Detection of Overexpression of Efflux Pump Expression in Fluoroquinolone-Resistant *Pseudomonas aeruginosa* Isolates

Abstract

Context: Fluoroquinolones are the most effective antibiotics against *Pseudomonas aeruginosa*; many strains, however, have shown resistance due to mutations in DNA gyrase, topoisomerase IV, or in the efflux pumps. Little is known about *P. aeruginosa* efflux pump resistance mechanisms in the Kingdom of Bahrain. **Aim:** The aim was to study efflux pump-mediated fluoroquinolone resistance among *P. aeruginosa* isolates using phenotypic (E-test and agar dilution) and genotypic (real-time-polymerase chain reaction [RT-PCR]) methods. **Materials and Methods:** Fifty ciprofloxacin-resistant *P. aeruginosa* isolates were included in this study. Genus and species of *P. aeruginosa* were confirmed by conventional PCR. The minimum inhibitory concentration (MIC) of ciprofloxacin with and without carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was determined by E-test and agar dilution test. The overexpression of genes *MexB*, *MexD*, *MexF,* and *MexY* was measured by RT-PCR. **Results:** All isolates were confirmed as *P. aeruginosa*. Among the fifty isolates, four showed reduction in ciprofloxacin MIC after addition of CCCP. These four isolates showed upregulation of expression of at least one of the four genes by RT-PCR. The mean gene expression of *MexB*, *MexD*, *MexF,* and *MexY* increased by 1.6, 4.65, 3.4, and 3.68-fold, respectively. **Conclusion:** The results demonstrate the presence and type of efflux pump overexpression, mandating for large multicentric studies.

Keywords: *Agar dilution, efflux pump, fluoroquinolone resistance, Pseudomonas aeruginosa, real-time-polymerase chain reaction*

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Introduction

Pseudomonas aeruginosa is the most common nosocomial pathogen with high rates of morbidity and mortality.[1] It is a common cause of health care-associated infections such as pneumonia, bloodstream infections, urinary tract infections, and surgical-site infections.[2] It is transmitted through contaminated hands or medical equipment and has an incubation time of 24–72 h.[3] Identification of *P. aeruginosa* can be achieved by routine culture of clinical samples on artificial media, biochemical tests, automated biochemical techniques, or molecular typing and the antibiotic susceptibility pattern was determined by disc diffusion test.[4]

P. aeruginosa infections are typically treated with fluoroquinolones, β-lactams, and aminoglycosides.[5] Many *P. aeruginosa* strains isolated from patients have shown resistance to fluoroquinolones due to mutations in the genes encoding DNA gyrase, topoisomerase IV, or the efflux pumps.[6] Mutations in the efflux pump system lead to overexpression of these pumps, thus reducing the accumulation of antibiotics in the bacterial cell contributing to multidrug resistance (MDR) in *P. aeruginosa*.[7] The efflux pumps present in the bacteria are transporter proteins localized in the cytoplasmic membrane that expel different types of antibiotics, dyes, and detergents.[8]

In general, the bacterial multidrug efflux pump (mex) transporters can be divided into five classes based on the substrate specificity, sequence, number of components, and energy source: (i) resistance nodulation cell division (RND), (ii) small MDR, (iii) major facilitator superfamily, (iv) multidrug and toxic compound extrusion, and (v) adenosine triphosphate (ATP)-binding cassette.[9] Here, we studied the mechanism of resistance nodulation cell division (RND) multidrug efflux pumps (MexCD-OprJ, MexEF-OprN, MexAB-OprM, and MexXY-OprM).

Inhibition of the efflux pump system is important for a successful fluoroquinolone therapy.[10] Efflux pump inhibitors (EPIs)

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are adjuvant therapies used to decrease resistance level and raise the intracellular concentration of drugs in order to decrease mortality rates.[10] The main challenge faced in the production of the EPIs is their toxicity.[10] Carbonyl cyanide m-chlorophenylhydrazone (CCCP) is used as one of the *in-vitro* EPIs for *P. aeruginosa* infections.[11] It is an oxidative phosphorylation uncoupler that belongs to the class of protonophores which reduce ATP production and increase bacterial membrane permeability by interfering with proton motive force.[11] We aimed to identify the efflux pump-mediated fluoroquinolone resistance among *P. aeruginosa* by phenotyping (using E-test and agar dilution) with and without CCCP and genotyping (using real-time-polymerase chain reaction [RT-PCR]).

