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ORGINAL PAPER

Association between Existence of Integrons and Multi-Drug Resistance in *Acinetobacter* Isolated from Patients in Southern Iran

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Abstract

Nosocomial infections caused by multi-drug resistant *Acinetobacter* pose a serious problem in many countries. This study aimed at determining the antibiotic susceptibility patterns and prevalence of different classes of integrons in isolated *Acinetobacter*. In addition, the association between production of specific bands in PCR assay and magnitude of multi-drug resistance was investigated. In total, 88 *Acinetobacter* strains were isolated from patients from October 2008 through September 2009. The Minimal inhibitory concentration (MIC) of 12 antibiotics conventionally used in clinics against the isolates, was determined by E-test method. The existence of integron classes was investigated by PCR assay through the amplification of integrase genes. The most effective antibiotic against *Acinetobacter* was colistin with 97.7% activity, followed by imipenem (77.3%) and meropenem (72.7%). The presence of integron classes 1 and 2 in 47 (53.4%) isolates was confirme, However, no class 3 was detected. The proportion of class 1, compared with class 2, was high (47.7% vs. 3.4%). The association between multi-drug resistance to norfloxacin, ceftazidime, gentamicin, ciprofloxacin, cefepime and amikacin and the presence of integrons was statistically significant. However, the association was not remarkable in many of the isolates which exhibited resistance to the rest of antibiotics. This may imply that in addition to integrons, other resistance determinants such as transposon and plasmid may also contribute to resistance. To reduce the pressure on sensitive isolates, comprehensive control measures should be implemented. Furthermore, wise application of effective antibiotics could help alleviate the situation. Colistin is the most effective antibiotic *in vitro* against *Acinetobacter.*

Key words: *Acinetobacter*, integrons, multi-drug resistance, PCR assay

Introduction

Acinetobacter is a gram-negative bacterium, which is a very effective human colonizer found in many health care environments (Bergogne-Berezin and Towner, 1996; Perez *et al.*, 2007). The combination of its environmental resilience and its wide range of resistance determinants renders it a successful nosocomial pathogen (Nordmann, 2004). Nowadays, *A. baumannii* is emerging as a cause of numerous global outbreaks, displaying ever-increasing rates of resistance (Villegas and Hartstein, 2003).

Although six classes of integrons exist (Neild *et al.*, 2001), three main classes have been described (Rowe-Magnus and Mazel, 1999). All integrons have a 5' conserved segment, including an *intI* gene encoding an integrase and an *attI* recombination site, but have distinct 3' conserved segments. As for the class 1 integrons, the 3' conserved segment includes three open reading frames (ORFs): *qacEΔ1*, a deletion derivative of the antiseptic resistance gene *qacE*; the *sul1* sulfonamide resistance gene; and ORF5, of unknown function (Radström *et al.*, 1994). The second class of integrons was found in transposon Tn7 and its derivatives, and its 3' conserved segment contains five *tns* genes involved in the movement of the transposon. A single class 3 integron has been reported to date, but its 3' conserved segment has not been characterized (Arakawa *et al.*, 1995). Class 1 integron is more frequent in *Acinetobacter* species (Koeleman *et al.*, 2000; Galleco and Towner, 2001; Gaur *et al.*, 2006; Ploy *et al.*, 2000; Xu *et al.*, 2008).

The objectives of this study were to determine the prevalence of different classes of integrons in isolated *Acinetobacter* and to find out the association between the presence of integrons and antibiotics resistance. Furthermore, the relationship between the productions of specific bands in PCR assay with the extension of multi-drug resistance was studied.

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Experimental

Material and Methods

Isolation of *Acinetobacter*. Eighty eight *Acinetobacter* strains were isolated from patients hospitalized in Nemazee hospital, affiliated with Shiraz University of Medical Sciences, Iran during the period from October 2008 to September 2009. Identification of the isolates was carried out using the API 20E system (bioMérieux, Marcy l'Etoile, France).

