Dissemination of Class 1 Integron among Different Multidrug Resistant *Pseudomonas aeruginosa* Strains

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ABSTRACT

Background and objectives: The present study was conducted to detect class 1 integrons and evaluate antibiotic susceptibility patterns among clinical isolates of *P. aeruginosa*.

Methods: Sixty clinical samples from blood, tracheal wounds, burns and urinary tract infections were collected from three general hospitals in Tehran, Iran. Culture of specimens was performed on common bacteriological culture media. Bacteria were identified based on mobility, pigment production, growth at 42 ºC, and oxidase and catalase tests. Overall, 21 *P. aeruginosa* strains were isolated. Antimicrobial susceptibility of was evaluated via the disk diffusion method (Kirby-Bauer) according to the CLSI guidelines. Presence of the *intI1*, *sul1*, *aadA2* and *aadB* gene cassettes was investigated using PCR. The collected data were analyzed using SPSS software (version 21).

Results: The most effective antimicrobial agents against *P. aeruginosa* isolates were tetracycline and gentamicin. All *P. aeruginosa* isolates were multidrug resistant. Moreover, the *intI1*, *sul1*, *aadA2* and *aadB* genes were found in 90.5%, 90.5%, 47.6% and 19% of the *P. aeruginosa* isolates, respectively.

Conclusion: The results indicate that the presence of *aadB*, *aadA2* and *sul1* gene cassetes may play an important role in the dissemination of antimicrobial resistance determinants.

Keywords: *Pseudomonas aeruginosa*, integron, multidrug resistance.

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INTRODUCTION

Pseudomonas aeruginosa is as a common nosocomial pathogen, which is naturally resistant to many antimicrobial agents. Several resistance mechanisms have been identified in this pathogen, including acquisition of resistance-encoding genes through mobile genetic elements (1, 2). These elements include integrons capable of integrating and mobilizing gene cassettes, most of which contain resistance-encoding genes. To date, nine classes of integrons have been recognized among which, class 1 integrons are the most prevalent among *P. aeruginosa*. Class 1 integrons located in plasmids and transposons are of particular importance since they can undergo horizontal transfer and contribute to rapid dissemination of antibiotic resistance genes among bacterial isolates (3).

Integrons may play a major role in the dissemination of multidrug resistance (MDR) genes in Gram-negative bacteria (4). The class 1 integron usually consists two conserved segments (CS) flanking the antibiotic-resistance gene cassette(s). The 5'-CS contains the *intI1* gene, an *attI* recombination site, and a strong promoter (5). The *intI* gene encodes integrase, which inserts gene cassettes at the *attI* site (6). The 3'-CS normally carries the antiseptic-resistance gene *qacED1* and the sulfonamide-resistance gene *sul*. On the other hand, the gene cassette is a small, mobile genetic element consisting of a single gene and a recombination site called the 59-base element (5, 7). More than 60 gene cassettes containing different resistance genes have been identified in Gram-negative bacilli (8), conferring resistance to different families of antibiotics such as aminoglycosides, β-lactams, chloramphenicol, trimethoprim and more recently to erythromycin (9) and rifampicin (10).

Resistance to various antibiotics is common among *P. aeruginosa* isolates in many parts of the world, including Iran (11-13). However, there is a relative paucity of data on integron-associated gene cassettes among MDR *P. aeruginosa* strains, particularly in developing countries. Aminoglycosides have been widely used for the treatment of *P. aeruginosa* infections, but increasing number of resistant strains have been reported in the recent years. Resistance to aminoglycosides in *P. aeruginosa* is primarily mediated by aminoglycoside - modifying enzymes including aminoglycoside phosphotransferases, acetyltransferases and nucleotidyl transferases (6). The genes encoding for these enzymes are often located on plasmids or transposons, enabling their rapid dissemination in a wide variety of bacterial species (14).

The *aadA2* gene encoding aminoglycoside adenyltransferase confers resistance to streptomycin and spectinomycin (15), whilst *aadB* encoding aminoglycoside adenylyltransferase confers resistance to kanamycin, tobramycin and gentamicin (16). Given the above, this study was conducted to evaluate antibiotic susceptibility patterns and presence of class 1 integrons and the *aadB, aadA2* and *sul1* gene cassetes among *P. aeruginosa* isolates.

