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ORIGINAL RESEARCH

Unraveling Mechanisms and Epidemic Characteristics of Nitrofurantoin Resistance in Uropathogenic *Enterococcus faecium* Clinical Isolates

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Purpose: Multidrug-resistant (MDR) *Enterococcus faecium* is an important nosocomial pathogen causing urinary tract infection, and the reapplication of nitrofurantoin (NIT) in the clinic has attracted great attention. This study aims to explore the NIT resistance mechanisms and epidemiological characteristics of *E. faecium* clinical isolates.

Patients and Methods: A total of 633 *E. faecium* clinical isolates was obtained from urine samples in a clinical teaching hospital during 2017–2018. Among them, 40 NIT-resistant strains, and a similar number of -intermediate and -susceptible strains were isolated. The minimum inhibitory concentrations (MICs) of NIT were detected by agar dilution method. The prevalence and mutations of nitroreductase-encoding genes *ef0404* and *ef0648* were explored by polymerase chain reaction (PCR), followed by efflux pump inhibition test and quantitative real-time PCR (qRT-PCR) to investigate the resistance mechanisms of NIT. Furthermore, the epidemiological characteristics were detected by multilocus sequence typing (MLST).

Results: The carrying rates of nitroreductase in NIT-susceptible, -intermediate, and -resistant isolates were 100%, 50%, and 20%, respectively. After exposure to the efflux pump inhibitor, the MIC of 12 *E. faecium* decreased by ≥4-fold. However, the efflux pump genes *efrAB, emeA*, and *oqxAB* were not overexpressed in NIT-resistant *E. faecium* isolates. Moreover, MLST analysis revealed that all the NIT-resistant isolates belonged to CC17, of which 30 (75%) were associated with ST78.

Conclusion: This study has established for the first time that the absence of EF0404 and EF0648 is the main mechanism of NIT resistance in *E. faecium*. Our findings are likely to fill the knowledge gap pertaining to the NIT resistance mechanism in *E. faecium* and provide important insights for molecular epidemiological characteristics analysis.

Keywords: *Enterococcus faecium*, nitrofurantoin, nitroreductase, resistance mechanism, epidemiology

Introduction

As a ubiquitous group of Gram-positive bacteria, *Enterococcus* is a leading cause of hospital-acquired infections and has therefore posed a serious threat to public health around the world.[1,2] The important infections most commonly caused by *Enterococcus* are urinary tract, device-associated, and soft-tissue infections as well as bacteremia.[3] In recent years, *Enterococcus faecium* has become the foremost Gram-positive pathogen

responsible for urinary tract infections.[4,5] In addition to intrinsic resistance and genetic diversity, its ability to recruit and express antimicrobial resistance determinants contributes to the rapid increase of multidrug resistance (MDR) in *E. faecium*.[6,7] The limited availability of novel antimicrobial agents has posed serious challenges to the clinical treatment of infectious diseases.[8,9]

As an age-old synthetic drug, nitrofurantoin (NIT) has been used for the prevention of urinary tract infection (UTI) for over 60 years.[10] Because of its considerable bactericidal activity and low resistance rate, the drug has attracted renewed clinical interest. The efficacy of NIT against high level of aminoglycoside-resistant (HLAR) *Enterococcus* and vancomycin-resistant *Enterococcus* (VRE) has led to it being considered as the last resort for the first-line therapy of uncomplicated lower urinary tract infection caused by several bacteria.[11–13]

However, the extensive use of NIT has resulted in the increased drug resistance of *E. faecium*. Resistance mechanisms of NIT previously studied are limited to *Enterobacteriaceae*. For instance, mutations in the nitroreductase-encoding gene *nfsAB* and the overexpression of the efflux pump gene *oqxAB* play important roles in NIT resistance in *Escherichia coli* and *Klebsiella pneumoniae*.[14–17] In addition, deletion in *ribE* (encoding lumazine synthase involved in the biosynthesis of flavin mononucleotide) is a key NIT resistance mechanism in *E. coli*.[13,18] However, the mechanism of NIT resistance in *E. faecium* remains poorly understood.

Previous studies have reported that EF0404, EF0648, EF0655, and EF1181 exhibit nitroreductase activity in *Enterococcus faecalis* V583.[19] Besides, Fatemeh Raci (2003) has suggested that the antimicrobial effect of nitro drugs is mediated by microbial nitroreductases that reduce the drug to a cytotoxic nitro radical and result in DNA damage.[20] Nonetheless, there is no evidence to establish that nitroreductase is responsible for NIT resistance in *E. faecium*.

