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ORIGINAL RESEARCH

Epidemiological characteristics and genetic structure of linezolid-resistant *Enterococcus faecalis*

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Objectives: The aim of this study was to investigate the mechanism of linezolid resistance and evaluate the risk factors for linezolid-resistant *Enterococcus faecalis* (LZR-Efa) infections.

Methods: A total of 730 *E. faecalis* isolates were collected, and whole-genome sequencing and bioinformatics analysis were performed. Meanwhile, risk factors related to linezolid resistance were analyzed by binary logistic regression.

Results: Twenty-six LZR-Efa were isolated from various clinical samples, and 24 isolates were multidrug resistant. Four isolates were daptomycin nonsusceptible, while all LZR-Efa were susceptible to vancomycin. Thirteen different sequence types (STs) were identified, and the most prevalent type was ST16 (23.1%). The genes *dfrE*, *lsaA,* and *emeA* were identified in all isolates. A total of 23 *E. faecalis* were positive for *optrA* gene, and six amino acids mutations were identified among 18 LZR-Efa in OptrA. The 23S rRNA mutation was found in 16 LZR-Efa isolates. However, the presence of *cfr* was not identified. Furthermore, there were 41 virulence genes detected, and 10 genes (*ace*, *bopD*, *cpsA*, *cpsB*, *ebpB*, *ebpC*, *efaA*, *fss1*, *fss2*, and *srtC*) were found in all isolates. A total of nine isolates were positive for multiple virulent factors (*ace*, *asa1*, *cylA*, *efaA*, *esp*, and *gelE*). There was no difference in the number of virulence factors among different specimens (P=0.825). It is of note that all patients had not been prescribed linezolid or traveled abroad previously. Moreover, previous use of carbapenems was a risk factor for LZR-Efa infections.

Conclusion: The main trends of LZR-Efa, with lower level of resistance, were sporadic mainly in the department of surgery. *optrA* and 23S rRNA were the main resistance mechanisms. In addition, carbapenems use was an independent predictor of LZR-Efa infections.

Keywords: linezolid, resistance mechanism, virulent factors, risk factors

Introduction

Enterococcus has become one of the most important opportunistic pathogens leading to nosocomial infections, posing significant challenge to clinicians. The vast majority of clinical enterococcal infections in humans are caused by *Enterococcus faecalis* and *E. faecium. E. faecium*, much less frequently isolated than *E. faecalis*, has a higher incidence of resistance to multiple antimicrobial agents.[1] According to the Zyvox Annual Appraisal of Potency and Spectrum (ZAAPS) program data, ampicillin, erythromycin, levofloxacin, and teicoplanin resistance rates for *E. faecalis* and *E. faecium* were 0% and 91.8%, 53.7% and 88.4%, 25.4% and 90.8%, and 0.4% and 14.7%, respectively.[2] In 2016, the European Union and European Economic Area population-weighted mean percentage for high-level gentamicin resistance in *E. faecalis* was 30.5%, with national percentages ranging from 12.5% to 56.3%.[3] It is of note that the number of

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vancomycin-resistant *Enterococcus* (VRE) has been increasing, resulting in the limited selection for treatment. Vega and Dowzicky showed that 40.8% (235/576) *E. faecium* and 1.6% (33/2,004) *E. faecalis* isolates were vancomycin-resistant.[4]

Linezolid was approved for management of VRE infections by the American Food and Drug Administration in 2000. Then linezolid-resistant *Enterococcus* was reported 1 year later. According to the ZAAPS and Linezolid Experience and Accurate Determination of Resistance program, the proportion of linezolid resistance in *Enterococcus* was 0.22% (21/9,417) and 0.78% (67/8,604), respectively. The main resistance mechanisms included alterations in the ribosomal proteins L3 and/or L4, mutations in domain V of the 23S rRNA, and/or presence of *cfr* or *optrA* gene. Resistance to linezolid in VRE is a result of decreased binding due to mutations at the 23S rRNA or acquisition of a *cfr* gene through horizontal transmission.[5,6]

The treatment and clinical outcomes regarding linezolid-resistant *Enterococcus* were limited. Older age, male gender, renal impairment, liver disease, diabetes, solid organ, and hematologic transplant have all been identified as risk factors for enterococcal blood stream infections in nonselected observational cohort studies. In addition, Billington et al[7] found that *E. faecalis* infections were associated with a urinary focus, genitourinary malignancy, and abnormal genitourinary anatomy. However, risk factors for linezolid resistance reported in the literature have been inconsistent.[8] The aim of this study was to evaluate the epidemiological, virulence factors, antibiotic resistance mechanism, and clinical profiles of LZR-Efa infections, gaining insights into control and prevention of resistance transmission.

Methods

Bacterial strains and clinical data

A total of 730 *E. faecalis* isolates were collected from patients hospitalized at The First Affiliated Hospital of Zhejiang University from January 2011 to December 2015. Species identification was conducted by API20 (bioMérieux, Durham, NC, USA) and MALDI-TOF technique (Bruker Diagnostics, Bremen, Germany). Linezolid susceptibility was screened via agar dilution method to determine minimal inhibitory concentration (MIC). The isolates with MIC of linezolid ≥8 mg/L were frozen at −80°C for further analysis.

The LZR-Efa infection group was compared with the linezolid-sensitive *E. faecalis* (LZS-Efa) infection group to evaluate the risk factors for linezolid resistance. The two groups were matched for age, same sex, specimen sources, and department (ratio: 1:2) from the same collection year. The medical records of patients were reviewed by two researchers. The included data were as follows: demographics, clinical characteristics (underlying diseases, comorbidities, invasive procedures, surgical procedures), laboratory examination, treatment history, hospitalization, and clinical outcomes. Antimicrobial drug exposure referred to the use of antibiotics for more than 72 hours at any point 2 weeks prior to diagnosis.

