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The influence of integrons on multidrug resistant *Acinetobacter* spp. isolated from environment and clinical samples

Shaheli, M.[1,2], Baseri Salehi, M.[3*] and Bahador, N.[2]
[1]Department of Microbiology, College of Science, Fars Science and Research Branch, Islamic Azad University, Fars, Iran
[2]Department of Microbiology, College of Science, Shiraz Branch, Islamic Azad University, Shiraz, Iran
[3]Department of Microbiology, Kazerun Branch, Islamic Azad University, Kazerun, Iran
[*]Corresponding author e-mail: Baserisalehi682@gmail.com
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Abstract. Antibiotic resistance is one of the biggest threats to global health. Recently *Acinetobacter* has been identified as an important bacteria to the acquisition of antibiotic resistance. The main objective of this study was to determine the antibiotic resistance in clinical and environmental *Acinetobacter* isolates and to determine the relation between antibiotic resistance and the presence of integrons. In total 83 clinical and 62 environmental samples were collected. Clinical samples were urine, skin, blood, sputum, wound, respiratory discharges and environmental samples were rhizosphere of wheat and corn and water (mud and swamps). *Acinetobacter* isolates were authenticated by molecular method (16SrRNA) and their antibiotic sensitivity was assessed using disk diffusion method. Furthermore, the presence of different classes of integrons (I-III) was evaluated using specific primers. The results obtained indicated that 56 and 34 *Acinetobacter* were isolated from clinical and environmental samples respectively. Clinical and environmental *Acinetobacter* isolates showed resistant characters against Cefotaxime, Ceftriaxone and Polymyxin B, Tetracycline, Ampicillin/Sulbactam respectively. In addition, 92.9% and 94.1% of clinical and environmental isolates were Multiple drug Resistant (MDR) group. Frequency of the presence of class I-III Integrons in clinical isolates was 48.2%, 9.0% and 7.1% and in environmental isolates was 41.2%, 5.9% and 8.8%. This study illustrated the resistant characters of clinical and environmental *Acinetobacter* to Meropenem and Imipenem. In addition, we found significant correlation between Cefotaxime and Ceftriaxone resistance among all isolates. Besides, class I –II Integrons existed simultaneously in the isolates and probably carriage of these integrons induced drug resistant character.

INTRODUCTION

Acinetobacter is an opportunistic pathogen that causes severe complications in hospitalized patients, especially those in intensive care units (ICU) due to acquired multidrug resistance (MDR). Several reports indicated that *Acinetobacter* spp. have potent activity for developing antibiotic resistant property especially against Amikacin, Tobramycin, Ceftazidime, Ciprofloxacin, Piperacillin/Tazobactam, Doxycycline, Carbapenem, Minocycline, Trimethoprim/Sulfametoxazol, Levofloxacin, imipenem and Sulbactam/Ampicillin (Barbe *et al*., 2004). Therefore, treatment of infection caused by MDR *Acinetobacter* are considered problematic. Although, several mechanisms have been introduced for developing antibiotic resistant property in bacteria, integrons have been identified as a primary source of resistance genes in *Acinetobacter*. Integrons are DNA elements capable of capturing genes by a site-specific recombination mechanism that often carry gene cassettes containing

