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Original Article

Evaluation of Antibiotic Resistance Pattern and Extended-Spectrum Beta-lactamases in *Pseudomonas aeruginosa* Isolates Obtained from Clinical Samples by Phenotypic and Genotypic Methods in Zabol, Iran

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Abstract

Background: *Pseudomonas aeruginosa* is a human opportunistic pathogen that is known to be responsible for various diseases. However, its antibiotic-resistant isolates often cause serious infections.

Methods: This study for the first time investigated a total of 80 *P. aeruginosa* isolates collected from patients admitted to Amir Al-Momenin hospital. The isolates were identified by biochemical assays. The combination disc test method was used to measure antibiotic susceptibility and confirm the presence of extended spectrum-beta lactamases-producing enzymes. Also, the presence of enzyme-producing genes *bla CTXM-1, bla CTXM-2, bla CTXM-3, bla SHV, and bla OXA* of the target enzymes was examined using *polymerase chain reaction*.

Results: Out of 80 *P. aeruginosa* isolates, 32 isolates (40%) were beta-lactamase generators. Resistance to the studied antibiotics was found to be 97.5%, 90%, 81.3%, 75%, 75%, 72.5%, 60%, 52.5%, 50%, 32.5%, 28.8%, and 0% for amoxicillin, amoxiclav cephalexin, nitrofurantoin, cotrimoxazole, azithromycin, ceftriaxone, cefotaxime, gentamicin, ceftazidime, ciprofloxacin, and imipenem, respectively. Therefore, the highest antibiotic resistance was against amoxicillin, co-amoxiclav, and cephalexin, respectively, while the lowest was detected for imipenem. Besides, 17.5% of the studied isolates were multidrug-resistant (MDR). Among extended-spectrum beta-lactamases-producing genes, *bla CTXM-3* displayed the highest frequency of 84.4%.

Conclusion: The findings demonstrated the wide resistance of *P. aeruginosa* isolates against various antibiotic classes. According to the results, it is suggested to identify different patterns of antibiotic resistance of *P. aeruginosa* isolates prior to the onset of treatment for any *P. aeruginosa*-related infections.

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

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Introduction

Pseudomonas aeruginosa is an opportunistic, aerobic, gram-negative, polar-flagella bacterium that is characterized by pili and exotoxin and is the third leading cause of nosocomial infections and the second leading cause of burn wound infections (1, 2). Excessive use of antibiotics in recent years has made this bacterium resistant to various broad-spectrum antibiotics from different groups, so the existence of multidrug-resistant (MDR) strains is currently the main problem in the treatment of related infections (3). This bacterium is present in main hospital wards, such as burn and intensive care units. The reduction of outer membrane permeability, production of chromosomal beta-lactamase, and changes in peritoneal systems are the main causes of the inherent resistance of this pathogen against antibiotics (4,5).

This bacterium has a strong secretory system and hydrolyzes antibiotics by reducing the absorption of drugs from the outer membrane and producing various enzymes (6). One of the mechanisms of antibiotic resistance in this bacterium is the production of beta-lactamases, which can inactivate many beta-lactam antibiotics, such as penicillin, cephalosporins, and carbapenem by hydrolysis of the central nucleus. The emergence of new antibiotics, such as broad-spectrum cephalosporins, and their widespread use for the treatment of infections has led to the rise of

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a new class of these enzymes called extended-spectrum beta-lactamases (ESBLs) (6,7). ESBLs are enzymes that inactivate beta-lactam antibiotics by opening the amide bond of their beta-lactam rings. ESBLs are commonly encoded on plasmids that are resistant to cephalosporins, including ceftazidime, cefotaxime, and ceftriaxone. Beta-lactamases are inhibited by beta-lactam inhibitors, such as sulbactam, tazobactam, and clavulanic acid (8,9). Beta-lactam agents such as penicillins, cephalosporins, monobactams, and carcinogens are among the most widely prescribed antibiotics (10).

