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Prevalence of Class 1 Integron, Resistance Gene Cassettes and Antimicrobial Susceptibility Profiles among Isolates of *Pseudomonas aeruginosa* in Iran

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Abstract

Pseudomonas aeruginosa is one of the most important opportunistic human pathogens worldwide. High prevalence of Multi Drug Resistant _P. aeruginosa_ (MDRPa) in Iran is a serious problem for antimicrobial therapy. Several studies have reported the MDRPa in Europe and Asia. Due to the use of broad-spectrum antibiotics, bacterial resistance is increasing in Iran, located in Middle East. The present cross-sectional study was designed to investigate the prevalence of class1 integron, resistance gene cassettes and antimicrobial susceptibility profiles among isolates of _P. aeruginosa_ in Al-Zahra Hospital, Isfahan City, central part of Iran from Jan-Sep 2014. The aim of this study was to determine the antimicrobial susceptibility, the prevalence of Class1 integron, resistance gene a measuring in Iran. A total of 231 _P. aeruginosa_ isolates were collected from clinical specimens including urine (50.6%), tracheal tube (25.5%), wound (13.4%), blood (6.1%), catheter (2.2%), cerebrospinal fluid (1.7%) and sputum (0.4%) isolates from hospitalized patients (mean age: 50.27 ± 24.12 years).The majority of patients (68%) were male. Isolates were collected from different parts of the hospital as follows: ICU, Internal Medicine, Emergency care, Pediatrics, Nephrology, Transplant Center, General surgery and Infectious. Revealed data show a high rate of MDR _P. aeruginosa_ isolates in the studied area; also, the result signifies the spread of _aadA6_ among clinical isolates in hospitalized patients.

Keywords

***Pseudomonas aeruginosa*, Resistance Gene Cassettes, Antibiotic Susceptibility**

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1. Introduction

Pseudomonas aeruginosa is one of the most important opportunistic human pathogens responsible for 10% - 15% of the nosocomial infections worldwide [1] [2]. This pathogen is naturally resistant to various antimicrobial agents, and also it has a distinctive valence through multiple mechanisms to become resistant to almost all the commercial antibiotics [3] [4]. High prevalence of Multi Drug Resistant (MDR) *P. aeruginosa* in Iran is a serious problem for antimicrobial therapy [5]-[10]. Infections caused by aforementioned drug resistant pathogens are dependent with notable increase in morbidity and mortality [7].

Plasmids, transposons and integrons are mobile genetic elements that they resort of earning resistance mechanisms cooperating to *P. aeruginosa* multidrug resistance [9]. The integron is potentially a major element in the spreading of multidrug resistance among gram-negative bacteria, particularly in genus *Pseudomonas* [4]. Integrons are one of the genetic elements have been found to be able to elevate absorption and expression of drug resistance genes located within gene cassettes [10]-[12]. Out of all sequenced bacterial genomes, integrons have been found in approximately 9% [11]. Various investigations revealed the presence of class 1 integron in 40% - 70% of gram-negativepathogens isolated from clinical setting. It is obvious that integrons have major antibiotic resistance roles, causing difficulties in bacterial infection control; so, it is important to understand the origin of these elements for exploring the means by which bacterial lateral gene transfer can seriously impact on, and be impacted by, human activities and for practical control of antibiotic resistance [11]-[19]. Class 1 itegron is made of a functional platform (5'CS), which contains the integrase gene (*intI*), the recombination site (*attI*) and a promoter region (P ant), and a (3'CS), which normally includes the *qacED*1and *sulI* genes [6]-[24]. The gene cassettes among integrons are variable in these structures, small, mobilegenetic elements, containing of a single gene (or sporadically two genes) in addition a recombination site named the 59-be [24]-[31]. Several resistance gene cassettes carried by class 1 integrons in *P. aeruginosa* encodes multiple resistance mechanisms such as resistance to beta-lactams, aminoglycosides and other antimicrobial agents [9]-[13]. Several studies have reported the multiple drug resistance in *P. aeruginosa* clinical strains in European and Asian countries [26] [27]. Due to the use of broad-spectrum antibiotics, bacterial resistance is increasing in Iran, bordered with European and Asian countries. The present study was designed to investigate the prevalence of class 1 integron, resistance gene cassettes and antimicrobial susceptibility profiles among isolates of *P. aeruginosa* in Al-Zahra Hospital, Isfahan City, central part of Iran.

