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Frequency of DNA gyrase and topoisomerase IV mutations and plasmid-mediated quinolone resistance genes among *Escherichia coli* and *Klebsiella pneumoniae* isolated from urinary tract infections in Azerbaijan, Iran

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ABSTRACT

Objectives: This study assessed genetic alterations in *gyrA*, *gyrB*, *parC* and *parE* and the prevalence of plasmid-mediated quinolone resistance (PMQR) genes among *Escherichia coli* and *Klebsiella pneumoniae* isolates from urinary tract infections (UTIs) in Azerbaijan, Iran.
Methods: A total of 205 clinical isolates of *E. coli* ($n = 177$) and *K. pneumoniae* ($n = 28$) were obtained from UTIs. Antimicrobial susceptibility was determined by disk diffusion and agar dilution assays. The presence of PMQR genes was determined by PCR, and sequencing of the *gyrA*, *gyrB*, *parC* and *parE* was performed.
Results: The rate of fluoroquinolone (FQ) resistance among the isolates was 77.1%. The Ser83Leu mutation in *gyrA* was observed in all 60 FQ-resistant isolates selected for direct sequencing. The second most common mutation in *gyrA* was Asp87Asn. Frequent mutations in *parC* were Ser80Ile and Glu84Val. Ser359Ala + Ser367Thr and Gly385Cys mutations in *gyrB* were identified in one isolate each of *K. pneumoniae* and *E. coli*, respectively. The *parE* gene had mutations at Ile529Leu, Ser458Ala and Leu416Phe. Overall, PMQR determinants were identified in 90% of *E. coli* and 100% of *K. pneumoniae*. The prevalence of PMQR genes was as follows: *aac(6′)-Ib*-cr, 71.7%; *oqxB*, 51.7%; *oqxA*, 36.7%; *qnrB*, 28.3%; *qnrS*, 21.7%; *qnrD*, 16.7%; *qepA*, 5.0%; *qnrA*, 1.7%; and *qnrC*, 1.7%.
Conclusions: FQ resistance rates were high. Mutations in DNA gyrase and topoisomerase IV and the prevalence of PMQR genes in *E. coli* and *K. pneumoniae* isolates were alarming. Moreover, the combination of these resistance mechanisms plays an important role in high-level FQ resistance.

1. Introduction

Escherichia coli is the most common bacterial agent causing urinary tract infections (UTIs) [1]. Fluoroquinolones (FQs) have been the most commonly used antibiotics to treat UTIs caused by Gram-negative bacteria [2]. However, the extensive use of FQs has led to increasing FQ resistance. FQs inhibit the activity of DNA gyrase and topoisomerase IV enzymes, which are essential for bacterial DNA replication and survival [3]. The main cause of resistance to FQs is mediated by mutations in the quinolone resistance-determining regions (QRDRs) of *gyrA*, *gyrB*, *parC* and *parE* genes that lead to alteration of the target proteins (DNA gyrase and topoisomerase IV) [4]. Other resistance mechanisms are plasmid-mediated quinolone resistance (PMQR) determinants, overexpression of efflux pumps and alteration in membrane permeability [5,6].

Three types of PMQR determinants have been characterised, namely Qnr proteins, AAC(6′)-Ib-cr modified acetyltransferase and the efflux pumps QepA and OqxAB [7]. The *qnr* genes, including *qnrA*, *qnrB*, *qnrC*, *qnrD* and *qnrS*, encode pentapeptide repeat proteins that protect DNA gyrase and topoisomerase IV and lead to resistance to FQs [8]. The *aac(6′)-Ib*-cr gene encodes an aminoglycoside acetyltransferase resulting in FQ inactivation by acetylation of these antimicrobial agents [9]. The multidrug efflux pumps

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OqxAB and QepA belong to the resistance–nodulation–cell division family (RND) and the major facilitator superfamily (MFS), respectively. These pumps confer reduced susceptibility to FQs and other antimicrobial agents by drug extrusion from the cell.

The aim of this study was to determine genetic mutations in *gyrA*, *gyrB*, *parC* and *parE* and the prevalence of PMQR genes among *E. coli* and *K. pneumoniae* isolates from UTIs in Azerbaijan, Iran.

