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Prevalence of Antibiotic Resistance and Class 1 Integrons in Clinical and Environmental Isolates of *Pseudomonas aeruginosa*

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Abstract

Background: Many clinical isolates of *Pseudomonas aeruginosa* exhibit antibiotic-resistance and it is the main cause of hospital infections.
Objectives: The current study aimed at evaluating the diversity of genes coding for antibiotic resistance in clinical and environmental isolates of *P. aeruginosa* in Hamadan, West of Iran.
Methods: In the current cross sectional study, 100 clinical and 50 environmental isolates of *P. aeruginosa* were collected from October 2013 to May 2014. The standard disk diffusion method (SDD) was performed to test antibiotic susceptibility. All isolates were evaluated by the polymerase chain reaction (PCR) for the presence of class 1 integrons and genes encoding resistance to aminoglycoside antibiotics. The relationship between antibiotic resistance and integrons was tested by the 2-tailed chi-square (χ^2) or the Fisher exact tests. P values ≤ 0.05 were considered statistically significant.
Results: The most common antibiotic resistance was to cotrimoxazole (61.3%). The least observed resistance was to meropenem (9.3%) and piperacillin /tazobactam (12%). The result of minimum inhibitory concentration (MIC) for amikacin showed that 20% of the strains were resistant, 15% had intermediate resistance, and 65% were sensitive. Class 1 integrons were found in 57% of the isolates. The aminoglycoside-resistance cassette included aadA (confers resistance to streptomycin and spectinomycin) and aphA1 (encoded aminoglycoside 3'-phosphotransferase) genes were the most prevalent in the isolates.
Conclusions: Antibiotic resistance had a high prevalence in clinical isolates of *P. aeruginosa* and was commonly associated with class 1 integrons. The knowledge of drug resistance patterns helps to apply effective antibiotic treatments and appropriate infection control measures to prevent the spread of infection in hospitals.

Keywords: Integron, Aminoglycoside, Resistance, *Pseudomonas aeruginosa*

1. Background

Pseudomonas aeruginosa is a Gram-negative, aerobic, non-fermentative, andopportunistic organism that causes severe and refractory infections especially in patients with weak immune system (1). This microorganism is the third most common cause of fatal hospital infections in patients with cystic fibrosis, neoplastic diseases, and severe burns (1, 2).

After the employment of antibiotics to treat infectious diseases, according to the law of natural selection, bacteria became resistant to them (3). Unfortunately, the indiscriminate use of antibiotics, particularly in recent years, created many problems, which mainly stem from their toxic effects and the emergence of resistant strains (4, 5).

Dissemination of antibiotic resistance by horizontal gene transfer leads to the rapid emergence of antibiotic resistance among clinical isolates of bacteria. Many mobile genetic elements such as plasmids, transposons, integrons, and phages are responsible for the horizontal gene transfer of antibiotic resistance. Recent studies suggest that integrons play a significant role in disseminating antibiotic resistance genes in clinical environments (6, 7). Moreover, their role as a mobile genetic element in horizontal transfer of antibiotic resistance is proved (8).

Integrons have various classes, but just 3 of them are studied in details. These 3 classes of integrons, known as class 1, 2, and 3, are the only ones that are clinically important (9-11).

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More than 60 different types of gene cassette are described that include gene cassettes resistant to aminoglycosides, penicillins, cephalosporins, carbapenems, trimethoprim, chloramphenicol, rifampin, erythromycin, and quaternary ammonium compounds (12). Moreover, more than 1 gene cassette can be found in integrons, which is why they can cause resistance to more than one antibiotic.

A better understanding of antibiotic resistance in this opportunistic pathogen and also the discovery of new emerging ones can help to determine its epidemiology in a particular geographic area and can be also a useful factor to develop new methods to treat patients affected by such infections (13).

2. Objectives

The current study aimed at investigating the diversity of genes coding antibiotic resistances in clinical and environmental isolates of *P. aeruginosa* in Hamadan, West of Iran.

3. Methods

3.1. Bacterial Isolates

The current cross sectional study investigated 100 *P. aeruginosa* strains isolated from clinical specimens such as chip (tracheal tube), blood, urine, sputum, and wound of patients hospitalized in educational hospitals and 50 environmental *P. aeruginosa* strains isolated from the same hospitals.

Presumptive identification of *P. aeruginosa* isolates collected over 9 months from April to December 2014 were carried out using the conventional phenotypic methods, including Gram staining, oxidase and catalase tests, pyocyanin production, growth at 41 - 42°C, oxidation/fermentation (OF) determination, and pigmentation test. Furthermore, the polymerase chain reaction (PCR) assay targeting PA-16S rRNA gene specific for *P. aeruginosa* were applied to confirm the results obtained from the presumptive phenotypic tests (14, 15).

