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ORIGINAL RESEARCH

Antimicrobial Resistance of *Clostridioides difficile* in Children from a Tertiary Pediatric Hospital in Shanghai, China

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Background: Our previous study reported a high rate of recurrence in children with *Clostridioides difficile* (*C. difficile*) infection (CDI) after conventional antibiotic therapy. Here, we aimed to explore whether metronidazole and vancomycin resistant *C. difficile* isolates are circulating in pediatric CDI.

Methods: Antimicrobial susceptibility testing (AST) using the agar dilution method according to the Clinical and Laboratory Standard Institute (CLSI) were performed on *C. difficile* isolates collected from children with CDI between 2019 and 2022 at the Shanghai Children's Hospital. Whole-genome sequencing (WGS) was performed on all *C. difficile* isolates, and the presence of antibiotic resistance genes (ARGs) were identified using Resfinder and the Comprehensive Antibiotic Resistance Database (CARD). The presence of plasmid pCD-METRO was detected using SRST2 (v0.2.0) against 8 pCD-METRO coding sequences.

Results: A total of 50 *C. difficile* isolates were collected from stools of CDI children. The overall resistance rate on all isolates was 30.00% for metronidazole, 6.00% for vancomycin, 0% for rifaximin, 2.00% for rifampin, 24.00% for meropenem, 100.00% for ceftriaxone and clindamycin, 86.00% for erythromycin, 30.0% for levofloxacin, and 50.0% for tetracycline. Multidrug-resistant (MDR) was presented in 44 isolates (88.00%). Sixteen reported potential ARGs relating with resistance to antibiotic classes of aminoglycoside (*AAC(6')-Ie-APH(2'')-Ia, aad(6), ANT(6)-Ib, APH(2'')-If, APH(3')-IIIa*), lincosamide-clindamycin-erythromycin (*ErmB, ErmQ*), fluoroquinolones (*CdeA*), glycopeptides (*vanRG*), nucleoside (*SAT-4*), tetracycline (*tetM, tetA(P), tetB(P), tetO*), and trimethoprim (*dfrF*) were identified. However, the pCD-METRO plasmid and *vanA/B* were not detected in any isolates.

Conclusion: *C. difficile* isolates from children with reduced susceptibility to metronidazole and vancomycin are emerging in pediatric CDI in China. The lack of pCD-METRO plasmid and *vanA/B* associated with reduced antibiotic susceptibility suggests there are additional mechanisms of resistance.

Keywords: *Clostridioides difficile*, antibiotic resistance, metronidazole, vancomycin, children

Introduction

Clostridioides difficile (*C. difficile*), an anaerobic, gram-positive, spore-forming bacillus, is a leading cause of healthcare-associated diarrhea in humans.[1] Clinical manifestations of *C. difficile* infection (CDI) range from asymptomatic colonization, or self-resolving diarrhea to colitis, severe pseudomembranous enterocolitis, toxic megacolon, and death.[2] The pathogenicity of *C. difficile* is mainly attributed to the two major virulence factors produced by toxigenic *C. difficile* strains, enterotoxin A (TcdA) and cytotoxin B (TcdB).[3] The development of CDI in children is major associated with an increased susceptibility of *C. difficile* colonization caused by an alteration of gut microbiota.[4] Events implicated in the gut microbiota disruption, including antibiotic

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exposure, proton pump inhibitors (PPI) use, and inflammatory bowel diseases (IBD), are recognized as the risk factors for pediatric CDI.[4] Discontinuation of antibiotics and appropriate rehydration are generally effective for symptoms resolution in children with CDI, and specific antibiotics are advised for the treatment of severe pediatric CDI, such as metronidazole, vancomycin, and fidaxomicin.[5] In addition, fecal microbiota transplantation (FMT) is recommended for the treatment of recurrent CDI (rCDI).

Multiple antibiotics resistance has been identified in *C. difficile*.[6,7] Several recent studies indicated that the susceptibility of clinical *C. difficile* isolates to metronidazole and vancomycin is also declining.[8,9] Our previous single center analysis showed that RCDI occurred in 48.53% (33/68) and 46.33% (19/41) of pediatric CDI cases initially treated with metronidazole and vancomycin, respectively.[10] Failure of antibiotics therapy implicates *C. difficile* strains with a reduced susceptibility to metronidazole and vancomycin may exist in our patients. Researches have shown that metronidazole resistance is associated with pCD-METRO,[11] heme responsive genes,[12] nitroimidazole reductase genes[8,13] and chromosomal loci.[14] For vancomycin resistance, recent evidence indicated drug-binding site alterations, efflux pumps, RNA polymerase mutations, plasmid acquisition and biofilm formation may play important roles.[15–17]

Given the high occurrence of RCDI post-antibiotic treatment in our center,[10] we aimed to explore whether metronidazole and vancomycin resistant *C. difficile* isolates are circulating in pediatric CDI by performing AST and WGS. Here, we show that the emergence of *C. difficile* isolates with reduced susceptibility to metronidazole and vancomycin may be associated with the failure of antibiotic therapy in CDI children.