2. Methodology

2.1. Bacterial Isolates

A cross-sectional study was conducted in Al-Zahra Hospital in Isfahan City, located in central part of Iran from Jan-Sep 2014. Two hundred and thirty one *P. aeruginosa* strains were obtained from different specimens of inpatients coming from clinical cases, including 117 urine (50.6%), 59 tracheal tube (25.5%), 31 wound (13.4%), 14 blood (6.1%), 5 catheter (2.2%), 4 cerebrospinal fluid (1.7%) and 1 sputum (0.4%) isolates. These samples were collected from different parts of the hospital as follows: ICU[1], Internal Medicine, Emergency care, Pediatrics, Nephrology, Transplant Center, General surgery and Infectious.

At first, primary isolation was performed by phenotypic characteristics on blood agar and McConkey(Merck, Germany), then the isolates identification was carried out and confirmed to the species level by standard biochemical tests including Gram stain, catalase and oxidase test, O/F (Oxidation Fermentation) test (Merck, Germany), pyocyanin pigment production and growth at 42˚C [8].

2.2. Antimicrobial Susceptibility Test

Antimicrobial susceptibility test was performed on isolates using the Kirby-Bauer disk-diffusion breakpoint assay on Mueller-Hinton agar (Merck, Germany); and the cultures were incubated for 24 h at 37˚C. The following antibiotic disks (Mast, Bootle, Merseyside, UK) were used for susceptibility test: imipenem (10 µg), meropenem (10 µg) aztreonam (30 µg), cefepime (30 µg), ceftazidime (30 µg), amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), norfloxacin (10 µg), t obramycin (30 µg), piperacillin/tazobactam (100/10 µg) and colistin (10 µg). *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922) were used as the quality

[1]Intensive care unit (ICU).

control strains in every susceptibility test [12]. MDR was defined as non-susceptible to at least one agent in three or more anti-pseudomonal antimicrobial categories, and as extensively drug- resistant *P. aeruginosa* (XDR-PA) if it was non-susceptible to at least one agent in all but two or less anti-pseudomonal antimicrobial categories.

2.3. DNA Extraction and PCR Amplification

DNA extraction was carried out on MDR *P. aeruginosa* by boiling method. The template DNA stored at −20˚C until polymerase chain reaction (PCR) amplification was performed.MDR *P. aeruginosa* isolates were screened for the presence of class 1 integron by using 4 specific primers located on intI1 gene, qacED, sulI and gene cassette regions. Master mix component was as follow: 10× PCR buffer in a final concentration of 1×, MgCl2 (50 mM) in a final concentration of 1.5 mM, 10 mM dNTPs Mix in a final concentration of 0.2 mM, Forward and Reverse primers in a final concentration of 0.4 µM. PCR amplification was performed in a total volume of 25 µl (24 µl of PCR master mix plus 1 µl of template DNA). Amplification was carried out in a thermocycler (Eppendorf Master cyclers, MA) using the published paper programs [6] [7]: The primers (obtained from Takapouzist Company, Iran) used for the analysis and PCR conditions are summarized in **Table 1**. Class1 integron variable regions were amplified using primers Int1, In5'cs and In3'cs (**Table 1**). After performing PCR reaction, amplification products were identified using 1.5% agarose gel electrophoresis stained with 0.5 µg/ml ethidium bromide solution. Then, the results visualized using UV trans-illuminator on the UV gel document (Ingenius, Syngene). The size of specific bands was compared with 100 bp DNA ladder (Bioneer, Korea).

