

Study of routine antibiotic Resistance and detection of class 1 integrons in *Pseudomonas aeruginosa* in clinical isolates collected from Hadishar Hospital

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ABSTRACT

Pseudomonas aeruginosa is one of the most important causative agents of nosocomial infections especially in ICU and burn units. In recent years, there are increasing reports of multidrug resistant *P. aeruginosa* outbreaks in clinical settings worldwide class 1 integrons have been found to be the most prevalent in clinical isolates of *P. aeruginosa* that confer resistance to known antibiotics. The aim of this study was to determine the frequency of class 1 integron among multidrug resistant *P. aeruginosa* isolates. One hundred non duplicated clinical isolates were collected from Hadishahr hospital. All isolates were identified using standard laboratory methods. Antimicrobial susceptibility profiles were determined against the selected antimicrobials using the standard Kirby Bauer disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guideline. PCR assay was performed for detection class 1 integron. The chi-square test was used to determine the association between integron carriage and antimicrobial susceptibility patterns.

Results: Among one hundred isolates that studied, 62 isolates exhibited the MDR pattern. Thirty six (58%) of MDR isolates were found to have the class 1 integron. Analysis of data revealed a significant association between MDR pattern and presence of class 1 integron ($p < 0.001$). The results also showed that integron-positive isolates were statistically more resistant to aminoglycoside, quinolons and beta-lactam compounds. This study showed high prevalence of class 1 integron among *P. aeruginosa* isolated from our hospital settings. Considering the significant association between integron carriage and reduced susceptibility to variety of antibiotics, use of appropriate infection control strategy and a regular surveillance system is necessary to prevent further spread of infection by these organisms.

KEYWORDS: *P. aeruginosa*, Multidrug resistant, Integron class 1

INTRODUCTION

Currently, Multi-Drug Resistance *Pseudomonas aeruginosa* has been regarded as one of the main causes of nosocomial infections especially in intensive care units that diagnosis of these resistant organisms is of great importance in avoidance of development of drug resistance and treatment of patients especially those who have been hospitalized. The uncontrolled use of antibiotics to treat bacterial infections has caused selection of resistant strains, which unfortunately the risk of transmission of resistance genes from aforementioned strains to susceptible bacteria keeps increasing. Hall & Collis [1] diagnosed another mechanism of transmission of antibiotic resistance gene, fulfilled via other elements called "integrons". Integrons may be found as part of mobile genetic elements containing promoter which enable to integrate mobile genetic elements called with gene cassette and move them. With regard to placement of this type of resistance genes on integrons paving the way for rapid dissemination of these genes among other types, diagnosis of these integrons can provide useful information on amount of prevalence of resistant *Pseudomonas aeruginosa* strains, tracking dissemination and developing resistance.

Aims of this research include:

Determination of the relationship between the presence of class 1 integron and Multidrug Resistance Phenotype in *Pseudomonas aeruginosa* isolates collected from hospitals across Hadishahr

Xu et al. [2] conducted a research entitled "occurrence and characteristics of class 1 and 2 integrons in *Pseudomonas aeruginosa* isolates from patients in southern China" deducing that

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54(45.8%) among 118 clinical isolates were positive in sake of presence of class 1 integron. In following, it was specified that 93.2% of the isolates containing presence of class 1 integron showed multidrug resistance pattern. Yet, isolates lacking integron showed 18.2% amount of multidrug resistance. On the whole, a significant difference on multidrug resistance was seen between isolates having and lacking presence of class 1 integron, indicating important role of this mobile genetic element in dissemination of different drug resistance patterns in *Pseudomonas aeruginosa* isolates [1].

Chen et al. [3] conducted a research entitled "Identification and characterization of class 1 integrons among *Pseudomonas aeruginosa* isolates from patients in Zhenjiang, China" and specified that 38% of the isolates have been positive in sake of presence of class 1 integron. In following, it was specified that there is a significant relationship between presence of class 1 integron in isolates under study and resistant isolates to antibiotics ceftazidime, gentamicin, cefepime, tobramycin, amikacin, levofloxacin and ciprofloxacin.

Poonsuk et al. [4] conducted a research entitled "Class 1 integrons in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* isolated from clinical samples" and examined 101 isolates of *Pseudomonas aeruginosa* and 176 isolates of *Acinetobacter baumannii* in Thailand, and specified that 69.3% of isolates of *Pseudomonas aeruginosa* and 31.8% isolates of *Acinetobacter baumannii* have been positive in sake of presence of class 1 integron. In this study, presence of class 2 & 3 integron was not separated.