Materials and Methods

The study was conducted following ethical approval from Arabian Gulf University (AGU) and Ministry of Health (Ref no. MA/EF/357/2017). Fifty nonduplicate ciprofloxacin-resistant *P. aeruginosa* isolates were collected from patients attending the Salmaniya Medical Complex, King Hamad University Hospital, and Bahrain Defense Force Hospital, Kingdom of Bahrain. The isolates were cultured in Luria–Bertani (LB) broth. The bacterial suspensions from LB broth were added to 1.5-ml Eppendorf tubes containing 20% skim milk with glycerol solution and were stored in a freezer at −80°C.

For the confirmation of *P. aeruginosa,* conventional PCR was done by using two primers I lipoprotein (*OprI*) and L lipoprotein (*OprL*) on all phenotypically identified strains of *P. aeruginosa* [Table 1]. *OprI* confirmed the genus and *OprL* confirmed the species. DNA extraction was done by boiling method.[4] Each DNA sample was amplified in 25-µl PCR master mix reaction Thermo Fisher Scientific (Waltham, Massachusetts, U.S). The total master mix for genus (*OprI*) or species (*OprL*) was composed of 12.5-µl Hot Start PCR Master Mix (Thermo Fisher Scientific), 9-µl DNase-/RNase-free water, 0.5-µl forward primer, 0.5-µl reverse primer, and 2.5-µl DNA template. The thermal cycle was optimized for 30 cycles starting from initial denaturation at 94°C for 5 min, followed by denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and the final extension at 72°C for 10 min. The amplified products were detected by gel electrophoresis.

For the determination of minimum inhibitory concentration (MIC), Mueller–Hinton agar (MHA) plates with and without CCCP were swabbed with the isolate for the E-test. The inoculum was prepared by emulsifying[4,5] the isolated colonies from MHA plates into 2 ml of Mueller–Hinton broth to get 10^4 colony-forming unit (CFU)/spot on the agar for all isolates. These inoculums were incubated for 2 h at 37°C in an incubator, and then each inoculum was compared to 0.5 McFarland turbidity standard. Sterilized cotton swabs were used to streak the bacterial suspension broth on plain MHA and MHA with CCCP. E-test strips of ciprofloxacin ranging from 0.002 to 32 µg/ml were placed on both the plates and were incubated at 37°C. After 18–24 h, the MIC with and without CCCP (EPI) was read. In general, the MIC *in vitro* for the sensitive strain is ≤1 µg/ml, intermediate susceptibility is 2 µg/ml, and resistance is ≥4 µg/ml.[12]

For the agar dilution test, ciprofloxacin stock solution was prepared. Two sets of nine serial concentrations of antibiotic were prepared from the main ciprofloxacin stock solution based on twofold dilutions from 2 mg/l up to 512 mg/l with and without CCCP. Each bacterial inoculum was prepared in the same way as for E-test and was compared with 0.5 McFarland standard turbidity. A volume of 1 µl of each inoculum was pipetted into serial agar plates which gave a density equivalent to 10^4 CFU/spot and was incubated at 37°C. After 18–24 h, MIC was read.

For the detection of *P. aeruginosa* gene expression, total RNA was extracted by Trizol RNA extraction method.[13] Complementary deoxyribonucleic acid (cDNA) was synthesized according to the manufacturer's instructions. The high-capacity cDNA reverse transcription kit Applied Biosystems (Foster city, California, U.S) was used. The thermal cycle temperature program was as follows: 10 min at 25°C, 120 min at 37°C, 5 min at 85°C, and finally cooling at 4°C. The cDNA tubes were then stored in the freezer at −20°C.[14]

Expression of *MexB*, *MexD*, *MexF*, and *MexY* genes was determined by RT-PCR by comparing with housekeeping gene (16S). Quantification of cDNA was determined by the Applied Biosystems (Foster city, California, U.S). The primers used for the PCR amplification of cDNA in the study are shown in Table 2. In thermal cycle, the initial denaturation was at 95°C for 10 min followed by 40 cycles with three cycling temperatures, namely, 95°C for 15 s, 58°C for 45 s, and 60°C for 1 min. Furthermore, it was continued with a melting curve at 61°C for 1 min and 95°C for 15 s. The results were analyzed with Manager 1.2

Table 1: Polymerase chain reaction primers for the amplification of I lipoprotein and L lipoprotein

Target	Primer	Oligonucleotide sequence (5'-3')	Amplicon size (bp)
OprI	*OprI-F*	ATGAACAACGTTCTGAAATTCTCTGCT	249
	OprI-R	CTTGCGGCTGGCTTTTTCCAG	
OprL	*OprL-F*	ATGGAAATGCTGAAATTCGGC	504
	OprL-R	CTTCTTCAGCTCGACGCGACG	