Methods

Clinical *C. difficile* Isolates

Clinical CDI is defined as patients with three or more liquid stools (Bristol 6–7) per day, and either a positive stool test for CD toxins or a PCR detection of toxigenic CD, or colonoscopic findings revealing pseudomembranous colitis according to the 2017 update clinical practice guidelines for CDI in children.[2] Stool samples were collected from pediatric patients with confirmed CDI at the Shanghai Children's Hospital from 2019 to 2022. About 1 mL (1 g) of stool was mixed with same volume of 95% ethanol for 30 min at room temperature for spore selection. The supernatant was discarded after centrifugation at 2500 g for 5 min. The lower sediment was serially diluted and plated on selective taurocholate cycloserine-cefoxitin fructose agar (CCFA, Oxoid, UK) plates supplemented with sheep blood (7%). Plates were placed in an anaerobic workstation (ELECTROTEK AW500 SG, UK) incubated for 48 h, at 37°C. Colony was picked based on typical morphology of *C. difficile* (yellowish/white with a ground-glass appearance, and has a horse-like odor). *C. difficile* isolates were further confirmed by PCR and Sanger sequencing of the housekeeping gene *tpi* and the 16S rRNA gene. Confirmed *C. difficile* colony was re-cultured on 5% Columbia blood agar twice to obtain pure *C. difficile* isolates. Each pure isolate was stored at −80°C in brain heart infusion (BHI) broth supplemented with 0.5% yeast extract (YBHI, Oxoid, UK) containing 50% glycerol.

This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013), and was approved by the Ethical Review Board of the Shanghai Children's Hospital (2022R043-E01), and informed consent was taken from parent or legal guardian of all the participates.

Antimicrobial Susceptibility Testing

All *C. difficile* isolates were tested for susceptibility using the agar dilution method according to the Clinical and Laboratory Standard Institute (CLSI) for the following 10 antibiotics: metronidazole, vancomycin, rifaximin, rifampin, ceftriaxone, clindamycin, meropenem, erythromycin, levofloxacin, and tetracycline.[18] All AST were performed on *Brucella* agar plates supplement with 1 mg/L of vitamin K, 5 mg/L of hemin, and 5.0% sheep blood at 37°C for 48 h under anaerobic condition in duplicate from independent cultures for 3 times. *C. difficile* ATCC 43255 strain was used as quality control and was included in each AST test. Antimicrobial susceptibility was mostly interpreted according to the minimal inhibitory concentrations (MIC) breakpoints of CLSI[18] and European Committee on Antimicrobial Susceptibility Testing (EUCAST).[19] Multidrug-resistant (MDR) was defined as an isolate with resistance to three or more classes of antibiotics.[18] All AST data were verified by three different people independently.

Whole-Genome Sequencing

All clinical *C. difficile* isolates were recovered on CCFA agar plates. Single colony was picked and grown in YBHI broth for 16 h at 37°C anaerobically. Genomic DNA was extracted from the bacteria pellet using the Wizard® Genomic DNA Purification Kit (Promega, USA). High quality genomic DNA (at least 1 μg) was used for generating libraries with ~400 bp insert sizes for paired-end sequencing using TruePrep DNA Library PrepTM Kit V2 (Illumina, USA). Whole-genome sequencing (WGS) was performed using the Illumina NovaSeq 6000 platform (Illumina, USA) with 150-base paired-end reads according to the standard protocol (Shanghai Winnerbio Technology Co., Ltd., China). Raw reads were processed and quality controlled by fastp (V0.22.0) and remove sequencing adapters and low-quality reads (reads containing more than 3 unknown bases, more than 50% of the bases scored below Q20, and read length less than 30 bp). Genome assembly was performed using SPAdes (V3.15.3). CDS was predicted using Glimmer (V3.02). Isolates with evidence of contamination were excluded. Sequence type (ST) and clade were determined by alignment of assembled scaffold to *C. difficile* PubMLST.[20] Prediction of non-redundant coding sequence (CDS) was performed using Glimmer (V3.02). Roary (v.3.12.0) was used for the identification of core genome.[21] The phylogenetic tree was first generated by a built-in Species Tree inference from all genes (STAG) algorithm in OrthoFinder based on FastTree. FastTree infers approximately maximum-likelihood (ML) phylogenetic trees from alignments with up to hundreds of thousands of sequences.[22] Beautification and visualized of the tree were performed using iTOL.[23] The toxin genes including *tcdA, tcdB, cdtA and cdtB* were detected using Virulence Factors of Pathogenic Bacteria database (VFDB).[24]

