Epidemiological characteristics and genetic structure of linezolid-resistant Enterococcus faecalis

Meijuan Chen,1 Hongying Pan,1 Yaling Lou,2 Zhe Wu,3 Jiajie Zhang,1 Yicheng Huang,1 Wei Yu,1,3,8 Yunqing Qiu3,8

1Department of Infectious Diseases, Zhejiang Provincial People’s Hospital, People’s Hospital of Hangzhou Medical College, Hangzhou, China; 2Department of Infectious Diseases, Dongyang People’s Hospital, Jinhua, China; 3State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China; 4State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China; 5Department of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China; 6Department of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China; 7Department of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, Hangzhou, China; 8These authors contributed equally to this work

Correspondence: Wei Yu
Department of Infectious Diseases, Zhejiang Provincial People’s Hospital, People’s Hospital of Hangzhou Medical College, No.158 Shangtang Road, Hangzhou City, Zhejiang Province 310014, China
Tel +86 571 8 589 3027
Fax +86 571 8 589 3027
Email wyu@zju.edu.cn

Yunqing Qiu
State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, College of Medicine, Zhejiang University, No. 79 Qingchun Road, Hangzhou City, Zhejiang Province 310029, China
Tel +86 571 8 723 6606
Fax +86 571 8 723 6606
Email qiuyn@zju.edu.cn

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, IsaA, and emcA 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, cplA, efaA, esp, and geE). 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 carbapenem 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, carbapenem 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. 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. 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%. It is of note that the number of
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 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.\(^7\) 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
The characterization of lZR-efa (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   | 6B-17| 24     | F   | Urinary infection | Improvement | Hangzhou | Worker | 01/2012 |
| 8,714    | Blood   | 6B-17| 62     | F   | Hepatitis     | 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   | Hepatobiliary carcinoma | 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   | Hepatitis     | Improvement | Huzhou | Farmer | 08/2013 |
| 13,470   | Bile    | 3-4  | 67     | M   | Nephrology | Improvement | Jiaxing | Farmer | 09/2013 |
| 13,484   | Fester  | 7-2  | 65     | F   | Mammary cancer | Improvement | Huzhou | 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   | Hepatobiliary | Improvement | Hangzhou | Worker | 12/2013 |
| 15,814   | Urine   | 3-3  | 70     | M   | Prostatic hyperplasia | Improvement | Wenzhou | Retired | 01/2014 |
| 17,838   | Urine   | 10-5 | 60     | 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   | Sacrococcygeal disease | Improvement | Shaoxing | 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-Eb