2. Materials and methods

2.1. Patients and bacterial strains

This study was carried out during April–October 2017 at hospitals of Tabriz and Urmia, in Iran. A total of 177 *E. coli* and 28 *K. pneumoniae* isolates were obtained from midstream urine samples of patients with UTIs. All urine samples were cultured on MacConkey agar and sheep blood agar. *E. coli* and *K. pneumoniae* isolates were identified using conventional biochemical tests in the Department of Microbiology, School of Medicine, Tabriz University of Medical Sciences (Tabriz, Iran).

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing of the isolates was performed by the Kirby–Bauer (disk diffusion) method according to Clinical and Laboratory Standards Institute (CLSI) recommendations [10] for the following antimicrobial agents: ampicillin (10 μg); cefotaxime (30 μg); cefuroxime (30 μg); cefazolin (30 μg); cefepime (30 μg); imipenem (10 μg); aztreonam (30 μg); nitrofurantoin (300 μg); gentamicin (10 μg); amikacin (30 μg); trimethoprim/sulfamethoxazole (30 μg); fosfomycin (200 μg); nalidixic acid (30 μg); ciprofloxacin (5 μg); levofloxacin (5 μg); ofloxacin (5 μg); gatifloxacin (5 μg); and moxifloxacin (5 μg). Minimum inhibitory concentrations (MICs) of nalidixic acid, ciprofloxacin and levofloxacin were determined by the agar dilution method according to CLSI guidelines [10]. *E. coli* ATCC 25922 was used as a quality control strain.

2.3. Detection of mutations in gyrA, gyrB, parC and parE

PCR amplification was carried out using primers previously described by Pitondo-Silva et al. [11]. A total of 60 FQ-resistant isolates (50 *E. coli* and 10 *K. pneumoniae*) were selected for direct sequencing based on the various MICs of nalidixic acid, ciprofloxacin and levofloxacin. Nucleotide sequencing of the PCR products was performed using an automated sequencer (Microsynth AG, Balgach, Switzerland). Mutations in *gyrA*, *gyrB*, *parC* and *parE* for the 50 FQ-resistant *E. coli* and 10 FQ-resistant *K. pneumoniae* were compared with the sequence of the reference genes *E. coli* K-12 (GenBank **NC_000913.3**) and *K. pneumoniae* ATCC 13883 (**DQ673325**). The sequences were aligned using ClustalX2 and MEGA4 multiple sequence alignment software.

2.4. Detection of plasmid-mediated quinolone resistance genes

Screening of PMQR genes [*aac(6′)-Ib*-cr, *oqxA*, *oqxB*, *qepA*, *qnrA*, *qnrB*, *qnrC*, *qnrD* and *qnrS*] was performed in 60 selected FQ-resistant isolates by PCR using the method described by Chen et al. [7].

2.5. Statistical analysis

Data analysis was performed using IBM SPSS Statistics for Windows v.22.0 (IBM Corp., Armonk, NY). A *P*-value of ≤0.05 was considered statistically significant.

3. Results

A total of 205 isolates, including 177 *E. coli* and 28 *K. pneumoniae*, were isolated from UTIs of 69 male patients (33.7%) and 136 female patients (66.3%). The mean ± standard deviation age of the patients was 50.4 ± 21.8 years. Samples were obtained from different hospital wards as follows: internal, 130 (63.4%); surgery, 41 (20.0%); intensive care unit, 20 (9.8%); and paediatric, 14 (6.8%).

Regarding antimicrobial resistance, 138/177 (78.0%) of the *E. coli* and 20/28 (71.4%) of the *K. pneumoniae* isolates were resistant to FQs. The highest rates of resistance were found to ampicillin (87%), cefazolin (79.5%), nalidixic acid (71.2%), moxifloxacin (66.8%) and trimethoprim/sulfamethoxazole (64%) (Fig. 1), whereas the lowest rates of resistance were detected to fosfomycin (2.4%), imipenem (5.4%) and amikacin (9.3%). Resistance to FQs was higher in Tabriz than Urmia (86.1% vs. 67%; $P \leq 0.05$). Based on the results of the agar dilution assay, the resistance rates to nalidixic acid, ciprofloxacin and levofloxacin were 70.2%, 64.4% and 58.5%, respectively. The MIC_{50} values (MIC required to inhibit 50% of the isolates) of nalidixic acid, ciprofloxacin and levofloxacin were 512, 128 and 128 μg/mL, respectively, and the MIC_{90} values (MIC required to inhibit 90% of the isolates) were 64, 32 and 16 μg/mL, respectively. Multidrug resistance (resistance to three or more classes of antimicrobial agents) was observed in 86.3% of isolates.