Fonseca et al. [5] conducted a research entitled "Class 1 integrons in *Pseudomonas aeruginosa* isolates from clinical settings in Amazon region, Brazil" and examined 101 clinical *Pseudomonas aeruginosa* isolates and specified that 44 isolates (41.5%) of *Pseudomonas aeruginosa* isolates have been positive in sake of presence of class 1 integron[4].

Peymani et al. [6] conducted a research entitled "Prevalence of Class 1 Integron among Multidrug-Resistant *Acinetobacter baumannii* in Tabriz" and specified that 80% of the isolates indicated multidrug resistance that 92.5% of them have been positive in sake of presence of class 1 integron. In following, it was specified that the isolate with presence of class 1 integron showed higher resistance than antibiotics including quinolones, beta-lactams, and aminoglycosides. In this study, a significant relationship was not observed between presence of class 1 integron and resistance to antibiotics including ampicillin and cefpodoxime, because all the isolates have been resistant to these two antibiotics [5].

RESEARCH METHOD

The population under study includes bacterial strains isolated from transmitted biological samples such as sputum, peritoneal fluid, urine, spinal fluid, and wound to laboratory of microbiology in hospitals across Hadishahr. Ratio estimate formula is used to estimate the sample size. Considering $\alpha=0.05$, prevalence of resistant isolates among the separated *Pseudomonas aeruginosa* isolates ($P=0.45$) and accuracy of 0.08, 100 positive *Pseudomonas aeruginosa* are considered. Separated *Pseudomonas aeruginosa* isolates will be kept in -70°C refrigerator.

The inclusion criteria include:

1-the transmitted samples which are positive in sake of growth of *Pseudomonas aeruginosa* isolates.

The exclusion criteria include:

1-the transmitted samples that their culture results in the bacteria than *Pseudomonas aeruginosa*

2-the patients that their transmitted samples appear repetitive

The present research was conducted on 100 clinical *Pseudomonas aeruginosa* samples. 100 clinical samples in sake of infection with *Pseudomonas aeruginosa* were collected from different sectors of hospitals across Hadishahr during 2014. The bacteria isolates were collected from clinical samples including blood, urine and urinary catheters, endotracheal, sputum and other clinical samples. The separated isolates in laboratory of hospitals were given on two environments including blood agar base and E.M.B Agar and were examined through the biochemical experiments pertaining to diagnosis of *Pseudomonas aeruginosa* after 24 incubation at temperature 35°C .

The biochemical tests which have been used to diagnose this organism include:

- 1- culturing on MacConkey agar (SMAC)
- 2- Gram staining
- 3- Oxidase Test
- 4- Culturing on Kligler Iron Agar (KIA)
- 5- Culturing on Cetrinide Aga

- 6- Fermentation/ oxidation test
- 7- Growth at 42 ° C.
- 8- Mobility Assessment Test
- 9- Citrate test

After identification of isolates to keep bacteria for a long term, firstly they have been cultured in vials containing Tryptic soy broth and then 2 drops of sterile 20% glycerol were added to them after 24 hours incubation at temperature 35°C under growth of bacteria, and then they were kept in -20°C refrigerator till doing the tests.

Determination of antibiotic susceptibility pattern via Disk Agar Diffusion

To do test, the instruction prevailing in The Clinical and Laboratory Standards Institute (CLSI) was used and conducted as follows [7].

1-firstly Müller-Hinton agar as a microbiological growth medium was prepared and PH was regulated between 7.2-7.4. These plates were incubated to control pollution for 24 hours at temperature 35°C.

2-at next stage, the containers containing antibiotic discs Gentamicin, amikacin, amoxicillin/clavulanic acid, piperacillin /tazobactam, Ticarcillin/clavulanic acid, cefepime, ceftriaxone, ceftazidime, imipenem, meropenem, ciprofloxacin and trimethoprim, Sulfamethoxazole were transmitted from refrigerator at -20°C to 4°C to do the test. Some minutes before doing test, the containers containing discs were placed at laboratory medium so as to reach to the temperature at room. Antibiotic discs were purchased from Mast Corporation Ltd.

3-at next stage, standard antimicrobial suspension was prepared to do test. Since the strains that their culture has not passed more than 24 hours are used to prepare suspension, the samples were cultured on simple gelose medium. Then they were incubated at temperature 35°C for 24 hours. An amount of colony was transmitted to the tube containing 2 ml Sterile Normal Saline, where a suspension was obtained after mixing it with mixer that the bacteria concentration equaled to half of McFarland concentration. Sterile Ear Suspension was spread on Müller-Hinton agar via cotton swab. 15 minutes after inoculated suspension, antibiotic discs which have been reached to room temperature were placed on plate to the distance of 1 cm from each other.

