

Phenotypic and genotypic characterization of linezolid-resistant *Enterococcus faecium* from the USA and Pakistan

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Objectives: Linezolid is an important therapeutic option for the treatment of infections caused by VRE. Linezolid is a synthetic antimicrobial and resistance to this antimicrobial agent remains relatively rare. As a result, data on the comparative genomics of linezolid resistance determinants in *Enterococcus faecium* are relatively sparse.

Methods: To address this knowledge gap in *E. faecium*, we deployed phenotypic antibiotic susceptibility testing and Illumina WGS on hospital surface (environmental) and clinical isolates from the USA and Pakistan.

Results: We found complete concordance between isolate source country and mechanism of linezolid resistance, with all the US isolates possessing a 23S rRNA gene mutation and the Pakistan isolates harbouring two to three acquired antibiotic resistance genes. These resistance genes include the recently elucidated efflux-pump genes *optrA* and *poxxA* and a novel *cfr*-like variant. Although there was no difference in the linezolid MIC between the US and Pakistan isolates, there was a significant difference in the geometric mean of the MIC between the Pakistan isolates that had two versus three of the acquired antibiotic resistance genes. In five of the Pakistan *E. faecium* that possessed all three of the resistance genes, we found no difference in the local genetic context of *poxxA* and the *cfr*-like gene, but we identified different genetic contexts surrounding *optrA*.

Conclusions: These results demonstrate that *E. faecium* from different geographical regions employ alternative strategies to counter selective pressure of increasing clinical linezolid use.

Introduction

Enterococcus faecium is a common gut commensal organism and an increasingly important cause of nosocomial infection.¹ One feature implicated in the success of *E. faecium* as a pathogen is its repertoire of acquired antibiotic resistance genes (ARGs) that enable evasion of antimicrobial therapy.¹ As an example, treatment of *E. faecium* infections with vancomycin has facilitated proliferation of the *vanA* gene cassette throughout *E. faecium*.² Due to the increase in vancomycin-resistant Gram-positive pathogens, newer therapeutics, notably the oxazolidinones linezolid and tedizolid,

have become important therapeutic agents for treating infections caused by this organism.³

Accordingly, sporadic resistance to linezolid has been identified in cohorts of *E. faecium* and other Gram-positive bacteria.^{4–6} These include vertically transmitted mutations in the linezolid target, the 23S rRNA gene sequence, and alterations in the ribosomal proteins L3, L4 and L22.^{7–9} Acquired plasmid-borne ARGs, including the 23S rRNA methyltransferases *cfr* and *cfr(B)*, have been previously identified in *E. faecium*.^{10–12} Newly identified efflux-pump genes, *optrA* and *poxxA*, have also been described in *E. faecium*.^{13,14}

Despite the identification of vertically and horizontally transferable linezolid resistance determinants, a comprehensive genomic survey of linezolid-resistant *E. faecium* isolates has not been performed. Additionally, there is a gap in knowledge on the relationship of established linezolid resistance determinants and their encoded phenotypic susceptibility to the newest oxazolidinone, tedizolid. To address this, we performed WGS and comparative analysis on 41 newly sequenced isolates from the USA and 8 newly sequenced isolates from Pakistan. To increase the number of isolates for analysis, we supplemented these data with 52 publicly available genomes of *E. faecium* isolated from the same locations in the USA and Pakistan. Our results indicate that the mechanism of linezolid resistance is more strongly associated with geography rather than *E. faecium* clade/phylogeny in this cohort, with resistant isolates from the USA harbouring the G2576T SNP in 23S rRNA loci and resistant isolates from Pakistan encoding combinations of *poxA*, *optrA* and *cfr*-like ARGs.

Materials and methods

Linezolid-non-susceptible *E. faecium* cohort

To understand the genotypic mechanism for linezolid resistance in two different geographies, we analysed a collection of banked linezolid-intermediate and linezolid-resistant *E. faecium* isolates recovered from cultures of environmental or clinical specimens between 2012 and 2018. Inclusion criteria include phenotypic resistance or intermediate resistance to linezolid using the Etest gradient diffusion assay (bioMérieux, Durham, NC, USA). We accessed 44 banked linezolid-non-susceptible environmental *E. faecium* and 3 linezolid-susceptible isolates from 2015 to 2016 that were sequenced in a previous analysis (BioProject PRJNA497126) from longitudinal surveillance of hospital surfaces in Pakistan. We newly sequenced four linezolid-non-susceptible and four linezolid-susceptible isolates collected from a previous analysis of clinical isolates obtained in 2012–13 from two hospitals in Pakistan.¹⁵ We additionally accessed 30 clinical isolates of linezolid-non-susceptible *E. faecium* banked from the clinical microbiology laboratory of Barnes-Jewish Hospital (St Louis, MO, USA) from 2015 to 2018. Finally, we accessed eight environmental linezolid-non-susceptible and three linezolid-susceptible *E. faecium* isolates obtained from environmental surfaces in the Barnes-Jewish Hospital during 2017–18. *E. faecium* Aus0004 (clade A1 reference), *E. faecium* E2134 (clade A2 reference) and *E. faecium* E1007 (clade B reference) were obtained from a previous genomic analysis of *Enterococcus* evolution.¹⁶ The linezolid-resistant isolate due to a 23S rRNA G2576T mutation, *E. faecium* VRE1558, and the linezolid-resistant isolate due to a 23S rRNA G2505A mutation, *E. faecium* E1644, were also included in the phylogenetic analysis.^{17,18}

