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The Study of Frequency of *SIM* and *AmpC* Genes in Clinical Isolates of Pseudomonas aeruginosa in Gilan, Iran

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ABSTRACT

Aims: This study aimed to determine the antibiotic resistance pattern of *Pseudomonas aeruginosa* clinical isolates and the frequency of *blaSIM* and *blaAmpC* genes in resistant strains.
Materials & Methods: In this cross-sectional study, 94 *P. aeruginosa* isolates were collected from the burn wards of Gilan province hospitals in 2018 and identified by biochemical methods. Strains producing β-lactamases and metallo-β-lactamases were detected by two methods: disk diffusion method and antibiotic resistance method in combination with disk diffusion method, respectively. The presence of *blaSIM* and *blaAmpC* genes in the resistant strains was investigated using PCR, and data analysis was performed.
Findings: Based on the obtained results, colistin was identified as the most effective antibiotic with a resistance rate of 27.7%, and the highest antibiotic resistance was observed against trimethoprim/sulfomethoxazole (83%). In the phenotypic test of 94 samples, 29 (30.9%) carbapenemase-producing isolates and 33 (35.1%) β-lactamase-producing isolates were identified. Based on the PCR results, among 44 (46.8%) samples containing β-lactamase and carbapenemase enzymes, the frequency of *blaSIM* gene was 9.1% (4 of 44, and 4.3% in all the studied isolates), and the frequency of *blaAmpC* gene was 15.9% (7 of 44, and 7.4% in the all studied isolates).
Conclusion: The results of this study indicated a high prevalence of drug resistance in clinical isolates of *P. aeruginosa*. In particular, there was an increasing rate of resistance to beta-lactam antibiotics, and the presence of MBL and ESBL associated genes was considerable, which limit the choice of suitable treatment for patients with severe infections.

Keywords: *Pseudomonas aeruginosa*, Antibiotic resistance, PCR, *blaSIM*, *blaAmpC*.

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Introduction

Pseudomonas aeruginosa is a non-fermenting Gram-negative bacillus, knows as an important leading cause of infection in hospitalized patients, especially in patients with weakened immune system (including patients with burns and cystic fibrosis). Despite the improvement of health and therapeutic methods, the risk of *P. aeruginosa* infection is high in ICU patients [1]. The mortality rate of patients with *P. aeruginosa*-induced bacteremia has been reported to be 30-50%. This bacterium is an important pathogen that is more resistant to antibiotics than other Gram-negative bacteria. The intrinsic resistance of this bacterium is associated with its outer membrane with low permeability, it could also develop an acquired resistance to antibacterial agents that are typically effective on this pathogen [2-3].

Antibiotic resistance is usually based on one of the following mechanisms, and *P. aeruginosa* is not an exception: 1) the microorganism produces an enzyme that causes active drug degradation, 2) microorganisms change their permeability to the drug, 3) microorganisms change their necessary receptors for medication, and 4) microorganisms find another metabolic pathway that compensates for the drug-inhibited reaction [4]. One of the most important cause of antibiotic resistance is the production of β-lactamase enzymes [5]. The currently accepted method to classify β-lactamases is the classification based on their molecular structure and amino acids sequence. Four classes of these enzymes (A to D) and their functions have been identified. Groups A, C, and D have a serine-based function, while Group B enzymes are called metallo-β-lactamases (MBL_s) because of their need for zinc metal [6].

One of the most important factors contributing to the drug resistance of *P. aeruginosa* to carbapenem antibiotics is the production of metallo-β-lactamase (MBL) enzymes. This has been widely studied, and different involved genes and alleles have been identified [7].

Pseudomonas carbapenemase-encoding genes are a serious clinical threat. The genes encoding these enzymes, which are located in the plasmids, could be easily transmitted to other bacteria. MBLs have a broad spectrum and are not inhibited by β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam; on the other hand, they could hydrolyze all β-lactam antibiotics except monobactam and aztreonam. MBL enzymes could be divided into *UIM, SIM, AIM, GIM, SPM, or VIM* according to their molecular structure [8-9]. *SIM* has been isolated from *Acinetobacter baumannii* strains and is a subclass of B1. SIM is very similar to IMP (69% to ISP12 and 64% to IMP9) and has the ability to hydrolyze a broad spectrum of β-lactams. *SIM* is located on Class I Integrons. Identifying antibiotic resistance and identifying strains harboring different *AmpC* gene families, as well as determining the prevalence and emergence of new strains, could reveal the relationship between resistance to broad-spectrum antibiotics and the presence of some of these genes [10].

