Plasmid-Mediated Fluoroquinolone Resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*

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Abstract

Introduction *Pseudomonas aeruginosa* and *Acinetobacter baumannii* are important pathogens in health care–associated infections. Fluoroquinolone resistance has emerged in these pathogens. In this study, we aimed to determine the occurrence of plasmid-mediated quinolone resistance (PMQR) determinants (*qnrA*, *qnrB*, *qnrS*, *aac(6′)-Ib-cr*, *oqxAB*, and *qepA*) by polymerase chain reaction (PCR) and the transmissibility of plasmid-borne resistance determinants in clinical isolates of *P. aeruginosa* and *A. baumannii*.

Materials and Methods The study included *P. aeruginosa* (85) and *A. baumannii* (45) which were nonduplicate, clinically significant, and ciprofloxacin resistant. Antibiotic susceptibility testing was done by disk diffusion method for other antimicrobial agents, namely amikacin, ceftazidime, piperacillin/tazobactam, ofloxacin, levofloxacin, and imipenem. Minimum inhibitory concentration of ciprofloxacin was determined. Efflux pump activity was evaluated using carbonyl-cyanide m-chlorophenylhydrazone (CCCP). The presence of PMQR genes was screened by PCR amplification. Transferability of PMQR genes was determined by conjugation experiment, and plasmid-based replicon typing was performed.

Results Resistance to other classes of antimicrobial agents was as follows: ceftazidime (86.9%), piperacillin/tazobactam (73.8%), imipenem (69.2%), and amikacin (63.8%). The minimal inhibitory concentration (MIC)50 and MIC90 for ciprofloxacin were 64 and greater than or equal to 256 µg/mL, respectively. There was a reduction in MIC for 37 (28.4%) isolates with CCCP. In *P. aeruginosa*, 12 (14.1%) isolates harbored *qnrB*, 12 (14.1%) *qnrS*, 9 (10.5%) both *qnrB* and *qnrS*, 66 (77.6%) *aac(6′)-Ib-cr*, and 3 (3.5%) *oqxAB* gene. In *A. baumannii*, *qnrB* was detected in 2 (4.4%), 1 (2.2%) harbored both the *qnrA* and *qnrS*, 1 isolate harbored *qnrB* and *qnrS*, 21 (46.6%) *aac(6′)-Ib-cr*, and 1 (2.2%) isolate harbored *oqxAB* gene. Notably, *qepA* gene was not detected in any of the study isolates. Conjugation experiments revealed that 12 (9.2%) were transferable. Of the transconjugants, seven (58.3%) belonged to IncFII type plasmid replicon, followed by four (33.3%) IncA/C and one (8.3%) IncFIC type.

Conclusion The plasmid-mediated resistance *aac(6′)-Ib-cr* gene is primarily responsible for mediating fluoroquinolone resistance in clinical isolates of *P. aeruginosa* and *A. baumannii*. The predominant plasmid type is IncFII.

Keywords
- *aac(6′)-Ib-cr*
- fluoroquinolones
- PMQR

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Introduction

Fluoroquinolones are synthetic antimicrobial agents with a broad spectrum of activity. They are effective against a wide range of gram-negative and gram-positive pathogenic bacteria. Over the past few years, fueled by their wide use, resistance to fluoroquinolones has raised globally.[1] An important resistance mechanism to fluoroquinolones is described by mutations in the quinolone resistance-determining regions of gyrase and topoisomerase encoding genes.[2] Another well-known fluoroquinolone resistance mechanism is the decreased intracellular drug accumulation by upregulation of efflux pumps or decreased expression of outer membrane porin.[3] The emergence of plasmid-mediated quinolone resistance (PMQR) has been reported since 1998. These are horizontally transferable and are referred to as "PMQR." The three PMQR genes include: (1) the *qnr*, (2) *aac(6')-Ib-cr* (aminoglycoside acetyltransferase), and (3) *oqxAB* and *qepA* (efflux pumps).[4]

