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Distribution of the *optrA* gene in *Enterococcus* isolates at a tertiary care hospital in China

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ABSTRACT

Objectives: Linezolid-resistant *Enterococcus* have spread worldwide. This study investigated the prevalence of linezolid-non-susceptible *Enterococcus* (LNSE) and the potential mechanism and molecular epidemiology of LNSE isolates from Nanjing, China.
Methods: Linezolid susceptibility of 2555 *Enterococcus* was retrospectively determined by Etest. Vancomycin and teicoplanin MICs were determined for LNSE by Etest. PCR and DNA sequencing were used to investigate the potential molecular mechanism. Clonal relatedness between LNSE isolates was analysed by MLST. WGS was also performed.
Results: A total of 27 *Enterococcus* isolates (24 *Enterococcus faecalis*, 3 *Enterococcus faecium*) with linezolid MICs of 4–48 μg/mL were identified, among which 20 *E. faecalis* and 3 *E. faecium* were positive for *optrA*. No mutations were found in genes encoding domain V of 23S rRNA or ribosomal proteins L3/L4; the *cfr* gene was not found. The 24 linezolid-non-susceptible *E. faecalis* were classified into eight STs (ST16, ST480, ST476, ST631, ST585, ST428, ST25 and ST689). The three linezolid-non-susceptible *E. faecium* were classified as ST17, ST400 and ST195. Comparison of the deduced OptrA amino acid sequences of the 23 *optrA*-positive isolates by PCR-based sequencing and WGS with that of the original OptrA from *E. faecalis* E349 revealed seven variants (KD, EDP, EDM, D, EDD, RDK and DP) in 16 isolates, with no mutations in the remaining 7 isolates. *optrA* was found downstream of *fexA* by searching the pE349 sequence based on WGS data.
Conclusions: Emergence of LNSE with *optrA*-mediated resistance and clonal dissemination of ST16 *E. faecalis* in our hospital may pose a potential public-health threat.

1. Introduction

Enterococci are a leading cause of nosocomial infections and are commonly recovered from bloodstream infections, intra-abdominal infections, surgical wounds and, in particular, urinary tract infections [1]. For enterococci, especially for *Enterococcus faecalis*, biofilms and other virulence factors can promote infections as well as increase tolerance to antimicrobial agents and host immune responses. Antimicrobial-resistant enterococci, particularly multidrug-resistant isolates, are now the leading cause of nosocomial infections worldwide, e.g. the emergence of vancomycin-resistant enterococci (VRE) and linezolid-resistant enterococci [1,2].

Linezolid is the first member of an entirely new class of antibiotics that inhibit bacterial protein synthesis by binding to the large (50S) subunit of the bacterial ribosome via interaction with the 23S rRNA of Gram-positive bacteria [3]. Based on the unique mechanism of action, it is highly effective in the treatment of serious infections caused by antimicrobial-resistant Gram-positive bacteria such as methicillin-resistant *Staphylococcus aureus* (MRSA) and VRE [3,4]. Following the introduction of linezolid, it was claimed that resistance to linezolid would be rare and difficult for bacteria to develop. However, linezolid-resistant staphylococci and enterococci have been increasingly reported in recent years

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[1,5–8] since the first case report on the emergence of linezolid-resistant MRSA in North America in 2001 [9]. We have reported that linezolid resistance mediated by the G2603T mutation accompanied the presence of the chloramphenicol–florfenicol resistance (*cfr*) gene in linezolid-resistant *Staphylococcus capitis* isolates and found clonal dissemination of a linezolid-resistant *S. capitis* strain among five patients at Nanjing Drum Tower Hospital (Nanjing, China) between September 2012 and February 2014 [10].

