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ORIGINAL RESEARCH

Co-Incidence of Type II Topoisomerase Mutations and Efflux Expression in High Fluoroquinolone Resistant *Enterococcus faecalis* Isolated from Urinary Tract Infections

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Introduction: *Enterococcus faecalis* is one of the most common pathogens in urinary tract infections (UTIs). Fluoroquinolones have been frequently used to treat *E. faecalis* UTIs, and the emergence of fluoroquinolone-resistant *E. faecalis* strains has recently been reported in several countries. This study aimed to elucidate the mechanisms involved in fluoroquinolone resistance in clinical *E. faecalis* isolates by analyzing mutations in quinolone- resistance-determining regions (QRDRs) of *gyr*A and *par*C and investigating the role of some efflux pumps.

Methods: In total, 70 clinical *E. faecalis* isolates collected from UTIs were identified by phenotypic and genotypic methods. Antimicrobial susceptibility testing was performed and multidrug-resistant (including ciprofloxacin resistant) isolates were studied for minimum inhibitory concentrations to ciprofloxacin, levofloxacin, and ofloxacin. In the following, mutations in QRDRs of *gyr*A and *par*C and expression of EfrA, EfrB, and EmeA efflux pumps were investigated in 20 high-level ciprofloxacin resistant and two ciprofloxacin susceptible isolates.

Results: High-level resistance to ciprofloxacin was detected in 97.5% of isolates. Sequencing of QRDRs revealed that 65% and 75% of isolates carried mutations in *gyr*A and *par*C, respectively. The presence of efflux genes was detected in all studied isolates, but expression of *efr*A, *eme*A, and *efr*B was demonstrated in 50%, 40%, and 30% of resistant isolates, respectively. Neither QRDR mutation nor the expression of efflux genes showed any significant association with MIC.

Conclusion: Co-incidence of mutation and efflux gene expression in more than half of isolates (13/20) suggests that both mechanisms may play a role in fluoroquinolone resistance. The other unknown mechanisms including different efflux pumps and probably other QRDRs mutations may contribute to fluoroquinolone resistance in *E. faecalis*.

Keywords: *E. faecalis*, UTI, *gyr*A, *par*C, efflux pump

Introduction

Fluoroquinolones, as broad-spectrum drugs with lesser side effects and favorable oral dosage, are of convenient therapeutic agents recommended for the treatment of urinary tract infections (UTIs) caused by both gram-positive and gram-negative organisms.[1]

Among nosocomial pathogens, enterococci have caused major concerns because of their typical antibiotic resistance. Within various body systems, urinary tract is a common site of enterococcal infections. Reportedly, *Enterococcus faecalis* is

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responsible for more than half of enterococcal UTIs.[2] Since these drugs can interact with various cellular components, different resistance strategies including intrinsic or acquired mechanisms may be proposed.[3]

Resistance to fluoroquinolones is mediated through chromosomes and/or plasmid by various mechanisms, including mutations in the structural genes targeted by fluoroquinolones, *gyr*A and *gyr*B coding for DNA gyrase, or *par*C and *par*E coding for topoisomerase IV, as well as some efflux pumps.[4]

Mutational changes in quinolone-resistance-determining region (QRDR) of type II topoisomerases, DNA gyrase and topoisomerase IV are among common resistance mechanisms. Both enzymes are critical during bacterial replication. Thus, it is vital to inhibit their enzymatic activities. While interaction of fluoroquinolones to these enzymes differs in various bacteria, association of higher-level fluoroquinolone resistance with mutations in both target enzymes has been demonstrated.[5]

Decreased accumulation of fluoroquinolones in bacterial cells mediated by efflux pumps is another major resistance mechanism. EmeA and EfrAB belong to Major Facilitator Superfamily (MFS) and ATP-binding cassette (ABC) family transporters, respectively, and are multidrug efflux pumps contributing in the extrusion of fluoroquinolones in enterococci.[3,6]

Having knowledge about resistance mechanisms will be crucial to find new suitable drug targets that can subvert established bacterial traits. Therefore, this study aimed to analyze QRDR mutations conferring resistance and the expression of some multidrug efflux pumps in a population of MDR *E. faecalis* isolated from UTIs.

