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ORIGINAL RESEARCH

Molecular Characterization of Linezolid-Non-Susceptible *Enterococcus faecium*: Identification of *optrA and vanM* Co-Harboring Strain in Clinical Isolate from China

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Purpose: This study aimed to explore the resistance mechanisms and molecular characteristics of linezolid-non-susceptible *Enterococcus faecium* isolates (LNSEFM) from a tertiary hospital in Beijing, China, focusing on novel findings with significant clinical and epidemiological implications.

Patients and Methods: LNSEFM strains isolated from clinical specimens between January 2011 and December 2023 were collected and screened for resistance genes, including *rplC, rplD, rplV*, 23s rRNA, *optrA, poxtA*, and *cfr* using polymerase chain reaction (PCR) and DNA sequencing. Molecular epidemiological analysis was performed using multi-locus sequence typing (MLST). Isolates carrying *optrA* and those harboring *poxtA* were subjected to whole-genome sequencing (WGS).

Results: Among 2384 clinical *E. faecium* isolates, 19 (0.80%) were linezolid-non-susceptible (MIC 4–32 mg/L). Among these, two vancomycin-resistant *Enterococcus* (VRE) strains exhibited an intermediate susceptibility to linezolid. Two distinct *optrA* variants (designated as KLDK and KLDP) were detected in separate LNSEFM isolates. The KLDK-positive isolate was found to co-harbor the *vanM* gene cluster despite maintaining vancomycin susceptibility. Additionally, one linezolid-resistant isolate carried a G2576T mutation in the 23S rRNA gene, whereas the other harbored the *poxtA* gene. MLST revealed 13 sequence types (STs) among the isolates, including a novel type ST2709.

Conclusion: This study identified key notable findings in LNSEFM: identification of linezolid intermediate VRE in China, clinical detection of the *optrA* KLDK variant in enterococci, *optrA-vanM* co-presence in vancomycin-susceptible *E. faecium*, and a novel sequence type (ST2709). These findings enrich our understanding of the molecular epidemiology of LNSEFM and provide critical insights into clinical antimicrobial management and infection control.

Keywords: linezolid-non-susceptible *Enterococcus*, *optrA*, *poxtA*, whole genome sequencing

Introduction

Enterococcus is a Gram-positive opportunistic pathogen that is a core member of the intestinal microbiota of humans and animals and can cause severe infections, including urinary tract infections, surgical wound infections, bacteremia, endocarditis, infections associated with catheters and other implanted medical devices, and pneumonia.[1,2] Notably, *Enterococcus faecium* is a member of the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, Enterobacter species) group prioritized by WHO for global AMR surveillance, and it plays a key role in difficult-to-treat nosocomial infections.[3,4] Furthermore, it shows intrinsic and acquired resistance to multiple antibiotic classes.[5] Specifically, *vanM*-type vancomycin-resistant *Enterococcus* (VRE)

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has been reported in *E. faecium* isolates in several Chinese studies.[6,7] In particular, the emergence and widespread dissemination of VRE have reduced the options for antibiotics. Linezolid is considered to be a last-resort antibiotic for treating multidrug-resistant Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA) and VRE.[8,9] Over the past few years, reports of linezolid-resistant enterococci (LRE) have emerged and are on the rise globally.[10] Although the current detection rate of LRE is low, the potential risk posed by its spread may make infections difficult to control.[11]

Given the clinical burden of *Enterococcus* infections, especially those caused by LRE, clarifying the underlying molecular resistance mechanisms is crucial for guiding clinical treatment and infection control. Several resistance mechanisms have been identified to be associated with LRE. Mutations in the domain V region of the 23S rRNA gene represent the most common mechanism, such as G2576T and G2505A substitutions.[12,13] Additionally, modifications in the 50S ribosomal subunit proteins L3, L4, and L22, encoded by genes *rplC, rplD*, and *rplV*, significantly contribute to reduced susceptibility to linezolid.[14] In addition to these mutations, the transferable gene *cfr*, which encodes a 23S rRNA methyltransferase, also confers the PhLOPSA phenotype (resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A compounds).[15] Notably, the increase in multidrug-resistant (MDR) microorganisms triggering infections is growing worldwide and becoming more serious in developing countries.[16,17] The *cfr* gene variants *cfr*(B) and *cfr*(D) have also been identified in enterococci isolates.[18,19] Furthermore, another transferable gene, *optrA*, encodes the ATP-binding cassette (ABC) protein, which was first identified in a clinical *Enterococcus faecalis* from China in 2015 and was later discovered in many countries.[20,21] Also reported have been *optrA* variants.[21] Another novel phenicol-oxazolidinone-tetracycline resistance gene, *poxtA*, was originally identified in an Italian clinical MRSA isolate in 2018.[22] Since its initial detection, it has also been found in enterococci isolates from animals, humans and environmental sources.[21]

Previous studies in China have mainly focused on short-term surveillance of LRE, particularly linezolid-resistant *Enterococcus faecalis*. In contrast, long-term molecular epidemiological data on linezolid-non-susceptible *Enterococcus faecium* isolates (LNSEFM) remain scarce, and their resistance mechanisms are not yet fully elucidated. Considering LRE as a potential emerging threat and these existing research gaps, this study consecutively collected LNSEFM isolates over a 12-year period at a tertiary hospital in China to investigate their molecular characteristics and resistance mechanisms.