Illumina WGS and genomic analysis

Stock cultures of the *E. faecium* isolates sequenced in this investigation were recovered from freezer vials and streaked out onto blood agar (Hardy Diagnostics). Approximately 10 colonies were suspended into 1 mL of nuclease-free water. Genomic DNA was extracted using the QIAamp Bacteremia DNA Kit (QIAGEN, Germantown, MD, USA). Genomic DNA was sequenced with Illumina WGS, producing short-read sequences. Illumina adapter sequences were removed using Trimmomatic (version 0.38) and sequence contamination was removed with DeconSeq (version 0.4.3).^{19,20} The processed reads were assembled into contigs using SPAdes (version 3.13.0).²¹ Isolates sequenced in this paper, as well as previously sequenced isolates (including outgroups E1007, Aus0004 and E2134, used for clade identification, and VRE1558 and E1644, positive for 23S rRNA mutations G2576T and G2505A, respectively), were annotated with Prokka (version 1.12).²² MLST STs were also determined using BLAST similarity ([\[github.com/tseemann/mlst\]\(https://github.com/tseemann/mlst\)\). Core-genome analysis was performed with Roary \(version 3.12.0\) on the .gff files from Prokka. The core-genome alignment with PRANK was converted to an approximate maximum-likelihood tree in FastTree \(version 2.1.9\). After determination that all of the isolates were from clades A1 or A2 we removed the clade B genome from analysis and performed parSNP \(version 1.2\) on the FASTA files of the isolates.²³ The Newick file for both trees were viewed in iTOL.²⁴ For detailed information on software parameters and commands used in this investigation, please see Appendix S1 \(available as Supplementary data at JAC Online\).](https://</p>
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Antibiotic susceptibility testing

Pure cultures of isolates had phenotypic antibiotic resistance determined using Kirby–Bauer disc diffusion assays and gradient diffusion (e.g. Etest) assays. Both assays were performed according to the manufacturers' instructions. The results were interpreted using the CLSI M100 criteria for *Enterococcus* species.²⁵ Linezolid (BD, Franklin Lakes, NJ, USA) and vancomycin (Hardy Diagnostics, Santa Maria, CA, USA) were tested using Kirby–Bauer discs. Strains were classified as linezolid susceptible at or above 23 mm, intermediate at 21–22 mm and resistant at or below 20 mm; similarly, strains were classified as vancomycin susceptible at or above 17 mm, intermediate at 15–16 mm and resistant at or below 14 mm. We additionally tested linezolid (bioMérieux), daptomycin (bioMérieux), dalbavancin (Liofilchem, Waltham, MA, USA) and tedizolid (Liofilchem) using quantitative gradient diffusion assays and interpreted the MIC value in accordance with 2019 CLSI standards: strains were classified as linezolid susceptible at or below 2 mg/L, intermediate at 4 mg/L and resistant at or above 8 mg/L; strains were classified as daptomycin susceptible at or below 1 mg/L, susceptible dose-dependent at 2–4 mg/L and resistant at or above 8 mg/L; and strains were classified as dalbavancin susceptible at or below 0.25 mg/L.²⁵ As there is currently an absence of *E. faecium* breakpoints for tedizolid, we used the *Enterococcus faecalis* breakpoint criteria for our cohort; strains were classified as tedizolid susceptible at or below 0.5 mg/L and non-susceptible above 0.5 mg/L. All interpretations of Etest MIC values were performed with clinical accuracy and read appropriately. Reported Etest MIC values were rounded up to the nearest doubling dilution.

