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ORIGINAL RESEARCH

Emergence of *vanA*-Type Vancomycin-Resistant *Enterococcus faecium* ST 78 Strain with a rep2-Type Plasmid Carrying a Tn1546-Like Element Isolated from a Urinary Tract Infection in China

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Purpose: The emergence of vancomycin-resistant enterococci (VRE) dramatically narrows therapeutic options. Although the prevalence of VRE in China has maintained a low level, VRE outbreaks have been reported in some tertiary hospitals in the developed areas of China. The clonal background of *vanA*-positive *Enterococcus faecium* strains has not been well characterized in China. Here, we report the whole-genome sequence of a *vanA*-type vancomycin-resistant *E. faecium* belonging to sequence type (ST) 78 isolated from a urinary tract infection in China.

Patients and Methods: A vancomycin-resistant *E. faecium* was isolated from a 66-year-old male patient diagnosed with brainstem hemorrhage. Antibiotic susceptibility assays were performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). Complete genome sequencing was performed using both the HiSeq^{TM} 4000 platform and the MinION platform. Plasmid, genomic and phylogenetic relationship analysis were further performed.

Results: *E. faecium* VRE1 was resistant to all antimicrobials tested except for tetracyclines and oxazolidinones. The whole genome of *E. faecium* VRE1 was composed of one chromosomal DNA and four plasmids. Two virulence genes and five antimicrobial resistance genes were identified. In silico multilocus sequence typing (MLST) showed that it belonged to ST78 (clonal complex CC17), a well-known epidemic clone that is widespread in Europe and the United States. Three antimicrobial resistance genes, including aminoglycoside resistance genes *ant(6)-Ia* and *aph(3')-III* ; and glycopeptide resistance gene *vanA*, were located on a *rep2*-type plasmid carrying a Tn*1546*-like element that has not been reported. The most closely related strain harboring a similar plasmid backbone was recovered from fodder sample in China that differed by 178 cgMLST loci.

Conclusion: Our study characterizes the genomic feature of a vancomycin-resistant *E. faecium* ST78 strain harboring a *vanA*-carrying plasmid in China. The ST78 clonal group possessed the potential to emerge as a successful *vanA*-carrying epidemic lineage in China.

Keywords: *Enterococcus faecium*, vancomycin-resistant enterococci, *vanA*, whole-genome sequencing, Tn*1546*-like element

Introduction

Enterococcus faecium has emerged as a leading cause of multidrug-resistant infections, such as bacteremia, intra-abdominal infections and urinary tract infections. Vancomycin is a glycopeptide antibiotic that plays an antibacterial role by combining with the peptidoglycan precursor and blocking synthesis of the cell wall. It is the first-line drug for the treatment of Gram-positive bacteria, including methicillin-resistant

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staphylococcus aureus (MRSA) and multidrug-resistant enterococci. Since the first report of vancomycin-resistant enterococci (VRE) in 1988,[1] it has been increasingly reported all over the world, representing a global public health concern.[2] Resistance to vancomycin dramatically narrows the therapeutic options in *E. faecium* infections.

The prevalence of VRE shows significant regional differences. In Europe, VRE are prevalent in the community, and the use of glycopeptide antibiotics (avoparcin) in agriculture is thought to be one of the major factors contributing to the further dissemination of VRE.[3] In the United States, because of the increasing clinical use of vancomycin, VRE isolated from hospitals has gradually increased and has become one of the most prevalent nosocomial pathogens.[4,5] Compared with Europe and the USA, China has maintained a low prevalence of VRE, with an isolation rate of 1.4% for *E. faecium* and 0.1% for *Enterococcus faecalis* in the CHINET surveillance in 2017.[6]

A total of eight acquired glycopeptide resistance determinants have been reported in enterococci (VanA, VanB, VanD, VanE, VanG, VanL, VanM and VanN), of which VanA is the most frequently encountered worldwide.[7] The *vanA* gene is traditionally associated with a plastic antimicrobial resistance transposon, Tn*1546*, which also contains the additional genes responsible for vancomycin resistance (*vanR, vanS, vanH, vanX, vanY* and *vanZ*). Tn*1546* is able to easily transpose into diverse conjugative plasmids, and the horizontal transfer of Tn*1546*-like elements also plays an important role in the dissemination of *vanA*-type VRE.[8–10]

Attributable to the narrow antibiotic spectrum of vancomycin and its highest limit level in the classification management system of antibiotics, VRE are still rare in China. However, the prevalence of VRE in some tertiary hospitals in Shanghai and Beijing has increased, and VRE outbreaks have been reported sporadically in China.[11] In this study, a *vanA*-carrying *E. faecium* strain was isolated from a male patient hospitalized in a tertiary hospital in Hangzhou, China. The whole genome of the strain was sequenced, and a *vanA*-harboring plasmid was also analyzed to elucidate its genomic epidemiological characteristics.

