

Original Article



Frequency of class I and II integrons in the clinical isolates of *Pseudomonas aeruginosa* with multidrug resistance in Shahrekord teaching hospitals and Isfahan Shahid Chamran hospital during 2016-2017

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Abstract

Background and aims: Increasing the prevalence of nosocomial infections by multidrug resistant (MDR) *Pseudomonas aeruginosa* has severely challenged the choice of treatment and led to an increased mortality rate. Thus, this study investigated the frequency of class I and II integrons and its association with MDR.

Materials and Methods: A total of 175 *P. aeruginosa* isolates were collected from Shahrekord teaching hospitals and Isfahan Shahid Chamran hospital during 12 months (from April 2008 to March 2009). Antibiotic susceptibility was determined by disc diffusion according to the Clinical and Laboratory Standards Institute (CLSI) Antimicrobial Susceptibility Testing. The E-test strips of imipenem, ciprofloxacin, and amikacin were used to identify the minimum inhibitory concentration and MDR bacteria. Finally, the frequency of class I and II integrons genes was evaluated by using the polymerase chain reaction test.

Results: The highest antibiotic resistance and the highest susceptibility belonged to meropenem (86.9%) and polymyxin B (96.0%) by disc diffusion, respectively. By the E-test, the highest and lowest resistance rates were reported for imipenem (97.2%) and ciprofloxacin (86.8%), respectively. The frequency of MDR strains was 82.3% as well. The frequency of class I and II integrons was 57.7% and 17.7% in all *P. aeruginosa* isolates, as well as 68.1% and 21.5% in the MDR isolates, respectively. There was also a significant correlation between I and II integrons and MDR.

Conclusion: Overall, the resistance to different antibiotics and the frequency of MDR strains among the studied *P. aeruginosa* isolates were very high. There was also a significant correlation between integrons and multidrug resistance. Regarding the role of integrons in the transfer of drug-resistant genes and the development of MDR strains, the use of appropriate diet and accurate determination of the susceptibility pattern of *P. aeruginosa* isolates are considered necessary.

Keywords: Integron, *Pseudomonas aeruginosa*, Multidrug resistance

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Introduction

Pseudomonas aeruginosa is an oxidase-positive gram-negative, mobile, spore-free, and aerobic bacterium. This bacterium is not able to ferment carbohydrates and grows at 37-42°C (1-3). In addition, *P. aeruginosa* accounts for 11%-13.8% of hospital-acquired infections, but in the intensive care unit, this rate reaches 13.8-22.6% (4). This organism is the third leading bacterial cause of the development of nosocomial infections, followed by *Staphylococcus aureus* and *Escherichia coli* (5). The rapid spread of multidrug resistant (MDR) bacteria is a serious public health problem (6). Therefore, the World Health Organization (WHO) has named the year 2011 as the year of antibiotic resistance (7).

The increased prevalence of nosocomial infections with

MDR *P. aeruginosa* strains has seriously challenged the treatment choice and thus has led to an increased mortality rate (8). *P. aeruginosa* is an important clinical pathogen that has an inherent resistance to various antimicrobial agents. Genes that increase antibiotic resistance of *P. aeruginosa* (e.g., beta-lactams, aminoglycosides, and fluoroquinolones) may be produced by this bacterium as well. The acquisition of genes in horizontal gene transfer is dependent on mobile genetic elements (MGEs) such as integron, transposon, and plasmid.

These MGEs play an important role in the spread of resistance genes among the bacteria. Further, the genes transferred by the integron cause the coding of multiple drug resistance mechanisms such as beta-lactams, aminoglycosides, and other antimicrobial agents (9-

12). So far, four classes of integrons have been identified in gram-negative bacterial isolates. However, class I integron has the highest prevalence among the clinical isolates and can carry one or more gene cassettes, which can result in resistance to aminoglycosides, beta-lactams, fluoroquinolones, macronutrients, and macrolides (12). Given the presence of antibiotic resistance genes on the integron and the possibility of the rapid spread of these genes among different bacterial species, identifying the integrons provides useful information on the prevalence of MDR *P. aeruginosa* and how resistance genes are spread.