3.2. Antimicrobial Susceptibility Testing

All isolated strains were tested by the Kirby-Bauer disk diffusion and MIC methodology to determine the antimicrobial susceptibility pattern, according to the recommendations of the clinical and laboratory standards institute (CLSI 2013) (16, 17). The antibiotic disks (Himedia Co., India) utilized in the current study for disk diffusion method were amikacin (30 μg), gentamicin (10 μg), tobramycin (10 μg), ciprofloxacin (5 μg), imipenem (10 μg), meropenem (10 μg), ceftazidime (30 μg), cefoperazone (75 μg), ceftazidime (30 μg), cefepime (30 μg), piperacillin (100 μg), piperacillin-tazobactam (110/10 μg), colistin (10 μg), cefoperazone-sulbactam (100/10 μg), and cotrimoxazole (10 μg). The MIC was performed using amikacin powder (Himedia Co., India), separately. The microplates containing Mueller-Hinton broth were used for this purpose. Different serial dilutions for amikacin including 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 μg/mL were prepared and bacterial standard suspension was added to them. The microplates were incubated for 18 hours at 37°C. The wells with no turbidity were considered as MIC (18).

3.3. DNA Extraction

DNA was extracted using the simple boiling method. In practice, 2 - 3 colonies of an overnight cultured *P. aeruginosa* isolates were suspended in 400 μL of 1X TE buffer (10 mM Tris, 1mM ethylenediaminetetraacetic acid (EDTA) by vortexing. The suspension was heated in a boiling bath at 95°C for 10 minutes. Then, cellular debris was removed by centrifugation at 12,000 rpm for 10 minutes. One microliter of supernatant was used as a template DNA in PCR reactions. The quality of extracted DNA was assessed spectrophotometrically by the Nanodrop ND-1000 (Nanodrop Technologies, Inc., Wilmington, DE, USA) and electrophoresis using 2% agarose gel in 1X TBE buffer (18).

3.4. PCR Assay

Single PCR was applied to detect *int-1*, *aphA-1*, *aphA-6*, *aacC-1*, *aadB*, and *aadA* genes. The primer sequences used in the current study are presented in Table 1. The reaction mixture for PCR contained 10 μL of 2X Taq premix Mastermix (Parstous Biotech CO. Iran), 5 μL sterile double distilled water, 1 μL of each forward and reverse primers, and 3 μL of extracted DNA. The optimum conditions for PCR were obtained by the following steps: an initial denaturation for 5 minutes at 94°C; 30 cycles at 94°C for 30 seconds; 55°C for 30 seconds; 72°C for 1 minute, and a final cycle of 72°C for 10 minutes in a Bio-Rad Thermal Cycler.

PCR products and a 50-bp DNA marker (Fermentase Co, USA) were run simultaneously on 1.5% agarose gel stained with DNA safe stain (SinaClon Co, Iran) at 80 V for 1 hour. Finally, the PCR final products were visualized and illustrated under an ultraviolet (UV) transilluminator (Vilbert-Lourmat Co., Japan) (22).

3.5. Statistical Analysis

In the current study, the possible association between antibiotic susceptibility patterns and carrying various genes were examined. All categorical variables were compared using the 2-tailed chi-Square (χ^2) or the Fisher exact

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Table 1. The Sequence of Primers Used in the Study

Target Gene	Sequences	Gene Accession No.	Product, bp	Annealing Temperature, °C	Reference
Int-1	F: CAG TGG ACA TAA GCC TGT TC	AB777501	160	55	(19)
	R: CCC GAC GCA TAG ACT GTA				
aphA-1	F: CGAGCATCAAAT-GAAACTGC	CP002522	623	54	(20)
	R: GCGTTGCCAATGATGT-TACAG				
aphA-6	F: ATGGAATTGCC-CAATATTATTC	JF949760	797	54	(21)
	R: TCAATTCAATTCAT-CAAGTTTTA				
aacC-1	F: GACATAAGCCTGTTCG-GTT	X15852	372	49	(21)
	R: CCCGCTTTCTCGTAGCA				
aadB	F: ATCTGCCGCTCTGGAT	U17586	404	50	(21)
	R: CGAGCCTGTAGGACT				
aadA	F: ATGAGGGAAGCGGT-GATCG	JX861889	792	50	(21)
	R: TTATTTGCCGACTAC-CTTGGTG				

test. P values $\leq$ 0.05 were considered statistically significant. All statistical analyses were performed with SPSS version 20.