Among 60 FQ-resistant *E. coli* and *K. pneumoniae* isolates selected for direct sequencing, the Ser83Leu substitution in *gyrA* was identified in all 60 FQ-resistant isolates, among which 43/50 *E. coli* (86%) and 6/10 (60%) *K. pneumoniae* co-harboured an Asp87Asn substitution in the same gene. A genetic alteration of Ser80Ile in *parC* was detected in 42/50 (84%) FQ-resistant *E. coli* and 6/10 (60%) FQ-resistant *K. pneumoniae* isolates, among which 17/50 (34%) *E. coli* and 3/10 (30%) *K. pneumoniae* isolates co-harboured the Glu84Val substitution. One each of the FQ-resistant *K. pneumoniae* and *E. coli* were found to carry Ser359Ala + Ser367Thr and Gly385Cys substitution in the *gyrB* gene. Alteration of Ile529Leu and Ser458Ala in *parE* was identified in 12/50 (24%) and 8/50 (16%) *E. coli* and in 2/10 (20%) and 1/10 (10%) *K. pneumoniae* isolates, respectively. A Leu416Phe mutation in *parE* was detected in 4/50 (8%) *E. coli* isolates (Table 1).

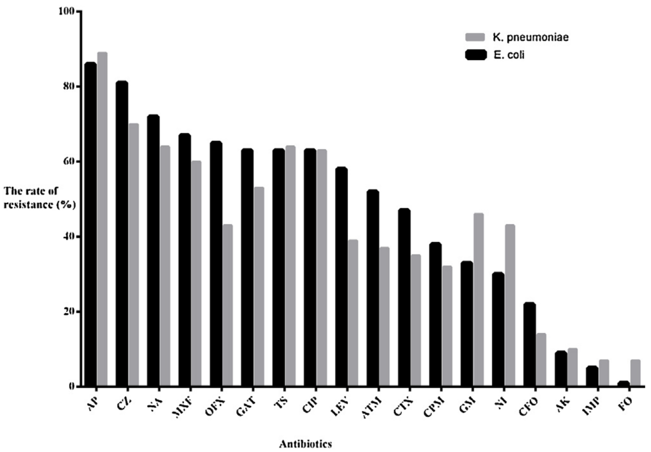

Fig. 1. Antimicrobial susceptibility patterns of *Escherichia coli* and *Klebsiella pneumoniae* isolates from urinary tract infections in Iran (April–October 2017). AP, ampicillin; CZ, cefazolin; NA, nalidixic acid; MXF, moxifloxacin; OFX, ofloxacin; GAT, gatifloxacin; TS, trimethoprim/sulfamethoxazole; CIP, ciprofloxacin; LEV, levofloxacin; ATM, aztreonam; CTX, cefotaxime; CPM, cefepime; GM, gentamicin; NI, nitrofurantoin; CFO, cefuroxime; AK, amikacin; IMP, imipenem; FO, fosfomycin.

Table 1
Distribution of DNA gyrase and topoisomerase IV mutations, based on minimum inhibitory concentrations (MICs) of fluoroquinolones, in selected *Escherichia coli* and *Klebsiella pneumoniae* isolates from urinary tract infections in Iran (April–October 2017).

