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RESEARCH ARTICLE

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Investigation of plasmid-mediated quinolone resistance genes among clinical isolates of *Klebsiella pneumoniae* in southwest Iran

Nabi Jomehzadeh | Khadijeh Ahmadi | Mozhdeh Amiri Bahmanshiri

Department of Microbiology, School of Medicine, Abadan University of Medical Sciences, Abadan, Iran

Correspondence
Nabi Jomehzadeh, Department of Microbiology, School of Medicine, Abadan University of Medical Sciences, Abadan, Iran.
Emails: n.jomehzadeh@abadanums.ac.ir; jomehzadeh.n@gmail.com

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Abstract

Background: Extensive and inappropriate use of quinolones has led to growing resistance rates to these broad-spectrum antibiotics. The present study purposed to investigate the prevalence of plasmid-mediated quinolone resistance (PMQR) genes in *Klebsiella pneumoniae* clinical isolates.

Method: Ninety-two non-repetitive *K. pneumoniae* clinical isolates were confirmed by standard microbiological methods. Antibacterial susceptibility of isolates toward seven agents from the quinolone family was evaluated by the disc diffusion method. Ciprofloxacin minimum inhibitory concentrations (MICs) were determined using the standard agar dilution method. PCR amplification was used to detect the existence of PMQR genes in the studied isolates.

Results: In the present study, significant quinolones' resistance (40%) was observed in *K. pneumoniae* isolates, and most of the strains were resistant to nalidixic acid (94.6%) and ofloxacin (45.6%). MIC analysis showed 15 strains were resistant to 6–128 μg/ml of ciprofloxacin, and five were intermediately-resistant. PMQR genes were detected in 88% of all isolates. *Acc(6')-Ib-cr* was constituted half of the total PMQR genes detected among ciprofloxacin non-susceptible isolates. Of 20 ciprofloxacin non-susceptible isolates, 65% (n = 13) harbored multiple PMQR determinants, and 15 strains were determined as integron carriage.

Conclusion: The findings of this study indicated considerable resistance against quinolones, which could be correlated with the extensive and inappropriate use of this class of antibiotics as empirical treatment.

KEYWORDS
integrons, *Klebsiella pneumoniae*, minimum inhibitory concentration, PMQR, quinolones

1 | INTRODUCTION

Klebsiella pneumoniae is one of the pioneer pathogens in causing a wide range of nosocomial and community-acquired infections, including septicemia, pneumonia, meningitis, soft tissue infections, and urinary tract infections.[1,2] Global concerns about this pathogen are growing due to the increased incidence of resistance to quinolones and other classes of antibiotics, as well as the declining effectiveness of treating infections.[3,4] Fluoroquinolones are a class of broad-spectrum bactericidal agents that have been raised

as a suitable therapy option for a variety of infectious diseases.[5] However, various studies have revealed that as a consequence of widely non-prescribed usage, a significant percentage of *K. pneumoniae* strains have become resistant to these antimicrobials.[6] To date, several mechanisms have been described for fluoroquinolone resistance. The main mechanism involves mutations in the chromosomal genes encoding quinolones target-site, including DNA gyrase and topoisomerase IV.[7] The other mechanism attributed to fluoroquinolones resistance is plasmid-mediated quinolone resistance (PMQR), three types have been described so far: (i) genes encoding qnr proteins (*qnrA*, *qnrB*, *qnrC*, *qnrD*, and *qnrS*), which protect the quinolone target enzymes, (ii) *aac (6′)-Ib-cr* a variant of aminoglycoside acetyltransferase, which modifies ciprofloxacin and norfloxacin causing reduced susceptibility against them, (iii) quinolone efflux pumps (*qepA* and *OqxAB*), which excrete fluoroquinolone from cells.[8] Integrons are the primary source of resistance genes and are known as the repository of antibiotic-resistance genes in microbial populations. These mobile genetic elements, usually carried by conjugated plasmids, have been shown to confer in the dissemination of antibiotic-resistance genes and the development of multiple drug resistance (MDR).[9] Furthermore, integrons can be also acquired, harbored, and horizontally spread PMQR genes in the form of gene cassettes.[10] So far, no comprehensive investigation on the frequency of PMQR determinants in *K. pneumoniae* clinical isolates in this region. Hence, this research was undertaken to evaluate the distribution of integron and PMQR genes among quinolone and fluoroquinolone-resistant strains recovered from clinical specimens in southwest Iran.