4. Results

A total of 150 *P. aeruginosa* isolates were collected from April to December 2014including 100 strains isolated from various clinical specimens such as blood, urine, and wound, and 50 strains from different sectors of educational hospitals including infectious diseases, burn, and the intensive care unit (ICU).

4.1. Antibiotic Susceptibility Results

The analysis of the results showed that the highest level of resistance was to co-trimoxazole (92 isolates, 61.3%). Moreover, the lowest level of resistance was to meropenem (14 isolates, 9.3%) and piperacillin/tazobactam (18 isolates, 12%). The resistance to various antibiotics in isolated strains from clinical and environmental specimens is shown in Table 2.

The result of MIC for amikacin showed that 30 (20%) isolates were resistant, 23 (15.3%) intermediate, and 97 (64.6%) sensitive.

Table 2. Antibiotic Resistance Pattern in Clinical and Environmental Isolates of *Pseudomonas aeruginosa*

Antibiotic	Resistance of Clinical Isolates	Resistance of Environmental Isolates	Total
Imipenem	20 (20)	4 (8)	24 (16)
Meropenem	10 (10)	4 (8)	14 (9.3)
Cefepime	16 (16)	15 (30)	31 (20.7)
Piperacillin	10 (10)	18 (36)	28 (18.7)
Piperacillin/tazobactam	4 (4)	14 (28)	18 (12)
Amikacin	24 (24)	21 (42)	45 (29.3)
Gentamicin	18 (18)	17 (34)	35 (23.3)
Colistin sulfate	11 (11)	12 (24)	23 (15.3)
Cotrimoxazole	62 (62)	30 (60)	92 (61.3)
Ceftazidime	17 (17)	11 (22)	28 (18.7)
cefoperazone	28 (28)	14 (28)	42 (28)
cefoperazone/sulba	22 (22)	14 (28)	36 (24)
Tobramycin	25 (25)	20 (40)	45 (30)
Ciprofloxacin	72 (48)	39 (26)	39 (26)

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

4.2. Identification of Class 1 Integron and Aminoglycoside-Resistant Cassettes

The results of a single PCR reaction revealed that out of 150 *P. aeruginosa* strains, 85 isolates (56.6%) contained class

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1 integron. The *aadA* gene was the most prevalent one with 81 (54%) and alpha-1 was the least prevalent one observed in only 19 (12.7%) aminoglycoside-resistant cassettes. Table 3 shows the results of PCR for all isolates.

5. Discussion

Integrons are the moving agents that can be located in plasmids, chromosomes, or transposons (6). These agents, similar to plasmid and transposons, play an important role in the development of multiple drug resistances. Nowadays, more than 21 different gene cassettes are recognized that are capable of providing the prerequisites needed to become resistant to antibiotics such as aminoglycosides, penicillin, cephalosporin, carbapenems, trimethoprim, chloramphenicol, rifampin, erythromycin and the quaternary ammonium compounds. Furthermore, it was reported by many studies that integrons containing more than 1 gene cassette enable the carrying bacteria to develop multi drug resistances (21). Among the discovered integrons, class 1 integrons are the most prevalent and the most important ones to transfer drug resistant genes.

The current study aimed at investigating the antimicrobial resistance pattern of *P. aeruginosa* strains isolated from clinical and environmental specimens. Among the 150 *P. aeruginosa* strains analyzed in the current study, the highest susceptibility was observed to imipenem and meropenem antibiotics (103 strains, 68.7%), and the highest resistance was to cotrimoxazole (92 strains, 61.3%) and the lowest resistance was to piperacillin/tazobactam (Table 2). The resistance of clinical and environmental isolates was also separately assessed; accordingly, it was determined that clinical isolates had the highest susceptibility to the piperacillin (69 out of 100 clinical strains, 69%).

Many studies are performed in this area, but the obtained results are not consistent according to the geographical regions and the date of the studies. The current study results were compatible with the results obtained by Mostafa Hosseini et al. (18). They reported that *P. aeruginosa* isolates had a low resistance to imipenem (9.6%) and amikacin (12.9%) in Hamadan, Iran. Since both studies were conducted in the same geographical region, it can be concluded that antimicrobial resistance patterns of *P. aeruginosa* are also similar in other similar geographical regions. The study conducted by Rajat M et al. in India reported that the level of resistance to ciprofloxacin, tobramycin, levofloxacin, gentamicin, and imipenem were 49%, 68%, 25%, 63%, and 14%, respectively (23). Another study carried out by Jioaguo Chen et al. (24) in China demonstrated that the highest level of resistance was to cefoxitin (90%), ceftriaxone (88.7%), cotrimoxazole (84.5%), and piperacillin (69%), and the lowest level of resistance was to tobramycin (35.2%), amikacin (31%), ceftazidime, and imipenem (29.6%). It was also outlined by Bing et al. that Pseudomonas spp. had the highest resistance to cefotaxime, cotrimoxazole, and chloramphenicol (25). The resistance to amikacin and ciprofloxacin were reported 17.25% and 27.59%, respectively by Chander and Raza et al. (26). The study was conducted in Nepal and it can be inferred that poor hygienic conditions in this country is a possible reason for higher resistance to antibiotics. Another study carried out in India reported the level of resistance to ciprofloxacin and meropenem 59% and 11%, respectively (27).