Materials and Methods

Bacterial Strains and Antimicrobial Susceptibility Testing (AST)

A total of 2384 non-duplicated *Enterococcus faecium* strains (isolated from unique patients, except 2 sequential linezolid resistant strains from the same patient at different sites and different time points) were collected from the teaching hospital of Capital Medical University in Beijing between January 2011 and December 2023. Strains were derived from clinical specimens including urine, bloodstream, wounds, drainage fluid, secretions, pleural effusion, ascites, semen, and catheter-related sources. Species identification was performed using an automated VITEK 2 system and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) (VITEK-MS; bioMerieux, France; IVD version 3.0). Antimicrobial susceptibility tests were initially performed using AST-GP67 cards (BioMérieux) on a VITEK-2 system (bioMérieux, Lyon, France), including linezolid (LZD), vancomycin (VAN), penicillin (PEN), tetracycline (TET), ampicillin (AMP), ciprofloxacin (CIP), levofloxacin (LEV), and tigecycline (TGC). Isolates that showed elevated minimum inhibitory concentration (MIC) values of linezolid (MIC $\geq$ 4 mg/L) were further confirmed using the broth microdilution method. MICs of vancomycin and teicoplanin were further confirmed using the E-test. *E. faecalis* ATCC 29212 was used as the quality control strain. Susceptibility of TGC was determined according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2023). The classifications of susceptibility (S), intermediate (I), and resistance (R) for all antimicrobials except TGC were determined according to the *CLSI M100-S34* guidelines (2024). MIC interpretation of linezolid was based on the CLSI breakpoint criteria: $\leq$2 mg/L was susceptible, 4 mg/L was intermediate and $\geq$8 mg/L was resistant.

Molecular Epidemiology Investigation

Multi-locus sequence typing (MLST) of *E. faecium* isolates was performed according to a previously described method.[23,24] Seven housekeeping genes (*adk, pstS, gyd, purK, gdh, ddl, atpA*) were amplified and sequenced. Primer sequences and polymerase chain reaction (PCR) product sizes for each gene are listed in Supplementary Table S1. The reaction volume was 50 μL, including 2×Taq PCR Mix 25μL (TIANGEN, Beijing, China), DNA template 4μL, forward and reverse primers (10μmol/L) each 2μL, and ddH2O 17μL. PCR conditions for all amplification reactions were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30s, annealing at 50°C for 30s, and extension at 72°C for 30s, and a final extension at 72°C for 5 min. PCR products were visualized on 1.5% agarose gels stained with GeneRed (TIANGEN, Beijing, China) to confirm amplicon size and purity. Sterile nuclease-free water was used as a no-template control in all PCR reactions to rule out contamination. Positive amplicons were submitted to Beijing Ruibiotech Co., Ltd. for Sanger sequencing. Sequence quality control was performed using Phred scores (threshold ≥20) and ambiguous base calls (N) exceeding 5% in any region were re-sequenced. The sequences were analyzed using the MLST website (http://pubmlst.org/efaecium), and sequence types (STs) were assigned only when all seven loci matched the existing alleles in the database. Novel STs were submitted to the curator for validation and assignment of new identifiers. Ambiguous results were resolved by repeated PCR and sequencing from independent colonies. A minimal spanning tree was constructed using PHYLOViZ v2.0 with the goeBURST algorithm to visualize clonal relationships among STs.

Molecular Detection of Resistance Genes and Mutations

The genomic DNA of each LNSEFM isolate was extracted using a TIANamp Bacterial DNA Kit (TIANGEN, Beijing, China), following the manufacturer's protocol without any modifications. Genomic DNA quality and quantity were assessed using Nanodrop spectrophotometry (Thermo Fisher Scientific, USA) to determine the OD260/OD280 ratio (purity) and concentration, and 1% agarose gel electrophoresis was conducted to verify DNA integrity. Extracted DNA was stored at −20°C in aliquots to prevent repeated freeze–thaw cycles until further use. To investigate the linezolid resistance mechanisms, mutations in domain V of the 23S rRNA gene, the gene encoding proteins L3 (*rplC*), L4 (*RplD*), and L22 (*rplV*), and the presence of *cfr, optrA*, and *poxtA* were identified by PCR using a previously described method.[24] Primer sequences and PCR product sizes for each gene are listed in Supplementary Table S1. The PCR reaction matrix was the same as that described above. The 23S rRNA gene and genes encoding ribosomal proteins L3 (*rplC*), L4 (*rplD), optrA*, and *poxtA* were amplified using the following PCR conditions: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30s, annealing at 55°C for 30s, and extension at 72°C for 30s; and a final extension at 72°C for 7 min. For the L22 (*rplV*) and *cfr* genes, the annealing temperature was adjusted to 50°C, while the other cycling parameters remained constant. The methods used for assessing the PCR product and sequence quality were consistent with those described in the MLST protocol. The sequences of 23S rRNA, L3 (*rplC*), L4 (*RplD*), and L22 (*rplV*) were blasted against reference sequences from *E. faecium* (GenBank accession no. CP003583.1). The complete *optrA* and *poxtA* sequences were compared with the wild-type *optrA* sequence (GenBank accession no. NG_048023.1), and *poxtA* (GenBank accession No. MF095097.1), respectively. Sequence similarity was determined using BLAST at the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast).