The work was in accordance with the Declaration of Helsinki. This study was approved by the recommendations of the Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University, with reference number 2018502. To protect personal privacy, identifying information of each patient in the electronic database was encrypted. Informed consent was waived by the Clinical Research Ethics Committee because no intervention was involved and no patient-identifying information was included.

Antibiotic susceptibility test

Antibiotic agents (oxacillin, cefazolin, cefuroxime, clindamycin, erythromycin, levofloxacin, moxifloxacin, tetracycline, tigecycline, amikacin, fosfomycin, trimethoprim-sulfamethoxazole, linezolid, vancomycin, daptomycin, rifampin) were purchased from Dalian Meilun Biotech (Dalian, China). Glucose-6-phosphate was obtained from Sigma-Aldrich Co. (St Louis, MO, USA). Tigecycline and daptomycin susceptibility test were done via broth dilution method according to CLSI recommendations. The MICs of the other 15 antibiotics were determined using the twofold serial agar dilution method.[9] *E. faecalis* ATCC 29212 were used as quality-control strains.

Multilocus sequence typing (MLST)

MLST was performed on all LZR-Efa isolates using the scheme of Institute Pasteur, and the sequence types (STs) were assigned using the MLST database (http://pubmlst.org/ecloacae). A minimal spanning tree was generated by using BioNumerics v7.0 to provide a graphical representation of the clonal distribution of LZR-Efa.

Whole-genome sequencing (WGS) and data analysis

WGS was carried out for 26 LZR-Efa with further analysis of gene environment. Genomic DNA was extracted by QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and sequenced using HiSeq 2000 (Illumina, San Diego, CA, USA) with constructing 2×125 bp paired-end libraries. De novo assembly was done using the CLC Workbench v8.0

(QIAGEN, Hilden, Germany). The resistance genes were identified by BLAST against the ResFinder 2.1 database (https://cge.cbs.dtu.dk/services/ResFinder/). The bioinformatics tools used in this study were available at the following web platforms: National Center for Biotechnological Information, Sequence Manipulation Suite, and European Bioinformatics Institute.

This Whole Genome Shotgun BioProject for LZR-Efa has been deposited at GenBank under the accession QNGG00000000–QNHF00000000 (Table S1).

Statistical analysis

Continuous variables were presented as mean ± SD (x±SD), and independent samples *t*-tests were used for comparisons. Data with non-normal distribution were expressed as medians with corresponding IQRs and compared using the Wilcoxon rank-sum test. Categorical variables presented as numbers and percentages and compared percentages using the chi-squared test. For multivariate analysis, binary logistic regression was used to identify risk factors. A two-tailed *P*-values <0.05 were considered to be statistically significant. Data analysis was performed using SPSS Statistics for Windows, version 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Detection of linezolid-resistant clinical isolates and their clinical characteristics

Among 730 clinical *E. faecalis* isolates over the study period, 26 (3.5%, 26/730) were resistant to linezolid. The incidences of LZR-Efa were 0%–1.2%, 4.4%–5.07%, and 2.5% from 2011 to 2015. The patients ranged in age from 18 to 70 years (Table 1). There were 11 males and 15 females, 4 of whom were outpatients. These samples obtained from culture included urine (n=13), ascites (n=5), bile (n=3), fester (n=3), and blood (n=2). The patients were from multiple cities in the Zhejiang Province, China. Medical records were reviewed, and patient histories confirmed that they had not been prescribed linezolid or traveled abroad previously. With the exception of one patient who died of multiple organ dysfunction syndrome, all the other patients recovered.

Characteristics of the antibiotic susceptibility and sequence types (STs)

The susceptibility test results for the 26 strains are shown in Table 2. All isolates were resistant to cefazolin, cefuroxime, clindamycin, and amikacin, whereas 46.1% (12/26), 69.2%

Table 1 Clinical features of LZR-Efa

Isolates	Sources	Ward	Age, y	Sex	Diagnosis	Prognosis	Region	Job	Year
6,888	Urine	Outpatients	24	F	Urinary infection	Improvement	Hangzhou	Worker	01/2012
8,714	Blood	6B-17	62	F	Hepatolithiasis	Improvement	Jinhua	Retired	01/2013
10,938	Fester	5-1A	62	M	Metastatic carcinoma of bladder	Improvement	Huzhou	Worker	05/2013
11,340	Ascites	6B-18	68	F	Hepatocholangiocarcinoma	Improvement	Huzhou	Retired	06/2013
11,382	Urine	Outpatients	41	F	Urinary infection	Improvement	Hangzhou	Worker	06/2013
12,654	Bile	6B-18	59	M	Hepatolithiasis	Improvement	Huzhou	Farmer	08/2013
13,470	Blood	3-4	67	M	Nephrostomy	Improvement	Jiaxing	Farmer	09/2013
13,484	Fester	7-2	65	F	Mammary cancer	Improvement	Hangzhou	Farmer	09/2013
14,980	Urine	3-4	62	M	Prostatic cancer	Improvement	Hangzhou	Retired	11/2013
15,224	Urine	9-5	40	F	Nephrotic syndrome	Improvement	Shaoxing	No	12/2013
15,407	Bile	6B-18	51	F	Hepatolithiasis	Improvement	Hangzhou	Worker	12/2013
15,814	Urine	3-3	70	M	Prostatic hyperplasia	Improvement	Wenzhou	Retired	01/2014
17,838	Urine	10-5	64	F	Renal calculus	improvement	Hangzhou	Retired	03/2014
18,026	Urine	6B-7	32	M	IgA nephropathy	Improvement	Hangzhou	Worker	04/2014
19,663	Urine	Outpatients	23	F	Urinary infection	Improvement	Hangzhou	Worker	06/2014
19,910	Fester	3-2	18	M	Sacroiliac disease	Improvement	Hangzhou	Student	07/2014
23,903	Ascites	6B-12	60	F	Pancreatic cancer	Improvement	Jiaxing	Retired	07/2014
23,967	Bile	6A-4	63	M	Acute obstructive suppurative cholangitis	Improvement	Shaoxing	Worker	07/2014
24,393	Urine	10-5	57	F	Ureteral calculus	Improvement	Shaoxing	Farmer	08/2014
26,167	Urine	5-2	37	F	Renal calculus	Improvement	Hangzhou	Worker	11/2014
27,149	Ascites	9-3	65	M	HIV	Died	Shaoxing	Farmer	12/2014
27,451	Urine	Outpatients	46	F	Urinary infection	Improvement	Hangzhou	Worker	01/2015
31,890	Urine	10-1	69	M	Urethral stricture	Improvement	Shaoxing	Retired	07/2015
32,142	Urine	6A-8	64	F	Renal calculus	Improvement	Jinhua	Retired	07/2015
32,633	Ascites	7-2	68	F	Malignant tumor of abdominal wall	Improvement	Ningbo	Farmer	08/2015
33,710	Ascites	5-1	41	M	Colonic tumor	Improvement	Hangzhou	Worker	09/2015