Furthermore, the results of MIC indicated that most of *P. aeruginosa* strains (64.6%) were susceptible to amikacin. The statistical analysis did not find any significant correlation between the frequencies of aminoglycoside genes and MIC of amikacin ($P > 0.05$), while the levels of resistance significantly varied among the isolated strains ($P = 0.04$).

Another objective of the current study was to investigate the frequency of aminoglycoside-resistant gene cassettes. Among 100 *P. aeruginosa* strains isolated from clinical specimens, 56 strains (56%) harbored int-1 gene. Likewise, out of 50 strains isolated from environmental samples, 29 strains (58%) contained *int-1* gene.

The frequencies of *aphA-1*, *aphA-2*, *aadA*, *aadB*, and *aacC-1* gene cassettes in 150 strains of the study were 12.6%, 16%, 54%, 36.6%, and 42%, respectively.

There are at least 22 different types of genes known as *aadA-1* to *aadA-24* in GenBank, but some of them are missed over time. The *aadA-1* is a gene cassette and a part of integrons, plasmid, and transposons (28).

Khosravi et al. reported the frequency of class 1 integrons in *Pseudomonas* spp. isolated from burning victims, which was higher than that of the current study. This discrepancy can be attributed to different places these isolates were collected (29).

The results of the current study were consistent with those of the study by Ruiz et al. in Spain (30). In that study, 26 out of 56 clinical isolated *P. aeruginosa* strains contained class 1 integron that had the highest prevalence in the *aadB* gene cassette. The interesting point of that study, contradictory with the results of the current study, was that they did not find any integron genes in the environmentally isolated strains that can be due to the lower prevalence of resistance to antibiotics in European countries than Iran.

Yan et al. conducted another study on the presence of integron genes in 118 bacterial strains including both Gram-positive and -negative ones; moreover, they assessed antibiotic resistances among these strains and the association between the presence of resistant genes and antibiotic resistance (20). The results of their study demonstrated that 100 out of 118 strains (84.7%) were resistant to more than 3 antibiotics, and then, categorized as multidrug re-

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Table 3. The Prevalence of Class 1 Integron and Gene Cassettes in Clinical and Environment Isolates of *Pseudomonas aeruginosa*

Source		Integron Class 1	Cassette Gene				
			aphA-1	aacC-1	aadB	aadA	aphA-6
Clinical (100 Isolates)	Urine	29 (73)	6 (15)	20 (50)	18 (45)	28 (70)	12 (30)
	Trachea-tube	14 (40)	3 (8)	11 (34)	8 (22.9)	12 (34.3)	2 (5.7)
	Blood	5 (41.7)	2 (16.7)	7 (58.3)	5 (41.7)	5 (41.7)	2 (16.7)
	Sputum	6 (75)	0 (0)	3 (37.5)	4 (50)	5 (62.5)	0 (0)
	Wound	2 (50)	2 (50)	2 (50)	0 (0)	1 (25)	2 (50)
	Eye	0 (0)	0 (0)	0 (0)	0 (0)	1 (100)	0 (0)
Environment (50 isolates)	29 (58)	6 (12)	20 (40)	20 (40)	29 (48)	6 (12)	
Total (150 isolates)	85 (56.6)	19 (12.6)	63 (42)	55 (36.6)	84 (54)	24 (16)	

sistance. In the comparison of strains carrying integron genes with the strains that did not contain such genes, it was found that the level of resistance in integron-positive strains were higher than that of integron-negative strains. In the current study, 90 strains contained class 1 integron gene, 1 strain contained class 2 integron, and 4 strains contained both class 1 and class 2 integrons, and class 3 integron was not found in any of the strains.

5.1. Conclusions

Overall, the results of the current study demonstrated a high level of class 1 integron in *P. aeruginosa* strains isolated from the selected hospitals. Since the presence of class 1 integron can spread drug resistance against important antibiotics such as β-lactams, aminoglycosides, and tetracycline, more attention should be paid to this issue.

Footnotes

Competing Interests: Authors declared no conflict of interest.

Ethical Approval: Not applicable.

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