2 | MATERIALS AND METHODS

2.1 | Bacterial isolation

From January 2021 to June 2021, 92 non-duplicate *K. pneumoniae* isolates were collected from clinical specimens of patients admitted to affiliated hospitals of the Abadan University of Medical Sciences. The isolates were accurately identified by standard microbiological and biochemical tests[11] and stored in trypticase soy broth (TSB; Merck) containing 20% glycerol at −70°C.

2.2 | Susceptibility tests

The antimicrobial susceptibility testing was performed for *K. pneumoniae* strains by the Kirby-Bauer disc diffusion technique under the Clinical and Laboratory Standards Institute (CLSI) guidelines[12] for the following standard antibiotics (Roscoe): nalidixic acid (NA; 30 μg) ciprofloxacin (CIP; 5 μg), ofloxacin (OFX; 5 μg), levofloxacin (LEV; 5 μg), gatifloxacin (GAT; 5 μg), norfloxacin (NOR; 10 μg), and moxifloxacin (MOX; 5 μg). Minimum inhibitory concentration (MIC) was also performed according to CLSI guidelines for ciprofloxacin non-susceptible isolates by the standard agar dilution method.[12] *E. coli* ATCC 25922 was used as a quality stander strain.

2.3 | Detection of PMQR and integrase genes by PCR

Crude DNA of the isolates was extracted by a commercial extraction kit (SinaClon BioScience Co.) following the manufacturer's protocol. The quality of DNA was assessed by a Nanodrop spectrophotometer (Nanodrop One; Thermo Scientific, Wilmington) then stored at −20°C until use. The frequency of PMQR genes was measured using three separate multiplex PCR, the first for *qnr* genes (A, B, and S), the second for *aac(6′)-Ib-cr*, and *qepA* genes, and the third for *oqxAB*. Moreover, PMQR-positive isolates were screened for class I and class II integrons (*intI1* & *intI2*) using a multiplex PCR. Each reaction mixture was prepared in a final volume of 25 μl containing 12.5 μl of Master mix Red (SinaClon Co.), 0.5 μl (25 pmol) of each primer, 1 μl of template DNA, and 10.5 μl sterile distilled water. The primer sequences and PCR conditions are listed in Table 1. The reactions were conducted in C1000 Bio-Rad Thermal Cycler (Bio-Rad Laboratories, Inc.). The PCR products were separated on 1.5% agarose gel stained with ethidium bromide (0.5 mg/ml).

2.4 | Conjugation test

Plasmid transferability from PMQR-positive isolates that served as donors was investigated using a phenotypic conjugation test as previously described.[16] *Escherichia coli* J53 AziR (sodium azide-resistant) strain was applied as the recipient cells. Mating was conducted in the Log phase by the addition 0.5 ml of both donor and recipient cells in 3 ml of LB (Luria-Brittany; Himedia Laboratories) broth and incubated at 37°C for 24 h. Transconjugants cells were cultured on MacConkey agar plates containing 100 μg/ml sodium azide and 0.5 μg/ml ciprofloxacin. To detect transferable antibiotic-resistance determinants, the probable transconjugants were tested for their resistance to all seven antibiotics. Finally, transconjugants were assessed by PCR test for the determination of transferability of PMQR genes.

3 | RESULTS

Of the 92 *K. pneumoniae* species, 78 (84.8%) were isolated from urine, 5 (5.4%) from the wound, 5 (5.4%) from blood, and 4 (6.5%) from respiratory tract samples. The investigated isolates displayed different fluoroquinolones resistance activities. The highest rates of resistance were observed toward nalidixic acid (94.6%) and ofloxacin (45.6%) (Table 2). The MIC was assessed for 20 (21.7%) ciprofloxacin non-susceptible isolates and the results showed 15/20 (75%) were resistant to 6–128 μg/ml of ciprofloxacin, while 5/20 (25%) were intermediately-resistant (MIC = 1.5–3 μg/ml) (Table 3). Molecular analysis revealed 81 (88%) isolates harboring at least one PMQR determinant: 67/81 (82.7%) *acc(6′)-Ib-cr*, 29/81 (35.8%) *oqxA*, 22/81 (27.1%) *oqxB*, 20/81 (24.7%) *qnrB*, 14/81 (17.3%) *qnrS*, and 10/81 (12.3%) *qnrA* (Figure 1). None of the isolates carried *qepA* genes.

