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Genetic Cassettes Profiling of Class I Integron and Antimicrobial Susceptibility Profiles Among *Pseudomonas aeruginosa* Isolates Collected from Patients in North of Iran

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Abstract

Background: *Pseudomonas aeruginosa* is an opportunistic nosocomial bacterium, especially in infection wards and among patients with burns. The present study addressed the molecular investigation of the gene cassettes of Class I integrin (*intI*) and its relationship with multiple drug resistance in clinical samples of *P. aeruginosa* isolated from Babol hospitals in north of Iran.
Objectives: This study aimed to detect the frequency of *intI* and gene cassettes in the clinical isolates of *P. aeruginosa*.
Methods: In this study, from 75 clinical samples, 30 strains were identified using specific biochemical methods. After determining antibiotic susceptibility using disk diffusion and agar dilution, the frequency of the *intI* gene and its gene cassettes were determined using the polymerase chain reaction (PCR) method.
Results: The highest resistance rate was observed for cefotaxime, ampicillin, and nitrofurantoin using disk diffusion and agar dilution methods. The molecular analyses revealed that 60% of the isolates had the *intI* genes. The frequency of the *aadB*, *dfrA1*, and *bla-$_{OXA30}$* genes were 61, 66, and 33%, respectively.
Conclusions: The high resistance of *Pseudomonas* isolates is due to the presence of *intI* and its gene cassettes. Considering their high resistance to cefotaxime, gentamicin, ampicillin, and imipenem in hospitals, selecting appropriate drugs or generally changing the treatment course for patients is possible to prevent the spread of resistance inducing genes and the development of nosocomial infections.

Keywords: *Pseudomonas aeruginosa*, Class 1 Integron, Gene Cassette, Antibiotic Resistance

1. Background

Pseudomonas aeruginosa obligates aerobic Gram-negative bacillus and is the second most common cause of infective diseases in different hospitals' wards including intensive care units. This organism becomes resistant to antimicrobial agents via various mechanisms, including altering the microorganism's permeability against drugs, enzymatic resistance, efflux pump, receptor altering for drugs, and achieving a secondary metabolic pathway (1). Resistance to antibiotics in this bacterium occurs through mutation or further horizontal transfer through conjugation by plasmid and the transposons carrying antibiotic resistance genes (1, 2).

In this case, the plasmids and transposons carry antibiotic resistance genes and transfer them from one cell to another. In 1986, a DNA sequence with genes for resistance to different antibiotics was found. These regions have been identified at the other sites of various plasmids and are similar to transposons. Integrons are genetic units encompassing genes and transferring them while placed inside mobile factors, called gene cassettes (3). They have an integrase gene (*int1*), one member of the tyrosine recombinase family (4, 5). The integron structure consists of two fixed protected areas, called 3' and 5', located on both sides of a variable region (genetic cassette) containing one or several antibiotic resistance genes (6, 7). Gene cassettes usually contain one drug resistance gene and one protected site, providing the grounds for integrase identification through attachment and cutting processes.

Further, various studies have reported that integrons usually have more than one gene cassette, in which case these bacterial isolates have the multidrug resistance

(MDR) pattern (4, 8). Gram-negative bacilli containing *int1*and its gene cassettes have aroused concerns among physicians and infection specialists regarding infection control. The resistance of this bacterium to a wide range of antibiotics, especially the common antibiotics used in hospitals, has complicated the detection of proper therapeutic methods and the use of infection control instruments (8, 9).

2. Objectives

This study aimed to detect the frequency of *int1* and gene cassettes in the clinically isolates of *P. aeruginosa* from Babol, north of Iran, and investigate their correlations with antibiotic resistance patterns in hospitalized patients.

3. Methods

3.1. Clinical Samples

In this cross-sectional study, 30 clinical samples of *P. aeruginosa* were collected from patients admitted to Ayatollah Rohani Hospital, Babol, north of Iran, from 2016 to 2017. This project was a part of another project (10) (Code of Ethics: MUBABOL. REC.1394.162). The bacterial isolates were collected from the hospital laboratory. *Pseudomonas aeruginosa* was recognized using conventional biochemical and microbiological tests.