Patients and Methods

Patient and Bacterial Isolate

A 66-year-old male patient diagnosed with brainstem hemorrhage was long-term hospitalized in the Department of Rehabilitation Medicine in a tertiary hospital in Hangzhou, Zhejiang Province, China. During his hospitalization, the patient received multiple antimicrobial treatments, including teicoplanin, ceftazidime, sulperazone and meropenem. A vancomycin-resistant *E. faecium* was isolated from his urine sample on December 3, 2018. The strain was preliminarily identified using the VITEK MS system (bioMérieux, France) and was further confirmed by 16S rRNA gene sequencing.

Antimicrobial Susceptibility Testing

Antibiotic susceptibility assays were performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI). In total, ten categories including sixteen antibiotics were tested. They were penicillins (penicillin and ampicillin), aminoglycosides (gentamicin and streptomycin), quinolones (ciprofloxacin, levofloxacin and moxifloxacin), macrolides (erythromycin), lincosamides (clindamycin), tetracyclines (tetracycline and tigecycline), glycopeptides (vancomycin and teicoplanin), nitrofurantoins (nitrofurantoin), lipopeptides (daptomycin) and oxazolidinones (linezolid). The minimal inhibitory concentrations (MICs) of penicillin, vancomycin, teicoplanin, tetracycline and tigecycline were determined using the Etest method. The minimum inhibitory concentrations (MICs) of gentamicin, streptomycin and daptomycin were determined using standard broth microdilution tests. The MICs of other antimicrobial agents were determined using a VITEK 2 system (bioMérieux, France) with Gram-positive antimicrobial susceptibility testing cards (AST-GP67). Antimicrobial susceptibility was determined using the breakpoints approved by the CLSI.[12]

Genomic DNA Extraction and Whole-Genome Sequencing

Genomic DNA of *E. faecium* VRE1 was extracted using a QIAamp DNA MiniKit (Qiagen, USA) and then subjected to whole-genome sequencing. Whole-genome sequencing was performed using both the HiSeq™ 4000 platform (Illumina, San Diego, CA, USA), with a 150 bp paired-end protocol, and the MinION (Nanopore, Oxford, UK) platform. The short reads generated by the HiSeq™ 4000 were *de novo* assembled into contigs using SPAdes. Long Nanopore reads were generated by a MinION Sequencer. Hybrid assembly of both short Illumina reads and long MinION reads was performed using Unicycler v 0.4.7 under conservative mode.[13] Complete circular contigs generated were then corrected using Pilon v 1.23 with Illumina reads for several rounds. Then, the whole-genome sequence of *E. faecium* VRE1 was

generated. We obtained five circular contigs, which were represented by a complete chromosome and four plasmids. The whole-genome sequence was annotated by the NCBI Prokaryotic Genomes Annotation Pipeline (PGAP) server.

Identification of Antimicrobial Resistance Genes and Virulence Genes

Acquired antimicrobial resistance genes were identified using the ResFinder 2.1 server by uploading the entire genome sequence to the database. Virulence genes were analyzed using VirulenceFinder 2.0 with a 98% threshold for gene identification and an 80% minimum length.[14]

Plasmid Analysis

The plasmid sequences were annotated by the NCBI Prokaryotic Genomes Annotation Pipeline (PGAP) server. A graphical map of the *vanA*-carrying plasmid was converted by CGView Server, complete with labels and footnotes.[15] Circular comparisons between pVRE1-VanA and similar plasmids were conducted by BLAST Ring Image Generator (BRIG) as concentric rings.[16]

Genomic Analysis and Phylogenetic Relationship Analysis

In silico multilocus sequence typing (MLST) analysis was performed using the MLST 2.0 server with the entire genome sequence.[14] Plasmid replicon types were analyzed using PlasmidFinder 1.3.[14] Identification of insertion elements (ISs) was predicted by the application of ISfinder.[17] The phylogenetic relationship between *E. faecium* VRE1 and other *E. faecium* strains using a core genome multilocus sequence typing (cgMLST) strategy was performed by BacWGSTdb server. The database currently contains 1758 *E. faecium* strains, including 61 ST78 strains.[18,19]

Nucleotide Sequence Accession Numbers

The whole-genome sequence of the strain *E. faecium* VRE1 and the plasmids was submitted to GenBank under accession number CP040740-CP040744.