Hence, we aimed to investigate the main mechanism of NIT resistance in *E. faecium*, which is mediated by nitroreductases and multidrug resistance efflux pumps. Besides, multilocus sequence typing (MLST) was performed to reveal the homology among the NIT-resistant isolates.

Materials and Methods

Bacterial Strains

A total of 633 non-duplicated *E. faecium* strains was isolated from the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) in 2017–2018. Bacterial identification was performed using Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF MS; BioMérieux, Lyons, France). *E. faecium* ATCC 29212 was used as a quality control strain in antimicrobial susceptibility testing experiments.

Antimicrobial Susceptibility Testing

Agar dilution method was applied to determine the minimum inhibitory concentration (MIC) of NIT, and the results were interpreted according to the recommendations of the Clinical and Laboratory Standards Institute (CLSI, 2020) guidelines. The breakpoints of NIT for the agar dilution method were as follows: susceptible ≤32 mg/L; intermediate = 64 mg/L; and resistant ≥128 mg/L. All strains were tested in three biologically independent experiments.

PCR Amplification and DNA Sequencing

Genomic DNA of the experimental strains was extracted using the Bioflux Bacterial DNA Extraction Kit (Bioflux, Tokyo, Japan) as per the instructions of the manufacturer. Polymerase chain reaction (PCR) was employed to detect NIT resistance-related genes (*ef0404, ef0648, ef0655*, and *ef1181*) and efflux pump genes (*oqxA, oqxB, efrA, efrB*, and *emeA*). The positive PCR products were sent to Shanghai Majorbio Bio-Pharm Technology Co. (Shanghai, China) for sequencing. The sequences were compared with the standard strain *E. faecium* DO (Accession number: CP003583) deposited in the National Center for Biotechnology Information (NCBI) database using BLASTn and BLASTx programs (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The online PROVEAN platform (http://provean.jcvi.org/seq_submit.php) was used to predict the alterations in the biological functions of the proteins. Primers used for amplification and sequencing are furnished in Table 1.

Effect of Efflux Pump Inhibitor

Several studies have revealed the presence of 34 potential drug-efflux genes in the *E. faecalis* genome, and the pumps have been shown to exhibit differing but somewhat overlapping broad substrate profiles.[21] Therefore, the efflux pump inhibition test was performed to identify which efflux pump is responsible for NIT resistance in *E. faecium*. The MICs of *E. faecium* with or without the efflux pump inhibitors carbonyl cyanide m-chlorophenylhydrazone (CCCP, 6 μg/mL),

Table 1 Primers Used in This Study

Genes	Primer Sequence (5′→3′)	Tm (°C)	Product size (bp)
Primers related to resistance mechanisms			
ef0404	F:ACAACATATACAACGAATGATTTTTCAG R:TTTTATTGCCTATTCAAATGTCGTG	59	660
ef0648	F:ATGTATCAAGATGTTGTTCGCAGC R:CAATCACTTTGGATGTTTGTTCC	58	701
efrA	F:ACGCCAGTGATGTTTATTGC R:ACGAATAGCTGGTTCCATGT	57	543
efrB	F:AGTTACTATGTGGTTGCTGG R:GGACATCACTACGGTTCATT	57	439
oqxA	F:GACAGCGTCGCACAGAATG R:GGAGACGAGGTTGGTATGGA	56	339
oqxB	F:CGAAGAAAGACCTCCCTACCC R:CGCCGCCAATGAGATACA	58	240
emeA	F:GTGACAGCCTTTGTGGCAGCT R:TAGTCCGTTGATGGTTCCTTG	57	687
Primers used for qRT-PCR			
16S rRNA (qRT-PCR)	F:AGAGCAAGCGGACCTCATAAA R:AACGTATTCACCGTGACATTCTG	55	
oqxA (qRT-PCR)	F:CGCAGCTTAACCTCGACTTCA R:ACACCGTCTTCTGCGAGACC	60	141
oqxB (qRT-PCR)	F:TCCTGATCTCCATTAACGCCCA R:ACCGGAACCCATCTCGATGC	60	131
efrA (qRT-PCR)	F:TTGGCTTTATGACGCCAGT R:ATGCGCGTATTACCCGCAA	57	225
efrB (qRT-PCR)	F:TAGTGATGATGTTCTTAATCAA R:ATTGACTTGTTTAAAGCCTTCA	55	233
emeA (qRT-PCR)	F:AGCCCAAGCGAAAAGCGGTTT R:CCATCGCTTTCGGACGTTCA	57	128
Multilocus sequence typing (MLST) PCR primers			
adk	F:TATGAACCTCATTTTAATGGG R:GTTGACTGCCAAACGATTTT	55	437
atpA	F:CGGTTCATACGGAATGGCACA R:AAGTTCACGATAAGCCACGG	55	556
ddl	F:GAGACATTGAATATGCCTTATG R:AAAAAGAAATCGCACCG	55	465
gdh	F:GGCGCACTAAAAGATATGGT R:CCAAGATTGGGCAACTTCGTCCCA	55	530
gyd	F:CAAACTGCTTAGCTCCAAGGC R:CATTTCGTTGTCATACCAAGC	55	395
purK	F:GCAGATTGGCACATTGAAAGT R:TACATAAATCCCCCTGTTTY	55	492
pstS	F:TTGAGCCAAGTCGAAGCTGGAG R:CGTGATCACGTTCTACTTCC	55	583