The activity of in vitro class A enzymes is inhibited by beta-lactam inhibitors, such as clavulanic acid, sulbactam, and tazobactam (11,12). Beta-lactamases are inhibited by beta-lactam inhibitors such as clavulanic acid, sulbactam, and tazobactam. The high resistance of *P. aeruginosa* to various antibiotics makes it difficult to treat *P. aeruginosa* infections. Besides, the antibiotic resistance patterns of *P. aeruginosa* strains isolated from clinical specimens vary with the geographical region around the world.

The present research aimed to determine the antibiotic resistance pattern and frequency of the wide-spectrum beta-lactamases in the *P. aeruginosa* strains isolated from clinical specimens by phenotypic and genotypic methods in the Sistan region, Iran.

Methods

Sample collection and storage

The *P. aeruginosa* specimens were collected from the clinical urine samples of the patients admitted to hospitals in Zabol over a six-month period. The samples were transferred to the laboratory at the university and their identities were determined again. The confirmed samples were stored at -70°C and 3-4 colonies of the bacteria were transferred into micro-tubes filled with 1.5 µL of Müller-Hinton broth containing glycerol 20% with a sterilized loop. Then, the micro-tubes were kept at 42°C for 18 hours. After the samples became blurred due to the bacteria growth, they were first kept at 4°C for some hours to prevent cold shock and then stored at -70°C.

Purification and identification of P. aeruginosa isolates

The microorganisms were streaked on the MacConkey agar and pseudomonas agar media with a loop. They were then incubated at 42°C for 24 hours after which they were checked for the fruit odor (grape) and the production of blue-green pigments. Next, they were subjected to diagnostic tests of oxidase, catalase, OF, and SIM to confirm the existence of *P. aeruginosa* and their single colony on the samples (13,14).

Investigation of antibiotic susceptibility pattern

The antibiotic resistance pattern of the *P. aeruginosa* isolates was determined with the Antibiogram (disc diffusion) test, which is the most common test used to study antibiotic resistance on agar. The test was conducted based on diffusion in the disc and according to the guideline of the National Committee of Clinical Laboratory Standards (14), using 12 antibiotic discs (made by ROSKO) including ceftazidime (30 µg), ciprofloxacin (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), co-trimoxazole (30 µg), cephalexin (30 µg), azithromycin (30 µg), gentamicin (30 µg), imipenem (30 µg), amoxiclav (30 µg), amoxicillin (30 µg), nitrofurantoin (30 µg), and ceftazidime/clavulanic acid (CAZ: 30 µg/CV: 10 µg).

Confirmation of ESBL-producing organisms among screened strains using a phenotypic method

The target organisms were cultured on Müller-Hinton agar and investigated by the CMD method. Finally, the results were interpreted according to the CLSI guidelines. In the disc diffusion, a combination of ceftazidime antibiotics and clavulanic acid was used on the Müller-Hinton agar. Beta-lactamase generator is considered extended-spectrum if the diameter of the non-growth halo around the discs containing the composition of antibiotic with clavulanic acid is 5 mm larger than the diameter of the non-growth halo around the corresponding disc (9).

Polymerase chain reaction (PCR)

DNA was extracted from the *P. aeruginosa* isolates by boiling method. Then, the PCR technique was performed with specific primers on the ESBL genes, including *bla CTXM-1, bla SHV, bla CTXM-2, bla CTXM-3, and bla OXA.*

Data analysis

The collected data were entered into the SPSS (ver. 18) software package. They are tabulated in the paper. They were also subjected to the χ^2 test at the $P < 0.05$ level.

Results

Antibiotic resistance pattern

Among the 80 samples studied, the highest antibiotic resistance was related to amoxicillin (97.5%), amoxiclav (90%), and cephalexin (81.3%). The lowest antibiotic resistance was attributed to imipenem (0%), ciprofloxacin (28.8%), and ceftazidime (32.5%) (Table 1).

Based on the combination method disk (CMD) test, the frequency of antibiotic resistance in ESBL samples is 32 (40%). The highest resistance is to amoxicillin (93.8%), amoxiclav (90.6%), and cephalexin (90.6%), and the lowest to imipenem (0%), ciprofloxacin (46.9%), and nitrofurantoin (53.1%) (Table 2).