The plasmid *qnr* genes (*qnrA*, *qnrB*, and *qnrS*) encode for proteins of the pentapeptide repeat family that protect DNA gyrase and topoisomerase intravenous from fluoroquinolone inhibition. The *aac(6')-Ib-cr* is a bifunctional variant of aminoglycoside acetyltransferase capable of modifying the fluoroquinolones that have an amino nitrogen on the C7 of piperazinyl ring, such as ciprofloxacin and norfloxacin, thereby reducing their activity.[5] Other fluoroquinolones lacking an unsubstituted piperazinyl nitrogen are not affected.[6] The plasmid-mediated *qepA* efflux pump belongs to the major facilitator superfamily that decreases susceptibility to hydrophilic fluoroquinolones, especially ciprofloxacin.[7] The *oqxAB* encodes for efflux pumps belonging to the resistance nodulation division family and is a multidrug efflux pump.[8]

Acinetobacter baumannii and *Pseudomonas aeruginosa* are well recognized representatives of nonfermenting gram-negative pathogens which are responsible for health care-acquired infections. In both species, resistance to fluoroquinolones has been a recognized problem due to their ready ability to acquire resistance determinants.[2] Most studies on prevalence of PMQR genes are focused on Enterobacteriaceae.[9–11] Data on the prevalence of PMQR genes among clinical isolates of *P. aeruginosa* and *A. baumannii* are scarce.[12]

The presence of fluoroquinolone resistance genes on plasmid enables their spread to other bacterial species by horizontal gene transfer. The identification of related plasmids associated with specific resistance genes helps track the spread of resistant plasmids. Hence, polymerase chain reaction (PCR)-based replicon typing (PBRT) has been adopted worldwide as the method for plasmid identification and typing.[13]

In this study, we aimed to determine the occurrence of PMQR determinants (*qnrA*, *qnrB*, *qnrS*, *aac(6')-Ib-cr*, *oqxAB*, and *qepA*) by PCR and the transmissibility of these plasmid-borne resistance determinants in clinical isolates of *P. aeruginosa* and *A. baumannii.*

Materials and Methods

Bacterial Isolates

The study included *P. aeruginosa* (85) and *A. baumannii* (45) which were nonduplicate, clinically significant and ciprofloxacin resistant (as determined by disc diffusion test) and obtained from clinical specimens of hospitalized patients at university teaching hospital in South India. They were collected over a period of 1 year from July 2014 to June 2015. They were obtained from clinical specimens such as urine (5), exudative samples (66), respiratory secretions (47), and blood stream (12). The isolates were identified up to species level by automated system (VITEK2 GN-card; BioMerieux, Brussels, Belgium) and/or standard biochemical tests.

Antimicrobial Susceptibility Testing

Antibiotic susceptibility testing was done by Kirby–Bauer disc diffusion method for the following antimicrobials: ceftazidime (30 µg), piperacillin/tazobactam (30 µg), imipenem (10 µg), amikacin (30 µg), levofloxacin (5 µg), and ofloxacin (5 µg) (Himedia Laboratories, India). The minimal inhibitory concentration (MIC) of ciprofloxacin was determined by agar dilution technique according to CLSI 2017 guidelines.[14] ATCC *Escherichia coli* 25922 was used as control for both disc diffusion method and MIC determination.

Phenotypic Detection of Efflux Pump Activity

To detect the presence of efflux pump mechanism, carbonylcyanide m-chlorophenylhydrazone (CCCP), the efflux pump inhibitor was added to each Muller–Hinton (MH) agar plate containing 0.125 to 256 µg/mL of ciprofloxacin. The fixed concentration of CCCP in the MH agar was 20 µg/mL. The MIC with CCCP incorporated was determined in twofold serial dilutions as for the antibiotic (CLSI 2017).[14] A plate without antibiotic and containing only CCCP inhibitor was used as control. The criteria for the presence of efflux pump activity was based on a fourfold decrease in MIC of ciprofloxacin on addition of CCCP.[15]

