



Original Article

# Genotypic Investigation of Antibiotic Resistant *blaOXA-4* Gene in Clinical Isolates of *Pseudomonas aeruginosa*

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**Abstract**

**Background:** *Pseudomonas aeruginosa*, an opportunistic Gram-negative bacterium, is responsible for 10-15% of hospital infections worldwide. The acquisition of resistance genes is one of the important mechanisms that causes the spread of resistance in this bacterium. This study aimed to conduct a phenotypic and genotypic investigation of the *blaOXA-4* resistance gene in *P. aeruginosa* isolated from clinical samples.

**Methods:** In this study, 110 *P. aeruginosa* strains were isolated from various clinical samples. The disk diffusion method was applied to reveal the resistance pattern in the isolates. Moreover, the combined disk method was used for the phenotypic analysis of extended-spectrum beta-lactamases (ESBL). Finally, the presence of the *blaOXA-4* beta-lactamase gene was analyzed genotypically by polymerase chain reactions (PCR) method.

**Results:** The highest sensitivity and resistance of the isolates were related to amikacin (65.45%) and ceftazidime (86.36%), respectively. The phenotypic analysis indicated that 72 isolates (65.45%) of *P. aeruginosa* are ESBL-producing. Furthermore, the presence of *blaOXA-4* was approved genotypically in 33 *P. aeruginosa* isolates (45.83%).

**Conclusion:** This study revealed a high prevalence of antibiotic-resistant isolates of *P. aeruginosa* in the East Azerbaijan population that may be associated with the presence of the *blaOXA-4* gene. However, further studies are necessary to identify other resistant genes in ESBL-producing isolates and other geographical areas with larger sample size.

**Keywords:** *Pseudomonas aeruginosa*, Antibiotic resistance, Extended spectrum beta-lactamase, *blaOXA-4* gene



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## Introduction

*Pseudomonas aeruginosa* is the most pathogenic member of the Pseudomonadaceae family that includes gram-negative, non-fermenting, obligatory aerobic, oxidase-positive bacilli with mobility and growth ability in various environments (1,2). This bacterium is responsible for serious infections (e.g., otitis, keratitis, endocarditis, septicemia, and pneumonia) in the world that cause high rates of mortality in patients with neoplastic disease, cystic fibrosis, and severe burns (3,4).

The spread of antibiotic-resistant strains of *P. aeruginosa* is the major cause of failure in infection control and the main reason for lethality in patients with defects in the immune system (5). This bacterium has an inherent resistance against various antiseptic and antimicrobial compounds that may be due to outer

membrane impermeability to antibiotic penetration by active transmission (6,7). Furthermore, *P. aeruginosa* can acquire drug resistance through the increased production of secretory pumps as well as the production of carbapenemase and beta-lactamase enzymes (6,7).

Beta-lactam antibiotics family with beta-lactam rings are the most common antibacterial compounds worldwide that include carbapenems, monobactams, cephalosporins, and penicillin (8). Some bacteria produce beta-lactamase enzymes that cause the destruction or inactivation of beta-lactam antibiotics via hydrolysis central core of the beta-lactam ring and antibiotic resistance as a result (9,10). The production of new and widely used antibiotics such as broad-spectrum cephalosporins has led to the emergence of extended-spectrum beta-lactamases (ESBL) as a new class of beta-lactamases enzymes (11). Evidence suggested



that Gram-negative bacteria, especially *P. aeruginosa*, encodes metallo- $\beta$ -lactamase enzyme by several transferable genes (12,13). Therefore, the continuous increase in the prevalence of antibiotic-resistant strains has become an important concern (14,15). In the Ambler classification scheme,  $\beta$ -lactamases of classes A, C, and D are serine  $\beta$ -lactamases. In contrast, the class B enzymes are metallo- $\beta$ -lactamases. Except OXA-type enzymes (which are class D enzymes), the ESBLs are of molecular class A. Moreover, class D enzymes such as OXA-type carbapenemases that can be encoded by chromosome or plasmid are proven to have a vital role in resistance to carbapenem (16,17).

Given the significance of increased antibiotic resistance in opportunistic microorganisms such as *P. aeruginosa* as well as the importance of resistance mechanisms knowledge to deal with infection by these bacteria, this study aimed to conduct a phenotypic and genotypic investigation of antibiotic resistance *bla*OXA-4 gene in clinical isolates of *P. aeruginosa* in East Azerbaijan population.

## Materials and Methods

### Collection of Isolates

We collected 110 *P. aeruginosa* isolates from different clinical sources such as burn wounds, tracheal tubes, urine, and blood samples of patients referred to Asadabadi hospital, Tabriz, Iran, during 2020-2021. Various standardized methods and biochemical analyses such as Gram staining, H<sub>2</sub>S production, urease production, **Voges-Proskauer**, hemolysis, catalase, oxidase, and indole were applied to identify the isolates. The approved isolates were then preserved by Tryptic Soy Broth (Merck, Germany) and 15% glycerol at -70 °C.

