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Comparison of Efflux Pump Involvement in Antibiotic Resistance Among *Pseudomonas aeruginosa* Isolates of Burn and Non-Burn Patients

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Abstract

Background: *Pseudomonas aeruginosa* is an important cause of hospital-acquired infections that can create serious problem for patients and physicians. Many factors are associated with the antibiotic resistance of *P. aeruginosa*, such as efflux pumps.
Objectives: The aim of this study was the phenotypic and molecular detection of efflux pumps in our clinical *P. aeruginosa* isolates in a comparison between burn and non-burn specimens.
Materials and Methods: In this study, clinical strains of *P. aeruginosa* were collected from burn and non-burn specimens from April - July 2013. Antibiotic susceptibility testing of the isolates was performed after biochemical and molecular identification. The minimum inhibitory concentration (MIC) of imipenem, cefepime, gentamycin, and ciprofloxacin, with and without carbonyl cyanide 3-chlorophenylhydrazone (CCCP), was determined for phenotypic detection of efflux pumps.
Results: Our results confirmed 203 and 60 *P. aeruginosa* isolates from burn and non-burn specimens, respectively. The most antibiotic resistance was observed against tobramycin in both group of specimens, and no resistance was seen to colistin. Phenotypic detection of efflux pumps was determined to correlate to a > 4-fold decrease in the MICs of the tested antibiotics with CCCP compared to without CCCP in 57 strains.
Conclusions: High-level antibiotic resistance can occur as a result of multidrug efflux pumps combined with other mechanisms of resistance. However, the association between over-expression of these genes and highly resistant clinical isolates cannot be ignored.

Keywords: Efflux Pumps, CCCP, Burn, Non-Burn, *P. aeruginosa*

1. Background

Pseudomonas aeruginosa is a major cause of nosocomial infections, especially in burn patients (1-4). One of the important characteristics of *P. aeruginosa* is high antimicrobial resistance to antimicrobial agents used in clinical settings (5). Some of this high antimicrobial resistance is related to OprD protein in *P. aeruginosa*, with less permeability, the potential to produce several types of carbapenemase enzymes, and variable efflux pumps (6-8). Efflux pumps are antibiotic-resistance mechanisms that are related to the inability of antibiotics to permeate bacterial cells, keeping them out of the cell and decreasing antibiotic concentrations inside the cell (8). The resistant-nodulation-division (RND) family efflux pump is an intrinsic antibiotic resistance mechanism that can create antibiotic cross-resistance in *P. aeruginosa*, particularly in clinical isolates recovered from burn patients (8-10). Four of these efflux pumps are more prevalent in *P. aeruginosa*: MexXY-OprM can cause resistance to aminoglycosides, beta-lactams, and fluoroquinolones; MexEF-OprN is related to resistance to fluoroquinolones; and MexCD-OprJ and MexAB-OprM can cause resistance to beta-lactams and fluoroquinolones (9-11). Therefore, detection of MexXY-OprM in aminoglycoside-, beta-lactam-, and fluoroquinolone-resistant bacteria can be helpful for identification of the mechanism of antibiotic resistance in these bacteria, as this efflux pump group can create resistance to these three antibiotic families (9). Efflux pump inhibitors are widely used to detect various active efflux pumps (12). Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (an uncoupler of oxidative phosphorylation, which disrupts the proton gradient of the membranes) is a conventional efflux pump inhibitor that can increase the sus-

ceptibility of a number of multi-drug resistant (MDR) bacteria (12).

2. Objectives

The aim of this study was the phenotypic detection of efflux pumps in antibiotic-resistant *P. aeruginosa* by using CCCP as an efflux pump inhibitor in clinical *P. aeruginosa* isolates in a comparison between burn and non-burn samples.

3. Materials and Methods

3.1. Bacterial Isolates

Bacterial isolates were collected from burn-wound infections at Motahari hospital (n = 203) and from different clinical specimens, such as urine and blood cultures, at Milad hospital (n = 60) from April to July 2013. Identification of the isolates was performed by phenotypic testing and PCR as a molecular method at the pediatric infectious research center of the Mofid hospital laboratory. PCR amplification of I lipoprotein (*oprI*) was performed for detection of genus, and of L lipoprotein (oprL) was performed for detection of species of this organism. The sequences of primers and PCR conditions were described previously (5). Direct sequencing of PCR-amplified products was carried out using an ABI 3730X capillary sequencer (Genfanavaran, Macrogen, Seoul, Korea).