Mutations in the central loop of the domain V region of the 23S rRNA gene have been well recognised as the main mechanism mediating resistance to linezolid [6,7]. Acquisition of the *cfr* gene encoding a 23S rRNA methyltransferase and modifying adenosine at position 2503 in 23S rRNA has been frequently reported [11]. In addition, mutations or deletions in genes encoding the 50S ribosomal subunit proteins L3 or L4 also play an important role [12,13]. In 2015, another gene, named *optrA*, which mediates linezolid resistance in *Enterococcus*, was first discovered in China [14] and was later detected in Europe (Demark [15], Germany [16], Italy [17], Poland [18], Ireland [19]), Asia (South Korea [20], Taiwan [21], China [22,23]), the Americas (USA, Colombia) [24] and Africa [25]. The *optrA* gene (oxazolidinone phenicol transferable resistance) encodes an ATP-binding cassette (ABC) transporter resulting in resistance or elevated minimum inhibitory concentrations (MICs) for oxazolidinones (linezolid and tedizolid) and phenicols (chloramphenicol and florfenicol), and the *optrA*-carrying plasmid pE349 can easily be transferred among *Enterococcus* spp. [14]. The domestic detection rate is approximately 2% in China, but in Jiangsu Province, especially in Nanjing City, there has been no report on linezolid resistance in *Enterococcus* or on the prevalence of the *optrA* gene. In the present study, the susceptibility of *Enterococcus* isolates to linezolid over a 3.5-year period at a tertiary care hospital in Nanjing, China, was retrospectively determined. A total of 27 linezolid-non-susceptible *Enterococcus* (LNSE) isolates were identified. The *optrA* gene was the main gene mediating linezolid non-susceptibility among 23 *Enterococcus* isolates, with mainly sequence type 16 (ST16) *E. faecalis* clonal dissemination in our hospital.

2. Materials and methods

2.1. Bacterial strains and data collection

A total of 2555 *Enterococcus* isolates recovered at Nanjing Drum Tower Hospital between April 2014 and November 2017 were selected for screening for a linezolid-non-susceptible phenotype. A total of 27 (1.1%) *Enterococcus* isolates (24 *E. faecalis* and 3 *Enterococcus faecium*) were included in this study, which were recovered from urine ($n = 7$), blood ($n = 6$), bile ($n = 5$), secretions ($n = 4$), ascites ($n = 3$) and drainage ($n = 2$) from 27 hospitalised patients in nine different wards. Confirmation of *E. faecalis* and *E. faecium* was performed using a VITEK®2 Compact GP card (bioMérieux, Marcy-l'Étoile, France) combined with additional sequencing of the 16S rRNA gene [10]. Clinical data such as clinical features, laboratory results and treatment were retrieved from the medical records department. The study protocol was approved by the Ethics Committee of Nanjing Drum Tower Hospital, and written informed consent was obtained from all patients included in the study.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using VITEK®2 Compact GP67 cards (bioMérieux) according to the manufacturer's instructions. MICs of vancomycin, teicoplanin and linezolid were determined by Etest (bioMérieux) for re-examination and were further confirmed by the broth dilution method for linezolid. *E. faecalis* ATCC 29212 and *S. aureus* 29213 were used as quality control strains in parallel. Antimicrobial susceptibility testing results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [26].

2.3. Molecular detection of resistance genes

Amplification for the *cfr* gene, domain V of the 23S rRNA gene and the genes encoding ribosomal proteins L3 and L4 was performed as previously described [6,10]. The *optrA* gene was first screened with primers 5′-AGG TGG TCA GCG AAC TAA-3′ and 5′-ATC AAC TGT TCC CAT TCA-3′, which generated a 1395-bp amplicon [14], and subsequently with primers 5′-TAG GAG GTA GAA GCA AAT GA-3′ and 5′-CGG CAA ACT CAA AAG GTC-3′ for whole sequence amplification, which generated a 1968-bp amplicon [22]. Amplicons were purified using a QIAGEN DNA Purification Kit (QIAGEN, Hilden, Germany) and were subjected to sequencing. Sequence similarity was determined using the BLAST program from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST).

2.4. Multilocus sequence typing (MLST)

MLST of the *Enterococcus* isolates was performed according to the reference method [22,23]. Briefly, seven housekeeping genes of *E. faecalis* and *E. faecium* were PCR-amplified and sequenced. The PCR reaction system was as described previously and the cycling conditions were in accordance with the MLST database (https://pubmlst.org/efaecalis/; https://pubmlst.org/efaecium/). STs were assigned to the isolates according to those published in the MLST database.