Objectives: The present study was carried out to detect the frequency of *SIM* and *AmpC* genes in clinical samples of MBL-producing *P. aeruginosa* strains isolated from burn wounds of patients hospitalized in the hospitals of Gilan province.

Materials and Methods

Isolation and Identification of Bacteria: In this descriptive cross-sectional study, conducted from August 2018 to January 2019, 94 *P. aeruginosa* clinical strains were isolated from burn wounds of patients hospitalized in the burn wards of Gilan

hospitals. The specimens were placed in plastic microtubes containing BHI transport medium (Merck, Germany) and transferred to the microbiology laboratory for complementary tests. They were cultured on MacConkey, TSI, and SIM environments (Merck, Germany). Biochemical tests including oxidase, catalase, urease, TSI, oxidation and fermentation (O/F), and growth ability of the bacteria at 42 °C were performed according to the standard detection table [11].

Determination of antibiotic resistance pattern of the isolates: Antibiotic resistance patterns of 94 *P. aeruginosa* isolates were determined by disk diffusion method according to the CLSI standard. Antibiotic discs used were as follows: ciprofloxacin (5 µg), colistin (10 µg), gentamicin (10 µg), imipenem (10 µg), cefotaxime (30 µg), ceftazidime (30 µg), kanamycin (30 µg), and trimethoprim/sulfamethoxazole (1.25/23.75 µg) (Padtan Teb).

The phenotype of β-lactamase and carbapenemase production in the isolates was ascertained by the combined disk diffusion method according to the CLSI procedure. Ceftazidime and ceftazidime + clavulanic acid were used to evaluate the strains ability to produce broad-spectrum β-lactamase, and imipenem and imipenem + EDTA discs were used to examine carbapenemase production. The standard strain of *P. aeruginosa* ATCC 27853 was used as a control. If the difference between the inhibition zone diameter of a single disc and a disc containing an inhibitor was equal to or greater than 5 mm, that strain was identified as enzyme producer [12].

Frequency of blaSIM and AmpC genes: To identify two *blaSIM* and *AmpC* genes, the bacterial genomic DNA was extracted using the Boiling method. Briefly, bacterial isolates were cultured in LB broth medium. After reaching an appropriate turbidity, 100 µL of the culture was mixed with 400 µL of TE buffer and boiled at 95 °C for 15 min and placed at -20˚C for 5 min. The vials were then centrifuged at 14000 × g for 10 min, and the supernatant was transferred from each vial to a new sterile microtube. For definitive confirmation of the extracted DNA, they were transferred onto 1% agarose gel and electrophoresed [13].

The amplification of *blaSIM* and *AmpC* genes was performed using specific primers (Table 1) and Polymerase Chain Reaction (PCR) method. PCR was performed in a total volume of 25 µL, containing 12.5 µL of PCR mixture, forward and reverse primers (10 pmol), template DNA (2 µL), and 12.5 µL sterile distilled water. The microtubes were then transferred to a thermocycler with an appropriate program described by Cayci et al. (2014) [14]. The PCR products were detected by electrophoresis on agarose gel 1%.

Findings

The antibiotic resistance pattern of 94 *P. aeruginosa* clinical isolates to 8 different antibiotics was as follows: trimethoprim/sulfamethoxazole 83%, cefotaxime 73.4%,

Table 1) Primers used for PCR detection of *SIM* and *AmpC* genes

Target Gene	Primer Sequence (5' → 3')	Product Size (bp)	Reference
blaSIM-F *blaSIM-R*	TAC AAG GGA TTC GGC ATC G TAA TGG CCT GTT CCC ATG TG	570	32
blaAmpC-F *blaAmpC-R*	CTT CCA CAC TGC TGT TCG CC TTG GCC AGG ATC ACC AGT CC	1063	26

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ceftazidime 35.1%, Kanamycin 57.4%, ciprofloxacin 50%, gentamicin 45.7%, imipenem 40.4%, and colistin 27.7%. Colistin was recognized as the most potent antibiotic against clinical isolates of *P. aeruginosa,* while the highest resistance was observed to trimethoprim/sulfamethoxazole (Table 2). In phenotypic assays, 30.9 and 35.1% of the isolates were positive for metallo-β-lactamases (MBL) and extended-spectrum β-lactamases (ESBL) production, respectively. Out of 44 MBL and/or ESBL producing strains examined in PCR assay, *blaSIM* and *blaAmpC* genes were identified in 4 (4 of 44, 9.1%; 4 of 94, 4.3%) and 7 samples (4 of 44,15.9%; 4 of 94, 7.4%), respectively (Figures 1 and 2).