Abbreviations: F, forward primer; R, reverse primer; Tm, melting temperature.

verapamil (100 μg/mL), chlorpromazine (20 μg/mL), reserpine (20 μg/mL), omeprazole (100 μg/mL), and Phe-Arg-β-naphthylamide (PAβN) (20 μg/mL) were compared to measure the efflux activities. The phenotype test is regarded to be positive when the MIC of the strain decreases by ≥4-fold after the supplementation of the efflux pump inhibitor.[16]

NIT-susceptible and -intermediate *E. faecium* strains were used as negative controls.

Quantitative Real-Time PCR (qRT-PCR) of Efflux Pump Gene

To evaluate the relative expression levels of efflux pump genes *efrAB, emeA*, and *oqxA*, qRT-PCR was performed on NIT-resistant and -susceptible *E. faecium* isolates before and after induction with NIT of 1/2 MIC based on a 7500 RT-PCR system (Thermo Fisher Scientific, Marsiling, Singapore) with an SYBRTM Green RT-PCR Kit (TOYOBO, Osaka, Japan). The primers used are listed in Table 1. To extract the RNA of the experimental strains, a single colony was selected and inoculated overnight in blood agar plates and added to fresh Luria broth (LB) medium with shaking at 180 rpm to logarithmic phase (OD_{600} value of approximately 0.5) at 37°C. The bacterial culture (3 mL) was centrifuged at 14,000 × g for 5 min, and the supernatant was discarded. Total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The concentration and purity of the extracted RNA were estimated, and the samples were stored at −80°C for further experiments. The purified RNA was then reverse transcribed to cDNA for qRT-PCR analysis with a cDNA Synthesis Kit (Takara, Japan) based on the manufacturer's manual. The *16S rRNA* gene was used as the reference gene to normalize the data. The $2^{-\Delta\Delta Ct}$ method was utilized for determining the expressions of *efrAB, emeA*, and *oqxA*.

MLST Typing of NIT-Resistant Isolates

In this experiment, clone correlation analysis of the 40 NIT-resistant isolates was carried out with MLST by amplifying seven housekeeping loci (*adk, atpA, ddl, gdh, ggd, pstS*, and *purK*) of *E. faecium*. The database available at Institut Pasteur's *E. faecium* MLST website (http://efaecium.mlst.net/) was checked to obtain the corresponding allelic profiles and subsequently their sequence type (ST). The primers used are given in Table 1.

Statistical Analysis

All data were analyzed using the GraphPad Prism v8.01 statistical software package (GraphPad Software, La Jolla, CA, USA). The Chi-Square test was used to compare the significance of gene carriage rate. Unpaired Student's *t*-test (two-tailed) was performed for comparing the significance of gene expression in qRT-PCR. *p*-values of <0.05 were considered to be statistically significant. For all analyses, * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

Results

Antimicrobial Susceptibility Testing

Among the 633 *E. faecium* isolates collected from the First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China) in 2017–2018, 50.9% (322/633) were resistant to NIT. As shown in Table 2, the NIT-resistant strains had higher drug resistance rates than the NIT-intermediate and -susceptible strains. Based on the results of antimicrobial susceptibility testing, 40 each of NIT-resistant, -intermediate, and -susceptible *E. faecium* strains isolated from the urine samples were selected for further research. The NIT-resistant isolates demonstrated high resistance rates toward ampicillin, penicillin, fluoroquinolones (ciprofloxacin and levofloxacin), and tetracycline. Besides, all strains were highly susceptible to linezolid and glycopeptides (vancomycin and teicoplanin) (Table 3).

