

Antibiotic resistance and frequency of class 1 integrons among *Pseudomonas aeruginosa* isolates obtained from wastewaters of a burn center in Northern Iran

M. Ebrahimpour¹, I. Nikokar¹, Y. Ghasemi¹, H. Sedigh Ebrahim-Saraie¹, A. Araghian¹, M. Farahbakhsh¹, F. Ghassabi²

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Parole chiave: *Pseudomonas aeruginosa*, Antibioticoresistenza, Intergroni di classe 2

Abstract

Background. *Pseudomonas aeruginosa*, being responsible of a broad variety of infections, is considered an important nosocomial pathogen. The emergence of multiple-drug resistance among strains of *P. aeruginosa* appeared as a further public health concern. Due to the considerable ability of multiple-drug resistant *P. aeruginosa* strains to transmit themselves in the environment, we aimed to investigate the association of class 1 integrons with the antibiotic resistance profile of *P. aeruginosa* strains isolated from hospital wastewaters.

Methods. In this cross-sectional study, a total of 100 *P. aeruginosa* isolates were obtained from raw wastewater samples from February 2010 to January 2011 in a Teaching Burn Hospital in Guilan province. All isolates were identified as *P. aeruginosa* using standard microbiological tests. Antibiotic susceptibility was investigated using the disk diffusion method according to Clinical and Laboratory Standards Institute recommendations. All isolates were assayed for the presence of the class 1 integrons gene by PCR.

Results. Overall, 30 (30%) *P. aeruginosa* isolates were positive for the presence of class 1 integrons. The highest antibiotic resistance rates in both integron-positive and -negative isolates belonged to cephalexin and cephazolin, with 100% resistance. Amikacin and ciprofloxacin with the lowest level of resistance (13.3%) were the effective antibiotics against integron-positive isolates. The rates of MDR isolates were significantly higher among integron-positive isolates with 43.3% compared to negative isolates with 22.9% ($P < 0.05$).

Conclusion. The results highlight the importance of class 1 integrons in multiple antibiotic resistance among *P. aeruginosa* isolates. Moreover, the spread of hospital derived wastewaters in the environment can be regarded as the origin of significant reservoirs for antibiotic-resistant pathogens.

Introduction

Pseudomonas aeruginosa is a ubiquitous Gram-negative bacterium causing a broad spectrum of infections and frequently considered as a nosocomial pathogen (1).

Due to its ability to produce a variety of virulence factors, this organism causes a wide range of diseases, from superficial skin infections to acute and chronic general infections (2, 3). Some vulnerable groups, such as immunocompromised or burned

¹Department of Biotechnology, Laboratory of Microbiology and Immunology of Infectious Diseases, Para Medicine Faculty, Guilan University of Medical Sciences, Rasht, Iran

²Department of Bacteriology and Virology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

patients, are at higher risk of *P. aeruginosa* colonization and infections (4).

The remarkable ability of natural and acquired antibiotic resistance of *P. aeruginosa* limits therapeutic options (5). Therefore, *P. aeruginosa* related infections are usually associated with high morbidity and mortality (6). Emergence and spread of multiple drug resistance (MDR) of *P. aeruginosa* appear as a great public health concern in many parts of the world, Iran as well (7). Strains of *P. aeruginosa* are considered as MDR when they are characterized by resistance to at least three groups of antibiotics among -lactams, carbapenems, aminoglycosides, and fluoroquinolones (8). In the recent past, a number of studies have reported an increased prevalence of MDR strains of *P. aeruginosa* in Iranian healthcare settings (9-11).

Out of the various mechanisms which are involved in antimicrobial resistance of *P. aeruginosa*, the acquisition of resistance genes by horizontal transfer mediated by mobile genetic elements (MGE) play a crucial role (12, 13). These elements contain integrons able to integrate and mobilize gene cassettes and most of which contain resistance-encoding genes (13). Plasmids or transposons carrying integrons have an important role in the dissemination of resistance genes in the environments (14). Several classes of integrons have been identified, of which class 1 integrons are the most prevalent in *P. aeruginosa*, and mostly associated with β -lactamases resistance (15, 16). Previous studies indicated that class 1 integrons are widespread among Gram-negative bacteria in different environments, including wastewaters (17, 18).

Due to the considerable ability of integrons-containing *P. aeruginosa* strains to disseminate in hospital and community environment, understanding the distribution of resistant *P. aeruginosa* is important to optimize infections-control approaches. Therefore, we aimed to investigate the presence and dissemination of class 1

integrons and antibiotic resistance profile of *P. aeruginosa* isolates obtained from wastewaters of a burn center in Guilan, North of Iran.