| Isolates   | OXA | CFZ | CXM | ERY | CLI | LVX | MXP | TC | TGC | AMK | FM  | SXT | LZ  | VAN | DAP | RIF | MLST | optRA   | 23S rRNA                  |
|-----------|-----|-----|-----|-----|-----|-----|-----|----|-----|-----|-----|-----|-----|-----|-----|-----|------|---------|--------------------------|
| 6,888     | 8   | 32  | 32  | >32 | >32 | 1   | 0.5 | >32| >32 | 1   | 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                        |
| 10,938    | 8   | 32  | 32  | >32 | >32 | 32  | 32  | >32| >32 | 1   | 32  | >8/152 | 8   | 1   | 4   | 2   | 16   | –                        |
| 11,340    | 8   | >32 | >32 | >32 | >32 | 32  | >32 | 0.5| >32 | 64  | >8/152 | 16  | 1   | 4   | 1   | 476   | –                        |
| 11,382    | 8   | 32  | 16  | >32 | >32 | 16  | >32 | 1  | >32 | 64  | 0.015/0.25 | 8   | 1   | 2   | 1   | 856*  | Thr95Ile, Pro463Thr       |
| 12,654    | 8   | 16  | 16  | 2   | 32  | 2   | 1   | 1  | 0.5 | >32 | 32  | 2/38   | 8   | 2   | 2   | >4  | 857*  | NA                        |
| 13,470    | 16  | 32  | >32 | >32 | >32 | 32  | >32 | 0.5| >32 | 32  | 0.8/152 | 8   | 1   | 4   | 1   | 480   | Asp158Tyr, Pro463Thr       |
| 13,484    | 16  | >32 | >32 | >32 | 4   | >32 | 1   | >32| >32 | 32  | >8/152 | 8   | 2   | 8   | 2   | 21    | Asp158Tyr, Pro463Thr       |
| 14,980    | 16  | 32  | >32 | >32 | >32 | >32 | 16  | 0.5| >32 | 64  | >8/152 | 8   | 1   | 4   | 0.5 | 585   | NA                        |
| 15,224    | 16  | 32  | >32 | >32 | >32 | >32 | >32 | 0.8| >32 | 64  | >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                        |
| 15,814    | 8   | 32  | 16  | >32 | >32 | 1   | 0.5 | >32| >32 | 32  | >8/152 | 8   | 1   | 4   | 1   | 16    | ile86Arg, Glu238Lys, Pro463Thr |
| 17,838    | 16  | 32  | >32 | >32 | >32 | >32 | >32 | 8  | >32 | 0.25/4.75 | 8   | 1   | 4   | 2   | 480   | Thr95Ile, Glu260Lys        |
| 18,026    | 16  | 32  | >32 | >32 | >32 | >32 | >32 | 16 | >32 | 0.0315/0.59375 | 8   | 1   | 4   | 4   | 476   | –                        |
| 19,663    | 16  | 32  | >32 | >32 | >32 | 32  | >32 | 0.5 | >32 | 64  | >8/152 | 8   | 1   | 2   | 2   | 480   | Asp158Tyr, Pro463Thr       |
| 19,910    | 16  | 32  | >32 | >32 | >32 | >32 | >32 | 16 | 0.4 | >32 | 0.8/152 | 8   | 2   | 4   | >4  | 116   | Thr95Ile, Glu260Lys        |
| 23,903    | 16  | 32  | >32 | >32 | >32 | >32 | >32 | 16 | >32 | 0.8/152 | 8   | 1   | 4   | 0.5 | 585   | Asp158Tyr, Pro463Thr       |
| 23,967    | 16  | 32  | >32 | >32 | >32 | >32 | >32 | 16 | >32 | 0.8/152 | 8   | 1   | 4   | 0.5 | 585   | Asp158Tyr, Pro463Thr       |
| 24,393    | 8   | 16  | 16  | 2   | 32  | 1   | 0.5 | >32| >32 | 64  | 0.0625/1.1875 | 16  | 1   | 4   | 4   | 81    | Asp158Tyr, Pro463Thr       |
| 26,167    | 8   | 16  | 16  | >32 | >32 | >32 | >32 | 1  | >32 | 64  | 0.015/0.25 | 8   | 1   | 2   | 2   | 619   | Asp158Tyr, Pro463Thr       |
| 27,149    | 8   | 32  | 32  | >32 | >32 | >32 | >32 | 32 | 1   | 0.5 | >32 | 32  | >8/152 | 8   | 1   | 4   | 2   | 858*  | Asp158Tyr, Pro463Thr       |
| 27,451    | 16  | 32  | >32 | >32 | >32 | >32 | >32 | 16 | >32 | 0.8/152 | 8   | 1   | 4   | 0.25 | 585   | –                        |
| 31,890    | 16  | 32  | >32 | >32 | >32 | >32 | >32 | >32| >32 | 128 | >8/152 | 8   | 1   | 4   | 1   | 16    | Asp158Tyr, Pro463Thr       |
| 32,424    | 8   | 32  | 16  | >32 | >32 | >32 | >32 | >32| >32 | 128 | >8/152 | 8   | 1   | 4   | 1   | 16    | Asp158Tyr, Pro463Thr       |
| 32,633    | 16  | 32  | 32  | >32 | >32 | >32 | >32 | >32| >32 | 128 | >8/152 | 8   | 1   | 4   | 1   | 16    | Asp158Tyr, Pro463Thr       |
| 33,710    | 8   | 32  | 32  | >32 | >32 | >32 | >32 | >32| >32 | >32 | >32 | >8/152 | 8   | 1   | 4   | 1   | 16    | Asp158Tyr, Pro463Thr       |

Note: New ST types.

Abbreviations: AMK, amikacin; CFZ, ceftazolin; CLI, clindamycin; CXM, cefuroxime; DAP, daptomycin; ERY, erythromycin; FM, fosfomycin; LVX, levofloxacin; LZ, linezolid; LZR-Eb, linezolid-resistant Enterococcus faecalis; MLST, multilocus sequence typing; MXP, 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.