In silico oxazolidinone resistance determinant identification

ResFinder annotation of known resistance genes was used to identify isolates that harboured *optrA*, *poxA* and *vanX*.²⁶ We used Roary to assemble the pangenome of the isolates and found that a *cfr*-like gene had been annotated in the genes_presence_absence output of the program.²⁷ The gene sequence was compared with *cfr* and variant *cfr*(B) sequences using BLAST.^{11,28}

Following published suggestions for determining linezolid resistance mutations, the reads of processed isolates were aligned using Bowtie2 to a reference 23S rRNA sequence of Aus0004.²⁹ The 23S rRNA sequence of Aus0004 (NCBI Reference Sequence: NR_103056.1) did not harbour any of the mutations associated with linezolid resistance. SNPs that did not match the Aus0004 reference sequence were identified using a custom Python 3 script. From this alignment, the site of the SNP that correlated with the G2576T mutation (using *Escherichia coli* numbering) responsible for linezolid resistance was identified. Isolates found to be positive for the mutation by this method had the SNP in at least 50% of reads. To identify all isolates that had the G2576T mutation at any frequency, a second script was run to extract isolates with an SNP at the respective site. All isolates having the mutation at a frequency of at least 17% of reads, which is regarded as the minimum frequency for phenotypic linezolid resistance, were considered to be resistant by ribosomal mutation.²⁹ Other published mutations responsible for linezolid resistance were sought out, but not identified in any of the isolates; these included the G2505 23S rRNA gene mutation and mutations in the L3, L4 and L22 proteins.^{18,30,31}

Data availability

All genomes sequenced in this study have been uploaded to the NCBI WGS database associated with BioProject PRJNA517335.

Results

Acquired linezolid resistance genes (*optrA*, *poxxA* and *cfr*-like) were found exclusively in the *E. faecium* isolates recovered from Pakistan, regardless of clade

We accessed banked environmental and clinical isolates of linezolid-non-susceptible *E. faecium* isolates from the USA and Pakistan as well as several known linezolid-susceptible isolates from both locations to perform genomic analysis of linezolid resistance determinants. We used Illumina WGS to construct draft genomes for 49 isolates (Table S1) and obtained 52 publicly available *E. faecium* genomes isolated from the same locations in the USA and Pakistan (Table S2). We used Kirby–Bauer disc diffusion and gradient diffusion methods in conjunction with CLSI interpretive guidelines to assign phenotypic resistance criteria to linezolid (resistant, intermediate or susceptible) and tedizolid (using *E. faecalis* breakpoints for non-susceptible or susceptible). Initially, we constructed a core-genome phylogenetic tree on the 1691 core genes between all genomes. Phylogenetic comparison of the cohort with reference isolates from *E. faecium* clades A1, A2 and B determined that all isolates in the cohort belong to clades A1 and A2, characteristic of human pathogens (Figure S1).¹⁶ To gain further resolution on the relatedness of the *E. faecium* isolates, we excluded the clade B isolate E1007 and constructed a recombination-free phylogenetic tree using parSNP (Figure 1). The phylogeny of the isolates was generally geographically stratified, as 80.5% (33/41) of *E. faecium* from the USA were in clade A1 and 90.9% (50/55) of *E. faecium* from Pakistan were in clade A2. The isolate cohort represented 11 identifiable MLST STs. Of the US isolates, 70.7% (29/41) were resistant to linezolid and, of these, 100% (29/29) were positive for the G2576T 23S rRNA SNP using Bowtie2 alignment of Illumina reads to the Aus0004 reference sequence (Figure 1).²⁹ A comparable proportion of the *E. faecium* isolates from Pakistan, 72.7% (40/55), were also resistant to linezolid; however, in contrast, 97.5% (39/40) of these isolates were positive for an acquired linezolid resistance gene identified by ResFinder or Prokka, but negative for the G2576T SNP. The canonical 23S rRNA methyltransferase gene *cfr* was not identified in our isolates; however, a variant of the *cfr* family was annotated by Prokka in 76.4% (42/55) of *E. faecium* isolates from Pakistan (Figure 1). BLASTP query and comparison with previously characterized sequences of the *cfr* gene, the *cfr*(B) variant and the ancestral *rlmN* gene determined that the *cfr*-like gene shared 64% identity over 95% of query length with the original *cfr* gene and 65% identity over 97% of the length of *cfr*(B) (Figure S2). An identity of 74.9% over 99.7% was previously used to classify *cfr*(B) as unique from *cfr*, therefore the gene we have described fits within the category of other emerging *cfr*-like family members.^{32,33} Of the isolates from Pakistan, 78.2% (43/55) and 61.8% (34/55) contained the linezolid ABC transporters *poxxA* and *optrA*, respectively. Of the isolates with gene-based resistance, 76.7% (33/43) harboured all three of the resistance genes identified in the cohort; 20.9% (9/43) of the isolates harboured only *poxxA* and the *cfr*-like gene and 2.33% (1/43) harboured only *optrA* and *poxxA*. Of the

isolates from both Pakistan and the USA, 90.6% (87/96) and 88.5% (85/96) of the isolates were resistant to vancomycin and dalbavancin, respectively. Only 3.13% (3/96) of the isolates were resistant to daptomycin, another therapeutic agent commonly used to treat VRE in the USA; however, an additional 68.8% (66/96) had MIC values in the susceptible dose-dependent classification range. These results indicate that while clade A1 and clade A2 *E. faecium* isolates can be found in both the USA and Pakistan, there is a differential burden in the mechanism of linezolid resistance between the surveyed isolates from these locations.

