



# The Impact of Mutations in Topoisomerase Genes and the Plasmid-Mediated Quinolone Resistance (PMQR) Determinants on the Resistance to Fluoroquinolones in *Klebsiella pneumoniae*

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## Abstract

**Background:** *Klebsiella pneumoniae* causes a variety of infections including community acquired pneumonia and nosocomial infections.

**Objectives:** The aim of this study was to determine the plasmid-associated quinolone-resistance (PMQR) genes and mutations in quinolone-resistance determining region (QRDR) of topoisomerase genes in *K. pneumoniae*.

**Methods:** One hundred clinical *K. pneumoniae* isolates were collected from Kermanshah hospitals for this study. The minimum inhibitory concentrations (MIC) for levofloxacin and ciprofloxacin was determined by broth microdilution method. The presence of PMQR genes (*qnrA*, *qnrB*, *qnrS*, *aac* (6')-Ib, and *qepA*) among isolates resistant to fluoroquinolones was detected by PCR. All PCR products for topoisomerase genes (*gyrA*, *gyrB* and *parC*) were sequenced and analyzed.

**Results:** Sequence analysis showed mutations in *gyrA* at codons 83 and 87 in 24 (85.7%) and 20 (71.4%) of isolates, respectively. For *parC* mutations were detected at codons 80 and 84 in 15 (53.6%) and 7 (25%) isolates, respectively. At the outside of QRDR in *gyrA* and *gyrB*, mutations were also observed. The mutation was not detected in the QRDR of *gyrB*. The *qnrB*, *qnrS*, and *aac* (6')-Ib-cr were found in 1 (3.6%), 11 (39.3%) and 25 (89.3%) of isolates, respectively. However, the *qnrA* and *qepA* genes were not detected among isolates.

**Conclusions:** The resistance to fluoroquinolones is relatively high among *K. pneumoniae* and mutations in QRDR of topoisomerase genes play an important role for this resistance. The plasmid-mediated genes also increase the level of resistance. The mutations outside the QRDR of topoisomerase genes also occur, but their exact role in resistance needs to be clarified.

**Keywords:** Fluoroquinolones Resistance, QRDR Mutations, PMQR Determinants, *Klebsiella pneumoniae*

## 1. Background

*Klebsiella pneumoniae* causes a variety of infections, in particular, the community-acquired pneumonia and nosocomial infections. Hospital-acquired infections caused by this organism include urinary tract infection, pneumonia, septicemia, and wound infection. It has been estimated that about 8% of nosocomial infections are caused by *K. pneumoniae* (1, 2). Given the acquisition of various resistant genes, the choice of effective antibiotics to treat infections caused by this bacterium has been limited. The broad-spectrum fluoroquinolones have been used to treat a wide range of bacterial infections (3). Fluoroquinolones bind to and inhibit the enzyme activity of topoisomerase II (DNA gyrase) and topoisomerase IV required for appropriate bacterial DNA functions. DNA gyrase is a tetramer enzyme consisting of two subunits A and two subunits B,

which are coded by the *gyrA* and *gyrB* genes, respectively. Topoisomerase IV is also coded by two genes, *parC* and *parE* (4). DNA gyrase is involved in creating the negative super coiled DNA and the role of topoisomerase IV is to separate daughter chromosomes at the end of DNA replication cycles (5, 6).

Given the widespread use of fluoroquinolones, resistance to these drugs has been reported worldwide (7). So far, several mechanisms of resistance to fluoroquinolones have been identified which include mutations in topoisomerase genes (8) and reduce intracellular drug accumulation within the bacterial cell and the acquisition of plasmid-mediated quinolone-resistance genes (PMQR) (8). The mutation in topoisomerase genes is the common mechanism of resistance to fluoroquinolones and usually occurs in a certain quinolone-resistance determining re-

gion of DNA (QRDR). Mutations in *gyrA* are more common than *gyrB* gene (9). In *gyrA*, mutations usually occur at nucleotide 201 to 320 encoded amino acids 67 to 106, in particular at serine 83 and aspartate 87 (10). Mutations at codon 426 and 447 of *gyrB* usually make a low-level of resistance to fluoroquinolones (11, 12). Mutations in the amino acid 78, 80 and 84 positions of *parC* in *K. pneumoniae* resistant to fluoroquinolones have also been reported worldwide although with different rates (10, 13).