Materials and Methods

In this descriptive-analytical study, 175 *P. aeruginosa* isolates were collected from blood, urine, ulcers, burns, respiratory secretions, peritoneum, and cerebrospinal fluid specimens in the teaching hospitals affiliated with Shahrekord University of Medical Sciences and Isfahan Shahid Chamran hospital from April 2016 to March 2017. After completing the checklist of patient characteristics, the specimens were transferred to the Microbiology Laboratory of the Shahrekord University of Medical Sciences. The specimens were then retested for the presence of *P. aeruginosa* strains by using certain standard laboratory tests such as hot dyeing, oxidase and catalase tests, citrate mobility and consumption test, pigment production, cultivation on a specific cetrinide agar medium (Merck, Germany), and oxidative fermentative test.

Next, antibiotic susceptibility was determined by disc diffusion (Kirby Bauer) according to the CLSI Antimicrobial Susceptibility Testing (13). For this purpose, the Mueller-Hinton agar (Merck, Germany) was first prepared and then its PH was adjusted to 2.7-7.2. Then, a standard microbial suspension was prepared based on the 0.5 McFarland standard and cultured by using a cotton swab. Furthermore, piperacillin (100 µg), piperacillin-tazobactam (10/100 µg), ceftazidime (30 µg), cefepime (30 µg), aztreonam (30 µg), imipenem (10 µg), meropenem (10 µg), doripenem (10 µg), gentamicin (10 µg), amikacin (30 µg), tobramycin (10 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), colistin (10 µg), and polymyxin B (300 units, Mast, England) were placed at a distance of at least 2 cm apart. Then, the diameter of the inhibition zone around each disc was measured and the results were recorded after 24-hour incubation at 37°C. *Escherichia coli* (ATCC25922) was utilized as the standard strain for qualitative control. The E-test strips of imipenem, ciprofloxacin, and amikacin (Liofichem, Italy) were employed to determine the minimum inhibitory concentration and the MDR strains. Genomic DNA was extracted by using boiling. To this end, 3-5 colonies of the 24-hour culture of the bacteria were first dissolved in 500 ml sterile distilled water and the suspension was stained for 15 minutes at 95°C. After centrifuging the suspension at 13000 rpm for 10 minutes at 4°C, spectrophotometry (NanoDrop) was used to determine the

quality of the extracted DNA. If the concentration of DNA was appropriate, the specimen was frozen at -20°C and then utilized in all stages of the study.

Using the DNA extracted from the bacteria in the previous steps, the polymerase chain reaction was performed to determine the frequency of class I and II integrons, and 16S ribosomal RNA in a final volume of 25 µL was considered as the internal control for all specimens. The sequences for the genes of interest were obtained from the National Center for Biotechnology Information (NCBI) and the primers were designed using the DNASIS MAX 3.0 (Table 1). After preparing the master mix and adding DNA of each specimen, the microtubes were placed in the Mastercycler (Eppendorf, Germany) under the specified temperature (Table 2). To control the contamination, tubes without DNA (containing master mix and distilled water) were applied as the negative control in all stages of the study. Eventually, the confirmed *P. aeruginosa* strains containing the integron were used as the positive control to confirm the precision of the test (13-15).

Data analysis

In this study, data analysis was performed by SPSS (version 18) using descriptive statistics (i.e., frequency, percentage, along with mean and standard deviation) and inferential statistics (i.e., the chi-square and Fisher exact tests). Additionally, the correlation was used to investigate the relationship between the components if necessary and $P < 0.05$ was considered statistically significant.

Results

Totally, 89 (50.9%) out of 175 *P. aeruginosa* specimens were collected from female patients. The mean age of

Table 1. Sequences of the primers used to detect class I and II integrons

Primer	Primer Sequence	Fragment Length (bp)
Int-1(F)	5'-TCTCGGGTAACATCAAGG-3'	234
Int-1(R)	5'-AGGAGATCCGAAGACCTC-3'	234
Int-2(F)	5'-CACGGATATCGCAGAAAAAG-3'	787
Int-2(R)	5'-GATGACAACGAGTGACGAAATG-3'	787

Table 2. Temperature protocol and the number of cycles in thermocycler for performing PCR for class I and II integrons

Reaction Step	Gene	Temperature (°C)	Time	Number of Cycle
Hot Star	Int-1	95	5 min	1
	Int-2	95	5 min	1
Denaturation	Int-1	95	30 s	35
	Int-2	95	60 s	35
Annealing	Int-1	55	30 s	35
	Int-2	53	60 s	35
Extension	Int-1	72	30 s	35
	Int-2	72	60 s	35

Note. PCR: Polymerase chain reaction.