OprI: I lipoprotein; *OprL*: L lipoprotein

Table 2: Polymerase chain reaction primers for the amplification of *MexB, MexD, MexF, MexY*, and *16S*

Target	Primer	Oligonucleotide sequence (5'-3')	Amplicon size (bp)
MexB	MexBMRTup	5'-ACTTCTTCAGCTTCAAGGAC-3'	155
	MexBMRTdown	5'-GAGCATGAGGAACTTGTTG-3'	
MexD	MexDRTup	5'- CTACCCTGGTGAAACAGC-3'	250
	MexDRTdown	5'-AGCAGGTACATCACCATCA-3'	
MexF	MexFRTup	5'-CATCGAGATCTCCAACCT-3'	350
	MexFRTdown	5'-GTTCTCCACCACCACGAT-3'	
MexY	MexYMRTup	5'-GCTACAACATCCCCTATGAC-3'	445
	MexYMRTdown	5'-AACTGGCGGTAGATGTTG-3'	
16S	16S RNA-F	5'-AGGCCCGGGAACGTATTCAC-3'	198
	16S RNA-R	5'-GAGGAAGGTGGGGATGACGT-3'	

software (Applied Biosystems, 2008) using ΔCT equation followed by ΔΔCT and relative quantification (RQ) equations.

The total RNA was measured quantitatively by RT-PCR for each gene (*MexB*, *MexD*, *MexF,* and *MexY*). The expression level was measured by comparing the isolates' genes with *P. aeruginosa* ATCC27853 (reference strain) by using RQ equation ($RQ = 2^{-\Delta\Delta CT}$), the RQ value of each gene was equal to 1. Therefore, any gene with RQ value >1 was considered as upregulated which means it was overexpressed. If the RQ value was <1, then the gene was downregulated which means normal gene expression.

Statistical analysis

For statistical analysis, the Statistical Package for the Social Science (SPSS) version 20 (IBM, Armonk, NY, United States of America) program was used, and comparison of the tests was done by Fisher's exact test.

Results

All the fifty isolates were confirmed as *P. aeruginosa* by conventional PCR and visualization on 1.5% agarose gel. Figure 1 depicts some of the PCR products with controls. In the present study, 26% of the isolates were recovered from swabs, 22% from deep tracheal aspiration, 16% from endotracheal tube, 10% from drain fluid aspiration, 8% from urine, 8% from sputum, and least from blood (6%) and tissues (4%). The antibiotic susceptibility pattern of the isolates to other antibiotics was determined according to the Clinical and Laboratory Standards Institute guidelines 2017 by disc diffusion test and is summarized in Table 3.[12] Of the isolates, 43 (86%) were MDR, and all the isolates were sensitive to colistin 50 (100%).

Of the fifty isolates, three (6%) isolates showed reduction in MIC on MHA with CCCP in comparison to plain MHA by E-test [Table 4 and Figure 2]. *P. aeruginosa* ATCC27853 strain was used as control on plain MHA and MHA with CCCP (MIC result 0.125 µg/ml) in both plates. All the fifty isolates were also tested by agar dilution test on two sets of nine serial concentrations (2–512 mg/l) of antibiotics prepared in MHA with and without CCCP. Four (8%) isolates showed twofold reduction in MIC on MHA-containing CCCP [Table 5]. The remaining 46 (92%) isolates did not show any reduction in MIC.

Table 3: Antibiotic susceptibility pattern of the isolates to various antibiotics

Antibiotic	Number of sensitive isolates	Number of resistant isolates
Ciprofloxacin (S ≥21 mm R ≤15 mm)	0	50
Norfloxacin (S ≥29 mm R ≤22 mm)	5	45
Meropenem (S ≥30 mm R ≤24 mm)	5	45
Imipenem (S ≥27 mm R ≤19 mm)	6	44
Ceftazidime (S ≥34 mm R ≤23 mm)	7	43
Cefotaxime (S ≥22 mm R ≤18 mm)	7	43
Tigecycline (S ≥16 mm R ≤12 mm)	12	38
Piperacillin (S ≥36 mm R ≤22 mm)	5	45
Gentamicin (S ≥26 mm R ≤15 mm)	12	43
Amikacin (S ≥30 mm R ≤19 mm)	14	36
Colistin (S ≥17 mm R ≤11 mm)	50	0