The acquisition of PMQR genes by *K. pneumoniae* also plays an important role in the resistance to fluoroquinolones. Three PMQR genes have been reported which include *qnr*, *aac(6′)-Ib-cr* and *qepA*. The *qnr* gene family encodes proteins that protect topoisomerase II and IV from quinolones impacts. The *aac(6′)-Ib-cr* gene, which was first discovered in 2003, decreases the activity of fluoroquinolones including ciprofloxacin and norfloxacin (14, 15). The *qepA* gene causes a high level of resistance to hydrophilic fluoroquinolones (16).

## 2. Objectives

To better understand the role of these mechanisms in antibiotic resistance, this study aimed to determine mutations in the QRDR of *gyrA*, *gyrB* and *parC* genes and the frequency of PMQR genes in *K. pneumoniae* isolates resistant to fluoroquinolones.

## 3. Methods

### 3.1. Bacterial Isolates

In this experimental study, One hundred non-duplicate isolates of *K. pneumoniae* were collected from 2013 to 2014 in three general hospitals in Kermanshah during an increase of *K. pneumoniae* infection among hospitalized patients. The clinical samples included urine (n = 54, 54%), burn (n = 15, 15%), respiratory tract secretions (n = 15, 15%), and others (blood, wound and ascitic fluid) (n = 16, 16%). They were collected from 59 (59%) female and 41 (41%) male people with the average age of  $39.5 \pm 26$  years old. All isolates were identified by bacteriological and biochemical tests followed by confirmation using API20E Kit (bio-Merieux, France).

### 3.2. Antibiotic Susceptibility Testing

Minimum inhibitory concentrations (MIC) of levofloxacin and ciprofloxacin (Sigma, USA) were determined by a standard broth microdilution method according to CLSI protocols (17). Briefly, Mueller-Hinton broth (Merck, Germany) with a final inoculum of  $5 \times 10^5$  CFU/mL in 96-well plates were incubated at 37°C for 24 hours. *E. coli*

strain ATCC 25922 was used as the standard drug-susceptible control for MIC measurements. The breakpoints recommended by CLSI were used for ciprofloxacin (susceptible  $\leq 1 \mu\text{g/mL}$ ; resistant  $\geq 4 \text{ mg/mL}$ ) and levofloxacin (susceptible  $\leq 2 \mu\text{g/mL}$ ; resistant  $\geq 8 \text{ mg/mL}$ ).

### 3.3. DNA Extraction and PCR Assay

The DNA of isolates resistant to fluoroquinolones was extracted using the boiling method. Amplification of the QRDR of *gyrA*, *gyrB*, and *parC* genes was carried out by PCR using specific primers (SinaClon, Iran) (Table 1). The PMQR genes (*qnrA*, *qnrB*, *aac(6′)-Ib* and *qepA*) were amplified using specific primers by PCR (SinaClon, Iran) (Table 1). After electrophoresis of PCR products on 1% agarose gel (Merck Co, Germany) and staining with ethidium bromide, the gels were visualized by gel-Documentation apparatus (Bio-Rad, USA). PCR products were sequenced and analyzed by BLAST tools (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

### 3.4. Restriction Digestion

RFLP was used to differentiate the naive *aac(6′)-Ib* gene from those of the *cr* variant of this gene involved in fluoroquinolones resistance. All PCR products for *aac(6′)-Ib* gene were digested by BtsCI restriction endonuclease (Fermentase, England). The presence of two DNA fragments with 272 and 210 bp by digestion indicated of *aac(6′)-Ib*, whereas an undigested DNA indicated of *aac(6′)-Ib-cr* variant.

### 3.5. Sequencing and Data Analysis

All PCR products for *gyrA*, *gyrB*, and *parC* genes were purified using a DNA purification kit and sequenced (SinaClon, Iran). The DNA sequence was performed using an ABI 3730XL DNA analyzer (Macrogen Inc, Korea). Sequence data were analyzed for homology using the national center for biotechnology information GenBank database (<http://www.ncbi.nlm.nih.gov/>). The nucleotide sequence data have been deposited in the pubmed genebank nucleotide sequence database (<http://www.ncbi.nlm.nih.gov/>).

All data were statistically analyzed using SPSS version 20 for associations between mutations with the PMQR genes and the level of fluoroquinolone resistance by Kruskal-Wallis and Mann-Whitney tests.

## 4. Results

The antibiotic susceptibility and MIC of ciprofloxacin and levofloxacin for 100 *K. pneumoniae* isolates have been shown in Table 2 and Figure 1.