As per the CLSI guideline, the isolates that were resistant to at least three antibiotics from different families were considered MDR. Among all isolates, 14 isolates (17.5%) were identified as MDR. In this group (ESBL isolates based on the CMD test) the highest rate of resistance to the antibiotic was 100%, attributed to amoxicillin, amoxiclav, gentamicin, ciprofloxacin, and cephalexin.

The lowest rate of resistance in ESBL isolates based on the CMD test was related to imipenem (0%), ceftazidime (35.7%), and nitrofurantoin (57.1%) (Table 3).

Among the studied MDR isolates, 92.9% had ESBL whereas 7.1% did not, showing a significant relationship ($P=0.000$). Among the ESBL isolates, the number of the MDR isolates (amoxicillin + gentamicin + ciprofloxacin) was 13 (40.6%); also, the results revealed that the frequency distribution of broad-spectrum beta-lactamases was 13 (92.9%) among the ESBL isolates. The frequency of the MDR isolates (amoxicillin + gentamicin + ciprofloxacin) among the ESBL isolates was 13 (40.6%) according to the CMD test, and the frequency of the MDR isolates (amoxicillin + gentamicin + ciprofloxacin) among all isolates was 14 (17.5%).

Frequency of ESBL encoding genes

The genes *bla SHV, bla OXA, bla CTXM-1, bla CTXM-2, and bla CTXM-3* were identified in the wide-spectrum beta-lactamase-generating strains using specific primers and the PCR method. Based on the results, 15.6% of the ESBL isolates contain *bla SHV, bla OXA, bla CTXM-1, bla CTXM-2, and bla CTXM-3.* Among the isolates, *bla CTXM-3* had the highest frequency rate (84.4%) (Table 4 and Figure 1).

The comparison between the MDR and non-MDR groups in terms of the frequency of the ESBL encoding genes showed that 85.7% of the MDR isolates contain the CTXM-3 gene, whereas only 62.5% of the non-MDR isolates have this gene. According to the statistical analysis, this difference is not significant at the 95% confidence level, but it is significant at a lower confidence level (about 88%).

The two above-said groups did not significantly differ in resistance to azithromycin ($P=0.6$), amoxicillin ($P=0.157$), cephalexin ($P=0.280$), and amoxiclav; however, significant differences were observed between these two groups in terms of resistance to gentamicin ($P=0.000$), ciprofloxacin ($P=0.000$), cefotaxime ($P=0.000$), ceftazidime ($P=0.000$), ceftriaxone ($P=0.017$), nitrofurantoin ($P=0.000$), and cotrimoxazole ($P=0.033$). The comparison of the two groups revealed that 84.4% of the ESBL isolates were resistant to gentamicin, whereas only 27.1% of the non-ESBL isolates were resistant to this antibiotic. Also, 46.9% of the ESBL isolates were

Table 1. Frequency distribution of antibiotic resistance of the studied *Pseudomonas aeruginosa* isolates

Antibiotic	Resistant No. (%)	Semi-sensitive No. (%)	Sensitive No. (%)
Azithromycin	58 (72.5)	0 (0)	22 (27.5)
Amoxicillin	78 (97.5)	0 (0)	2 (2.5)
Amoxiclav	72 (90)	6 (7.5)	2 (2.5)
Gentamicin	40 (50)	14 (17.5)	26 (32.5)
Ciprofloxacin	23 (28.8)	13 (16.3)	44 (55)
Cefotaxime	42 (52.5)	38 (47.5)	0 (0)
Imipenem	0 (0)	15 (18.8)	65 (81.3)
Cephalexin	65 (81.3)	15 (18.8)	0 (0)
Ceftazidime	26 (32.5)	4 (5)	50 (62.5)
Ceftriaxone	48 (60)	5 (6.3)	27 (33.8)
Cotrimoxazole	60 (75)	15 (18.8)	5 (6.3)
Nitrofurantoin	60 (70)	5 (6.3)	15 (18.8)