Materials and Methods

Bacterial Population and Antimicrobial Susceptibility Testing

During 2 years, *E. faecalis* isolates were collected from patients with clinical symptoms of UTIs at two university-affiliated hospitals (Kerman, Iran). Following identification and confirmation,[7,8] disk diffusion test was performed to detect susceptibility, in order to find MDR isolates (i.e. resistance to more than three different classes of antibiotics). Next, for defined MDR isolates (n= 40), minimal inhibitory concentrations (MICs) were determined for ciprofloxacin, levofloxacin, and ofloxacin (obtained from Exir pharmaceutical company, Iran) using two-fold agar dilution method. Results were interpreted according to susceptibility breakpoints defined by the Clinical Laboratory Standard Institute (CLSI) guidelines.[9] At last, 20 MDR isolates (with high-level resistance to ciprofloxacin; MIC ≥ 64 µg/mL) and two susceptible isolates (non-MDR) were randomly selected for the next phases.

Sequencing the QRDR Coding Regions in *gyr*A and *par*C

Bacterial DNA was extracted using DNA extraction kit (Sinaclon, Iran) according to the manufacturer's instructions. Amplification of DNA fragments containing QRDRs of *gyr*A and *par*C genes was carried out using specific primers as described previously.[10] Amplicons were sequenced using the same primers by Bioneer Company on an applied Biosystems 3730/3730X1 DNA analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were compared with *E. faecalis* V583 (GenBank Accession No. NC_004668.1) using Vector NTI Advance™ 10.

Expression of EmeA, EfrA/B Efflux Pumps

RNA Extraction and cDNA Synthesis

Overnight grown cultures of *E. faecalis* isolates were diluted (1:50) and inoculated to fresh brain heart infusion broth media (Merck, Darmstadt, Germany) as the following: one containing ciprofloxacin (at concentration of 1/2 MIC) and the other without any antibiotic supplementation. Following incubation with gentle shaking until reaching the late exponential phase (OD600 = 0.8),1 mL of each culture was harvested by centrifugation (10,000 rpm/2 min) at 4°C and the bacterial pellet was immediately used for RNA extraction using RNA extraction Kit (Yekta Tajhiz, Tehran, Iran) following manufacturer's instruction. Residual chromosomal DNA was removed by treating samples with the DNase I, RNase-free kit (Sinaclon, Iran). RNA quantification and quality assessment were carried out by NanoDrop NDe1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Then, cDNA was synthesized using the cDNA Synthesis Kit (Yekta Tajhiz, Tehran, Iran) according to the manufacturer's recommended protocol.

Quantitative RT-PCR

Real-time PCR was carried out in an ABI Step One plus Real-time PCR system with Eva Green PCR Master Mix (Applied Biosystems) (90ºC for 15 min, 40 cycles of 95ºC for 20 s, 57ºC (for *efrA*), 56ºC (for *efrB*)[11] and 60ºC (for both *emeA* and *gyrB*). Real-time PCR reactions were carried out in duplicate. The primer pairs used for *eme*A in real-time PCR were different from those in conventional PCR.[10,12]

Threshold values were calculated at a constant level of fluorescence. To determine the relative expression level of *emeA, efrA,* and *efrB* genes, the method described by Pfaffl[13] was employed using *gyrB* as housekeeping gene.[10]

Results

Totally, 40 isolates (40/70, 57%) were defined as MDR. As shown in Table 1, all these isolates were included in the resistant category for ciprofloxacin, levofloxacin, and ofloxacin according to CLSI standards.[9] In this study, ciprofloxacin MIC ≥64 µg/mL was considered high-level ciprofloxacin resistance. In the following, 20 MDR *E. faecalis* isolates which showed high-level resistance to ciprofloxacin and two ciprofloxacin susceptible isolates were randomly selected for further investigations.

Sequence Variations in QRDRs of *gyr*A and *par*C

Regarding two susceptible isolates, 100% similarity was observed with the reference sequence (*E. faecalis* V583) in both QRDRs of *gyr*A and *par*C.