2.4. DNA Sequencing and Sequence Analysis

Sequencing was done by Sanger's method (Applied Biosystems 3730/3730xl DNA Analyzers Sequencing; Bioneer). The sequences were analyzed using Chromas Pro version 1.7.5 Technelysium (http://www.technelysium.com.au/). Nucleotide sequences were compared using online BLAST software (http://www.ncbi.nlm.nih). The sequences obtained from the gene cassette analysis have been deposited in GenBank under accession numbers FJ908755 for *aadB*, FJ908756 for *aadA6* and *orfD*genes, KJ679405 for *aacA*7, bla_{OXA-2}, *aacA*8 and HF546976 for bla_{NDM-1} gene.

3. Results

The mean age of the studied patients was 50.27 ± 24.12 years, which ranged from under one to 91 years. Demographic information of patients in this study has been summarized in **Table 2**. The majority of patients (68%) were male, 157 versus 74 females. The number and rate of isolates from different parts of the hospital were as follows: ICU, 71 (30.7%); Internal Medicine, 38 (16.5%); Emergency care, 14 (6.1%); Pediatrics, 17 (7.3%); Nephrology, 9(3.9 %); Transplant Center, 28 (12.1%); General Surgery, 49 (21.2%) and Infectious, 5 (2.2%).

3.1. Antimicrobial Resistance Pattern

The resistance pattern of *P. aeruginosa* isolates in this study revealed that 192 (83.1%) were Multi Drug Resis-

Table 1. Primer sets and PCR conditions used in this study.

Primer	Sequence (5′ → 3′)	PCR conditions	PCR product (bp)	Reference
IntI f	AAA ACC GCC ACT GCG CCG TTA	94˚C, 30 s; 55˚C, 30 s; 72˚C, 1 min (35 cycles)	This work	6
IntI r	GAA GAC GGC TGC ACT GAA CG			
qacED1-f	ATCGCAATAGTTGGCGAAGT	94˚C, 1 min; 48˚C, 45 s; 72˚C, 1 min (35 cycles)	236	7
qacED1-r	CAAGCTTTTGCCCATGAAGC			
SulI-f	CTTCGATGAGAGCCGGCGGC	94˚C, 1 min; 48˚C, 45 s; 72˚C, 1 min (35 cycles)	437	7
SulI-r	GCAAGGCGGAAACCCGCGCC			
In5'CS	GGCATCCAAGCAGCAAG	94˚C, 1 min; 48˚C, 45 s; 72˚C, 1 min (35 cycles)	Variable	7
In3'CS	AAGCAGACTTGACCTGA			

Table 2. Factors associated with class 1 integron among *Pseudomonas aeruginosa* isolates.

Patient characteristic	IntI positive n (%) **N** = 146 **(63.2)**	IntI negative n (%) **N** = 85 **(36.8)**	*P* value
Age			
< 40 Years	33 (43.4%)	43 (56.6%)	<0.001
≥40 Years	113 (72.9%)	42 (27.1%)	
Sex			
Male	102 (65%)	55 (35%)	
Female	44 (59.5%)	30 (40.5%)	
Ward			
Transplant center	28 (96.6%)	1 (3.4%)	<0.001
ICU	48 (67.6%)	23 (32.4%)	
Internal medicine	20 (52.6%)	18 (47.4%)	
Infectious	3 (60%)	2 (40%)	
General surgery	26 (53.1%)	23 (46.9%)	
Paediatrics	4 (25%)	12 (75%)	0.02
Emergency	10 (71.4%)	4 (28.6%)	
Nephrology	7 (77.8%)	2 (22.2%)	
Sample type			
Urine	83 (70.9%)	34 (29.1%)	0.01
Catheters	3 (60%)	2 (40%)	
Blood	6 (42.9%)	8 (57.1%)	
Wound	17 (54.8%)	14 (45.2%)	
Tracheal tube	35 (59.3%)	24 (40.7%)	
CSF	1 (25%)	3 (75%)	
Sputum	1 (100%)	0 (0%)	
Drug resistance			
Multi drug-resistance	146 (76%)	46 (24%)	<0.001
Extensively-drug resistance	78 (88.6%)	10 (11.4%)	<0.001

tant (MDR). Eighty (38.1%) *P. aeruginosa* isolates were resistant to all antibioticstested in this study. Resistanceor intermediate resistance (non-susceptible) was mostly observed toimipenem (63.6%), meropenem (63.6%), gentamicin (81.4%), amikacin (69.7%), ceftazidime (61.9%),ciprofloxacin (69.7%), tobramycin (74.5%), cefepime (61.9%), norfloxacin (68.4%), aztreonam (60.6%), colistin (58.9%) and piperacillin-tazo- bactam (56.7%) (**Figure 1**).