2.5. Whole-genome sequencing (WGS)

Genomic DNA was prepared using a QIAamp DNA Kit (QIAGEN) from the 27 LNSE isolates and was subjected to WGS using an Illumina HiSeq PE150 System (Illumina Inc., San Diego, CA). Raw data were processed, including removing reads with 5 bp of ambiguous bases, reads with 20 bp of low quality (≤Q20) bases, adapter contamination and duplicate reads. Library construction and sequencing reactions were performed according to the manufacturer's instructions, and a 350-bp paired-end library was generated. The resulting sequences were de novo assembled using SPAdes v.3.9.0 (http://cab.spbu.ru/software/spades/) [27]. For the prokaryotic organism, the ab initio prediction method was used to get gene models for the strains. Mauve Contig Mover was utilised to arrange and orient the scaffolds against the reference strain *E. faecalis* V583 (GenBank accession no. NC_004668.1) for the 24 linezolid-non-susceptible *E. faecalis* (LNSEfs) isolates, and reference strain *E. faecium* DO (GenBank accession no. NC_017960.1) for the 3 linezolid-non-susceptible *E. faecium* (LNSEfm) isolates. Average sequencing coverage of the 24 LNSEfs and 3 LNSEfm isolates were 348.42× and 262.12×, respectively. Information on the number of contigs, assembly size and percent GC of all 27 LNSE isolates are shown in Supplementary Table S1. Identification of resistance-associated genes present in the LNSE isolates was performed using ResFinder 3.0 (http://cge.cbs.dtu.dk/services/ResFinder) [28]. Phylogenetic analysis was conducted by the neighbour-joining method using MEGA6 (https://www.megasoftware.net) [29]. All contigs of the strains were searched against the pE349 sequence (accession no. KP399637) using BLAST analysis with default parameters (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Draft sequence data have been submitted to GenBank under sample accession nos. RPCC00000000–RPCW00000000, QDDM00000000, QDDN00000000, QDDO00000000 and QDDP00000000.

3. Results

3.1. Emergence of linezolid-non-susceptible enterococci in Nanjing, China

A total of 27 LNSE were recovered from 2555 enterococci isolates over a 3.5-year surveillance period at a tertiary care hospital in China. Further 16S rRNA sequencing and analysis confirmed the identification of all 24 *E. faecalis* and 3 *E. faecium* isolates in accordance with conventional methods. As shown in Table 1, the 27 isolates were obtained from nine different wards, including the department of general surgery ($n = 7$), gastroenterology ($n = 5$), urology ($n = 4$), intensive care unit ($n = 3$), infectious diseases ($n = 3$), gynaecology ($n = 2$), endocrinology ($n = 1$), rheumatology ($n = 1$) and oncology ($n = 1$). Medical records showed that none of the 27 patients had received linezolid for treatment before isolation of the LNSE strains. Annual comparison of LNSE isolates revealed a slight decrease from 9 (1.9%) and 8 (1.2%) isolates in 2014 and 2015, to 4 (0.6%) and 6 (0.8%) isolates in 2016 and 2017, respectively.

The MICs of linezolid ranged from 4 μg/mL to 48 μg/mL among the 27 *Enterococcus* isolates in this study, including 22 isolates that were resistant to linezolid and 5 isolates that were intermediate-resistant, which was further confirmed by Etest and broth dilution method (Table 1). However, the MICs of vancomycin and teicoplanin among these isolates were ≤2 μg/mL and the MICs of tigecycline were ≤0.12 μg/mL. Susceptibility to other tested antibiotics is shown in Table 1.

