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ORIGINAL RESEARCH

Characterization of High-Linezolid-MIC *Clostridioides difficile* Isolated from a Chinese Hospital: First Genomic Evidence of *Cfr*(B) Transmission and Tn*6218* Association

Xuyan Fu[1,*], Xiajing Bi[2,*], Tao Lv[1], Yunbo Chen[1]

[1]State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, 310003, People's Republic of China; [2]Department of Nursing, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, 310003, People's Republic of China

*These authors contributed equally to this work

Correspondence: Yunbo Chen, Email chenyunbo@aliyun.com

Background: *Clostridioides difficile* (*C. difficile*) exhibiting high linezolid minimum inhibitory concentration (>4 μg/mL) remains infrequently reported in clinical settings. Notably, the prevalence of linezolid-resistant *C. difficile* is exceptionally low (<3% in Chinese isolates), and the underlying genetic determinants are poorly characterized.

Methods: We conducted a genomic study to investigate the genetic characteristics of *C. difficil*e with high linezolid MIC. To determine the MIC of linezolid and delineate antimicrobial resistance profiles, these isolates were systematically subjected to antimicrobial susceptibility testing. Multilocus sequence typing, antimicrobial resistance genes, and the characteristics of the *cfr* gene in linezolid-resistant *C. difficil*e strains were analyzed following whole-genome sequencing. Roary was used to construct a pangenome phylogenetic tree, and a Bayesian evolutionary analysis was performed using BEAST.4.

Results: Among 421 screened *C. difficile* isolates, nine isolates (2.1%) exhibited high-linezolid MICs (≥16 μg/mL), including six ST37 (A-B+) and three ST3 strains (two A-B-). All harbored *cfr*(B) on Tn*6218*, sharing homology with *E. faecium* (NG_050395.1).

Conclusion: This study underscores the risk of *cfr*(B) dissemination via mobile genetic elements in clinical settings, urging surveillance of co-occurrence in *Enterococcus* and *C. difficile* to curb resistance spread.

Keywords: *Clostridioides difficile*, linezolid, *cfr*(B), transposon, horizontal transmission

Introduction

Linezolid is the first member of the class of oxazolidinone antibiotics and is one of the most important antimicrobial agents for infections caused by Gram-positive bacteria.[1,2] In China, linezolid has been widely used in the treatment of severe infections such as pneumonia, intra-abdominal infections, and skin infections.[3] However, several nationwide and global surveillance programs have reported very low percentages of linezolid-resistant target bacteria,[4] with reports of such resistance primarily documented in human isolates of *Staphylococcus aureus, Enterococcus faecalis*, and *Enterococcus faecium* (*E. faecium*).[5]

Resistance can arise through diverse mechanisms, including point mutations in the 23S rRNA (particularly G2576U), modifications in the genes coding for the ribosomal proteins L3 (*rplC*) and L4 (*rplD*), and the presence of mobile oxazolidinone resistance genes.[4,6] However, genes such as *cfr, optr*A, *poxt*A, and *poxt*A2 represent the emergence of a new non-mutational and transmissible mechanism of linezolid resistance, which has raised great concern. The enzyme encoded by *cfr*, rRNA methyltransferase, confers resistance to a spectrum of antibiotics classified as $PhLOPS_A$ (phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A).[7]

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Initially identified on a *Mammaliicoccus sciuri* plasmid in 2000, the *cfr* gene has since been detected in nearly 20 distinct genetic environments.[6,8] Homologs of *cfr* have been identified in *E. faecalis, Bacilus spp*., and *Enterobacteriaceae* of animal origin.[5,9] Putative *cfr*-like genes, known as *cfr*(B), *cfr*(C), *cfr*(D), and *cfr*(E), share over 50% protein sequence homology with the original *cfr* and have been identified within the Firmicutes phylum.[10–12]

Clostridioides difficile (*C. difficile*) is the primary pathogen responsible for antibiotic-associated diarrhea.[13] Antibiotic use disrupts the normal gastrointestinal flora and is one of the leading risk factors for *C. difficile* infection (CDI).[14] Although linezolid is not included in clinical guidelines for CDI treatment, it has demonstrated modest activity against the majority of clinical *C. difficile* isolates.[15–17] Some pivotal clinical trials and observations suggest that linezolid may reduce *C. difficile* toxin levels or provide a potential protective role against CDI.[15,18–20] Furthermore, the emergence of high-linezolid-MIC strains remains a concern, as it may facilitate gut colonization and the potential transfer of resistance genes to other clinically relevant pathogens, even if linezolid is not a first-line treatment for *C. difficile* infections.