3.2. Antibiotic Susceptibility Test

Following the Clinical and Laboratory Standards Institute (CLSI document M100) guideline (11), antimicrobial susceptibility was determined by the Mueller-Hinton agar plates (Merck, Germany) using the standard disk diffusion (DD) method for antimicrobials under gentamicin (GM, 10 μg), cefepime (CPM, 30 μg), amikacin (AK, 30 μg), ciprofloxacin (CIP, 5 μg), imipenem (IMI 10 μg), cefotaxime(CTX, 30 μg), ampicillin (AP, 10μg), trimethoprim (TM, 5 μg), and nitrofurantoin (NI, 300μg) (MAST Diagnostics, Merseyside, UK). *Pseudomonas aeruginosa* ATCC 27853 was used as a positive quality control (11-13).

3.3. Antibiotic Susceptibility Assay by Agar Dilution Method

After preparing stock solution according to the CLSI 2020 (11) standard for antibiotics, 1.5×10^8 CFU/mL of microbial suspensions were cultured in specimens on Muller-Hinton agar containing the concerned antibiotics (MAST Diagnostics, Merseyside, UK) and incubated at 37°C for 18 - 24 h. A plate containing medium without antimicrobial agents was considered a negative quality control, and *P. aeruginosa* ATCC 27853 strain was used as a positive quality control. The results were evaluated according to the table of the CLSI2020 standard (11-14).

3.4. DNA Extraction

The DNA extraction of all strains was performed using the PCR kit (Roche, Germany). The extracted DNAs were stored at -20°C for subsequent steps (12, 13, 15, 16).

3.5. Polymerase Chain Reaction Method (PCR)

Genomic DNA was extracted from *P. aeruginosa* colonies using the DNA purification kit (Roche, Germany). Table 1 presents the primer sequences. The total volume of the PCR reaction mixture was 60 μL encompassing 10 μL of extracted template DNA, 5.0 μL of 10x buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 50 pMole of each primer (Copenhagen, Denmark),1.5 U of Taq DNA polymerase (Amplicon Co., Denmark), and 21.8 μL ddH_2O. Amplification was performed in a thermocycler (Corrbet, Australia) with a specific program (Table 1) and subjected to electrophoresis in 1.5% agarose gel. Consequently, the DNA standards of the PCR product were sequenced (Forster, USA). Standard strain ATCC1209 was used as a positive quality control and ATCC1053 as a negative quality control (15-18).

3.6. Sequencing of Class 1 Integron Gene

The data of variable region of type *1* integron amplicons and its nucleotide sequences were sequenced and analyzed, respectively, by the National Center for Biotechnology Information (NCBI) (Retrieved from: http://www.ncbi.nlm.nih.gov/BLAST/).

3.7. Statistical Analysis

Paired-sample *t* test and Spearman's correlation tests were used to analyze the collected data with SPSS software version 21.01. In this study, $P < 0.05$ was set as the significance level.

4. Results

4.1. Bacterial Isolation

During one year (2016 - 2017), 30 clinical isolates of *P. aeruginosa* were collected from patients admitted to Ayatollah Rohani Hospital (Babol, north of Iran).

4.2. Antibiotic Resistance Profile

All samples were examined for resistance to nine antimicrobials using DD. The resistance rates to NI, AP, CTX, CPM, TM, GM, CIP, IMI, and AK were 100, 100, 100, 93.3, 9, 13.7, 10.3, and 3.4%, respectively (Table 2).

The results of the agar dilution method rates for NI, AP, CTX, TM, GM, CPM, CIP, IMI, and Ak were 100, 96.7, 96.7, 90, 63.3, 56.7, 40, 40, and 3.3%, respectively (Table 3). The multidrug resistance phenotype of *P. aeruginosa* clinical isolates for *intI* and gene cassettes of *aadB*, *dfrA1*, and *bla-$_{OXA30}$* are presented in Table 4.

Table 1. Primer Sequences and PCR Programs for Some Genes

Gene	Sequence Primer PCR Product	PCR Product	PCR Program
IntI	F: TCTCGGGTAACATCAAGG; R: AGGAGATCGGAAGACCTC	243 bp	5 min at 94°C; 35 cycles (1 min at 94°C, 1 min at 53°C, and 30 sec at 72°C); 5 min at 72°C
aadB	F: GGGCGCGTCATG GAG GAGTT; R: TATCGCGACCTGAAAGCGGC	329 bp	5 min at 94°C; 35 cycles (1 min at 94°C, 1 min at 65°C, and 30 sec at 72°C); 5min at 72°C
DfrA1	F: GGAGTGCCAAAGGTGAACAGC; R: GAGGCGAAGTCTTGGGTAAAAAC	367 bp	5 min at 94°C; 35 cycles (1 min at 94°C, 1 min at 45°C, and 30 sec at 72°C); 5 min at 72°C
bla-$_{OXA30}$	F: ATTATCTACAGCAGCAGCGCCAGTGCATC; R: TTCGACCCCAAGTTTCCTGTAAGTGC	716 bp	5 min at 94°C; 35 cycles (1 min at 94°C, 1 min at 50°C, and 30 sec at 72°C); 5 min at 72°C