antibiotic resistance genes. However six classes of integrons (based on *intl* gene) have been introduced, classes 1, 2 and 3 showed relatively more implicated in dissemination of antibiotic resistance (Bou *et al*., 2000; Bergogne-Berezin & Towner, 1996). Of these classes of integrons, class I integrons play an important role in the distribution of antibiotic resistance. *Staphylococcus*, *Corynebacterium*, *Citrobacter*, *Campylobacter*, *Burkholderia*, *Serratia*, *Shigella*, *Salmonella*, *Alcaligenes*, *Aeromonas*, *Acinetobacter*, *Klebsiella*, *Pseudomonas*, *Escherichia* and *Vibrio* are Gram positive and negative bacteria that carried class I integrons. Class I integrons carry 40 resistant genes associated with aminoglycosides, bata-lactams, chloramphenicol, macrolides, and sulfonamides (Chen *et al*., 2015). In addition, class II integrons followed by class I integrons, have shown high prevalence in clinical isolates of Gram negative bacteria viz., *Acinetobacter*, *Shigella*, *Salmonella* (Dijkshoorn *et al*., 2007). Class III integrons similar to class I contain several genetic cassettes such as *Imp bla-1* and *aacA4* coding metalobetalactamase and aminoglycosides resistance (Carvalho *et al*., 2011). Based on foregoing evidence this study attempts to elucidate the antibiotic resistance potential in clinical and environmental *Acinetobacter* isolates. In addition, the existence of integrons in the isolates was assessed to achieve maximum information concerning the pattern of antibiotic resistance in clinical and environmental *Acinetobacter*.

MATERIALS AND METHOD

Sample collection

The present study was conducted from February 2015 to May 2016. A total of 83 clinical and 62 environmental samples were collected. Clinical samples were collected from Namazi and Shiraz Ghotbodin Hospitals and environments samples collected from soil and water. The clinical samples were urine, skin, blood, sputum, wound, respiratory discharges and environmental samples collected from rhizosphere of wheat and corn plants. Furthermore, water samples were collected from mud and swamps of Jahrom, Sepidan and Kamfiruz fields, Fars province. All samples were transferred to the laboratory within two hours and subjected to microbiological analysis.

Phenotype and genotype diagnosis: All samples cultivated on MacConkey agar produced colorless colonies and were evaluated by Gram stain, oxidase and catalase tests and biochemical tests viz., O/F, TSI, Urease, motility and Nitrate. The presumptive *Acinetobacter* was authenticated by using DNA gene sequencing (16SrRNA) method (Table 1). To perform the molecular method, PCR thermal program adjusted on primary denaturation at 94ºC for 5 min, denaturation at 94ºC for 45 sec, annealing at 58ºC for 30 sec, elongation at 72ºC for 50 sec and final elongation for 7 min with 35 cycles. PCR products were electrophoresed using 1.0% agarose gels (Chen *et al*., 2007).

Antibiotic susceptibility of *Acinetobacter* isolates: Antibiotic susceptibility of *Acinetobacter* isolates was carried out using the disk diffusion assay. To perform the experiment, the isolates were cultivated on Muller Hinton agar and the antibiotic disks included: Ceftazidime, Cephotoxim, Ceftriaxone, Gentamicin, Amikacin, Tetracycline, Ciprofloxacin, Imipenem, Tobramycin, Meropenem, Polymyxin B, Cotrimoxazole, Ampicillin/

Table 1. Universal primer for polymerase Chain Reaction of 16SrRNA

Gene	Primer	Product length	Reference
16S rRNA	GTCGTAACAAGGTAGCCGTA GGGTTCCCCCATTCAGACAT	798	Chen *et al.* (2007)

Table 2. Primers of *Int1*, *Int2*, *Int3* genes for detection of integrons

Gene	Primer	Product length	Reference
Int1	CAGTGGACATAAGCCTGTTC CCCGAGGCATAGACTGTA	160	Chen *et al.* (2015)
Int2	TTGCGAGTATCCATAACCTG TTACCTGCACTGGATTAAGC	288	Chen *et al.* (2015)
Int3	AGTGGGTGGCGAATGAGTG TGTTCTTGTATCGGCAGGTG	600	Yaqoob *et al.* (2011)

Table 2. Determination of frequency of rising antibiotic resistant *Acinetobacter* isolates based on the one sample T test relationship and at 0.05 significance level