Table 1 The Fluoroquinolone MIC Distribution for 40 Clinical MDR *E. faecalis* Isolated from UTIs

Fluoroquinolones	MIC (µg/mL)						
	8	16	32	64	128	256	>256
Ciprofloxacin	1	0	1	10	16	7	5
Levofloxacin	1	5	17	13	4	0	0
Ofloxacin	1	0	4	18	13	4	0

Abbreviations: MIC, minimum inhibitory concentration; UTI, urinary tract infection.

For gyrA, amino acid mutations were seen in 65% of the studied isolates (13/20). Among these isolates, two types of mutations were observed: serine 84 to isoleucine (n=12, 92%) and serine 84 to tyrosine (n=1, 7.6%) (Figure 1). Notably, seven isolates did not possess any mutational changes.

For *par*C QRDR, totally three types of mutations were found among 75% of resistant isolates (15/20). In 86.6% of isolates (n =13), only one amino acid substitution, serine 82 to isoleucine, was detected. The two other mutation types were seen among two different isolates as follows: isolate 39: amino acid changes at positions serine 82 to leucine, serine 83 to arginine, tyrosine 85 to phenylalanine, aspartic acid 94to asparagine and lysine 96 to asparagine, and isolate B128: amino acid changes at positions serine 82 to leucine, serine 83 to threonine, arginine 90 to leucine, aspartic acid 94 to tyrosine, isoleucine 102 to phenylalanine and asparagine 106 to lysine (Figure 2). Interestingly, the two latter isolates exhibited high MIC to all tested quinolones.

Herein, 75% of isolates (n= 15), carried mutations in one or both QRDRs of DNA gyrase (GyrA) and topoisomerase IV (ParC). As shown in Table 2, co-incidence of mutations in QRDRs of *gyr*A and *par*C was detected in 65% of isolates (n= 13). Only two isolates contained mutation in *par*C, and for five other isolates, no mutation was found. Statistical analysis did not show any significant association between mutations and MICs level ($P > 0.05$).

Expression of *emeA, efrA* and *efrB* Genes

The *emeA, efrA* and *efrB* genes were detected in all studied isolates. The ratio of relative expression of *emeA, efrA* and