Linezolid resistance differs by genes present, not by mechanism

The geometric mean linezolid MIC (21.83 mg/L) was greater than the geometric mean tedizolid MIC (2.87 mg/L) (Figure 2a and b). There was minimal difference between the geometric mean linezolid MIC for isolates with gene-based linezolid resistance (40.75 mg/L) and isolates with mutation-based linezolid resistance (40.32 mg/L) (Figure 2c). However, the geometric mean linezolid MIC for isolates with all three observed resistance genes (64 mg/L) was significantly greater ($P < 0.0001$) than the geometric mean linezolid MIC for isolates that harboured only *poxxA* and the *cfr*-like gene (6.86 mg/L) (Figure 2d). Our results demonstrate that while tedizolid resistance and linezolid resistance may be related, there are several instances in our cohort where they are independent of one another (Figure S3). Of the 96 isolates, 22 (22.9%) were neither susceptible to both antibiotics nor resistant to linezolid and non-susceptible to tedizolid (Figure S3). Of these, 40.9% (9/22) of isolates had intermediate linezolid resistance, but were susceptible to tedizolid, 36.4% (8/22) of isolates were linezolid intermediate and non-susceptible to tedizolid and 22.7% (5/22) of isolates were susceptible to linezolid, but non-susceptible to tedizolid (Figure S3). The previously identified 23S rRNA G2505A linezolid resistance mutation was not identified within the isolates from our cohort.^{18,29} However, heterogeneity at site 1232 in the aligned 23S rRNA gene of *E. faecium* Aus0004 was observed in all isolates from our cohort (with >17% frequency in 76 isolates). This site has not previously been associated with linezolid resistance and the mutation was observed in both linezolid-resistant and -susceptible isolates, therefore it likely does not contribute to phenotypic linezolid resistance. Within the population of *E. faecium* that contained the G2576T mutation at >17%, there was not a correlation between frequency of the G2576T SNP and phenotypic linezolid resistance (Figure S4).

Different genetic platforms of *optrA* in linezolid-resistant *E. faecium* from Pakistan

We used EasyFig to analyse the genetic context of *optrA*, *poxxA* and the *cfr*-like gene in five isolates that harboured all three genes (Figure 3). The visualized genetic context of *optrA* was identical in Pakistan isolates EF_48, EF_111 and EF_181, as well as partially in EF_325. These segments harboured a *fexA* phenicol resistance gene adjacent to *optrA*. The context of *optrA* in EF_155 differed from the others and contained the *erm*(A1) methyltransferase gene. The *optrA* contigs also contained several transposase-associated and phage-associated transposase genes, which could enable horizontal transfer of the *optrA* gene. The contig from