Table 2. Antibiotic Susceptibility of Integron-Positive and Integron-Negative Strains of *Pseudomonas aeruginosa* Compared to Disk Diffusion [a]

Antibiotics	Disk Diffusion Method (n = 30)		Integron-Positive (n = 18)		Integron-Negative (n = 12)		P-Value
	Susceptibility	Resistant	Susceptibility	Resistant	Susceptibility	Resistant	
Gentamicin	14 (46.6)	16 (53.4)	7 (38.9)	11 (61.1)	7 (58.3)	5 (41.7)	0.296
Ciprofloxacin	23 (76.7)	7 (23.3)	13 (72.2)	5 (27.8)	10 (83.3)	2 (16.7)	0.481
Cefepime	2 (6.7)	28 (93.3)	1 (5.6)	17 (94.4)	1 (8.3)	11 (91.7)	0.795
Trimethoprim	3 (10)	27 (90)	0	18 (100)	3 (25.0)	9 (75.0)	0.025*
Cefotaxime	0	30 (100)	0	18 (100)	0	12 (100)	0.00*
Ampicillin	0	30 (100)	0	18 (100)	0	12 (100)	0.00*
Imipenem	23 (76.7)	7 (23.3)	14 (77.8)	4 (22.2)	9 (75.0)	3 (25.0)	0.860
Amikacin	27 (90.0)	3 (10)	14 (77.8)	4 (22.2)	11 (91.7)	1 (8.3)	0.804
Nitrofurantoin	0	30 (100)	0	18 (100)	0	12 (100)	0.00*

[a] Values are expressed as No. (%).

Table 3. Antibiotic Susceptibility of Integron-Positive and Integron-Negative Strains of *Pseudomonas aeruginosa* Compared to Agar Dilution Method [a]

Antibiotic	Agar Dilution Method (n = 30)		Integron-Positive (n = 18)		Integron-Negative (n = 12)		P-Value
	Susceptibility	Resistant	Susceptibility	Resistant	Susceptibility	Resistant	
Gentamicin	11 (36.7)	19 (63.3)	3 (16.7)	15 (83.3)	8 (66.6)	4 (33.4)	0.005 *
Ciprofloxacin	18 (60)	12 (40)	8 (44.4)	10 (55.6)	10 (83.3)	2 (16.7)	0.033*
Cefepime	13 (43.3)	17 (65.7)	3 (16.7)	15 (83.3)	10 (83.3)	2 (16.7)	0.000*
Trimethoprim	3 (10)	27 (90)	0	18 (100)	3 (25)	9 (75)	0.025*
Cefotaxime	1 (3.3)	29 (96.7)	0	18 (100)	1 (8.3)	11 (91.7)	0.213
Ampicillin	1 (3.3)	29 (96.7)	0	18 (100)	1 (8.3)	11 (91.7)	0.213
Imipenem	18 (60)	12 (40)	11 (61.1)	7 (38.9)	7 (58.3)	5 (41.7)	0.879
Amikacin	29 (96.7)	1 (3.3)	17 (94.4)	1 (5.6)	12 (100)	0	0.406
Nitrofurantoin	0	30 (100)	0	18 (100)	0	12 (100)	0.000*

[a] Values are expressed as No. (%).

4.3. Gene Cassettes

60% (n : 18) carried *intI* gene among 30 *P. aeruginosa* isolates. The prevalence of *aadB*, *dfrA1*, and *bla-$_{OXA30}$* genes were 11 (61%), 12 (66%), 6 (33%), and 0 (0%), respectively.

4.4. Nucleotide Sequence Accession Number

The positive *aadB* and *dfrA1*strains were sequenced and established in the Gene Bank database as MH708573 and MH708574, respectively.