MATERIAL AND METHODS

This study was carried out in the microbiology laboratory of Department of Microbiology at Islamic Azad University, North Tehran Branch (Iran) during April 2012-January 2013. Sixty samples from blood, tracheal wounds, burns and urinary tract infections were collected from three general hospitals in Tehran. The samples were immediately cultured on MacConkey agar (Merck, Germany) and incubated at 37 °C for 18-24 hours. Standard microbiological tests including Gram staining, oxidase test, catalase test, citrate test, urea test, nitrate test, gelatin hydrolysis test, indole production test, methyl red and Voges-Proskauer test and growth at 42 °C were performed for identification of the *P. aeruginosa* strains. All isolates were kept at -20 °C until used (17). Antimicrobial susceptibility testing was performed using the disk agar diffusion method according to the CLSI recommendations (2013 M100-S23) (18). In brief, an inoculum of 10^8 cfu/mL bacteria equivalent to 0.5 McFarland was inoculated on Mueller-Hinton agar (Merck, Germany). Subsequently, discs containing the following antibiotics were added to the plates: ampicillin (10 µg), cefixime (5 µg), cephalothin (30 µg), nalidixic acid (30 µg), carbenicillin (100 µg), clindamycin (2 µg), chloramphenicol (30 µg), trimethoprim-sulfamethoxazole (25 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), gentamicin (10 µg), amikacin (30 µg), streptomycin (10 µg) and tetracycline (30 µg) (Pad-Tan Teb Co.,

Tehran, Iran). The plates were incubated at 37 °C for 24 hours. Diameter of inhibition zone was measured based on the the CLSI guidelines. *P. aeruginosa* ATCC 27853 was used as the quality control strain.

DNA was extracted using a DNA isolation kit (MBST, Iran) according to the manufacturer's instructions. A single colony of each isolate was inoculated into 10 mL of Luria–Bertani broth (Merck, Germany). After 18 hours, two mL of each sample was used for DNA extraction using a commercial kit. Briefly, the samples were lysed in 180 µl lysis buffer, and proteins were degraded with 20 µl proteinase K at 55 ^{o}C for 10 min. After adding 360 µl of binding buffer, each sample was incubated at 70 °C for 10 min. Then, 270 µl of ethanol (96%) were added and the solution was vortexed. The content of tubes was transferred to a MBST-column. The MBST-column was centrifuged and washed twice with 500 µl of washing buffer. DNA was eluted from the carrier using 100 µl of elution buffer. After removing the DNA, the samples were stored at -20 °C until PCR analysis.

All PCR reactions were carried out using a 25 µL reaction solution containing 18.5 µL of water, 2 µL of a 10X reaction buffer, 0.3µL of $MgCl_2$ (50 mM), 1 µL of dNTPs (10 mM), 1 µL of each primer (50 mM), 0.2 µL of Taq DNA polymerase (mi-E8001S, Metabion, Planegg/Martinsried, Germany), and 1 µL of template DNA. The presence of class 1 integrons was evaluated by PCR using primers specific for the integron-integrase gene (*intI1*). All isolates were screened for the presence of *intI1* (19), *aadB* (20), *aadA2* (21) and *sul1* (21). Table 1 represents the PCR annealing temperature, primer (Metabion, Germany) sequences and size of amplicons. The reaction was performed in a thermocycler (Eppendorf Mastercycler®, MA) using the following thermocycling conditions: initial denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 30 s, annealing for 60 s and extension at 72 °C for 60 seconds, and final extension at 72 °C for 10 min (Table 1).

Four µL of PCR products were electrophoresed on 2% agarose gel in a TAE buffer at 45V. After ethidium bromide staining for 15 min, the results were visualized and photographed using a gel documentation system. DNA molecular weight marker (300 bp – 10000 bp) was used as the standard (mi-E8201 Metabion, Planegg/Martinsried, Germany).

Table 1- The sequence of primers, annealing temperatures (°C) and size of amplicons for each target gene

Target gene	Forward	Reverse	Annealing Temperature (°C)	Size of amplicon (bp)	Reference
intI1	**5′-ACGAGCGCAAGGTTTCGGT-3′**	**5′-AAAGGTCTGGTCATACATG-3′**	**53**	**565**	**19**
sul1	**5′-TCACCGAGGACTCCTTCTT-3′**	**5′-AATATCGGGATAGAGCGCA-3′**	**55**	**317**	**21**
aadA2	**5′-TGTTGGTTACTGTGGCCGTA-3′**	**5′-GATCTCGCCTTTCACAAAGC-3′**	**50**	**623**	**21**
aadB	**5′-ATGGACACAACGCAGGTCGC-3′**	**5′-TTAGGCCGCATATCGCGACC-3′**	**55**	**534**	**20**

Data were analyzed with SPSS (version 21) using Chi-Square test. A P-value of less than 0.05 was considered as statistically significant.