3.2. The optrA gene may contribute to linezolid resistance of enterococci in Nanjing, China

Previous studies suggested that either mutation in domain V of 23S rRNA [7] or presence of the *cfr* gene [11] are the main mechanisms contributing to linezolid resistance. Therefore, in this study gene sequencing and PCR of the 23S rRNA and *cfr* gene of the LNSE isolates was performed. Notably, the most frequent mutation G2576T [7] could not be detected among these 27 isolates. In addition, none of the isolates carried the *cfr* gene or had mutations in ribosomal proteins L3 or L4 [11,12] in the current study. Interestingly, 23 LNSE isolates were detected to have the *optrA* gene, except for EF332, EF348, EF427 and EF433. Furthermore, the full length open reading frame of the *optrA* gene (1968 bp) among the LNSE isolates was confirmed either by direct Sanger sequencing or WGS (see below). The translated OptrA amino acid sequences of the LNSE isolates compared with OptrA in *E. faecalis* E349 type strain revealed seven types of mutations (Table 2), including KD (EF438, EF5152, EF1765, EF5505, EF2469, EF3157 and EF2389), EDP (EF437 and EM2369), EDM (EM333 and EM429), D (EF5473 and EF4106), EDD (EF5141), RDK (EF2597) and DP (EF353) in 16 isolates, with no mutations in the remaining 7 isolates (EF294, EF2916, EF346, EF349, EF439, EF4946 and EF6735).

3.3. Three distinct clones (ST16, ST480 and ST476) are dominant in linezolid-non-susceptible Enterococcus faecalis isolates in Nanjing, China

MLST was performed to further investigate the clonal relationship among the 27 LNSE isolates. All LNSEfs isolates were classified into eight known STs, including ST16 (EF2597, EF348, EF5152, EF5473, EF1765, EF2469, EF3157 and EF2389), ST480 (EF438, EF437, EF5505 and EF6735), ST476 (EF439, EF4106, EF5141 and EF2916), ST631 (EF294, EF349 and EF346), ST585 (EF353 and EF427), ST428 (EF332), ST25 (EF433) and ST689 (EF4946). As shown in Table 1, three distinct clones including ST16 ($n = 7$), ST480 ($n = 4$) and ST476 ($n = 4$) were the main STs among the LNSEfs isolates that harboured the *optrA gene*. In parallel, three linezolid-resistant *E. faecium* isolates were identified as ST17 (EM333), ST400 (EM429) and ST195 (EM2369) (Table 1).

To further investigate the genetic environment and potential regulation mechanism, all LNSE isolates were submitted into high-throughput sequencing. WGS confirmed the presence of the *optrA* gene by searching ResFinder 3.0 in all isolates except for EF332, EF348, EF427 and EF433, which was consistent with the initial PCR results. In addition, all *optrA*-positive isolates harboured the *fexA* gene. Intriguingly, BLAST analysis with the pE349 sequence also indicated the *fexA* gene located upstream of *optrA* in their genome contigs, suggesting that *optrA* may be located in a plasmid resembling pE349. Furthermore, phylogenetic analysis of these LNSEfs isolates indicated a distinct clustering profile (Fig. 1).

4. Discussion

With the frequent use of linezolid in clinical treatment, linezolid-resistant coagulase-negative staphylococci and linezolid-resistant enterococci strains have been increasingly isolated from healthcare settings [1,30,31]. The proportion of linezolid resistance in *Enterococcus* was 0.22% (21/9417) in the Zyvox® Annual Appraisal of Potency and Spectrum (ZAAPS) study and 0.78% (67/8604) in the Linezolid Experience and Accurate Determination of Resistance (LEADER) study, which are two linezolid surveillance programmes monitoring the susceptibility of common pathogenic bacteria to linezolid [1,30,31]. In the current study, 1.1% (27/2555) of enterococci isolates with a linezolid-non-susceptible phenotype were identified, of which 22 showed a resistant phenotype, which was similar to the above surveillance programmes but lower than reports from nationwide surveillance in China from 2004–2014, which found a rate of 1.5% (34/2201) [22].

