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Original Article

Role of efflux pump inhibitor in decreasing antibiotic cross-resistance of *Pseudomonas aeruginosa* in a burn hospital in Iran

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Abstract

Introduction: Multidrug resistance in *Pseudomonas aeruginosa* may be due to efflux pump overexpression. This study phenotypically examined the role of efflux pump inhibitors in decreasing antibiotic cross-resistance between beta-lactams, fluoroquinolones, and aminoglycosides in *P. aeruginosa* isolates from burn patients in Iran.

Methodology: A total of 91 phenotypically and genotypically confirmed *P. aeruginosa* samples were studied. Multidrug cross-resistance was determined using the disk diffusion method and minimum inhibitory concentration (MIC) test. The contribution of efflux pumps was determined by investigating MIC reduction assay to markers of beta-lactams, fluoroquinolones, and aminoglycosides in the absence and presence of an efflux pump inhibitor. All the isolates were also tested by polymerase chain reaction for the presence of *mexA, mexC*, and *mexE* efflux genes.

Results: Of the isolates, 81 (89%) and 83 (91.2%) were multidrug resistant according to the disk diffusion and MIC method, respectively. Cross-resistance was observed in 67 (73.6%) and 68 (74.7%) of isolates according to the disk diffusion and MIC method, respectively. In the presence of the efflux pump inhibitor, twofold or higher MIC reduction to imipenem, cefepime, ciprofloxacin, and gentamicin was observed in 59, 65, 55, and 60 isolates, respectively. Except for two isolates that were negative for *mexC*, all isolates were positive for *mexA, mexC*, and *mexE* genes simultaneously.

Conclusion: Efflux pumps could cause different levels of resistance based on their expression in clinical isolates. Early detection of different efflux pumps in *P. aeruginosa* could allow the use of other antibiotics and efflux pump inhibitors in combination with antibiotic therapy.

Key words: burn patients; Pseudomonas aeruginosa; cross-resistance; efflux pump.

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Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that causes a variety of infections, especially in immunocompromised patients such as burn patients. Infections caused by *P. aeruginosa* are related to significant morbidity and mortality [1,2]. The organism exhibits a high level of intrinsic resistance and only a limited number of antimicrobial agents are active against it [3]. In addition, *P. aeruginosa* has a remarkable ability to acquire further resistance mechanisms to multiple groups of antimicrobial agents, including beta-lactams, aminoglycosides, and fluoroquinolones [4]. *P. aeruginosa* represents a phenomenon of bacterial resistance, since practically all known mechanisms of antimicrobial resistance, including import of resistance mechanisms on mobile genetic elements, the chromosomally encoded AmpC cephalosporinase, the outer membrane porin OprD, and the multidrug efflux pumps can be seen in it [5,6]. Among these resistance mechanisms, overexpression of efflux systems has been implicated in multidrug-resistant phenotypes in *P. aeruginosa* clinical isolates [7]. Beta-lactams, fluoroquinolones, and aminoglycosides are three important substrates of efflux pumps which could be overexpressed in *P. aeruginosa* strains. To recognize the presence of acquired multidrug resistance due to efflux pump overexpression, phenotypic and genotypic tests may be used in laboratory practice [3]. Increased expression of efflux pumps could increase the minimum inhibitory concentrations (MICs) of antimicrobials agents [3]. A series of different compounds have been identified as efflux pump inhibitors with the ability to broadly inhibit several identified multidrug efflux pumps in *P. aeruginosa* [6]. In this study, multidrug resistance due to overexpression of the efflux systems was evaluated