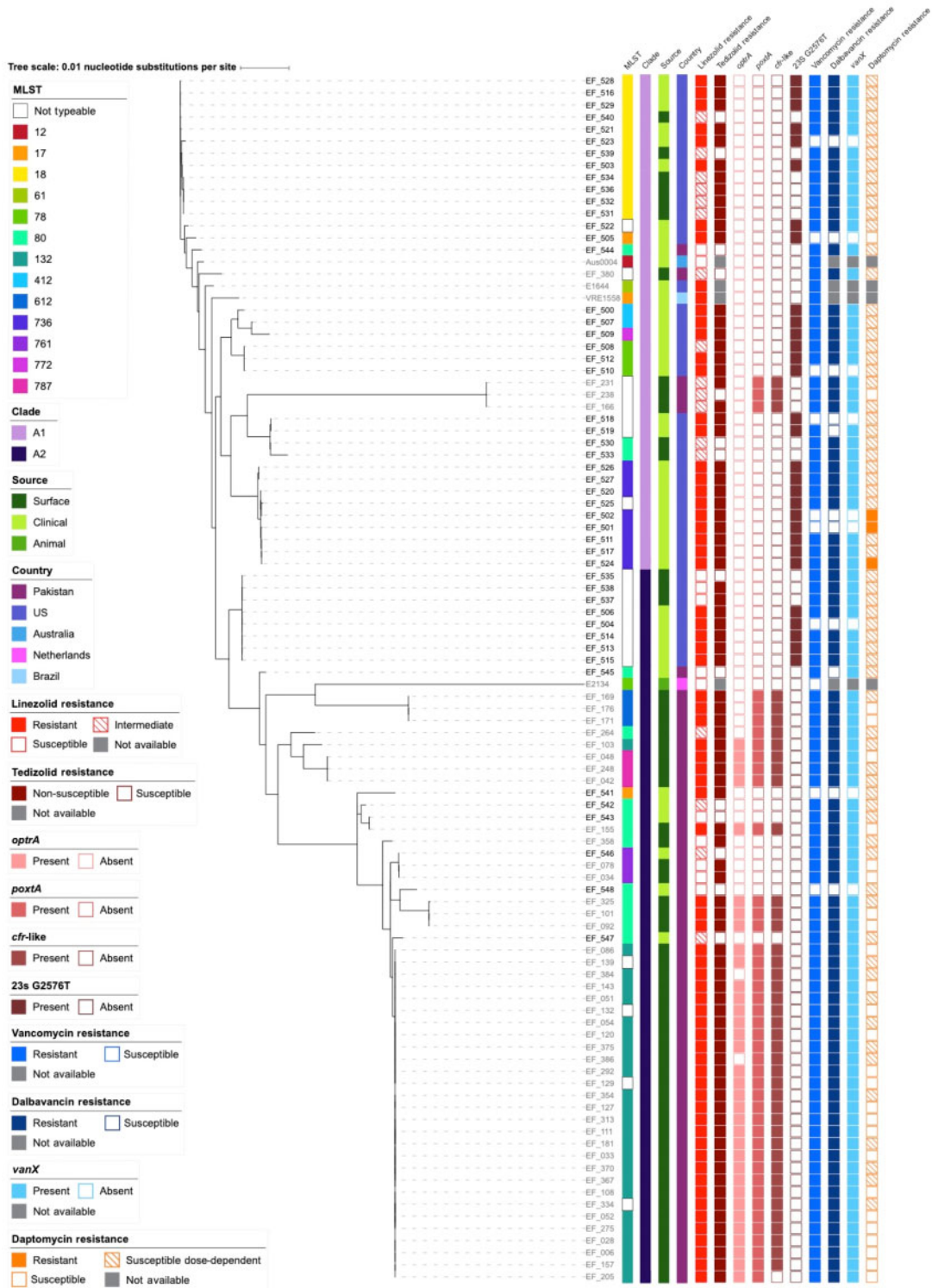


Figure 1. Recombination-free phylogenetic tree including MLST, country, source, resistance, resistance gene and mutation data. Linezolid resistance in US isolates was attributed solely to the G2576T mutation of the 23S rRNA gene sequence. In contrast, linezolid resistance in Pakistan isolates resulted from different combinations of the acquired resistance genes *optrA*, *poxxA* and a *cfr*-like gene. Vancomycin resistance was observed in 90.6% (87/96) of the isolates and dalbavancin resistance was observed in 88.5% (85/96). Daptomycin resistance was observed in 3.13% (3/96) of the isolates with an additional 68.8% (66/96) classified as susceptible dose-dependent.