MIC determination. Minimal inhibitory concentrations (MICs) of 12 antibiotics including, ciprofloxacin, colistin, ceftazidime, ampicillin/sulbactam, imipenem, meropenem, gentamicin, norfloxacin, amikacin, cefepeme, tobramycin and cefoperazon/sulbactom against the isolates were determined by the E-test method and the results were interpreted as recommended by the manufacturer's instructions.

DNA extraction and PCR assay. Bacteria DNA were harvested by conventional phenol-chloroform extraction method. The quantity of DNA was determined by Nanodrop (NanoDrop Technologies, Wilmington, Delaware USA) and adjusted 50 ng μl^{-1}. Determination of integron classes was performed by multiplex PCR using the primers described in Table I. The primers were obtained from TIB MOLBIOL Syntheselabor GmbH (Berlin, Germany). PCR assay was performed in 20 µl volume, containing 0.4 mM deoxynucleoside triphosphate (dNTP), 2 ml of 10X PCR buffer, 1 U of *Taq* polymerase (Fermentas, Lithuania), 0.6 mM $MgCl_2$, 0.25 mM of each primer and 250 ng DNA in 5 µl volume were added to the reaction mixtures. PCR was performed under pre-denaturation at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds ending with a final extension step at 72°C for 5 min and held at 8°C. Products were electrophoresed in 2% agarose, stained by ethidium bromide and video images were obtained by gel documentation (Uvtec, Sigma, Germany) system.

Primers *Int1F* and *Int1R* were used to amplify a 160 bp fragment of the *intI1* gene for the class 1 integrase and the primers *Int2F* and *Int2R* amplified a fragment of 288 bp, specific for the *intI2* gene. Primers *Int3F* and *Int3R* were used to amplify a specific *intI3* gene.

Detection of the complete gene composition of class 1 integrons was performed with primers for the 5' and 3' conserved segments. This PCR also permitted the determination of the size of any inserted gene cassette. The size range of the detected inserted gene cassette varied from 500 to > 3000 bp.

PCR assay to detect complete gene makeup of class 1 integron was carried out in 20 µl volume with the same concentration mixture mentioned above. The only modification was increased to 3U amount of Smart Taq polymerase (Fermentas, Lithuania). PCR amplification program was as follows: 5 minutes of initial denaturation at 94°C , 1 min of denaturation at 94°C , 1 min of annealing at 55°C, and 30 seconds of extension at 72°C for a total of 35 cycles. Five seconds were added to the extension time at each cycle.

Statistical analysis. Correlation between antibiotic resistance patterns and presence of different classes of integron was determined by Chi-square and Fisher's Exact test by SPSS version 15 software. The significant level was defined as $P < 0.05$.

Results

Acinetobacter spp was mostly isolated from the blood, 35 (39.8%) and predominantly in men (70%), than in women (30%). *A. baumannii* was dominant in the isolated bacteria 79 (89.8%), followed by *Acinetobacter lwoffii* 8 (9.1 %). 97.7% of *Acinetobacter* isolates were susceptible to colistin, 77.3% to imipenem, 72.7% to meropenem, 67% to cefoperazon/sulbactam, 63.6% to tobramycin, 61.4% to ampicillin/sulbactam, 26.1% to ciprofloxacin, 25% to amikacin, 23.8% to norfloxacin, 20.4% to gentamicin, 19.3% to cefepime and 18.2 % to ceftazidime.