### Antibiotic Resistance Analysis

The antimicrobial-resistant pattern of the approved isolates was evaluated by the disk diffusion method as described by the Clinical and Laboratory Standards Institute. We used numerous antibiotic discs (Padtanteb, Iran), including gentamicin (10 µg), tobramycin (5 µg), ciprofloxacin (5 µg), amikacin (30 µg), ceftazidime (30 µg), piperacillin (100 µg), imipenem (30 µg), and cefepime (30 µg). Furthermore, *P. aeruginosa* ATCC 27853 was considered as the control strain (5).

### Phenotypic Detection of Extended-Spectrum $\beta$ -Lactamase-Producing Isolates

The combination disc method was applied for phenotypic detection of ESBL  $\beta$ -lactamases by cefotaxime-clavulanic acid (30 µg-10 µg), ceftazidime-clavulanic acid (30 µg-10 µg), cefotaxime (30 µg), and ceftazidime (30 µg) antibiotic discs (Padtanteb, Iran). The isolates with simultaneous resistance against at least 3 antibiotics were considered multidrug-resistant (MDR), while the isolates with  $\geq 5$  mm growth inhibition halo by combined discs were considered ESBL-positive as compared to growth inhibition halo by

alone discs (6).

### Genotypic Detection of Extended-spectrum Beta-lactamase-producing Isolates

The extraction of genomic DNA from the isolates was conducted by a specific kit (Invitex Stratec Business, Canada) according to instructions of the manufacturer. The polymerase chain reactions (PCR) method was applied for the genotypic detection of *bla*OXA-4 as a  $\beta$ -lactamases coding gene. Amplification was conducted in 25 µL total volume (12.5 µL master mixture, 1 µL each primer, 1 µL extracted DNA, and 9.5 µL sterile distilled water) followed by 1 cycle initial denaturation (95 °C for 5 minutes), 45 cycles denaturation (94 °C for 30 seconds), annealing (50 °C for 30 seconds), extension (72 °C for 1 minute), and 1 cycle final extension (72 °C for 5 minutes). The used primer sequence was forward 5'-ATGAAAAACACAATACATATC-3' and reverse 5'-TTATAAATTTAGTGTGTTTAG-3' with 830 bp product size. The amplified products were separated by electrophoresis on 2% agarose gel and photographed by gel document (Syngene, India), and *P. aeruginosa* ATCC 27853 was considered the control strain (9).

### Statistical Analysis

The obtained raw data were analyzed statistically by SPSS statistical software (version 16). The association between antibiotic resistance and the presence of the *bla*OXA-4 gene was evaluated by Fisher and chi-square ( $\chi^2$ ) tests. Moreover, the significance level was considered *P* value < 0.05.

## Results

### Bacterial Isolates

We collected 110 clinical isolates of *P. aeruginosa* out of 622 specimens identified by biochemical analysis. The clinical isolates of *P. aeruginosa* included 34 cases (30.90%) from wound samples, 26 cases (23.65%) from tracheal aspirate samples, 27 cases (24.55%) from urine samples, and 23 cases (20.90%) from blood samples.

### Antibiotic Resistance Pattern

The results of the disk diffusion method demonstrated that *P. aeruginosa* isolates are most sensitive to amikacin (65.45%) antibiotic and most resistant to ceftazidime (86.36%), ciprofloxacin (80.00%), and tobramycin (76.36%) antibiotics (Table 1).

### Phenotypically Detected Extended-Spectrum $\beta$ -Lactamase-Producing Isolates

The results of phenotypic detection of ESBL-producing *P. aeruginosa* demonstrated that out of 110 isolates, 72 cases (65.45%) are ESBL-positive. Moreover, out of 72 ESBL-positive isolates, 24 cases were from burn wound samples, 14 cases from tracheal aspirate samples, 17 cases from urine samples, and 17 cases from blood samples (Table 2).