Materials and Methods

Study design and setting

In this cross-sectional study, a total of 100 *P. aeruginosa* isolates were obtained from raw wastewater samples during the period February 2010 to January 2011 in a teaching burn hospital in Guilan province, Iran. Samples were collected in sterile containers and transferred to the laboratory in an insulated box with cooling packs and were analyzed immediately after arrival. The specimens were first inoculated into Tryptic Soy Broth (TSB) and the primary isolation was performed on blood agar and McConkey agar. All isolates were identified as *P. aeruginosa* using standard microbiological tests including colony morphology, Gram staining, pigment production, Catalase and oxidative-fermentative (OF) tests, and reactions on triple sugar-iron agar (TSI).

Antibiotic susceptibility testing

Antibiotic susceptibility was tested on Mueller-Hinton agar (Merck, Germany) using the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) recommendations (19). The antibiotics tested were the following: amikacin (AK, 30 μ g), ceftazidime (CAZ, 30 μ g), carbenicillin (PY, 30 μ g), gentamicin (GM, 10 μ g), imipenem (IMI, 10 μ g), piperacillin (PYR, 100 μ g), ciprofloxacin (CIP, 5 μ g), cephazolin (CZ, 30 μ g), tobramycin (TN, 10 μ g), and cephalexin (CFX, 30 μ g) (all from Mast Diagnostics, Mast Group Ltd, Merseyside, UK). *P. aeruginosa* ATCC 27853 was used as the control organism. Intermediate-resistant isolates were considered as resistant. In this study, multi-drug resistant isolates were

defined as those resistant against at least three of following groups: 1) β -lactams; 2) carbapenems; 3) ceftazidime; 4) piperacillin; 5) fluoroquinolones and 6) aminoglycosides (8).

Polymerase chain reaction (PCR) assay

Genomic deoxyribonucleic acid (DNA) was extracted from *P. aeruginosa* isolates by boiling method as described previously (20). All the isolates were screened by PCR to amplify the class 1 integrons cassette using the following primer: 5'- GGC ATC CAA GCA GCA AG -3', and as reverse primer: 5'- AAG CAG ACT TGA CCT GA -3' (21). Amplification was performed for 35 cycles as initial denaturation at 94° C for 5 min, denaturation at 94° C for 1 min, primer annealing at 57° C for 1 min, extension at 72° C for 2 min and a final extension at 72° C for 5 min. The PCR products were electrophoresed on agarose gel and gels were stained in ethidium bromide solution for 15 minutes and finally visualized in gel documentation system.

Statistical analysis

Data were analyzed using the SPSSTM software (IBM Corp., USA) version 21.0. The

results are presented as descriptive statistics in terms of relative frequency. Values are expressed as the mean \pm standard deviation (continuous variables) or percentages of the group (categorical variables). Chi-square or Fisher's exact tests were used to estimate any statistical association. Statistical significance was regarded as P values < 0.05.

Results

All together, 100 *P. aeruginosa* isolates, obtained from wastewaters of the burn center, were examined. Antibacterial susceptibility tests revealed that all isolates were resistance against cephazolin and cephalexin, and showed a high rate of antibiotic resistance to carbenicillin (64%). On the other hand, the lowest antibiotic resistance rates were seen toward amikacin (7%), followed by ciprofloxacin (14%). The overall results of antibiotic susceptibility pattern for *P. aeruginosa* isolates are shown in Table 1.

Thirty (30%) *P. aeruginosa* isolates were positive for the presence of class 1 integrons. The highest antibiotic resistance rates in both integron-positive and -negative isolates

Table 1 - Antibiotic resistance pattern of studied *P. aeruginosa* isolates

Antibiotics ^a	Integron Positive Total No. 30	Integron Negative Total No. 70	Total No. 100	Significance level
AK	4 (13.3)	3 (4.3)	7 (7)	P = 0.19
TN	6 (20)	13 (18.6)	19 (19)	P = 0.86
GM	7 (23.3)	9 (12.9)	16 (16)	P = 0.24
CIP	4 (13.3)	10 (14.3)	14 (14)	P = 0.89
IMI	7 (23.3)	9 (12.9)	16 (16)	P = 0.24
PYR	7 (23.3)	14 (20)	21 (21)	P = 0.79
CAZ	12 (40)	18 (25.7)	30 (30)	P = 0.16
PY	22 (73.3)	42 (60)	64 (64)	P = 0.26
CFX	30 (100)	70 (100)	100 (100)	P = 1.00
CZ	30 (100)	70 (100)	100 (100)	P = 1.00

^a AK: amikacin; TN: tobramycin; GM: gentamicin; CIP: ciprofloxacin; IMI: imipenem; PYR: piperacillin; CAZ: ceftazidime; PY: carbenicillin; CFX: cephalexin; CZ: cephazolin

belonged to cephalexin and cephalazolin with 100% resistance. Amikacin and ciprofloxacin with the lowest level of resistance (13.3%) were the effective antibiotics against integron-positive isolates. Despite the highest relative frequency of antibiotic resistance in integron-positive isolates compared to negative isolates, the observed differences were not statistically significant.