	Antibiotic	Resistance zone	Mean of inhibitory zone	P value	Mean of inhibitory zone	P value
1	Ceftazidime	≤14	2.02	0.000	1.97	0.000
2	Cefotaxime	≤14	2.7	0.000	2.4	0.000
3	Ceftriaxone	≤13	2.14	0.000	2.4	0.000
4	Tobramycin	≤12	6.5	0.000	6.4	0.000
5	Gentamicin	≤12	5.05	0.000	4.6	0.000
6	Amikacin	≤14	7.7	0.000	7.06	0.000
7	Tetracycline	≤11	11.95	0.466	10.8	0.919
8	Ciprofloxacin	≤15	3.16	0.000	3.2	0.000
9	Piperacillin	≤17	7.7	0.000	7.53	0.000
10	Ampicillin-sulbactam	≤11	9.9	0.248	10.2	0.596
11	Meropenem	≤13	7.8	0.000	6.7	0.000
12	Imipenem	≤13	3.8	0.000	4.8	0.000
13	Polymyxin B	≤11	12.1	0.854	10.7	0.721
14	Co-trimoxazole	≤10	2.2	0.000	2.7	0.000

Sulbactam and Pieracillin/Tazobactam were used for evaluation of antibiotic resistant patterns (Andrews, 2009). The the plates were kept in incubator at 37°C. After 24h zone of inhibition for each disk was measured (mm) according to CLSI protocol sensitivity, intermediate and resistance responses of the isolates against antibiotics were recorded. In addition, Multidrug Resistant (MDR), Extensively Drug Resistant (XDR) and Pan Drug Resistant (PDR) of the isolates were recognized based on resistance to penicillin or cephalosporin, aminoglycosides and quinolones (MDR), extra carbapenem (XDR) and extra polymyxin and tigecycline (PDR).

Detection of integrons in *Acinetobacter* isolates: Detection of class I-III integrons in the isolates carried out by molecular method using specific primers (Table 2). PCR thermal program for detection of the genes adjusted on primary denaturation at 94ºC for 5 min, denaturation at 94ºC for 30 sec, annealing at 55-56ºC for 30 sec, elongation at 72ºC for 30 sec and final elongation for 7 min with 35 cycles and PCR products were electrophoresed on 1.0% agarose gels (Yaqoob *et al.*, 2011).

Statistical analysis

The statistical test of significance viz., Chi square was used to determine relation between the existence of intergrons and

resistance to antibiotics in clinical and environmental *Acinetobacter* isolates.

RESULTS

Isolation and identification of *Acinetobacter*: the results obtained from isolation and phenotypic and genotypic identification of *Acinetobacter* indicated that 56 and 34 *Acinetobacter* were isolated and identified from clinical and environmental samples respectively (Figure 1).

Antibiotic sensitivity of *Acinetobacter* isolates: The results of antibiotic susceptibility of *Acinetobacter* isolates showed in (Figures 2 & 3). As shown in these figures the *Acinetobacter* isolates from clinical and environmental sample were resistance to Polymyxin B and Tetracycline and sensitive to Cefotaxime and Ceftriaxone. However, clinical and environmental isolates of *Acinetobacter* showed different responses against the rest of antibiotics. Statistical analysis of data obtained from antibiotic sensitivity of *Acinetobacter* isolates showed in Table 2.

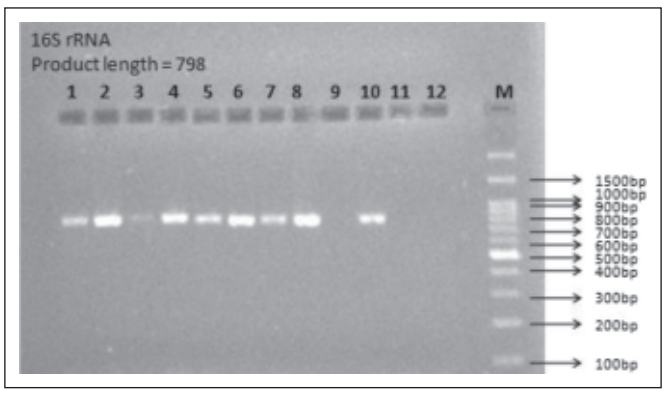

Figure 1. Agarose gel electrophoresis analysis of PCR amplicons specific for 16S rRNA
(M: Marker, 1: positive control, 2-8&10: positive, 9&11: Negative, 12: Negative control)