DNA sequence analysis of the QRDR of *gyrA* showed mutations at codon 83 of 24 (85.7%) and at codon 87 of 20

**Table 1.** Primers Used in This Study

Primer	DNA Sequence 5'-3'	Target Site	Amplicon Size, bp	Reference
<i>gyrA</i> -F	CGA CCT TGC GAG AGA AAT	QRDR of <i>gyrA</i>	626	(18)
<i>gyrA</i> 63R	GTT CCA TCA GCC CTT CAA			
<i>gyrB</i> -F	TCTACTGCTTYACCAACAACA	QRDR of <i>gyrB</i>	704	(19)
<i>gyrB</i> -R	CGTCCGATCGGTATGAT			
<i>parC</i> -F	ATGGCAGAGCGCTTGGG	QRDR of <i>parC</i>	437	(19)
<i>parC</i> -R	GCCGTCRAAGTTGGCAC			
<i>qnrA</i> -F	ATTCTCACGCCAGATTGG	<i>qnrA</i>	516	(20)
<i>qnrA</i> -R	GATCGCAAAGTTAGTCA			
<i>qnrB</i> -F	GATCGTGAAGCCAGAAGG	<i>qnrB</i>	469	(20)
<i>qnrB</i> -R	ACGATGCTGTTAGTTGTC			
<i>qnrS</i> -F	ACGACATCGTCACTGCAA	<i>qnrS</i>	417	(20)
<i>qnrS</i> -R	TAAATTGGCACCTGTAGGC			
<i>aac(6')-Ib</i> -F	TTGGATGCTCTATGAGTGGTA	<i>aac(6')-Ib</i>	482	(15)
<i>aac(6')-Ib</i> -R	CTCGAATGCCTGGCGTGT			
<i>qepA</i> -F	GGACATCTACGGCTCTTCG	<i>qepA</i>	720	(14)
<i>qepA</i> -R	AGCTGACGTAICTGCGTCAT			

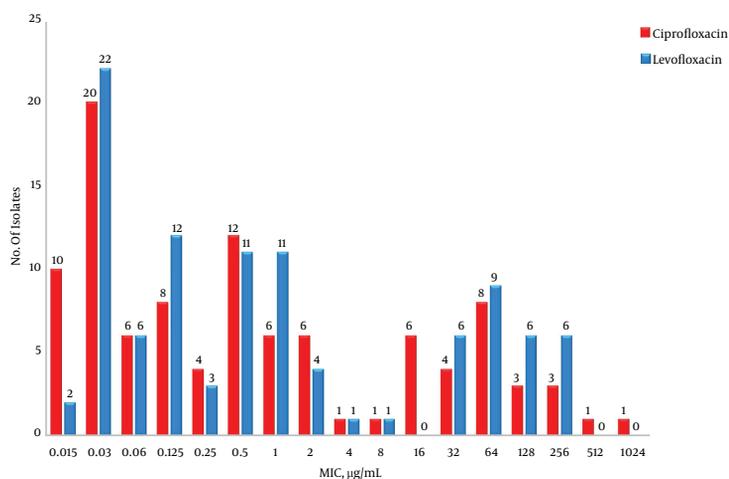
Abbreviations: bp, base pair; F, forward primer; R, reverse primer.

**Table 2.** Susceptibility of *K. pneumoniae* Isolates to Flouroquinolones

Antibiotic	Sensitive, %	Intermediate, %	Resistance, %	MIC <sub>50</sub> <sup>a</sup>	MIC <sub>90</sub> <sup>b</sup>	MIC Range
<b>Ciprofloxacin</b>	66	6	28	0.5	64	0.015 - 1024
<b>Levofloxacin</b>	71	1	28	0.5	128	0.015 - 256

<sup>a</sup>The MIC at which 50% of the isolates are inhibited.

<sup>b</sup>The MIC at which 90% of the isolates are inhibited.



**Figure 1.** The Distribution of Isolates According to Their MIC ( $\mu\text{g/mL}$ )

(71.4%) isolates (Table 3). Sequence analysis of *parC* showed 15(53.6%) isolates with a mutation at codon 80 and 7 (25%) isolates with a mutation at codon 84.