Antibiotic Resistance Genes Analysis

Antibiotic resistance genes (ARGs) in the obtained *C. difficile* genome were identified using Resfinder (v4.1)[25] and Comprehensive Antibiotic Resistance Database (CARD 2.0.0) databases with cutoff values of 70% nucleotide identity and 90% coverage of the gene query length.[26] Reads were compared using SRST2 (v0.2.0) against 8 pCD-METRO (GenBank accession: CAADHH010000057) coding sequences to find the presence of plasmid pCD-METRO.[27]

Results

Clinical *C. difficile* Isolates and Patient Characteristics

A total of 50 clinical *C. difficile* isolates were obtained from stools collected from 50 unique pediatric CDI patients enrolled in our center during 2019 to 2022. The characteristics of patients with positive *C. difficile* cultures are shown in Table 1. The median age of the 50 CDI children was 5 years (interquartile range (IQR), 2.50–9.30 y). Thirty-six cases (72.00%) were community-acquired CDI and 13 (28.00%) patients were hospital-acquired CDI. Exposure history included previous antibiotics exposure (29/50, 58.00%) and PPI use (8/50, 16.00%). The main clinical symptoms were diarrhea (29/50, 58.00%), and hematochezia (16/50, 32.00%). For the initial specific antibiotic treatment, 18 (36.00%) patients received metronidazole and 15 (32.00%) patients received vancomycin. After following up for 8 weeks, the total sustained clinical remission rate of the first round of metronidazole and vancomycin therapy was 77.78% and 81.25%, respectively.

Antimicrobial Susceptibility Testing

Susceptibility tests of 10 antibiotics using the agar dilution method were performed for all 50 clinical *C. difficile* isolates according to the CLSI standard performed in the microbiology laboratory of Department of Clinical Laboratory, Shanghai Children's Hospital. As shown in Table 2, the metronidazole MIC_{50} was 0.25 μg/mL, and MIC_{90} was 128 μg/mL; the vancomycin MIC_{50} was 1 μg/mL, and MIC_{90} was 8 μg/mL; the rifaximin MIC_{50} was <0.064 μg/mL, and MIC_{90} was 2 μg/mL; the rifampin MIC_{50} was <0.064 μg/mL, and MIC_{90} was 8 μg/mL; the ceftriaxone, clindamycin, and erythromycin MIC_{50} was >128 μg/mL, and MIC_{90} was >128 μg/mL; the levofloxacin MIC_{50} was 4 μg/mL, and MIC_{90} was 32 μg/mL; the meropenem MIC_{50} was 8 μg/mL, and MIC_{90} was 32 μg/mL; the tetracycline MIC_{50} was 8 μg/mL, and MIC_{90} was 64 μg/mL for all isolates. The overall resistance rate was 30.00% for metronidazole, 6.00% for vancomycin, 0% for rifaximin, 2.00% for rifampin, 24.00% for meropenem, 100.00% for ceftriaxone and clindamycin, 86.00% for

Table 1 Characteristics of the Pediatric Patients with *C. Difficile* Infection

Characteristics	Overall (N = 50)
Age (year, median, IQR)	5 (2.50, 9.30)
Sex, male, n (%)	25 (50.00)
Community-acquired CDI, n (%)	36 (72.00)
Hospital-acquired CDI, n (%)	14 (28.00)
Outpatients, n (%)	8 (16.00)
Inpatients, n (%)	42 (84.00)
Exposure history, n (%)	
Antibiotics	29 (58.00)
PPI	8 (16.00)
Prior CDI episode, n (%)	
Yes	14 (28.00)
Comorbidity, n (%)	
IBD	7 (14.00)
Immunodeficiency	3 (6.00)
Symptoms, n (%)	
Diarrhea	29 (58.00)
Hematochezia	16 (32.00)
Fever	14 (28.00)
Abdominal pain	13 (26.00)
Vomit	11 (22.00)
Pseudomembrane	4 (8.00)
Treatment and response, n (%)	
Metronidazole	18 (36.00)
Response	14 (77.78)
Failure	4 (22.22)
Vancomycin	16 (32.00)
Response	13 (81.25)
Failure	3 (18.75)

Abbreviations: IQR, interquartile range; PPI, proton pump inhibitor; CDI, *C. difficile* infection; IBD, inflammatory bowel disease.

erythromycin, 30.00% for levofloxacin, and 50.00% for tetracycline (Table 2). MDR was presented in 44 isolates (88.00%) according to CLSI. AST data for all *C. difficile* isolates were summarized in Table S1.

