 bp to 3000 bp).

kacin 38% ($n = 38$) and tetracycline 31% ($n = 31$). Most isolates showed high resistance to piperacillin (100%) and cephalosporin drugs (more than 95%).

The REP- fingerprinting of some *A. baumannii* isolates are shown in Fig. 1. All the isolates not previously compared with REP or other typing methods were revealed to have 20 REP patterns. No reliable REP pattern was observed among 15 isolates.

PCR detecting integrase gene showed that 58% ($n=58$) of all the isolates had *intI 1*; however, *intI 2* was only identified in 14% ($n = 14$) of the isolates and *intI 3* was not revealed in any of the clinical isolates. The coexistence rate of *intI 1/intI 2* was 9% ($n = 9$). The relationship between antibiotic resistance and the existence of different integrons is shown in Table II. More than 50% of penicillin and cephalosporin resistant isolates har-

Table II
Distribution of *intI 1* and *intI 2* among *A. baumannii* isolates resistant to different antibiotic agents

Antibiotic agent	Number of resistant isolates t	Class 1 integron n (%)	Class 1&2 integrons n (%)	Class 2 integron n (%)t
Piperacillin	100	58 (58)	14 (14)	9 (9)
Ampicillin- sulbactam	62	24 (37.5)	3 (4.83)	1 (1.61)
Ciprofloxacin	85	51 (60)	14 (16.4)	9 (10.58)
Amikacin	62	27 (43.54)	10 (16.12)	7 (11.29)
Imipenem	53	24 (45.28)	7 (13.20)	4 (7.54)
Cefotaxime	97	57 (58.76)	13 (13.4)	9 (9.27)
Cefepime	99	58 (58.58)	14 (14.14)	9 (9.09)
Ceftazidime	97	57 (58.76)	13 (13.4)	9 (9.27)
Ceftriaxone	97	56 (57.73)	14 (14.43)	9 (9.27)
Tetracycline	31	16 (51.6)	7 (22.58)	5 (16.12)
Gentamicin	45	18 (40)	8 (17.7)	4 (8.88)

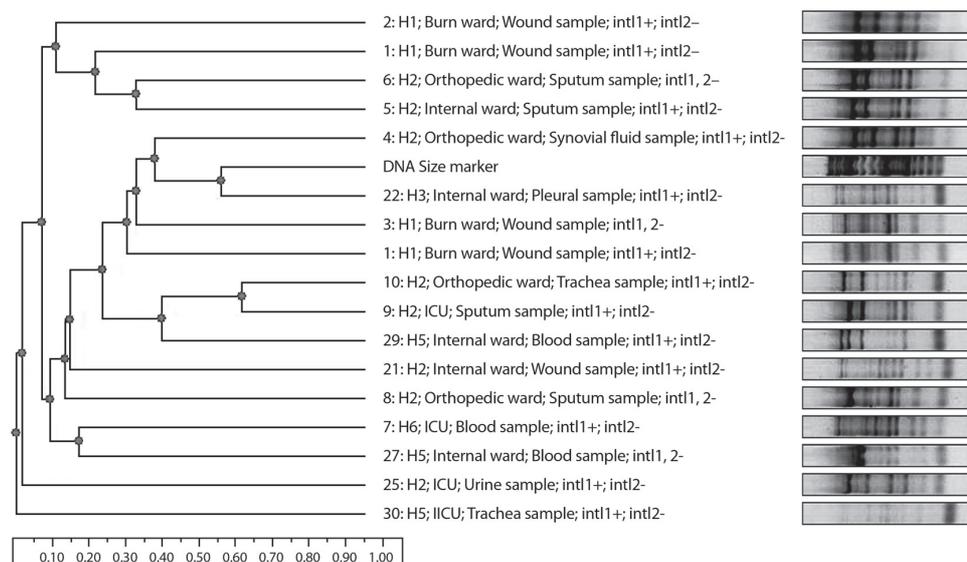


Fig. 5. UPGMA dendrogram illustrating the relationships between sample origins, hospitals, wards and the existence of class 1 and 2 integron genes among the genotypes of *A. baumannii* isolates.

bored different integrons. Ceftriaxone and cefotaxime resistant isolates were the most common *intI* 1 harboring isolates (58.76%), followed by ceftriaxone and cefepime with 58.73% and 58.58%, respectively. However, tetracycline resistant isolates were the most common *intI* 2 harboring isolates (22.58%), followed by gentamicin and amikacin with 17.7% and 16.12%, respectively (Figs 3, 4). Amplification of the integron gene cassettes of the integrase positive isolates gave PCR products of various sizes ranging from 100 bp to 2.5 kb (Fig. 4). Distribution of the integron gene cassettes among integrase positive isolates was accounted for 10% ($n = 10$). Various amplicons of integron gene cassettes were not seen among all integrase positive isolates. The correlations between REP-genotype, different hospitals, wards, sample origins and the existence of integrons are shown in dendrogram (Fig. 5).