Polymerase Chain Reaction

The DNA of the study isolates was extracted by the boiling method. The amplification of *qnr* genes (*qnrA*, *qnrB*, and *qnrS*) was performed by multiplex PCR using the cyclic profile: initial denaturation at 94°C for 7 minutes; denaturation at 94°C for 50 seconds, annealing at 53°C for 40 seconds, and elongation at 72°C for 60 seconds, repeated for 35 cycles, and a final extension at 72°C for 5 minutes.[16] The PCR conditions for *acc(6')-Ib-cr* were: initial denaturation at 94°C for 7 minutes, denaturation at 94°C for 50 seconds, annealing at 55°C for 40 seconds, and elongation at 72°C for 60 seconds, repeated for 35 cycles, and a final extension at 72°C for 5 minutes.[17] The PCR cyclic parameters for *oqxAB* were as follows: initial denaturation at 95°C for 15 minutes; 30 cycles of 94°C for 30 seconds, 63°C for 90 seconds, and 72°C for 90 seconds, followed by a final extension at 72°C for 10 minutes. The PCR conditions used for *qepA* were as follows: initial denaturation at 96°C for 1 minute, followed by 30 cycles of amplification at 96°C for 1 minute, annealing

Table 1 Primers used in this study

PMQR gene	Primers	Product size	Reference
qnrA	5′-TCAGCAAGAGGATTTCTCA-3′ 5′-GGCAGCACTATTA CTCCCA-3′	516	[16]
qnrB	5′-GATCGTGAAAGCCAGAAAGG3′ 5′-ACGATG CCTGGTAGTTGTCC-3′	469	[16]
qnrS	5′-ACGACATTCGTCAACTGCAA-3′ 5′-TAAATTGGCACCCTGTAGGC-3′	417	[16]
acc(6')-Ib-cr	5′-TTGGAAGCGGGGACGGAM-3′ 5′-ACACGGCTGGACCATA -3′	260	[17]
oqxAB	5′-CCGCACCGATAAATTAGTCC-3′ 5′-GGCGAGGTTTTGATAGTGGA-3′	313	[18]
qepA	5-GCA GGT CCA GCA GCG GGT AG-3 5-CTT CCT GCC CGA GTA TCG TG-3	218	[18]

Abbreviation: PMQR, plasmid-mediated quinolone resistance.

at 60°C for 1 minute, extension at 72°C for 1 minute, and the final extension step was at 72°C for 5 minutes.[18] The primers used is given in ►**Table 1**.[16–18] The PCR by-product was examined by electrophoresis in agarose gel containing ethidium bromide and visualized by gel documentation system.

DNA Sequencing

The PCR positive amplicons were sequenced at SciGenome Labs Pvt, Ltd., India and analyzed with BLAST tools (www.ncbi.nim.nih.gov). The assigned GenBank accession number for the submitted sequences are: (1) MH709266 (*qnrA*); (2) KY130487 (*qnrB*); (3) KY130488 (*qnrS*); (4) MH709269 (*acc(6')-Ib-cr*), and (5) MN273774 (*oqxAB*).

Conjugation

Conjugation experiments were performed for all PMQR positive isolates. *Escherichia coli* J53 AziR strain was used as the recipient and PMQR positive isolates as donor. The donor and recipient cells (0.5 mL each) in logarithmic phase were added to 3 mL of LB broth and incubated at 37°C overnight. Transconjugants were selected by plating on MacConkey agar plates containing sodium azide (100 µg/mL) and ciprofloxacin (0.5 µg/mL).[19] The transfer of PMQR genes in transconjugants was confirmed by PCR.

Incompatibility Grouping of Plasmid Encoding Resistance for PMQR Genes

Plasmid Inc group for the transconjugants was determined by PBRT. Five sets of multiplex PCR ([HI1, HI2, I1]; [X, L/M, N]; [FIA, FIB, W]; [YP FIC]; [A/C, T, FII$_S$]) and three simplex PCR (F$_{repB}$, K/B, B/O) were performed.[13] The primers employed is depicted in ►**Table 2**.[13]

Results

Antimicrobial Susceptibility Testing

All the study isolates were resistant to other fluoroquinolones—levofloxacin and ofloxacin. Resistance to other classes of antimicrobial agents was as follows: ceftazidime (86.9%), piperacillin/tazobactam (73.8%), imipenem (69.2%), and amikacin (63.8%). The MIC of ciprofloxacin ranged from 4 to greater than or equal to 256 µg/mL. The MIC$_{50}$ and MIC$_{90}$ values were 64 and greater than or equal to 256 µg/mL, respectively.