Abbreviations: F, female; LZR-Efa, linezolid-resistant *Enterococcus faecalis*; M, male.

Table 2 The antibiotic susceptibility and resistance mechanism of 26 LZR-Efa

Isolates	OXA	CFZ	CXM	ERY	CLI	LVX	MXF	TC	TGC	AMK	FM	SXT	LZ	VAN	DAP	RIF	MLST	optrA	23S rRNA
6,888	8	32	32	>32	>32	1	0.5	>32	1	>32	64	>8/152	8	1	8	2	16	Ile86Arg, Glu238Lys, Pro463Thr	–
8,714	8	16	8	1	8	2	1	1	0.25	>32	32	0.25/4.75	8	1	8	>4	674	NA	Glu26Gln, Leu221Val, Glu260Lys
10,938	8	32	32	>32	>32	>32	32	>32	1	>32	32	>8/152	8	1	4	2	16	–	–
11,340	8	32	>32	>32	>32	>32	32	>32	0.5	>32	64	>8/152	16	1	4	1	476	–	–
11,382	8	32	16	>32	>32	>32	16	>32	1	>32	64	<0.015/ 0.25	8	1	2	1	856[a]	Asp158Tyr, Pro463Thr	Leu156Phe, Arg172Gln
12,654	8	16	16	2	32	2	1	1	0.5	>32	32	2/38	8	2	2	>4	857[a]	NA	Val30Leu, Gly57Ser, Pro236Ser, Ile269Val
13,470	16	32	>32	>32	>32	>32	16	>32	0.5	>32	64	>8/152	8	1	4	1	480	Asp158Tyr, Pro463Thr	Thr95Ile, Glu260Lys
13,484	16	32	>32	>32	>32	4	1	>32	1	>32	32	>8/152	8	2	8	2	21	Asp158Tyr, Pro463Thr	Val30Leu, Gly57Ser, Pro236Ser, Ile269Val
14,980	16	32	>32	>32	>32	>32	16	>32	0.5	>32	64	>8/152	8	1	4	0.5	585	–	Arg172Gln, Pro236Ser
15,224	8	16	>32	>32	>32	32	16	32	0.5	>32	32	>8/152	8	1	2	1	16	Asp158Tyr, Pro463Thr	–
15,407	8	16	32	>32	>32	1	0.25	1	0.25	>32	64	0.25/4.75	8	0.5	4	2	479	NA	Pro81Ser, Ala177Ser
15,814	8	32	16	>32	>32	1	0.5	>32	1	>32	32	>8/152	8	1	4	1	16	Ile86Arg, Glu238Lys, Pro463Thr	–
17,838	16	32	>32	>32	>32	32	8	>32	0.5	>32	8	0.25/4.75	8	1	4	2	480	–	Thr95Ile, Glu260Lys
18,026	16	32	>32	>32	>32	32	16	>32	0.5	>32	32	0.0315/ 0.59375	8	1	4	4	476	–	–
19,663	16	32	>32	>32	>32	32	8	>32	0.5	>32	64	>8/152	8	1	2	2	480	Asp158Tyr, Pro463Thr	Thr95Ile, Glu260Lys
19,910	16	32	>32	>32	>32	16	4	>32	0.5	>32	32	>8/152	8	2	4	>4	116	Asp158Tyr, Pro463Thr	Arg172Gln
23,903	16	32	>32	>32	>32	>32	16	>32	0.5	>32	64	>8/152	8	1	4	0.5	585	Pro463Thr, Ile604Met	Arg172Gln, Pro236Ser
23,967	16	32	>32	>32	>32	>32	16	>32	0.5	>32	64	>8/152	8	1	4	0.5	585	Pro463Thr, Ile604Met	Arg172Gln, Pro236Ser
24,393	8	16	16	2	32	1	0.5	>32	0.5	>32	32	0.0625/ 1.1875	16	1	4	4	81	Asp158Tyr, Pro463Thr	Glu154Gln, Pro236Ser, Ile269Val
26,167	8	16	16	>32	>32	1	0.5	>32	1	>32	64	<0.015/ 0.25	8	1	2	2	619	Asp158Tyr, Pro463Thr	Glu154Gln, Pro236Ser, Ile269Val
27,149	8	32	32	>32	>32	>32	32	1	0.5	>32	32	>8/152	8	1	4	2	858[a]	Asp158Tyr, Pro463Thr	–
27,451	16	32	>32	>32	>32	>32	16	>32	0.5	>32	32	>8/152	8	1	4	0.25	585	–	Arg172Gln, Pro236Ser
31,890	16	32	>32	>32	>32	>32	32	>32	0.5	>32	32	>8/152	8	1	8	4	585	Pro463Thr, Ile604Met	Arg172Gln, Pro236Ser
32,142	8	32	16	>32	>32	>32	32	>32	0.5	>32	128	>8/152	8	1	4	1	16	Asp158Tyr, Pro463Thr	–
32,633	16	32	32	>32	>32	>32	32	>32	0.5	>32	32	>8/152	8	1	4	1	476	Asp158Tyr, Pro463Thr	–
33,710	8	32	32	>32	>32	>32	32	>32	0.5	>32	32	>8/152	8	1	4	1	16	Asp158Tyr, Pro463Thr	–

Note: [a]New ST types.