TABLE 1 Multiplex PCR primers used to detect PMQR and integrase genes

Gene	Primer Sequence (5′→3′)	Size of product	PCR conditions	Reference
qnrA	TCAGCAAGAGGATTTCTCA GGCAGCACTATTACTCCCA	516	94°C, 7 min; 35 cycles of 94°C for 50 s, 53°C, 40 s, 72°C, 1 min, final extension 72°C, 5 min.	11
qnrB	GATCGTGAAAGCCAGAAAGG ACGATG CCTGGTAGTTGTCC	469		
qnrS	ACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC	417		
aac(6′)-Ib-cr	TTGGAAGCGGGGACGGAM ACACGGCTGGACCATA	260	94°C, 2 min; 35 cycles of 94°C for 30 s, 55°C, 30 s, 72°C, 2 min, final extension 72°C, 5 min.	13
qepA	GCAGGTCCAGCAGCGGGTAG CTTCCTGCCCGAGTATCGTG	199		
oqxA	AACCTCGTCTCCCGTGAAGAGTGG TGAACGCTCTCCACCGCTTCAA	392	95°C, 10 min; 30 cycles of 94°C for 1 min, 55°C, 2 min, 72°C, 4 min, final extension 72°C, 10 min.	14
oqxB	CAGCTCAACAATAAGGATGCGGTC GGAGATCAGGAAATCGCTCTCCTG	512		
intI1	GTTCGGTCAAGGTTCTG GCCAACTTTCAGCACATG	920	94°C, 2 min; 30 cycles of 94°C for 1 min, 50°C, 1 min, 72°C, 30 s, final extension 72°C, 5 min.	15
intI2	ATGTCTAACAGTCCATTTTT AAATCTTTAACCCGCAAAC	420		

TABLE 2 Antibiotic susceptibility pattern of *Klebsiella pneumoniae* isolates

Antibiotics	Susceptible (%)	Intermediate (%)	Resistance (%)
Nalidixic Acid	5 (5.4)	0	87 (94.6)
Ciprofloxacin	72 (78.3)	3 (3.2)	17 (18.5)
Ofloxacin	42 (45.6)	8 (8.7)	42 (45.6)
Levofloxacin	57 (62)	7 (7.6)	28 (30.4)
Gatifloxacin	63 (68.5)	3 (3.2)	26 (28.3)
Norfloxacin	50 (54.3)	2 (2.2)	40 (43.5)
Moxifloxacin	75 (81.5)	0	17 (18.5)

Among the 20 ciprofloxacin non-susceptible strains 10 (50%) carried *acc(6′)-Ib-cr*, which was the most frequent either alone or in co-existence with other PMQR genes. This was followed by *oqxA* and *qnrB* (n = 8, 40%), *oqxB* (n = 7, 35%), *qnrS* (n = 3, 15%), and *qnrA* (n = 1, 5%) (Table 3). Notably, the vast majority (13/20, 65%) of CIP-non-susceptible strains were found to harbor multiple PMQR determinants, whiles the remaining seven strains had only one gene. As shown in Table 3, MIC levels were higher in isolates with multiple PMQR genes. Also, strains with a MIC > 128 showed resistance to the majority of antibiotics used. Remarkably, the two strains isolated from the sputum, in addition to containing three PMQR genes simultaneously, were resistant to most antibiotics and had a MIC > 128. The presence of integrons genes (*intI1* & *intI2*) was assessed among CIP-non-susceptible strains by PCR. The results showed 11 strains carried class 1 integron, 3 strains had class 2 integron, and one strain contained both classes. Interestingly, none of the intermediately-resistant isolates carried integron genes. Conjugation testing was performed for all ciprofloxacin non-susceptible strains, but only seven isolates were transferable. All transconjugants were resistant to tested quinolone agents. PCR tests confirmed that the transconjugants harbored identical multiple PMQR genes as their donors (Table 3). It is worth noting that only strains with MICs >32 μg/ml were determined to be transconjugant.