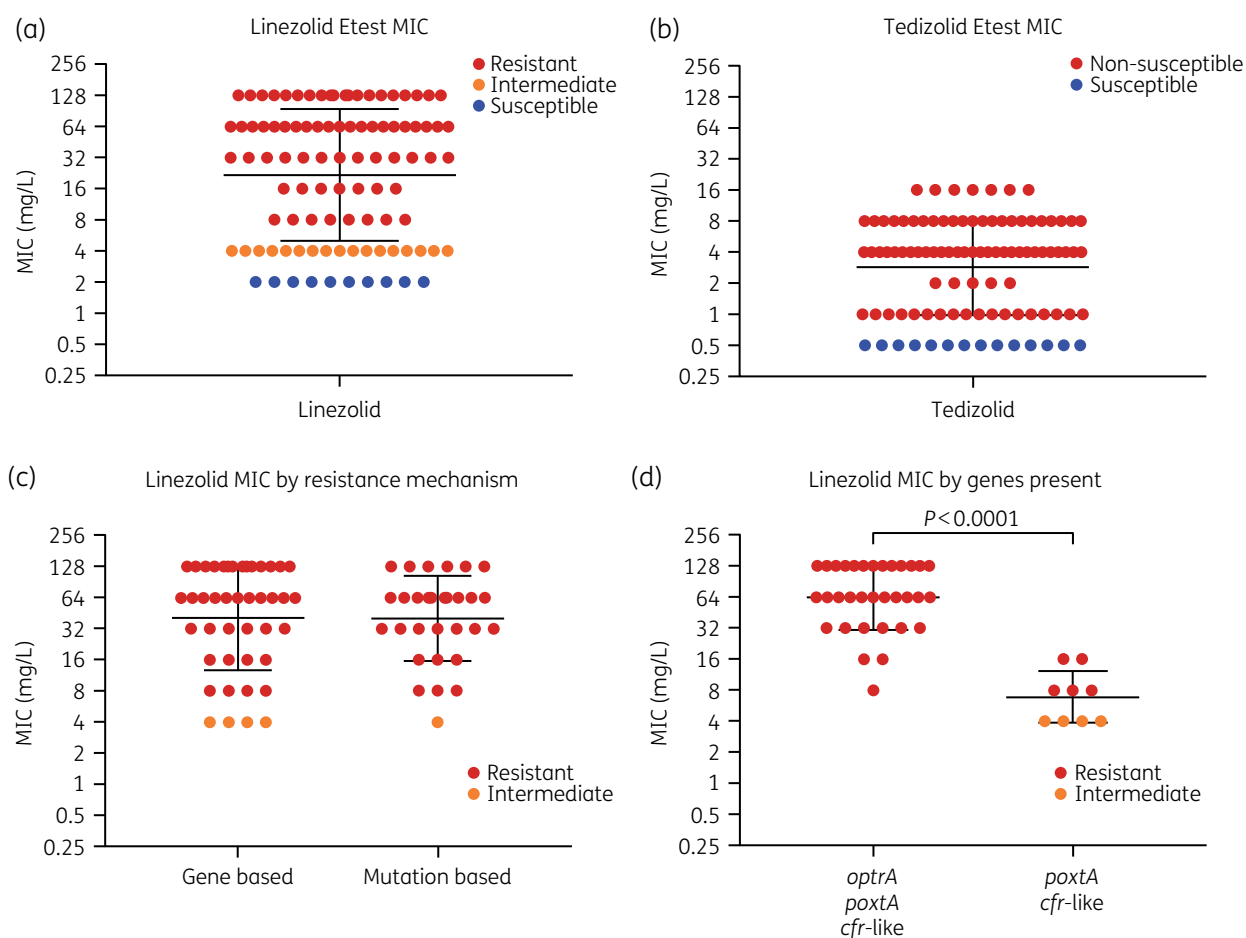


Figure 2. Linezolid and tedizolid MICs and comparisons by basis of resistance mechanism. The geometric mean MIC of linezolid (a) is higher than the geometric mean MIC of tedizolid (b) at 21.83 and 2.87 mg/L, respectively. There was no difference in linezolid resistance between isolates with gene- or mutation-based resistance mechanisms (c). However, isolates that harboured all three linezolid resistance genes (d); statistical analysis was done using the unpaired *t*-test in Prism v8. Please note, y-axis values for all graphs are \log_2 scaled for visual acuity.

EF_103 contained only the *optrA* gene. In all cases *poxtA* was assembled on a short contig with no other flanking genes and the genetic context around the *cfr*-like gene was identical in the isolates we observed (Figure S5).

Discussion

The molecular epidemiology of linezolid resistance in VRE is largely uncharacterized, but linezolid resistance is rapidly increasing.⁵ Consistent with earlier reports on the distribution of isolates in *E. faecium* clades, all of our isolates were in the A1 or A2 group.¹⁶ Nearly 72% (69/96) of the isolates in this study were linezolid resistant, with an additional 18% (17/96) having intermediate linezolid resistance. Additionally, 85% (82/96) of the isolates were non-susceptible to tedizolid, with much lower MIC values than observed for linezolid, as has been previously observed in linezolid-resistant *E. faecium* from Germany.³⁴ In our cohort, linezolid resistance can be attributed to a combination of resistance genes or the G2576T mutation in the 23S rRNA gene. While the resistance mechanism differs between geographical locations, with

resistance in the strains recovered from Pakistan containing gene-mediated resistance determinants and US isolates harbouring 23S rRNA gene mutation(s), both groups displayed similar phenotypic MIC distributions. Possibly due to differences between short-read Illumina and longer-read Sanger sequencing, we did not observe a correlation between the linezolid MIC and the proportion of the G2576T mutation 23S rRNA allele, as has been identified previously.³⁵ Limiting linezolid use may partly curtail the spread of resistance, as the G2576T resistance mutation can arise in pathogens due to prolonged drug exposure and the *cfr*, *optrA* and *poxtA* resistance genes identified have historically been capable of horizontal transfer through situation on mobile genetic elements.^{14,36–38} Tedizolid holds promise for treatment of MDR infections.³⁹ However, we found that 100% (69/69) of linezolid-resistant isolates were also non-susceptible to tedizolid and 47% (8/17) of linezolid-intermediate isolates were tedizolid non-susceptible. Unexpectedly, five isolates were linezolid susceptible but tedizolid non-susceptible, although the MIC distributions for these isolates were near the resistance breakpoint for both antimicrobials. The MIC breakpoints published by the CLSI for non-susceptibility to