by phenotypic tests in the presence of carbonyl cyanide 3-chlorophenylhydrazone (CCCP) as an efflux pump inhibitor. CCCP is a known proton motive force and efflux pump inhibitor [8] that can be added in Mueller-Hinton agar during its preparation. The MICs of antibiotics for strains overexpressing efflux pumps are usually twofold or higher than those strains of species that do not overexpress efflux pumps. This phenotypic test is a valuable method to detect efflux pump overexpression that contributes to multidrug cross-resistance among bacteria. We hypothesized that part of the antibiotic resistance among *P. aeruginosa* isolates could be due to overexpression of efflux pumps. We used CCCP as a screening agent to assess (i) the prevalence of efflux pump overexpression among multidrug-resistant isolates of *P. aeruginosa*; (ii) the contribution of efflux pump overexpression as the supposed mechanism for the multidrug cross-resistance between beta-lactams, fluoroquinolones, and aminoglycosides in *P. aeruginosa*; and (iii) MIC reduction of beta-lactams, fluoroquinolones, and aminoglycosides simultaneously in the presence of an efflux pump inhibitor.

Methodology

Bacterial isolates

The study included a total of 91 *P. aeruginosa* isolates recovered consecutively from burn wound infections of patients hospitalized at a burn hospital in Tehran, Iran. *P. aeruginosa* isolates were first identified based on standard biochemical tests [9]. Then phenotypic identification was confirmed at the species level by using polymerase chain reaction (PCR) amplification of *oprI* and *oprL* genes [10] (Table 1). Bacterial genomic DNA was extracted by the boiling method. For this purpose, all isolates were inoculated aerobically on nutrient agar (Merck, Darmstadt, Germany) for 18–24 hours at 37°C. Depending on colony size, three to six colonies were picked from plates and mixed in 0.1 mL DNase/RNase-free water in sterile 1.5 mL tubes to obtain a turbid suspension of bacteria (~1–2×10^9 cells/mL). The cell suspensions were held in a boiling water bath for 10 minutes to lyse the cells and then centrifuged at 10,000 g at 4°C for 10 minutes. Then in sterile conditions, the supernatant liquid was transferred into another tube. The tubes were stored at -20°C prior to being used in PCR amplification as a DNA template. Also, all the confirmed *P. aeruginosa* isolates were stored at -70°C in trypticase soy broth (Merck, Darmstadt, Germany) supplemented with 10% glycerol until ready for further experiments. Control strains included PAO1 (wild type) and *P. aeruginosa* ATCC 27853 [11].

In vitro susceptibility testing

Antibiotic susceptibility testing of *P. aeruginosa* species was done using the disk diffusion method on Mueller-Hinton agar (Merck, Darmstadt, Germany). All isolates were tested for susceptibility to imipenem (10 μg), cefepime (30 μg), ticarcillin (75 μg), aztreonam (30 μg), tobramycin (10 μg), gentamicin (10 μg), colistin (25 μg), amikacin (30 μg), ciprofloxacin (5 μg), piperacillin (100 μg), and piperacillin-tazobactam (110 μg) (Mast, Merseyside, UK). All isolates were then subjected to determination of MICs for imipenem (Sigma-Aldrich, Stelnhelm, Germany) and cefepime (Sigma-Aldrich, Stelnhelm, Germany) as markers of beta-lactams, ciprofloxacin (Sigma-Aldrich, Stelnhelm, Germany) as a marker of fluoroquinolones, and gentamicin (Sigma-Aldrich, Stelnhelm, Germany) as a marker of aminoglycosides. Isolates showing resistance to one or more antibiotics from three or more antimicrobial classes were considered to be multidrug resistant. Since the efflux pump inhibitors could reduce the MICs required to kill the resistant organisms, the MIC analyses of the mentioned antibiotics were performed again in the presence of CCCP (Sigma-Aldrich, St. Louis, USA), for all resistant strains. CCCP was incorporated in Mueller-Hinton agar (Merck, Darmstadt, Germany) at concentrations of 12.5 μM, and

Table 1. Primers used in this study.