Whole-Genome Sequencing (WGS) and Bioinformatic Analysis

Whole-genome sequencing of isolates carrying *optrA or poxtA* was performed using the DNBSEQ platform at the Beijing Genomics Institute (Shenzhen, China). Genomic DNA concentration was measured using a Qubit fluorometer with a Qubit dsDNA HS Assay Kit, and its integrity was verified by 1% agarose gel electrophoresis prior to library preparation. Qualified DNA was fragmented using a Covaris sonicator to generate short DNA fragments, which were size-selected using an Agencourt AMPure XP-Medium kit to enrich 300–400 bp fragments, and purified DNA was quantified using Qubit. The library preparation involved end repair of double-stranded DNA, 3′-end A-tailing, adapter ligation, and PCR amplification of ligated products, with fragment size selection using Agencourt AMPure XP-Medium and quality assessment by Agilent 2100 Bioanalyzer. The amplified products were denatured into single strands and circularized, and uncircularized linear DNA was

digested to obtain final libraries, which were evaluated for fragment size and concentration using an Agilent 2100 Bioanalyzer (DNA 1000 Reagents). Single-stranded circular DNA molecules were converted into DNA nanoballs (DNBs) via rolling circle replication, loaded onto a high-density DNA nanoball chip, and sequenced using combinatorial probe–anchor synthesis (cPAS) technology. Raw reads were filtered using Fastp v0.23.2, to remove adapters, low-quality bases (Phred score < 20), and reads with >10% ambiguous bases or <5× coverage of consecutive bases. The filtered reads were assembled using SOAPdenovo v1.05 software. Antibiotic resistance genes were identified using CARD v3.2.6 and ResFinder 4.1, with thresholds of ≥90% nucleotide identity, ≥80% query coverage, and ≤1e-10 e-value. Genes that met the criteria for both databases were identified. Draft sequence data were submitted to GenBank under accession numbers JBJMAS000000000, JBJOUF000000000, and JBJPIB000000000.

Results

Clinical Characteristics and Antimicrobial Susceptibility Testing

From 2011 to 2023, 19 LNSEFM isolates were recovered from 2384 clinical *E. faecium* isolates from a Chinese tertiary hospital with an overall prevalence rate of 0.80% (19/2384; 95% confidence interval [CI], 0.51–1.25%). Most of the strains were isolated in 2020 (Figure 1). The clinical characteristics of patients are presented in Table 1. Most of the patients were female (n=11/19, 57.9%) and ranged from 27 to 90 years of age (median age, 74 years). Isolates were collected from different departments, with the highest number collected from the ICU (n=7, 36.8%), followed by the emergency (n=4, 21.1%) and pneumology (n=3, 15.8%) departments. The sample types included urine (most common), catheter tips, wound secretions, and drainage fluids. Medical records showed that two patients received linezolid treatment before LNSEFM strain isolation. The clinical outcomes included 10 discharges (52.6%), 3 deaths (15.8%), and 1 transfer (5.3%).

The antimicrobial susceptibility results of the 19 LNSEFM strains are presented in Table 2. The MICs of linezolid ranged from 4 to 32 mg/L, including 6 isolates that were resistant to linezolid (31.6%) and 13 isolates that were intermediate (68.4%). All isolates were resistant to ciprofloxacin and levofloxacin and susceptible to tigecycline. The highest rates of drug resistance were observed for penicillin (89.5%, 17/19), and ampicillin (89.5%, 17/19), followed by tetracycline (47.4%, 9/19). Notably, two vancomycin-resistant isolates (EF1 and EF8) concurrently exhibited intermediate susceptibility to linezolid, suggesting a potential association between vancomycin resistance and reduced linezolid susceptibility in clinical *E. faecium* isolates. From the MLST results summarized in Table 2, the predominance of ST78 (3/19 isolates, 15.8%) within the CC17 lineage indicates clonal dissemination of hospital-adapted strains.