The rates of MDR isolates were significantly higher among integron-positive isolates with 43.3% (n=13) compared to negative isolates with 22.9% (n=16). Overall, 46.1% of integron-positive isolates were MDR to at least 3 antibiotics, 23.1% to 4 antibiotics, 23.1% to 5 antibiotics and 7.7% to 6 antibiotics. The full MDR pattern of *P. aeruginosa* isolates in the separation of integron positivity are shown in Table 2.

Table 2 - MDR pattern of studied *P. aeruginosa* isolates based on integron positivity

MDR No. ^a	Integron Positive		Integron Negative		Significance level
	Total No. 30	No. (%)	Total No. 70	No. (%)	
3	IMI, CAZ, CIP	1 (7.7)	CAZ, CIP, PY	1 (6.3)	
	CAZ, PY, PYR	4 (30.8)	CIP, PY, PYR	1 (6.3)	
	GM, PY, TN	1 (7.7)	CAZ, PY, PYR,	1 (6.3)	
4	GM, IMI, CAZ, AK	1 (7.7)	IMI, CAZ, PY, TN	3 (18.8)	
	GM, CIP, PY, PYR	1 (7.7)	GM, PY, PYR, TN	3 (18.8)	
	CAZ, AK, PY, PYR	1 (7.7)	IMI, CAZ, PY, PYR	1 (6.3)	
5	-	0	GM, CAZ, CIP, PY, PYR	1 (6.3)	
			GM, CAZ, CIP, PY, TN	1 (6.3)	
6	GM, IMI, CAZ, PY, PYR, TN	3 (23.1)	GM, IMI, CAZ, AK, PY, TN	2 (12.5)	
	GM, IMI, CAZ, AK, PY, PYR, , TN	1 (7.7)	GM, IMI, CAZ, CIP, PY, PYR, TN	1 (6.3)	
7			GM, CAZ, AK, CIP, PY, PYR, TN	1 (6.3)	
	Total	13 (43.3)		16 (22.9)	P = 0.039

^a AK: amikacin; TN: tobramycin; GM: gentamicin; CIP: ciprofloxacin; IMI: imipenem; PYR: piperacillin; CAZ: ceftazidime; PY: carbenicillin; CFX: cephalexin; CZ: cephalazolin

Discussion

Resistance to antimicrobial agents is a major concern in clinical settings and the emergence of resistant *P. aeruginosa* infections with high morbidity and mortality in burn patients has become a serious challenge for clinicians (1, 15). It is assumed that both hospitals and municipal wastewaters can serve as a route for the spreading of bacteria and antibiotic resistant genes into the natural environment (22). In this regard, Aali et al. (23) showed a high concentration of antibiotic resistant bacteria and antibiotic resistant genes in hospital wastewaters (23). Class 1 integrons are considered among the most important elements in the worldwide antibiotic resistance problem, due to the high mobilization ability of these elements into a wide range of pathogens (24). In the present study, we detected the presence of class 1 integrons in 30/100 (30%) of *P. aeruginosa* isolates obtained from wastewaters of a burn center in Guilan province, Northern Iran.

Few studies have investigated the presence of class 1 integrons in bacterial isolates from the wastewaters, being the majority of studies devoted to clinical isolates. In a previous work of the authors, class 1 integrons were detected in 37 out of 182 (43%) isolates of *P. aeruginosa* collected from patients with infected burn wound from the same burn center of the present study (15). In other Iranian studies from different regions of the country, variable rates of class 1 integrons among clinical *P. aeruginosa* isolates were reported. Rajabnia et al. from Babol City (Northern Iran), Yousefi et al. from Tabriz City (Northwestern Iran), and Goudarzi et al. from Tehran (Central Iran) showed integrons positivity 39.4%, 56.3%, and 57.2%, respectively (25-27). This variability in the frequency of class 1 integrons has been seen in other parts of the world as well (16, 28, 29). Despite the lack of data on the prevalence of class 1 integrons among bacterial strains from wastewaters

in Iran, previous reports from other parts of the world prove differences in integron gene content in various geographic regions and among pathogens (17, 22, 30, 31).