4 | DISCUSSION

The extensive usage of fluoroquinolones in recent years has led to the development of Enterobacteriaceae isolates that are resistant to them.[8] Among Enterobacteriaceae, *K. pneumoniae* is a pioneer species in resistance to various classes of antibiotics, including quinolones. This multidrug-resistant opportunistic bacterium constitutes a solemn challenge to infectious disease clinicians across the world.[7] To the best of our knowledge, this is the first survey that reports the prevalence of PMQR determinants among quinolone-resistant *K. pneumoniae* strains in Abadan, southwest Iran. In the present study, the rate of quinolones resistance was approximately 40%, and the majority of isolates were resistant to nalidixic acid and ofloxacin. This comes around consistent with the reported rate by Shokoohizadeh et al. (38%).[17] Our results for ciprofloxacin resistance in *K. pneumoniae* isolates (18.5%) were not in agreement with other reports, which have shown higher resistance.[18,19] Nevertheless, in previous studies performed by Razavi et al. and Priyadarshini et al. the prevalence of ciprofloxacin resistance was 19.6% and 19.09%, respectively, which was well in line with our results.[4,20] Although the exact reasons for these conflicting rates of ciprofloxacin resistance are unclear, differences in the number and type of specimens, geographic area, and methodology may explain the disparities. On a worldwide scale, the prevalence rate of quinolones resistance in *K. pneumoniae* isolates might be differed by geographic area; for example, resistance rates in Togo are 85.8%, 62.8% in Iraq, and

TABLE 3 Summary of phenotypic and genotypic features of ciprofloxacin non-susceptible isolates

Strains (n = 20)	Sample type	PMQR determinant	Integrase gene	FRP	CIP MIC (μg/ml)	TG
K14	Urine	*oqxA*, *oqxB*	*intI1*	NA, CIP, OFX	8	–
K5	Urine	*aac(6′)-Ib-cr*, *qnrS*	*intI1*	NA, CIP, LVX, NOR	32	+
K12	Urine	*qnrB*, *oqxA*	*intI1*	NA, CIP, OFX, NOR	6	–
K55	Urine	*qnrB*, *aac(6′)-Ib-cr*	*intI2*	NA, CIP, OFX, LVX, NOR	64	+
K24	Urine	*oqxA*, *oqxB*	*intI1*	NA, CIP, LVX	8	–
K30	Respiratory	*qnrA*, *qnrB*, *aac(6′)-Ib-cr*	*intI1*	CIP, OFX, LVX, NOR, GAT	>128	+
K16	Urine	*oqxB*	–	NA, CIP	1.5	–
K62	Urine	*aac(6′)-Ib-cr*, *oqxB*, *qnrB*	*intI2*	NA, CIP, NOR, GAT	32	+
K23	Urine	*aac(6′)-Ib-cr*	*intI1*	NA, CIP, NOR	16	–
K47	Urine	*qnrB*, *oqxA*, *oqxB*	*intI1*	NA, CIP, OFX, LVX	16	–
K48	Urine	*qnrB*, *qnrS*	*intI1*	CIP, OFX, NOR	6	–
K74	Urine	*oqxA*	–	CIP, OFX	1.5	–
K80	Urine	*qnrB*	–	NA, CIP, NOR	3	–
K7	Wound	*qnrB*, *qnrS*, *aac(6′)-Ib-cr*	*intI1*	CIP, OFX, LVX, NOR, GAT	>128	+
K36	Blood	*oqxA*, *oqxB*	*intI1*	CIP, OFX, LVX, NOR	6	–
K89	Blood	*aac(6′)-Ib-cr*, *oqxA*	*intI2*	CIP, LVX, NOR, GAT, MOX	64	+
K51	Respiratory	*aac(6′)-Ib-cr*, *oqxA*, *oqxB*	*intI1*, *intI2*	CIP, OFX, LVX, NOR, GAT, MOX	>128	+
K58	Urine	*aac(6′)-Ib-cr*	*intI1*	NA, CIP, LVX	16	–
K76	Urine	*qnrS*	–	NA, CIP, OFX	3	–
K66	Urine	*aac(6′)-Ib-cr*	–	NA, CIP	1.5	–

Abbreviations: CIP, ciprofloxacin; FRP, Fluoroquinolone Resistance pattern; GAT, gatifloxacin; LVX, levofloxacin; MOX, moxifloxacin; NA, nalidixic acid; NOR, norfloxacin; OFX, ofloxacin; TG, Transconjugant.