RESULTS

Gram-negative, catalase-positive and oxidase-positive bacilli able to grow at 42 °C were identified as *P. aeruginosa* strains. Overall, 21 *P. aeruginosa* strains were identified among different samples. The strains were able to produce acid from glucose and were positive in the urea test, gelatin hydrolysis test, citrate test, nitrate reduction. The strains were found negative in the indole production, methyl red and Voges-Proskauer tests (17).

All *P. aeruginosa* isolates were 100% resistant to ampicillin, cefixime, cephalothin, nalidixic acid, clindamycin and chloramphenicol. The most effective antimicrobial agent against *P. aeruginosa* was tetracycline (Table 2).

The class 1 integrons was found in 19 (90.5%) *P. aeruginosa* isolates. The *sul1*, *aadA2* and *aadB* genes were detected in 19 (90.5%), 10 (47.6%) and 4 (19%) *P. aeruginos* isolates, respectively. The *aadA2* gene was present in 10 (52.6%) integron-positive *P. aeruginosa* strains but absent in integron-negative strains. Moreover, the *aadB* gene was present in 4 (21%) integron-positive *P. aeruginosa* strains but absent in integron-negative strains.

Figure 1- PCR amplification of the class I integron *intI1* gene in *P. aeruginosa* isolates. Column M: DNA size marker (mi-E8201), columns 1-3: negative isolates, columns 4-6: positive isolates (clinical isolates of *P. aeruginosa*), column 7: negative control

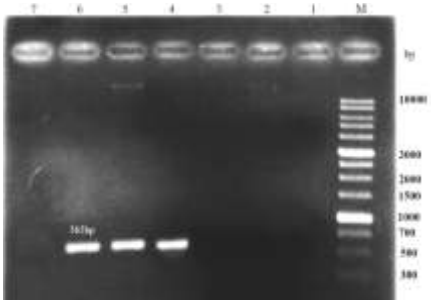

Table 2- Association between antibiotic resistance pattern and presence of integrons in *P. aeruginosa* isolates

Antibiotic	Antibiotic susceptibility (*n*=21)		Integron-positive (*n*= 19)		Integron-negative (*n*=2)		P-value
	*Resistant (%)	Sensitive (%)	Resistant (%)	Sensitive (%)	Resistant (%)	Sensitive (%)	
Gentamicin	18 (85.7)	3 (14.3)	17 (89.5)	2 (10.5)	1 (50)	1 (50)	0.129
Amikacin	16 (76.2)	5 (23.8)	15 (78.9)	4 (21.1)	1 (50)	1 (50)	0.361
Streptomycin	18 (85.7)	3 (14.3)	18 (94.7)	1 (5.3)	0	0	0.000
Tetracycline	15 (71.4)	6 (28.6)	14 (73.7)	5 (26.3)	1 (50)	1 (50)	0.481
Carbenicillin	18 (85.7)	3 (14.3)	17 (89.5)	2 (10.5)	1 (50)	1 (50)	0.12
Ciprofloxacin	17 (80.9)	4 (19.1)	17 (89.5)	2 (10.5)	0	0	0.002
Norfloxacin	17 (80.9)	4 (19.1)	17 (89.5)	2 (10.5)	0	0	0.002
Trimethoprim-Sulfamethoxazole	20 (95.2)	1 (4.8)	19 (100)	0	1 (50)	1 (50)	0.002

***Number of resistant and intermediate isolates according to the CLSI guidelines**

DISCUSSION

In this study, we evaluated antibiotic susceptibility pattern and presence of class 1 integrons along with associated gene cassetes in *P. aeruginosa* isolates. The results showed that all isolates were MDR. Multiple antibiotic resistance is defined as resistance to more than three antibiotics from different classes (22). The MDR rate in our study was higher than that in previous studies in Iran. Study of Mirsalehian et al. in Tehran reported that 87.05% of *P. aeruginosa* isolates were MDR (23).

The emergence of resistant *P. aeruginosa* strains has limited the antibiotic treatment options, leading to an increased risk of treatment failure. These results might be due to inadequate use of antimicrobial drugs in different clinical settings and the spread of resistance determinants.

Multiple classes of integrons have been identified in Gram-negative bacteria according to their distinct integrase genes. Class 1 integrons are the most prevalent in clinical isolates and carry single or multiple gene cassettes (24). PCR detection of the integrase gene has some advantages over the PCR detection of integron cassette for screening of integrons because it is designed to give a small product, which is easily amplified. PCR detection of the integron cassette can give false negative results when integrons are present, the cassette array is difficult to amplify and cassettes are absent. PCR detection of the integrase gene is simple, reliable and easy to perform (25).