No mutations in the genes encoding 23S rRNA or the ribosomal proteins L3 and L4 were detected in the 27 LNSE isolates, and the *cfr* gene, which has been previously reported to cause high-level resistance to linezolid in *Staphylococcus* and *Enterococcus* spp., was not detected. In 2015, a novel linezolid resistance gene named *optrA* was first confirmed in China [14] and was later detected internationally [15–25]. Overall, the detection rate of the *optrA* gene was 2.0% in China, with positivity rates ranging from 0.4% to 3.9% from 2004 to 2014 [22]. In the current study, 23 isolates (0.9%; 23/2555), including 20 *E. faecalis* and 3 *E. faecium*, were positive for the *optrA* gene, which is lower than the above mentioned overall detection rate in China [14,22,23] although it is consistent with the overall detection rates of the *optrA* gene being higher in *E. faecalis* than in *E. faecium* [14,22,23]. The mechanism mediating resistance to linezolid in the remaining four isolates (EF332, EF348, EF427 and EF433) was uncertain [30]. Even with WGS technology, we failed to find any reported determinants mediating resistance to linezolid in these four isolates, among which three isolates showed an intermediate-resistant phenotype to linezolid (Table 1). Thus, there may be other mechanisms involved, i.e. the observed phenotype could be related to cell wall thickness or biofilm formation [32].

The OptrA protein encoded by *optrA* is composed of 655 amino acids and a previous report showed that there were variants in the amino acid sequences compared with the original OptrA from *E. faecalis* E349 type strain. Some variants have been observed among linezolid-susceptible isolates, and some variants have been shown to cause a 2–8-fold increase in the MICs for oxazolidinones in *S. aureus* and *E. faecalis* [22,33]. After deducing the amino acid sequence for OptrA, no mutation was observed in seven isolates compared with the *E. faecalis* E349 type strain, whilst seven variants, including KD, EDD, EDP, EDM, RDK, DP and D (Tables 1 and 2), were detected in the other 16 isolates in this study, for which

Table 1
Clinical characteristics and antimicrobial susceptibility phenotype of linezolid-non-susceptible *Enterococcus* isolates.