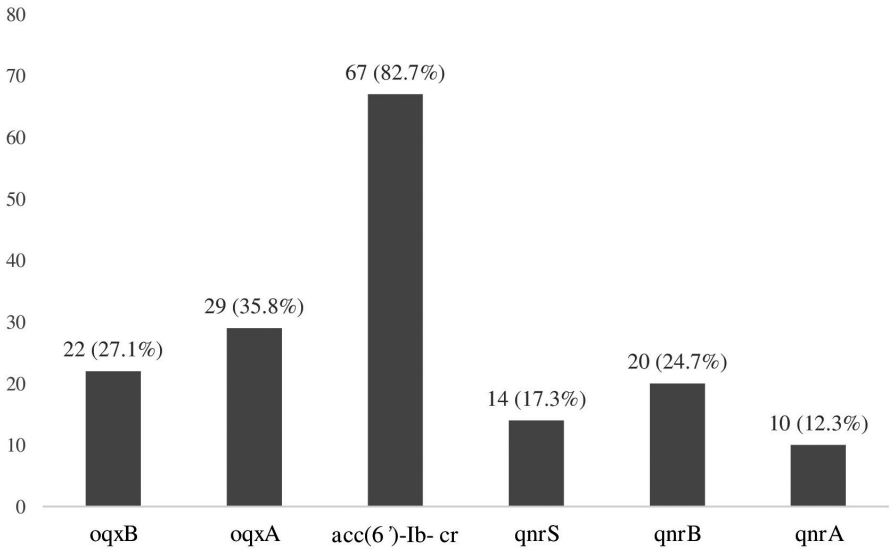

FIGURE 1 Distribution of plasmid-mediated quinolone resistance (PMQR) genes among *Klebsiella pneumoniae* isolates

34.8% in Iran.[7,21,22] Although the main cause of quinolones resistance is a mutation in chromosomal topoisomerases, PMQR genes have been believed that play a significant role in the development of quinolone-resistant isolates due to their high horizontal transferability.[23] This study reports the high prevalence (88%) of PMQR genes in isolates, in which *aac(6′)-Ib-cr* was detected as the most common gene. These results are in line with other studies conducted in Iraq,[7] Iran,[24] Korea,[25] and Thailand.[26] The *aac(6′)-Ib-cr* gene has been detected widely in Enterobacteriaceae isolates throughout the world, which demonstrates its enormous dissemination to other Gram-negative organisms.[27] The second most common PMQR genes detected in our isolates were the *oqxA* (35.8%) and oqxB (27.1%). However, the frequency of these genes is lower than that reported by Rodriguez-Martinez et al.[28] and Goudarzi et al.[24] These quinolone efflux pumps (*oqxAB*) confers reduce susceptibility to nalidixic acid, ciprofloxacin, norfloxacin, and some other antibiotics.[29] The finding showed that 20 (21.7%) of *K. pneumoniae* isolates were not susceptible to ciprofloxacin (MIC = 1.5–128 g/ml)

and frequently contained multiple PMQR determinants. We also observed that 75% of these twenty strains, contained integrons (mostly class 1) and showed a higher ciprofloxacin MIC (≥6–128 μg/ml) than those with only PMQR genes. Similarly, Jamshidi et al. disclosed that 82.3% of the ciprofloxacin-resistant *K. pneumoniae* isolates carried *intI1* (mostly along with PMQR genes) and discerned a significant correlation between integron carriage and decreased susceptibility to fluoroquinolones.[9] In another study conducted in Brazil, the predominance coexistence of PMQR genes and class 1 integron, were reported in *K. pneumoniae* and *E. coli* isolates.[30] Conjugation test results for ciprofloxacin-resistant isolates showed that all seven transconjugant strains not only carried multiple PMQR genes but remarkably also had a MIC ≥32 μg/ml. This was a lower rate of transferability than reported by Geetha et al.[8] In another study, Yang et al. demonstrated that PMQR determinants were transferable and that the MICs of ciprofloxacin for transconjugant strains harboring multiple PMQR genes were higher than for unconjugated recipient strains.[25] The lack of molecular typing and sequencing of PMQR genes was the study's primary limitation. In addition, chromosomal mutations in quinolone resistance-determining regions, notably in the *gyrA* and *parC* genes, have not been investigated. However, with the future assessment of more clinical isolates for these mutations, the influence of PMQR in quinolone resistance could be better illustrated. The findings of this investigation revealed a considerable prevalence of PMQR genes in *K. pneumoniae* clinical isolates in our region. The prevalence of clinical isolates of *K. pneumoniae* with multiquinolone-resistance, multiple PMQR determinants, and integrons in community and hospitals settings is very alarming because dissemination of these opportunistic pathogens could interfere with the treatment of common infections.

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CONFLICT OF INTEREST

The authors state that there is no conflict of interest in this work.

CONSENT TO PARTICIPATE

Conscious written consent was obtained from all participants.

CONSENT FOR PUBLICATION

The final manuscript was read and approved by all the authors.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article. For other data, these may be available through the corresponding author upon reasonable request.

ORCID

Nabi Jomehzadeh https://orcid.org/0000-0001-6379-3827

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