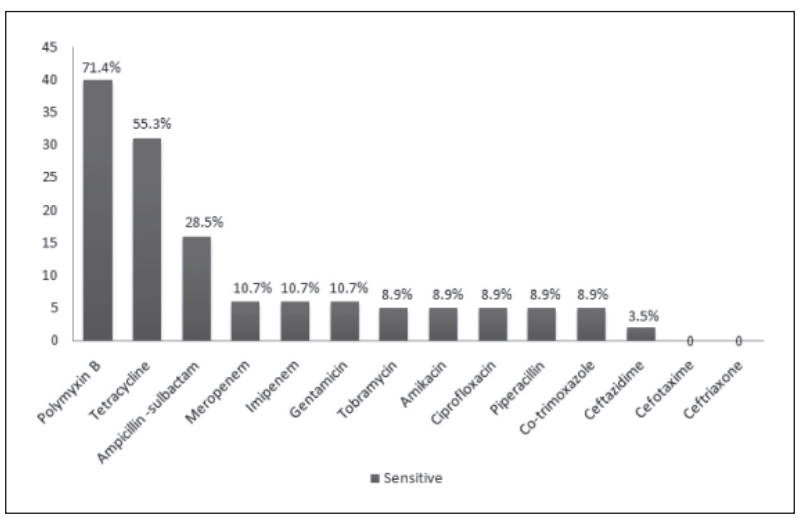

Figure 2. Antibiotic susceptibility of clinical isolates of *Acinetobacter* spp.

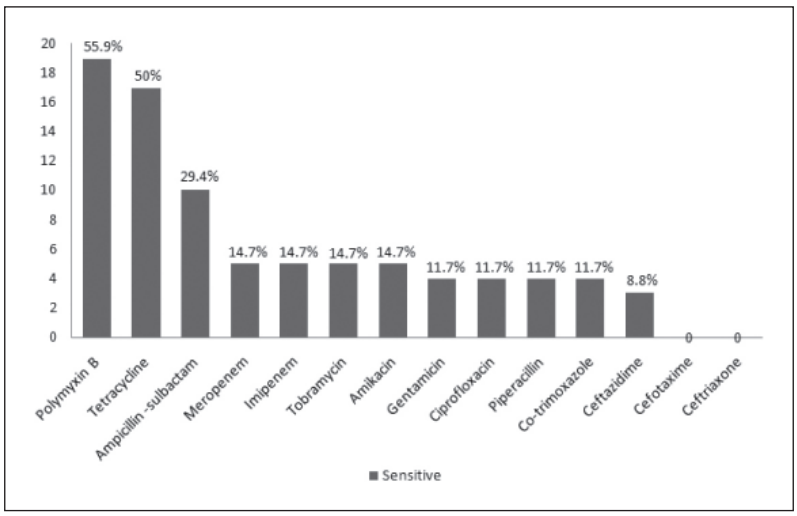

Figure 3. Antibiotic susceptibility of environmental isolates of *Acinetobacter* spp.