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.

Characteristics of virulence genes

There were 41 virulence genes detected and 10 genes (ace, bopD, cpsA, cpsB, ebpB, ebpC, efA, 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 prgBasc10), 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, efA, esp, and gelE). There was no relationship between the number of virulence factors and clinical specimens (P=0.825).
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.

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.

The majority of LZR-Efa in our study was from urinary source, in keeping with prior observations. With an intrinsic and acquired resistance to some antimicrobial agents, enterococci have become important nosocomial pathogens.

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. 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.

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. The same types of E. faecalis, ST16, ST27, ST116, ST256, ST476, ST480, and ST593, were detected in samples from animals and meat as well. 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. 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. 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. 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). Interestingly, the optrA gene was detected in an E. faecium isolate 2 years before linezolid approval applications in China. 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. It has been demonstrated that 23S rRNA mutations could revert to a susceptible phenotype when selective drug pressure was removed, while rapid resis-

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

| Risk Factor          | 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, glycopeptid; ICU, intensive care unit; LZR-Efa, linezolid-resistant E. faecalis; LZS-Efa, linezolid-sensitive E. faecalis; NIT, nitromidazoles; QUI, quinolones; SXT, trimethoprim-sulfamethoxazole.
tance selection emerged when selective pressure returned.\textsuperscript{18} Meanwhile, Alonso et al\textsuperscript{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 \textit{cfr} gene in LZR-Efa. Although linezolid nonsusceptible clinical isolates almost exclusively harbored 23S rRNA alterations in the early 2000s, the detection rates of \textit{optrA} and \textit{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 \textit{E. faecalis}. Our present study found 10 virulence genes (\textit{ace}, \textit{bopD}, \textit{cpsA}, \textit{cpsB}, \textit{ebpB}, \textit{ebpC}, \textit{efA4}, \textit{fss1}, \textit{fss2}, and \textit{srtC}) in all isolates, and 9 isolates were positive for multiple virulent factors (\textit{ace}, \textit{asa1}, \textit{cylA}, \textit{efA4}, \textit{esp}, and \textit{gelE}). These virulence factors were mainly associated with bacterial adherence, evasion of phagocytosis, and biofilm formation. Several proteins, such as \textit{Cyl} and \textit{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. \textit{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.\textsuperscript{20,21} Capsular polysaccharide (eps) has a crucial role in pathogenesis, mediating evasion of phagocytosis by polymorphonuclear neutrophils, and stimulating cytokine production.\textsuperscript{22} \textit{Ebp} pili are important for biofilm formation and for the pathogenesis of experimental endocarditis and UTIs.\textsuperscript{23} Zheng et al\textsuperscript{12} demonstrated a positive association between linezolid resistance in \textit{E. faecalis} and robust biofilm formation. They found that the \textit{cylA} gene was associated with weak biofilm formation, while the \textit{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 \textit{Enterococcus}, such as exposure to other antibiotics, long-term hospitalization, and prolonged stay in the intensive care unit.\textsuperscript{5,23} 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 \textit{E. faecium} acquisition was the previous use of carbapenems (OR=10.24; 95\% CI=1.35–77.66).\textsuperscript{26} Other reports showed that the use of carbapenems is an independent predictor of \textit{E. faecium} bacteremia.\textsuperscript{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 \textit{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 IZ 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 (n=26) | 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         | 99.6%       | 97.7%       | 98.9%       | NA          | 95.5%            | 100.0%      | 100.0%      | 99.8%       |
| 13,484         | 100.0%      | 98.2%       | 98.8%       | NA          | 90.9%            | 100.0%      | 100.0%      | 95.2%       |
| 15,224         | 99.3%       | 99.4%       | 97.7%       | 98.9%       | 100.0%            | 95.5%       | 100.0%      | 100.0%      | 99.8%       |
| 15,407         | 98.8%       | 97.5%       | 98.8%       | NA          | NA                | NA          | 100.0%      | 99.8%       |
| 15,814         | 99.3%       | 98.8%       | 97.7%       | NA          | NA                | NA          | NA          | NA          | NA          |
| 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                | 95.6%       | 100.0%      | 100.0%      | 99.8%       |
| 19,663         | 99.3%       | 96.6%       | 97.7%       | 98.9%       | 100.0%            | 95.5%       | 99.4%       | 100.0%      | 99.8%       |