Patient	Strain ID	Isolation (yyyy/mm/dd)	Ward	Resource	ST	MIC (μg/mL)												Genes[a]	
						LNZ	VAN	TEC	LVX	CIP	ERY	AMP	TGC	PEN	TET	HLG	HLS	*optrA* presence	*optrA* mutation(s)[b]
1	EF2597	2014/04/09	Rheumatology	Blood	16	24	1	1	≥8	≥8	≥8	≤2	≤0.12	2	≥16	R	R	+	I104R, Y176D, E256K (RDK)
2	EF294	2014/05/19	Gastroenterology	Bile	631	16	1	2	2	1	≥8	≥32	≤0.12	≥64	≥16	S	R	+	None
3	EF332	2014/07/02	General surgery	Bile	428	4	1	1	2	1	≥8	≥32	≤0.12	≥64	≥16	R	R	–	
4	EF2916	2014/07/04	Gastroenterology	Blood	476	16	1	1	≥8	≥8	≥8	≤2	≤0.12	2	≥16	R	R	+	None
5	EF349	2014/09/24	General surgery	Urine	631	12	1	1	≥8	≥8	≥8	≤2	≤0.12	2	≥16	S	S	+	None
6	EF346	2014/10/19	ICU	Ascites	631	24	1	2	0.5	1	≥8	≤2	≤0.12	2	≥16	S	R	+	None
7	EF348	2014/10/22	IDD	Urine	16	4	1	2	1	1	≥8	≤2	≤0.12	2	≥16	R	R	–	
8	EF353	2014/12/29	Endocrinology	Secretions	585	24	1	2	≥8	≥8	≥8	≤2	≤0.12	2	≥16	R	R	+	Y176D, T481P (DP)
9	EF427	2015/05/05	ICU	Bile	585	4	1	1	2	1	0.5	≤2	≤0.12	4	≤1	S	S	–	
10	EF433	2015/05/27	General surgery	Ascites	25	8	1	2	2	1	1	≥32	≤0.12	≥64	2	S	S	–	
11	EF438	2015/06/24	IDD	Urine	480	48	1	2	≥8	≥8	1	≤2	≤0.12	1	≥16	S	S	+	T112K, Y176D (KD)
12	EF4106	2015/07/05	Gastroenterology	Blood	476	4	1	1	≥8	≥8	≥8	≤2	≤0.12	2	≥16	R	R	+	Y176D (D)
13	EF439	2015/09/03	Gastroenterology	Drainage	476	16	1	1	≥8	≥8	≥8	≤2	≤0.12	0.5	≥16	R	R	+	None
14	EF437	2015/09/09	Gynaecology	Drainage	480	16	1	2	≥8	≥8	≥8	≤2	≤0.12	2	≥16	S	R	+	K3E, Y176D, T481P (EDP)
15	EF4946	2015/12/28	Urology	Urine	689	32	1	1	≥8	≥8	≥8	≤2	≤0.12	2	≥16	R	S	+	None
16	EF5152	2016/02/12	General surgery	Ascites	16	24	1	1	1	0.5	2	≤2	≤0.12	4	≥16	S	S	+	T112K, Y176D (KD)
17	EF5141	2016/02/15	Oncology	Blood	476	4	1	1	≥8	≥8	≥8	≤2	≤0.12	2	≤1	R	S	+	K3E, Y176D, G393D (EDD)
18	EF5473	2016/05/20	Urology	Urine	16	16	1	1	2	2	≥8	≤2	≤0.12	2	≥16	R	S	+	Y176D (D)
19	EF5505	2016/06/12	Gynaecology	Secretions	480	24	1	1	≥8	≥8	≥8	≤2	≤0.12	2	≥16	R	R	+	T112K, Y176D (KD)
20	EF6735	2017/04/19	ICU	Blood	480	24	1	1	≥8	≥8	≥8	≤2	≤0.12	1	≥16	R	R	+	None
21	EF1765	2017/08/17	Urology	Urine	16	16	1	1	≥8	≥8	≥8	≤2	≤0.12	1	≥16	R	R	+	T112K, Y176D (KD)
22	EF2469	2017/09/24	General surgery	Bile	16	24	1	1	≥8	≥8	≥8	≤2	≤0.12	2	≥16	R	S	+	T112K, Y176D (KD)
23	EF3157	2017/10/31	IDD	Urine	16	12	1	1	≥8	≥8	≥8	≤2	≤0.12	4	≥16	S	S	+	T112K, Y176D (KD)
24	EF2389	2017/11/23	General surgery	Secretions	16	16	1	1	≥8	≥8	≥8	≤2	≤0.12	1	≥16	R	S	+	T112K, Y176D (KD)
25	EM333	2014/09/10	Gastroenterology	Bile	17	12	≤0.5	1	≥8	≥8	≥8	≥32	≤0.12	≥64	≥16	R	S	+	K3E, Y176D, I622M (EDM)
26	EM429	2015/05/08	Urology	Secretions	400	8	≤0.5	1	≥8	≥8	≥8	≥32	≤0.12	≥64	≥16	R	R	+	K3E, Y176D, I622M (EDM)
27	EM2369	2017/11/23	General surgery	Blood	195	12	≤0.5	1	≥8	≥8	≥8	≥32	≤0.12	≥64	≥16	R	S	+	K3E, Y176D, T481P (EDP)

ST, sequence type; MIC, minimum inhibitory concentration; LNZ, linezolid; VAN, vancomycin; TEC, teicoplanin; LVX, levofloxacin; CIP, ciprofloxacin; ERY, erythromycin; AMP, ampicillin; TGC, tigecycline; PEN, penicillin; TET, tetracycline; HLG, high-level gentamicin; HLS, high-level streptomycin; R, resistant; S, susceptible; ICU, intensive care unit; IDD, infectious diseases department.

[a] None of the isolates carried the *cfr* gene and no mutations in genes encoding domain V of 23S rRNA or ribosomal proteins L3/L4 were found.