3.2. Antibiotic Susceptibility Testing

The antibiotic susceptibility testing was performed by the disc diffusion method on Mueller-Hinton agar (MHA) using ceftazidime (30 μg), cefepime (30 μg), imipenem (10 μg), ticarcillin (75 μg), ticarcillin-clavulanic acid (75/10 μg), piperacillin (100 μg), piperacillin-tazobactam (100/10 μg), ciprofloxacin (5 μg), gentamicin (10 μg), tobramycin (10 μg), amikacin (30 μg), tetracycline (30 μg), trimethoprim (5 μg), trimethoprim-sulfamethoxazole (1.25/23.75 μg), and colistin (10 μg). According to the clinical and laboratory standards institute (CLSI) 2013 (13). The antibiotic disks used in this study were purchased from Mast Diagnostics (UK). *P. aeruginosa* ATCC 27853 was used as the control strain for the antibiotic susceptibility testing. Resistance to at least three families of tested antibiotics indicated MDR.

3.3. Treatment With Efflux Pump Inhibitor

For detection of the efflux pump mechanism, efflux pump inhibitor CCCP was added to each of the MHA plates, containing 0.5 - 1024 μg/mL gentamicin, 0.5 - 1024 μg/mL cefepime, 0.5 - 256 μg/mL imipenem, and 0.5 - 128 μg/mL ciprofloxacin, as representatives of three important antibiotic families. The final concentration of CCCP (C2759 Sigma-Aldrich, France) in the MHA with and without antibiotics was 25 μg/mL (12, 14) simultaneously for each antibiotic. The positive criterion for the presence of active efflux pumps in the isolates was the MIC of the tested antibiotics decreasing at least 4-fold when CCCP was added (12, 14). The control strains included PAO1 (wild type).

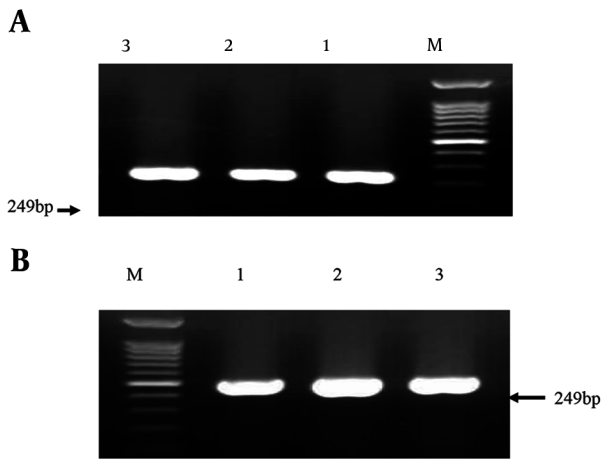
3.4. Statistical Analysis

The involvement of active efflux pumps in the burn and non-burn specimens was analyzed using SPSS software, version 18.0 The Chi-square of all antibiotics was determined between the burn and non-burn specimen isolates, and a $P < 0.05$ was considered significant.

4. Results

In this cross-sectional study, 203 *P. aeruginosa* isolates were confirmed from burn-wound infections and 60 were collected from different clinical specimens at Milad hospital. The isolates were detected by phenotypic and PCR methods (Figure 1). The age of the patients was between 2 and 73 years.

Figure 1. Phenotypic and PCR Methods

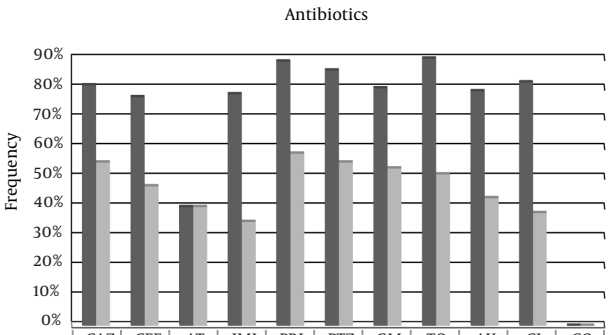


A) PCR amplification of *oprI* gene among suspected isolates for detection of *Pseudomonas* spp. M, 1 kb DNA size marker; lane 1, positive control *P. aeruginosa* ATCC 27853; lanes 2 and 3, positive isolates. B) PCR amplification of *oprL* gene among suspected isolates for detection of *P. aeruginosa*. M, 1 kb DNA size marker; lane 1, positive control *P. aeruginosa* ATCC 27853; lanes 2 and 3, positive isolates.