Species	Isolate	MIC (μg/mL)			Topoisomerase mutations[a]			
		NAL	CIP	LVX	GyrA	GyrB	ParC	ParE
E. coli ($n = 50$)	E1	32	0.25	0.5	Ser83Leu	–	–	–
	E2	64	0.25	0.5	Ser83Leu	–	–	–
	E3	64	1	1	Ser83Leu	–	–	–
	E4	64	0.25	4	Ser83Leu	–	–	–
	E5	64	1	8	Ser83Leu	–	–	–
	E6	128	4	0.5	Ser83Leu	–	–	–
	E7	128	32	0.5	Ser83Leu	–	–	–
	E8	64	4	8	Ser83Leu, Asp87Asn	–	–	Leu416Phe
	E9	64	4	8	Ser83Leu, Asp87Asn	–	Ser80Ile	Leu416Phe
	E10	64	8	8	Ser83Leu, Asp87Asn	Gly385Cys	Ser80Ile	Leu416Phe
	E11	128	16	8	Ser83Leu, Asp87Asn	–	Ser80Ile	
	E12	512	32	8	Ser83Leu, Asp87Asn	–	Ser80Ile	–
	E13	256	32	16	Ser83Leu, Asp87Asn	–	Ser80Ile	–
	E14	64	16	16	Ser83Leu, Asp87Asn	–	Ser80Ile	–
	E15	128	8	16	Ser83Leu, Asp87Asn	–	Ser80Ile	Leu416Phe
	E16	512	64	64	Ser83Leu, Asp87Asn	–	Ser80Ile	–
	E17	256	32	32	Ser83Leu, Asp87Asn	–	Ser80Ile	–
	E18	256	32	32	Ser83Leu, Asp87Asn	–	Ser80Ile	Ser458Ala
	E19	256	64	64	Ser83Leu, Asp87Asn	–	Ser80Ile, Glu84Val	–
	E20	256	32	32	Ser83Leu, Asp87Asn	–	Ser80Ile	Ile529Leu
	E21	256	128	128	Ser83Leu, Asp87Asn	–	Ser80Ile, Glu84Val	Ser458Ala
	E22	512	32	32	Ser83Leu, Asp87Asn	–	Ser80Ile	–
	E23	512	64	64	Ser83Leu, Asp87Asn	–	Ser80Ile, Glu84Val	Ile529Leu
	E24	512	64	64	Ser83Leu, Asp87Asn	–	Ser80Ile, Glu84Val	Ile529Leu
	E25	512	32	64	Ser83Leu, Asp87Asn	–	Ser80Ile	Ser458Ala
	E26	512	64	32	Ser83Leu, Asp87Asn	–	Ser80Ile	–
	E27	512	64	32	Ser83Leu, Asp87Asn	–	Ser80Ile	–
	E28	512	64	64	Ser83Leu, Asp87Asn	–	Ser80Ile, Glu84Val	Ile529Leu
	E29	512	64	64	Ser83Leu, Asp87Asn	–	Ser80Ile	–
	E30	512	64	64	Ser83Leu, Asp87Asn	–	Ser80Ile	Ile529Leu
	E31	512	64	64	Ser83Leu, Asp87Asn	–	Ser80Ile	Ile529Leu
	E32	512	64	128	Ser83Leu, Asp87Asn	–	Ser80Ile	–
	E33	512	64	128	Ser83Leu, Asp87Asn	–	Ser80Ile, Glu84Val	Ser458Ala
	E34	512	64	128	Ser83Leu, Asp87Asn	–	Ser80Ile, Glu84Val	–
	E35	512	128	64	Ser83Leu, Asp87Asn	–	Ser80Ile, Glu84Val	–
	E36	512	128	64	Ser83Leu, Asp87Asn		Ser80Ile, Glu84Val	Ile529Leu
	E37	512	128	64	Ser83Leu, Asp87Asn		Ser80Ile, Glu84Val	Ile529Leu
	E38	512	128	64	Ser83Leu, Asp87Asn		Ser80Ile, Glu84Val	–
	E39	512	128	128	Ser83Leu, Asp87Asn	–	Ser80Ile, Glu84Val	–
	E40	512	128	128	Ser83Leu, Asp87Asn	–	Ser80Ile	Ile529Leu
	E41	256	128	128	Ser83Leu, Asp87Asn	–	Ser80Ile, Glu84Val	Ser458Ala
	E42	512	128	128	Ser83Leu, Asp87Asn	–	Ser80Ile, Glu84Val	–
	E43	512	128	128	Ser83Leu, Asp87Asn	–	Ser80Ile, Glu84Val	Ile529Leu
	E44	512	128	128	Ser83Leu, Asp87Asn	–	Ser80Ile	Ser458Ala
	E45	512	128	128	Ser83Leu, Asp87Asn	–	Ser80Ile	–
	E46	512	128	128	Ser83Leu, Asp87Asn	–	Ser80Ile	Ile529Leu
	E47	512	128	128	Ser83Leu, Asp87Asn	–	Ser80Ile, Glu84Val	Ser458Ala
	E48	512	128	128	Ser83Leu, Asp87Asn	–	Ser80Ile, Glu84Val	–
	E49	512	128	128	Ser83Leu, Asp87Asn	–	Ser80Ile	Ser458Ala
	E50	512	128	128	Ser83Leu, Asp87Asn	–	Ser80Ile	Ile529Leu
K. pneumoniae ($n = 10$)	E51	32	0.25	1	Ser83Leu	–	–	–
	E52	32	0.5	1	Ser83Leu	–	–	–
	E53	64	8	0.5	Ser83Leu	Ser359Ala, Ser367Thr	–	–
	E54	32	4	8	Ser83Leu, Asp87Asn	–	Ser80Ile	–
	E55	128	8	8	Ser83Leu		–	–
	E56	256	64	32	Ser83Leu, Asp87Asn	–	Ser80Ile	–
	E57	512	128	64	Ser83Leu, Asp87Asn	–	Ser80Ile, Glu84Val	–
	E58	512	128	128	Ser83Leu, Asp87Asn	–	Ser80Ile	Ile529Leu
	E59	512	128	128	Ser83Leu, Asp87Asn	–	Ser80Ile, Glu84Val	Ser458Ala
	E60	512	128	128	Ser83Leu, Asp87Asn	–	Ser80Ile, Glu84Val	Ile529Leu