Table 4. Multidrug Resistance Phenotype of *Pseudomonas aeruginosa* Clinical Isolates for *IntI* and Gene Cassettes of *aadB*, *dfrA1*, and *bla*-$_{OXA30}$

Antibiotics Resistant Pattern	Number of Isolates	*IntI*	*aadB*	*DfrA1*	*bla*-$_{OXA30}$
CTX/SXT/AM/IPM/FM/GM/CP/FEP	1	1	-	1	-
CTX/SXT/AM/AN/FM/GM/FEP	2	2	2	1	1
CTX/SXT/AM/IPM/FM/GM/FEP	2	2	2	2	-
CTX/SXT/AM/IPM/FM/GM/CP	1	1	-	1	-
CTX/SXT/AM/FM/GM/CP/FEP	1	-	-	-	-
CTX/SXT/AM/FM/GM/FEP	8	5	3	2	2
CTX/SXT/AM/AN/FM/FEP	1	1	1	1	-
CTX/SXT/AM/FM/CP/FEP	3	3	-	1	-
CTX/SXT/AM/IPM/FM/FEP	3	1	1	1	1
CTX/SXT/AM/FM/FEP	5	2	2	2	2
CTX/AM/FM/CP/FEP	1	-	-	-	-
CTX/AM/FM/GM	1	-	-	-	-
CTX/AM/FM/FEP	1	-	-	-	-

5. Discussion

Pseudomonas aeruginosa is an important opportunistic nosocomial bacterium with high resistance to most common antibiotics. For this reason, treating infections induced by this bacterium is challenging. The innate and acquired resistance to antimicrobial agents is involved in the mortality rate of patients suffering from infections induced by this bacterium (19). Integrons have been known as the primary mechanism to detect the gene of resistance to antibiotics in Gram-negative bacteria (20). The integrase gene sets the ground for resistance to common antibiotics, including ampicillin, gentamicin, trimethoprim, and cefotaxime (5). The findings of this study indicated that 18 isolates (60%) had the *int1* gene. Further, out of the samples with the *int1* gene, 61, 62, and 33% had the gene cassettes of *aadB*, *dfrA1*, and *bla*-$_{OXA30}$.

The correlation between the presence of the *intI* gene and inducing resistance to each antibiotic was examined with SPSS software version 21.0 using the chi-square test. In the DD method, there is a significant correlation between the presence of *intI* gene and resistance to trimethoprim, cefotaxime, ampicillin, and nitrofurantoin. In the agar dilution method, there is a significant correlation between the presence of *intI* gene and resistance to gentamicin, ciprofloxacin, cefepime, trimethoprim, and nitrofurantoin separately (Table 2,3). In Brazil, Fonseca et al. (21) documented 45.5% of *P. aeruginosa* strains had the *int1* gene, and out of 29 isolates, 66% and 52% samples had *aacA* and *bla*-$_{OXA30}$ genes. In another study, the *intI* gene frequency was 57.1%, and there was also a significant relationship between the gene cassettes in *int1* and antibiotic resistance (22). In another study by Nikokar et al. (23) on antibiotic resistance of the *Int1* gene in *P. aeruginosa*, 47% of the strains were resistant to the antibiotic. However, most of the strains were insensitive to imipenem. The PCR results indicated that 45.3% of the isolates had the *intI* gene and that 69% of the strains were antibiotic-resistant. All studies suggest a significant relationship between the presence of *int1* and gene cassettes with antibiotic resistance (24).

5.1. Conclusions

The high resistance of *P. aeruginosa* isolates is due to the presence of *int1* and its gene cassettes. Considering their high resistance to cefotaxime, gentamicin, ampicillin, and imipenem in hospitals, selecting appropriate drugs or generally changing the treatment course for patients is possible to prevent the spread of resistance inducing genes and the development of nosocomial infections.

Footnotes

Authors' Contribution: Study concept and design: E. F. Sh. and R. E. Sh.; analysis and interpretation of data: E. F. Sh. and R. E. Sh.; drafting of the manuscript: E. F. Sh., R. E. Sh., and P. S.; critical revision of the manuscript for important intellectual content: E. F. Sh., R. E. Sh., and P. S.; statistical analysis: R. E. Sh.

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Ethical Approval: This project was a part of another project (Code of Ethics: MUBABOL. REC.1394.162); hence, there was no need to receive another code of ethics.

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