Discussion

P. aeruginosa is one of the main leading causes of nosocomial infections and mortality in patients with leukemia (lymphoma), severe burns, and cystic fibrosis [15]. Due to its high drug resistance, this bacteria by itself accounts for 30% of all nosocomial infections and has made the treatment of these infections a serious problem in the health system [16].

MBLs are enzymes that confer bacterial resistance to Imipenem [15]. The over-use of antibiotics plays a key role in the emergence of MBL-producing strains [17]. Epidemiological studies around the world have demonstrated that drug resistance pattern of *P. aeruginosa* strains could be different from country to country, from one geographical area to another, and even between different hospitals in a geographical area.

Figure 1) Electrophoresis of PCR product for *blaSIM* gene. M: 100-bp DNA marker; 4: C- ; 5: C+; 1-3: the *blaSIM* gene PCR product, with an approximate length of 570 bp.

In this research, the antibiotic resistance

Table 2) Antibiotic susceptibility pattern of *P. aeruginosa* isolates.

Antibiotics	Resistant Number (%)	Intermediate Number (%)	Sensitive Number (%)
Cefotaxime	69 (73.4)	11 (11.7)	14 (14.9)
Ceftazidime	33 (35.1)	41 (43.6)	20 (21.3)
Ciprofloxacin	47 (50)	27 (28.7)	20 (21.3)
Colistin	26 (27.7)	60 (63.8)	8 (8.5)
Gentamicin	43 (45.7)	7 (7.5)	44 (46.8)
Imipenem	38 (40.4)	4 (4.3)	52 (55.3)
Kanamycin	54 (57.4)	25 (26.6)	15 (16)
Trimethoprim/ Sulfamethoxazole	78 (83)	2 (2.1)	14 (14.9)

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pattern of the isolates was as follows: trimethoprim/sulfamethoxazole 83%, cefotaxime 73.4%, kanamycin 57.4%, ciprofloxacin 50%, gentamicin 45.7%, imipenem 40.4%, ceftazidime 35.1%, and colistin 27.7%.

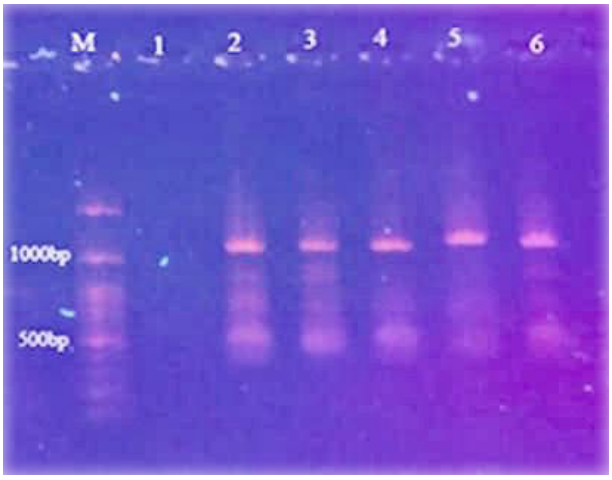

Figure 2) Electrophoresis of PCR product for *blaAmpC* gene. M: 100-bp DNA marker; 1: C- ; 2: C+; 3-6: the *blaAmpC* gene PCR product with an approximate length of 1063 bp.