However, there are no clinical resistance breakpoints for linezolid in *C. difficile* according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST, http://www.eucast.org/clinical_breakpoints/) and the Clinical and Laboratory Standards Institute (CLSI).[21] Despite this, there have been some reports on *C. difficile* with high linezolid minimum inhibitory concentration (MIC), defined as MIC > 4μg/mL.[22,23] While *C. difficile* strains with high linezolid MIC are less frequent,[24] the routine application of linezolid in clinical practice may pose a risk for CDIs. In our previous molecular epidemiology study of CDI, 1.5% of the isolates had high linezolid MIC, demonstrating varying MIC values.[17] Another study in China reported that 2.5% of *C. difficile* isolates showed high linezolid MICs.[25] However, this study did not explore the mechanisms of high-linezolid MIC in *C. difficile*.

This study aimed to investigate the genomic characterization and environment of high-linezolid MIC in clinical isolates of *C. difficile*.

Materials and Methods

Bacterial Isolates

This study was conducted at the First Affiliated Hospital, School of Medicine, Zhejiang University, a tertiary academic medical center, commencing in September 2009 ending in December 2022. The diagnosis of CDI was based on clinical indications, using culture and toxin gene assays for *C. difficile*.

Patients presenting with diarrhea and suspected CDI had unformed stool specimens collected and forwarded to the clinical microbiology laboratory. Aliquots of 0.5 mL or 0.5 g samples were homogenized with equal volumes of 95% ethanol and incubated at room temperature for 30 minutes for spore selection.[26] The selective medium used was cycloserine-cefoxitin-taurocholate agar (CCFA-TA; Oxoid) enriched with 7% sheep blood. These samples were then cultured under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) at 37 °C for 48 hours. Isolated colonies were identified as *C. difficile* using MALDI-TOF MS analysis using the Bruker Daltonics Microflex LT system (Bruker Daltonik GmbH, Bremen, Germany) using a published protocol.[17]

Antimicrobial Susceptibility

To characterize the antimicrobial susceptibility of *C. difficile* strains, the MIC of ten antibiotics was determined by agar-dilution or broth-dilution methods, including metronidazole, vancomycin, clindamycin, erythromycin, linezolid, moxifloxacin, levofloxacin, rifampicin, and tetracycline, according to CLSI guidelines.[27] Brucella agar (BBL BD, USA) with 5% fibrotic sheep blood, vitamin K (10 μg/mL), and hemin (5 μg/mL) was used for cultures. The incubation duration for antimicrobial susceptibility testing strictly followed CLSI guidelines with all *C. difficile* isolates incubated for 48 hours at 35±1°C under anaerobic conditions. The resistance breakpoint for vancomycin (>2 μg/mL) which was based on epidemiological cut-off values (ECOFFs) according to EUCAST guidelines (www.eucast.org). Due to the absence of breakpoints or ECOFFs for rifampicin in *C. difficile*, the EUCAST breakpoint for *Staphylococcus aureus* was used (>0.06 μg/mL) (www.eucast.org). *C. difficile* ATCC 700057 (ribotype 038) served as the quality control in this assessment. Throughout the entire study period (2009–2022), all bacterial culture and antimicrobial susceptibility testing procedures were maintained under strictly standardized protocols.

Genome Sequencing and Assembly

Based on the antimicrobial susceptibility results, the genomic DNA of the strains with high-linezolid MIC strains was extracted using the FastDNA® Spin Kit for Soil (MP Biomedicals, Illkirch, France) to investigate their genomic characteristics. Whole-genome sequencing was performed on the Illumina NovaSeq 6000 platform using the Rapid Plus DNA Library Prep Kit for Illumina (RK20208; Illumina, San Diego, CA, USA), achieving 200× sequencing coverage. Sequence data were processed and quality-controlled using FastQC and adapter regions were trimmed using Trimmomatic.[28]

After preprocessing, high-quality trimmed reads were assembled using the SPAdes genome assembler v.3.6 with the default parameters, with a scaffold N50 length of approximately 0.5 Mb and a guanine-cytosine (GC) content of about 28.93%.[29] However, minor fragments of putative mobile genetic elements were observed in the assembled sequences, though their exact nature requires further investigation.