We found high frequency of *intI1* among *P. aeruginosa* isolates. In other studies in Iran, the frequency of class 1 integrons are ranging from 27.5% to 95.7%. In a study conducted by Mobaraki et al., 27.5% of *Psudomonas* strains contained the class 1 integrons (26). Higher rates were observed in studies of Yousefi et al. (56.3%), Mirahsani et al. (76%), Hosseini et al. (90%), Hosseini Pour et al. (92%) and Khosravi et al. (95.7%) (27-31). The frequency of class 1 integrons was found to be 41.5% and 82% among *P. aeruginosa* isolates in Brazil and Thailand, respectively (32, 33). However, the frequency of class 1 integrons was very low (4.5%) in a multicenter study in Turkey (34). The frequency of *intI1* in our study was similar

to that of two other studies (35, 36). The *sul1* gene that codes for sulfamethoxazole resistance is located on the 3'conserved segment of the integron. This gene was detected in all integron-positive *P. aeruginosa* strains. In line with findings of previous studies, our results indicated that *sul1* is strongly linked to integrons (37).

The results also showed a significant association between integrons and resistance to streptomycin**,** ciprofloxacin**,** norfloxacin and trimethoprim-sulfamethoxazole. The significant correlation between the presence of integrons and antibiotic resistance among the MDR *P. aeruginosa* isolates suggests that integrons might be responsible for the distribution of antibiotic resistance genes among MDR strains.

Studies conducted in China and Spain reported a significant association between resistance genes and resistant to some antibiotics such as aminoglycosides, beta-lactams and quinolons (3, 38). In another study conducted in Iran, a significant correlation was found between the presence of integrons and resistance against ceftazidime, piperacillin and ciprofloxacin in *P. aeruginosa* isolates (39).

Aminoglycosides are highly potent, broad-spectrum antibiotics with favorable properties for the treatment of life-threatening infections. The emergence of resistant strains has somewhat limited the potential of aminoglycosides in empiric therapies (40). One of the most common resistance mechanisms against aminoglycosides is the production of aminoglycoside-modifying enzymes, such as aminoglycoside acetyltransferases, aminoglycoside phosphorylases and aminoglycoside adenyltransferases (41). In this study, two aminoglycoside resistance genes were investigated; the *aadA2* gene encoding an aminoglycoside adenyltransferase, which confers resistance to streptomycin and spectinomycin (42) and the *aadB* gene encoding an aminoglycoside adenylyltransferase, which confers resistance to gentamicin, kanamycin and tobramycin (43). In a study conducted in China, several aminoglycoside resistance genes including *aadA1*, *aadA2*, *aadA5*, *aadA6*, *aadB* and *accA4* within the integron structures were detected among *A. baumannii* and *P. aeruginosa* strains. The most frequently detected resistance genes (*aadA* family) were aminoglycoside adenyltransferase-encoding genes that confer resistance to streptomycin and spectinomycin (3). In a study carried out in northwest of Iran, the *aad* and *aac* were the most common genes found in the cassettes, which are associated with resistance to aminoglycosides (26).

In this study, the *aadA2* and *aadB* genes were present only in the integron-positive strains of *P. aeruginosa*. In addition, all *P. aeruginosa* strains with the *aadA2* and *aadB* genes were completely resistant to streptomycin and gentamicin. However, strains without these genes showed high level of resistance to streptomycin and gentamicin. Resistance gene cassettes found in the class 1 integrons cannot cover all resistance phenotypes in this pathogen, indicating the existence of other resistance mechanisms. Several other resistance mechanisms have been reported in *P. aeruginosa*, such as multidrug efflux systems and other gene cassettes (35, 44). However, such mechanisms were not investigated in the present study.

CONCLUSION

Our results confirm the high prevalence of class 1 integrons and their important role in the dissemination of antimicrobial resistance genes among *P. aeruginosa* isolates. The high rate of antimicrobial resistance among *P. aeruginosa* isolates highlights the need for urgent reconsideration of antibiotics use at clinical settings. Therefore, it is important to perform antibiotic surveillance programs for appropriate empirical therapy and infection control practices. In addition, monitoring of drug resistance using gene integrase PCR is crucial for infection control planning against multidrug resistance *P. aeruginosa* in hospitals.

Nevertheless, further studies should be performed on the prevalence of integrons in other parts of the country.

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CONFLICT OF INTEREST

All authors declare that there is no conflict of interest.

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