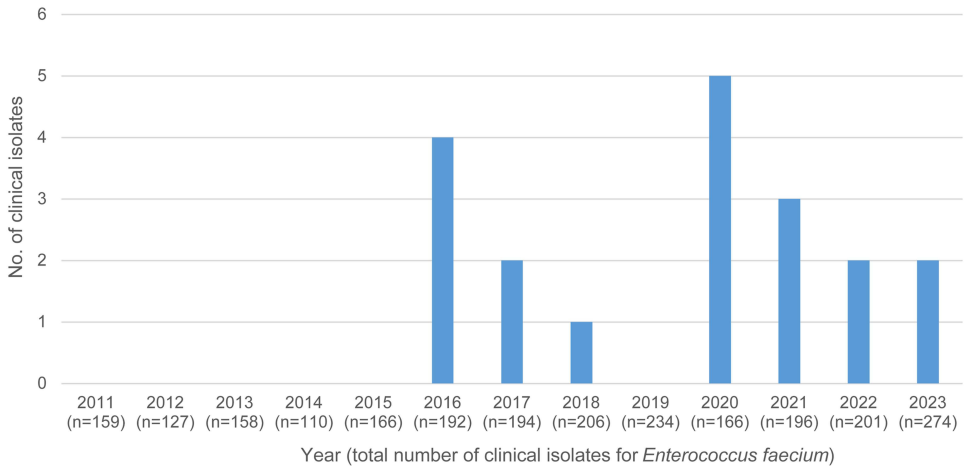

Figure 1 Temporal distribution of LNSEFM isolates collected at a tertiary hospital in China.

Table 1 Characteristics of LNSEFM Isolates

Strain	Date of Isolation (yyyy/mm/dd)	Samples	Age/Sex	Department	Diagnosis	Outcomes	Prior LZD use
EF1	2016/07/26	Urine	82/F	ED	–	–	–
EF2	2016/12/02	Urine	65/F	General	Urinary infection	Discharge	No
EF3	2016/12/13	Urine	81/F	Pneumology	Pulmonary infection	Discharge	No
EF4	2016/12/13	Urine	59/M	Orthopedics	Lumbar instability	Discharge	No
EF5	2017/01/22	Wound	67/M	ICU	Bacterial pneumonia	Discharge	No
EF6	2017/05/18	Urine	81/F	ED	–	–	–
EF7	2018/10/01	Catheter	30/F	ICU	Placenta previa	Discharge	No
EF8	2020/02/18	Urine	77/F	Pneumology	Urinary infection	Transfer	No
EF9	2020/07/16	Urine	74/M	ICU	Multiple abscess, Urinary infection	Discharge	Yes
EF10	2020/08/03	Wound	74/M	ICU	Multiple abscess, Urinary infection	Discharge	Yes
EF11	2020/09/22	Urine	85/F	Pneumology	Cerebral infarction	Discharge	No
EF12	2020/11/24	Urinary Catheter	66/F	General	Endometrial malignancy, Urinary infection	Discharge	No
EF13	2021/01/28	Urine	86/M	ED	Chronic obstructive pulmonary disease	–	–
EF14	2021/09/16	Urine	45/F	Outpatients	–	–	–
EF15	2021/12/07	Urine	81/F	ICU	Cerebral hemorrhage	Death	No
EF16	2022/05/06	Urine	66/M	ED	Severe pneumonia, Urinary infection	Death	No
EF17	2022/06/19	Urine	82/M	ICU	Urinary infection	Discharge	Yes
EF18	2023/05/30	Drainage fluid	27/M	ICU	Severe acute pancreatitis	Death	No
EF19	2023/11/14	Urine	90/F	Outpatients	–	–	–

Abbreviations: LZD, linezolid; ED, emergency department; ICU, intensive care unit.

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Table 2 The Antibiotic Susceptibility and Resistance Mechanism of 19 LNSEFM Isolates