4-after placing disc, plates were incubated for 24 hours at temperature 35°C. Then, diameter of the zone for lack of growth around each disc was measured via ruler and the results were written down in the prepared forms. In this test, strain *E.coli* ATCC 25922 was used to control doing test.

Molecular study on prevalence of class 1 integron

To determine prevalence of class 1 integron, polymerase chain reaction (PCR) is used. In this method, internal control gene primer of class 1 integron was used that presence or lack of presence of class 1 integron will be specified through gene amplification and electrophoresis of products on agarose gel.

DNA extraction

The stages for DNA extraction from ESBL-producing *E.coli* strains via boiling method were conducted as follows:

1-Then, 3 to 5 colonies of each sample were solved into an Eppendorf reaction-vials (15008) 1.5 ml containing 200 µl distilled water.

2-the samples are shaken via Shaker to solve the colonies thoroughly.

3-vials were placed in Boiled Water Bath for 10-15 minutes, so that the boiling water surface spans two third of vial.

4-at this stage, vials are centrifuged for 5-10 minutes at round 14000, that Eppendorf Centrifuge was used at this stage.

5- The supernatant vials were transferred to sterile Eppendorf to do the reaction PCR.

6-at this stage, NanoDrop® devices at two wavelengths 280/260 nm were used after extraction.

Preparation of primers

1-firstly, primer sequences were delivered to Sinajen Company. Primers were received in lyophilization form after designing. To prepare the solution, the instruction below was considered:

1- primer sequence was used as follow:

IntF, 5'-ATCATCGTCTAGAGACGTCGG

IntR, 5'-GTCAAGTTCTGGACCAGTTGC (69)

2-at next stage, vials containing primer were placed at temperature 37°C for 0.5 hours.

3-at this stage, 100 μmol stock solutions was prepared and kept at temperature -20°C .
4-dependant on daily work to prepare solution, primers with concentration $10\mu\text{mol}$ from two gene strings were prepared and used for each series of PCR reactions.

Doing PCR test

At this stage, firstly matermix was prepared (Table 1). Then, amplification and detection of presence of class 1 integron via primers were come to realize. To do PCR reaction, final volume of each reaction has been 25 microliter. To obtain the best amount of the used compositions (Mgcl_2), a gradient of different amounts of these compositions was conducted during several reactions.

Table 1. optimal amount to prepare master mix for reaction PCR

compositions	Volume (ml)
DNTP mix 10 mmol	2
PCR Buffer 10 X	10
Mgcl_2 50 mmol	3
D.H ₂ O	73

Preparation of PCR reaction

Considering final volume of each PCR reaction which has been 25 ml, volume of primers, RNA polymerase and DNA must be added to master mix, represented in table 2 as follows:

Table 2. The required molecular materials to do PCR reaction

compositions	Volume (ml)
Master mix	22
DNA Template	1
Primer F	1
Primer R	1
Taq pol 5 u/ μl	0/25

Thermal cyclers planning

After putting vials in Thermal cycler, different temperature conditions and times at PCR reaction for genotype PER-1 were implemented as follow:

Table 3. The planning conditions in Thermal cycler at PCR reaction

	Temperature	Time
First denaturation	95	5 min
Cycle(30)	95	1 min
	55	sec 30
	72	1 min
Final extension	72	10 min

Electrophoresis of PCR products

For electrophoresis, 1% agarose gel was used on the PCR products. 1 gr agarose powder was dissolved in 100 cc 1X TBE Buffer and 1 microliter ($10\mu\text{g} / \text{ml}$) SYBR green was added to it after heating and cooling. After mixing, the solution was poured into the mold and then was extruded from the mold after shaping the gel and transferred into the electrophoresis tank. 7 microL of the PCR product was mixed with 3 microL of 6 X loading Buffer and placed in the gel wells for electrophoresis. For electrophoresis, voltage was regulated on 100 V, that when the sample passed third fourth of gel, gel was extruded from tank and observed with UV lamp. Under appropriateness of the bands undergoing electrophoresis, gel was observed with UVP device and a photo was taken from the gel.

Qualitative control

To assure from accuracy of test, *Acinetobacter baumannii* strain containing class 1 integron was used as positive control and *E. coli* ATCC 25922 was used as negative control. Further, to control doing test, microtubes containing reaction materials without DNA were used.

Overview of the relationship between presence of integrons and antimicrobial resistance

In following, this relationship was examined via chi-square test concerning the relationship between presence of integrons and antimicrobial resistance using software SPSS. P-value under 0.05 was considered significant statistically.

Conclusion

In the present research, 100 isolates of *Pseudomonas aeruginosa* strains after doing standard microbiological laboratory tests were collected (image 1-3). In doing oxidase test, standard strains of *Pseudomonas aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were used (Image 1).