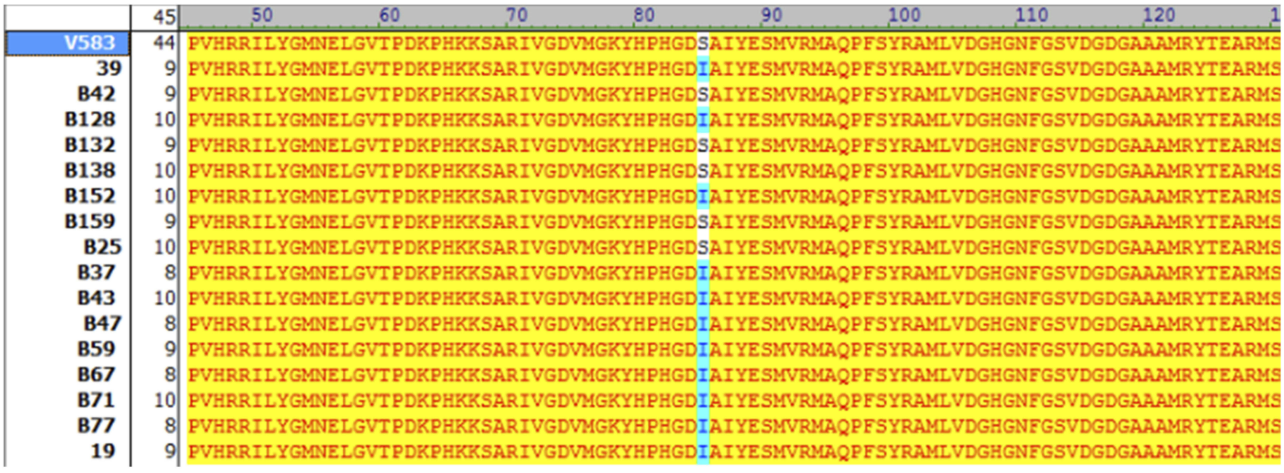

Figure 1 Amino acid sequences of QRDR domain of *gyr*A in ciprofloxacin-resistant *Enterococcus faecalis* isolates with different MIC values. The amino acid substitutions are marked with different colors. Sequences of *gyr*A were compared with reference sequence *(E.feacalis* V583) using Vector NTI AdvanceTM 10. Amino acids identical to the corresponding reference sequence are indicated by yellow color. S=Serine (Ser); I=Isoleucine (Ile); L=Leucine (Leu); R=Arginin (Arg); Y=Tyrosine (Tyr); F= Phenylalanine (Phe); D= Aspartic acid (Asp); N= Asparagine (Asn); T= Threonine (Thr); K= Lysine (Lys).

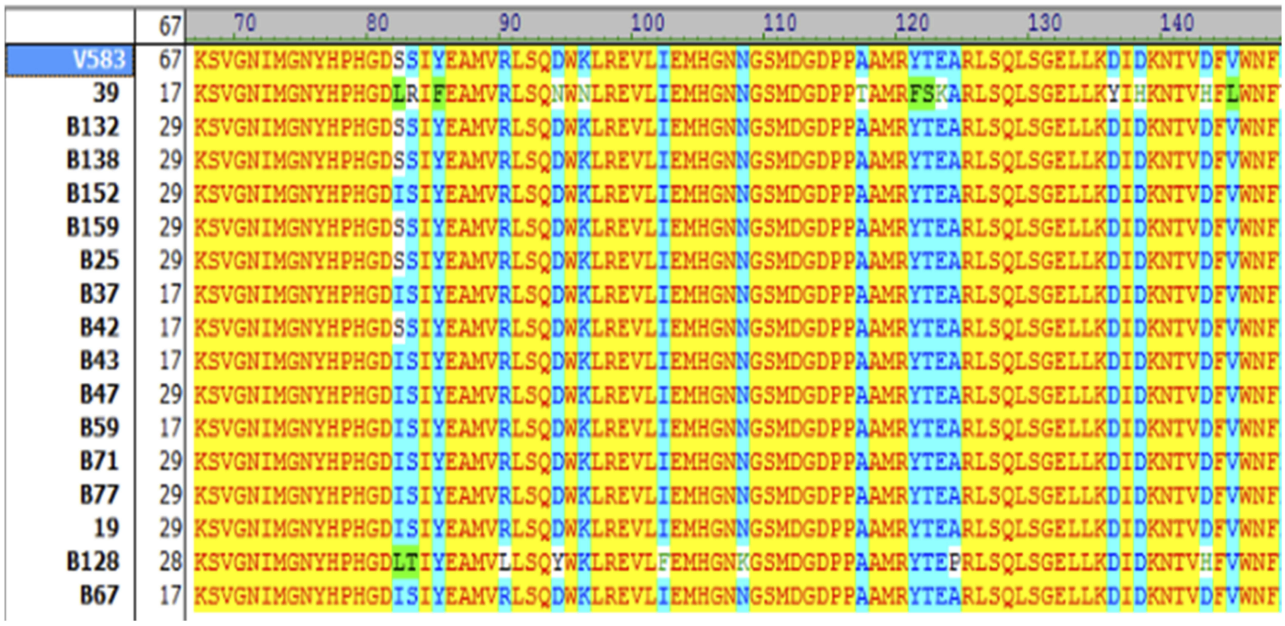

Figure 2 Amino acid sequences of QRDR domain of *par*C in ciprofloxacin-resistant *Enterococcus faecalis* isolates with different MIC values. The amino acid substitutions are marked with different colors. Sequences of *parC* were compared with reference sequence *(E.feacalis* V583) using Vector NTI AdvanceTM10. Amino acids identical to the corresponding reference sequence are indicated by yellow color. S=Serine (Ser); I=Isoleucine (Ile); L=Leucine (Leu); R=Arginin (Arg); Y=Tyrosine (Tyr); F= Phenylalanine (Phe); D= Aspartic acid (Asp); N= Asparagine (Asn); T= Threonine (Thr); K= Lysine (Lys).

efrB mRNA among the resistant isolates varied from 0.026 to 2.004 for *emeA*, 0.062 to 7.621 for *efrA*, and 0.008 to 4 for *efrB* (Table 2). The abundance of the *emeA, efrA* and *efrB* mRNA expression varied considerably among the isolates with 1.88-fold, 2.08-fold and 2.69-fold difference between the least to the most expressed ones, respectively.