Diagnosis of integrons was carried out by multiplex PCR assay. The presence of *intI1* and *intI2* was confirmed while *intI3* was not detected. Figure 1 demonstrates the presence of integrase 1 and 2. In total,

Table I
Primers used in this study

Primer	Nucleotide sequence	References
Int1-F	5' CAG TGG ACA TAA GCC TGT TC 3'	Koeleman *et al*., 2000
Int1-R	5' CCC GAG GCA TAG ACT GTA 3'	
Int2-F	5' TTG CGA GTA TCC ATA ACC TG 3'	
Int2-R	5' TTA CCT GCA CTG GAT TAA GC 3'	
Int3-F	5' ACG GAT CTG CCA AAC CTG ACT 3'	Ploy *et al*., 2000
Int3-R	5' GCC TCC GGC AGC GAC TTT CAG 3'	
CS-F	5' GGC ATC CAA GCA GCA AG 3'	Lévesque *et al.,* 1995
CS-R	5' AAG CAG ACT TGA CCT GA 3'	

Table II
Prevalence of different classes of integrons in *Acinetobacter* isolates

Percentage	Number	Integron
47.7	42	Class I
3.4	3	Class II
2.3	2	Class I & Class II
0	0	Class III
46.6	41	Without integron
100	88	Total

47 (53.4%) *Acinetobacter* strains exhibited either a class 1 integrase or class 2 integrase or both of them. Table II shows the prevalence of different classes of integron in *Acinetobacter* isolates.

Forty five (57%) of the 79 *A. baumannii* strains, and 2 (25%) of the 8 *A. lwoffii* strains carry an integron. The frequencies of the different antibiotic resistance patterns and their association with integrons were assessed. Based on this evaluation, strains exhibiting resistance to the panel of antibiotics including norfloxacin, ceftazidime, gentamicin, ciprofloxacin, amikacin, cefepime and norfloxacin, ceftazidime, gentamicin, ciprofloxacin, ampicillin/sulbactam, amikacin, cefoperazon/sulbactom, cefepime showed high prevalence of class 1 integrons.

Data also indicate an association between the panel of antibiotics including norfloxacin, ceftazidime, gentamicin, ciprofloxacin, amikacin, tobramycin, cefepime and class 2 integron (Table III). Amplification of integron class 1 produced bands ranging between 500 to

Table III
Association between Antibiotic resistance and integron classes

Integron	Antibiotic resistance pattern			
	Total	Class I & II	Class II	Class I
TZ	1	–	–	1
TZ–AK	–	–	1	1
AB-PM	–	–	–	1
AK-TM	–	–	–	–
GM-TM	–	–	–	1
AB-IP-MP	–	–	–	1
NX-TZ-GM-TM-PM	–	–	–	1
TZ-GM-AK-TM-PM	1	–	–	1
NX-TZ-GM-CI-AK-PM	11	–	–	13
NX-TZ-GM-CI-AB-PM	2	–	–	2
NX-TZ-GM-AK-TM-PM	–	–	–	1
NX-TZ-CI-AK-MP-PM	–	–	–	1
NX-TZ-GM-CI-AK-CPS-PM	1	–	–	2
NX-TZ-GM-CI-AK-TM-PM	4	3	–	14
NX-TZ-GM-CI-AK-PM-CO	–	–	–	1
NX-TZ-GM-AK-IP-TM-MP-PM	1	–	–	1
NX-TZ-GM-CI-AB-AK-CPS-PM	8	–	–	11
NX-TZ-GM-CI-AK-TM-MP-PM	1	–	–	2
NX-TZ-GM-CI-AB-AK-TM-PM	1	–	–	1
TZ-GM-AB-IP-TM-MP-PM-CO	–	–	–	1
TZ-GM-CI-AK-IP-TM-MP-PM	1	–	–	1
NX-TZ-GM-CI-AB-AK-TM-MP	1	–	–	1
NX-TZ-GM-CI-AB-IP-CPS-MP-PM	2	–	–	2
NX-TZ-GM-CI-AB-AK-IP-CPS-MP-PM	5	–	1	6
NX-TZ-GM-CI-AB-AK-IP-TM-CPS-MP-PM	1	–	–	8
Sensitive	1	–	–	12
Total	42	3	2	88

Abbreviations: CI – ciprofloxacin; CO – colistin; TZ – ceftazidime; AB –ampicillin/sulbactam; IP – imipenem; MP – meropenem; GM – gentamicin; NX–norfloxacin; AK – amikacin; PM – cefepime; TM – tobramycin; CPS – cefoperazon/sulbactom.

