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Original Article

Investigating Class I, II and III Integrons in Multidrug Resistance in *Pseudomonas aeruginosa* Isolated from Hospital Infections in Ahvaz

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Background and Aims: The indiscriminate use of antibiotics can lead to antibiotic resistance in the treatment of infections caused by bacteria such as *Pseudomonas aeruginosa*. The presence of integrons in Pseudomonas is clearly associated with multidrug resistances. Therefore, this study aimed at tracking class I, II and III integrons of antibiotic-resistant isolates of *Pseudomonas aeruginosa* that were isolated from nosocomial infection.

Materials and Methods: In this study, 51 isolates of *Pseudomonas aeruginosa* were collected from different wards of Imam Khomeini hospital of Ahvaz since October of 2014 until March of 2015. After identification test and antibigram, coding genes of antibiotic resistance and class I, II and III integrons were detected by polymerase chain reaction (PCR) method.

Results: Tetracycline revealed the most resistance with 84% frequency in discreted isolates. In the encoding antibiotic resistance genes with a frequency of 94% was the most common blaTEM. Class I integron had 92% prevalence, class II Integron showed 52% prevalence and class III Integron demonstrated 17% prevalence.

Conclusions: In *Pseudomonas aeruginosa*, class I integron was more prevalent than other integrons and the integrase gene was probably one of the causes of multiple antibiotic resistance in this bacteria. Moreover, frequency of integron III was reported 17%.

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Introduction

Pseudomonas aeruginosa is a non-fermentative Gram-negative bacillus which rarely causes infection in the natural host [1]. *Pseudomonas* is found in large quantities in water, soil, plants and animals, which lives as normal flora on the skin, nose and respiratory systems of humans [2]. Since the bacteria have a low need to grow, it remains in the environment and can be easily transmitted to susceptible patients [3]. As a matter of fact, *Pseudomonas* has an outer membrane with low permeability, multidrug discharger pumps, lactamase and the outer membrane purine degradation set which could be the reason for the resistance of these microorganisms during the treatment [2]. Coding genes of antibiotic resistance are often transported by mobile genetic elements called integrons [1] that can be placed in plasmids, chromosomes or transposons. These elements are very important in the development of multiple drug resistance, such as plasmids and transposons. The overall structure of integrons, resistance genes are on determined gene cassettes. The transfer of resistance genes occurs due to the connection ability of cassette in the integron set during a specific recombination process [4]. At the end of 3' and 5' integrons, two nucleotide sequences are protected. Essential components of area 5 in all integrons consist of: 1. integrase gene that is site-specific for recombinase enzymes, 2. attI sequence is a specific recombination place located in the vicinity of *intI*, utilized as a receiver for the gene cassette, 3. The promoter is required for expression of available gene cassette, integrated between sector 3' and 5' integrons [5]. So far, more than 60 different genes cassettes have been identified that provide important arrangements concerning antibiotic resistance including aminoglycosides, cephalosporins and carbapenems. The role of these elements in the development of multiple resistance as well as resistance to a wide range of antibiotics, specifically antibiotics used in hospitals, makes it difficult to find appropriate treatment solutions and infection control tools [4]. Regarding the important role of this bacterium in nosocomial infections and the role of integron gene cassettes based on the transfer of antibiotic resistance, this study intended to trace the class I, II and III integrons in isolates of *P. aeruginosa* of nosocomial infection in order to determine the antibiotic resistance pattern of this bacterium.

Materials and Methods

In this cross-sectional study, 51 *P. aeruginosa* clinical isolates of infections were collected including infected wounds, bedsores, burns, urinary tract infections and respiratory infections being previously coordinated with patients of the hospitals located in Ahvaz. The isolates have been prepared in the hospital clinical laboratory identified by the biochemical tests [1]. The study samples were collected over a period of 5 months (from October 2014 to March 2015), which were transferred to the

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microbiology laboratory of Jahad Daneshgahi clinic of Ahvaz. The studied isolates were re-identified after the restoration and re-cultivation on the blood agar medium using biochemical tests such as Gram stain, catalase test and oxidase test [6, 7]. The bacteria grown in the TSP in a 1.5 ml micro-tubes in the rpm 9000 were deposited for 3 minutes, and the DNA was extracted according to the manufacturer's instructions (Fermentas, German Company). Agarose gel electrophoresis was used to assess the quality of extracted DNA from the analyzed samples. Biophotometer devices were used to DNA amount in the sample at a light wavelength of 280 nm. After extracting DNA using primers pair corresponding to the *nanI* gene (Table 1), *P. aeruginosa* isolates were confirmed by polymerase chain reaction (PCR) method [8].