NAL, nalidixic acid; CIP, ciprofloxacin; LVX, levofloxacin.

[a] — Indicates isolates with no mutations.

PMQR genes were identified in 54 (90%) of the 60 FQ-resistant isolates as follows: *aac(6)-Ib*-cr, 43 (71.7%); *oqxB*, 31 (51.7%); *oqxA*, 22 (36.7%); *qnrB*, 17 (28.3%); *qnrS*, 13 (21.7%); *qnrD*, 10 (16.7%); *qepA*, 3 (5.0%); *qnrA*, 1 (1.7%); and *qnrC*, 1 (1.7%) (Table 2). The results showed that 11, 18, 12, 8 and 5 of the isolates were positive for one, two, three, four and five PMQR genes, respectively.

4. Discussion

Following the widespread use of FQs in the therapy of UTIs, resistance to FQs has been increasing [12]. In the current study, a high FQ resistance rate was observed among *E. coli* (78.0%) and *K. pneumoniae* (71.4%) isolated from UTIs. Multidrug resistance was high in our region. The frequency of resistance to FQs is reported to

Table 2
Prevalence of plasmid-mediated quinolone resistance (PMQR) determinants in selected *Escherichia coli* and *Klebsiella pneumoniae* isolates from urinary tract infections in Iran (April–October 2017).

Species	No. (%) of isolates								
	qnrA	*qnrB*	*qnrC*	*qnrD*	*qnrS*	*aac(6′)-Ib*-cr	*oqxA*	*oqxB*	*qepA*
E. coli (*n* = 50)	1 (2)	13 (26)	1 (2)	6 (12)	11 (22)	36 (72)	12 (24)	21 (42)	3 (6)
K. pneumoniae (*n* = 10)	0	4 (40)	0	4 (40)	2 (20)	7 (70)	10 (100)	10 (100)	0
Total (*n* = 60)	1 (1.7)	17 (28.3)	1 (1.7)	10 (16.7)	13 (21.7)	43 (71.7)	22 (36.7)	31 (51.7)	3 (5.0)

vary among regions, e.g. Egypt (41.3%), Iran (60.4%) and India (89%) [3,13,14]. FQ resistance is mostly attributed to mutations targeting the QRDRs of DNA gyrase and topoisomerase IV [15]. In the current study, all of the tested FQ-resistant *E. coli* and *K. pneumoniae* isolates exhibited a Ser83Leu mutation in the QRDR of *gyrA*, which is consistent with previous studies [13,16]. Strains with MICs in the range of 0.25–4 μg/mL for ciprofloxacin and levofloxacin possessed only a *gyrA* mutation. In addition, an Asp87Asn substitution in *gyrA* was detected in 86% of *E. coli* and 60% of *K. pneumoniae* isolates with high MICs to FQ (ciprofloxacin, 4–128 μg/mL; nalidixic acid, 32–512 μg/mL; and levofloxacin, 8–128 μg/mL). These results ae similar to previous studies conducted by Namboodiri et al. [17]. In the current study, a Ser80Ile substitution in the QRDR of *parC* was found in 84% of *E. coli* and 60% of *K. pneumoniae* FQ-resistant isolates, which is associated with high levels of resistance to FQs. The data in this study indicated that 34% of *E. coli* and 30% of *K. pneumoniae* isolates were characterised as having an additional mutation Glu84Val in *parC* with higher levels of FQ resistance (MICs ≥256, ≥64 and ≥64 μg/mL for nalidixic acid, ciprofloxacin and levofloxacin, respectively). In a study performed in Algeria by Betitra et al., the Ser80Ile and Glu84Val substitutions were reported as the most prevalent mutations in the *parC* gene [18].