Detection of Efflux Pump Activity

Among 130 isolates, twofold reduction was evident in 46 (35.8%) and fourfold or more reduction was observed in 37 (28.4%). Fourfold was evident in 11 (12.9%), 8-fold in 5 (5.8%), 16-fold in 7 (8.2%), 32-fold in 3 (3.5%), and 128 fold in 2 (2.3%) among *P. aeruginosa.* In *A. baumannii*, 4-fold reduction was observed in one (2.2%) isolate, 8-fold in three (6.6%), 16-fold in two (4.4%), and 64-fold in three (6.6%), respectively (►**Table 3**).

Polymerase Chain Reaction

Among *P. aeruginosa*, *qnr* genes were detected in 36 (27.6%) isolates, of which 12 (14.1%) isolates harbored *qnrB*, 12 (14.1%) carried *qnrS* gene, and 9 (10.5%) isolates harbored both *qnrB* and *qnrS* genes. Among *A. baumannii*, *qnrB* was detected in two (4.4%) isolates and only one (2.2%) harbored both the *qnrA* and *qnrS*; 77.6% (66) of *P. aeruginosa* and 46.6% (21) of *A. baumannii* isolates harbored *aac(6′)-Ib-cr* gene; 3.5% (3) of *P. aeruginosa* and 2.2% (1) of *A. baumannii* isolates harbored *oqxAB* gene. *qepA* gene was not detected in any of the study isolates. The PMQR genes encountered is depicted in ►**Table 4**.

PMQR Gene Transfer and Distribution of Plasmid Replicons

In *P. aeruginosa*, 9.2% (12/130) were transferred successfully. All the 12 transconjugants were positive only for *aac(6′)-Ib-cr* gene. In *A. baumannii*, none of them was transferable.

The plasmid incompatibility types of the transconjugants were recognized by PBRT. Of the 12 transconjugants, 7 (58.3%) belonged to IncFII type plasmid replicon, 4 (33.3%) were IncA/C, and 1 (8.3%) IncFIC type.

Table 2 Primers for PCR-based replicon typing[13]

Replicon type	Primer sequence (5′–3′)	Amplicon size (bp)
HI1	F-GGAGCGATGGATTACTTCAGTAC R-TGCCGTTTCACCTCGTGAGTA	471
HI2	F-TTTCTCCTGAGTCACCTGTTAACAC R-GGCTCACTACCGTTGTCATCCT	644
I1	F-CGAAAGCCGGACGGCAGAA R-TCGTCGTTCCGCCAAGTTCGT	139
X	F-AACCTTAGAGGCTATTTAAGTTGCTGAT R-TGAGAGTCAATTTTTATCTCATGTTTTAGC	376
L/M	F-GGATGAAAACTATCAGCATCTGAAG R-CTGCAGGGGCGATTCTTTAGG	785
N	F-GTCTAACGAGCTTACCGAAG R-GTTTCAACTCTGCCAAGTTC	559
FIA	F-CCATGCTGGTTCTAGAGAAGGTG R-GTATATCCTTACTGGCTTCCGCAG	462
FIB	F-GGAGTTCTGACACACGATTTTCTG R-CTCCCGTCGCTTCAGGGCATT	308
W	F-CCTAAGAACAACAAAGCCCCCG R-GGTGCGCGGCATAGAACCGT	242
Y	F-AATTCAAACAACACTGTGCAGCCTG R-GCGAGAATGGACGATTACAAAACTTT	765
P	F-CTATGGCCCTGCAAACGCGCCAGAAA R-TCACGCGCCAGGGCGCAGCC	534
FIC	F-GTGAACTGGCAGATGAGGAAGG R-TTCTCCTCGTCGCCAAACTAGAT	262
A/C	F-GAGAACCAAAGACAAAGACCTGGA R-ACGACAAACCTGAATTGCCTCCTT	465
T	F-TTGGCCTGTTTGTGCCTAAACCAT R-CGTTGATTACACTTAGCTTTGGAC	750
FII_{S}	F-CTGTCGTAAGCTGATGGC R-CTCTGCCACAAACTTCAGC	270
F_{repB}	F-TGATCGTTTAAGGAATTTTG R-GAAGATCAGTCACACCATCC	270
K/B	F-GCGGTCCGGAAAGCCAGAAAA R-TCTTTCACGAGCCCGCCAAA	160
B/O	F-GCGGTCCGGAAAGCCAGAAAA R-TCTGCGTTCCGCCAAGTTCGA	159

Abbreviation: PCR, polymerase chain reaction.