To ensure a comprehensive genomic analysis, Prokka v1.124 was used for genome annotation.[30] All of the aforementioned software programs were used the default parameters for genomic data analysis. The ST, multilocus sequence typing (MLST) clade classification, and toxin genes were determined based on assembled sequences using the PubMLST sequence query page (http://pubmlst.org/cdifficile/).

Identification of Antimicrobial Resistance Genes and Transposons

Antimicrobial resistance genes were identified using the Comprehensive Antibiotic Resistance Database Resistance Gene Identifier software (https://card.mcmaster.ca/analyze/rgi). For the detection of transposons, a stringent approach was applied using the basic local alignment search tool to profile against the VRprofile2 database (https://tool2-mml.sjtu.edu.cn/VRprofile), with sequence identity and coverage thresholds set at greater than 80%.

Phylogenetic Analyses of High-Linezolid-MIC Strains

To determine the evolutionary relationships between these strains exhibiting elevated linezolid MICs, we performed pangenome analysis and reconstructed a phylogenetic tree using Roary with default parameters.[31] The Interactive Tree of Life web platform was then used to visualize the phylogenetic relationships.[32]

Single nucleotide polymorphisms (SNPs) between isolates were also analyzed to investigate the clonal characteristics of the SNP phylogeny. SNP identification and analysis were conducted using Snippy (https://github.com/tseemann/snippy) with default parameters, which facilitated mapping against the whole-genome sequences of reference strain CD630 (GenBank accession number AM180355). The minimum spanning tree was constructed in PHYLOViZ 2.0 based on a pairwise comparison of core-genome SNPs.[32]

Bayesian Evolutionary Analysis of Cfr Genes

To analyze the evolutionary relationships among different *cfr* genes from various species, Bayesian evolutionary analysis was performed using BEAST as previously described.[33,34] Each combined model was run three times for 100 million generations, with sampling performed every 10,000 states. Good convergence of chains and effective sample size values were examined using Tracer v1.7.2. TreeAnnotator v1.10.4 was used to construct an evolution tree file. Bayesian evolutionary tree was generated using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Comparative Genomic Analysis

Contigs harboring *cfr* and *cfr*-like genes were subjected to comparative analysis against the sequences in the GenBank database using the BLASTn algorithm to elucidate their genomic context. Comparative analysis of *cfr* and *cfr*-like genes was performed using Geneious Prime software (https://www.geneious.com/). Linear genomic comparison visuals were generated using Easyfig to clearly represent the genetic environments in which *cfr* resides.[35]

Results

Epidemiological Characterization of C. difficile Isolates

In total, 455 (9.1%) non-duplicate *C. difficile* isolates were identified from 5012 patients suffering from diarrhea. Among these 455 strains, 421 were successfully resuscitated and subjected to antimicrobial susceptibility testing. A total of nine strains (2.1%) with linezolid MICs of 16 μg/mL or greater were detected from nine different patients (Table 1). Within this subset of high-linezolid-MIC isolates, six were classified as sequence type 37 (ST37), exhibiting a *tcdA* gene-negative and *tcdB* gene-positive phenotype (A-B+). The remaining three isolates were categorized as ST3; of these, two lacked *tcdA* and *tcdB* genes (A-B-), while one isolate possessed both genes (A+B+).

These high-linezolid-MIC isolates were all susceptible to vancomycin and metronidazole, but most of them were resistant to fluoroquinolones and rifampicin. All ST37 strains in our study demonstrated high-level moxifloxacin resistance, consistently carrying a *gyr*A mutation at codon 82 that results in the Thr82Ile (T82I) amino acid substitution (Figure 1). One of the ST3 isolates had a MIC of 32 μg/mL for levofloxacin and carried a similar mutation at codon 82, leading to another amino acid substitution (threonine→valine). Other resistance genes, including *cat*P, *mef*H, *tet*M, *erm*B, and *erm*G, were detected in some isolates, while *aac(6')-Ie-aph(2")-Ia, cdt*A, and *clc*D (*cfr*B) were detected in all isolates (Figure 1). Furthermore, aside from *cfr*(B), no other known resistance mechanisms were identified, such as point mutations in the 23S rRNA gene (G2576U) or mutations or deletions in ribosomal proteins L3 (*rplC* gene) and L4 (*rplD* gene).