Primer	5'-sequence-3'	Product length (bp)	Reference
oprI-F	ATGAACAACGTTCTGAAATTCTCTGCT	249	[10]
oprI-R	CTTGCGGCTGGCTTTTTCCAG		
oprL-F	ATGGAAATGCTGAAATTCGGC	504	[10]
oprL-R	CTTCTTCAGCTCGACGCGACG		
mexA1	CGACCAGGCCGTGAGCAAGCAGC	316	[17]
mexA2	GGAGACCTTCGCCGCGTTGTCGC		
mexC3	GTACCGGCGTCATGCAGGGTTC	164	[17]
mexC4	TTACTGTTGCGGCGCAGGTGACT		
mexE4	CCAGGACCAGCACGAACTTCTTGC	114	[17]
mexE5	CGACAACGCCAAGGGCGAGTTCACC		

MIC reduction testing was performed by the twofold serial dilution method using a final inoculum of 10^6 cells/mL in agar plates with CCCP [12]. *P. aeruginosa* ATCC 27853 and PAO1 were used as reference strains. Both methods were carried out and interpreted according to the guidelines established by the Clinical and Laboratory Standards Institute (CLSI; M100-S24, 2014). The second MIC tests were performed to check the role of efflux pumps in resistance to beta-lactams, fluoroquinolones, and aminoglycosides. These antibiotics selectively extracted by different efflux systems in pseudomonads [13].

PCR for mexA, mexC, and mexE genes

To investigate the presence of different efflux genes, all the isolates were tested by PCR for the presence of *mexA*, *mexC*, and *mexE* genes, which are representative of MexAB-OprM, MexCD-OprJ, and MexEF-OprN efflux systems, respectively, using previously described primers [12,14]. Primer designations and their sequences are shown in Table 1.

Results

A total of 91 strains of *P. aeruginosa* were isolated from wound infections of hospitalized burn patients and confirmed using biochemical tests and confirmatory PCR assays. The results of the disk diffusion antibiogram showed that 81 (89%) of the *P. aeruginosa* isolates exhibited resistance to three or more antibiotics. According to the antibiogram results, aside from colistin, to which all the isolates were susceptible, the highest rate of resistance was seen against imipenem and tobramycin (88%) and the lowest rate of resistance was seen against aztreonam (76%). According to the disk diffusion method, 81 (89%) isolates were multidrug resistant, of which 67 (83%) isolates were resistant to imipenem, cefepime, ciprofloxacin, and gentamicin simultaneously. Figure 1 summarizes the susceptibility status of isolates against 11 studied antibiotics according to the disk diffusion method. MIC reduction tests to markers of beta-lactams, fluoroquinolones, and aminoglycosides were done in the absence (as control) and presence of CCCP.

Figure 1. Susceptibility status of the isolates against 11 studied antibiotics according to disk diffusion. IMI: imipenem; CEF: cefepime; TC: ticarcillin; AZ: aztreonam; TOB: tobramycin; GN: gentamicin; CO: colistin; AK: amikacin; CIP: ciprofloxacin; PTZ: piperacillin-tazobactam; PIP: piperacillin

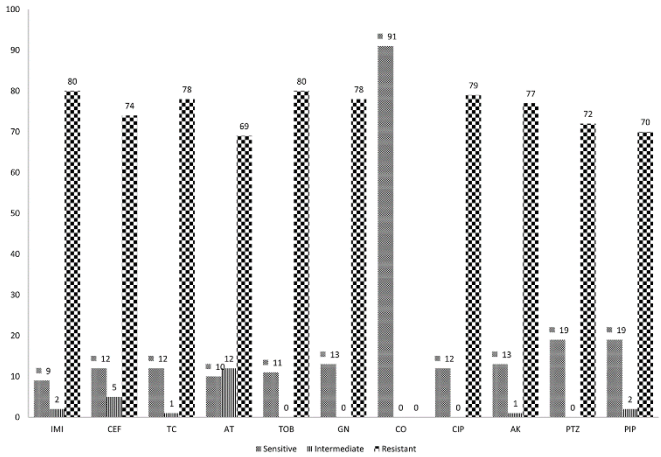

Table 2. Distribution of isolates by level of resistance to tested antibiotics in the absence/presence of carbonyl cyanide 3-chlorophenylhydrazone (CCCP). The number of resistant isolate according to Clinical and Laboratory Standards Institute breakpoints are shown in bold.