Whole-Genome Sequencing

All 50 clinical *C. difficile* isolates underwent WGS. As shown in Table S2, clean data of86950Mbp with an average of 1739 Mbp (range, 743–7043 Mbp) were obtained after the quality control process. The average read coverage was 414× (range, 177–1607×) across the assembly genome (about 4.2 Mbp). *De novo* assembly of the reads generated an average scaffold of 87 (range, 28–994), and the average N50 was207182bp (range, 8937–448 661). The average G + C content was 28.60% (range, 28.10–29.30%). A total of191036CDSs (average, 3820; range, 3573–4138) were identified, out of which 2950 CDSs were identified as core genes (genes present in 100.00% of the 50 genomes) by pan-genome analysis, accounting 77.20% of the average number of CDS per genome (3820 CDS). Multi-locus sequence typing (MLST) analysis identified 19 STs, including ST2, ST3, ST8, ST15, ST26, ST33, ST35, ST37, ST39, ST42, ST43, ST44, ST51, ST54, ST69, ST81, ST102, ST103, and ST129. The phylogenetic tree generated by a STAG algorithm in OrthoFinder separated our clinical pediatric *C. difficile* isolates into 2 of the 5 *C. difficile* clades, and the majority of the isolates belongs to clade 1 (45/50, 90.00%, Figure 1). For toxin gene identification, 31 (62.00%) isolates carried *tcdA, tcdB, cdtA* and *cdtB*, 9 (18.00%) isolates carried *tcdA* and *tcdB*, and 10 (20.00%) isolates did not harbor neither *tcdA, tcdB* nor *cdtA, cdtB* (Figure 1). Antibiotic resistance rates of each antibiotic

Table 2 Summary of Antimicrobial Susceptibility Testing Data for *C. Difficile* Isolates (N = 50)

Antimicrobial Agent[a]	MIC Range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	Breakpoints (μg/mL)[b]			Susceptible		Intermediate		Resistant	
				Susceptible	Intermediate	Resistant	N	%	N	%	N	%
Metronidazole	≤0.064–>128	0.25	128	≤8	16	≥32	35	70	0	0	15	30
Vancomycin	≤0.064–>128	1	8	≤2	4	≥8	46	92	1	2	3	6
Rifaximin	≤0.064–4	<0.064	2	–	–	≥8	50	100	–	–	0	0
Rifampin	≤0.064–8	<0.064	8	–	–	≥8	49	98	–	–	1	2
Meropenem	4.0–16.0	8	32	≤4	8	≥16	24	48	14	28	12	24
Ceftriaxone	>128	>128	>128	≤16	32	≥64	0	0	0	0	50	100
Clindamycin	16–>128	>128	>128	≤2	4	≥8	0	0	0	0	50	100
Erythromycin	0.5–>128	>128	>128	–	–	≥8	7	14	–	–	43	86
Levofloxacin	1–>128	4	32	–	–	≥8	16	32	–	–	15	30
Tetracycline	≤0.064–>128	8	64	≤4	8	≥16	20	40	5	10	25	50

Notes: [a]Tested drug ranges: 0.064–128 μg/mL. [b]Breakpoints mostly according to CLSI and EUCAST.
Abbreviation: MIC, minimum inhibitory concentration.