Molecular Mechanisms of NIT Resistance

A significant positive correlation was observed between NIT resistance and the prevalence of *ef0404* and *ef0648* genes. In our study, all the susceptible isolates were found to carry at least one nitroreductase gene (*ef0404* 75.0% and *ef0648* 72.5%), and the carriage rates of the nitroreductase genes among the NIT-intermediate (*ef0404* 20.0%

Table 2 Percentage of Antimicrobial Agent Resistance Rates Among Nitrofurantoin-Resistant, -Intermediate and -Susceptible *E. Faecium* Isolates

Isolates	Resistance Rate (%)								
	AMP	PEN	CIP	LVX	TCY	ERY	TEC	VAN	LNZ
Resistant (n=322)	98.8	99.4	96.6	95.3	24.8	95.0	0.6	0.6	0.3
Intermediate (n=143)	79.0	81.8	75.5	74.8	39.2	90.7	0.7	0.7	0.7
Susceptible (n=168)	87.5	88.1	84.5	85.1	13.7	86.3	0	0	0.6

Abbreviations: AMP, ampicillin; PEN, penicillin; CIP, ciprofloxacin; LVX, levofloxacin; TCY, tetracycline; ERY, erythromycin; TEC, teicoplanin; VAN, vancomycin; LNZ, linezolid.

Table 3 Minimum Inhibitory Concentrations (MICs) and Sequence Types (STs) of 40 Nitrofurantoin-Resistant *E. Faecium* Isolates

Isolates	MICs (μg/mL)										
	STs	NIT	AMP	PEN	CIP	LVX	TCY	ERY	TEC	VAN	LNZ
SC-1182	**ST1822**[a]	128	≥128	≥128	≥64	≥64	≤1	≥64	1	1	2
SC-1183	ST761	256	≥128	≥128	≥64	≥64	≤1	≥64	1	1	2
SC-1209	ST78	256	≥128	≥128	≥64	≥64	≤1	≥64	1	1	2
SC-1218	ST78	256	≥128	≥128	≥64	≥64	≤1	≥64	0.5	1	2
SC-1221	ST230	256	≥128	≥128	≥64	≥64	≤1	≥64	2	1	2
SC-1245	ST78	256	≥128	≥128	≥64	≥64	2	≥64	0.5	1	2
SC-1306	ST78	256	≥128	≥128	≥64	≥64	≤1	≥64	0.5	1	2
SC-1307	ST78	256	≥128	≥128	≥64	≥64	≤1	≥64	1	1	2
SC-1310	ST78	256	≥128	≥128	≥64	≥64	≤1	≥64	0.5	1	2
SC-1312	ST249	256	≥128	≥128	32	≥64	≤1	≥64	0.5	1	2
SC-1319	ST78	256	≥128	≥128	≥64	≥64	≤1	≥64	1	1	2
SC-1324	ST555	256	≥128	≥128	≥64	≥64	≤1	≥64	1	1	2
SC-1331	ST78	256	≥128	≥128	≥64	≥64	64	≥64	0.5	1	2
SC-1334	ST17	256	≥128	≥128	≥64	≥64	≤1	≥64	1	1	2
SC-1351	ST230	256	≥128	≥128	≥64	≥64	≤1	≥64	0.5	1	2
SC-1355	ST78	256	≥128	≥128	≥64	≥64	≤1	≥64	1	1	2
SC-1393	ST78	256	32	≥128	≥64	≥64	32	≥64	≤0.125	1	2
SC-1394	ST78	256	≥128	≥128	16	≥64	≤1	≥64	0.5	1	2
SC-1408	ST78	256	≥128	≥128	≥64	≥64	≤1	≥64	1	1	2
SC-1430	ST78	256	≥128	≥128	≥64	≥64	64	≥64	0.5	1	2
SC-1597	ST78	128	≥128	≥128	≥64	≥64	≤1	≥64	1	1	2
SC-1607	ST78	128	≥128	≥128	≥64	≥64	64	≥64	0.5	≤0.5	2
SC-1610	ST555	128	≥128	≥128	≥64	≥64	≤1	≥64	0.5	≤0.5	2
SC-1618	ST78	128	≥128	≥128	≥64	≥64	≤1	≥64	0.5	1	2
SC-1643	ST78	128	≥128	≥128	≥64	≥64	≤1	≥64	1	1	2
SC-1645	ST78	128	≥128	≥128	≥64	≥64	4	≥64	1	1	2
SC-1672	ST78	128	≥128	≥128	≥64	≥64	≤1	≥64	0.5	1	2
SC-1673	ST497	256	≥128	≥128	≥64	≥64	≤1	≥64	1	1	2
SC-1701	ST78	128	≥128	≥128	≥64	≥64	≤1	≥64	1	≤0.5	2
SC-1702	ST78	128	≥128	≥128	≥64	≥64	8	≥64	2	≤0.5	2
SC-1731	ST78	128	≥128	≥128	≥64	≥64	≤1	≥64	0.25	1	2