According to our results, the most frequent expressed efflux gene was *efr*A (10/20, 50%), followed by *eme*A (8/20, 40%) and *efr*B (6/20, 30%). Furthermore, co-expression of efflux genes was mostly detected for *efr*A+ *efr*B (6/20, 30%), followed by *efr*B+ *eme*A (4/20, 20%) and *efr*A+ *eme*A (3/20, 15%). Altogether, co-expression of two efflux pumps was seen in 13 isolates. In addition, in three isolates (B43, B47, B67), three efflux pumps were co-expressed simultaneously. All of these isolates contained similar mutations both in *gyr*A and *par*C (Figure 3).

As shown in Table 2, no expression of efflux genes was found in six high-level ciprofloxacin-resistant isolates (6/20). Interestingly, four of these isolates did not have any mutations in *gyr*A and *par*C. Statistical analysis did not show any significant association between efflux gene expression and MICs (P> 0.05).

Discussion

Resistance to fluoroquinolones in bacteria is mainly mediated by spontaneous mutations in the QRDRs of DNA gyrase and topoisomerase IV especially at the highly conserved residues.[5] Whether *gyr*A mutation is more important in fluoroquinolone resistance or mutation in *par*C, is controversial. Leavis et al[14] found *par*C mutation in all ciprofloxacin resistant isolates while additional mutation in *gyr*A was detected only in 42% of them. Thus, they concluded that the main target in mutation-mediated ciprofloxacin resistance in enterococci is mutation in *par*C. On the other hand, López et al[15] suggested DNA gyrase as the primary target for resistance to levofloxacin and ciprofloxacin in *E. faecalis* isolates. In contrast to the mentioned studies and similar to Yasufuku et al,[16] our results did not show any significant difference between the frequency of mutations in *gyr*A and *par*C (65% versus 75%, respectively).

Different frequencies of mutations have also been described: Jafari-Sales et al[17] reported *gyr*A mutation in 92.5% of ciprofloxacin resistant isolates, while in the study by Piekarska et al,[18] 19% and 36% of isolates and in a report by Yasufuku et al,[16] 28% and 18% of isolates harbored mutations in *gyr*A and *par*C, respectively.

The type of mutations is also different: In a study conducted by Lopez et al,[15] all strains with ciprofloxacin MIC >32 mg/l presented amino acid changes in GyrA protein (S83I, S83Y, S83R or S83I, and E87G) with/without changes in ParC protein (S80I or S80R or S80I). A report by Kanematsu et al[19] demonstrated the amino