Table 1. Primers sequencing related to detecting genes in *P. aeruginosa*

Gene	(Sequence) 3 '- 5'	Length(bp)	Refrence
NanI	F: ATGAATACTTATTTTGATAT R: CTAAATCCATGCTCTGACCC	228	8
GyrA	F: GTGTGCTTTATGCCATGAG R:GGTTTCCTTTTCCAGGTC	287	11
ParC	F:CATCGTCTACGCCATGAG R:AGCAGCACCTCGGAATAG	267	11
blaTEM	F: ATGAGTATTCAACATTTCCG R: CTGACAGTTACCAATGCTTA	867	12
β- ***blaSHV***	F: GGTTATGCGTTATATTCGCC R: TTAGCGTTGCCAGTGCTC	867	12
blaOXA	F: ACACAATACATATCAACTTCGC R: AGTGTGTTTAGAATGGTGATC	814	12
blaCTX-M	F: ATGTGCAGYACCAGTAARGT R: TGGGTRAARTARGTSACCAGA	593	12
blaDHA	F: CACACGGAAGGTTAATTCTGA R: CGGTTARACGGCTGAACCTG	970	12
blaVEB	F: CGACTTCCATTTCCCGATGC R: GGACTCTGCAACAAATACGC	642	12
IntI	F: 5'- CAGTGGACATAAGCCTGTTC-3' R: 5'- CCCGAGGCATAGACTGTA-3'	160	5
IntII	F: 5'- CACGGATATGCGACAAAAAG-3' R: 5'-GATGACAACGAGTGACGAAATG_3'	787	5
IntIII	F: 5'-GCCTCCGGCAGCGACTTTCAG_3' R: 5'-ACGGATCTGCCAAACCTGACT_3'	980	10

At all stages of PCR testing, the standard strains of *P. aeruginosa* (ATCC 27853) were used as a positive control. The thermal program applied for genes amplification consisted of initial denaturation stage (at 95ºC, 6 min.), one-cycle denaturation (at 95ºC, 45 seconds), the annealing (at 51ºC, 45 seconds), the extension (at 72ºC, 1 min.), step 2 to 4, 35 cycles were repeated, and then followed by a terminal extension at 72ºC for 7 min.). Primer

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pairs shown in table 1 were used in regard with detection of coding genes of class *I*, *II* and *III* Integrons in *P. aeruginosa* isolates. PCR reactions were performed in a volume of 25 µl. 10 µl of PCR products was mixed with 3 µl loading buffer for electrophoresis and was transferred to gel well. Electrophoresis of samples was conducted at a constant voltage of 90 volts for about 1 hour. After electrophoresis, the results were analyzed by gel transition to the gel reading devices (Gel documentation). The gel photo and its record on a heat-sensitive paper were used to interpret the study results [9], and antibiogram was performed using simple disc diffusion method (Kirby Baeur) according to CLSI tables (2012). *P. aeruginosa* isolates were grown in BHI medium at overnight, and then a density of culture equivalent to 0.5 McFarland dense was prepared to be presented to the Mueller Hinton solid medium in the presence of antimicrobial discs, including tetracycline (30 mg/disc), streptomycin (10 mg/disc), sulfamethoxazole (25 mg/disc), gentamicin (10 mg/disc), enrofloxacin (5 mg/disc), cephalothin (3 mg/disc), ciprofloxacin (5 mg/disc), trimethoprim (5 mg/disc), ampicillin (10 Iu/disk). After 24 hours of incubation at 37°C, bacterial sensitivity or resistance to antibiotics were determined and notified by measuring the diameter of growth inhibition around each disc. In this experiment, the standard strains of *P. aeruginosa* (ATCC 10145) were studied as a positive control in regard with determining the antibiotic susceptibility of isolates [10].

This study was approved by Ethics Committee of Islamic Azad University of Shahrekord branch. All ethical issues were considered, according to which this study was performed obtaining the permission of hospitals.