Table 2. The frequency distribution of antibiotic resistance among the ESBL isolates based on the CMD test

Antibiotic	Resistant No. (%)	Semi-sensitive No. (%)	Sensitive No. (%)
Azithromycin	24 (75)	0 (0)	8 (25)
Amoxicillin	30 (93.8)	0 (0)	2 (6.3)
Amoxiclav	29 (90.6)	2 (6.3)	1 (3.1)
Imipenem	0	10 (31.3)	22 (68.8)
Gentamicin	27 (84.4)	1 (3.1)	4 (12.5)
Cotrimoxazole	24 (75)	4 (12.5)	4 (12.5)
Ciprofloxacin	15 (46.9)	8 (25)	9 (28.1)
Cefotaxime	28 (87.5)	0 (0)	4 (12.5)
Cephalexin	29 (90.6)	0 (0)	3 (9.4)
Ceftriaxone	26 (81.3)	0 (0)	6 (18.8)
Ceftazidime	18 (56.3)	3 (9.4)	11 (34.4)
Nitrofurantoin	17 (53.1)	0 (0)	15 (46.9)

Table 3. The frequency distribution of antibiotic resistance among the MDR isolates of *Pseudomonas aeruginosa*

Antibiotic	Resistant No. (%)	Semi-sensitive No. (%)	Sensitive No. (%)
Azithromycin	11 (78.6)	0 (0)	3 (21.4)
Amoxicillin	14 (100)	0 (0)	0 (0)
Amoxiclav	14 (100)	0 (0)	0 (0)
Gentamicin	14 (100)	0 (0)	0 (0)
Ciprofloxacin	14 (100)	0 (0)	0 (0)
Cefotaxime	11 (78.6)	0 (0)	3 (21.4)
Ceftazidime	5 (35.7)	2 (14.3)	4 (50)
Ceftriaxone	13 (92.9)	0 (0)	1 (7.1)
Cotrimoxazole	12 (85.7)	1 (7.1)	7 (7.1)
Cephalexin	14 (100)	0 (0)	0 (0)
Imipenem	0 (0)	4 (28.6)	10 (71.4)
Nitrofurantoin	8 (57.1)	0 (0)	6 (6.42)

Table 4. The frequency distribution of the ESBL encoding genes among the ESBL isolates

Gene	Number (%)
OXA	22 (68.8)
SHV	15 (46.9)
CTXM-1	17 (53.1)
CTXM-2	0
CTXM-3	27 (84.4)

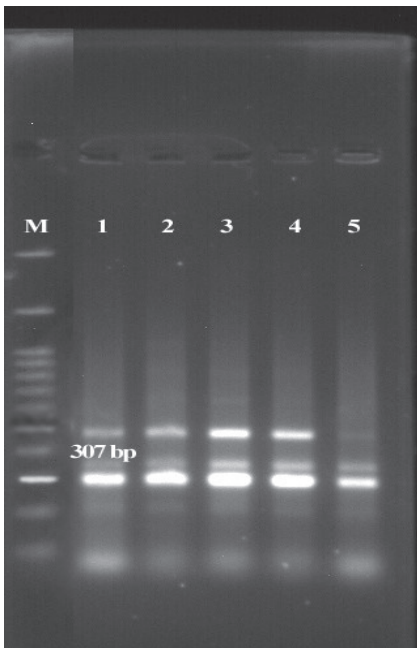

Figure 1. Image of the agar gel related to *bla CTXM-3* (each band is 307 bp in size and the bands are related to isolates 1, 2, 3, 4, and 5)

resistant to ciprofloxacin, whereas only 16.7% of the non-ESBL isolates were resistant to it. Among the ESBL and non-ESBL isolates, 87.5% and 29.2% showed resistance to cefotaxime, respectively. These figures were 56.3% and 16.7% for ciprofloxacin, respectively. Furthermore, 81.3% of the ESBL isolates were found to be resistant to ceftriaxone, while only 52.1% of the non-ESBL isolates were resistant to this antibiotic. Interestingly, the non-ESBL isolates were more resistant to nitrofurantoin and cotrimoxazole than the ESBL isolates (89.6% vs. 53.1% for nitrofurantoin and 79.2% vs. 75% for cotrimoxazole) (Figure 2).