Discussion

Acinetobacter baumannii is typically resistant to various antimicrobial agents such as penicillins, cephalosporines, macrolides, aminoglycosides, tetracyclines and fluoroquinolones (Wang *et al.*, 2007).

Because of the multidrug resistance and tendency to spread in hospital population, *A. baumannii* has a special clinical significance, requiring epidemiologic monitoring as a measure for control of nosocomial infection.

On the basis of the sequence of integrase gene, integrons are divided into at least six classes, with class 1 integron being the most common among the clinical isolates of Gram-negative bacteria, including acineto-

bacters (Koeleman *et al.*, 2001; Turton *et al.*, 2005). It seems that class 2 integrons were rarely detected in *Acinetobacter* spp., but class 3 integrons were not detected in those bacteria at all (Ploy *et al.*, 2000; Koeleman *et al.*, 2001; Turton *et al.*, 2005).

PCR detecting integrase gene used has advantages over the integron cassette PCR in screening for integrons, in that it is designed to give a small product which is easily amplified. Integron cassette PCR can give a negative result even when the integrons are present, if the cassette array is difficult to amplify or if there are no cassettes present. The PCR detecting integrase gene was simple, reliable, and easy to perform (Turton *et al.*, 2005).

In the current study a high prevalence of *intI* 1 and *intI* 2 was found among multidrug resistant *A. baumannii* strains, isolated from different specimens. The results related to class1 integron were in concordance with other studies (Gonzalez *et al.*, 1998; Seward 1999). Although some studies clarified the presence of *intI* 2 among *A. baumannii* strains, only 14% of the isolates in this study seemed to harbor this class of integrons. These findings are relatively significant since most studies report that class 2 integrons are not found or are found in low rates among *A. baumannii* strains (Ploy *et al.*, 2000; Turton *et al.*, 2005). Class 3 integron was not found among *A. baumannii* strains, which was in accordance with other reports (Ploy *et al.*, 2000). Although amplicons with variable sizes were found in integron gene cassette, in agreement with other studies, these variable amplicon could not be detected among all integrase positive strains (Turton *et al.*, 2005).

Antibiotic resistance is an important factor in the spread of nosocomial infection. It is generally considered that the existence of integrons confers the advantage of antibiotic resistance upon the strains. Among the multiresistant strains described in this study, there were still some strains which did not contain integrons (of classes 1 and 2 at least); however, most of them were susceptible to gentamicin, imipenem, and ampicillin-sulbactam.

Integrons containing the same organization of cassettes were found in various REP genotypes, suggesting horizontal transfer of integrons, also reported in other studies (Sallen *et al.*, 1995; Seward 1999). In addition, the promoter sequences were mostly conserved, even in isolates from different countries with distinct selective pressure, suggesting that acquisition of resistance is likely due to transfer of entire integrons *via* plasmids and/or transposons rather than of individual cassettes (Ploy *et al.*, 2000). In concordance with other studies, it was proven that the isolates of the same genotype possess different integrons and in the same way, the unrelated isolates with different genotypes could contain the same integrons.

Similar 2.5-kb integrons, with integron cassettes, found by PCR in the present study, have been widely found in isolates of European clones I and II from many countries (Nemec *et al.*, 2004; Turton *et al.* 2005). The 2.5-kb integron has also been found in a number of outbreak strains of *A. baumannii* of different genotypes in Italy, Russia and Ireland (Gombac *et al.*, 2002; Zarrilli *et al.*, 2004; Turton *et al.*, 2005). In conclusion, integrons could be a feature of epidemic strains or clones of *A. baumannii* currently found in Iran. Information on both the genotype and integron type is useful in epidemiological studies. The association of integrons with epidemic behaviors merits further studies.

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