3.2. Detection and Characterization of Integrons

PCR assay was performed to detect integrin integrase genes (*intI1*), *qacED*, and *sulI* genes, and gene cassette regions (5CS/3CS) among 192 clinical MDR *P. aeruginosa* isolates. Out of 192 isolates, 146 (76%) were positive for class 1 integron by amplifying the *intI1* gene. Nucleotide sequence analysis of the class 1 integron variable regions revealed the presence of 3 different fragment sizes of approximately 0.8, 1.2 and 2.5 kb (**Figure 2**). 120

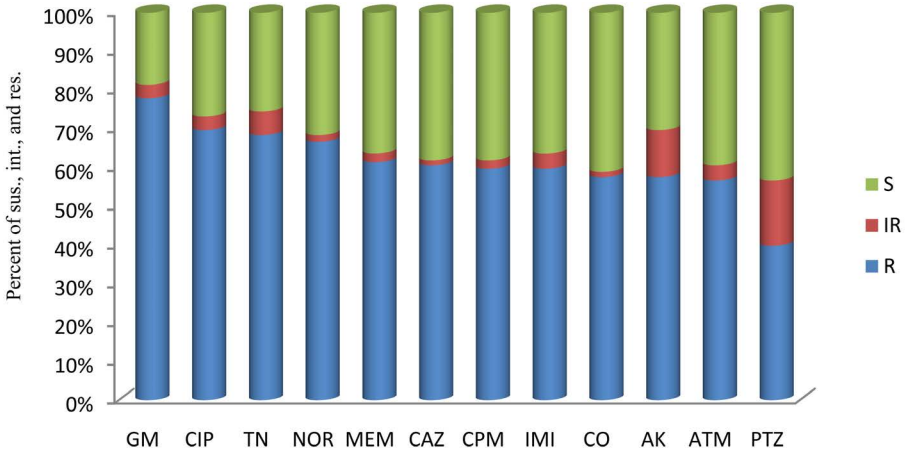

Figure 1. The percentage rate of antibiotic resistance of 192 MDR *P. aeruginosa* isolates from hospitalized patients in Alzahra Hospital in Isfahan, Iran. GM: gentamicin; CIP: ciprofloxacin; TN: tobramycin; NOR: norfloxacin; CAZ: ceftazidime; CPM: cefepime; IMI: imipenem; MEM: meropenem; Co: colistin; Ak: amikacin; ATM: aztreonam; PTZ: Piperacillin-tazobactam. S: sensitive; IR: intermediate; R: resistant.