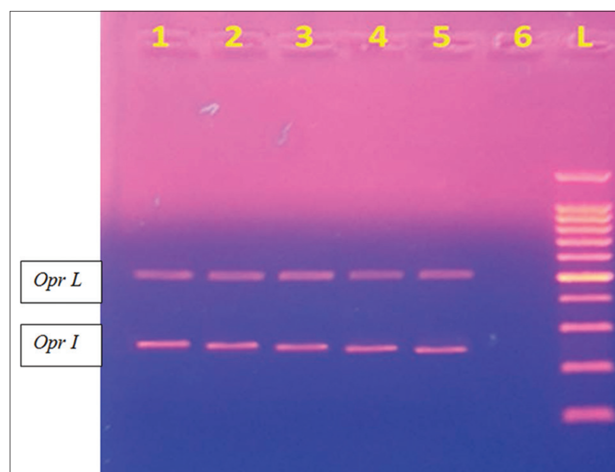

Figure 1: Polymerase chain reaction products on agarose gel electrophoresis of conventional polymerase chain reaction. Lanes (1–4) are two bands for isolate numbers 1–4, lane 5 is *Pseudomonas aeruginosa* ATCC27853 strain (positive control), lane 6 is water (negative control), and lane 7 is the ladder

Of the fifty isolates, four isolates showed upregulation in at least one of the four genes, and 46 isolates showed downregulation in all the four genes. Isolate no. 25 showed upregulation of all the four genes. In isolate no. 29, three genes were upregulated (*MexD*, *MexF,* and *MexY*), whereas isolate no. 16 showed two genes (*MexB* and *MexD*), and in isolate no. 50, only one gene was upregulated (*MexF*) [Figure 3]. The results showed that the mean of *MexB* gene significantly increased by 1.6 folds, *MexD* gene by 4.65 folds, *MexF* gene by 3.4 folds, and *MexY* increased by 3.68 folds.

The results of both the E-test and agar dilution test were analyzed for correlation with RT-PCR results. We found statistically significant correlations ($P < 0.001$) between the phenotype and genotype results.

Discussion

Treatment of *P. aeruginosa* infection is considered a challenge in the medical practice due to a high incidence of mortality in the hospitals.[15] Ciprofloxacin has been successfully used for urinary tract infections, pneumonia, bloodstream infections, and surgical-site infections, but recently, resistance levels have increased.[15] In this study, notable resistance was seen with other class of antibiotics as well. Nearly 72% of isolates showed resistance to amikacin; 76% to tigecycline; 86% to ceftazidime, cefotaxime, and gentamicin; 88% to imipenem; and 90% to norfloxacin, meropenem, and piperacillin. A review by Aly and Balkhy in 2012 showed the prevalence of antibiotic resistance in Gulf Corporation Council countries from 1990 to 2011.[16] They reported that *P. aeruginosa* resistance was 2.6% in Kuwait, 92.3% in Saudi Arabia, 4.2% in Emirates, 0.3% in Oman, and 0.6% in Qatar, but there was no report from the Kingdom of Bahrain.[16]

Resistance in *P. aeruginosa* may occur due to low permeability of the outer membrane, production of inactivating enzymes, efflux pump overexpression, target mutation, and mutation in genes such as AmpC cephalosporinase.[6,17] Fluoroquinolone resistance may be due to target mutation and/or efflux pump overexpression, sometimes at the same time.[15]

In this study, four isolates showed twofold reduction in MIC on addition of EPI, which is in corroboration with other studies that have reported reduction in MIC from 2 to ≥32 folds in *P. aeruginosa*.[6] There were also other studies conducted by Nikaido and Pagès in 2012 and Talebi-Taher *et al*. in 2016 which reported a positive correlation between efflux pump expression and MIC level, which is consistent with this study.[6,18] The MIC of isolate no. 25 was 128 mg/l with overexpression of four genes, whereas isolate no. 29 showed MIC result of 32 mg/l with overexpression of three genes. Furthermore, isolate no. 16 (overexpression of two genes) and isolate no. 50 (overexpression of one gene) showed the same MIC value (8 mg/l).

We observed that the MIC results of agar dilution were higher than that of E-test by 1–2 dilution levels, which is similar to the study by Liu *et al*. in 2014.[19] In the E-test, only three isolates were determined to be resistant, whereas

Table 4: Minimum inhibitory concentration reduction on the addition of carbonyl cyanide 3-chlorophenylhydrazone

Number	MIC without CCCP (mg/l)	MIC with CCCP (mg/l)	Fold reduction
16	4	2	2
29	12	6	2
50	8	4	2

MIC: Minimum inhibitory concentration; CCCP: Carbonyl cyanide 3-chlorophenylhydrazone

Table 5: Minimum inhibitory concentration reduction level of the four resistant isolates