The pangenome phylogenetic tree revealed two clades: the ST3 clade and the ST37 clade (Figure 1). A minimum spanning tree was then constructed to analyze the relationship between these high-linezolid-MIC *C. difficile* isolates based on core-genome SNPs (Figure 2). Based on genetic correlation (SNP ≤ 2),[36] all isolates exhibited a divergence of greater than two SNPs from one another. This genetic heterogeneity strongly suggests that the high-linezolid-MIC *C. difficile* isolates are genetically distinct entities, precluding the presence of a clonal outbreak and transmission of linezolid-resistant *C. difficile* within our healthcare facility.

Genomic Characterization in C. difficile Isolates with High-Linezolid-MIC

To investigate the genetic basis of high-linezolid MIC, we downloaded 13 other *cfr* genes from different species for comparison (Supplementary Table 1). Compared with other species, the strains in this study shared very little homology in *cfr*(B) gene with other species, except *E. faecium* (Supplementary Figure 1).

A Bayesian evolutionary tree was used to analyze the relationship between different *cfr* genes from different species (Figure 3). *cfr*(B) genes from *C. difficile* shared a common ancestor and were closely related to the *cfr*(B) gene in *C. difficile* Ox2167 (Accession number: NG_065840.1) and *E. faecium* (Accession number: NG_050395.1). Therefore, based on the results of the above analyses, we speculate that the *cfr*(B) gene or its environment carried by *C. difficile* is homologous to that of *E. faecium*, suggesting potential transfer between these organisms.

Table 1 Characteristics and Antimicrobial Susceptibility of the Nine C. Difficile Strains in This Study

NO.	Isolated Date	*tcdA*	*tcdB*	ST	Clade	VA	CM	RI	MX	MZ	LE	EM	LZ	TC
s10040802	2010.4	+	+	3	I	0.25 (S)	32 (R)	0.06 (S)	4 (I)	0.125 (S)	32 (R)	256 (R)	16 (R)	0.06 (S)
s10110802	2010.11			3	I	0.25 (S)	32 (R)	0.06 (S)	0.25 (S)	0.125 (S)	2 (S)	256 (R)	64 (R)	0.06 (S)
s16121604	2016.12			3	I	0.125 (S)	32 (R)	32 (R)	0.25 (S)	0.125 (S)	2 (S)	256 (R)	32 (R)	8 (I)
s10061704	2010.6		+	37	4	0.5 (S)	32 (R)	32 (R)	32 (R)	0.125 (S)	32 (R)	256 (R)	32 (R)	8 (I)
s11012504	2011.1		+	37	4	0.25 (S)	32 (R)	32 (R)	32 (R)	0.25 (S)	32 (R)	8 (R)	16 (R)	32 (R)
s11060309	2011.6		+	37	4	0.125 (S)	32 (R)	32 (R)	32 (R)	0.125 (S)	32 (R)	256 (R)	16 (R)	16 (R)
s12021701	2012.2		+	37	4	0.125 (S)	32 (R)	0.06 (S)	32 (R)	0.125 (S)	32 (R)	256 (R)	16 (R)	64 (R)
s12041902	2012.4		+	37	4	0.25 (S)	32 (R)	32 (R)	32 (R)	0.25 (S)	32 (R)	16 (R)	16 (R)	32 (R)
s18091102	2018.9		+	37	4	0.125 (S)	32 (R)	32 (R)	32 (R)	0.25 (S)	32 (R)	256 (R)	16 (R)	32 (R)
700057						0.25 (S)	4 (S)	0.06 (S)	1 (S)	0.25 (S)	2 (S)	1 (S)	0.06 (S)	32 (R)

Abbreviations: VA, vancomycin; CM, clindamycin; RI, rifampicin; MX, moxifloxacin; MZ, metronidazole; LE, levofloxacin; EM, erythromycin; LZ, linezolid; TC, tetracycline; S, Susceptible; I, Intermediate; R, Resistant.