According to sequence analysis of the *gyrB* gene, Ser359Ala and Ser367Thr substitutions were found in one *K. pneumoniae* isolate with MICs of 64, 8 and 0.5 μg/mL for nalidixic acid, ciprofloxacin and levofloxacin, respectively. These mutations were also previously reported in FQ-resistant isolates [19]. Mutation in the *gyrB* gene (Gly385Cys) was observed in one *E. coli* isolate with MICs of 64, 8 and 8 μg/mL for nalidixic acid, ciprofloxacin and levofloxacin, respectively.

In this study, three types of mutation in the *parE* gene were identified, including Ile529Leu (24% of *E. coli* and 20% of *K. pneumoniae*), Ser458Ala (16% of *E. coli* and 10% of *K. pneumoniae*) and Leu416Phe (8% of *E. coli*).

To our knowledge, such alterations in *parE* and *gyrB* are reported for Enterobacteriaceae isolates for the first time in Iran. Sorlozano et al. reported that the Ser458Ala mutation in *parE* was observed in isolates with high-level FQ resistance [16], which is in accordance with the current results. The Ile529Leu alteration was observed in isolates with high MICs to FQs as previously reported by Nam et al. [19]. The Leu416Phe substitution in *parE* occurred in isolates with low levels of FQ resistance, which is similar to the findings of a previous study [20]. As all of these substitutions in the *gyrB* and *parE* genes lie outside of the QRDR, their exact roles in FQ resistance cannot be clearly established.

The presence of PMQR determinants on mobile genetic elements may lead to their dissemination among the Enterobacteriaceae family [21]. The present study demonstrated a high prevalence (90%) of PMQR genes among FQ-resistant Enterobacteriaceae. The *aac(6′)-Ib*-cr gene was the most prevalent PMQR gene, in agreement with other studies [21,22]. In some studies, a low prevalence of *aac(6′)-Ib*-cr has been reported [8,9]. The *oqxA* and *oqxB* genes were reported all of the *K. pneumoniae* isolates in the current study, whilst 24% and 42% of the *E. coli* isolates carried these genes. In China, Yuan et al. reported that 6.6% of *E. coli* and 100% of *K. pneumoniae* harboured the *oqxAB* genes [23]. However, in another study carried out by Kim et al. [24], the prevalence of *oqxAB* was lower than the current results. The results reported here showed that only 6% of *E. coli* isolates carried the *qepA* gene, as noted previously, which is consistent with previous studies [7,25].

In the current study, *qnrB* was the most frequent *qnr* gene among the isolates, which is in agreement with other studies from Iran and Brazil [22,26]. In the present study, the prevalence of the *qnrS* determinant among Enterobacteriaceae isolates (21.7%) was higher than that in Iran (8%), Korea (3.9%) and China (2.5%) [3,9,21]. The data reported here revealed that *qnrA* and *qnrC* were detected only in one *E. coli* isolate each. In a previous study, the presence of *qnrA* and *qnrC* genes was not detected in any isolate [27]. The prevalence of *qnrD* in *K. pneumoniae* (40%) was significantly higher than that in *E. coli* (12%). In contrast to the present study, Staji et al. reported a high prevalence of *qnrD* in *E. coli* [28]. These differences in the incidence of PMQR determinants could be related to geographical area, study period and differences in FQ usage. PMQR genes can contribute to the selection of chromosomal mutations that result in higher levels of FQ resistance.

5. Conclusion

Due to the high prevalence of FQ resistance, determination of antimicrobial susceptibility before FQ administration is recommended in our region. Mutations in DNA gyrase and topoisomerase IV can confer reduced susceptibility of *E. coli* and *K. pneumoniae* to FQs, and the most common were mutations in *gyrA*, followed by *parC*, *parE* and *gyrB*. The prevalence of PMQR genes is high, which may contribute to the spread of FQ resistance in our setting. Furthermore, the combination of DNA gyrase and topoisomerase IV mutations and PMQR genes confers high-level resistance to FQs. This study suggests that other FQ resistance mechanisms, such as porin loss and efflux pumps, should be investigated in a future study.

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Competing interests

None declared.

Ethical approval

This project was approved by the Local Ethics Committee of Tabriz University of Medical Sciences [no. 5/4/10393]. Written consent of participants was received.

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