Strain	MIC (mg/L)									Linezolid resistance mechanism					MLST
	LNZ	VAN	TEC	PEN	TET	AMP	CIP	LEV	TGC	23S rRNA	*rplC*	*rplD*	*optrA*	*poxtA*	
EF1	4	256	8	0.5	≤1	8	≥8	≥8	≤0.12	–	T600C	C174T, C180T	–	–	341[b]
EF2	4	0.5	0.5	≥64	≤1	≥32	≥8	≥8	≤0.12	–	T600C	–	–	–	856[b]
EF3	4	0.5	1	≥64	≥16	≥32	≥8	≥8	≤0.12	–	T600C	–	–	–	17[b]
EF4	4	0.5	1	≥64	≤1	≥32	≥8	≥8	≤0.12	–	T600C	–	–	–	78[b]
EF5	4	1	0.5	≥64	≥16	≥32	≥8	≥8	≤0.12	–	T600C	–	–	–	192[b]
EF6	4	0.5	0.25	32	4	≥32	≥8	≥8	≤0.12	–	T600C	–	–	–	78[b]
EF7	4	0.5	0.25	32	≥16	≥32	≥8	≥8	≤0.12	–	T600C	–	–	–	359[b]
EF8	4	256	8	≥64	≤1	≥32	≥8	≥8	≤0.12	–	T600C	C174T, C180T	–	–	547[b]
EF9	16	0.5	0.25	≥64	≤1	≥32	≥8	≥8	≤0.12	–	T600C	C174T, C180T	–	–	547[b]
EF10	32	0.5	0.5	≥64	≤1	≥32	≥8	≥8	≤0.12	G2576T	T600C	C174T, C180T	–	–	547[b]
EF11	4	0.5	0.25	≥64	2	≥32	≥8	≥8	≤0.12	–	T600C	–	–	–	555[b]
EF12	4	1	0.25	≥64	≤1	≥32	≥8	≥8	≤0.12	–	T600C	–	–	–	555[b]
EF13	4	0.5	0.25	≥64	≥16	≥32	≥8	≥8	≤0.12	–	T600C	–	–	–	80[b]
EF14	8	1	0.25	≥64	≥16	≥32	≥8	≥8	≤0.12	–	T600C	–	–	+	25[b]
EF15	32	1	0.25	32	≥16	≥32	≥8	≥8	≤0.12	–	T600C	C174T, C180T	KLDK	–	976
EF16	4	0.5	0.25	≥64	≥16	≥32	≥8	≥8	0.25	–	T600C	–	–	–	363[b]
EF17	4	0.5	0.25	≥64	≥16	≥32	≥8	≥8	0.25	–	T600C	–	–	–	78[b]
EF18	32	1	0.25	≥64	≤1	≥32	≥8	≥8	≤0.12	–	T600C	C174T, C180T	–	–	976
EF19	16	1	0.5	1	≥16	8	4	≥8	≤0.12	–	T600C	G387A	KLDP	–	2709[a]

Notes: [a]New ST type. [b]Isolates belonging to CC17 clone.

Abbreviations: MIC, minimum inhibitory concentration; MLST, Multilocus sequence typing; rRNA, ribosomal RNA; S, susceptible; I, intermediate; R, resistant; PEN, Penicillin; AMP, Ampicillin; TGC, Tigecycline; TEC, Teicoplanin; VAN, Vancomycin; LEV, Levofloxacin; TET, Tetracycline; CIP, ciprofloxacin; LZD, linezolid; KLDK, Thr112Lys, Ser147Leu, Tyr176Asp, Ile287Lys; KLDP, Thr112Lys, Ser147Leu, Tyr176Asp, Thr481Pro.

Molecular Epidemiology Analysis

MLST was performed to further investigate clonal relationships among the 19 LNSEFM isolates. The isolates were classified into 13 sequence types (Table 2 and Figure 2). ST2709 was identified as a novel *E. faecium* sequence type. The most prevalent sequence type was ST78. Except for ST976 and ST2709, all sequence types belonged to the CC17 clone complex.

Identifying the Linezolid Resistance Mechanism

The molecular mechanisms of linezolid resistance in the LNSEFM isolates are shown in Table 2. Three isolates carried linezolid resistance genes (*optrA* or *poxtA*). Notably, the *cfr* gene was not detected in any of the 19 LNSEFM isolates, indicating that linezolid resistance in the studied strains is not mediated by the *cfr*-dependent mechanism. The two resistant isolates carried *optrA* (MIC=32 μg/mL and MIC=16 μg/mL). Compared with wild-type *optrA* from *E. faecalis* E349, two mutations were identified: EF15 exhibited mutations T112K, S147L, Y176D, and I287K (a KLDK variant), and EF19 displayed T112K, S147L, Y176D, and T481P (a KLDP variant). Both variants (KLDK and KLDP) have been previously reported, with the reported strains exhibiting high linezolid MIC values that align with our results (16–32 μg/mL).[25,26] Another linezolid resistance gene, *poxtA*, was detected in isolate EF14.

Additionally, resistance mutations in domain V of the 23S rRNA gene were detected in only one isolate that harbored a G2576T mutation (*Escherichia coli* numbering). Furthermore, we detected several mutations in *rplC* and *rplD*, but none led to alterations in the corresponding amino acid sequences. In total, 100% (19/19) and 36.8% (7/19) of LNSEFM isolates carried mutations in *rplC* and *rplD*, respectively. The T600C mutation in *rplC* was identified in all isolates. Mutations in *rplD* predominantly involved the C174T and C180T types (in six strains), and the G387A mutation was also observed (in one strain). The two VRE isolates (EF1 and EF8) contained only the *rplC* (T600C) and *rplD* (C174T and C180T) mutations. The mutations in *rplC* and *rplD* are synonymous substitutions and located within the functionally conserved domains of ribosomal proteins L3 and L4, respectively (annotated via NCBI Conserved Domain Database, CDD: rplC 439857, rplD 439858).

Identifying an optrA and vanM Co-Harboring Strain

During the sequencing analysis of bacterial isolates, strain E15 (previously found to harbor the optrA gene) also contained the *vanM* gene cluster, a genetic element typically associated with vancomycin resistance in enterococci.