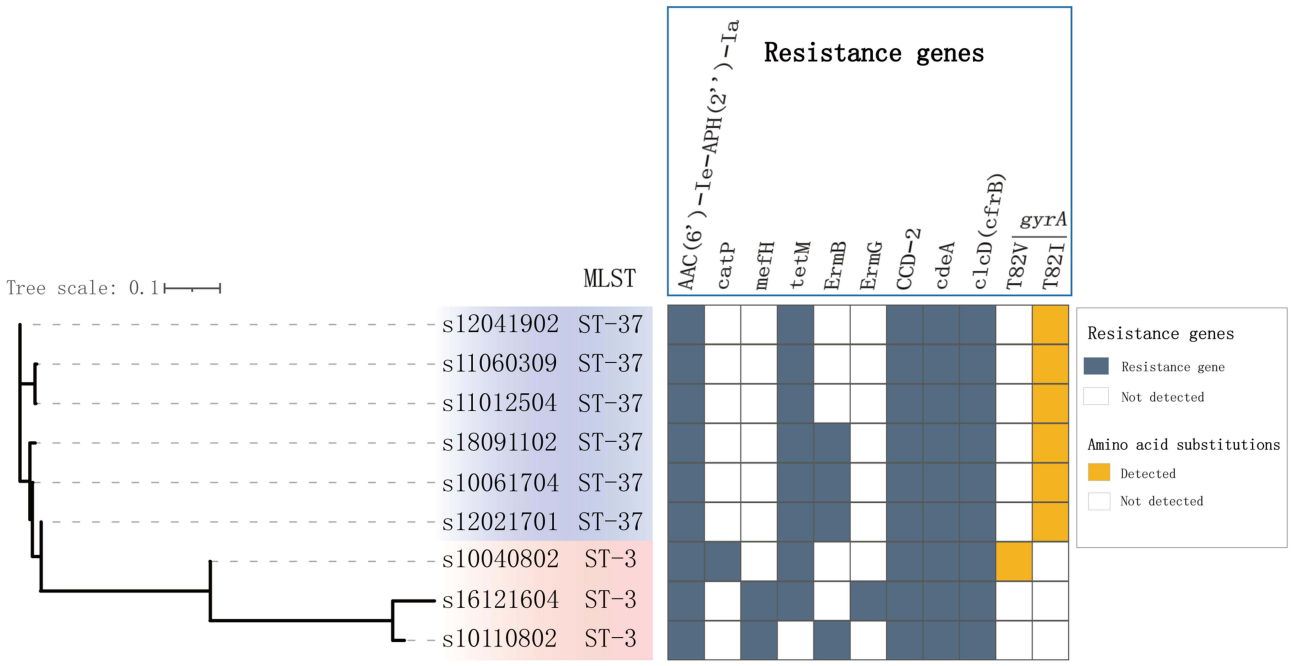

Figure 1 Pangenome phylogenetic tree and resistant genes of nine high-linezolid-MIC *C. difficile* strains.

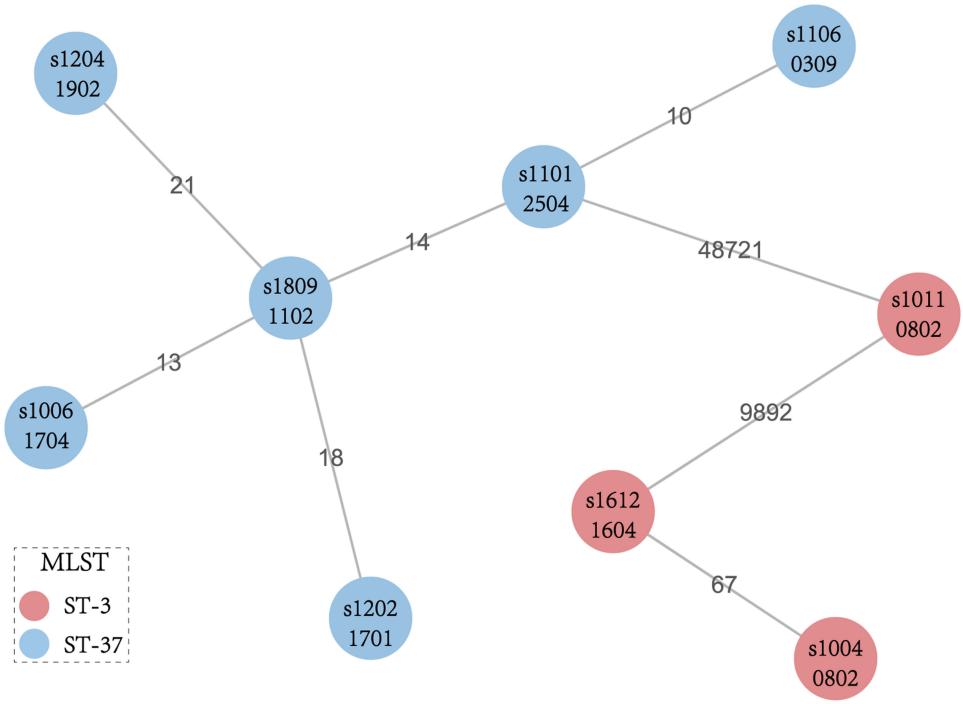

Figure 2 Minimum spanning tree of nine high-linezolid-MIC *C. difficile* strains based on SNPs.