Discussion

Fluoroquinolones are potent antibiotics active against a broad range of bacteria. The global increase in the prevalence of clinical strains with reduced susceptibility to fluoroquinolones constitutes a major concern.[20] The emergence of fluoroquinolone resistance among *P. aeruginosa* and *A. baumannii* presents a serious challenge in clinical management of bacterial infections.[21] In this study, antibiotic susceptibility testing revealed that all the test isolates were resistance to the other fluoroquinolones also: levofloxacin and ofloxacin. Resistance to other class of antibiotics was ceftazidime (86.9%), piperacillin/tazobactam (73.8%), imipenem (69.2%), and amikacin (63.8%). In this study, 32.9% (28) of *P. aeruginosa* and 37.7% (17) of *A. baumannii* isolates had an MIC of greater than or equal to 256 µg/mL to ciprofloxacin and similar observation has also been documented by Zaki et al.[22]

In this study, the MIC decrease factor value of fourfold and more reduction was evident in 28.4% (37/130) of isolates. In one isolate (4.5%) (*P. aeruginosa*), it resulted in loss of ciprofloxacin resistance thus reflecting a highly active efflux activity as evidenced by the decrease in MIC from 256 to 0.5 µg/mL on addition of CCCP inhibitor. Similar inhibition of efflux pump activity has been described previously.[23,24] Researchers in the United States, Iran, and Bahrain have observed nonsignificant twofold reduction in MIC on addition of efflux pump inhibitor CCCP in *P. aeruginosa*.[25–27] Helmy et al in their study reported significant efflux pump activity in *A. baumannii* and *P. aeruginosa* (46.1 and 41.1%).[28] Gomaa et al in Egypt recorded a high percentage of efflux pump-mediated resistance (77.8%) in *A. baumanni*.[29] In contrast, in South Africa, CCCP inhibitors did not affect MIC in *A. baumanni*.[24]

In this study, among *qnr* genes, 14.1% (12) were *qnrB* and 14.1% (12) were *qnrS* in *P. aeruginosa* which is a higher rate compared with a previous study from Egypt, which documented the presence of *qnrB* and *qnrS* genes in 1.8 and 2.7% of *Pseudomonas* spp.[18] In contrast, El-Badawy et al and Rafiq et al documented high prevalence of *qnrS* (79.5 and 24%) gene, respectively. *qnrA* and *qnrB* were not detected in any of their isolates of *Pseudomonas* spp.[30,31] Similar to the earlier study, *qnrA* gene was not detected in any of the isolates of *P. aeruginosa* in the present study. In China, a single isolate of *P. aeruginosa* with *qnrA* has been observed.[32] In many other studies, *qnr* determinants were not detectable in clinical isolates of *P.aeruginosa*.[33,34]

Yang et al examined the prevalence of *qnr* genes among 39 isolates of *A. baumannii* where 7.7% (3/39) isolates harbored *qnrB* and 2.6% (1/39) *qnrS*.[12] Hamed et al also reported the presence of *qnrS* gene in one isolate of *A. baumannii*.[35] Touati et al observed *qnrA* gene in only one isolate in their study.[36] In the present study, *qnrB* was encountered in two isolates singly. One isolate harbored *qnrB* and *qnrS*, while another carried both *qnrA* and *qnrS*.

Table 3 Effect of CCCP on the ciprofloxacin MIC

Organism ($n = 130$)	Fold reduction in MIC + CCCP (µg/mL)							
	0	2	4	8	16	32	64	128
Pseudomonas aeruginosa ($n = 85$)	21	36	11	5	7	3	0	2
Acinetobacter baumannii ($n = 45$)	26	10	1	3	2	0	3	0

Abbreviations: CCCP, carbonyl-cyanide m-chlorophenylhydrazone; MIC, minimal inhibitory concentration.