In our findings, the comparison of MDR isolates with the presence of class 1 integrons showed higher rates of MDR among integron-positive isolates compared to negative isolates (43.3% vs. 22.9%, P <0.05). These findings are in agreement with other surveys which have shown higher rates of antibiotic resistance and MDR in association with the presence of class 1 integrons and confirm the importance of these elements in the dissemination of antimicrobial resistance among pathogens (31-33).

Many gene cassettes conferring resistance to beta-lactam and aminoglycosides are carried by class 1 integrons, therefore the majority of integron-positive isolates may become resistant to these antibacterial agents (34). In our experience, relative frequencies of antibiotic resistance were slightly higher in integron-positive than in integron-negative isolates, but the difference was not significant. However, in our previous work, clinical isolates of *P. aeruginosa* showed significant differences, in term of antibiotic resistance, between integron-positive and -negative isolates (15). Based on previous studies these findings are not unexpected, since - because of higher antibiotic use and selective pressure - antibiotic resistance rate in hospital environment is more noticeable (26). Finally, the lack of a typing method can be mentioned as one the main limitations of the present study, which could clarify the association of clinical and environmental isolates.

In summary, the results highlight the importance of class 1 integrons in multiple antibiotic resistance among *P. aeruginosa* isolates. Moreover, hospital derived wastewaters have been regarded as a significant reservoirs of antibiotic-resistant pathogens in the environment and

responsible for risks for human and zoonotic public health. The occurrence of these pathogens emphasizes the need for a more rational behavior in antibiotics prescription at the clinical level. Therefore, it is essential to optimize infection control policies and safe management of hospitals wastes.

Acknowledgment

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Conflict of Interest

None to be declared

Riassunto

Antibioticoresistenza e frequenza degli integroni di classe 1 tra i ceppi di *Pseudomonas aeruginosa* isolati da rifiuti liquidi ospedalieri di un Centro Ustionati dell'Iran settentrionale

Premessa. *Pseudomonas aeruginosa*, in quanto responsabile di una larga gamma di infezioni, è considerata un importante patogeno nosocomiale. L'emergere della sua multiresistenza agli antibiotici appare come un ulteriore problema per la Sanità Pubblica. Data la facilità con cui i ceppi multi-resistenti si diffondono nell'ambiente, abbiamo voluto verificare l'eventuale associazione tra gli integroni di classe 1 ed i profili di antibiotico-resistenza dei ceppi di *P. aeruginosa* isolati dai rifiuti liquidi degli ospedali.

Metodi. Nel presente studio trasversale sono stati impiegati 100 ceppi di *P. aeruginosa* isolati tra il Febbraio 2010 ed il Gennio 2011 dagli scarichi fognari di un ospedale d'insegnamento che tratta ustionati nella Provincia di Guilan. L'identificazione dei ceppi è stata effettuata con i test microbiologici standard. La resistenza antibiotica è stata determinata con il metodo della diffusione da disco secondo le raccomandazioni dell'Istituto degli Standard Clinici e di Laboratorio, e per tutti i ceppi si è cercata la presenza dei geni integron di classe 1 usando il metodo PCR.

Risultati. In totale, il 30% dei ceppi è risultato positivo per l'integron di classe 1. I più elevati tassi di resistenza antibiotica nei ceppi integrone-positivi ed integrone-negativi sono risultati essere quelli verso la Carbenicillina (73.3% e 40%) ed il Cefatidime (60% vs 25.7%). Amikacina e Ciprofloxacina, entrambe con l'86.7%, mostravano la più elevata frequenza di positività nei ceppi integrone-positivi.

La frequenza di multiresistenza era significativamente

più alta nei ceppi integrone-positivi (43.3%) rispetto ai ceppi integrone-negativi (22.9%, $P < 0.05$).

Conclusioni. I nostri risultati sottolineano l'importanza degli integroni di classe 1 nella comparsa di multiresistenza nei ceppi di *P. aeruginosa*. In più, la dispersione nell'ambiente delle acque di scarico ospedaliero appare responsabile della creazione di significativi serbatoi di patogeni antibiotico-resistenti.

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Corresponding author: Iraj Nikokar, Department of Biotechnology, Laboratory of Microbiology and Immunology of Infectious Diseases, Para Medicine Faculty, Guilan University of Medical Sciences, Rasht, IR Iran.
e-mail: Nikokariraj@yahoo.com