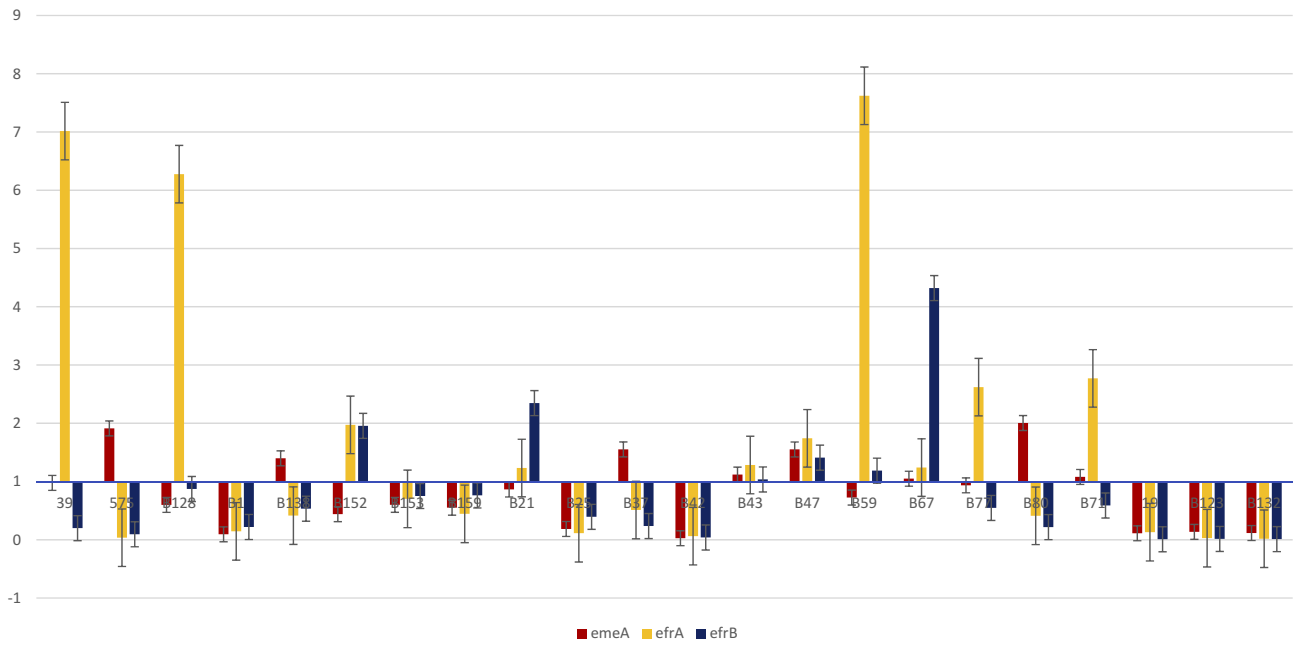

Figure 3 Fold changes expression of three different efflux genes (*eme*A, *efr*A, *efr*B) in 22 clinical MDR *E. faecalis* isolates.

Table 2 Characteristics of *Enterococcus faecalis* Isolated from UTIs with Different MICs to Ciprofloxacin: Polymorphisms in the QRDRs of the *gyr*A and *par*C Genes and Relative Quantities of the *eme*A, *efr*A, and *efr*B Expression

	Samples	MIC(μg/mL)			Amino Acid Changes in[a]:		*emeA*[b]	*efrA*[b]	*efrB*[b]
		Ciprofloxacin	Levofloxacin	Ofloxacin	*gyrA*	*parC*			
1	39	256	>64	>128	S83 I	S82L, S82L, S83R, Y85F, D94N, K96N	0.976	7.015	0.199
2	575	256	32	32	S83 I	S82 I	1.911	0.035	0.094
3	B128	>256	32	128	S83 I	S82L, S83T, R90L, D94Y, I102F.N108K	0.598	6.276	0.871
4	B1f	64	32	64			0.093	0.144	0.219
5	B138	>256	32	8			1.398	0.414	0.533
6	B152	256	32	32	S83 I	S82 I	0.439	1.972	1.955
7	B153	>256	32	64	S83T	S82 I	0.599	0.702	0.751
8	B159	256	32	64			0.55	0.444	0.761
9	B21	64	16	64	S83 I	S82 I	0.863	1.231	2.346
10	B25	64	16	64			0.185	0.112	0.393
11	B37	128	32	64	S83 I	S82 I	1.551	0.51	0.235
12	B42	64	32	64			0.026	0.062	0.039
13	B43	128	32	64	S83 I	S82 I	1.119	1.283	1.035
14	B47	128	32	64	S83 I	S82 I	1.55	1.741	1.409
15	B59	128	32	64	S83 I	S82 I	0.724	7.621	1.186
16	B67	128	32	64	S83 I	S82 I	1.048	1.239	4.32
17	B77	128	64	128	S83 I	S82 I	0.934	2.62	0.546
18	B80	128	>64	128		S82 I	2.004	0.411	0.217
19	B71	128	64	128	S83I	S82 I	1.078	2.77	0.588
20	19	256	64	64		S82 I	0.111	0.13	0.008
21	B123	1	2	1			0.135	0.027	0.014
22	B132	1	1	1			0.115	0.017	0.011