Antibiotic	≤ 0.5 µg/mL	1 µg/mL	2 µg/mL	4 µg/ml	8 µg/mL	16 µg/mL	32 µg/mL	≥ 64 µg/mL
Imipenem	0 / 0	0 / 0	2 / 8	12 / 19	**2 / 12**	**4 / 5**	**10 / 16**	**61 / 31**
Cefepime	0 / 0	0 / 0	0 / 10	13 / 14	1 / 4	1 / 1	**1 / 4**	**75 / 58**
Ciprofloxacin	7 / 29	2 / 1	1 / 1	**1 / 4**	**8 / 31**	**53 / 18**	**10 / 5**	**9 / 2**
Gentamicin	0 / 0	1 / 10	1 / 12	6 / 14	5 / 2	**0 / 5**	**1 / 8**	**77 / 40**

Table 3. Role of carbonyl cyanide 3-chlorophenyl hydrazine (CCCP) in reduction of minimum inhibitory concentration (MIC) of antibiotics on resistant *Pseudomonas aeruginosa.*

Antibiotic	Fold MIC reduction						Reversed to susceptible breakpoint
	No change	2	4	8	16	≥ 32	
Imipenem (n = 77)	18 (24%)	33 (42%)	8 (10%)	4 (6%)	6 (8%)	8 (10%)	4 (4%)
Cefepime (n = 76)	12 (16%)	25 (33%)	14 (19%)	8 (11%)	1 (1%)	15 (20%)	5 (6%)
Ciprofloxacin (n = 81)	25 (31%)	28 (35%)	7 (9%)	1 (1%)	5 (6%)	15 (18%)	20 (24.5%)
Gentamicin (n = 78)	23 (30%)	21 (27%)	7 (9%)	2 (3%)	2 (3%)	23 (28%)	24 (26%)

According to CLSI guidlines, the *P. aeruginosa* isolates with MIC ≥ 8 mg/L, MIC ≥ 32 mg/L, MIC ≥ 4 mg/L, and MIC ≥ 16 mg/L are considered as imipenem, cefepime, ciprofloxacin, and gentamicin resistant, respectively. The preliminary results of the MIC method showed that 68 (75%) isolates were resistant to imipenem, cefepime, ciprofloxacin, and gentamicin simultaneously. Table 2 summarizes the distribution of studied isolates by level of resistance to the tested antibiotics. By comparing the MIC results, with and without CCCP, twofold or higher MIC reduction to imipenem, cefepime, ciprofloxacin, and gentamicin was observed in 59, 65, 55, and 60 isolates, respectively, out of 91 samples on the CCCP-supplemented plate. Table 3 shows the role of CCCP in the reduction of MICs for the different antibiotic class markers. PCR revealed that, except for two isolates that were negative for *mexC*, all the isolates were positive for *mexA*, *mexC*, and *mexE* genes as representative of MexAB-OprM, MexCD-OprJ, and MexEF-OprN efflux systems, respectively.