Ethical Approval

This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Zhejiang Provincial People's Hospital. Written informed consent from the patient was exempted by the Ethics Committee of Zhejiang Provincial People's Hospital because the present study only focused on bacteria. The clinical isolate *E. faecium* VRE1 was part of the routine hospital laboratory procedure.

Results

MICs of Antimicrobial Susceptibility Testing

The MICs of the antibiotics tested are presented in Table 1. *E. faecium* VRE1 was resistant to multiple antimicrobials including penicillin, ampicillin, gentamicin, streptomycin, ciprofloxacin, levofloxacin, moxifloxacin, erythromycin, clindamycin, nitrofurantoin, vancomycin, teicoplanin and daptomycin. This strain was susceptible to only three antibiotics, ie, tetracycline, tigecycline and linezolid. *E. faecium* VRE1 was highly resistant to vancomycin, with an MIC exceeding 256 mg/L.

Genome Characteristics of *E. faecium* VRE1

The whole-genome sequence of *E. faecium* VRE1 was composed of one chromosomal DNA comprising 2,718,395 bp and four plasmids with sizes of 238,664 bp, 132,733 bp, 6175 bp and 2056 bp. In the chromosomal DNA, a total of 69 tRNA genes, 3 rRNA operons and 2950 protein-coding sequences were identified by the NCBI Prokaryotic Genomes Annotation Pipeline (PGAP) server. Several IS elements were found in the genome,

Table 1 Minimal Inhibitory Concentrations (MICs) of the Antibiotics for *Enterococcus faecium* VRE1

Antibiotics	MIC (mg/L)
Penicillin [b]	>256
Ampicillin [a]	≥32
Streptomycin [c]	≥1024
Gentamicin [c]	32
Ciprofloxacin [a]	≥8
Levofloxacin [a]	≥8
Moxifloxacin [a]	≥8
Erythromycin [a]	≥8
Clindamycin [a]	≥8
Tetracycline [b]	0.5
Tigecycline [b]	0.125
Vancomycin [b]	>256
Teicoplanin [b]	128
Nitrofurantoin [a]	128
Linezolid [a]	2
Daptomycin [c]	8

Notes: [a]Tested by an AST-GP67, [b]Tested by the Etest method, [c]Tested by standard broth microdilution tests.

with the majority belonging to the IS*3* and IS*6* families. In silico MLST analysis revealed that *E. faecium* VRE1 belongs to ST78 (clonal complex CC17).

The distributions of the antimicrobial resistance genes and virulence genes in the genome of *E. faecium* VRE1 are presented in Table 2. Two putative virulence genes, *acm* and *espfm*, were detected in the chromosomal DNA, encoding a collagen adhesin precursor and an enterococcal surface protein, respectively. Five acquired antimicrobial resistance genes were identified, ie, macrolide resistance gene *msr(C)*; aminoglycoside resistance genes *aac(6')-Ii, ant(6)-Ia* and *aph(3')-III*; and glycopeptide resistance gene *vanA*. Except for *aac(6')-Ii* and *msr(C)* located on the chromosomal DNA, other antimicrobial resistance genes, including *vanA*, were all located on a 132,733 bp-sized plasmid, pVRE1-VanA. Six additional genes involved in vancomycin resistance, namely, *vanR-A, vanS-A, vanH-A, vanX-A, vanY-A* and *vanZ-A*, could also be identified on this plasmid.

Genetic Organization of vanA-Carrying Plasmid

The genetic organization of plasmid pVRE1-VanA is presented in Figure 1. It is a *rep2*-type plasmid. Several insertion sequences could be identified in this plasmid, including IS*1216*, IS*1542*, IS*6*, IS*3*, IS*30*, IS*Efm1*, IS*Efa11*, IS*Efa4* and IS*200/IS605*. Among them, IS*1216* appeared more frequently than the others. Genome alignment of the complete sequence of pVRE1-VanA with the NCBI GenBank database indicated that pVRE1-VanA appeared to be a novel plasmid.