In three isolates, an extra mutation outside QRDR in *gyrA* at position 161 was detected which did not make significant changes in MIC of ciprofloxacin and levofloxacin

**Table 3.** The Frequency of Mutations and Amino Acid Changes Within *gyrA*, *gyrB* and *parC* of 28 Isolates Resistant to Fluoroquinolones<sup>2</sup>

Gene	Amino Acid Position	Nucleotide Changes	Amino Acids Substitute	No. of Isolates (%)
<i>gyrA</i>	Serine 83/	TCC → TTC	Phenylalanine	12 (42.9)
	Asp87	GAC → GCC	Alanine	
	Serine 83/	TCC → TTC	Phenylalanine	1 (3.6)
	Asp87/	GAC → GCC	Alanine	
	Glu94	CAG → CAC	Histidine	
	Serine 83/	TCC → TTC	Phenylalanine	4 (14.3)
	Asp87/	GAC → AAC	Asparagine	
	Serine 83/	TCC → TTC	Phenylalanine	3 (10.7)
	Asp87/	GAC → AAC	Asparagine	
	Thr161	ACC → ATC	Isoleucine	
	Serine 83	TCC → ATC	Isoleucine	2 (7.1)
	Serine 83	TCC → TAC	Tyrosine	2 (7.1)
	Glu94	CAG → CAC	Histidine	1 (3.6)
	WT	-	-	3 (10.7)
<i>gyrB</i>	Asp368	GAC → AAT	Asparagine	1 (3.6)
	WT	-	-	27 (96.4)
<i>ParC</i>	Ser80	AGC → ATC	Isoleucine	15 (53.6)
	Glu84	GAA → AAA	Lysine	7 (25)
	WT	-	-	6 (21.4)

<sup>2</sup>Abbreviation: WT, wild type.

in comparison with isolates without this mutation ( $P = 0.645$ ). Similarly, one isolate with an extra mutation outside QRDR in *gyrA* at codon 94 had no significant impact on MIC compared with the isolates without this mutation for both antibiotics tested ( $P = 0.610$ ). Sequence analysis of *gyrB* showed a mutation outside QRDR in one isolates at codon 368, which apparently did not increase bacterial MIC.

The *qnrB* and *qnrS* were found in 11 (39.3%) and 1 (3.6%) isolates, respectively. Of the 28 isolates selected for detection of the *aac(6')-Ib-cr*, 25 of 28 (89.3%) isolates were positive for *aac(6')-Ib* and all of them was the *aac(6')-Ib-cr* variant. PMQR genes enhanced the resistance of isolates to fluoroquinolones (Table 4).

## 5. Discussion

The results of previous studies indicated that the rate of resistance to ciprofloxacin is higher than levofloxacin among Enterobacteriaceae (21, 22). However, in our study, all isolates resistant to ciprofloxacin were also resistant to levofloxacin but the average MIC for ciprofloxacin

was higher than levofloxacin that indicate levofloxacin is slightly more effective against *K. pneumoniae* isolates.

The impact of mutations in DNA gyrase and topoisomerase IV on the resistance to fluoroquinolones in bacteria have previously been studied (23). In Gram-negative bacteria, mutations usually occur in subunit *GyrA* protein of DNA gyrase which is the first target of fluoroquinolones. DNA sequence analysis of our data showed 5 types of mutations in *gyrA* gene with amino acid changes among *K. pneumoniae* isolates which is consistent with the results of previous studies (13, 19). Mutation at codon 83 in *gyrA* was the most frequent one among fluoroquinolone resistant *K. pneumoniae* and substitution of the serine by phenylalanine was the most common changes. This result is consistent with the results of other studies (24). Moreover, mutation outside QRDR of *gyrA* was also detected in three strains at codon 161. Another Iranian paper has reported this mutation among *K. pneumoniae* isolates (24).

The MIC of fluoroquinolones for isolates with two mutations in *gyrA* gene was higher than isolates with only one mutation that is similar to other studies' results (25-28). Sequence analysis of QRDR in *parC* gene revealed two muta-

**Table 4.** Fluoroquinolone (FQ) Resistance Determinants Detected in the *K. pneumoniae* and corresponding Minimum Inhibitory Concentration (MIC) of Ciprofloxacin (CIP) and levofloxacin (LVX)