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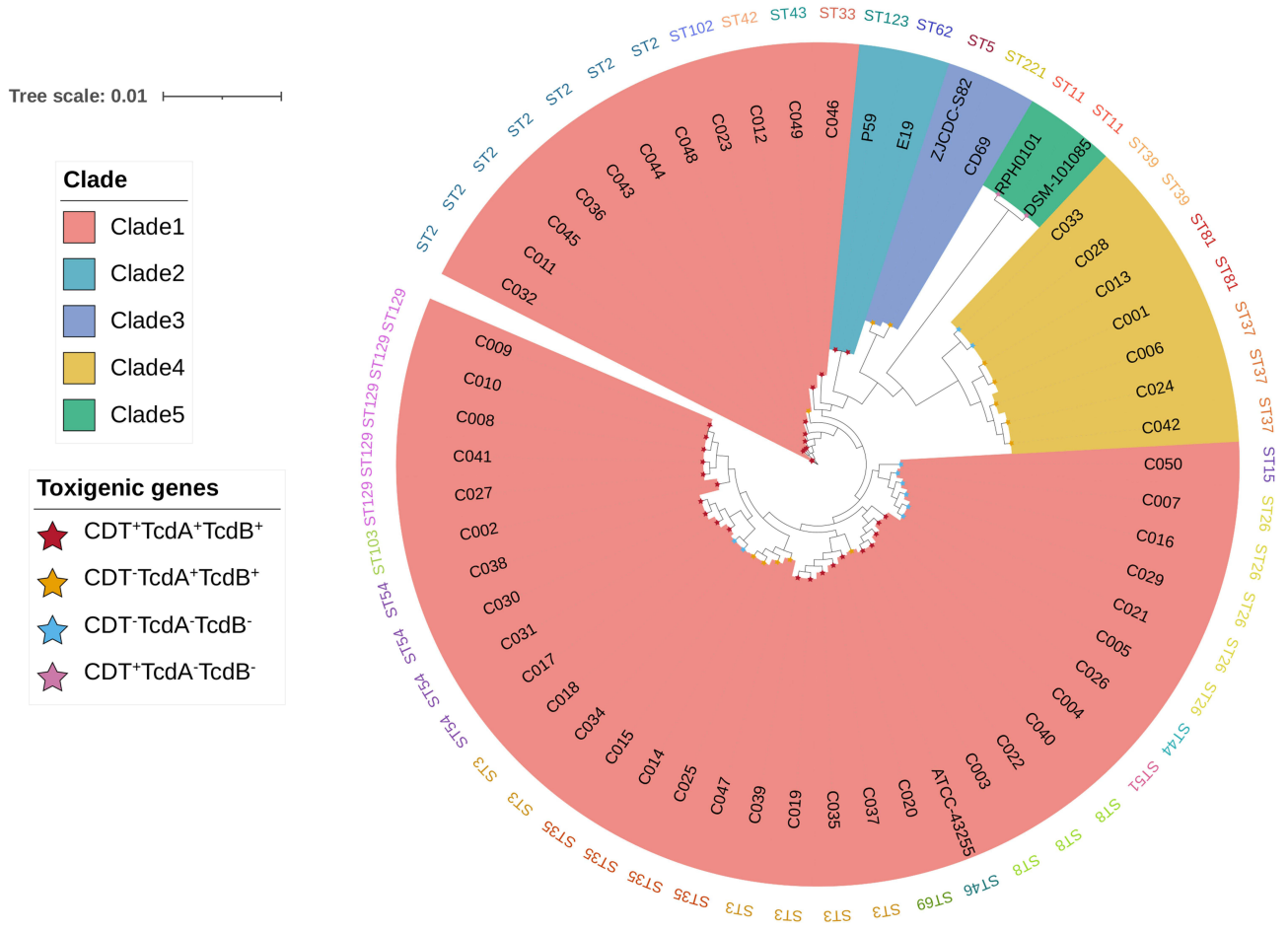

Figure 1 The phylogenetic tree of clinical *C. difficile* isolates collected from children with CDI. The phylogenetic tree was generated by a STAG algorithm in OrthoFinder including 50 genomes sequenced in this study and 7 reference genomes. STs and clades are labeled on the outer circle. The background shading represents the MLST clades. Different toxin profiles are indicated as colored stars at the start of the branches.

Abbreviations: CDI, *C. difficile* infection; STAG, Species Tree inference from all genes; ST, sequence type; MLST, multi-locus sequence typing; CARD, Comprehensive Antibiotic Resistance Database.

based on in vitro MIC data across STs and corresponding clades were shown in Figure 2A. Most of the isolates with metronidazole and vancomycin resistances belong to clade 1.

Antibiotic Resistance Genes

A total of 16 ARGs were identified according to the CARD database, which relating resistance to antibiotic classes of aminoglycoside, erythromycin, fluoroquinolone, glycopeptide, lincosamide, nucleoside, tetracycline, and trimethoprim (Table S3). Multiple ARGs conferred resistance to antibiotic aminoglycoside class were detected, including *AAC(6')-Ie-APH(2'')-Ia, aad(6), ANT(6)-Ib, APH(2'')-If*, and *APH(3')-IIIa*. *ErmB* conferred resistance to macrolide-lincosamide-streptogramin B (MLSB) class of antibiotics was detected in 35 isolates (70.00%) with both erythromycin and clindamycin resistance. In addition, all 5 isolates of ST129 belong to clade 1 with both erythromycin and clindamycin resistance carried *ErmQ*. *CdeA* relating resistance to fluoroquinolone class was detected in all 50 isolates. *VanRG* associated with acquired vancomycin resistance was detected in 43 isolates (86.00%), including 3 isolates with resistance to vancomycin. Several ARGs of tetracycline resistance were identified in isolates with resistance to tetracycline, including *tetM, tetA(P), tetB(P)*, and *tetO* in 23 (46.00%), 7 (14.00%), 7 (14.00%), and 1(2.00%) isolates, respectively. Seven isolates with resistance to tetracycline carried both *tetA(P)* and *tetB(P)*. The pCD-METRO plasmid associated with metronidazole resistance, and ARGs related to cephalosporin resistance were not identified in any isolates. The distribution of ARGs in different clades of clinical *C. difficile* isolates was presented in Figure 2B.