Notes: Ciprofloxacin MIC ≥ 64 μg/mL was considered as high-level resistance to ciprofloxacin. B123 and B132 are ciprofloxacin susceptible. [a]Amino acid changes with respect to the reference sequence of *E. faecalis* V583 (GenBank Accession No. NC_004668.1). [b]mRNA expression levels as measured by real-time PCR.
Abbreviations: MIC, minimum inhibitory concentration; S, Serine; Y, Tyrosine; N, Asparagine; I, Isoleucine; K, Lysine; F, Phenylalanine; L, Leucine; D, Aspartic acid.

acid mutation of S83I in GyrA in 28.3% and the amino acid mutation of S80I in ParC in 17.9% of clinical strains of *E. faecalis*. In addition, Kim and Woo[20] reported that all high-level ciprofloxacin-resistant enterococcus isolates recovered from chicken meat in Korea showed mutations in both *par*C and *gyr*A consisting of S83I-S80I (94.2%), S83F-S80I (2.3%), S83Y-S80I (2.3%), and S83Y-S80F (1.2%). However, in our study, 92% of mutations in QRDR *gyr*A and 80% of *par*C mutations were related to S83I and S82I, respectively.

In this study, similar to Kim et al in 2017 and Oyamada et al in 2006, no correlation was found between mutation pattern and the MIC.[10,20] This is in contrast to Yasufuku et al[16] who found that mutations were significantly correlated with the MICs of levofloxacin.

Correlations between mutations and their effects on drug–target interactions have not been fully explored. Published crystallographic structures suggest that Serine and acidic residues anchor the water–metal ion bridge that coordinates drug binding.[21] Accordingly, the number of mutations is a determining factor in reduced affinity between the drug and target enzymes resulting in drug resistance. In this context, Urushibara et al in 2014 found that MIC elevations significantly correlated with the number of QRDR mutations which cannot be generalized to our study with limited number of mutations in QRDRs.[5]

Resistance through active efflux of quinolones and increased expression of endogenous efflux pumps are alternative quinolone resistance mechanisms reported for *E. faecalis*.[3,6] In our study, the presence of *eme*A, *efr*A/B was detected in all ciprofloxacin resistant and susceptible isolates. Wei Jia et al detected *eme*A gene in 73.8% of ciprofloxacin-resistant (but not susceptible) enterococci and suggested that the distribution of the *eme*A gene is associated with the resistance to fluoroquinolones in enterococcus species.[12] In contrast, Shiadeh et al in 2018 and Valenzuela et al in 2013 detected *efr*A and *efr*B in 100% and 96% of isolates, respectively.[4,22]

In this study, different levels of efflux gene expression were obtained which can be attributable to bacterial isolates as reported by Lerma et al in 2014.[11] In addition, no significant relationship was found between the expression of efflux pumps and level of MIC. However, Lubelski et al in 2007 reported that elevated drug resistance was only observed when *efrA* and *efrB* genes were co-expressed.[23]

It is yet unclear which mechanism is more effective in development of resistance because there are resistant isolates with no expression of efflux genes but the presence of QRDR mutations and the presence of one isolate without any mutation with an expression of an efflux gene. Bioinformatic approaches have demonstrated 34 potential multidrug efflux pump genes, nine of which, including *eme*A and a *bmr* homologue, belong to the MF superfamily. In addition, 23 ABC transporter homologues have been detected in *E. faecalis* isolates.[24] Thus, it is suggested that another pump of MF superfamily rather than EmeA, or ABC transporter rather than EfrA/B may be involved in fluoroquinolone resistance. Also, other mechanisms such as mutation in *par*E or *gyr*B may be proposed. However, it has already been established that alterations in GyrB and ParE are associated with fluoroquinolone resistance in some Gram-positive bacteria such as *S. aureus, S. pneumoniae* and *E. faecium*, but not in *E. faecalis*.[25]

In conclusion, co-incidence of mutation and efflux gene expression in 65% of isolates suggests that both mechanisms may be important. More investigations are required to detect different strategies involved in this process, which will be critical to tackle antimicrobial resistance problem in clinical care, using novel therapeutic agents such as efflux pumps inhibitors to control multidrug antibiotic resistance.

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Disclosure

The authors report no conflicts of interest in this work.

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