Genetic Environment of vanA

The genetic environment of *vanA* was analyzed, which spanned from 82,560 bp to 95,279 bp of the plasmid pVRE1-VanA. We identified a Tn*1546*-like element in pVRE1-VanA (Figure 2). Three complete IS*1216* sequences and one incomplete IS*1216* (IS*1216* partial sequence) were found in the element. Among the three complete IS*1216* elements, two were distributed on both ends of the element and one was inserted into the *vanX-vanY* intergenic region. There were IS*1542* residues (IS*1542* partial sequence) located at the downstream of the first IS*1216* and IS*1216* residues (IS*1216* partial sequence) located at the upstream of the tail IS*1216*.

Phylogenetic Analysis of *E. faecium* VRE1

The phylogenetic relationship between *E. faecium* VRE1 and other *E. faecium* strains deposited in the NCBI GenBank database was analyzed using the BacWGSTdb server. One phylogenetically related strain was identified in the database: *E. faecium* strain SC4, with a difference of 178 alleles. *E. faecium* SC4 was isolated from fodder in 2016 in Beijing, China. *E. faecium* SC4 also harbors the macrolide resistance gene *msr(C)* and the virulence genes *acm* and *espfm* in its chromosomal DNA. Two additional plasmids could be identified in *E. faecium* SC4; among them the plasmid p2 (a *rep2*-type plasmid comprising 142,988 bp, accession no. CP025427) carrying the resistance genes *vanA, ant(6)-Ia, aph(3')-III* and *erm(B)*. Genome alignment showed that the plasmid p2 had 80% coverage and 98.92% identity to pVRE1-VanA (Figure 3).

Discussion

Since the first clinical VRE isolate was detected in Hangzhou, China, in April 2006, VRE have aroused our serious attention.[20] Several studies on VRE from China have been reported, and ST78 was the predominant sequence type in China.[21–26] However, the number of

Table 2 Antimicrobial Resistance and Virulence Encoded Genes of *Enterococcus faecium* VRE1

Antimicrobial Resistance Gene	Contig	Identity (%)	Position	Antimicrobial Resistance Category	Accession Number
msr(C)	VRE1	98.92	2,541,808.2543286	Macrolide	AY004350
aac(6')-Ii	VRE1	99.64	2,136,309. 2136857	Aminoglycoside	L12710
ant(6)-Ia	pVRE1-VanA	100	110,643. 111551	Aminoglycoside	AF330699
aph(3')-III	pVRE1-VanA	100	114,692. 115486	Aminoglycoside	M26832
vanA	pVRE1-VanA	100	86,599. 87630	Glycopeptide	M97297
Virulence Gene	Contig	Identity (%)	Position	Functional Annotation	Accession Number
acm	VRE1	100	2,147,759. 2149924	Collagen adhesin precursor	CP003351.1
espfm	VRE1	99.49	2,609,888. 2615815	Enterococcal surface protein	25,187,962

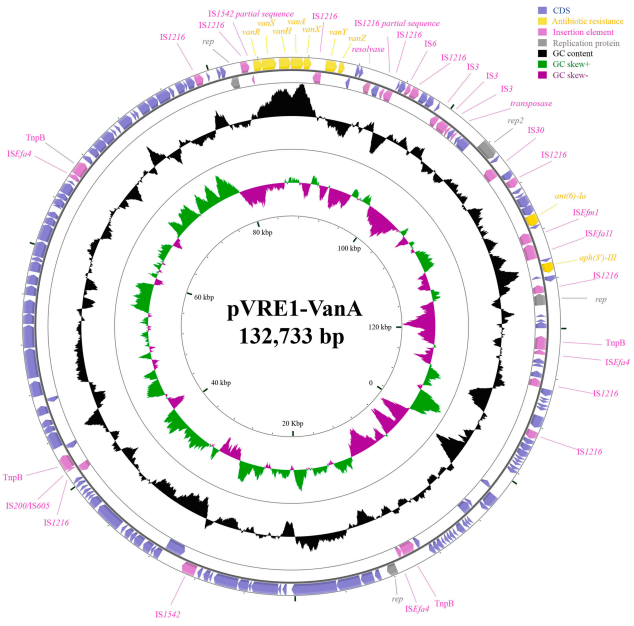

Figure 1 Circular representation of the *vanA*-encoding plasmid pVRE1-VanA.

studies examining the clonal background of *vanA*-positive *E. faecium* strains is still low in China.[27]

We report the complete genome sequence of a clinically isolated *E. faecium* ST78 strain harboring *vanA* from a patient in Hangzhou, China. Genome alignment of the whole sequence of pVRE1-VanA with the NCBI GenBank database indicated that pVRE1-VanA was a novel plasmid. The most closely related plasmid (*E. faecium* strain E7098 plasmid 3, accession no. LR135256) found in the database had 86% coverage and 99.13% identity to pVRE1-VanA (Figure 3).[28] *E. faecium* strain E7098 was isolated in the Netherlands. Two plasmid replicons (*rep2* and *rep22*) and six resistance genes (*ant (6)-Ia, aph(3')-III, erm(B), lnu(B), tet(L)* and *vanA*) could be identified in plasmid 3.