According to the results of antibiotic susceptibility testing, the most resistance was observed against tobramycin, and none of the strains showed resistance to colistin in either the burn or the non-burn isolates. The antibi-

otic susceptibility test results for the burn and non-burn specimens are shown in Figure 2.

Figure 2. Results of Antibiotic Susceptibility Testing by Disc Diffusion Method



	CAZ	CEF	AT	IMI	PRL	PTZ	GM	TO	AK	CI	CO
Series1	81%	77%	40%	78%	89%	86%	80%	90%	79%	82%	0%
Series2	55%	47%	40%	35%	58%	55%	53%	51%	43%	38%	0%

CTX, cefotaxime; CAZ, ceftazidime; CEF, cefepime; IMI, imipenem; PTZ, piperacillin-tazobactam; PRL, piperacillin; TC, ticarcillin; TC-c, ticarcillin-clavulanic acid; GM, gentamicin; AK, amikacin; TO, tobramycin; TM, trimethoprim; SXT, trimethoprim-sulfamethoxazole; CI, ciprofloxacin; T, tetracycline; CO, colistin. Series 1, burn isolates; Series 2, non-burn isolates.

The MIC range for the *P. aeruginosa* isolates is shown in Table 1, based on the sources of the bacterial isolates and on the tested antibiotics. Seventy-eight percent, 82%, 80% and 78% of the burn isolates showed MICs in the resistance range for imipenem, ciprofloxacin, gentamicin, and cefepime, respectively. On the other hand, 35%, 38%, 53%, and 47% of the non-burn isolates showed MICs in the resistance range for imipenem, ciprofloxacin, gentamicin and cefepime, respectively. Overall, resistance to the tested antibiotics was significantly lower in specimens isolated from non-burn patients than in those from burn patients ($P \leq 0.05$).

4.1. Effects of Efflux Pump Inhibitors on Antibiotic Resistance

The decreased MICs of antibiotics with CCCP in comparison to antibiotics alone are shown in Table 2, based on burn and non-burn specimens. A 4-fold or greater decrease in the MIC of the antibiotic with CCCP against the antibiotic alone was the criterion for significance (12-14). Decreased MICs of tested antibiotics in the presence of CCCP was significantly more common in the burn isolates than in the non-burn isolates ($P \leq 0.05$). Therefore, the burn isolates became significantly less resistant with efflux pump inhibitors ($P \leq 0.05$).

All bacteria grew well on the MHA plates with CCCP that did not contain antibiotics, indicating that 25 μg/mL of CCCP did not have an antibacterial effect by itself.