Statistical Analysis

In order to analyze the study data, SPSS software (ver.16) was utilized via Chi-square and Fisher exact tests. In fact, the relationship between the presence of class *III*, *II*, *I* integrons and antibiotic resistance in isolates was assessed based on the location of the bacteria at 95% confidence.

Results

The distribution and number of studied *P. aeruginosa* isolates in different nosocomial infections, 51 isolates were obtained from hospitals in Ahvaz containing 18 samples of purulent wounds, 13 samples of burning cases, 8 samples of bedsores, 7 samples of urinary tract infections and 5 samples of respiratory tract infections (sputum). All 51 isolates were identified in terms of molecular detection using *nanI* genes. Antibiotic susceptibility of studied isolates was related to 9 common antibiotics used in treating clinical infections in humans applying the simple disk diffusion method. All isolates were reported to have multiple antibiotic resistance (MDR). Tetracycline had the highest antibiotic resistance (84%) and enrofloxacin revealed the least resistance to the antibiotic (19.6%) (Table 2).

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Table 2. Antibiotic resistance pattern of *P. aeruginosa* isolates isolated from nosocomial infections in Ahvaz

Infection site	Number of isolates	TE30	S10	SXT	GM10	NFX5	CF30	CIP5	MP5	AM10
Infected wounds	18	15	13	8	8	4	5	3	7	6
Respiratory infections	5	2	3	3	3	2	3	1	2	-
UTI	7	7	4	3	1	1	2	3	3	2
Bedsore	8	7	3	1	2	1	1	2	3	3
Burn	13	12	7	5	7	2	2	2	4	5
Total	51	43	30	20	21	10	13	11	19	16

Table 3 demonstrates the distribution of coding genes regarding antibiotic resistance in *Pseudomonas aeruginosa* isolates. As it can be observed, almost all studied genes have been traced in different clinical infection isolates, among which bla_{TEM} gene with 94% frequency was the most common gene and *parC* gene with 9.8% frequency was the rarest coding gene of antibiotic resistance in these isolates. The statistical analysis carried out at 95% confidence detected a statistically significant difference between the presence of bla_{TEM} gene in *Pseudomonas aeruginosa* with other coding genes concerning antibiotic resistance (p=0.03). Since the integrons presence is one of the most important mechanisms of antibiotic resistance detection in *P. aeruginosa*, class *I*, *II* and *III* integrons of the bacteria was analyzed by PCR method. The highest incidence was related to Integrons *I* with a frequency of 92% and the lowest belonged to Integrons *III* with a frequency of 17% (Table 4). The study results revealed significant differences between class *I* and the other two integrons classes in the observed isolates (p= 0.04). As it is indicated in Table 3, except the respiratory tract isolates, each Integrons classes were presented in other studied isolates.

Table 3. Coding genes distribution of antibiotic resistance in *P. aeruginosa* isolates isolated from nosocomial infection

site of infection	Number of isolates	bla_{TEM}	bla_{SHV}	bla_{OXA}	$bla_{CTX\text{-}M}$	bla_{DHA}	bla_{VEB}	*GyrA*	*parC*
Infected wounds	18	17	4	1	2	2	1	2	1
Respiratory infections	5	4	2	2	1	1	-	1	-
UTI	7	7	3	1	3	1	-	2	1
Bedsore	8	8	1	3	3	2	2	3	1
Burn	13	12	3	2	4	5	7	4	2
Total	51	48	13	9	13	11	10	12	5

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Table 4. The integrons frequency in *P. aeruginosa* isolates isolated from nosocomial infection

Site of infection	Number of isolated	*IntIII*	*intII*	*intI*
Infected wounds	18	1	5	18
Respiratory infections	5	-	-	3
UTI	7	1	1	5
Bedsore	8	3	4	8
Burn	13	4	8	13
Sum	51	9	18	47

