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Detection of DNA Gyrase Mutation and Multidrug Efflux Pumps Hyperactivity in Ciprofloxacin Resistant Clinical Isolates of *Pseudomonas aeruginosa*

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Target modification and reduced drug accumulation are the main resistance mechanisms against fluoroquinolone antibiotics in *Pseudomonas aeruginosa*. We performed a genotypic characterization of three major Mex multidrug efflux pumps (MexAB-OprM, MexXY-OprM and MexCD-OprJ) in ciprofloxacin resistant clinical isolates of *P. aeruginosa*, collected from Tehran, Iran; this was followed by sequencing and analyzing the type II topoisomerases encoding *gyrA*, *gyrB*, *parC*, and *parE* genes. Reverse transcription PCR (RT-PCR) and semi-quantitative RT-PCR methods were used to analyse the transcription of efflux pumps. Topoisomerase mutation analysis was carried out through PCR amplification and sequencing of the quinolone resistance determining region (QRDR) of the topoisomerases encoding genes. Some 11.1% of the strains actively expressed MexCD-OprJ and 15.5% hyperexpressed MexXY-OprM efflux pumps. No overexpression was detected for MexAB-OprM, whereas 4.4% of strains showed simultaneous expression of MexCD-OprJ and MexXY-OprM. In the sequencing results, a single point mutation in the QRDR of *gyrA* was detected in all tested strains, where Isoleucine was substituted by Threonine at position 83. No mutations were detected in QRDR of *gyrB*, *parC* and *parE* genes. We are the first to report the genotypic analysis of ciprofloxacin mediated efflux pump resistance from Iran. These findings emphasize the clinical significance of multidrug efflux pumps and topoisomerases mutations activity in conferring resistance to fluoroquinolone antibiotics, and support the use of genotypic methods for analysis of resistance elements in *P. aeruginosa* in developing countries such as Iran.

Key words: **Ciprofloxacin, Multidrug efflux pumps, *Pseudomonas aeruginosa*, Topoisomerase.**

INTRODUCTION

Pseudomonas aeruginosa is a serious nosocomial bacterial pathogen, mainly due to the high level of antibiotic resistance, which is the result of several mechanisms such as outer membrane low permeability [1], acquisition of antibiotic modifying enzymes [2, 3], and overexpression of multidrug efflux pumps [4, 5]. Fluoroquinolones have been extensively used against various infections both in human and veterinary cases [6, 7]. Ciprofloxacin is historically known as the most effective fluoroquinolone antibiotic against *P. aeruginosa* infections [8, 9]. The main resistance mechanisms to fluoroquinolones include multidrug efflux pumps and drug target modifications. Mutations in type II topoisomerase encoding genes, including DNA gyrase (*gyrA*, *gyrB*) and DNA topoisomerase IV (*parC*, *parE*) in so-called quinolone resistance determining region (QRDR) [10], play a significant role in conferring fluoroquinolone resistance in clinical isolates of *P. aeruginosa* [11-13].

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MexAB-OprM, MexCD-OprJ and MexXY-OprM are among the well described multidrug efflux pumps of *P. aeruginosa*. MexAB-OprM is constitutively expressed in wild-type strains and overproduced in *nalB* and *nalC* mutants conferring resistance to fluoroquinolones, tetracycline and beta-lactams [14, 15]. The overexpression of the substrate-inducible pump, MexXY-OprM, results in resistance to some drugs such as fluoroquinolones, tetracycline, aminoglycosides and erythromycin [16, 17]. MexCD-OprJ pump has no expression under standard conditions, while expression occurs in *nfx*B mutant that is resistant to fluoroquinolones, tetracycline, chloramphenicol and trimethoprim [18, 19]. So far, there has been no valid evidence of genotypic analysis of fluoroquinolone-mediated efflux pump resistance in *P. aeruginosa* from Iranian medical settings. The aim of this study was to investigate the two main fluoroquinolone resistance mechanisms through transcriptional analysis of the three efflux pumps MexAB-OprM, MexCD-OprJ and MexXY-OprM and sequencing analysis of type II topoisomerase QRDR, in order to elucidate a possible correlation between the emergence of multidrug efflux pumps hyperactivity, topoisomerase mutations and resistance to fluoroquinolones in clinical isolates of *P. aeruginosa* from Iran.