Table 4 Distribution of PMQR genes

PMQR genes	*Pseudomonas aeruginosa* ($n = 85$)	*Acinetobacter baumannii* ($n = 45$)	Total prevalence ($n = 130$)
aac(6′)-Ib-cr	31 (36.4%)	17 (37.7%)	48 (36.9%)
oqxAB	1 (1.1%)	1 (2.2%)	2 (1.5%)
qnrB + *aac(6′)-Ib-cr*	12 (14.1%)	2 (4.4%)	14 (10.7%)
qnrS + *aac(6′)-Ib-cr*	12 (14.1%)	0	12 (9.2%)
qnrA + *qnrS* + *aac(6′)-Ib-cr*	0	1 (2.2%)	1 (0.7%)
qnrB + *qnrS* + *aac(6′)-Ib-cr*	9 (10.5%)	1 (2.2%)	10 (7.6%)
qnrB + *qnrS* + *oqxAB* + *aac(6′)-Ib-cr*	1 (1.1%)	0	1 (0.7%)
oqxAB + *aac(6′)-Ib-cr*	1 (1.1%)	0	1 (0.7%)
Total	67 (78.8%)	22 (48.8%)	89 (68.5%)

Abbreviation: PMQR, plasmid-mediated quinolone resistance.

In Brazil, a low prevalence of *aac(6′)-Ib-cr* gene (2.6%) was found in *P. aeruginosa*.[37] Studies from Turkey and Egypt reported a high prevalence 56.4 and 79.5% in *P. aeruginosa*, respectively. This is similar to the findings of the present study (66.9%).[38] In this study, only four (3%) isolates harbored *oqxAB.* Notably, *qepA* gene was not encountered. *oqxAB* and *qepA* genes were not identified in many other studies too.[39–41]

Conjugation experiments demonstrated that in 14.1% (12/85) of *P. aeruginosa*, PMQR determinants were successfully transferred and all the transconjugants harbored the *aac(6′)-Ib-cr* gene. In *A. baumannii*, none of them was transferable. Jiang et al in their study documented that in 33.3% of nonfermenting gram negative bacteria (NFGNB), the transconjugants harbored the same PMQR determinants as their donors.[42] In this study, more than one half of PMQR determinants, 59.2% were non-conjugative, and this suggests that these genes may be of chromosomal location. Among the PMQR genes, high incidence of *aac(6′)-Ib-cr* (66.9%) was encountered and when conjugated, the transferability rate was 100% for this gene. This emphasizes that *aac(6′)-Ib-cr* gene plays a major role in mediating fluoroquinolone resistance. In the present study, of the 12 transconjugants, 33.3% belonged to IncA/C type plasmid replicon. In Nigeria, IncF plasmid harboring *aac(6′)-Ib-cr* gene has been described in *P. aeruginosa*.[43] A recent study in Argentina has demonstrated plasmid IncR group in *P. aeruginosa*.[44]

In this study, the prevalence rate of PMQR determinants is (68.5%), which is higher than the rates in China (1.7%), Egypt (4.5%), and Nigeria (61%).[18,42,45] However, increasing rates of fluoroquinolone resistance have limited the treatment option. The approach of combined antibiotic therapies is an alternative to this phenomenon.[46]

The ciprofloxacin resistance isolates which were negative for PMQR genes in our study may probably harbor the chromosomal mutation genes (*gyrA*, *gyrB*, *parC*, and *parE*). These genes were not looked for in the present study.

Conclusion

Plasmid-mediated fluoroquinolone resistance is encountered in (78.8%) of *P. aeruginosa*, while in *A. baumannii*, it is present in a proportion of 48.8% of clinical isolates. Single clinical isolate can harbor multiple PMQR genes. Plasmid-mediated efflux fluoroquinolone resistance is responsible only for a small proportion of resistance to fluoroquinolone in clinical isolates of *P. aeruginosa* and *A. baumannii*. Of the plasmid efflux pump genes, only *oqxAB* is present in 3% of isolates. It is reasonable to assume that of the plasmid-mediated resistance, *aac(6′)-Ib-cr* is primarily responsible for mediating a major proportion of resistance to fluoroquinolones. IncFII is the predominant plasmid type followed by IncA/C and IncFIC type.

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Conflict of Interest
None declared.

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