Table IV
Size of amplicons when primers used to amplify integron class 1

Pattern of integron I bands (pb)	No. of isolates
500, 600, 1300	1
500, 800, 1200	1
500, 600, 800, 1200	13
500, 600, 800, 1200, 2500	5
500, 600, 800, 1200, >3000	1
500, 800, 900, 1200, 2500	1
500, 800, 1000, 1200, 2500	2
620, 900, 1300, 1700, >3000	1
500, 600, 800, 900,1200, 2500	2
500, 600, 800, 1000, 2300, 2500	1
500, 600, 800, 1200, 1500, 2500	2
500, 600, 800, 1200, 2500, 3000	2
500, 800, 1000, 1200, 1500, 2500	1
500, 600, 800, 900, 1200, 1500, 2500	4
500, 600, 800, 1000, 1200, 1500, 2500	4
500, 600, 800,1200, 1500, 2400, 2500	1
500, 600, 800, 900, 1200, 1500, 1700, 2500, >3000	1
500, 600, 750, 800, 900, 1200, 1300, 1500, 2500, >3000	1

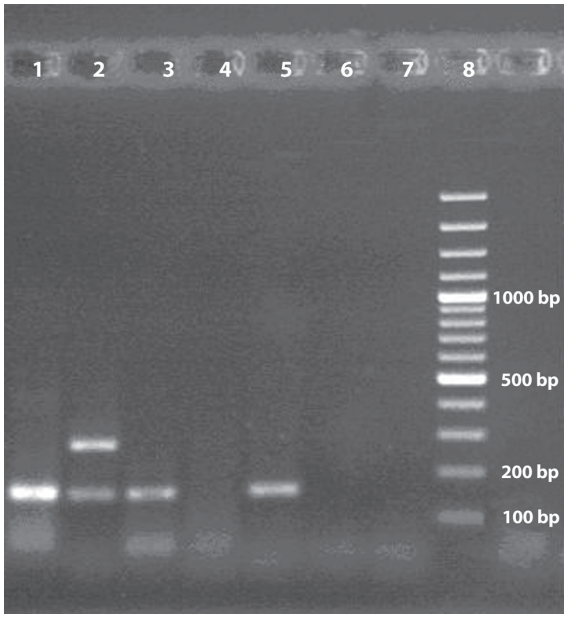

Fig. 1. Detection of integrons by amplification of integrase.
Lane 8, 100 bp DNA ladder (MBI Fermentas, Hanover, MD); lanes 1, 3, 5, integrase 1 amplicons (160 bp); lane 2, both integrase 1(160 bp) and integrase 2 amplicons (288 bp). Lane 4, 6 and 7 were integron negative.

>3000 bp and the strains containing bands with 500, 600, 800 and 1200 bp were more frequent (Table IV).

The association between drug resistance to norfloxacin, ceftazidime, gentamicin, ciprofloxacin, cefepime, amikacin and the presence of integrons was statistically significant, while no association was observed between colistin, imipenem, meropenem, cefoperazon/sulbactom, tobramycin, ampicillin/sulbactam and integron (Table V).

Discussion

Acinetobacter infections are complicated in hospitalized patients due to the acquisition of multi-drug resistance. Most samples in the present study were isolated from the blood (39.8%). Similar data were obtained previously in the same region (Feizabadi *et al.*, 2008). Dissemination of *Acinetobacter* through blood may

Table V
Association between the existence of integron and antibiotic resistance in 88 *Acinetobacter* isolates