No statistically significant differences were observed between the MDR group and the other isolates in terms of resistance to azithromycin ($P=0.155$), amoxicillin ($P=0.679$), cephalexin ($P=0.103$), ceftazidime (P = 0.182), cotrimoxazole ($P=0.511$), and amoxiclav ($P=0.443$), whereas the two groups differed significantly concerning resistance to gentamicin ($P=0.000$), ciprofloxacin ($P=0.000$), cefotaxime ($P=0.030$), ceftriaxone ($P=0.043$), and nitrofurantoin ($P=0.030$). The comparison of these groups revealed that all MDR isolates were resistant to gentamicin, whilst only 39.4% of the non-MDR isolates were resistant to this antibiotic. Also, 100% of the MDR isolates showed resistance to ciprofloxacin, but only 13.6% of the non-MDR isolates were resistant to his antibiotic. Similarly, 78.6% of the MDR isolates and 47% of the non-MDR isolates were found to be resistant to cefotaxime. In the case of ceftriaxone, 92.9% of the MDR isolates and 57.6% of the non-MDR isolates showed resistance. The resistance to nitrofurantoin was 57.1% among the MDR isolates and 78.6% among the non-MDR isolates (Figure 3).

The comparison of the frequency of ESBL encoding genes between the ESBL and non-ESBL groups showed that there were mostly no statistically significant differences except for *CTXM-3* ($P=0.012$). Nearly 84.4% of the ESBL isolates have this gene whereas only 58% of the non-ESBL isolates have it. It should be noted that CTXM-2 was not found in any of the isolates. The highest frequency of encoding genes among the MDR isolates was 85.7%, related to *bla CTXM-3*.

Discussion

To determine the antibiotic resistance and the frequency of wide-spectrum beta-lactamases, the research focused on 80 isolates of *P. aeruginosa* isolated from clinical samples in the Sistan region. The antibiotics used in the research included penicillin (amoxicillin and amoxiclav), cephalosporins (cephalexin, cefotaxime, ceftazidime, ceftriaxone, and ceftazidime + clavulanic acid), carbapenems (imipenem), aminoglycosides (gentamicin), azalides (azithromycin), cotrimoxazole, quinolones (ciprofloxacin), and nitrofurantoin.

The results show that *P. aeruginosa* is the most resistant to amoxicillin, amoxiclav, and cephalexin and the least resistant to imipenem, ciprofloxacin, and ceftazidime. Also, the highest frequency of the wide-spectrum beta-

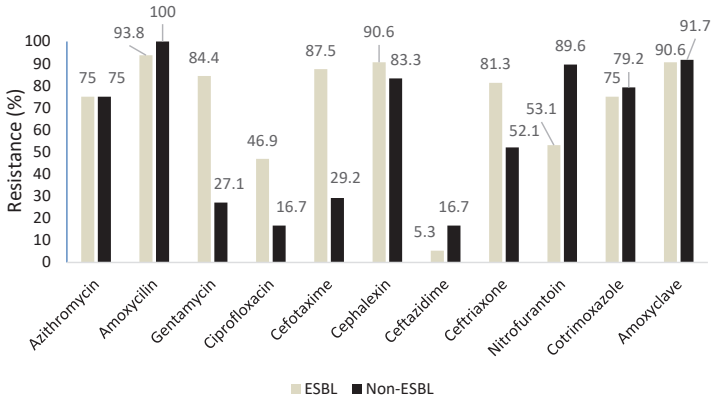

Figure 2. The frequency of antibiotic resistance in two groups of wide-spectrum beta-lactamase generating groups and other isolates