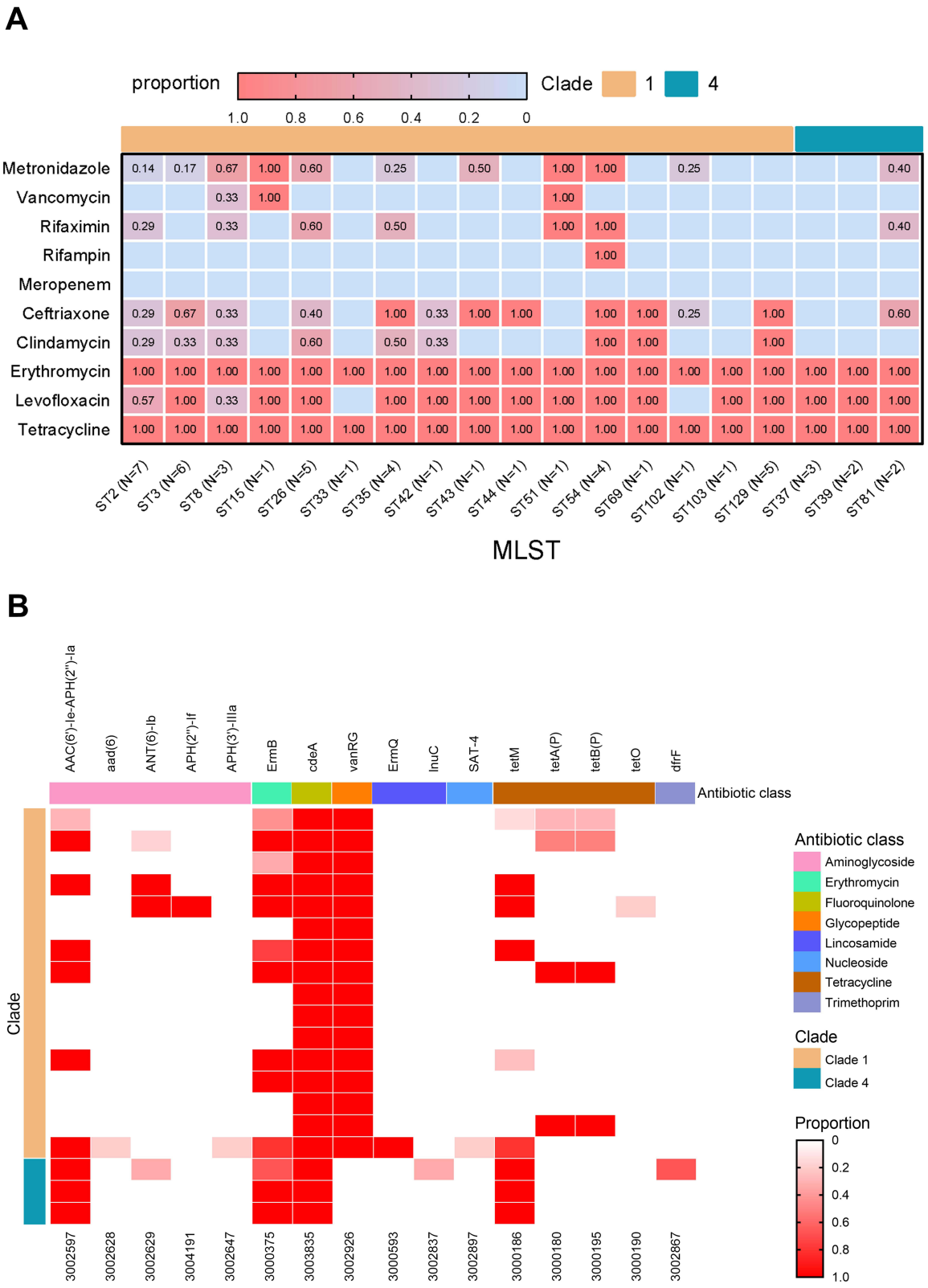

Figure 2 Resistance rates based on in vitro MIC data and distribution of in silico predicted ARGs. (**A**) Antibiotic resistance rate across ST and MLST clades. The heatmaps present proportions of antibiotic resistant samples for each antibiotic from top to bottom against MLST and color-coded according to clade. The number is the proportion of antibiotic resistance rate. (**B**) The presence of ARGs identified using the CARD indicated with a red line. The ARGs are implied by gene name (top) and Antibiotic Resistance Ontology accession number (bottom), and the relevant antibiotic class and clade are separately showed by the color-coded X and Y axes. The heatmaps represent the proportions of antibiotic resistant isolates.