**Table 1.** Antibiotic-Resistant Pattern of Clinical *Pseudomonas aeruginosa* Isolates

| Antibiotics   | Abbreviation | Dose (µg) | Resistance Patterns |                |             |
|---------------|--------------|-----------|---------------------|----------------|-------------|
|               |              |           | Sensitive           | Semi-sensitive | Resistant   |
| Ceftazidime   | CAZ          | 30        | 8 (7.27%)           | 7 (6.36%)      | 95 (86.36%) |
| Ciprofloxacin | CIP          | 5         | 20 (18.18%)         | 2 (1.81%)      | 88 (80.00%) |
| Tobramycin    | TOB          | 10        | 19 (17.27%)         | 7 (6.36%)      | 84 (76.36%) |
| Cefepime      | CEP          | 30        | 21 (19.01%)         | 6 (5.45%)      | 83 (75.45%) |
| Piperacillin  | PIP          | 100       | 36 (32.72%)         | 2 (1.81%)      | 72 (65.45%) |
| Imipenem      | IPM          | 30        | 51 (46.36%)         | 9 (8.18%)      | 50 (45.45%) |
| Gentamicin    | GEN          | 10        | 71 (64.54%)         | 3 (2.72%)      | 36 (32.72%) |
| Amikacin      | AMK          | 30        | 72 (65.45%)         | 5 (4.55%)      | 33 (30.00%) |

**Table 2.** Frequency of ESBL-Producing *Pseudomonas aeruginosa* Isolates

| Clinical Specimens | ESBL Positive | ESBL Negative | Total       |
|--------------------|---------------|---------------|-------------|
| Blood              | 17 (15.45%)   | 6 (5.45%)     | 23 (20.90%) |
| Urine              | 17 (15.45%)   | 10 (9.09%)    | 27 (24.55%) |
| Tracheal tube      | 14 (12.72%)   | 12 (10.90%)   | 26 (23.63%) |
| Burn wound         | 24 (21.83%)   | 10 (9.09%)    | 34 (30.90%) |
| Total              | 72 (65.45%)   | 38 (34.55%)   | 110 (100%)  |

Note. ESBL: Extended-spectrum beta-lactamases.

### Genotypically Detected Extended-Spectrum $\beta$ -Lactamase-Producing Isolates

The results of genotypic detection of ESBL-producing *P. aeruginosa* demonstrated that out of 72 isolates, 23 cases (31.95%) carried the *blaOXA-4* gene (Table 3). Additionally, the statistical analysis indicated that the presence of the *blaOXA-4* gene is associated with resistance to cefepime antibiotic (Table 4).

### Discussion

The high prevalence of *P. aeruginosa* with intrinsic resistance has led to the failure in the control and treatment of hospital infections by current antibacterial compounds. Therefore, the identification of antibiotic-resistant strains and various resistance factors provides a clear view for this problem (5). Resistant strains of *P. aeruginosa* commonly produce ESBL for the destruction of the beta-lactam chain (18). In this regard, the wide spread of ESBL-producing bacterial strains, especially *P. aeruginosa*, has increased hospital infections worldwide (19).

In the present study, we evaluated the phenotypic and genotypic resistance of 110 *P. aeruginosa* strains isolated from clinical samples such as burn wounds, tracheal aspirate, urine, and blood. The results revealed that the highest antibiotic resistance of *P. aeruginosa* is related to ceftazidime (86.36%), ciprofloxacin (80.00%), and tobramycin (76.36%), respectively, whereas the highest sensitivity of the isolates was related to amikacin (65.45%).

So far, numerous similar studies have been reported in different geographic areas in Iran. Fazeli et al in Isfahan reported that all clinical isolates (100%) of *P. aeruginosa*

**Table 3.** Frequency of the *blaOXA-4* Gene in ESBL-Producing *Pseudomonas aeruginosa* Isolates

| Clinical Specimens | Positive    | Negative    | Total       |
|--------------------|-------------|-------------|-------------|
| Blood              | 5 (6.95%)   | 12 (16.66%) | 17 (23.61%) |
| Urine              | 9 (12.50%)  | 8 (11.11%)  | 17 (23.61%) |
| Tracheal aspirate  | 5 (6.95%)   | 9 (12.50%)  | 14 (19.44%) |
| Burn wound         | 4 (5.55%)   | 20 (27.77%) | 24 (33.33%) |
| Total              | 23 (31.95%) | 49 (68.05%) | 72 (100%)   |

Note. ESBL: Extended-spectrum beta-lactamases.

**Table 4.** Association between Antibiotic Resistance and Presence of the *blaOXA-4* Gene in ESBL-Producing *Pseudomonas aeruginosa* Isolates

| Antibiotic    | Abbreviation | Resistant Isolates (n = 72) | Presence of <i>blaOXA-4</i> | P Value |
|---------------|--------------|-----------------------------|-----------------------------|---------|
| Ceftazidime   | CAZ          | 68 (94.44%)                 | 12                          | 0.871   |
| Ciprofloxacin | CIP          | 63 (87.50%)                 | 23                          | 0.412   |
| Tobramycin    | TOB          | 57 (79.16%)                 | 23                          | 0.217   |
| Cefepime      | CEP          | 55 (76.38%)                 | 50                          | 0.001   |
| Piperacillin  | PIP          | 54 (75.00%)                 | 22                          | 0.233   |
| Imipenem      | IPM          | 54 (75.00%)                 | 19                          | 0.791   |
| Gentamicin    | AMK          | 39 (54.16%)                 | 12                          | 0.118   |
| Amikacin      | GEN          | 38 (52.77%)                 | 20                          | 0.562   |

Note. ESBL: Extended-spectrum beta-lactamases.

are resistant to ticarcillin and ceftazidime (20). In another study, Ranjbar et al in Tehran demonstrated that all strains (100%) of *P. aeruginosa* isolated from burn wounds are MDR, and more than 90% of the isolates are resistant to imipenem and amikacin (21). As can be seen, the amount of resistance in the results of the two mentioned studies is greater than that in our results.