We also identified a Tn*1546*-like element in pVRE1-VanA (Figure 2). Compared with the typical Tn*1546* element (*E. faecium* strain BM4147 plasmid pIP816, accession no. KX976485),[9] there was an absence of the transposase ORF1 and resolvase ORF2 in pVRE1-VanA. The present Tn*1546*-like element was similar to the Tn*1546-B2*-type element, which has the characteristic of the presence of IS*1216* upstream of *vanR*.[3,10,29] A similar Tn*1546*-like element with IS*1216* inserted in the *vanX-vanY* intergenic region was found in the NCBI GenBank database (*E. faecium* strain AUSMDU00004167 plasmid unnamed3, accession no. CP027500).[7] The structure of the present Tn*1546*-like element was similar to that strain but seems to be more complex (Figure 2). We also found a Tn*1546*-like element carrying IS*1216* and IS*1542* elements in similar position to that previously reported in *E. gallinarum*.[30] The most closely related Tn*1546*-like

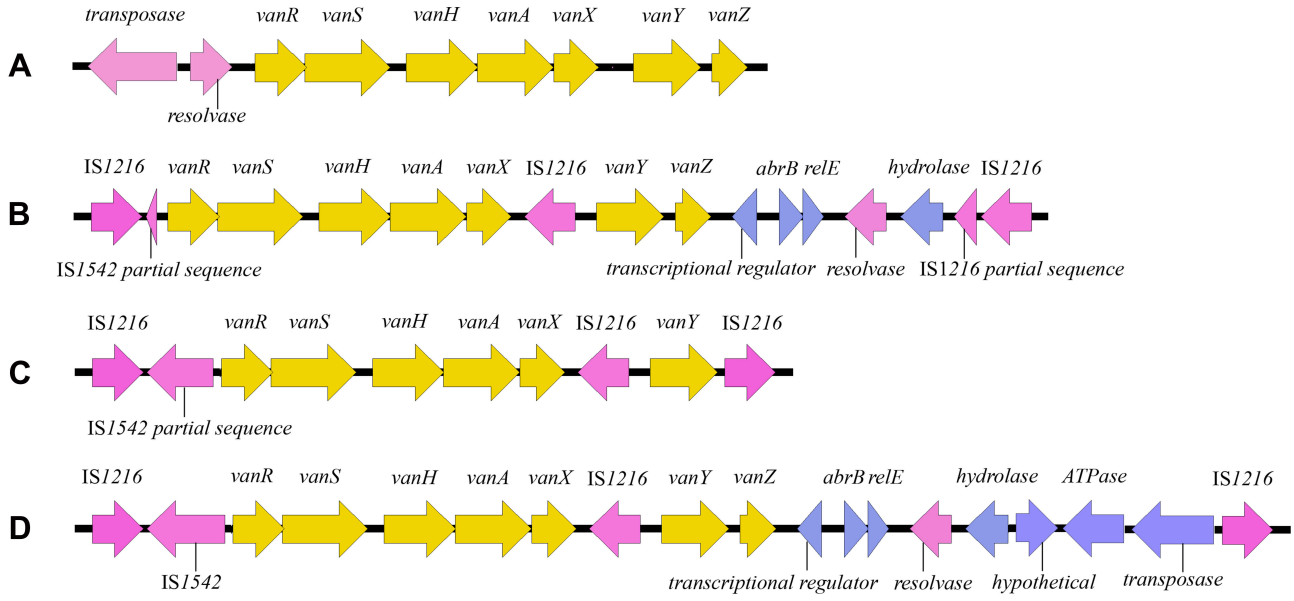

Figure 2 Comparison of the homologous regions shared by different types of *vanA*-carrying plasmids. (**A**) Typical Tn*1546* (*E. faecium* strain BM4147 plasmid pIP816, accession no. AM932524). (**B**) Tn*1546*-like element in this study (*E. faecium* strain VRE1 plasmid pVRE1-VanA, accession no. CP040742). (**C**) Similar Tn*1546*-like element with IS*1216* inserted in the *vanX-vanY* intergenic region (*E. faecium* strain AUSMDU00004167 plasmid unnamed3, accession no. CP027500). (**D**) Tn*1546*-like element most closely related to that in the present study (*E. faecium* strain 2014-VREF-63 plasmid p63–1, accession no. CP019989).