Abbreviations: MIC, minimum inhibitory concentration; ARGs, antibiotic resistance genes; ST, sequence type; MLST, multi-locus sequence typing; CARD, Comprehensive Antibiotic Resistance Database.

Discussion

A few antibiotics are conventionally recommended as the treatment for CDI, such as metronidazole, vancomycin, and fidaxomicin.[2] Treatment failure of oral metronidazole is associated with its low stool concentration and interaction with gut microbiota. *C. difficile* strain can easily overcome this insufficient concentration by higher MIC. Studies showed that an increasing MIC of metronidazole in several epidemic *C. difficile* ribotypes lead to a high rate of treatment failure.[28] In addition, the emergence of plasmid-mediated resistance to metronidazole further increases the risks of treatment failure.[28] Although oral vancomycin can quickly reduce *C. difficile* shedding through reaching high concentration in the stool, several mechanisms of vancomycin resistance in *C. difficile* were identified, include drug-binding site alterations, efflux pumps, RNA polymerase mutations, and biofilm formation.[16] Fidaxomicin has the lowest rate of recurrence of CDI, which might be associated with its narrow spectrum of antimicrobial activity and persistence on spores.[28] A high recurrence rate of CDI has been reported (5–50%) previously, and it can be even higher (40–75%) after the first recurrence.[1,29,30] RCDI can further aggravate the burden both to patients and healthcare system by causing increased morbidity and mortality of CDI.[31,32] Multiple factors are involved in the occurrence of RCDI, such as gut microbiota dysbiosis, continued *C. difficile* exposure, an incomplete host immune response, and reduced susceptibility to antibiotics.[33]

In this study, we showed that *C. difficile* isolates with reduced susceptibility to both metronidazole and vancomycin were emerging in the pediatric patients at a tertiary pediatric hospital in Shanghai, China. AST performed in clinical *C. difficile* isolates from 50 pediatric CDI patients indicated that 15 (30.00%) harbored *C. difficile* isolates with resistance to metronidazole, and 3 (6.00%) of them also resistant to vancomycin. The resistance rates of metronidazole and vancomycin were significantly higher in comparison with recent reports conducted in China,[34,35] as well as other reports from worldwide[7,36] suggesting a low resistance rate to metronidazole and vancomycin. Another adult study in China found that the resistance rate to metronidazole was 23.1%, which was similar with our study.[37] A recent systematic review and meta-analysis included a total of 111 studies published between 1992 and 2019 revealed that the weighted pooled resistance (WPR) for metronidazole and vancomycin was 1.0% and 1.0% for the breakpoint > 2 μg/mL and 0% for breakpoint ≥ 32 μg/mL.[7] Gargis et al showed that among 593 clinical *C. difficile* isolates, 98.50% and 97.30% were sensitive to vancomycin (MIC ≤ 2 μg/mL) and metronidazole (MIC ≤ 2 μg/mL), respectively.[36] In contrast, several studies reported high rates of clinical *C. difficile* isolates with decreased susceptibility to metronidazole and vancomycin.[8,9] For example, Darkoh et al showed that 29% (128/438) and 26% (114/438) of Houston diarrheal stool samples (114/438), 85% (83/98) and 67% (66/98) of Nairobi diarrheal stool samples harbored metronidazole and vancomycin non-susceptible *C. difficile* isolates, respectively.[9] However, Greentree et al did not yield vancomycin non-susceptible *C. difficile* isolates by screening of 176 CDI stool specimens in a Cleveland-area hospital, which highlighting the potential for false-positive results due to contamination with vancomycin-resistant enterococci.[38] The overall reduced susceptibility/resistance rates of 1501 clinical *C. difficile* isolates from MODIFY I and II studies were 14.5% for metronidazole, and 32.8% for vancomycin.[8] For other tested antibiotics conducted in this study, high resistant rates were observed in ceftriaxone and clindamycin (100%), erythromycin (85.7%), levofloxacin (30.6%), meropenem (24.5%), and tetracycline (50.0%). No rifaximin resistant *C. difficile* isolate was observed, which is different with a previously study showing a high reduced susceptibility/resistance rates to rifaximin (99.5%) in adult patients.[8] It may be associated with less usage of rifaximin in children in China. Taken together, differences in antibiotics resistance of clinical *C. difficile* isolates were reported, which can likely be explained by studies conducted in different location, different populations (age, race, etc.), antibiotic exposure history, or specific *C. difficile* STs. AST data need independent verification and need to be split out per isolate in relation to appropriate reference strains, particularly the doubly reduced susceptible strains of metronidazole and vancomycin. Given the majority of the reports were focus on the adults CDI patients, studies in pediatric CDI are needed to be conducted in the future.