(*Continued*)

Table 3 (Continued).

Isolates	MICs (μg/mL)										
	STs	NIT	AMP	PEN	CIP	LVX	TCY	ERY	TEC	VAN	LNZ
SC-1745	ST78	128	≥128	≥128	≥64	≥64	4	≥64	0.5	1	2
SC-1748	ST78	128	≥128	≥128	≥64	≥64	≤1	≥64	1	1	2
SC-1752	ST78	256	≥128	≥128	≥64	≥64	≤1	≥64	0.5	1	2
SC-1779	ST78	128	≥128	≥128	≥64	≥64	≤1	≥64	0.5	≤0.5	2
SC-1782	ST78	128	≥128	≥128	≥64	≥64	≤1	≥64	1	1	2
SC-1788	ST78	128	≥128	≥128	≥64	≥64	≤1	≥64	1	1	2
SC-1797	ST78	128	≥128	≥128	≥64	≥64	≤1	≥64	1	1	2
SC-1802	ST203	128	≥128	≥128	≥64	≥64	32	≥64	0.5	1	2
SC-1808	ST78	128	≥128	≥128	≥64	≥64	≤1	≥64	1	1	2

Notes: [a]New sequence types report in this study.
Abbreviations: AMP, ampicillin; PEN, penicillin; CIP, ciprofloxacin; LVX, levofloxacin; TCY, tetracycline; ERY, erythromycin; TEC, teicoplanin; VAN, vancomycin; LNZ, linezolid.

and *ef0648* 35.0%) and -resistant isolates (*ef0404* 5.0% and *ef0648* 17.5%) were 50% and 20%, respectively (Table 4). Furthermore, our study revealed that 20.8% (25/120) of the *E. faecium* isolates carried *efrAB* and 10.0% (12/120) carried *emeA*. One unanticipated finding was that the *oqxA* gene was present in only two (SC-1245 and SC-1325) out of 120 *E. faecium* isolates, and none had the *oqxB* gene (Figure S1).

While further exploring the resistance mechanisms of NIT, mutations of the resistance determinants *ef0404* and *ef0648* were detected in 12 NIT-resistant *E. faecium* isolates. Most of these were nonsense mutations, while missense mutation 478G > A and 52C > T of the nitroreductase-encoding gene *ef0648* was observed in four *E. faecium* strains. Moreover, base insertion (69–70insC) and amino acid mutations (52C > T) in *ef0648* were considered deleterious by PROVEAN. No missense mutation was found in the nitroreductase-encoding gene *ef0404* (Table 5).

The Potential Effect of the Efflux Pump Mechanism

The correlation between NIT resistance and efflux pump was tested. The results suggested that after exposure to the efflux inhibitors CCCP, verapamil, and chlorpromazine, the MICs of the 12 NIT-resistant *E. faecium* strains decreased by ≥4-fold. However, when used in combination with reserpine, omeprazole, and PAβN, the efflux pump inhibition test was negative. The MIC showed either no change or <4-fold decrease in NIT-susceptible (SC-1177), and NIT-intermediate (SC-1178) *E. faecium* strains (Table 6). Our findings allude that the overexpression of the efflux pump may influence NIT resistance in *E. faecium*.

Analysis of the Expression Level of the Efflux Pump Gene

Previous studies have indicated that efflux pumps EfrAB and EmeA are present in *E. faecium* and lead to multidrug resistance.[22,23] PCR results exposed that efflux pumps EfrAB and EmeA existed in 12 and 5 NIT-resistant *E. faecium* strains, respectively. The OqxAB efflux pump was present in only one NIT-resistant and one NIT-intermediate *E. faecium* strain. We examined the effect of efflux pump overexpression on NIT resistance. As depicted in Figure 1, the expression levels of *efrAB, emeA*, and *oqxA* in four NIT-resistant strains after induction by NIT of 1/2 MIC were not significantly increased in comparison with the control strain ATCC 29212. Hence, our results indicate that the efflux pumps EfrAB, EmeA, and OqxAB do not play a significant role in NIT resistance in the isolated *E. faecium* strains.