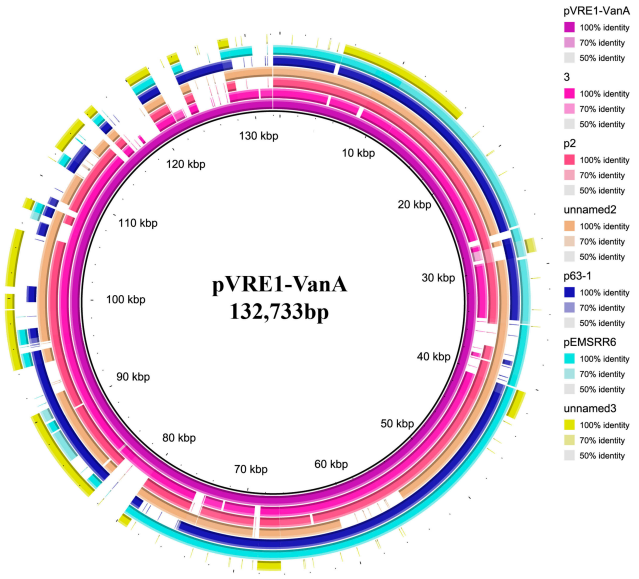

Figure 3 Plasmid sequence alignment of vanA-carrying plasmids that revealed partial sequence identity to pVRE1-VanA. 3: *E. faecium* E7098 plasmid 3, accession no. LR135256. p2: *E. faecium* SC4 plasmid p2, accession no. CP025427. Unnamed2: *E. faecium* VB3025 plasmid unnamed2, accession no. CP040238. p63–1: *E. faecium* 2014-VREF-63 plasmid p63–1, accession no. CP019989. pEMSRR6: *E. faecium* SRR6 plasmid pEMSRR6, accession no. MG640601. Unnamed3: *E. faecium* AUSMDU00004167 plasmid unnamed3, accession no. CP027500.

element to that reported in the present study was the one located on a 287,502 bp *repUS15*-type plasmid (p63–1) identified in *E. faecium* strain 2014-VREF-63 in Korea (*E. faecium* strain 2014-VREF-63 plasmid p63–1, accession no. CP019989).

According to the phylogenetic analysis, the most closely related vancomycin-resistant *E. faecium* strain, SC4, was found in fodder from Beijing, China with a transferable *vanA*-carrying plasmid.[31] In Hangzhou, the first report of vancomycin-resistant *E. faecium* was published in 2007 by Qu et al.[20] Twenty-one *vanA*-carrying *E. faecium* isolates were obtained from inpatients among five hospitals in Hangzhou, from April 2006 to April 2007. The predominant ST was ST78, and a Tn*1546*-like element with IS*1485* inserted between *vanXY* was found in those isolates. Another research on VRE conducted by Qu et al was published in 2012.[23] They collected 45 vancomycin-resistant *E. faecium* strains from different cities (including Hangzhou) of Zhejiang Province in 2009. In agreement with our results, ST78 resulted to be the predominant ST and all strains carried Tn*1546*-like elements, although of variable type and different from that described here. These findings highlight the spread also in China of the epidemic ST78. Besides clonal dissemination, horizontal transfer of Tn*1546*-like elements can contribute to the spread of vancomycin resistance to different enterococcal lineages. Moreover, the presence of additional resistance genes on the Tn*1546*-carrying pVRE1-VanA plasmid could contribute to the emergence of multidrug-resistant strains in mainland China. Therefore, more studies are required to illuminate the epidemic clones of VanA-positive *E. faecium* in China.

Conclusion

In summary, our study reports the emergence of a vancomycin-resistant *E. faecium* ST78 strain with a rep2-type *vanA*-carrying plasmid in Hangzhou, China. The ST78 lineage possesses the potential to emerge as a successful *vanA*-carrying epidemic clone. Further studies involving more VanA-producing isolates are warranted to identify reservoirs and monitor the transmission dynamics of *vanA* genes in China.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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