Discussion

P. aeruginosa is regarded as an opportunistic pathogen that contains the whole range of human infection. It is genetically resistant to many antibiotics that treating its infections is regarded rather impossible over time. In this regard, the *P. aeruginosa* is recognized as a multi-drug resistance bacteria. This study presents three main components aiming to trace the coding genes for antibiotic resistance, antibiotic susceptibility patterns and distribution of class *I*, *II* and *III* integrons in *P. aeruginosa* isolates isolated from nosocomial infection. Similar studies were conducted by Ren in 2012 in America [13], Cholly in 2011 in France [14], Taccone in 2012 in Brooklyn [15] as well as Taghvaei in 2013 in Iran [16]. In the present study, the most isolates were related to infected wounds and burns with a frequency of 35% and 25%, respectively. In the study of Zareei Yazdeli, the highest number of infections with *P. aeruginosa* was related to burns with 43.8% frequency rate [5]. Shahcheraghi (2009) reported 37% frequency of *P. aeruginosa* in the wound infections [17]. Moreover, Habib Babay (2006) conducted a study in Saudi Arabia, who managed to separate the most isolates from the wounds [18]. In the first part of this study, the antibiotic pattern of *Pseudomonas aeruginosa* was examined, which was found to have over 40% resistance to more than 5 antibiotics. Existence of this resistance may lie in the intractable consumption of some antibiotics including tetracycline. A study by Poonsuk was conducted in Thailand in 2012 demonstrating an increase in resistance of *Pseudomonas aeruginosa* strains. This study results revealed a resistance of 92.1%, 96%, 99% and 95% to amikacin, ceftazidime, gentamicin and ciprofloxacin respectively[19]. While the most resistance was related to streptomycin (84%) and tetracycline (58.8%), Fazeli (2012) showed that *P. aeruginosa* isolates were resistant to ciprofloxacin (29%) and gentamicin (32.2%), and in the present study, this figure dropped to 21% and 41%, respectively [20]. Ciprofloxacin is one of the strongest medications available for the treatment of infections caused by *P. aeruginosa*, specifically the treatment of

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urinary tract infections [21]. *P. aeruginosa* resistance to ciprofloxacin has been reported 26.8% in Latin America and 10-32% in Europe [22-25]. Regarding the second goal of this study, the presence of coding genes was studied in *P. aeruginosa* concerning antibiotic resistance. bla_{SHV} (25%), bla_{OXA} (17%), $bla_{CTX\text{-}M}$ (25%), bla_{DHA} (21%), bla_{VEB} (19%) and bla_{TEM} (94.2%) genes related to beta-lactam drugs as well as *gyrA* (23%) and *parC* (8.9%) genes related to fluoroquinolones were detected in the isolates. The findings of the studies conducted in Korea by Lee [26] and Kim (2004) [27], and by Luzzaro in Italy [28], reported MBL VIM as one of the most important MBLs within *P. aeruginosa* which was reported with higher frequency. Furthermore, in another study, Yu in Taiwan proposed that the most common beta-lactamase coding genes in *P. aeruginosa* were $bla_{SHV\text{-}5}$ and $bla_{SHV\text{-}12}$ genes [29]. In the present study, bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ genes with the frequency of 94%, 25% and 25% were the most common coding genes for antibiotic resistance that the presence of these genes is justified according to the pattern. Ultimately, integrons *I*, *II*, *III* of the isolates were studied, and the main three classes of integrons were detected with frequency of *I* (92%), *II* (35%) and *III* (17%). In a study conducted in 2010 by Yosefi, the prevalence of Integron gene *I* was reported 56.3% [30]. Moreover, Fonseca (2005) reported 41.5% [31], in China in 2009, it was reported 38% [32], and in the Gu study, it was reported 40.8% [33]. In a study by Shibata (2003) in Japan, integron *I* was reported to be more prevalent, whereas integron *III* was observed to be sporadic [34]. The prevalence of Integron *II* in the study of Keramati was reported 9% in 2014 in Zanjan [1]. Khosravi also reported, 5.3% in 2011 [35].

Conclusion

The findings of the present study revealed that all studied isolates which were MDR, had multiple antibiotic resistance and were generally separated from two important clinical infections of purulent wounds and burns. Phenotypic and genotypic evaluation of antibiotic resistance remarks *P.aeruginosa* resistance to the penicillin and tetracycline antibiotics and the presence of bla_{TEM} and bla_{SHV} genes. Almost all samples isolated from clinical infections had class *I*, *II* and *III* Integrons as one of the important mechanisms for acquisition and dissemination of antibiotics resistance mechanisms in the bacteria including *Pseudomonas aeruginosa*. Therefore, in state hospitals, it is essential to utilize management practices in order to optimize the use as well as of correct administration of antibiotics, preferably based on the results of antibiogram and tracking coding genes for antibiotic resistance.

Conflict of interest

The authors report no conflicts of interest.

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