Molecular Epidemiological Analysis

Amplification of the seven housekeeping genes of the 40 NIT-resistant *E. faecium* isolates by PCR and MLST

Table 4 Distribution of Resistance-Related Genes in Nitrofurantoin Resistant (NIT-R), -Intermediate (NIT-I) and -Susceptible (NIT-S) *Enterococcus Faecium* Isolates

Genes	% (n) of Isolates							
	Nitroreductase-Coding Genes			Efflux Pump Genes				
	ef0404	*ef0648*	Total[a]	*oqxA*	*oqxB*	*efrA*	*efrB*	*emeA*
NIT-R (n=40)	5 (2)	17.5 (7)	20 (8)	2.5 (1)	0	12.5 (5)	22.5 (9)	12.5 (5)
NIT-I (n=40)	20 (8)	35 (14)	50 (20)	2.5 (1)	0	2.5 (1)	12.5 (5)	5 (2)
NIT-S (n=40)	75 (30)	72.5 (29)	100 (40)	0	0	15 (6)	12.5 (5)	12.5 (5)

Notes: [a]Total carriage rate of *ef0404* and *ef0648*.

Table 5 Analysis the Mutations of Nitroreductase in Nitrofurantoin-Resistant Isolates

		Amino Acid Substitution(s)[a]	
Isolates	**MICs (μg/mL)**	***ef0404***	***ef0648***[b]
SC1218	256	N/d	**69–70 insC, 478G > A**
SC1245	256	126C > T, 333G > A, 339G > A, 588T > C	**478G > A**
SC1310	256	N/d	**478G > A**
SC1324	256	126C > T, 268T > C, 294G > A	N/d
SC1393	256	N/d	267C **>** A, 417G **>**A
SC1430	256	N/d	69T **>** A, 417G **>** A, 483A **>** G, 549C **>** T
SC1597	128	N/d	489T > G
SC1672	128	N/d	267C > A, 417C > A, 489T > G
SC1779	128	N/d	**52C > T**

Notes: [a]N/d, failed to amplify; [b]bold fonts represent missense mutations, others are nonsense mutations.

analysis revealed that they belonged to the same subtype, CC17. ST78 (30/40) was the predominant ST, accounting for 75.0%, followed by ST230 (2/40) and ST555 (2/40). Moreover, our investigation revealed a new sequence type (ST1822) for the first time (yet to be registered in the MLST database), and four individual isolates were assigned to ST761, ST17, ST249, ST497, and ST203 (Table 3). These results are consistent with Djahmi's findings, which showed that the most important STs present in *E. faecium* belong to the clonal complex CC17 and that the major ST of *E. faecium* is ST78 in Europe and Asia.[24] These results prove that the clonal cluster CC17 associated with nitrofurantoin-resistant *E. faecium* might originate from a single clonal lineage, which is likely to provide important insights for molecular epidemiological analysis.

Discussion

Prior studies have documented that *E. faecium* is an important pathogen causing urinary tract infection.[4,5] Its Intrinsic resistance and the capability to acquire resistance genes pose restrictions on therapeutic options.[25] NIT is an effective antimicrobial agent used for the treatment of urinary tract infections, and its application has increased exponentially in recent years.[26] More importantly, NIT can also be used in extended spectrum beta-lactamase-producing and carbapenem-resistant bacterial infections.[27,28] The European Association of Urology guidelines recommend the use of NIT as a first-line treatment for acute uncomplicated cystitis, especially in women.[29] In this study, majority of the NIT-resistant strains were isolated from urine samples, which might be explained by the fact that NIT is mainly used for the

Table 6 Minimum Inhibitory Concentrations (MICs) of Nitrofurantoin (NIT) in Nitrofurantoin-Resistant (n = 12) -Intermediate (n = 1), and -Susceptible (n = 1) Isolates with or without the Efflux Pump Inhibitors