Abbreviations: AMK, amikacin; CFZ, cefazolin; CLI, clindamycin; CXM, cefuroxime; DAP, daptomycin; ERY, erythromycin; FM, fosfomycin; LVX, levofloxacin; LZ, linezolid; LZR-Efa, linezolid-resistant *Enterococcus faecalis;* MLST, multilocus sequence typing; MXF, moxifloxacin; NA, not available; OXA, oxacillin; RIF, rifampin; SXT, trimethoprim-sulfamethoxazole; TC, tetracycline; TGC, tigecycline; VAN, vancomycin.

(18/26), 69.2% (18/26), 73.1% (19/26), 84.6% (22/26), 88.5% (23/26), and 92.3% (24/26) of strains were resistant to oxacillin, trimethoprim-sulfamethoxazole, moxifloxacin, levofloxacin, tetracycline, erythromycin, and tigecycline, respectively. Rifampin resistance was seen in 30.8% (8/26) of the isolates. Only one strain showed intermediate sensitivity to fosfomycin. However, 15.4% (4/26) isolates were daptomycin nonsusceptible. In total, all these linezolid-resistant isolates retained susceptibility to vancomycin. Besides 12,654 and 24,393, other isolates were multidrug-resistant LZR-Efa.

A total of 13 different STs were identified from 26 LZR-Efa isolates. The most prevalent type was ST16 (six isolates, 23.1%), followed by ST585 (five isolates, 19.2%), ST476 (three isolates, 11.5%), and ST480 (three isolates, 11.5%). Three new STs were identified (ST856–ST858) (Figure 1).

Antibiotic resistance mechanism of LZR-Efa

In an attempt to link the phenotypic resistance data with genotypic evidence of resistance, we searched for mutations known to confer antimicrobial resistance. The genes *dfrE*, *lsaA*, and *emeA* were identified in all isolates (Table S2). Among the 26 LZR-Efa isolates examined in this study, 23 *E. faecalis* were positive for *optrA* gene. Six amino acids mutations were identified among the 18 LZR-Efa isolates, and of which Pro463Thr (463 tryptophan acid was changed to be threonine) was most frequently detected in isolates (Table 2). Both Pro463Thr and Asp158Tyr mutations of OptrA were the most abundant in LZR-Efa (12/18). Besides other two sites, Pro463Thr and Ile604Met mutations in three ST585 isolates, three mutational loci (Pro463Thr, Glu238Lys, and Ile86Arg) were found in two ST16 isolates. In addition, the 23S rRNA mutation was found in 16 LZR-Efa isolates (Table 2). It is of note that 10 isolates had both OptrA and 23S rRNA mutations. Mutations of specific amino acids were found in same type ST or ST with one allele difference. However, the presence of plasmid-borne ribosomal methyltransferase gene, *cfr*, was not identified in 26 LZR-Efa.

Characteristics of virulence genes

There were 41 virulence genes detected and 10 genes (*ace*, *bopD*, *cpsA*, *cpsB*, *ebpB*, *ebpC*, *efaA*, *fss1*, *fss2*, and *srtC*) were found in all isolates (Table S3). Of all the LZR-Efa isolates, 96.2% (25/26) were positive for *ebpA*, 88.5% (23/26) were positive for *EF3023*, 69.2% (18/26) were positive for nine genes (*asa1*, *cpsC*, *cpsD*, *cpsE*, *cpsG*, *cpsH*, *cpsI*, *cpsJ*, and *cpsK*), 69.2% (18/26) were positive for five genes (*esp*, *fsrB*, *fsrC*, *gelE*, and *prgB/asc10*), and 69.2% (18/26) were positive for three genes (*cpsF*, *fsrA*, and *sprE*). The *hyl* and *agg* genes were not detected in any LZR-Efa isolates. The *bsh* gene was found in 15,224 isolated from bile. A total of nine isolates were positive for multiple virulent factors (*ace*, *asa1*, *cylA*, *efaA*, *esp*, and *gelE*). There was no relationship between the number of virulence factors and clinical specimens (P=0.825).

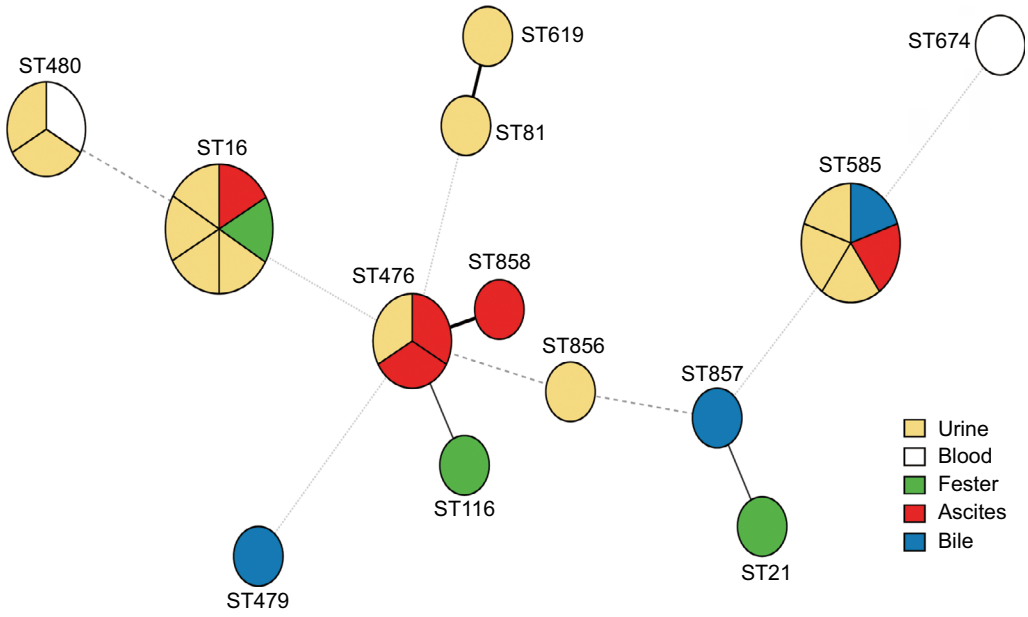

Figure 1 Minimum spanning tree of LZR-Efa. Solid line indicates one allele difference and dashed line indicates differences in two alleles.
Abbreviation: LZR-Efa, linezolid-resistant *Enterococcus faecalis*.