Isolate number	MIC without CCCP (mg/l)	MIC with CCCP (mg/l)	Fold reduction
16	8	4	2
25	128	64	2
29	32	16	2
50	8	4	2

MIC: Minimum inhibitory concentration; CCCP: Carbonyl cyanide 3-chlorophenylhydrazone

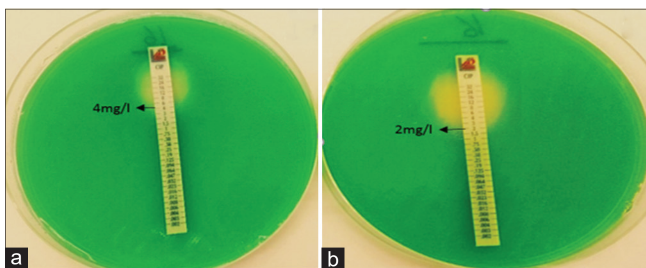

Figure 2: Comparison of E-test strip result between plain Muller–Hinton Agar (a) and Muller–Hinton Agar with carbonyl cyanide 3-chlorophenylhydrazone (b) for isolate no. 16

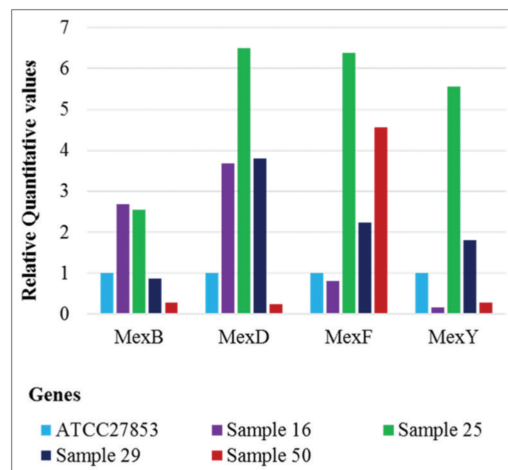

Figure 3: Relative quantification results of the four isolates compared to ATCC27853 strain

four isolates were determined to be resistant by agar dilution test, which makes the agar dilution test a better detection method compared to E-test, which was similar to the outcome of the study by Talebi-Taher *et al*., where they concluded that agar dilution test was a better method for detecting MIC because the E-test can only detect up to a limited MIC value (32 mg/l) for ciprofloxacin.[6]

By RT-PCR, four isolates (8%) showed overexpression of at least one of the four genes. *MexD* (6%) and *MexF* (6%) were overexpressed in three isolates, whereas *MexB* (4%) and *MexY* (4%) were overexpressed in only two isolates. One isolate showed overexpression of all the four genes, whereas a study by El-Said *et al*. from Egypt concluded that 78.6% of the isolates showed overexpression in at least one of the four genes with *MexB* (75%) followed by *MexY* (10.7%) and *MexF* (3.5%). One isolate showed simultaneous overexpression of *MexB* and *MexY* and one isolate overexpressed *MexF* together with both *MexB* and *MexY,* but no isolates showed overexpression of the *MexD* efflux pump gene. Another multicentric study in Spain reported overexpression of *MexY* (13.2%), *MexB* (12.6%), *MexF* (4.2%), and *MexD* (2.2%). The overexpression of *MexB* plus *MexY* was present in 5.3% of the isolates.

All the genotypic test results were similar to the phenotypic test results in this study; however, El-Said *et al*. failed to detect the same results in the phenotyping and genotyping tests.[20] They concluded that sometimes there are difficulties in the detection of the *P. aeruginosa* isolates resistant to ciprofloxacin by either phenotyping or genotyping tests.[20] In their work, among 28 isolates, 17 isolates showed reduction in MIC with at least one efflux pump gene overexpressed in the presence of EPI, whereas three isolates showed reduction in MIC without any efflux pump gene overexpression. Five isolates showed efflux pump gene overexpressed without reduction in MIC, whereas three isolates did not show any reduction in MIC and did not overexpress any efflux pump genes.

Conclusion

This is the first study in the Kingdom of Bahrain exploring the efflux pump mechanism in fluoroquinolone resistance. These results show the occurrence and the type of efflux pump overexpression. Knowledge of the mechanisms of antibiotic resistance will allow the usage of alternative antibiotics in health-care settings where needed and thus reduce the rate of antibiotic resistance. Furthermore, it is important to determine the prevalence of this efflux mechanism in antibiotic resistance among *P. aeruginosa* isolates. Therefore, multicentric studies with the use of various EPIs for other antibiotics as well as other antibiotic resistance mechanisms are highly recommended.

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Conflicts of interest

There are no conflicts of interest.

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