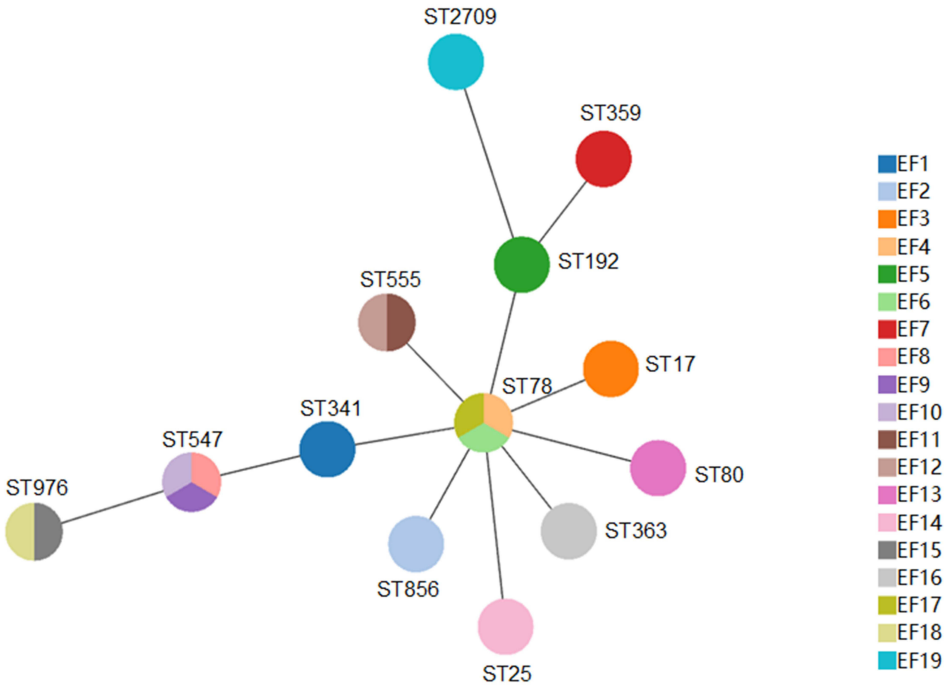

Figure 2 Minimum spanning tree of LNSEFM isolates.

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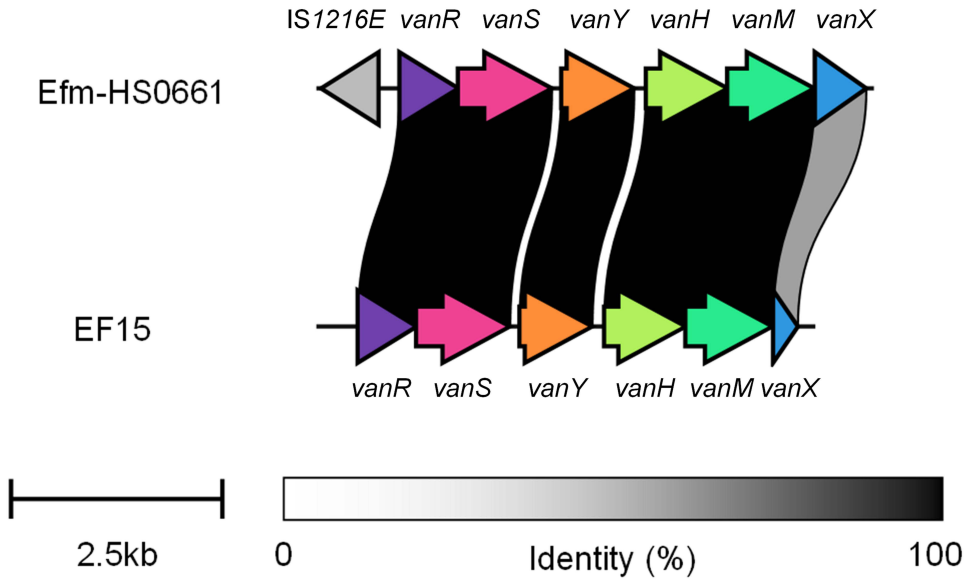

Figure 3 Schematic map of *vanM* gene clusters in *vanM*-carrying linezolid-resistant *E. faecium* EF15. The *vanM* gene cluster of vancomycin-resistant *E. faecium* Efm-HS0661 (GenBank accession no. FJ349556) are shown at the top.

Notably, despite the presence of the *vanM* gene cluster, antimicrobial susceptibility testing revealed that E15 was susceptible to vancomycin (MIC = 1μg/mL). To investigate the molecular basis of this discordance between genotype and phenotype, the *vanM* gene cluster of strain E15 was aligned with the reference sequence of the vancomycin-resistant *Enterococcus faecium* strain, Efm-HS0661. Sequence comparison identified two critical genetic alterations in the E15's *vanM* gene cluster: a complete deletion of the insertion sequence IS1216 and a partial deletion within the *vanX* gene (Figure 3). These genetic alterations render the *vanM* gene cluster nonfunctional.