Table 3. Frequency of Multidrug Resistance (MDR) of clinical and environmental *Acinetobacter* isolates

Number of antibiotic classes	Number and Percentage of clinical resistant strains	Number and Percentage of environmental resistant strains
1	2 (3.57%)	1 (2.94%)
2	2 (3.57%)	1 (2.94%)
3	1 (1.79%)	–
4	1 (1.79%)	2 (5.88%)
5	2 (3.57%)	1 (2.94%)
6	2 (3.57%)	4 (11.76%)
7	19 (33.93%)	9 (26.47%)
8	24 (42.86%)	8 (23.53%)
9	3 (5.36%)	8 (23.53%)
Total	56 (100%)	34 (100%)

Table 4. Frequency of Extensively Drug Resistant (XDR) and Pan Drug Resistant (PDR) of clinical and environmental *Acinetobacter* isolates

	Clinical isolates	Environment isolates
XDR	50 (89.3%)	29 (85.3%)
PDR	15 (26.8%)	13 (38.24%)

The results obtained from determination of frequency of rising antibiotic resistant *Acinetobacter* isolates indicated that *Acinetobacter* isolates showed antibiotic resistant property against all antibiotics other than Tetracycline, Polymyxin B and Ampicillin-Sulbactam.

Recognition of Multidrug resistant *Acinetobacter* isolates: The results obtained illustrated that 92.87% of clinical and 94.11% of environmental isolates were MDR. Furthermore, 89.3% of clinical and 85.3 of environmental isolates were XDR and 26.8 and 38.24 of clinical and environmental isolates were PDR respectively (Tables 3 & 4).

Statistical analysis of the results opined that all isolates from clinical and environmental samples were resistance and sensitive to all antibiotics tested (Tables 5 & 6).

Table 5. Antibiotic resistance pattern of clinical *Acinetobacter* isolates

Antibiotics	Antibiotics No.	Number of antibiotics resistant isolates	Total number of resistant isolates (%)
Cefta, Cefo, Ceftri, Tobra, Genta, Ami, Tetra, Cipro, Pipera, Ampi, Mero, Imi, Poly, Co-tri	14	3	3 (5.35%)
Cefta, Cefo, Ceftri, Tobra, Genta, Ami, Tetra, Cipro, Pipera, Ampi, Mero, Imi, Co-tri	13	15	23 (41.06%)
Cefta, Cefo, Ceftri, Tobra, Genta, Ami, Cipro, Pipera, Ampi, Mero, Imi, Poly, Co-tri		7	
Cefta, Cefo, Ceftri, Tobra, Genta, Ami, Tetra, Cipro, Pipera, Mero, Imi, Poly, Co-tri		1	
Cefta, Cefo, Ceftri, Tobra, Genta, Ami, Cipro, Pipera, Ampi, Mero, Imi, Co-tri	12	12	21 (37.5%)
Cefta, Cefo, Ceftri, Tobra, Genta, Ami, Tetra, Cipro, Pipera, Mero, Imi, Co-tri		4	
Cefta, Cefo, Ceftri, Tobra, Genta, Ami, Cipro, Pipera, Mero, Imi, Poly, Co-tri		3	
Cefta, Cefo, Ceftri, Genta, Ami, Tetra, Cipro, Pipera, Ampi, Mero, Imi, Co-tri		1	
Cefta, Cefo, Ceftri, Tobra, Ami, Tetra, Cipro, Pipera, Ampi, Mero, Imi, Co-tri		1	
Cefta, Cefo, Ceftri, Tobra, Genta, Ami, Cipro, Pipera, Mero, Imi, Co-tri	11	2	2 (3.57%)
Cefta, Cefo, Ceftri, Tobra, Genta, Ami, Cipro, Mero, Imi, Poly	10	1	1 (1.79%)
Cefta, Cefo, Ceftri, Tobra, Genta, Ami, Cipro, Pipera	8	1	1 (1.79%)
Cefo, Ceftri, Tobra, Pipera, Poly, Co-tri	6	1	1 (1.79%)
Cefta, Cefo, Ceftri, Co-tri	4	1	2 (3.57%)
Cefta, Cefo, Ceftri, Ampi		1	
Cefta, Cefo, Ceftri	3	1	1 (1.79%)
Cefo, Ceftri	2	1	1 (1.79%)

Abbreviations: Ceftazidime (cefta), Cephotoxim (cefo), Ceftriaxone (cefti), Gentamicin (Genta), Amikacin (Ami), Tetracycline (Tetra), Ciprofloxacin (Cipro), Imipenem (Imi), Tobramycin (Tobra), Meropenem (Mero), Polymyxin B (Poly), Cotrimoxazole (Co-tri), Ampicillin/Sulbactam (Ampi), Pieracillin/Tazobactam (Pipera).