MATERIALS AND METHODS

Bacterial strains, plasmid and growth conditions. A total number of 133 *P. aeruginosa* isolates were collected from patients in burn units of 3 major hospitals in Tehran, Iran. The antimicrobial susceptibility of these isolates was tested against 10 different antibiotics. Some 45 (35%) ciprofloxacin-resistant (Cip^R) strains were examined for phenotypic indication of efflux pumps overexpression, using an efflux inhibitor. The test resulted in 10 strains that phenotypically hyperexpressed these pumps (published elsewhere) [20]. These strains were tested for transcription activity of MexAB-OprM, MexCD-OprJ, and MexXY-OprM, using genotypic methods. The criterion of examining the relatively low number of strains for further analysis was comparable with other similar studies, investigating efflux pumps expression/activity and topoisomerase QRDR analysis in clinical isolates of *P. aeruginosa* [21-23]. *P. aeruginosa* wild-type strain PAO1 and laboratory mutants named as JFL-30, JFL28 and JFL10 that hyperexpressed MexAB-OprM, MexCD-OprJ and MexXY-OprM pumps, were also used respectively as controls in this study [24]. The *Escherichia coli* strain DH5α and plasmid pBluescript sk (-) were used as host cell and vector for cloning and *in vitro* transcription of *mexA* and *mexX*. Bacterial cells were routinely cultured in Luria-Bertani (LB) broth medium at 37°C. The DH5α cells were grown in LB broth, supplemented with 50 µg/ml of ampicillin and 8 µg/ml of tetracycline at 37°C.

Cloning and transcription analysis. Due to the presence of basic expression of two efflux pumps MexAB-OprM and MexXY-OprM, the transcription level of these pumps was tested using a semi-quantitative Reverse transcription PCR (RT-PCR) method. A regular RT-PCR protocol was also set up for transcription analysis of MexCD-OprJ efflux pump. PCR amplification of *mexA* and *mex*X was carried out using *P. aeruginosa* PAO1 genomic DNA and the primers: *mex*A (GeneBank: AAG03814.1) F: (5′-CCAACCCGAACAACGAGC-3′), R: (5′-TTGCTGTCGGTTTTCGCC-3′) (349 bp) and *mexX* (GeneBank: AB015853.1) F: (5′-TGGTCGCC-CTATTCCTGC-3′), R: (5′-ACGCCTTGAGCTTGTC-GG-3′) (336 bp). The genes were cloned into the Eco321 (RV) -digested pBluescript sk (-) (3 kbp) vectors and subsequently transformed into DH5α cells. The *in vitro* transcription assays were started using EcoR1-digested linear plasmid DNA of *mexA*/*mexX* and T3 RNA polymerase (Fermentas Inc.). Ten continuous dilutions (index: ½) were prepared from the synthesized RNA for *mexA* and *mexX*. RNA concentrations of serial dilutions were measured by spectrometry at A_{260} nm and reverse-transcribed into their respective complementary DNA (cDNA) using the Moloney murine leukemia virus reverse transcriptase (MMLV), according to the supplier's instructions (Fermentas Inc). The second strand PCR was followed by using template cDNA with the oligonucleotide primers as described above. The PCR products were visualized on agar gel electrophoresis and a UV transilluminator. The intensity of the PCR bands was measured using Gel analyzer software (BioDoc. GMBH) and combined with the serial RNA dilution spectrometry data to draw the *mexA*/*mexX* corresponding standard transcription plots.