Discussion

Linezolid is an effective antibiotic for treating infections caused by multidrug-resistant Gram-positive cocci. Since the introduction of linezolid in clinical practice, linezolid-resistant *Enterococcus* strains have emerged, and the rate of resistance has steadily increased in recent years, posing a significant threat to public health. A recent meta-analysis has estimated the global prevalence of linezolid-resistant *E. faecium* to be approximately 1.1%.[11] This study also revealed regional variations, indicating a prevalence of linezolid-resistant *E. faecium* strains of 0.9% in Asia, which is lower compared to Europe's 1.8% and America's 3.4%. Six-year surveillance conducted at a teaching hospital in China demonstrated that 0.24% (2/834) of *E. faecium* isolates exhibited resistance to linezolid.[27] In contrast, another study from 2011 to 2022 revealed a resistance rate of 0.2% (4/2114) among *E. faecium* isolates. In our study, the 12-year prevalence rate of linezolid-resistant *E. faecium* in our hospital was 0.25% (6/2384), aligning with previous studies in China and exhibiting lower than the global prevalence rates.[26] The relatively low linezolid resistance rate may be attributed to strict antibiotic stewardship, enhanced infection control, and limited clinical usage of linezolid in China. As shown in Tables 1 and 2, only two linezolid-resistant *E. faecium* strains were isolated in 2020, 2021, and 2023 respectively, with no resistant isolates detected in other years. This data indicates that the resistance is sporadic in our hospital (no continuous growth or sudden surge) in recent years. Notably, the 12-year prevalence of LNSEFM in our institution was 0.80% (19/2384), a value close to the upper limit of the 0–0.6% range reported by the China Antimicrobial Surveillance Network (CHINET) across multi-center Chinese hospitals during 2011–2023.[28] This local epidemiological data is further contextualized by the rising global prevalence of linezolid-resistant *E. faecium* (~1%). Of note, the emergence of LNSEFM in 2020 was sporadic, possibly linked to individual patient risk factors and environmental exposure rather than clonal outbreaks, as supported by the diverse MLST profiles of the isolates. Although the prevalence of linezolid resistance remains low, the emergence and spread of linezolid-resistant enterococci restrict the

therapeutic options for effectively treating VRE infections. Notably, through the analysis of antimicrobial susceptibility results, we discovered two linezolid-intermediate *Enterococcus* strains that were resistant to vancomycin, which, to our knowledge, have not been previously reported in China. There is a concern that the emergence of linezolid-resistant VRE strains (LR-VRE) will further shorten therapeutic options, making the treatment of such infections challenging. Therefore, intensive surveillance is necessary to prevent the emergence and rapid expansion of enterococci that are resistant to both vancomycin and linezolid.

Consistent with previous findings that *Enterococcus* strains are frequently associated with urinary tract infections (UTIs),[29–32] the majority of *Enterococcus* isolates in our study were obtained from urine samples (14/19,73.7%). Previous studies have indicated that prior exposure to linezolid and the duration of linezolid treatment were correlated with the development of resistance to linezolid.[33–35] However, in the present study, only two patients received linezolid treatment, while the others did not, highlighting the contribution of environmental and person-to-person spread, as previously described.[36] In this study, the clones of the 19 LNSEFM strains isolated at different time periods were diverse, suggesting that the cases in our hospital were sporadic rather than an outbreak. *E. faecium* clonal complex 17 (CC17) is a polyclonal group comprising multiple sequence types (STs). The epidemiology of enterococcal infections has been significantly influenced by the enhanced capability of a genogroup of *E. faecium*, associated with the pathogen designated as CC17, to colonize the human gastrointestinal tract and cause severe diseases.[37] ST78, belonging to CC17, was the most common type identified in this study and has previously been reported as the predominant ST in *vanA*- and *vanM*-type VRE*fm* strains in China.[7,38] In line with prior reports of ST78 as a prevalent lineage in China and the circulation of *vanM*, our findings also document an isolate in which *optrA* was identified within a *vanM* background, highlighting the potential convergence of glycopeptide and oxazolidinone resistance determinants in clinically important clones. The sequence types ST78 and ST80 identified in our study were also detected in another study on linezolid resistance in *E. faecium* in China.[24] In contrast, Ping's study revealed seven distinct sequence types that were entirely different from those observed in our research.[31] Furthermore, MLST demonstrated a novel STs (ST2709). These studies highlight the significant geographical variation that exists among linezolid-resistant isolates.