| 19,910         | 100.0%      | 99.0%       | 97.7%       | 98.9%       | NA                | 95.5%       | 100.0%      | 100.0%      | 99.8%       |
| 23,903         | 100.0%      | 99.4%       | 97.7%       | 98.9%       | NA                | 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%       | 98.8%       | 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%       | NA                | 95.5%       | NA          | 100.0%      | 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')-Ile-APH(2'')-Ia (n=17) | cat (n=17) | satNA4 (n=16) | ErmA (n=18) | InuB (n=12) | IsaE (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                            | NA         | 97.2%         | NA          | 85.2%       | NA          | NA          | NA          | NA          |
| 100.0%           | 100.0%                        | NA         | 99.4%         | 85.2%       | NA          | NA          | NA          | NA          | NA          |
| NA               | NA                            | NA         | NA            | 100.0%      | 100.0%      | 99.6%       | 95.7%       |
| 100.0%           | 100.0%                        | 97.2%      | 99.4%         | 85.2%       | 100.0%      | 100.0%      | 99.6%       |
| NA               | NA                            | NA         | NA            | NA          | NA          | NA          | NA          | NA          |
| 100.0%           | 100.0%                        | 97.2%      | 99.4%         | 85.2%       | NA          | NA          | NA          | 100.0%      | NA          |
| NA               | NA                            | NA         | NA            | NA          | NA          | NA          | NA          | NA          |
| 100.0%           | 100.0%                        | 97.2%      | 99.4%         | 85.2%       | 100.0%      | 99.8%       | 100.0%      | NA          | NA          |
| NA               | NA                            | NA         | NA            | 100.0%      | 95.6%       | NA          | NA          | NA          |
| 100.0%           | 100.0%                        | 97.2%      | 99.4%         | 85.2%       | NA          | NA          | NA          | 100.0%      | NA          |
| NA               | NA                            | NA         | NA            | NA          | NA          | NA          | NA          | NA          |
| 100.0%           | 100.0%                        | 97.2%      | 99.4%         | 85.2%       | 100.0%      | 99.8%       | 100.0%      | NA          | NA          |
| NA               | NA                            | NA         | NA            | 100.0%      | NA          | NA          | NA          | NA          |
| 100.0%           | 100.0%                        | 97.2%      | 99.4%         | 85.2%       | 100.0%      | 99.8%       | 100.0%      | NA          | NA          |
| NA               | NA                            | NA         | NA            | 100.0%      | NA          | NA          | NA          | NA          |
| 100.0%           | 100.0%                        | 97.2%      | 99.4%         | 85.2%       | 100.0%      | 99.8%       | 100.0%      | NA          | NA          |
| NA               | NA                            | NA         | NA            | 100.0%      | NA          | NA          | NA          | NA          |
| 100.0%           | 100.0%                        | 97.2%      | 99.4%         | 85.2%       | 100.0%      | 99.8%       | 100.0%      | NA          | NA          |
| NA               | NA                            | NA         | NA            | 100.0%      | 95.6%       | NA          | NA          | NA          |
| 100.0%           | 100.0%                        | 97.2%      | 99.4%         | 85.2%       | 100.0%      | 99.8%       | 100.0%      | NA          | NA          |
| NA               | NA                            | NA         | NA            | 100.0%      | NA          | NA          | NA          | NA          |
| 100.0%           | 100.0%                        | 97.2%      | 99.4%         | 85.2%       | 100.0%      | 99.8%       | 100.0%      | NA          | NA          |
| NA               | NA                            | NA         | NA            | 100.0%      | 95.6%       | NA          | NA          | NA          |
| 100.0%           | 100.0%                        | 97.2%      | 99.4%         | 85.2%       | 100.0%      | 99.8%       | 100.0%      | NA          | NA          |
| NA               | NA                            | NA         | NA            | 100.0%      | NA          | NA          | NA          | NA          |
| 100.0%           | 100.0%                        | 97.2%      | 99.4%         | 85.2%       | 100.0%      | 99.8%       | 100.0%      | NA          | NA          |
| NA               | NA                            | NA         | NA            | 100.0%      | 95.6%       | NA          | NA          | NA          |
| 100.0%           | 100.0%                        | 97.2%      | 99.4%         | 85.2%       | 100.0%      | 99.8%       | 100.0%      | NA          | NA          |
| NA               | NA                            | NA         | NA            | 100.0%      | 95.6%       | NA          | NA          | NA          |
| 100.0%           | 100.0%                        | 97.2%      | 99.4%         | 85.2%       | 100.0%      | 99.8%       | 100.0%      | NA          | NA          |
| NA               | NA                            | NA         | NA            | 100.0%      | 95.6%       | NA          | NA          | NA          |