In order to characterize the genetic determinants of the antibiotic resistance, we performed WGS in all 50 clinical *C. difficile* isolates. A total of 19 STs belong to clades 1 and 4 were identified based on the MLST analysis. Reduced susceptibility to metronidazole was more likely among the isolates belong to clade 1, as well as reduced susceptibility to vancomycin. The three STs with the highest metronidazole and vancomycin resistance rates were ST15, ST43, and ST18. Identifications of known genetic determinants conferred antibiotics resistance by CARD observed 16 ARGs among all 50 isolates. However, the pCD-METRO plasmid associated with metronidazole resistance[11] was not identified in our genome

collection, implicating other additional genetics determinants may be involved in the reduced susceptibility to metronidazole. The *van* operons, such as *vanA, vanB, vanC, vanD, vanE, vanG, vanL, vanM* and *vanN*, are recognized as the genetic basis of acquired vancomycin resistance in *Enterococci*.[39] In our *C. difficile* genome collection, a high carry rate (42/50, 84.00%) of *vanRG*, a *vanR* variant in the *vanG* gene cluster were observed. The *vanG* operons correspond to a low-level of vancomycin resistance,[39] which can explain the low reduced susceptibility to vancomycin in this study. Furthermore, the observations of ARGs mediating resistance to antibiotic classes of the lincosamide-clindamycin-erythromycin (*ErmB, ErmQ*),[40] and tetracycline (*tetM, tetA(P), tetB(P*), and *tetO)*[41] in our genome collection may be responsible for the high antibiotic resistance rates to erythromycin and clindamycin, and tetracycline, respectively. Several determinates were reported to be associated with fluoroquinolone resistance, such as *CdeA*, mutations in *gyrA*, and *gyrB*.[42,43] Our results showed that *CdeA* was detected in both resistance and susceptible strains to levofloxacin, which implicating the mutations in *gyrA*, and *gyrB* are more likely the cause of the resistance. However, *gyrA*, and *gyrB* were not detected in the genome collection of *C. difficile* strains isolated from our pediatric patients. Taken together, further studies are warranted to investigate the additional determinates associated with drug resistance of *C. difficile*.

The current study has several limitations. First, this is a single-center study with a small cohort. Studies with large number of clinical *C. difficile* isolates form pediatric patients are further needed. Second, we collected only one colony from each sample for AST and WGS, other *C. difficile* strains might be missed in patients with mixed infections. Particularly, toxigenic (TCD) strain that cause the disease were most likely missed in case with a non-toxigenic (NTCD) isolate. Third, fidaxomicin was not included in the AST because the antibiotic was not available at the time of testing. It is important to evaluate the susceptibility to fidaxomicin in clinical *C. difficile* isolates from pediatric patients to guide the future treatment choice. Finally, the clinical implications of the *C. difficile* isolates with reduced susceptibility to metronidazole and vancomycin are not determined due to the inconsistence between the antibiotic resistance and treatment outcome.

In summary, our results indicate that clinical *C. difficile* isolates with reduced susceptibility to metronidazole and vancomycin are emerging in a group of pediatric patients, which may explain a decline effectiveness of antibiotic-based therapy. The lack of pCD-METRO plasmid and *vanA/B* consistently associated with reduced susceptibility to metronidazole and vancomycin suggests additional mechanisms of resistance. Studies of susceptibility for fidaxomicin are necessary to guide its use for future therapy in pediatric CDI.

Abbreviations

CDI, *C. difficile* infection; RCDI, recurrent CDI; CDT, binary toxin; TcdA, enterotoxin A; TcdB, cytotoxin B; IBD, inflammatory bowel diseases; ARGs, antibiotic resistance genes; CARD, Comprehensive Antibiotic Resistance Database; AST, antimicrobial susceptibility testing; WGS, whole-genome sequencing; CCFA, cycloserine-cefoxitin fructose agar; MIC, minimal inhibitory concentrations; CDS, coding sequence; BHI, brain heart infusion; CLSI, Clinical and Laboratory Standard Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing; MDR, multi-drug-resistant; ST, sequence type; MLST, multi-locus sequence typing.

Data Sharing Statement

Raw sequencing data have been deposited in NCBI SRA database under BioProject number PRJNA1047048.

Ethical Considerations

This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013), and was approved by the Ethical Review Board of Shanghai Children's Hospital [2022R043-E01], and informed consent was taken from parent or legal guardian of all the participates.

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Disclosure

No potential conflict of interest was reported by the author(s).

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