Diversity of the Genetic Environment of Cfr(B)

Figure 4 displays the genetic environment of *cfr*(B) genes. Genome annotation and alignment showed that the *cfr*(B) gene in these nine *C. difficile* with high-linezolid-MIC genomes was located in the Tn*6218* transposon. The Tn*6218* structure included the *int* gene (transposase/integrase), the *xis* gene (excisionase), and the *rep* gene (regulatory proteins).

https://doi.org/10.2147/IDR.S523333

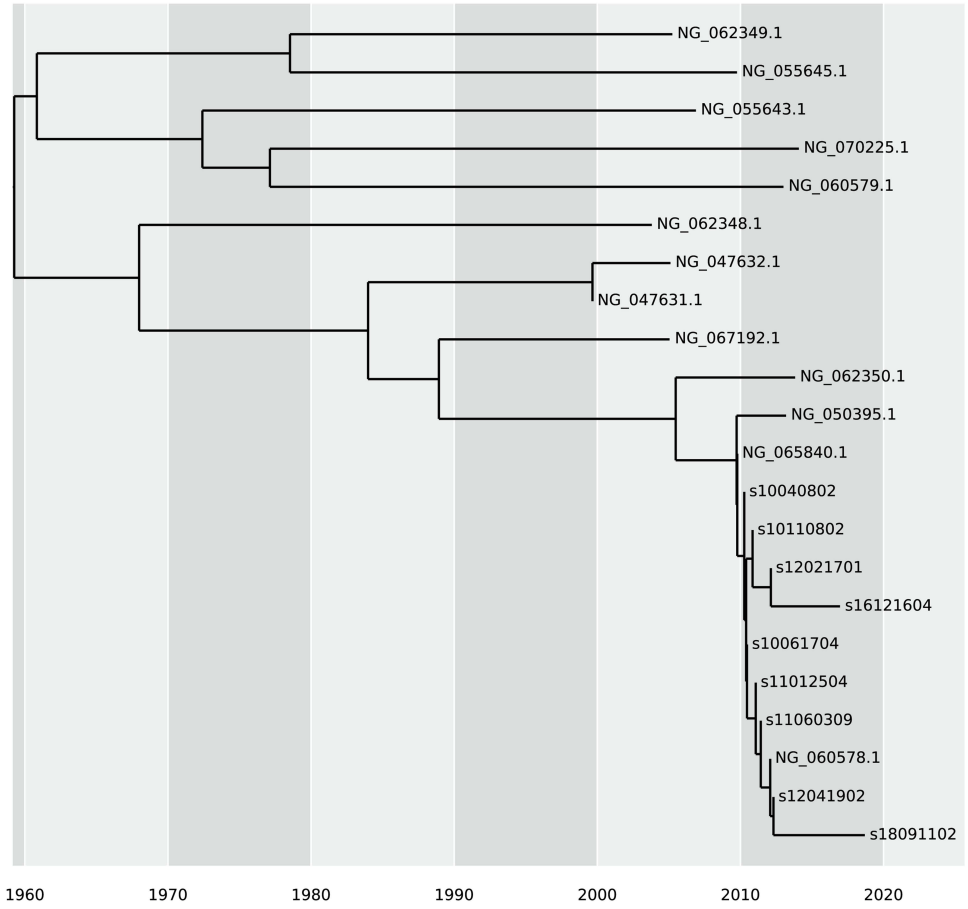

Figure 3 Bayesian evolutionary tree of *cfr* genes between studied strains and other different species.