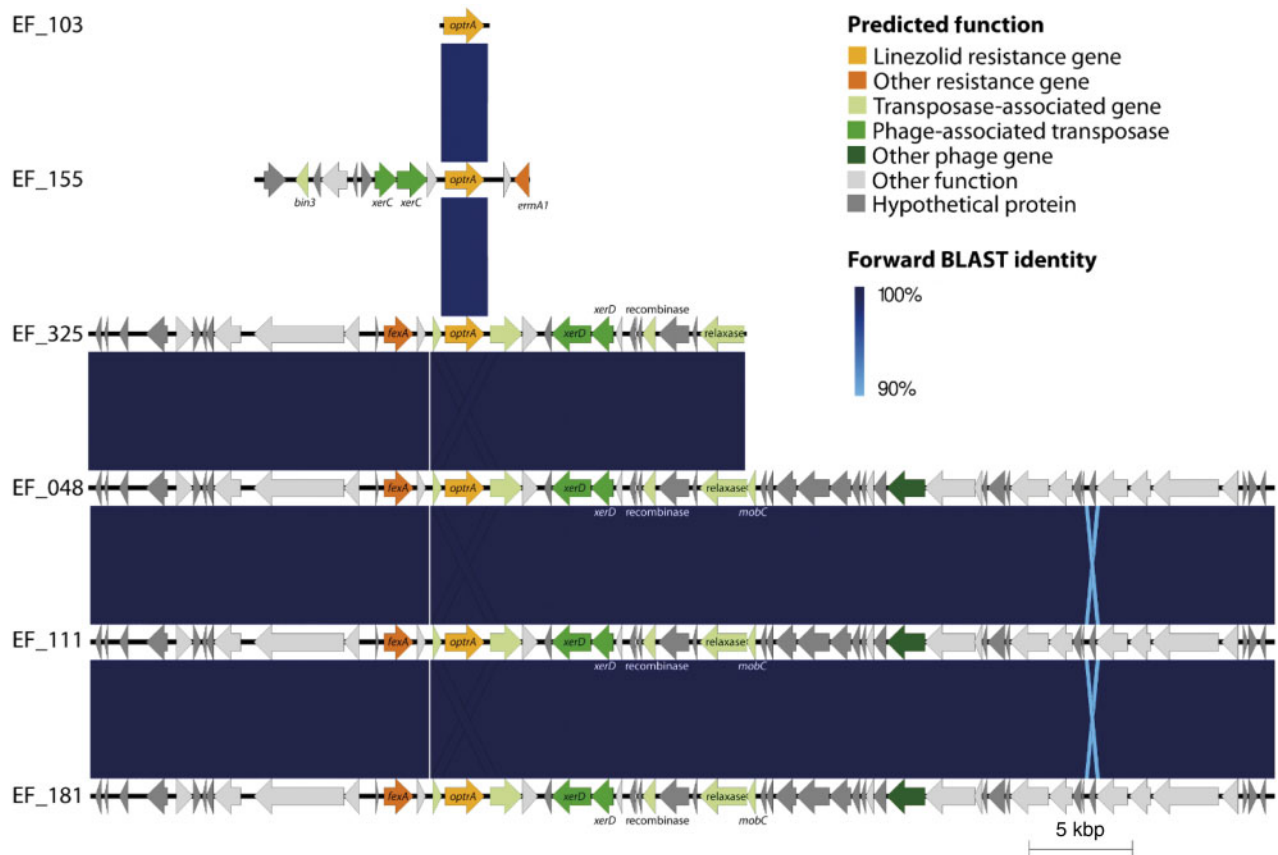


Figure 3. Genetic context of *optrA* in isolates that harbour *optrA*, *cfr*-like and *poxtA* genes. In isolates EF_325, EF_048, EF_111 and EF_181, *optrA* is downstream of the resistance gene *flexA* and in isolate EF_155 it is upstream of an *ermA1* resistance gene. These contexts are similar to those that *optrA* was in when it was first identified. However, the mobile elements surrounding *optrA* in our isolates differ from those previously identified. *optrA*'s location near mobile elements may allow it to be transferable.

tedizolid are lower than for linezolid based on pharmacokinetic and pharmacodynamic properties.⁴⁰ Future investigations to examine tedizolid-specific resistance determinants and suitable breakpoints specifically for *E. faecium* are warranted.³⁹

To the best of our knowledge, the *cfr* 23S rRNA methyltransferase family and the *optrA* and *poxtA* efflux pump genes are the only known acquired ARGs against linezolid.^{10,13,14} These genes can also confer resistance to other antibiotics, including chloramphenicol and clindamycin, complicating treatment options. *cfr*, *cfr(B)*, *cfr(C)* and unnamed *cfr*-like genes have previously been identified in linezolid-resistant strains of *Staphylococcus aureus*, *Clostridioides (Clostridium) difficile*, *Enterococcus* spp., *E. faecalis* and *E. faecium*.^{10,11,41-44} Interestingly, these genes do not appear to be restricted to pathogens, but can be found in a diverse number of Gram-positive species, indicating that multiple opportunities for horizontal gene transfer may arise.³³ Previously, *cfr* and its variants have been identified in isolates from countries including the USA, Germany, Spain, Italy, China, France, Denmark and the UK, but, to the best of our knowledge, this is the first report from Pakistan. In all isolates in which we observed the *cfr*-like gene, we also identified *poxtA* or both *poxtA* and *optrA*. Among isolates that only harboured the *cfr*-like gene and *poxtA*, the geometric mean MIC (6.86 mg/L) was ~10 times lower than that for those that