Table 6. Antibiotic resistance pattern of environmental *Acinetobacter* isolates

Antibiotics	Antibiotics No.	Number of antibiotics resistant isolates	Total number of resistant isolates (%)
Cefta, Cefo, Ceftri, Tobra, Genta, Ami, Tetra, Cipro, Pipera, Ampi, Mero, Imi, Poly, Co-tri	14	8	8 (23.53%)
Cefta, Cefo, Ceftri, Tobra, Genta, Ami, Tetra, Cipro, Pipera, Ampi, Mero, Imi, Co-tri Cefta, Cefo, Ceftri, Tobra, Genta, Ami, Cipro, Pipera, Ampi, Mero, Imi, Poly, Co-tri	13	6 2	8 (23.53%)
Cefta, Cefo, Ceftri, Tobra, Genta, Ami, Cipro, Pipera, Ampi, Mero, Imi, Co-tri Cefta, Cefo, Ceftri, Tobra, Genta, Ami, Tetra, Cipro, Pipera, Mero, Imi, Co-tri Cefta, Cefo, Ceftri, Tobra, Genta, Ami, Cipro, Pipera, Mero, Imi, Poly, Co-tri	12	5 2 2	9 (26.47%)
Cefta, Cefo, Ceftri, Tobra, Genta, Ami, Cipro, Pipera, Mero, Imi, Co-tri Cefta, Cefo, Ceftri, Tobra, Genta, Ami, Cipro, Pipera, Mero, Imi, Poly	11	2 1	3 (8.83%)
Cefta, Cefo, Ceftri, Tobra, Genta, Ami, Cipro, Mero, Imi	9	1	1 (2.94%)
Cefta, Cefo, Ceftri, Pipera, Ampi, Poly, Co-tri Cefo, Ceftri, Tetra, Pipera, Ampi, Poly, Co-tri	7	1 1	2 (5.88%)
Cefta, Cefo, Ceftri, Genta, Cipro, Ampi	6	1	1 (2.94%)
Cefo, Ceftri, Co-tri	3	1	1 (2.94%)
Cefo, Ceftri	2	1	1 (2.94%)

Abbreviations: Ceftazidime (cefta), Cephotoxim (cefo), Ceftriaxone (cefti), Gentamicin (Genta), Amikacin (Ami), Tetracycline (Tetra), Ciprofloxacin (Cipro), Imipenem (Imi), Tobramycin (Tobra), Meropenem (Mero), Polymyxin B (Poly), Cotrimoxazole (Co-tri), Ampicillin/Sulbactam (Ampi), Pieracillin/Tazobactam (Pipera).

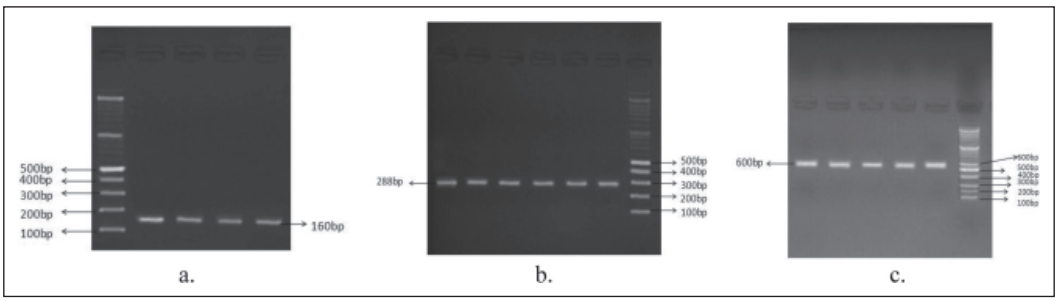

Figure 4. Agarose gel electrophoresis analysis of PCR amplicons specific for a. *Int*1, b. *Int*2, c. *Int*3