Table 3. Antibiotic resistance pattern of *pseudomonas aeruginosa* isolates with respect to the presence and absence of class I and II integrons

Antibiotic	Type of Integrin	Presence of Integrin No. (%)	Lack of Integrin No. (%)
Imipenem	Class 1	92 (62.2)	56 (37.8)
	Class 2	30 (20.3)	118 (79.7)
Meropenem	Class 1	98 (64.5)	54 (35.5)
	Class 2	29 (19.1)	123 (80.9)
Tobramycin	Class 1	96 (68.1)	45 (31.9)
	Class 2	28 (19.9)	113 (80.1)
Ciprofloxacin	Class 1	93 (65)	5 (35)
	Class 2	30 (21)	113 (79)
Levofloxacin	Class 1	90 (60.8)	58 (39.2)
	Class 2	30 (20.3)	118 (79.7)
Polymyxin B	Class 1	2 (100)	0 (0)
	Class 2	0 (0)	2 (100)
Colistin	Class 1	2 (100)	0 (0)
	Class 2	0 (0)	2 (100)
Gentamicin	Class 1	92 (66.2)	47 (33.8)
	Class 2	92 (62.2)	47 (33.8)

Table 4. Frequencies of integrons in multidrug resistant and non-multidrug resistant *Pseudomonas aeruginosa* isolates

Integron	Multidrug Resistant Strains No. (%)	Non-multidrug Resistant Strains No. (%)
Class 1	98 (68.1)	3 (9.7)
Class 2	31 (21.5)	0 (0)
Class 1 and 2	26 (18.1)	0 (0)

the patients from whom the specimens were collected was 43.9 ± 21.9 (range; 4-89) years. In addition, the highest numbers of specimens were collected from ICU patients with 55 (26.9%) isolates. Further, most *P. aeruginosa* isolates (26.9%, n=47) were taken from urine specimens, followed by wound burn specimens (26.3%) and respiratory secretions (22.9%). The highest antibiotic resistance was observed for meropenem (86.9%, n=152), imipenem and levofloxacin (84.6%, n=148), followed by ciprofloxacin (81.7%, n=143) and tobramycin (80.6%, n=141). Besides, the highest susceptibility was obtained for polymyxin B (96.0%, n=168) and then colistin (95.4%, n=167), the related data are displayed in Figure 1.

Based on the results, 144 (82.3%) *P. aeruginosa* isolates were confirmed as MDR by the E-test after they were also found to be MDR by the antibiogram method. Further,

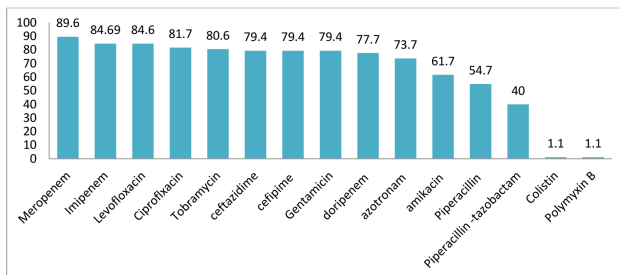


Figure 1. Antibiotic resistance percent in *Pseudomonas aeruginosa* isolates.

Fisher exact test showed that there was a significant relationship between class I integron and resistance against imipenem, meropenem, tobramycin, ciprofloxacin, levofloxacin, and gentamicin ($P < 0.05$). Furthermore, a significant relationship was observed between class II integron and resistance to imipenem ($P < 0.05$) and class I and II integrons were more prevalent in imipenem-resistant strains (Table 3).