Imani Foolad et al. (2010) in Zanjan reported the antibiotic resistance pattern of *P. aeruginosa* isolates as follows: tetracycline (86.4%), ceftriaxone (66.4%), cefotaxime (43.6%), gentamicin (25.5%), ceftazidime (20.9%), and amikacin (17.3%) [18]. In a study by Sadeghi et al. (2012) in Markazi province, the rate of resistance to imipenem was reported as 37%, of which 50% were MBL positive [19]. In another research by Taghvaee et al. (2013), examining 108 *P. aeruginosa* strains collected from Arak health centers, the resistance pattern was reported as follows: ceftazidime (33.3%), imipenem (22.2%), and meropenem (24%). Out of 36 ceftazidime-resistant strains, 32 (88.8%) strains were ESBL positive [20]. In a study in Ahvaz by Mardaneh et al. (2013), colistin exhibited the highest anti-*Pseudomonas* activity (78.3%), and there was a high resistance to beta-lactam antibacterials [21]. In another study by Rajaei (2017), out of 103 isolates studied, 26 (25.2%) strains showed high resistance to meropenem and imipenem, of which 19 (73%) isolates were MBL producer [22].

In all of these reports, the rate of resistance to the antibiotics used in this study (ceftazidime, cefotaxime, and gentamicin in Imani's study, imipenem in Sadeghi's study, and ceftazidime and imipenem in Taghvaee's study) was lower than that found in this research, which could be due to the fact that those studies were performed almost a decade ago. In the study conducted in Ahvaz, colistin was reported as the most effective antibiotic against *Pseudomonas*, consistent with this research result.

Sadri et al. (2010) reported high levels of antibiotic resistance in Tehran as follows: gentamicin (86%), ceftazidime (73%), amikacin (73%), and ciprofloxacin (55%) [23]; in their study, resistance to gentamicin, ceftazidime, and ciprofloxacin was higher than that found in this study. Their study results are not similar to this research findings, which could be due to factors such as statistical population, sample size, and type of diagnostic method used.

Radan et al. (2016) showed multi-drug resistance in 38% of the isolates and extensive drug resistance in 62% of the isolates from burns in Isfahan. The highest resistance rate was observed to amikacin, cefepime, ceftazidime, ciprofloxacin, imipenem, meropenem, and tobramycin (more than 90% of the isolates), while the resistance rate to aztreonam and piperacillin/tazobactam was reported as 86 and 70.7%, respectively. The most effective antibiotics were colistin and polymyxin B, and *blaIMP* gene was detected in 74.3% of the metallo-β-lactamase producing isolates [24]. Therefore, there is a high level of resistance to a variety of

antibiotics in Isfahan, and the prevalence of *blaIMP* gene is very high. However, colistin was found to be an effective antibiotic against *Pseudomonas*, consistent with the present study result.

Nikookar et al. (2013) showed the resistant rate of *P. aeruginosa* strains to imipenem to be 23.3% in Gilan province [25], which is less than the present study finding, suggesting further expansion of enzyme-producing strains in Gilan province over this period.

In this survey, out of 94 *P. aeruginosa* isolates, 44 strains were identified as β-lactamase and carbapenemase producers, among which 4 (4 of 44, 9.1%) samples were positive for *SIM* gene, and 7 (7 of 44, 15.9%) samples were positive for *AmpC* gene (4.3% and 7.4% in all the studied isolates, respectively).

Rafiee et al. (2014) reported the frequency of *AmpC* gene in all the studied isolates of *P. aeruginosa* to be 5% [26]. The frequency of *SIM* gene in 6771 samples was shown to be 14% by Alhaki et al. (2017) [27]. In another study, the frequency of *blaSIM* gene in 120 samples was determined as 17.1% by Haghi et al. (2017) [28]. Adabi et al. (2017) showed that out of 100 samples, 20 (20%) samples were positive for *AmpC* gene [29]. The frequency of *AmpC* gene in 114 samples was shown to be 85% by Tahmasebi et al. (2018) [30]. In the present study, the frequency of *SIM* and *AmpC* genes was reported as 4.3 and 7.4%, respectively. Except for Rafiee's study, the frequency of *SIM* and *AmpC* genes in this study was less than that reported in the previous studies. The discrepancy in this and other studies results could be due to geographical variation, so that even in some cases, variations in reports from various hospitals in the same city may be quite persistent and extensive [31]. It is recommended that further studies be performed to investigate the presence of other MBL coding genes and to determine the genetic origin of these genes in clinical isolates of *P. aeruginosa*.

Conclusion

This study results indicated a high prevalence of drug resistance in clinical isolates of *P. aeruginosa* collected from Gilan hospitals. In particular, there was an increasing rate of resistance to beta-lactam antibiotics, and the presence of MBL and ESBL associated genes was considerable. This limits the choice of suitable treatment for patients with severe infections.

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