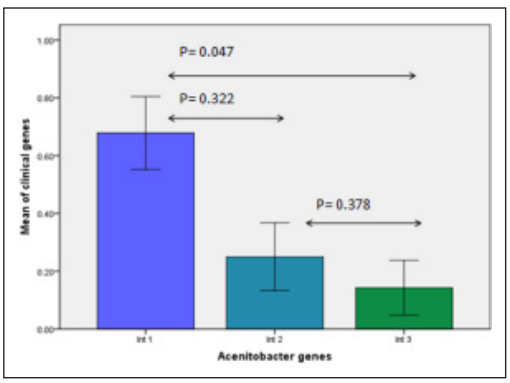

Figure 5. Determination of relation among integrons in clinical *Acinetobacter* isolates

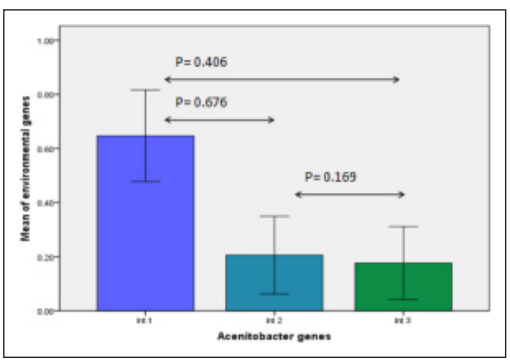

Figure 6. Determination of relation among integrons in environmental *Acinetobacter* isolates

Detection of integrons in *Acinetobacter* isolates: The results obtained from detection of class I, II and III integrons showed in Figure 4. As mentioned in this figure *Acinetobacter* isolates carriage class I integrons followed by class III. A mentioned in tables 8&9 statistical analysis based on the Chi square test at 0.05 significance level elucidated the presence of integrons and some antibiotic resistance in clinical and environmental *Acinetobacter* isolates. In addition, the frequency of integron genes in clinical and environmental *Acinetobacter* isolates showed in Figure 5 & 6. As seen in these figures class I integron in clinical isolates was related to class III (p=0.047). However, no relation was found among other integrons in these isolates.

DISCUSSION

The genus *Acinetobacter* comprises of heterogeneous bacteria with different characters. At present *Acinetobacter* genus has 32 species, some of them live on animate and some on inanimate surfaces. Recently *Acinetobacter* genus is considered as an opportunistic bacterial pathogen associated with nosocomial infections. These bacteria showed increasing antibiotic resistance (Chen *et al.*, 2015). Clinical *Acinetobacter* showed resistance against many antibiotics *viz.*, carbapenem and polymyxins (Falagas *et al.*, 2006). Akbari *et al.* (2010) determined that sensitivity rate towards Gentamicin, Imipenem and Ampicillin/Sulbactam and Amikacin in *Acinetobacter* was 55.0%, 45.0%, 38.0% and 38.0%. Our finding showed that clinical *Acinetobacter* isolates were sensitive to Gentamicin, Imipenem and Ampicillin/Sulbactam and Amikacin with frequency of 91.1%, 71.4%, 89.3%, 89.3% and environmental isolates were sensitive to

Table 7. Frequency of integron genes in the clinical and environmental *Acinetobacter* isolates

Genes	Clinical isolates	Environmental isolates
Int1	27 (48.21%)	14 (41.18%)
Int2	5 (8.93%)	2 (5.88%)
Int3	4 (7.14%)	3 (8.82%)
Int1-Int2	8 (14.29%)	5 (14.7%)
Int1-Int3	3 (5.36%)	3 (8.82%)
Int2-Int3	1 (1.79%)	–
None	8 (14.29%)	7 (20.59%)