harboured all three identified resistance genes (64 mg/L), with one of the two-gene isolates achieving only intermediate resistance. The genes *optrA* and *cfr* have previously been reported colocalized on plasmids in hospital-borne vancomycin-resistant *E. faecium*.⁴⁵ Upon its discovery, there was doubt as to whether *cfr(B)* granted the same resistance phenotype in *Enterococcus* as it does in *Staphylococcus* or if the *cfr*-like gene from *C. difficile* also confers antibiotic resistance.^{11,46} Additionally, a recent study using a mouse peritonitis model found that tedizolid underperformed compared with linezolid and daptomycin in bacterial clearance of *cfr(B)*-positive *E. faecium*.⁴⁷ Treatment of *cfr(B)*-positive *E. faecium* infection with linezolid garnered 86% survival in a mouse peritonitis model, despite presenting MICs that would suggest linezolid resistance.⁴⁷ Our data, coupled with these observations, suggest that the relative contribution of the *cfr*-like gene to phenotypic resistance may be less significant than that of other resistance genes and could be attributed to significant genotypic divergence from the canonical *cfr* gene. These phenotypic discrepancies may be exacerbated by synergistic effects occurring between the *optrA* and *poxtA* transporters and the *cfr*-like methyltransferase that are not occurring when *poxtA* and the *cfr*-like gene contribute to resistance in the absence of *optrA*. Therefore, while it is possible the *cfr*-like gene, *poxtA* and *optrA* contribute equally to linezolid

resistance, further investigation is necessary to determine their individual impacts on the observed resistance phenotypes.

Notably, *optrA* resided in different contexts within our isolates. Comparing the ARG genetic contexts of isolates randomly selected from different branches of the phylogenetic tree, we found several isolates with contexts similar to those in which *optrA* was originally identified—having either the *fxeA* phenicol exporter gene upstream of *optrA* or an *erm(A1)* ARG downstream of *optrA* (Figure 3).³⁷ However, the mobile elements identified in our isolates (several of which are phage-associated) differed from those previously observed near *optrA*. Although the limitations of short-read sequencing prevented us from obtaining longer genetic contexts of the *poxxA* and *cfr*-like genes (Figure S5), *poxxA*, *optrA* and *cfr* variants have previously been observed near mobilizing elements, with the *cfr* variants and *optrA* residing on plasmids.^{10,11,14,37}

This study aimed to characterize the molecular epidemiology and investigate the differential burden of linezolid resistance mechanisms in *E. faecium* from two geographically distinct locations. We found that all US isolates have the 23S rRNA G2576T mutation, while isolates from Pakistan harbour combinations of a *cfr*-like gene, *optrA* and *poxxA*. While geometric mean MIC values for these groups did not differ greatly (40.75 mg/L for gene-based resistance and 40.32 mg/L for mutation-based resistance), there was a difference between isolates that harboured *poxxA* and *optrA* compared with those isolates that had all three putative ARGs. Daptomycin is the antimicrobial agent evaluated in this study with the highest rate of susceptibility based on *in vitro* testing; 3.13% (3/96) of isolates in this study are phenotypically resistant; however, 68.8% (66/96) of isolates are susceptible dose-dependent to daptomycin. Of note, daptomycin therapy is not a viable option for pulmonary infections, but *Enterococcus* spp. are very uncommon causes of pneumonia.^{48,49} Additionally, in the case of isolate EF_524, therapeutic options would be extremely limited as the isolate is resistant to linezolid, tedizolid, vancomycin, dalbavancin, daptomycin and ampicillin, the primary antibiotics available for *Enterococcus* infection treatment. In five isolates that harboured all three ARGs, *optrA* was observed in different genetic contexts, while the *cfr*-like gene and *poxxA* were observed in similar contexts or were assembled on contigs that were too short to identify flanking genes. The major limitation of this study is that by using Illumina sequencing we are unable to resolve plasmid versus chromosomal segments. The use of long-read sequencing may further provide context for the genetic environment surrounding *cfr*, *poxxA* and *optrA* in the isolates from Pakistan. Nevertheless, our results indicate that *E. faecium* isolates can use distinct genetic strategies to achieve comparable *in vitro* linezolid resistance. Continued investigation of linezolid resistance in *E. faecium* and antibiotic stewardship of linezolid are advised to prevent the spread of resistance to this last-resort antibiotic.

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Transparency declarations

None to declare.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agencies.

Supplementary data

Appendix S1, Tables S1 and S2 and Figures S1 to S5 are available as Supplementary data at JAC Online.

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