Isolates	Nitrofurantoin MICs (μg/mL)[a]			
	NIT	NIT+CCCP (6μg/mL)	NIT+Verapamil (100μg/mL)	NIT+Chlorpromazine (20μg/mL)
Resistant isolates				
SC1218	256	128	128	**64**
SC1306	256	**64**	128	128
SC1310	256	128	128	**64**
SC1312	256	128	**64**	**64**
SC1351	256	**64**	**64**	128
SC1355	256	128	**64**	128
SC1393	256	**64**	**64**	**64**
SC1430	256	**64**	**64**	**64**
SC1731	128	**16**	64	**32**
SC1779	128	**16**	**32**	**32**
SC1802	128	**8**	64	**32**
SC1808	128	**32**	64	64
Intermediate isolate				
SC1178	64	32	32	32
Susceptible isolate				
SC1177	32	32	32	32
Negative control				
ATCC-29212	8	8	8	8

Notes: [a]Bold data represent the MICs of nitrofurantoin decreased ≥4-fold.

treatment of urinary tract infections.[30] The drug possesses several antibacterial mechanisms, the most important of which is the reduction of its nitro group by the bacterial nitroreductases, producing toxic products and thus affecting the cell's metabolism.[31] However, the resistance mechanisms of NIT in *E. faecium* are yet to be well understood, and further investigations are needed to prevent the spread of NIT resistance.

Significantly, we proved for the first time that nitroreductases EF0404 and EF0648 were responsible for NIT resistance in *E. faecium* and that deletion in the nitroreductase-encoding gene is the main mechanism involved. As expected, while the nitroreductase-encoding gene was found to exist in 100% of the NIT-sensitive *E. faecium* strains, it occurred in only 20% of the NIT-resistant strains. However, previous studies have asserted that four nitroreductases (EF0404, EF1181, EF0648, and EF0655) are present in *E. faecalis*.[19] However, only EF0404 and EF0648 were detected in our study. This may be the reason for the huge difference in the NIT resistance rates between *E. faecalis* and *E. faecium* (0.6% vs 50.9%). Besides, to the best of our knowledge, this is the first time that the plasmid-borne NIT resistance gene *oqxAB* has been detected in clinical *E. faecium* isolates, which mediates resistance to NIT in *E. coli* and *K. pneumonia*.[15–17] However, our result disagrees with the findings of Li Yuan et al,[32] who reported that the carriage rates of *oqxA* and *oqxB* (79.3% and 65.5%, respectively) in *Enterococcus* were significantly higher than that in this study (1.7% and 0, respectively). We speculate that this variation might have been caused by the difference in sample sources since the strains were isolated from swine manure in the earlier study. Although the prevalence of the *oqxAB* gene was low in our study, screening for the gene should be implemented to prevent its spread among *E. faecium*.[33]

Based on the results of antimicrobial susceptibility testing, many *E. faecium* isolates were found to be resistant to most of the antimicrobial agents in clinical use, probably due to genes encoding MDR efflux pumps in *Enterococcus*.[22] To ascertain the role of efflux pumps in the NIT-resistant phenotype of the 40 *E. faecium* isolates, the most extensively studied efflux pumps EmeA (a member of the major facilitator superfamily) and EfrAB