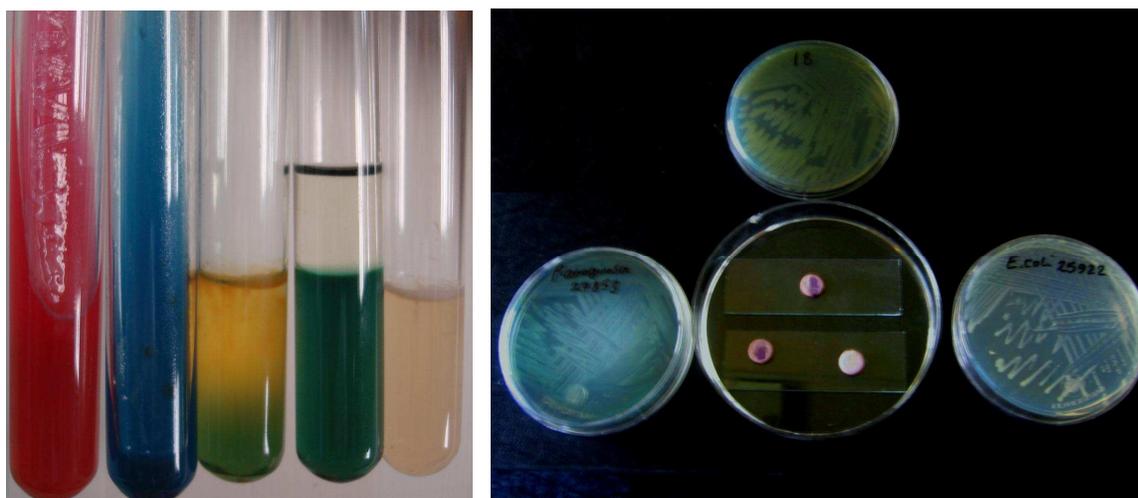


Image 1. Right side: result of oxidase test; left side: result of phenotypic identification tests

Clinical specimens were collected from patients hospitalized in ICU (44%), Hospital Internal Medicine Ward (28 %) , neurosurgery (10 %) , infection (10 %) , surgery (4 %) and Neurosurgery (4 %) , respectively. 38 isolates from blood samples, 17 isolates of endotracheal samples, 15 samples of urine, 12 isolates from wound samples, 7 isolates from sputum and 5 isolates from catheter, 6 isolates from cerebrospinal fluid were collected. In following, with overview of patients' information, it was specified that 58% and 42% of patients have been male and female, respectively.

Antibiotic susceptibility pattern

Through antibiotic susceptibility pattern via DAD method, it was specified that 62 isolates showed multiple drug resistance pattern and showed thorough drug resistance to antibiotic classes of Betalactams, Aminoglycosides, quinolones. 16 isolates showed thorough resistance to Imipenem, 12 isolates showed average resistance and 34 isolates showed susceptibility. Further, it was specified that 14 isolates showed thorough resistance to Meropenem, 13 isolates showed average resistance and 35 isolates showed susceptibility.

Molecular identification of class 1 integron

Doing PCR test via specific primers of class 1 integron on 100 isolates of *Pseudomonas aeruginosa*, it was specified that 41 isolates had class 1 integron. In following, it was specified that among 62 isolates of multidrug resistance pattern, 36 isolates had class 1 integron. Further, with regard to the results from chi-square test, a significant relationship was reported between presence of class 1 integron and multidrug resistance pattern.



Image class 1 integron gene PCR electrophoresis results

The relationship between antibiotic susceptibility pattern and presence of class 1 integron

In the present research, a significant relationship was reported between presence of class 1 integron and resistance to antibiotics Gentamicin, amoxicillin / clavulanic acid, cefotaxime, ceftriaxone, ceftazidime, Ticarcillin, imipenem, meropenem, cefpodoxime, aztreonam, piperacillin, amikacin, tetracycline, levofloxacin, trimethoprim - Sulfamethoxazole, ciprofloxacin, piperacillin/tazobactam. Results from this study indicated that isolates of *Pseudomonas aeruginosa* containing class 1 integron had a huge prevalence in hospital centers. With regard to the results from this study, 62% of isolates of *Pseudomonas aeruginosa* with multidrug resistance pattern separated from different sectors of hospital have been positive in sake of class 1 integron. Since isolates containing integron have been collected from ICU sector at hospitals under study, it is hoped to witness reduction of presence of these resistant organisms at different sectors of hospital via suitable instruments, use of proper antibiotic consumption pattern and minimization of use of β -lactam antibiotics especially cephalosporins.

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