5. Discussion

P. aeruginosa can cause healthcare-associated infections. In recent years, increased rates of MDR and/or extensively-drug resistant (XDR) isolates of this Gram-negative bacterium have become a challenging issue worldwide (15, 16). The resistance rate to most antibiotics, such as beta-lactams, gentamycin, and fluoroquinolones, which are used clinically to treat *P. aeruginosa* infections, are increasing throughout the world, including Iran (1, 4, 5). In a survey conducted in Mexico, more than 50% of *P. aeruginosa* isolates were MDR, with resistance to 12 tested antibiotics, including beta-lactams, aminoglycosides, and ciprofloxacin (17). Additionally, recent studies in Iran have found increased MDR and/or XDR of *P. aeruginosa* in burn infections (5). Similar to these previous studies, all of our strains isolated from burn wounds showed greater than 70% and 79% resistance to broad-spectrum cephalosporins and aminoglycosides, respectively (compared to 47% and 43% in non-burn specimens). However, the resistance rate observed to imipenem in the isolates included in the present investigation was higher than in studies from India and Tunisia (18.9% and 35%, respectively) (18, 19). This discrepancy could be related to differences in the quality programs of antimicrobial therapies, patterns of antibiotic resistance, geographic conditions, and environmental factors in various countries. According to the results of recent studies and the present one, the emergence of highly resistant *P. aeruginosa* strains to tested antibiotics among hospitalized burned patients in Tehran should be considered. In the current study, the MICs of the tested antibiotics in burn specimens were significantly higher than in non-burn specimens ($P \leq 0.05$). This can be related to the use of broad-spectrum antibiotics in burn patients more often than in non-burn patients, and the selection of antibiotic-resistant bacteria in burn patients. Some recent studies have indicated that antibiotic efflux pumps can be an important mechanism of resistance, sometimes including cross-resistance, in a number of clinically important bacteria, including *P. aeruginosa* (12, 14). Generally, high-level resistance occurs as a result of multidrug efflux pumps with another mechanism of antibiotic resistance, but an association with over-expression of these genes among highly resistant clinical isolates cannot be ignored.

Efflux pump inhibitors have been shown to reverse MDR in *P. aeruginosa* and other bacteria (8, 10). The effects of these compounds, such as CCCP, on MIC were examined in the same studies (12, 14). Rajamohan et al. (20) found that the addition of CCCP at a final concentration of 25 μg/mL greatly reduced the MIC of various biocides, from 2- to 12-fold. These results suggest that antibiotic efflux pumps are involved in the resistance to many antibi-

Table 1. MIC Range of *P. aeruginosa*

Value	Antibiotic MIC, (%)											
	Imipenem			Ciprofloxacin			Gentamicin			Cefepime		
	S	I	R	S	I	R	S	I	R	S	I	R
Burn specimens	6	16	78	13	5	82	12	8	80	18	4	78
Non-burn specimens	55	10	35	43	19	38	43	4	53	51	2	47

Table 2. Decrease of MICs of Antibiotics With CCCP, in % (at Least 4-Fold)

MIC Increase	Cefepime		Imipenem		Ciprofloxacin		Gentamicin	
	Burn	Non-Burn	Burn	Non-Burn	Burn	Non-Burn	Burn	Non-Burn
4-fold	13	9	5	4	8	5	9	6
8-fold	7	4	5	3	5	3	1	1
16-fold	1	1	6	4	5	3	3	2
32-fold	3	-	2	1	8	5	3	2
64-fold	-	-	3	1	1	1	2	1
128-fold	3	1	-	-	-	-	3	1
256-fold	-	-	-	-	-	-	2	-
1,024-fold	-	-	-	-	-	-	1	-

otics that are used for the treatment of clinical *P. aeruginosa* isolates. The results of CCCP testing in our study indicated that efflux pumps are present in burn specimens significantly more often than in non-burns ($P \leq 0.05$). Choudhury et al. (3) conducted a study to detect active efflux pumps in ciprofloxacin-resistant *P. aeruginosa* isolates from different clinical specimens, and found that 23% of isolates showed a positive reaction with CCCP and were considered to be strains with active efflux pumps. In the current study, this rate was 17% for the non-burn specimens and 27% for the burn samples. Ardebili et al. (12) detected active efflux pumps in *Acinetobacter baumannii* in burn isolates by CCCP; according to their results, 86% of these strains become less resistant to ciprofloxacin in the presence of CCCP as an efflux pump inhibitor. This is a higher rate than found in the current study (27%), which can be related to the differentiation between types of bacteria. These results may indicate the greater frequency of active efflux pumps in antibiotic-resistant *A. baumannii* in comparison with *P. aeruginosa*.

In conclusion, the Mex family of efflux pumps are described in *P. aeruginosa*, with over-expression conferring resistance to clinically used antibiotics and other antimicrobial agents.

Footnotes

Authors' Contribution: Leila Azimi: collection of laboratory data; Abdolaziz Rastegar Lari: creation of the idea and correction of the manuscript; Sadaf Jamali: English correction of the manuscript; Aslan Bijari: collection of laboratory data; Elnaz Rastegar Lari: correction of the manuscript.

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