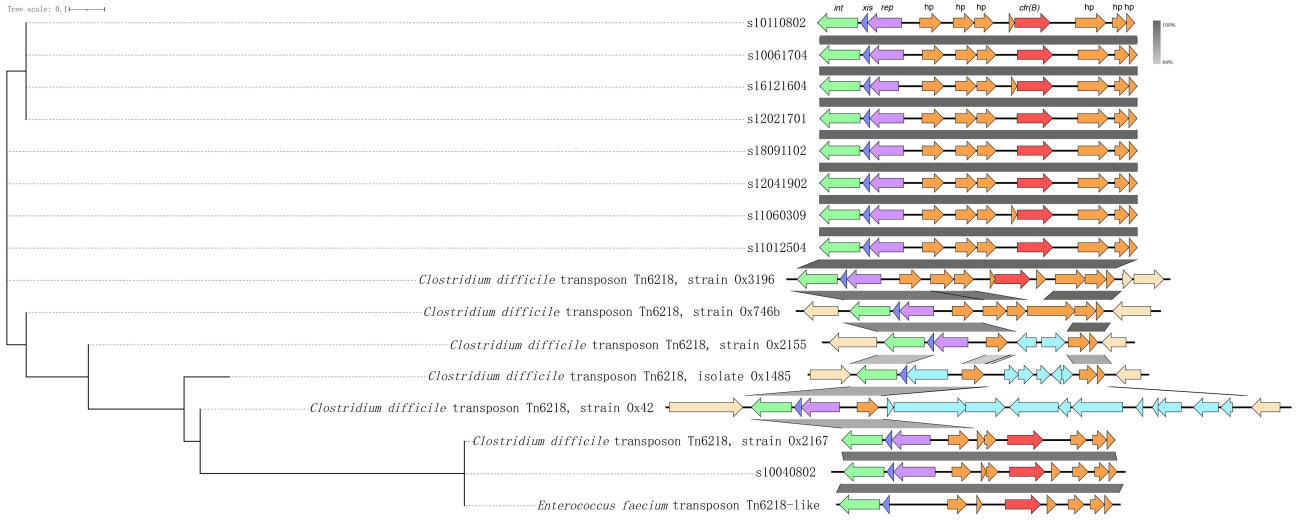

Figure 4 Genetic environment of *cfr*(B) genes in Tn*6218* between studied strains and other different species.

Additionally, the element contains a solitary open reading frame that encodes a putative efflux pump belonging to the multidrug and toxic compound extrusion family, the functional dynamics of which warrant further investigation.[37] Most Tn*6218* of the isolates in this study were similar to that of *C. difficile* Ox3196, while the s10040802 strain was similar to

C. difficile Ox2167 and *E. faecium*. These results revealed the possibility of horizontal transmission within and between different species.

Discussion

This study represents the first comprehensive characterization of clinical *C. difficile* strains exhibiting elevated linezolid MICs in one Chinese hospital, with particular emphasis on the *cfr*(B) gene and its associated transposon Tn*6218* among diverse linezolid-resistant bacterial species.[11] Our findings demonstrate that high-linezolid-MIC isolates commonly displayed multidrug-resistant phenotypes, particularly to rifampicin and fluoroquinolones. While alternative resistance mechanisms may coexist, *cfr*(B) has been established as the predominant determinant of high-linezolid MIC in Chinese clinical isolates. Importantly, our data highlight the potential for *cfr*(B) dissemination through mobile genetic elements within healthcare environments, emphasizing the critical need for enhanced surveillance of its co-occurrence in *Enterococcus* and *C. difficile* to mitigate resistance transmission.

Our previous study reported a low isolation rate of high-linezolid-MIC in *C. difficile*.[17] In contrast, rates exceeding 20% have been documented in Mexico.[22] Our results reveal substantial geographic variation in the epidemiology of high-linezolid-MIC strains, which may stem from differential distribution of sequence types across regions. This parallels observations from Mexico, where ST1 (NAP1/027) emerged as the predominant lineage among clinical isolates.[22] Therefore, a significant correlation exists between *C. difficile* STs and the specific *cfr* gene variants they carry. For instance, the *cfr*(E) gene variant was specifically identified in ST1 strains.[22] In this study, only ST3 and ST37 exhibited high-linezolid-MIC phenotypes, while the remaining 30 STs did not (data not shown). Notably, two of the three high-linezolid-MIC ST3 *C. difficile* strains were non-toxigenic. Similarly, Candela et al also identified *cfr*-like genes in the non-toxigenic strain *C. difficile*.[38] This finding raises concerns about the spread of high-linezolid-MIC non-toxigenic *C. difficile* as they are considered commensal members of the human microbiota. The identification of *cfr*(B) in non-toxigenic ST3 strains indicates that these commensals may act as silent resistance reservoirs, prompting consideration of active surveillance in high-risk clinical settings. Of particular significance is the transferability of the genes in question between *C. difficile* bacteria, which facilitate adaptive evolution, including through horizontal transfer.[39]