Among the 175 *P. aeruginosa* isolates, the frequencies of class I and II integrons were 57.7% (n: 101) 17.7% (n: 31), respectively, and 26 (14.9%) isolates were found to have both integrons. Among the 144 MDR *P. aeruginosa* isolates, the frequencies of class I and II integrons were 68.1% (n: 98) 21.5% (n: 31), respectively, and 26 (14.9%) isolates were found to have both integrons. Fisher's exact test showed that there was a significant relationship between class I and II integrons and multidrug resistance ($P < 0.05$). In addition, there was a significant relationship between the presence of both integrons and multidrug resistance ($P < 0.05$). Class I and II integrons were more prevalent among the MDR isolates (Table 4).

Note. From the left to the right: Column M: DNA ladder; Column 1: Positive control of class I integron; Columns 2-5, 7, and 8: Positive specimens containing class I integron with a product size of 234 bp; Column 6: Without class I integron; Column 9: Negative control.

Discussion

The presence of MGEs such as integrons in MDR isolates is one of the important reasons for the occurrence and transfer of resistance genes. Integrons have a high prevalence in gram-negative bacteria and cause resistance to most antibiotics used in the hospitals due to the acceptance of various drug-resistant cassettes (16).

In this study, the frequencies of class I and II integrons among all 175 isolates of *P. aeruginosa* were 57.7% and

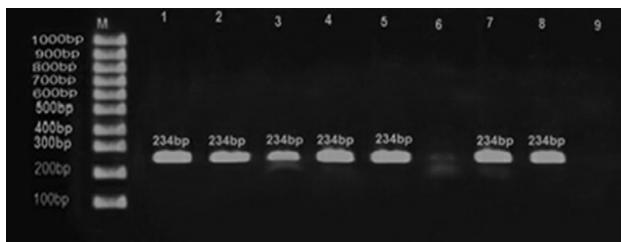


Figure 2. Agarose Gel (1%) of single-polymerase chain reaction of class I integron.
 Note. From the left to the right: Column M: DNA ladder; Column 1: Positive control of class I integron; Columns 2-5, 7, and 8: Positive specimens containing class I integron with a product size of 234 bp; Column 6: Without class I integron; Column 9: Negative control.

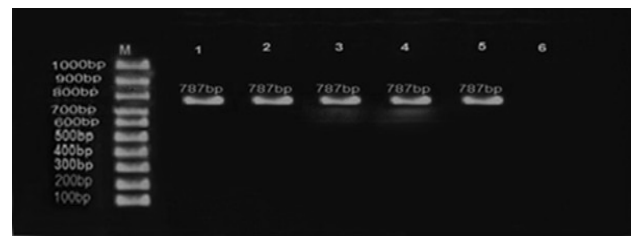


Figure 3. Agarose Gel (1%) of single-polymerase chain reaction of class II integron.
 Note. From the left to the right: Column M: DNA ladder; Column 1: Positive control of class II integron; Columns 2-5: Specimens containing class II integron with a product size of 787 bp; Column 6: Negative control.

17.7%, respectively, and 44.4% of the isolates contained integron. In addition, the frequencies of class I and II integrons in the 144 MDR isolates were 68.1% and 21.5%, respectively. In total, 89.6% of these isolates had integron. In the study of Fonseca et al in Brazil, the prevalence rate of class I integron among the MDR isolates was obtained 41.5 %, but no class II integron was observed in these isolates (17). Further, Yan et al showed that 76.3% of the MDR isolates contained class I integron and only 0.8% of them had class II integron (18). Similarly, Odumosu et al in Nigeria reported that the percentage of class I integron in MDR specimens was 57%, but no class II integron was observed in any of the isolates (9). In another study by Kor et al. in Malaysia, 45.6% of the MDR isolates included class I integron and only 2% of them contained class II integron (19). Likewise, Cicek et al in Turkey found that 4.87% of MDR strains contained class I integron, while none of the isolates had class II integron (11). In another study in China, Gu et al indicated that 40.8% and 0% of the isolates encompassed the class I and II integrons, respectively (20). In another study in China, the frequency of class I integron among *P. aeruginosa* isolates was 45.8% (21). Moreover, Poonsuk et al in Thailand concluded that the frequency of class II integron in all *P. aeruginosa* isolates was 69.3% (22). Compared to non-MDR isolates, most studies reported high-level frequencies of integrons in MDR isolates, suggesting the presence of drug-resistant genes and the production of MDR strains by integrons. In addition, the integrons in different parts of the world have different prevalence rates, which can be due to a different pattern of drug resistance across the world. Additionally, the percentage of class I integron was found to be extremely higher than that of class II integron in all studies, which implies the greater role of class I integron in the transfer of resistance genes.