Risk factors associated with the development of LZR-Efa

There were a total of 20 patients with LZR-Efa infections, and 40 patients with LZS-Efa were enrolled by reference to the matching criteria in the Methods section. Compared with patients with LZS-Efa, univariate analysis showed that those with LZR-Efa were more likely to exposure to carbapenem (*P*=0.007) (Table 3). The logistic regression analysis indicated that the previous use of carbapenems may be an independent risk factor for LZR-Efa infection (OR=6.631; 95% CI=1.489–29.522; *P*=0.013).

Discussion

Since linezolid launched on the market, the detection rate of LZR-Efa and even the incidence of MDR are increasing.[8] Although the prevalence of linezolid resistance is still low, emergence and dissemination of linezolid-resistant *enterococci* limit the therapeutic option for successful treatment of VRE infections. In the present study, with the analysis of LZR-Efa, we found MDR LZR-Efa were not spread by clonal strains. In addition, *optrA* and 23S rRNA were the main resistance mechanisms. Notably, all linezolid-resistant vancomycin-susceptible *E. faecalis* emerged without linezolid treatment. The virulence factors, associated with bacterial adherence, evasion of phagocytosis, and biofilm formation, were identified, while the stress response proteins were not found in any isolates. Furthermore, data from 60 patients were evaluated, and the results showed previous carbapenems exposure to be an independent risk factor for LZR-Efa infections.

Table 3 Risk factors for patients with LZR-Efa at univariate analysis

	LZR-Efa (n=20)	LZS-Efa (n=40)	*P*-value
CP	4 (20%)	7 (17.5%)	0.813
CEP	5 (25%)	14 (35%)	0.432
BL/BLI	7 (35%)	8 (20%)	0.206
CAR	7 (35%)	3 (7.5%)	0.007
NIT	2 (10%)	6 (15%)	0.591
QUI	6 (30%)	6 (15%)	0.171
AMI	2 (10%)	4 (10%)	1
GLY	2 (10%)	1 (2.5%)	0.209
FM	1 (5%)	0	0.154
SXT	0	2 (5%)	0.309
Bowel preparation	7 (35%)	15 (37.5%)	0.85
Operation	11 (55%)	20 (50%)	0.715
Indwelling catheter	9 (45%)	20 (50%)	0.715
ICU	2 (10%)	1 (2.5%)	0.209

Abbreviations: AMI, aminoglycoside; BL/BLI, β-lactams and β-lactamase inhibitors; CAR, carbapenems; CEP cephalosporin; CP, cephamicins; FM, fosfomycin; GLY, glycopeptide; ICU, intensive care unit; LZR-Efa, linezolid-resistant E. faecalis; LZS-Efa, linezolid-sensitive E. faecalis; NIT, nitromidazoles; QUI, quinolones; SXT, trimethoprimsulfamethoxazole.

The majority of LZR-Efa in our study was from urinary source, in keeping with prior observations.[7] With an intrinsic and acquired resistance to some antimicrobial agents, *enterococci* have become important nosocomial pathogens.[10] Although the prevalence of linezolid-resistant enterococci is currently very low, the incidence of LZR-Efa in the hospital setting, which was 3.6% (26/730) in this study, was higher than that in Europe.[3] Of concern, 15.4% (4/26) isolates were daptomycin nonsusceptible. Note that, 1.8% (7/389) vancomycin-resistant *E. faecium* isolates were found to be resistant to linezolid in South Korea, which severely restricted the treatment options.[11] Fortunately, all LZR-Efa isolates in our study were susceptible to vancomycin without creating a worse crisis in clinical treatment.

Overall, the clones of 26 LZR-Efa isolated in different time periods were diverse. The dominance of ST16 among our samples is somewhat consistent with prior studies in Malaysia and China.[12] The same types of *E. faecalis*, ST16, ST27, ST116, ST256, ST476, ST480, and ST593, were detected in samples from animals and meat as well.[13,14] The data presented here indicated that animal origin seemed to constitute a reservoir of LZR-Efa. Further investigations are needed to assess whether these LZR-Efa was indeed associated with the isolates from animals.

In the present study, *optrA* and 23S rRNA were the main resistance mechanisms for linezolid. The gene *optrA* could be horizontally transmitted among *enterococci*.[5] In parallel, these surveillance data suggest that *optrA* may be disseminating in *E. faecalis* more rapidly than *cfr* in *Staphylococcus aureus*, implying a greater transferability of *optrA*.[15] In addition, the *optrA*-positive incidence increased over time from 0.4% (1/262) in 2004–2005 to 3.9% (26/668) in 2013–2014.[16] Previous studies found that *optrA* gene was more frequently seen in *E. faecalis* than in *E. faecium* and in food-producing animals than in humans (15.9% and 2%–2.9%, respectively).[14,17] Interestingly, the *optrA* gene was detected in an *E. faecium* isolate 2 years before linezolid approval applications in China.[16] Therefore, the species and geographies cross transmission might be one of the major reasons.