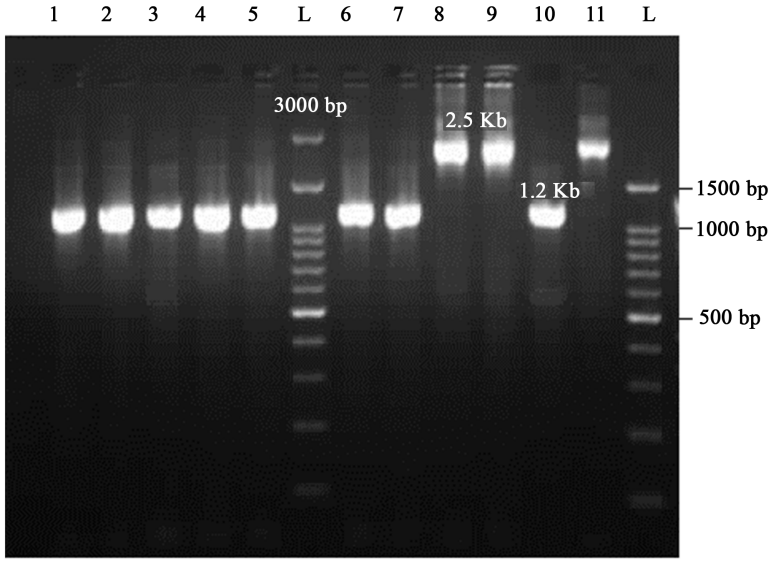

Figure 2. PCR amplification products of class 1 integron variable regions using primers In5' and In3' CS. L: 100 bp DNA ladder (Bioneer, Korea); lanes 1- 11, integron profiles 1 - 11 respectively among 192 MDR *P. aeruginosa* isolates in Al-Zahra Hospital in Isfahan, Iran.

isolates (62.5%) carried class 1 integron with sizes of approximately 1.2 kb, twenty three isolates (12%) with sizes of approximately 2.5 kb and three isolate (1.5) with sizes of approximately 0.8 kb.

The isolation sites and samples found in this study have been summarized in **Table 3**; also, the percentage rate of antibiotic resistance and presence of class 1 integrons among 192 MDR *P. aeruginosa* strains isolated from patients hospitalized in this study has been summarized in **Table 4**, **Figure 1** and **Figure 3**.

4. Discussion

Multi Drug Resistant *P. aeruginosa* is a serious challenge for treatment of nosocomial contagion, and a suitable antibiotic choice to initiate remedy is necessary for optimizing the clinical consequence [17]. As the results revealed, the most of isolates were resistant to several antimicrobial agents tested in this investigation and most of prevalence of class 1 integrons found in *P. aeruginosa* isolates consistent with those previously reported from

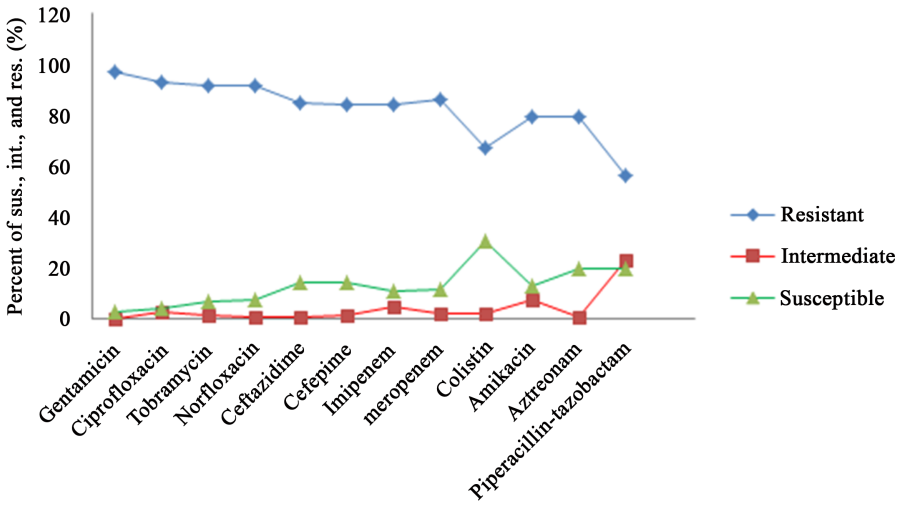

Figure 3. The percentage rate of antibiotic resistance and presence of class 1 integrons among 192 MDR *P. aeruginosa* isolates from hospitalized patients in Al-Zahra Hospital in Isfahan, Iran.

Table 3. Distribution of *Pseudomonas aeruginosa* isolates according to hospitals wards and sample types.