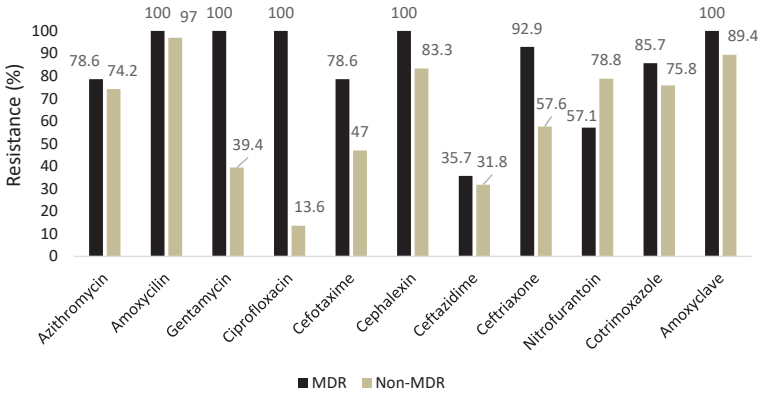

Figure 3. The frequency of antibiotic resistance in multi-drug resistant isolates and other isolates

lactamases-encoding genes in the *Pseudomonas* isolates is related to *bla CTXM-3* and the lowest to *bla CTXM-2.*

Since antibiotics such as gentamicin, amoxicillin, amoxiclav, ciprofloxacin, and cephalexin are widely used to treat various infections, and some are even prescribed in other cases, including veterinary and animal husbandry; therefore, the high resistance of MDR isolates to them is not surprising.

Elahi et al conducted a study on determining the prevalence of MDR, XDR, and PDR phenotypes among *P. aeruginosa* isolates collected from hospitalized patients. They reported that among the 80 isolates, the highest rate of specimens (58.75%) was collected from the intensive care unit. The highest antibiotic resistance was observed to ticarcillin/clavulanate (95%), meropenem (80%), and ceftazidime (75%). The antibiotics of piperacillin-tazobactam and cefepime had the highest inhibitory effect on the isolates so the sensitivity rates to them were 5.57% and 25.51%, respectively. Among the isolates, 75.88% were MDR strains and 25.46% were XDR strains. No PDR phenotype was detected among the clinical strains (15).

Shahraki Zahedani et al examined 200 *P. aeruginosa* isolates and found that strains isolated from urine (54%) and blood (23.5%) were the most frequent. The highest resistance level was seen against ciprofloxacin (37%) and the lowest against piperacillin-tazobactam and ceftazidime, with percentages of 6.5% and 6%, respectively. Furthermore, all strains were sensitive to colistin (16).

Beig et al reported that the highest level of resistance was found against ceftriaxone (63.63%) and the lowest against piperacillin (33.33%) (17).

Khosravi et al, in a study in Ahvaz, Iran, investigated the frequency of class 1 and 2 integrons in 90 *P. aeruginosa* isolates, collected from different clinical samples including blood, urine, lesion, and biopsy. They found that most isolates were highly resistant to gentamicin (94.6%) and ciprofloxacin (93.6%) (18).

Salehi et al explored the antibiotic resistance pattern of *P. aeruginosa* isolates collected from blood cultures in Tehran. Based on their results, the studied isolates were highly susceptible to the antibiotics consumed. The highest susceptibility was found to be for ciprofloxacin (97.4%), amikacin (93.4%), imipenem (89.4%), meropenem (87.2%), and ceftazidime (85.6%) (19).

Peymani et al studied the prevalence of some wide-spectrum beta-lactamases-encoding genes in the ESBL-generating *P. aeruginosa* isolates in Tehran, Iran, and reported that the frequency of the genes bla CTXM-1 and bla SHV-1 was 17.3% and 6.7%, respectively (20).

Rossi Gonçalves et al. (2017) conducted a research study in Brazil to study carbapenems-resistant *P. aeruginosa* in blood samples. They reported that the resistance rate was 65.2% to cefepime, 69.6% to ciprofloxacin, and 72.5% to amikacin and gentamicin (21).

Resistance to gentamicin is 51.56%; and in the present study, this resistance is 50%, which is consistent with the results of previous studies, considering that aminoglycosides such as gentamicin are among the main antibiotics for the treatment of *Pseudomonas* (15-20).