Association with integron[3]	% Resistance of total (total no)	% Resistance int-negative[2] (no)	% Resistance int-positive[1] (no)	Antibiotic
Colistin	0 (0)	2.3 (2)	2.3 (2)	0.126
Imipenem	13.6 (12)	9.1 (8)	22.7 (20)	0.501
Meropenem	15.9 (14)	11.4 (10)	27.3 (24)	0.571
Cefoperazone/sulbactam	21.6 (19)	11.4 (10)	33 (29)	0.110
Tobramycin	14.8 (13)	21.6 (19)	36.4 (32)	0.069
Ampicillin/sulbactam	25 (22)	13.6 (12)	38.6 (34)	0.092
Ciprofloxacin	48.9 (43)	25 (22)	73.6 (65)	**P< 0.05**
Amikacin	47.7 (42)	27.3 (24)	75 (66)	**0.001**
Norfloxacin	48.9 (43)	27.3 (24)	76.2 (67)	**P< 0.05**
Gentamicin	51.1 (45)	28.4 (25)	79.5 (70)	**P< 0.05**
Cefepime	51.1 (45)	29.5 (26)	80.6 (71)	**P< 0.05**
Ceftazidime	52.3 (46)	29.5 (26)	81.8 (72)	**P< 0.05**

[1] – int-positive: integron positive in multiplex PCR assay; [2] – int-negative: integron negative in multiplex PCR assay.
[3] – Significant values are in bold.

indicate the role of the bloodstream in spreading the infection (Gisneous and Rodriguez-Bano, 2002). Consistent with previous studies, *A. baumannii* is the predominant species in clinical isolates (Seifert *et al*., 1993; Towner, 2009).

The three most effective antibiotics against *Acinetobacter* were found to be colistin, imipenem and meropenem. Despite being the most effective antibiotic against *Acinetobacter in vitro*, colistin use is limited only to life threatening conditions due to its serious side effects (Reed *et al.*, 2001; Lewis and Lewis, 2004). Nevertheless, observation of high resistance rate of *Acinetobacter* to the majority of the tested antibiotics has limited the use of alternative effective antibiotics. More likely, resistance genes are acquired *via* genetic elements such as integrons, plasmids and transposons (Perez *et al.*, 2007). In this regard, the role of integron is remarkable due to possessing a strong capturing system (Gonzalez *et al*., 1998; Seward, 1999; Turton *et al.*, 2005). Continuous capturing of antibiotic resistance genes in *Acinetobacter* will extend quickly, so with more uncontrolled administration of antibiotics in hospitals and clinics, the possibility of acquiring resistance will be increased. To overcome progressive antibiotic resistance, rational and timely administration of effective antibiotics should be implemented. The present study on the existence of integron revealed that 53.4% of the isolates contained integron classes 1 or 2. These results are in agreement with published reports that *Acinetobacter* harbors high prevalence of integron class 1, lower class 2 and no class 3 (Koeleman *et al.*, 2000; Ploy *et al.*, 2000; Galleco and Towner, 2001; Gaur *et al.*, 2006; Xu *et al*., 2008). The lack of integron class 3 may indicate its null role in antibiotic resistance. As mentioned above, the prevalence of class 1 integron, as compared to class 2 may imply that class 1 integron is more important in capturing resistant determinants. Alternatively, both systems acquire the same resistance genes but class 1 integrons may express these genes more efficiently. To determine this possibility, sequencing and cloning of resistance genes of the isolates containing class 1 or 2 integrons might be helpful. Comparison of antibiotic resistance patterns and their association with class 1 and 2 integrons confirms that both classes of integrons exhibit similar resistance patterns to the tested antibiotics (Table III). However, class 1 integron is more likely involved in emerging resistance to antibiotics.

Data in the present study show a statistical association between the presence of integrons and resistance to 6 antibiotics. Because we did not detect any association between resistance to other antibiotics and the presence of integrons, this can implicate the role of other resistance determinants (Gaur *et al.*, 2006; Chen *et al.*, 2010).

In conclusion, *Acinetobacter* expressed high resistance to most of the prescribed antibiotics. To reduce the resistance rate, comprehensive control measures along with determination of periodical antibiotic sensitivity pattern may alleviate the situation to an acceptable level. Colistin, imipenem and meropenem are the most effective agents against *Acinetobacter*. However, the clinical application of colistin is limited due to its inappropriate side effects.

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