Table 8. Determination of relation between integrons and antibiotic resistance in clinical *Acinetobacter* isolates

	Ceftazidime	Cefotaxime	Ceftriaxone	Tetracycline	Co-trimoxazole
Int1	1.000	0.000	0.000	0.024	1.000
Int2	1.000	0.000	0.000	1.000	1.000
Int3	0.268	0.000	0.000	0.063	0.017
Int1-Int2	1.000	0.000	0.000	1.000	0.592
Int1-Int3	1.000	0.000	0.000	1.000	0.249
Int2-Int3	0.036	0.000	0.000	1.000	0.089

Table 9. Determination of relation between integrons and antibiotic resistance in environmental *Acinetobacter* isolates

	Cefotaxime	Ceftriaxone	Ampicillin-sulbactam
Int1	0.000	0.000	0.718
Int2	0.000	0.000	0.407
Int3	0.000	0.000	0.048
Int1-Int2	0.000	0.000	0.291
Int1-Int3	0.000	0.000	0.201

same antibiotics 85.3%, 70.6%, 85.3% and 88.2% respectively. On the other hand, *Acinetobacter* isolates showed antibiotic resistant property against all antibiotics other than the above mentioned antibiotics.

Mobile genetic elements including plasmids, transposons and integrons are pivotal in the spread of antibiotic resistance. Integrons could be considered capturing genes for acquisition of antibiotic resistance genes in *Acinetobacter* (Gillings *et al.*, 2008). Although six classes of integrons are existed, class I followed by class II have shown the major causes of rising antibiotic resistance in *Acinetobacter* (Kamalbeik *et al.*, 2013).

Sung *et al.* in 2014 assessed the role of integrons in formation of multidrug resistance. Based on their reports of 56 *Acinetobacter* isolates, 50 strains carriage class I integron. Our finding showed highest frequency of carriage in the clinical (48.2%) and environmental (41.2%) isolates that belonged to class I integron with 48.2% and 41,0% respectively. In addition, Class II integron carrying resistance genes present in clinical and environmental isolates with 14.3% and 14.7% respectively.

Enas *et al.* (2013) evaluated relation between clinical and environmental isolates of *Acinetobacter baumannii*. Their results opined that 29.2% and 33.3% of clinical and environmental isolates were resistance to Imipenem respectively. Our finding in this regard was 89.3% for clinical and 85.3% for environmental isolates. Sung in 2014 reported that class I integrons play an important role in the development of MDR. However this report supported our finding, Kamalbeik *et al.*, in 2013 reported that class II integron had major role in increasing antibiotic resistant *Acinetobacter* in intensive care unit (ICU). Chen *et al.* in 2015 showed that of 425 *Acinetobacter* isolates, 295 strains carried class I integron. In addition, they found a significant relationship between the presence of class I integron and some antibiotics such as Ceftazidime, Ceftriaxone and Cefepime. Our finding verified correlation between the presence of class I and III integrons and resistance to Ceftriaxone and Cefotaxime.

The present study showed that clinical and environmental *Acinetobacter* spp. isolates were resistance to Cefotaxime, Ceftriaxone. However, Marakiet *et al.* in 2016 showed most of the MDR *Acinetobacter baumannii* isolates was resistance to Ciprofloxacin, Ampicillin Meropenem and Imipenem (Maraki *et al.*, 2016).

CONCLUSION

The present study showed clinical or environmental *Acinetobacter* were resistance to Meropenem and Imipenem. In addition, a positive correlation of antibiotic resistance was found between clinical and environmental *Acinetobacter* isolates. On the other hand class I and II Integrons existed simultaneously in *Acinetobacter* isolates. Hence, carriage of integrons might be increased the frequency of occurrence of antibiotic resistant *Acinetobacter* in the hospitals.

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