This agrees with the fact that aminoglycosides, like gentamicin, are the key antibiotics used to treat *Pseudomonas* infections, especially urinary ones.

The mean antibiotic resistance to ciprofloxacin has been reported at 52.18% in previous studies , while it was 28.8% in the present study (15-20).

The mean antibiotic resistance to third-generation cephalosporins including cefotaxime, ceftazidime, and ceftriaxone has been found to be 66.04%, 54.83%, and 52%, respectively. We estimated it at 52.5%, 32.5%, and 60%, respectively, showing proximity. In the case of ceftazidime, since this compound has been cited as the most effective anti-*Pseudomonas* cephalosporin in most literature, resistance to it is expected to be low, which was supported by our findings.

The mean antibiotic resistance to cotrimoxazole has been estimated at 93.42% in the previous studies and 75% in the present study (in the Sistan region, Iran), showing consistency in the results (15-20). This medication is likely a common antibiotic prescribed in Iran, so it is used

as one of the main medications to treat *Pseudomonas* infections.

A comparison between the previous studies and the present work reveals that the mean antibiotic resistance to nitrofurantoin is 89.21%, reflecting the high resistance. It shows a relative consistency with the results of the previous studies.

In terms of ceftazidime, since this compound has been mentioned as the most effective anti-*Pseudomonas* cephalosporin in most literature and, yet its prescription is more restricted than other beta-lactams, the resistance to it is expected to be low, which was supported by our findings.

Given that imipenem is prescribed much less than other common antibiotics, the low resistance to this antibiotic revealed in the present study is not surprising.

Our results agree with the previous studies in terms of the resistance to ceftazidime, which is associated with the fact that this antibiotic is one of the main medications for the treatment of *Pseudomonas* infections. Resistance to ciprofloxacin has been reported at a higher level in some studies, probably due to the difference in the pattern and dosage of its consumption.

The mean resistance to imipenem agrees with our results, which can justify its prescription as a good option, especially for treatment-resistant *Pseudomonas* infections.

The results of the present study and previous works are inconsistent for nitrofurantoin. The resistance to this antibiotic is high in Iran and the Sistan region, perhaps due to this extensive application and the acquired resistance of the bacteria to this antibiotic.

The resistance to the three antibiotics of azithromycin, amoxicillin, and cephalexin has been reported to be high in the Sistan region. No similar results were found in research in other parts of the world.

The comparison of the frequency of beta-lactamase genes reveals that *bla CTXM-3* and *bla OXA* are the most frequent genes. Also, *bla CTXM-2* was not found in other studies, which is consistent with our findings.

The facts that the ESBL isolates are less resistant to cotrimoxazole than the other isolates and the MDR isolates are less resistant to nitrofurantoin than the other isolates may imply that antibiotics like cotrimoxazole and nitrofurantoin are good options for the treatment of infections induced by resistant *P. aeruginosa* isolates.

No previous report was found on amoxiclav, amoxicillin, azithromycin, and cephalexin. Nonetheless, high resistance has been presently recorded for these compounds. Since all these antibiotics are widely used, the resistance of *P. aeruginosa* strains to these compounds seems reasonable as supported by our findings.

Conclusion

Given the results, high resistance was detected to most studied antibiotics, implying their overuse in Iran. The results can be informative to researchers and medical staff.

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Conceptualization: Omid Tadjrobehkar.
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Formal Analysis: Omid Tadjrobehkar.
Investigation: Atefeh Kamali.
Methodology: Atefeh Kamali.
Project administration: Omid Tadjrobehkar.
Resources: Omid Tadjrobehkar.
Supervision: Omid Tadjrobehkar.
Writing – original draft: Atefeh Kamali.
Writing – review & editing: Omid Tadjrobehkar**,** Atefeh Kamali.

Conflict of Interests

The authors declare that they have no conflict of interest.

Ethical Approval

There was not any direct sampling from the patients and all samples were acquired as the anonymous bacterial samples on the agar media.

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