	ICU (No)	**Transplant center** (No)	**Nephrology** (No)	**General surgery** (No)	**Emergency care** (No)	**Infectious** (No)	**Internal medicine** (No)	**Paediatrics** (No)
Urine	24	23	9	24	12	2	18	5
Tracheal tube	39	0	0	3	0	2	11	4
Blood	4	2	0	4	0	1	3	0
Wound	1	2	0	15	2	0	6	5
Catheter	3	0	0	0	0	0	0	2
Sputum	0	1	0	0	0	0	0	0
CSF	0	0	0	3	0	0	0	1
Total	71	28	9	49	14	5	38	17

Table 4. The percentage rate of antibiotic resistance of class 1 integrons positive isolates among 192 MDR *P. aeruginosa* types.

	Resistance No. (%)	**Intermediate No. (%)**	**Sensitive No. (%)**
Gentamicin	97.3	0	2.7
Ciprofloxacin	93.2	2.7	4.1
Tobramycin	91.8	1.4	6.8
Norfloxacin	91.8	0.7	7.5
Ceftazidime	84.9	0.7	14.4
Cefepime	84.2	1.4	14.4
Imipenem	84.2	4.8	11
Meropenem	86.3	2.1	11.6
Colistin	67.1	2.1	30.8
Amikacin	79.5	7.5	13
Aztreonam	79.5	0.7	19.9
Piperacillin-tazobactam	56.8	23.3	19.9

Iran [1]-[21]. The high spread of MDR*P. aeruginosa* has become serious challenge in clinical settings. 192 isolates (83.1%) were considered as MDRPa (Multi Drug Resistant *P. aeruginosa*) which may lead to problems in treatment of *P. aeruginosa* infection. The special high rate of MDRPa found in this work could be due to the repeated and unsuitable use of antibiotics in Iran. The results of the present study indicated that the frequency of MDRPa was 83.1%; also, 88 (38.1%) isolates of *P. aeruginosa* were resistant to all the classes of antibiotics tested as well as strong relevancy intense was observed between integron presence and multiresistance.

The results of the present investigation showed thatthe screening for integrons in *P. aeruginosa* clinical isolates (192 MR and 39 Non-MR) from the Al-Zahra hospital; and146/231 isolates carry class 1 integrons. Out of these 192 MR isolates, 146 (76%) were positive for the presence of class 1 integrons. Several reports have revealed the presence of aminoglycoside resistance genes associated with integrons found in gram-negative bacteria [14]-[16] and also the present study revealed that aminoglycoside resistance was spread more than in the majority of isolates, including those of integron-positive. According to results, a significant difference was shown in distribution and frequency of class 1 integrons among MR and non-MR clinical strains of *P. aeruginosa* from Al-Zahra hospital, since only MR isolates (48.4%) harboured class 1 integrons carrying gene cassettes ($P < 0.001$). Among MDR isolates, 146 (76%) showed class 1 integrons. The rate of class 1 integrons observed in this study is higher than previously reported rates of 57.4% from Nigeria [9], 41.5% from Brazil [6], 45.8% from patients in southern china [4], 82% from Thai hospital in Thailand [7] and 69.2% from burn patients in Guilan, Iran [10]. In the current study a notable relevance was found between the presence of integrons and resistance against gentamicin, amikacin, imipenem, meropenem, ceftazidime, cefepime, tobramycin, norfloxacin, aztreonam and ciprofloxacin ($P < 0.001$).

The high resistance level against aminoglycoside antibiotics (gentamicin, amikacin and tobramycin) was observed in this study. Resistance to this class of antibiotics in *P. aeruginosa* is usually associated with the production of aminoglycoside adenylyltransferase which leads to resistance to aforementioned antibiotics [12]. Class 1 integrons have been dependent with different antibiotic resistance genes and organized important role in the expansion of antimicrobial resistance [7].