RT-PCR. The clinical strains were grown up to mid-exponential phase turbidity (A600=1.5-2) and the total RNA was isolated (RNeasy QIAGEN, GMBH). The reverse transcription reaction was followed by MMLV reverse transcriptase, converting approximately 2 µg of total RNA to cDNA. Secondary strand PCR was carried out using oligonucleotide primers of *mexA*, *mexX*, *mexC* (GenBank: AAG07987.1) F: 5′-CGTGCAATAGGAAGGATCGG-3′, R: 5′-GGATCTTCACCAGCGGCTC-3′ and *rpsL* (GenBank: AAG07656.1) F: 5′-AAGCGCATGGTCG-ACAAGA-3′, R: 5′-CTGTGCTCTTGCAGGTTGTGA-3′ (201 bp). The PCR results were analyzed on agarose electrophoresis gel and visualized through a UV transilluminator. mRNA level normalization of each strain was set according to that of the 16 s ribosomal *rpsL* housekeeping gene [25] and expressed as proportional to the value of *P. aeruginosa* strain PAO1, defined as 1. The bands densitometry of *mexA* and *mexX* was measured to derive a semi-quantitative level of these genes transcription for each strain. The strains were considered over expressing mutants if the levels of *mexA or mexX* transcription were higher than the minimum thresholds described by Mesaros *et al.* [26]. The simple detection of *mexC* expression on the electrophoresis analysis was thought valid enough to identify the strains as overproducing mutants.

Type II topoisomerase PCR amplification and QRDR sequencing analysis. Genomic DNA was isolated from clinical strains and used as template for PCRs. Sets of primers were designed for amplification of fragments including the putative QRDRs. *gyrA* (GeneBank ID AAG06556.1) F: 5′-AGCAGGTTGGGAATCTTGGT-3′ and R: 5′-C-CGCCGTGTGCTTTATG-3′ primers amplified 366 bp of QRDR from position 2273 to 2638 inside *gyrA*. *gyrB* (GeneBank ID AAG03394.1) primers F: 5′-TGCGGTGGAACAGGAGAT-3′ and R: 5′-CA-TCTGGCGGAAGAAGAAG-3′ amplified 514 bp region of QRDR from position 1047 to 1560. *parC* (GeneBank ID AAG08349.1, F:5′-AGTTCGCTG-AGCAGCACCT-3′ and R: 5′-ACCGAGCAGGCC-TATCTGAA-3′ primers amplified a 368 bp fragment of QRDR from position 1838 to 2205. Pairs of primers F: 5′-TCGAGGGCGTAGTAGATGTC-3′ and R: 5′-TCTCGGGCGTGGTGAAG-3′ were designed to amplify a 583 bp gene fragment of QRDR inside *parE* (GeneBank ID AAG08352.1) from position 275 to 857. The PCR sequencing procedure was followed by using dideoxy chain terminator method and directed through a BigDye Terminator v3.0 Chemistry DNA sequencing kit (Applied Biosystems, 33730xl, USA) according to the manufacturer's protocol. The sequencing results were analyzed using the Chromas sequence analyzer and CLCBio tools.

RESULTS

Analysis of *mexA*, *mexX* and *mexC* expression and selection of double-efflux expressing strains. Figure 1 (A to C) shows the RT-PCR results of *mexC, mexA* and *mexC* transcription analysis in 10 clinical strains of *P. aeruginosa*. Accordingly, 15.5% (7 of 45) of Cip^R strains showed a ≥3 times greater expression of MexXY-OprM efflux pump than wild type PAO1 strain, making them overexpressing mutants. For *mexA*, comparison of transcription level with that of *P. aeruginosa* PAO1 wild type did not show any significant (≥3 times) expression of MexAB-OprM in any of the studied clinical strains. Analysis of *mexC* expression in the Cip^R strains of *P. aeruginosa,* using traditional RT-PCR, showed that 11.1% (5 of 45) of the strains actively produced the MexC protein of the three component efflux pump MexCD-OprJ. According to our results, 4.4% (2 of 45) of the Cip^R strains showed simultaneous expression and overerexpression of *mexC* and *mexX* respectively (Fig. 1).