[b] Amino acid change corresponding to *optrA* gene mutations: D, Asp; E, Glu; K, Lys; M, Met; P, Pro; R, Arg; I, Ile; Y, Tyr; T, Thr; G, Gly.

the Y176D (D) variant has not been reported previously [22]. Based on comparison between *optrA* gene mutations and the deduced amino acids, the number of amino acid substitutions has little influence on the level of linezolid resistance [33]. In a nationwide surveillance of the *optrA* gene in *Enterococcus* spp. in China, 11 linezolid-susceptible isolates were observed with OptrA variants, including 9 *E. faecalis*, 1 *Enterococcus gallinarum* and 1 *E. faecium* [22]. We screened for the *optrA* gene in another 42 linezolid-

Table 2
OptrA variants in linezolid-non-susceptible *Enterococcus* isolates.

OptrA variant[a]	Mutation(s) compared with OptrA E349[b]	Strain(s)	Linezolid MIC range (μg/mL)
KD	Thr112Lys, Tyr176Asp	EF438, EF5152, EF1765, EF5505, EF2469, EF3157, EF2389	12–48
EDD	Lys3Glu, Tyr176Asp, Gly393Asp	EF5141	4
EDP	Lys3Glu, Tyr176Asp, Thr481Pro	EF437, EM2369	12–16
EDM	Lys3Glu, Tyr176Asp, Ile622Met	EM333, EM429	8–12
DP	Tyr176Asp, Thr481Pro	EF353	24
RDK	Ile104Arg, Tyr176Asp, Glu256Lys	EF2597	24
D	Tyr176Asp	EF4106, EF5473	4–16

MIC, minimum inhibitory concentration.
[a] D, Asp; E, Glu; K, Lys; M, Met; P, Pro; R, Arg.
[b] Original OptrA from *Enterococcus faecalis* E349 type strain.

susceptible *E. faecalis* isolates recovered from blood samples but no positive results were found (data not shown). The linezolid resistance breakpoint is still an unresolved issue: indeed, an *Enterococcus* with a linezolid MIC of 4 μg/mL is regarded as 'intermediate' according to the CLSI [26] but as 'susceptible' according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [34], which may be why strains EF332, EF348 and EF427 had a linezolid MIC of 4 μg/mL without any resistance-associated genes being detected. The low-range MIC phenomenon could be expected to make laboratory detection of *optrA*-positive isolates difficult. Although the 27 enterococci isolates showed a non-susceptible phenotype to linezolid, all isolates were susceptible to vancomycin, teicoplanin and tigecycline. It has been reported in the literature that enterococci isolates can show resistance to both linezolid and vancomycin, making the treatment of such infections challenging [16,20]. However, the strains isolated in the current study were susceptible to other drugs tested. Compared with the three *E. faecium* isolates, the *E. faecalis* isolates were still susceptible to conventional drugs such as penicillin and ampicillin (Table 1). The emergence of linezolid resistance is consistently related to prior linezolid exposure [1,5]. A previous report showed that linezolid-resistant enterococci strains were isolated with a mean time of 29.8 ± 48.8 days and 23.1 ± 21.4 days for *E. faecalis* and *E. faeciumț* respectively, after patients were treated with linezolid, although in a few cases resistant strains were acquired due to cross-infection [1]. In the current study, none of the 27 patients had been treated with linezolid before isolation of the LNSE even though the quantity of linezolid used in the studied hospital was increasing annually (data not shown).