In general, the 23S rRNA mutation was considered as the most common mechanism of linezolid resistance. The 23S rRNA mutation rate of LZR-Efa was 61.5% (16/26), which is consistent with previous study.[5] It has been demonstrated that 23S rRNA mutations could revert to a susceptible phenotype when selective drug pressure was removed, while rapid resis-

tance selection emerged when selective pressure returned.[18] Meanwhile, Alonso et al[19] validated that the isolates with high linezolid MIC value were strongly related to the high number of 23S rRNA mutation copies. However, all LZR-Efa with 23S rRNA mutations showed low-level resistance in our study. And no isolates carried *cfr* gene in LZR-Efa. Although linezolid nonsusceptible clinical isolates almost exclusively harbored 23S rRNA alterations in the early 2000s, the detection rates of *optrA* and *cfr* were increasing in recent years. Therefore, the changes associated with linezolid resistance mechanisms are worthy of great concern.

Previous studies have demonstrated that some virulence factors play important roles in the pathogenesis of *E. faecalis*. Our present study found 10 virulence genes (*ace*, *bopD*, *cpsA*, *cpsB*, *ebpB*, *ebpC*, *efaA*, *fss1*, *fss2*, and *srtC*) in all isolates, and 9 isolates were positive for multiple virulent factors (*ace*, *asa1*, *cylA*, *efaA*, *esp*, and *gelE*). These virulence factors were mainly associated with bacterial adherence, evasion of phagocytosis, and biofilm formation. Several proteins, such as CYL and GelE, secreted into the extracellular medium have been implicated in enterococcal virulence. Meanwhile, cell-surface determinants, including the family of aggregation substance proteins (AS proteins), pilin, polysaccharides, polysaccharide antigen, have been reported to contribute to the large bacterial aggregations and biofilm formation. Esp, as one of AS proteins, are anchored to the cell wall, affect biofilm formation, and have a role in experimental urinary tract infections (UTIs) and/or endocarditis model.[20,21] Capsular polysaccharide (cps) has a crucial role in pathogenesis, mediating evasion of phagocytosis by polymorphonuclear neutrophils, and stimulating cytokine production.[22] Ebp pili are important for biofilm formation and for the pathogenesis of experimental endocarditis and UTIs.[23] Zheng et al[12] demonstrated a positive association between linezolid resistance in *E. faecalis* and robust biofilm formation. They found that the *cylA* gene was associated with weak biofilm formation, while the *esp* gene was only associated with strong or medium biofilm formation. It is of note that some stress response proteins (Gls, Npr, Ahp, Tpx), being important for virulence, were not identified in this present study. In addition, various putative enterococcal virulence determinants can also be found in strains colonizing the gastrointestinal tract of healthy individuals.[24] The evidence of involvement of pathogenic factors still needs to be confirmed by further studies.

Usually, the emergence of linezolid-resistant enterococci was consistently related to prior linezolid exposure.[8] Of the 55 studies on LZR-Efa, 14 (25.5%) reported the duration of linezolid treatment, with a mean of 29.8±48.8 days.[5] Paradoxically, LZR-Efa could also develop in patients without prior exposure to linezolid in our study and previous studies. The similar studies suggested the presence of risk factors that predispose the linezolid resistance of the vancomycin-susceptible *Enterococcus*, such as exposure to other antibiotics, long-term hospitalization, and prolonged stay in the intensive care unit.[5,25] Notably, we identified previous use of carbapenems as the only independent risk factor associated with LZR-Efa infections. After applying a logistic regression model, an independent risk factor for *E. faecium* acquisition was the previous use of carbapenems (OR=10.24; 95% CI=1.35–77.66).[26] Other reports showed that the use of carbapenems is an independent predictor of *E. faecium* bacteremia.[27] Empirical treatment with broad spectrum antibiotic may facilitate colonization and infection by depleting the gastrointestinal tract of its normal anaerobic flora. Therefore, we speculate that the acquisition of linezolid resistance among *E. faecalis* clinical isolates in the present study was not associated with the consumption of linezolid. It is possible that LZR-Efa colonization without symptoms in the intestinal tract served as a reservoir to be transmitted to other patients or changed into pathogenic bacteria.

The study has several limitations, including its retrospective form and the relatively small number of LZR-Efa. However, this is a real-life clinical experience providing useful suggestions to clinicians about the clinical characteristics of infections caused by LZR-Efa.

Conclusion

The results of our study showed that LZR-Efa were sporadic with low-level linezolid resistance, while multidrug resistance was quite serious. In addition, carbapenems use was an independent predictor of LZR-Efa infections. Therefore, it is important to implement infection-control practices against these resistant strains, with an emphasis on contact precautions and more careful use of antibiotics to prevent selection of resistance.

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Author contributions

WY and YQ developed the concept and designed the experiments. MC, WY, YL, and ZW isolated bacteria and performed the laboratory measurements. HP and JZ collected and analyzed the epidemiological and clinical data

from the patient records. HP and YQ gave conceptual advice. WY and YH wrote the paper. All authors contributed toward data analysis, drafting and critically revising the paper, gave final approval of the version to be published 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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Supplementary materials