PMQR Genes	Mutations in <i>gyrA</i> and/or <i>parC</i>	MIC, µg/mL Range						No. of Isolates (%)
		CIP			LVX			
		4 - 16	32 - 128	> 256	4 - 16	32 - 128	> 256	
<i>qnrB</i> <sup>+</sup> <i>aac</i> (6')- <i>Ib-cr</i>	Ser83 + Asp87 + ser80 + Asp368			1			1	1 (3.6)
<i>qnrB</i> <sup>+</sup> <i>aac</i> (6')- <i>Ib-cr</i>	Ser83 + Asp87 + ser80/Glu84		4	2		4	2	6 (21.4)
<i>aac</i> (6')- <i>Ib-cr</i>	Ser83 + Asp87 + ser80/Glu84	3	3	1		5	2	7 (25)
-	Ser83 + Asp87 + ser80/Glu84	1	1			2		2 (7.1)
<i>aac</i> (6')- <i>Ib-cr</i>	Ser83 + Asp87 + Thr161 + Glu84		3			1		3 (10.7)
<i>aac</i> (6')- <i>Ib-cr</i>	Ser83 + Asp87 + Glu94 + ser80	1				1		1 (3.6)
<i>qnrB</i> <sup>+</sup> <i>aac</i> (6')- <i>Ib-cr</i>	Ser83 + ser80		1			1		1 (3.6)
-	Ser83 + ser80	1			1			1 (3.6)
<i>qnrB</i> <sup>+</sup> <i>aac</i> (6')- <i>Ib-cr</i>	Ser83		1			1		1 (3.6)
<i>aac</i> (6')- <i>Ib-cr</i>	Ser83	1				1		1 (3.6)
<i>aac</i> (6')- <i>Ib-cr</i> <sup>+</sup> <i>qnrS</i>	-			1			1	1 (3.6)
<i>qnrB</i> <sup>+</sup> <i>aac</i> (6')- <i>Ib-cr</i>	-		1			1		1 (3.6)
<i>aac</i> (6')- <i>Ib-cr</i>	-		1			1		1 (3.6)
<i>qnrB</i> <sup>+</sup> <i>aac</i> (6')- <i>Ib-cr</i>	Glu94	1				1		1 (3.6)

tions with amino acid changes which have been reported by several previous studies (19, 29). In the present study, all isolates with mutations in *parC* showed also mutations in *gyrA* that may suggest the topoisomerase IV is the second target for the fluoroquinolones (12). The isolates with mutations in both *gyrA* and *parC* simultaneously showed the higher MIC levels, which is compatible with the results of previous studies (13, 30). In three isolates, mutations in *gyrA* outside of QRDR were detected, but these mutations apparently do not significantly change the MIC value in comparison with isolates without mutations. In three isolates, MIC level was high, but without any mutations in the QRDR of *gyrA*, *gyrB* and *parC* genes. Since the plasmid genes can cause a low level of resistance, there may be other resistance mechanisms that involve such efflux pumps and mutation in *parE* gene as it has been reported by other studies. For instance, Al-Marzooq et al. and Park et al. have reported the *K. pneumoniae* isolates with resistant to ciprofloxacin but with no mutations in *gyrA* and *parC*. They suggested PMQR genes play an important role in the resistance to ciprofloxacin (13, 31).

The *aac* (6') -*Ib-cr* gene has recently been reported among bacteria and was the most common gene in our study. This is consistent with the results of other studies in Iran, South Korea and Sweden (32-34). In some studies in China and Thailand a low prevalence for *aac* (6') -*Ib-cr* has been reported (35, 36). Similarly, in these studies the frequency of *aac* (6') -*Ib-cr* was also higher than other genes. The frequency of *qnrB* gene in our study was high that is supported by the results of other studies (37, 38). On the other hand, the *qnrS* gene had a very low frequency, which is also similar to the results of previous studies, including studies in Iran, South Korea and China (33, 35). Furthermore, *qnrA* and *qepA* genes has not been found in several studies, including in Iran, Korea, Malaysia, which support our data for these genes (38, 39).

### 5.1. Conclusions

In conclusion, resistance to fluoroquinolones is relatively high among *K. pneumoniae* in Kermanshah. The MIC of isolates for fluoroquinolones increases by the accumulation of mutations within QRDR and harboring more PMQR genes. It seems that mutations in QRDR of topoisomerase

genes play an important role in *K. pneumoniae* resistant to fluoroquinolones. The plasmid mediated genes also increase the level of resistance. The mutations outside the QRDR of topoisomerase genes also occur in *K. pneumoniae* isolates but their exact role in resistance is not clear.

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## Footnotes

**Authors' Contribution:** Study concept and design: Alisha Akyaa, Keyghobad Ghadiri and Roya Chegene Lorestani; acquisition, analysis, and interpretation of data: Roya Chegene Lorestani and Azam Elahi; drafting of the manuscript: Alisha Akyaa, Roya Chegene Lorestani; critical revision of the manuscript for important intellectual content: Alisha Akyaa and Roya Chegene Lorestani; statistical analysis: Alisha Akyaa, Roya Chegene Lorestani and Azam Elahi; study supervision: Alisha Akyaa and Roya Chegene Lorestani.

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