High-linezolid-MIC clinical isolates of *C. difficile* have been associated with *cfr* genes.[11] In this study, all high-linezolid-MIC *C. difficile* carried the *cfr*(B) gene, which contrasts with other reports where high-linezolid-MIC strains carried the *cfr*(E) or *cfr*(F) genes.[22,40] Additionally, most high-linezolid-MIC *C. difficile* in that study were reported as ST1, which differs from our findings. This may contribute to explaining the different isolation rates of high-linezolid-MIC *C. difficile* across countries. Moreover, our results showed that all high-linezolid-MIC strains were resistant to erythromycin and clindamycin, which is consistent with previous observations.[41]

The *erm*(B) gene, which is responsible for the coding of the 23S rRNA methyltransferase, has been identified as a factor contributing to the development of erythromycin and clindamycin resistance.[42] However, we found that *cfr*(B) was situated within the genetic confines of the transposon Tn*6218*, and more than half of *C. difficile* isolates with high-linezolid-MIC did not carry the *erm*(B) gene. Additionally, other studies have reported that the *cfr* gene confers resistance to several antimicrobial classes, including erythromycin and clindamycin.[5,43] Given the differential phenomenon observed in this study, additional evidence is required to support the hypothesis that c*fr* causes multidrug resistance, including resistance to erythromycin and clindamycin.

A comparative analysis of *cfr* genes from different species was conducted, revealing similarities with those found in other *C. difficile* and *E. faecium* strains, but differences from other species such as *S. aureus, B. amyloliquefaciens, Paenibacillus* sp., *C. botulinum*, and *M. sciuri*. This finding demonstrated the variability of the *cfr* genes in different species. Of particular significance, *cfr*(B) was located within Tn*6218*, which exhibits structural conservation with the *cfr*-bearing transposon originally identified in *E. faecium*. The observed parallels in resistance gene carriage between *C. difficile* and *E. faecium* isolates strongly suggest phylogenetic relatedness of either the *cfr*(B) gene or its flanking genetic context across these taxonomically distinct organisms.

This study has several limitations that should be acknowledged. First, while we screened 421 clinical *C. difficile* isolates, only nine demonstrated elevated linezolid MICs, with *cfr*(B) identified as the sole resistance determinant. This relatively low prevalence nevertheless underscores the critical need for ongoing surveillance of high-linezolid MIC in

C. difficile, particularly targeting ST1/RT027 strains in Chinese hospitals. The limited number of linezolid-resistant isolates (n=9) from a single institution may constrain our ability to identify significant epidemiological associations and could affect the generalizability of our findings regarding *cfr*(B) distribution patterns. Second, we did not screen for linezolid-resistant *Enterococcus* species (particularly *E. faecium*) in our setting, which could have provided valuable evidence to support our hypothesis regarding potential interspecies transmission of *cfr*(B) between *C. difficile* and enterococci. Finally, the single-center design of our study may not fully capture the national epidemiology of linezolid-resistant *C. difficile* strains in China, suggesting the need for multicenter investigations to obtain more representative data.

Conclusions

In this study, we provide a systematic characterization of high-linezolid-MIC *C. difficile* strains isolated in a Chinese clinical setting, highlighting the critical role of the *cfr*(B) gene and the transposon Tn*6218* in high-linezolid MIC. The presence of *cfr*(B) in clinical isolates suggests potential selection pressure from linezolid use, warranting further investigation of prescribing patterns. Furthermore, the restricted homology of *cfr*(B) to *E. faecium* highlights the need for broader sequencing to trace evolutionary origins.

Data Sharing Statement

The genomic sequences of the nine *C. difficile* isolates characterized in this study, have been submitted and are publicly accessible in GenBank under the BioProject number PRJNA432876.

Ethics Approval

This study was approved by the Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine in 3 June 2024 (No. IIT20240504A) and reached a plan after discussion. The Ethics Committee believed that the protocol did not change the patient's treatment plan, did not disclose privacy, and did not cause adverse consequences; thus, consent was waived. This study complies with the Declaration of Helsinki.

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Disclosure

The authors report no conflicts of interest in this work.

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