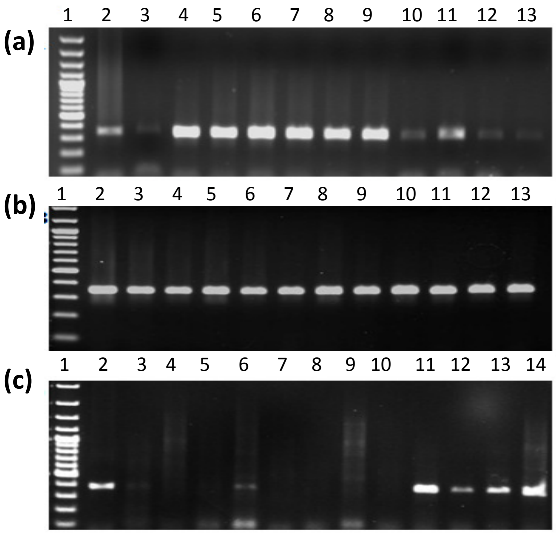

Fig. 1. Agar electrophoresis of RT-PCR against cDNA of *mexX, mexA,* and *mexC* in 10 Cip^R, efflux positive clinical strains of *P. aeruginosa*; **(a)** lane 1, 100 bp DNA marker; lane 2, MexXY-OprM hyper-expressing mutant (JFL-10); lane 3, *P. aeruginosa* PAO1 wild type strain; lanes 4-13, 10 clinical strains of *P. aeruginosa* (P1-P10) tested for expression of *mexX* gene (336 bp); **(b)** lane 1, 100 bp DNA ladder; lane 2, MexAB-OprM hypet-expressing mutant (JFL-30); lane 3, *P. aeruginosa* PAO1 wild type strain; lanes 4-13, 10 clinical strains of *P. aeruginosa* (P1-P10) tested for expression of *mexA* gene (349 bp); **(c)** lane 1, 100 bp DNA marker; lane 2, *P. aeruginosa* PAO1 wild type strain DNA PCR control; lane 3, MexCD-OprJ expressing mutant (JFL-28); lane 4, *P. aeruginosa* PAO1 wild type strain; lanes 5-14, 10 clinical strains of *P. aeruginosa* (P1-P10) tested for expression of *mexC* (388 bp).

Type II topoisomerase amino acid substitutions. PCR sequencing analysis of DNA gyrase and Topoisomerase IV genes detected a single point mutation in the QRDR of *gyrA* at nucleotide 249 that was spotted in all tested clinical strains of *P.aeruginosa* and led to the amino acid substitution Threonine 83 to Isoleucine (*P.aeruginosa* PAO1 numbering). Sequence analysis of *gyrB*, *parC* and *parE* genes did not show any mutations occurring in their respective amplified QRDR.

Contribution of efflux pump and topoisomerase activity to overall antibiotic susceptibility. The MIC (Minimum Inhibitory Concentration) distribution of three antibiotics (Imipenem, Gentamicin and Ceftazidime) for the studied Cip^R strains is shown in Table 1.

Table 1. The Antibiotic resistance, multidrug efflux pumps expression and type II topoisomerase mutation profile of 10 clinical strains of *P. aeruginosa*

Strains	Cip^R (≥4 mg/L)	IMI^R (≥4 mg/L)	GEM^R (≥16 mg/L)	CAZ^R (≥32 mg/L)	MDR Efflux pump	DNA gyrase	Topoisomerase IV
1	+	–	–	–	MexXY-OprM	gyrA (Thr-83►Ile)	–
2	+	+	+	+	MexXY-OprM MexCD-OprN	gyrA (Thr-83►Ile)	–
3	+	–	–	–	MexXY-OprM	gyrA (Thr-83►Ile)	–
4	+	–	+	+	MexXY-OprM	gyrA (Thr-83►Ile)	–
5	+	–	+	+	MexXY-OprM	gyrA (Thr-83►Ile)	–
6	+	–	+	+	MexXY-OprM	gyrA (Thr-83►Ile)	–
7	+	–	+	+	MexCD-OprN	gyrA (Thr-83►Ile)	–
8	+	–	–	–	MexXY-OprM MexCD-OprN	gyrA (Thr-83►Ile)	–
9	+	–	+	+	MexCD-OprN	gyrA (Thr-83►Ile)	–
10	+	–	–	–	MexCD-OprN	gyrA (Thr-83►Ile)	–