Table S1 Essential information of whole-genome sequencing for 26 LZR-Efa

Isolates	Accession	Clean reads	Clean bases	Length	Q30%	GC%
6,888	QNGG00000000	8,086,306	1,212,945,900	150	97.23	38.54
8,714	QNGH00000000	8,063,042	1,209,456,300	150	97.22	38.57
10,938	QNGI00000000	8,083,052	1,212,457,800	150	97.38	38.29
11,340	QNGJ00000000	8,081,886	1,212,282,900	150	97.32	38.26
11,382	QNGK00000000	8,087,010	1,213,051,500	150	97.46	38.54
12,645	QNGL00000000	8,067,526	1,210,128,900	150	97.14	38.92
13,470	QNGM00000000	8,084,470	1,212,670,500	150	97.49	38.73
13,484	QNGN00000000	8,079,076	1,211,861,400	150	97.40	38.69
14,980	QNGO00000000	8,084,456	1,212,668,400	150	97.25	38.12
15,224	QNGP00000000	8,048,514	1,207,277,100	150	97.36	37.96
15,407	QNGQ00000000	8,070,056	1,210,508,400	150	97.17	38.31
15,814	QNGR00000000	8,076,626	1,211,493,900	150	97.33	38.58
17,838	QNGS00000000	8,051,770	1,207,765,500	150	96.89	38.51
18,026	QNGT00000000	8,060,562	1,209,084,300	150	97.45	38.67
19,663	QNGU00000000	8,081,042	1,212,156,300	150	97.50	38.41
19,910	QNGV00000000	8,094,698	1,214,204,700	150	96.19	38.03
23,903	QNGW00000000	8,061,246	1,209,186,900	150	96.02	37.90
23,967	QNGX00000000	8,103,376	1,215,506,400	150	96.26	38.14
24,393	QNGY00000000	8,109,796	1,216,469,400	150	96.00	38.20
26,167	QNGZ00000000	8,097,538	1,214,630,700	150	96.20	38.21
27,149	QNHA00000000	8,116,074	1,217,411,100	150	96.26	38.84
27,451	QNHB00000000	8,111,306	1,216,695,900	150	96.16	38.37
31,890	QNHC00000000	8,085,996	1,212,899,400	150	96.18	38.19
32,142	QNHD00000000	8,069,526	1,210,428,900	150	96.02	37.82
32,633	QNHE00000000	8,104,740	1,215,711,000	150	96.22	38.21
33,710	QNHF00000000	8,104,866	1,215,729,900	150	96.22	38.01

Abbreviation: LZR-Efa, linezolid-resistant *Enterococcus faecalis*.

Table S2 Characteristics of resistance genes

Isolates	dfrE (n=26)	lsaA (n=26)	emeA (n=26)	fexA (n=23)	ANT(9) -Ia (n=12)	tetO (n=20)	dfrG (n=19)	ErmB (n=19)	tetK (n=18)
6,888	99.3%	98.8%	97.7%	98.9%	100.0%	NA	100.0%	NA	99.8%
8,714	100.0%	99.2%	97.7%	NA	NA	NA	NA	NA	NA
10,938	99.3%	98.8%	97.7%	98.9%	100.0%	95.6%	100.0%	100.0%	NA
11,340	100.0%	98.8%	97.7%	98.9%	NA	95.3%	100.0%	100.0%	99.8%
11,382	100.0%	99.4%	97.7%	98.9%	NA	95.5%	NA	100.0%	99.8%
12,645	99.3%	98.2%	97.7%	NA	NA	NA	NA	NA	NA
13,470	99.3%	99.6%	97.7%	98.9%	NA	95.5%	100.0%	100.0%	99.8%
13,484	100.0%	98.2%	97.7%	98.9%	NA	90.9%	100.0%	100.0%	95.2%
14,980	100.0%	99.4%	97.7%	98.9%	100.0%	95.5%	100.0%	100.0%	99.8%
15,224	99.3%	98.8%	97.7%	98.9%	NA	95.6%	100.0%	100.0%	NA
15,407	99.3%	98.8%	97.5%	NA	NA	NA	NA	NA	NA
15,814	99.3%	98.8%	97.7%	98.9%	100.0%	NA	100.0%	99.2%	99.8%
17,838	99.3%	99.6%	97.7%	98.9%	100.0%	95.5%	99.4%	100.0%	99.8%
18,026	100.0%	98.8%	97.7%	98.9%	NA	95.5%	NA	99.6%	99.8%
19,663	99.3%	99.6%	97.7%	98.9%	100.0%	95.5%	100.0%	100.0%	99.8%
19,910	100.0%	99.0%	97.7%	98.9%	NA	95.5%	100.0%	NA	99.8%
23,903	100.0%	99.4%	97.7%	98.9%	100.0%	95.5%	100.0%	100.0%	99.8%
23,967	100.0%	99.4%	97.7%	98.9%	100.0%	95.5%	100.0%	100.0%	99.8%
24,393	99.3%	99.6%	97.7%	98.9%	NA	95.5%	NA	NA	99.8%
26,167	99.3%	99.6%	97.7%	98.9%	NA	95.5%	NA	100.0%	99.8%
27,149	100.0%	98.8%	97.7%	98.9%	100.0%	NA	100.0%	NA	NA
27,451	100.0%	99.4%	97.7%	98.9%	100.0%	95.5%	100.0%	100.0%	99.8%
31,890	100.0%	99.4%	97.7%	98.9%	100.0%	95.5%	100.0%	100.0%	99.8%
32,142	99.3%	98.8%	97.5%	98.9%	NA	95.6%	100.0%	100.0%	NA
32,633	100.0%	98.8%	97.7%	98.9%	100.0%	95.3%	100.0%	100.0%	99.8%
33,710	99.3%	98.8%	97.5%	98.9%	NA	95.6%	100.0%	100.0%	NA

Abbreviation: NA, not available.

APH(3')-IIIa (n=17)	AAC(6')-Ie-APH(2")-Ia (n=17)	cat (n=17)	satNA4 (n=16)	ErmA (n=18)	lnuB (n=12)	lsaE (n=12)	aad(6) (n=9)	tetS (n=2)	tetM (n=2)
100.0%	100.0%	97.2%	99.4%	85.2%	100.0%	99.8%	100.0%	99.6%	95.7%
NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
100.0%	100.0%	NA	98.9%	85.2%	100.0%	100.0%	100.0%	NA	NA
100.0%	100.0%	100.0%	99.4%	85.2%	NA	NA	100.0%	NA	NA
NA	NA	97.2%	NA	85.2%	NA	NA	NA	NA	NA
NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
100.0%	100.0%	NA	99.4%	85.2%	NA	NA	100.0%	NA	NA
NA	99.7%	97.2%	NA	85.2%	NA	NA	NA	NA	NA
100.0%	100.0%	97.2%	99.4%	85.2%	100.0%	100.0%	NA	NA	NA
NA	NA	100.0%	NA	85.2%	NA	NA	NA	NA	NA
NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
100.0%	100.0%	97.2%	99.4%	85.2%	100.0%	100.0%	100.0%	99.6%	95.7%
100.0%	100.0%	100.0%	99.4%	85.2%	100.0%	100.0%	100.0%	NA	NA
100.0%	98.0%	97.2%	99.4%	85.2%	NA	NA	NA	NA	NA
100.0%	100.0%	100.0%	99.4%	85.2%	100.0%	99.8%	100.0%	NA	NA
100.0%	NA	NA	NA	85.2%	NA	NA	NA	NA	NA
100.0%	100.0%	97.2%	99.4%	NA	100.0%	100.0%	NA	NA	NA
100.0%	100.0%	97.2%	99.4%	NA	100.0%	100.0%	NA	NA	NA
NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
100.0%	100.0%	NA	99.4%	85.2%	NA	NA	100.0%	NA	NA
100.0%	100.0%	100.0%	99.4%	85.2%	100.0%	100.0%	NA	NA	NA
100.0%	100.0%	97.2%	99.4%	NA	100.0%	100.0%	NA	NA	NA
100.0%	100.0%	97.2%	99.4%	NA	100.0%	100.0%	NA	NA	NA
NA	NA	100.0%	NA	85.2%	NA	NA	NA	NA	NA
100.0%	100.0%	NA	99.4%	85.2%	100.0%	100.0%	100.0%	NA	NA
NA	NA	100.0%	NA	85.2%	NA	NA	NA	NA	NA