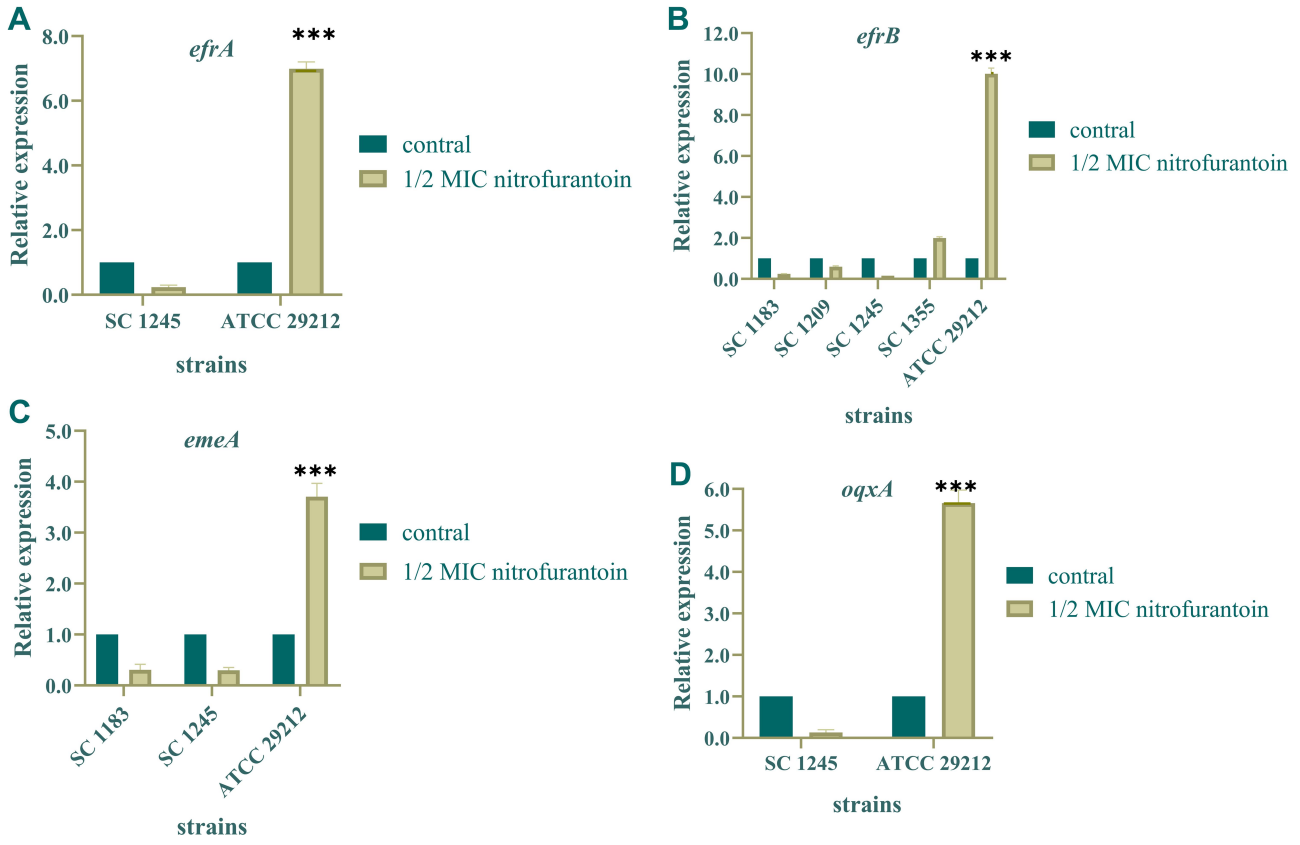

Figure 1 Relative expression levels of the efflux pump encoding genes in 4 nitrofurantoin-resistant *E. faecium* isolates. (**A**–**D**) The relative expression levels of efflux pump genes *efrA, efrB, emeA*, and *oqxA* in 4 nitrofurantoin-resistant *E. faecium* isolates before and after induced by nitrofurantoin of 1/2 MIC. $P < 0.05$ were considered to be statistically significant. ***$P < 0.001$ (Student's *t*-test).

(belonging to the ATP-binding cassette (ABC) superfamily) were amplified by PCR.[24,34] The results revealed that 12 and 5 NIT-resistant *E. faecium* strains exhibited the efflux pumps EfrAB and EmeA, respectively. Moreover, the effect of efflux inhibitors (including CCCP, verapamil, chlorpromazine, reserpine, omeprazole, and PAβN) on the activity of NIT against *E. faecium* was investigated. It was discerned that 12 of the 40 (40%) NIT-resistant isolates demonstrated a 4-fold decrease in the MIC of NIT in the presence of the efflux pump inhibitors CCCP, verapamil, and chlorpromazine. Moreover, according to qRT-PCR, efflux pump genes *efrAB, emeA*, and *oqxA* in the NIT-resistant strains were not overexpressed after induction by NIT. Our results imply that the overexpression of the pump leads to NIT resistance in the *E. faecium* isolates. However, which efflux pump plays a role warrants further research. Furthermore, the results of MLST alluded that the distribution of the STs was concentrated, suggesting that the NIT-resistant strains may spread horizontally among the nosocomial population. Although we have illuminated the main mechanisms of NIT resistance in *E. faecium*, more research needs to be undertaken to completely elucidate the concept.

Conclusions

Collectively, the findings of the present investigation provide convincing evidence that deletions in the nitroreductases-encoding genes *ef0404* and *ef0648* and the overexpression of the efflux pump genes are the main reasons for NIT resistance in *E. faecium*. Our results would be helpful in establishing a theoretical basis for the rational use of the drug and in adopting appropriate control measures to curb the increase in antimicrobial resistance.

Ethical Statement

The whole investigation protocols in this study were approved by The Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University. There are no studies with humans or animals performed by any of the authors in this article. Informed consent was waived

because this study with observational nature mainly focused on bacteria and did no interventions to patients.

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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