High resistance among *P. aeruginosa* strains against the following antibiotics: gentamicin, ciprofloxacin, tobramycin, norfloxacin, ceftazidime, cefepime, imipenem, meropenem, amikacin, aztreonam isolated from sputum, tracheal tube, urine and wounds was observed. The resistance rate of *P. aeruginosa* isolates from sputum, tracheal tube, urine, wound, blood, catheter andcerebrospinal fluid was reported as 100%, 89.8%, 83.8%, 80.6%, 64.3%, 60% and 50% respectively. In the present study, piperacillin/tazobactam and colistin was the most efficient antibiotics against *P. aeruginosa* and the antibiotic susceptibility test results showed 43.3% susceptible to piperacillin-tazobactam and 41.1% susceptible to colistin compared with other studies carried out on *P. aeruginosa* isolated from burn patients in Tehran in which reported 87.2% resistance to piperacillin-tazobactam [5]. This investigation reports 93 (48.4%) *P. aeruginosa* isolates containing class1 integron carrying resistance gene cassettes. Variable region genes of class 1 integrons found in our isolates were as follows: *aadB* gene (about 0.8 kb), *aadA*6, *orfD* (about 1.2 kb) and *aacA*7, *aacA*8, bla_{oxa2}, $bla_{NDM\text{-}1}$ gene (about 2.5 kb) and 120 clinical isolate have an *aadA*6-*orfD* array (about 1.2 kb). The *aadA*6-*orfD* had the maximum gene cassette in this investigation as well as the most prevalent gene cassette array in *P. aeruginosa* in Thai hospital in Thailand [7].

Genes giving resistance to aminoglycosides and ß-lactams are frequently found in integrons from *P. aeruginosa* isolates and the most popular aminoglycoside resistance gene cassettes related to *aad* and *aac* families [15]-[17]. The first report of identification and sequencing of a streptomycin/spectinomycinadenylyl transferase gene (*aadA*) was carried out in *Escherichia coli*and recently the crystal structure of an aminoglycoside adenylyl transferase (*AadA*) has been reported [22]. Aminoglycosides are a relatively large class of antibiotics and AAC (3)-II determines resistance to gentamicin, tobramycin and netilmicin [30]; also, AAC(6′)-II determines resistance to netilmicin, gentamicin and tobramycin. AAC(3)-I determines resistance to gentamicin and AAC(6′)-I determines resistance to tobramycin, netilmicin and amikacin. Finally, ANT (2′)-I determines resistance to gentamicin and tobramycin [31] [32].

This is noticeable that *orfD* has unknown functions and can potentially translate as a polypeptide chain. An ORF (open reading frame) is a portion of a DNA molecule that contains no stop codons, when translated into amino acids [25]. Twenty three of 192 (12%) clinical isolates have *aacA*7, *aacA*8, bla_{oxa2}, $bla_{NDM\text{-}1}$ (2.5 kb). bla_{oxa2} belongs to class D extendeds pectrum b-lactamases and hydrolyzes b-lactam antibiotics such as ceftazidime.

Class D *ß*-lactamases (oxacillinases) (OXA type enzymes) belongs to functional group 2d and molecular class

D [29]. OXA-1, OXA-2 and OXA-10 enzymes determine resistance to ureidopenicillins and carboxypenicillins but not to ceftazidime. Resistance, resulting from production of piperacillin and ticarcillin OXA-2 enzymes is lower than the resistance that develops when OXA-10 and OXA-1 oxacillinases are produced [29].

Study done among clinical isolates of *Pseudomonas aeruginosa* in 5hospitals Iran, Tehran reported class 1 integron containing *aadB*, *aadA*6-*orfD*, *aacA*4 and bla_{oxa10} [13]. Another study on gene cassette of Class 1 integrons in *P. aeruginosa* isolates from clinical settings in Amazon region, Brazil shoed that the most frequent gene cassette found was from the *aacA* family [6]. These our data indicate thatbla_{NDM-1}gene is the first report among class 1 integrons found in *P. aeruginosa* isolates from clinical settings in Iran.A member of a large gene family that encodes beta-lactamase enzymes called carbapenemases is NDM-1 gene [23].

5. Conclusion

In conclusion, our data show that high rate resistant to multiple drugs among *P. aeruginosa* signifies the spreads of *aadA*6 among clinical isolates in hospitalized patients in Al-Zahra hospital. There is a significant relationship to antibiotic resistance and class 1 integron and mobile genetic elements play a major role in the development of a resistance gene.

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