Discussion

The incidence of infectious diseases with high ranges of antibiotic resistance in developing countries is on the rise. *P. aeruginosa* is an opportunistic Gram-negative bacterium, resistant to multiple drugs. The organism exhibits a high level of intrinsic resistance and has also acquired multiple mechanisms of resistance to all available anti-pseudomonal agents [4]. The findings of the present study clearly show that antibiotic resistance rates are high among *P. aeruginosa* isolates in burn patients in Iran. Almost all isolates were resistant to three or more antibiotics tested except colistin, which showed the highest (100%) antibacterial activity. Only three (3.3%) *P. aeruginosa* isolates were fully susceptible. We observed a high rate of resistance to imipenem, cefepime, ciprofloxacin, and gentamicin as markers of beta-lactams, fluoroquinolones, and aminoglycosides, which are employed to treat *P. aeruginosa* infections. Most of the resistant *P. aeruginosa* isolates showed multidrug resistance to two or three tested antibiotic classes. We assumed that part of these multidrug cross-resistances among *P. aeruginosa* isolated from burn wound infections could be due to overexpression of multidrug efflux pumps, because efflux pumps have broad substrate specificity, and every efflux pump expels several antibiotic classes, including beta-lactams, quinolones, and aminoglycosides [12]. Widespread and improper use of antibiotics that are efflux pump substrates could be one of the main causes of inducing multidrug cross-resistance among *P. aeruginosa* in burn wound infections. Also, we assumed that it could have an adverse collateral effect on the susceptibility of *P. aeruginosa* to other existing anti-pseudomonal agents and more incidence of multidrug efflux pumps overexpression. To further investigate this assumption, we used the broad-spectrum efflux pump inhibitor compound, CCCP, and phenotypically investigated the efflux pump overexpression in clinical isolates of *P. aeruginosa*. We used this method because the susceptibility of *P. aeruginosa* strains to antimicrobial agents can be significantly enhanced by pump inactivation [13]. According to our present observations, many clinical isolates of *P. aeruginosa* with a wide range of resistant phenotypes showed increased susceptibility to imipenem, cefepime, ciprofloxacin, and gentamicin as makers of beta-lactams, fluoroquinolones, and aminoglycosides in the presence of efflux pump inhibitors. These results confirmed the simultaneous multidrug extrusion by the efflux systems. After adding CCCP, the accumulation of antibiotics increased and led to lower antibiotic MICs. Inhibition of efflux pumps by CCCP in some isolates brought MICs down even to the levels that make them sensitive strains of *P. aeruginosa.* Therefore, efflux pumps have an important role in the development multidrug-resistant *P. aeruginosa* isolates of burn patients in Iran. Based on genetic investigation to detect the presence of *mexA, mexC*, and *mexE* genes, we observed that, except for two isolates, the multidrug-resistant strains had these genes simultaneously, while according to phenotypic investigation to detect the efflux pump overexpression, twofold or higher MIC reduction to imipenem, cefepime, ciprofloxacin, and gentamicin was observed in only about 50% of the isolates. One possible explanation is that some of the efflux pumps are inactive now and could be overexpressed later. A major beneficial consequence of inhibition of efflux pumps demonstrated in this study is the notable decrease in the frequency of emergence of *P. aeruginosa* strains with clinically relevant levels of resistance to different antibiotic classes; also, further studies on efflux pump inhibitors appear to be an attractive approach to improving the clinical efficacies of antibiotics that are substrates of these pumps [15]. Since some antibiotic classes are substrates for several prevalent efflux pumps in *P. aeruginosa* [16], we could decrease the resistance pattern among *P. aeruginosa* isolates by inhibiting efflux pumps in combination with antibiotic therapy. We should consider the prevalence of efflux-mediated resistance apart from the other known resistance mechanisms in *P. aeruginosa*

isolates. These compounds may be very useful to study the contribution and prevalence of efflux pumps in intrinsic and acquired resistance to multiple antibiotics in other Gram-negative bacteria. Our efforts were focused on the evaluation of CCCP in *P. aeruginosa* strains; we suggest further studies to analyze the role of efflux inhibitors in different bacterial resistance.

Conclusions

It seems that multidrug resistance to most therapeutic antibiotics is common in *P. aeruginosa* isolates from burn patients in Iran. Since we observed resistance to unrelated antibiotic classes, it should be considered that improper use of antibiotics could causes resistance to other classes by triggering the overexpression of efflux pumps and select for mutants with multidrug cross-resistance. Efflux pump inhibitors could ideally increase the effectiveness of antibiotics in multidrug-resistant *P. aeruginosa* isolates.

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