| 100.0%           | 100.0%                        | 97.2%      | 99.4%         | 85.2%       | 100.0%      | 99.8%       | 100.0%      | NA          | NA          |
| NA               | NA                            | NA         | NA            | 100.0%      | 95.6%       | NA          | NA          | NA          |
| 100.0%           | 100.0%                        | 97.2%      | 99.4%         | 85.2%       | 100.0%      | 99.8%       | 100.0%      | NA          | NA          |
| NA               | NA                            | NA         | NA            | 100.0%      | 95.6%       | NA          | NA          | NA          |
| 100.0%           | 100.0%                        | 97.2%      | 99.4%         | 85.2%       | 100.0%      | 99.8%       | 100.0%      | NA          | NA          |
| NA               | NA                            | NA         | NA            | 100.0%      | 95.6%       | NA          | NA          | NA          | 95.7%       |
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) | +            | –            | +            | –            | –            | –            | –            | +            | –            | +            | +            | +            |
| cylD (n=14) | +            | –            | +            | –            | –            | –            | –            | +            | –            | +            | +            | +            |
| cylE (n=10) | +            | –            | –            | –            | –            | –            | –            | +            | –            | +            | +            | +            |
| cyLM (n=13) | +            | –            | –            | –            | –            | –            | –            | –            | +            | +            | +            | +            |
| cyLR1 (n=12) | +            | –            | –            | –            | –            | –            | –            | +            | –            | +            | +            | +            |
| cyLR2 (n=12) | +            | –            | –            | –            | –            | –            | –            | +            | –            | +            | +            | +            |
| cyLS (n=12) | +            | –            | –            | –            | –            | –            | –            | +            | –            | +            | +            | +            |
| ebpA (n=18) | +            | +            | +            | +            | –            | –            | +            | +            | +            | +            | +            | +            |
| ebpB (n=26) | +            | +            | +            | +            | +            | +            | +            | +            | +            | +            | +            | +            |
| ebpC (n=26) | +            | +            | +            | +            | +            | +            | +            | +            | +            | +            | +            | +            |
| E0149 (n=3) | –            | –            | –            | –            | –            | –            | –            | –            | –            | –            | –            | –            |
| E0485 (n=14) | +            | –            | +            | –            | –            | –            | –            | –            | +            | +            | +            | +            |
| E0818 (n=8) | –            | +            | –            | –            | –            | –            | –            | –            | –            | –            | –            | –            |
| E03023 (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) | –            | +            | –            | +            | +            | +            | +            | +            | –            | –            | –            | +            |
| prfB (n=26) | +            | +            | –            | –            | –            | –            | –            | –            | +            | +            | +            | +            |
| sprE (n=16) | –            | +            | –            | +            | –            | –            | +            | +            | –            | +            | –            | +            |
| srtC (n=26) | +            | +            | +            | +            | +            | +            | +            | +            | +            | +            | +            | +            |
| Characteristics of Virulence Genes | Table S3 |
|----------------------------------|----------|
| Genes 6,888                      |          |
| Genes 8,714                      |          |
| Genes 10,938                     |          |
| Genes 11,340                     |          |
| Genes 12,645                     |          |
| Genes 13,470                     |          |
| Genes 18,026                     |          |
| Genes 19,663                     |          |
| Genes 21,407                     |          |
| Genes 22,393                     |          |
| Genes 23,967                     |          |
| Genes 24,393                     |          |
| Genes 26,167                     |          |
| Genes 27,149                     |          |
| Genes 27,451                     |          |
| Genes 31,890                     |          |
| Genes 32,142                     |          |
| Genes 32,633                     |          |
| Genes 33,710                     |          |

The characterization of LZR-Efa