Resistance mechanisms to linezolid include mutations in the domain V of 23S rRNA; alterations in ribosomal proteins L3, L4, and L22 encoded by *rplC, rplD* and *rplV*; and the acquisition of transferable resistance determinants such as *optrA* and *poxtA*, or *cfr*. None of the isolates in our study had alterations in the ribosomal protein L22 or carried the *cfr* gene. Our study identified several *rplC* and *rplD* mutations in enterococci that may be associated with linezolid resistance. The T600C mutation in the *rplD* gene was identified in all LNSEFM isolates, and the *rplD* (C174T, C180T) mutations were detected in six isolates, as previously reported in Wang's study.[26] We also found a novel point mutation at G387A of the *rplD* gene. Interestingly, these mutations did not alter the corresponding amino acid sequences. These identified synonymous mutations may potentially contribute to linezolid resistance by affecting ribosome structure stability or translation efficiency, which warrants further experimental validation. Notably, two linezolid-resistant isolates displayed resistance exclusively via mutation-based mechanisms, and no other resistance-related mechanisms were identified. This finding suggests the presence of additional linezolid resistance mechanisms among the tested isolates, which require further experimental validation. Mutations in domain V of the 23S rRNA gene are considered the most common mechanism of linezolid resistance. EF10 was the sole isolate in which sequencing identified a G2576T substitution in the 23S rRNA. Both the EF9 and EF10 strains were isolated at separate times from the same patient with a history of linezolid use. Strain EF10 was isolated approximately one month after the isolation of EF9, and its MIC value was higher than that of EF9. EF10 displays an additional resistance mechanism involving the G2576T substitution in 23S rRNA, which is absent in EF9. This indicated that linezolid exposure may lead to the acquisition of additional resistance mechanisms.

In our study, linezolid resistance genes (*optrA* and *poxtA*) were identified in isolates that were resistant to linezolid but not in those that were intermediate. A similar pattern has also been reported in Yi's study.[24] Until now, several studies have showed that there were *optrA* variants in the amino acid sequences compared with the original *optrA* from *E. faecalis* E349 (designated as the wild type).[21] After deducing the amino acid sequence for *optrA*, we found two types of *optrA* variants, KLDK and KLDP in *E. faecium*. However, the wild type was not detected in our study. The KLDP variant has been previously identified.[26,39] The KLDK variant was first reported in non-enterococcal isolates, about *Vagococcus lutrae*, and was subsequently identified in *E. faecium* samples collected from hospital sewage.[25,40] The

novelty in our study is that the KLDK variant is reported in a clinical enterococcus isolate. Previous studies have revealed that distinct *optrA* variants may confer differential resistance to linezolid in enterococci.[39,41,42] However, due to the limited number of *optrA* variants, we were unable to ascertain a correlation between *optrA* variants and the MICs of linezolid. A *vanM* cluster was identified in the KLDK variant isolate. However, this isolate was susceptible to vancomycin and teicoplanin. We speculated that the impairment of *vanX* might be responsible for the silencing of the vancomycin-resistant phenotype, as previously reported.[6,43,44] Notably, *optrA* and *vanM* coexist in this strain. This co-occurrence enriches the genetic resistance reservoir of *E. faecium* and reflects potential adaptive evolution of *E. faecium* under antimicrobial selective pressure, with its functional relevance requiring further investigation. Another novel phenicol-oxazolidinone-tetracycline resistance gene, *poxtA*, was detected in one isolate. The IS1216E-*PoxtA*-IS1216E segment identified in our study was similar to that found in *Staphylococcus aureus* strain AOUC-0915.[22]

Notably, our study has minor limitations. The single-center design and small sample size reflect a limited scope of strain collection rather than impacting the identified resistance mechanisms, and functional validation of genetic variations will further strengthen our findings. Importantly, neither of these factors compromises the authenticity of our core discoveries.

Conclusions

Our study revealed multiple mechanisms underlying linezolid resistance in LNSEFM in China. Notably, the linezolid resistance genes *optrA* and *poxtA* were confined to linezolid-resistant isolates and completely absent from linezolid-intermediate isolates, which underscores that linezolid non-susceptibility arises via distinct pathways. However, our study also had some limitations. Although we conducted long-term surveillance of LNSEFM, the data were derived from a single hospital and involved a relatively small number of LNSEFM isolates. In addition, we identified *optrA* variants and mutations in *rplC* and *rplD*. However, the correlation between these discoveries and level of linezolid resistance needs to be confirmed by further experimentation. Finally, we documented the coexistence of the vanM and optrA genes in our linezolid resistant isolate. The presence of *vanM* and *optrA* on chromosomes and plasmids requires further experimental verification. Due to the single-center design and limited sample size of this study, we plan to actively promote multi-center collaboration in the future. Such efforts will include the integration of samples from various geographical regions and clinical backgrounds. The aim of this study was to further explore the resistance mechanisms and analyze the molecular basis of the specific resistance phenotypes discovered.

Ethical Approval

This study was approved by the Ethics Committee of the Beijing Chao-Yang Hospital, Capital Medical University (reference number: 2024-10-25-7). The study was conducted in accordance with local legislation and institutional requirements.

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Disclosure

The authors report no conflicts of interest in this work.

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