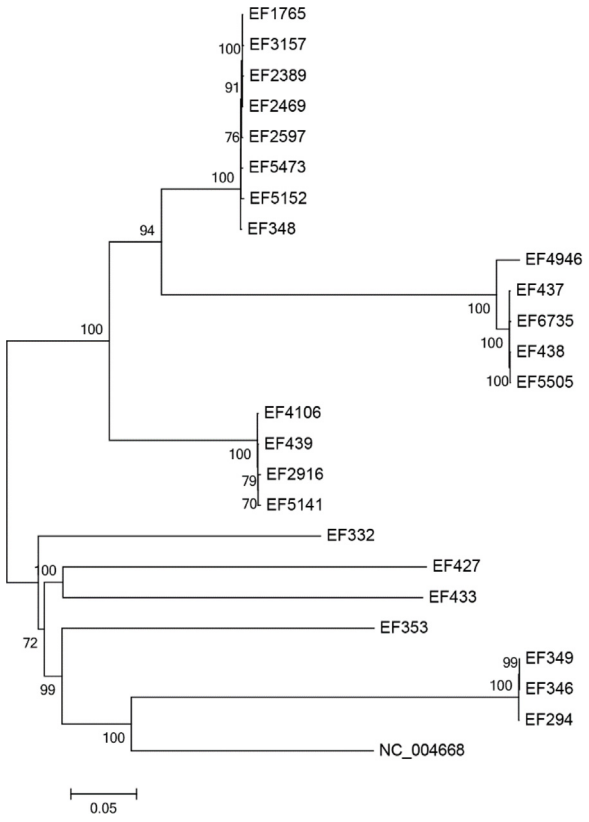

Fig. 1. Phylogenetic tree of linezolid-non-susceptible *Enterococcus faecalis* isolates based on whole-genome sequencing constructed by the neighbour-joining method. The scale bar shows the genetic distance. The number next to each node shows the percentage bootstrap value of 1000 replicates. NC_004668 indicates the *Enterococcus faecalis* V583 chromosome.

MLST analysis for the *optrA*-positive *Enterococcus* strains showed ST16 *E. faecalis* clonal dissemination in 2014, 2016 and 2017 in this survey, whilst the other isolates showed variable rates. In 2017, four *optrA*-positive *E. faecalis* isolates belonging to ST16 were identified, of which EF2469 and EF2389 were isolated from the same ward in two different months. Interestingly, seven isolates with a KD variant belonged to ST16 (5 isolates) and ST480 (2 isolates). Linezolid-resistant *E. faecalis* ST16 has been detected not only in China [23,35] but also in other countries [15], indicating a worldwide clone. The epidemic distribution of STs of linezolid-resistant *E. faecalis* in this study was similar to previous reports in China, such as ST16, ST480, ST631, and ST585 [23,35], but was different from South Korea, among others [20].

Previous reports showed that the *optrA* gene can be localised on plasmids and/or the chromosome detected in enterococci, *Staphylococcus sciuri* and *Staphylococcus suis* isolated both from humans and animals [36,37]. The first *optrA* gene found in *E. faecalis* E349 was located on a 36-kb pE349 plasmid in China [14]. Subsequently, *optrA*-positive enterococci were investigated in clinical and animal-derived enterococci in several countries across the globe, and the *optrA*-harbouring plasmid was highly similar to pE349, suggesting spread of the *optrA* gene between humans and animals [38]. It was found that the *optrA* gene carried by the enterococcal plasmid may be transmitted to different *Enterococcus* species through insertion sequence IS*1216*-mediated recombination [38,39]. After searching for resistance genes using WGS data of LNSE isolates, all *optrA*-positive isolates harboured *fexA* by searching in the ResFinder 3.0 antimicrobial resistance database, which was similar to the genetic environment published previously [38,39]. There is currently no detailed mechanism for how the gene is integrated into the chromosome. However, the localisation of the *optrA* gene identified in this study remains to be determined.

5. Conclusions

Linezolid is a last-resort drug for the treatment of MRSA and VRE. Although the prevalence of linezolid resistance in enterococci is lower than the national level, ST16 clonal dissemination

occurred in the tertiary care hospital in the current study. The *optrA* gene has been detected more frequently in *E. faecalis* and *E. faecium* isolated from food-producing animals (20.3% and 5.7%) than from humans (4.2% and 0.6%, respectively) [14], which is associated with inappropriate florfenicol use that could cause cross-resistance to linezolid. Possible transmission of the *optrA* gene between animals and humans and worldwide dissemination may pose a potential threat to public health. Prudent use of linezolid and appropriate infection control measures are needed to limit the emergence and spread of linezolid-resistant *Enterococcus* spp. and *Staphylococcus* spp.

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Competing interests

None declared.

Ethical approval

The study protocol was approved by the Ethics Committee of Nanjing Drum Tower Hospital, and written informed consent was obtained from all patients included in the study.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jgar.2019.01.001.

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