In two different studies, Salehi et al in Tehran and Mihani & Khosravi in Ahvaz reported that more than 70% of *P. aeruginosa* clinical isolates are resistant to ceftazidime (22,23). In another study, Fallah et al in Tehran reported that 83% of ESBL-producing *P. aeruginosa* strains isolated from wounds of burnt are resistant to imipenem (24). This rate of resistance in clinical isolates of *P. aeruginosa* is similar to neighboring countries and African and South American countries, including Pakistan and India, whereas it is significantly higher in North American and European countries (25,26). Differences in the results of various studies can be due to differences in sampling method and sample size. Moreover, differences in geographic area and public health level may be associated with the rate of resistance in *P. aeruginosa* isolates.

The phenotypic analysis revealed that 65.45% of the *P. aeruginosa* isolates are ESBL-producing strains which is considered a high ratio compared with other geographic areas in Iran. In three different studies in Iran, Mirsalehian et al, Shahcheraghi et al, and Shakibaie et al have reported that the frequency of ESBL-producing clinical isolates of *P. aeruginosa* is 40%, 39%, and 34%, respectively (27-29). The excessive use of broad-spectrum cephalosporins in our province (East Azarbaijan) may be an important

reason for the higher frequency of ESBL-producing isolates of *P. aeruginosa*.

*Pseudomonas aeruginosa* uses numerous mechanisms for the acquisition of drug resistance such as the production of efflux pumps and low membrane permeability. Therefore, the phenotypic identification of ESBL-producing isolates may present false results. In this regard, the molecular analysis of beta-lactamase genes is a precise method for the detection of ESBL-producing isolates of *P. aeruginosa*. So far, numerous types of beta-lactamase genes have been identified in *P. aeruginosa* isolates, including *VEB*, *TEM*, *GES*, *PER*, *SHV*, *CTX*, and *OXA* (3).

Furthermore, the genotypic analysis indicated that the frequency of the *blaOXA-4* gene in the detected ESBL-producing isolates of *P. aeruginosa* is 45.83%. Interestingly, it was found that the presence of the *blaOXA-4* gene is significantly associated with resistance to cefepime. In a study in Hamadan, Iran, Sezadehghani et al identified frequency carbapenem encoding genes (*OXA*), including *blaOXA-145* (27.5%), *blaOXA-224* (22.0%), *blaOXA-539* (20.1%), and *blaOXA-675* (11.9%) in clinical isolates of *P. aeruginosa*, which exhibited MDR (30). In a study by Radmehr et al in North Khorasan, Iran, the frequency of *blaOXA-23* gene was reported 61.42% (31). In another study, Bahrami et al reported that the frequency of the *blaOXA-48* gene is 12.5% in clinical isolates of *P. aeruginosa* in Bandar-Abbas, Iran (32). The beta-lactamase encoding genes are transferable between various bacterial strains, which can be a cause of the high prevalence of these genes in clinical isolates of *P. aeruginosa* in Iranian patients.

In addition to the small sample size of the present study, we only investigated the association between the *blaOXA-48* gene and antibiotic resistance of clinical *P. aeruginosa*. Moreover, we isolated *P. aeruginosa* strains from a limited type of clinical samples. **In addition, the investigation of ESBL-producing isolates of *P. aeruginosa* from other sources such as foods .**

Generally, the present study demonstrated that 65.45% of clinical isolates of *P. aeruginosa* are phenotypically ESBL producers, 45.83% of which carry the *blaOXA-4* gene. Furthermore, we suggest that the *blaOXA-4* gene may be associated with the resistance of *P. aeruginosa* isolates to cefepime antibiotics. Therefore, phenotypically and genotypically detection of ESBL-producing isolates of *P. aeruginosa* with MDR can be useful in the application of appropriate antibiotics and as a result a better management of hospital infections.

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#### Authors' Contribution

**Conceptualization:** Reza Shapouri.

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**Funding Acquisition:** Milad Gholampour Matin.

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#### Competing Interests

There is no conflict of interests as stated by the authors.

#### Ethical Approval

Not applicable.

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