Cip^R, Ciprofloxacin resistant; IMI^R, Imipenem resistant; GEM^R, Gentamicin resistant; CAZ^R, Ceftazidime resistant

Accordingly, 3 of 5 strains expressing MexCD-OprJ and also 5 of 7 strains overexpressing MexXY-OprM were resistant to Ceftazidime and Gentamicin. One MexCD-OprJ and MexXY-OprM double expressing strain was also shown to have triple resistance to gentamicin, Ceftazidime and Imipenem. In contrast, a similar single *gyrA* point mutation was detected in all Cip^R strains, regardless of their secondary antibiotic resistance profile.

DISCUSSION

Genotypic analysis of fluoroquinolone resistance has been previously reported in clinical isolates of *P. aeruginosa* [21, 28-30]. In this study, we reported the first genotypic analysis of major multi-drug efflux pumps in clinical isolates of *P. aeruginosa,* collected from public medical settings in Iran. Using a genotypic detection method, we analyzed three pumps: MexAB-OprM, MexXY-OprM and MexCD-OprJ on clinical isolates with significant hyperactive efflux phenotype [20]. Due to the upstream regulation of the genes, responsible for encoding the components of the Mex efflux pumps in *P. aeruginosa*, the indication of gene expression within the operon region implies the potential activity/hyperactivity of downstream genes that encode components of the efflux pump [31]. We detected 4.4% (2 of 45) of Cip^R isolates expressing at least two efflux pumps simultaneously. Emergence of simultaneous expression of different efflux pumps may be due to double mutations in gene regions regulating the operons that potentially confer higher level of antibiotic resistance to mutant strains [27]. This potentiality indicates a higher risk for emergence of multidrug resistance, with lower susceptibility to substrates of these pumps. We also attempted to sequence the type II topoisomerase subunits A and B genes *gyrA*, *parC* and *gyrB*, *parE* respectively. QRDR mutation analysis showed the replacement of Thr 83 with Ile in *gyrA* of all studied strains. This result is supported by previous reports on clinical strains of *P. aeruginosa* [30, 32]. The change of polar Threonine to the nonpolar and highly hydrophobic Isoleucine may affect the gyrase-quinolone interaction by loss of necessary enzyme-drug contacts or conformational changes that may eventually result in antibiotic resistance. The presence of unique *gyrA* mutation in all studied strains supports the fact that DNA gyrase is basically the first target enzyme of type II topoisomerase in ciprofloxacin resistance [32]. Regarding the relation of antibiotics MIC to efflux pumps and topoisomerase activity, the overexpression of MexCD-OprJ and MexXY-OprM potentially reduces the strains susceptibility to non-fluoroquinolone antibiotics Gentamicin (Aminoglycosides) and Ceftazidime (beta-lactam). In contrast, the *gyrA* single mutation (Thr 83) did not seem to confer resistance to any of tested non-fluoroquinolone antibiotics. The application of RT-PCR method in this study resulted in a more accurate analysis than that obtained from the phenotypic analysis of efflux activity by differential selection of active multidrug efflux pumps in studied strains. Real-Time PCR quantitation method has a higher specificity than the traditional RT-PCR, but it is still considered an expensive technique and not easily affordable for every laboratory routine use [33] in developing countries. Further work is needed to transfer the results of this study into a clinical setting, to provide information in regards to the choice of antibiotics dosage and to translate the results into improvements in hospitalization and healthcare policies.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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