Table S3 Characteristics of virulence genes

Genes	6,888 (n=33)	8,714 (n=28)	10,938 (n=38)	11,340 (n=24)	11,382 (n=18)	12,645 (n=17)	13,470 (n=16)	13,484 (n=18)	14,980 (n=39)	15,407 (n=21)	15,224 (n=40)	15,814 (n=34)
ace (n=26)	+	+	+	+	+	+	+	+	+	+	+	+
asa1 (n=18)	+	–	+	+	–	–	+	–	+	–	+	+
bopD (n=26)	+	+	+	+	+	+	+	+	+	+	+	+
bsh (n=1)	–	–	–	–	–	–	–	–	–	–	+	–
cpsA (n=26)	+	+	+	+	+	+	+	+	+	+	+	+
cpsB (n=26)	+	+	+	+	+	+	+	+	+	+	+	+
cpsC (n=18)	+	+	+	+	–	–	–	–	+	+	+	+
cpsD (n=18)	+	+	+	+	–	–	–	–	+	+	+	+
cpsE (n=18)	+	+	+	+	–	–	–	–	+	+	+	+
cpsF (n=17)	+	+	+	+	–	–	–	–	+	+	+	+
cpsG (n=18)	+	+	+	+	–	–	–	–	+	+	+	+
cpsH (n=18)	+	+	+	+	–	–	–	–	+	+	+	+
cpsI (n=18)	+	+	+	+	–	–	–	–	+	+	+	+
cpsJ (n=18)	+	+	+	+	–	–	–	–	+	+	+	+
cpsK (n=18)	+	+	+	+	–	–	–	–	+	+	+	+
cylA (n=14)	+	–	+	–	–	–	–	–	+	–	+	+
cylB (n=11)	+	–	+	–	–	–	–	–	+	–	+	+
cylI (n=14)	+	–	+	–	–	–	–	–	+	–	+	+
cylL (n=10)	+	–	+	–	–	–	–	–	+	–	+	+
cylM (n=13)	+	–	+	–	–	–	–	–	+	–	+	+
cylR1 (n=12)	+	–	+	–	–	–	–	–	+	–	+	+
cylR2 (n=12)	+	–	+	–	–	–	–	–	+	–	+	+
cylS (n=12)	+	–	+	–	–	–	–	–	+	–	+	+
ebpA (n=25)	+	+	+	+	+	–	+	+	+	+	+	+
ebpB (n=26)	+	+	+	+	+	+	+	+	+	+	+	+
ebpC (n=26)	+	+	+	+	+	+	+	+	+	+	+	+
EF0149 (n=3)	–	–	–	–	–	–	–	–	+	–	–	+
EF0485 (n=14)	+	–	+	+	–	–	–	–	+	–	+	+
EF0818 (n=8)	–	+	–	–	–	+	+	+	–	+	+	–
EF3023 (n=23)	+	+	+	+	+	+	+	+	+	–	+	+
efaA (n=26)	+	+	+	+	+	+	+	+	+	+	+	+
esp (n=17)	+	–	+	–	+	–	–	–	+	–	+	+
fsrA (n=16)	–	+	+	–	+	+	–	+	+	–	+	–
fsrB (n=17)	–	+	+	–	+	+	+	+	+	–	+	–
fsrC (n=17)	–	+	+	–	+	+	–	+	+	–	+	–
fss1 (n=26)	+	+	+	+	+	+	+	+	+	+	+	+
fss2 (n=26)	+	+	+	+	+	+	+	+	+	+	+	+
gelE (n=17)	–	+	+	–	+	+	+	+	+	–	+	–
prgB/asc10 (n=17)	+	+	+	+	–	–	–	–	+	–	+	+
sprE (n=16)	–	+	+	–	+	+	–	+	+	–	+	–
srtC (n=26)	+	+	+	+	+	+	+	+	+	+	+	+

17,838 (n=16)	18,026 (n=25)	19,663 (n=24)	19,910 (n=27)	23,903 (n=37)	23,967 (n=37)	24,393 (n=22)	26,167 (n=22)	27,149 (n=22)	27,451 (n=37)	31,890 (n=37)	32,142 (n=37)	32,633 (n=24)	33,710 (n=35)
+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	−	+	+	−	+	−	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+	+	+
−	−	−	−	−	−	−	−	−	−	−	−	−	−
+	+	+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+	+	+
−	+	−	+	+	+	−	−	+	+	+	+	+	+
−	+	−	+	+	+	−	−	+	+	+	+	+	+
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−	−	−	+	+	+	+	+	−	+	+	+	−	+
+	−	+	+	+	−	+	+	+	+	−	+	−	+
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+	+	+	+	+	+	+	+	+	+	+	+	+	+