Carbapenems such as imipenem and meropenem represent an important therapeutic regimen for resistant *P. aeruginosa* infection (23). The study of Peymani et al in Iran showed that the percentages of imipenem-resistant and susceptible isolates were 26% and 55%, respectively (24). Yan et al also demonstrated that the lowest resistance

was obtained against imipenem and vancomycin (18). In another study, Ninama et al reported 14% resistance to imipenem (25). The rates of resistance to meropenem and imipenem in this study were 86.6% and 84.6%, respectively, which was not consistent with the results of other studies. These drugs are not considered appropriate for treating these infections due to the high resistance of *P. aeruginosa* infections. The resistance rate to this type of antibiotics in our study was higher compared to those in other studies because most of the specimens in this study were collected from the patients in the burn units and the ICUs, and Carbapenems were routinely administered to the patients in these units. The excessive use of carbapenems to treat *P. aeruginosa* infections also led to widespread antibiotic resistance in these drugs.

The study of Fonseca et al. in Brazil showed that the highest antibiotic resistance (70%) was against aminoglycoside (17). Dubois et al in France reported 15.9% and 55.8% resistance to tobramycin and gentamicin, respectively (26). In Thailand, the rates of resistance to these two antibiotics were also reported to be 96% and 95%, respectively (22). In India, Ninama et al found 63% resistance to gentamicin (25). In the present study, the percentages of resistance to gentamicin, amikacin, and tobramycin were determined to be 79.4%, 61.7%, and 80.6%, respectively, representing a rise due to the misuse of these antibiotics in recent years. In addition, the resistance to aminoglycosides in developing countries is higher than that in developed countries due to comparatively higher consumption of aminoglycosides. In this study, 15 antibiotic discs were used to detect MDR strains, and the isolates found as resistant to at least six antibiotics were considered as MDR (18). Further, 144 out of 175 *P. aeruginosa* isolates were MDR with a prevalence rate of 82%. Shahcheraghi et al observed that the prevalence rate of MDR specimens among 750 *P. aeruginosa* isolates was 46.5% (27); it was reported to be 71%, 7.84%, and 18% by Fonseca et al. (17), Yan et al (18), and Cicek et al (11), respectively. In most studies, the strains with resistance to at least three antibiotics were identified as MDR, while in the present study, a strain could be considered MDR if it was resistant to at least six

antibiotics. However, the prevalence rate of MDR strains has increased compared to the past years due to the overuse of antibiotics in recent years. In general, the rate of MDR strains is higher in developing countries compared to developed ones. The relatively high frequency of the MDR isolates in our study could be attributed to the fact that half of the specimens were collected from the patients in the burn units and the ICUs.

Conclusion

The results of this study showed that resistance to various antibiotics and the frequency of MDR strains in *P. aeruginosa* isolates were very high. Based on the results, a remarkable prevalence of the integrons was found in the *P. aeruginosa* isolates and MDR strains. Given the role of integrons in the production of MDR strains and the challenges regarding the elimination of these strains, the use of appropriate diet and accurate determination of the susceptibility pattern of *P. aeruginosa* isolates seem necessary. Due to the ability of the integrons to interchange between different strains of bacteria involved in the development of nosocomial infections, it is necessary to use appropriate therapeutic strategies to prevent the spread of such infections and antibiotic resistance among different bacteria.

Conflict of Interests

The authors have no conflict of interests.

Ethical considerations

This project approved by the Research and Technology Deputy of the Shahrekord University of Medical Sciences with the ethics code of IR.SKUMS.REC.1395.79).

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