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Molecular epidemiology and antimicrobial susceptibility of human *Clostridium difficile* isolates from a single institution in Northern China

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Abstract

Because the epidemiology of *Clostridium difficile* infection (CDI) is region-specific, the present study was undertaken to examine the epidemiology of *C difficile* outbreaks in Beijing, China.

Eighty nonduplicate isolates were collected from March, 2016 to December, 2016. The molecular type and phylogenetic analysis were evaluated by multilocus sequence typing (MLST). The minimum inhibitory concentrations (MICs) for 11 antibiotics and the resistance mechanisms were investigated.

Sixty-five toxigenic strains (81.25%), including 22 $tcdA^-B^+CDT^-$ strains (27.5%) and 43 $tcdA^+B^+CDT^-$ strains (53.75%), and also 15 nontoxigenic strains ($tcdA^-B^-CDT^-$; 18.75%) were detected. MLST identified 21 different sequence types (STs), including 2 novel types (ST409 and ST416). All isolates were susceptible to metronidazole, vancomycin, fidaxomicin, piperacillin/tazobactam, and meropenem, and all were effectively inhibited by emodin (MICs 4–8 μg/mL). The resistance rates to rifaximin, ceftriaxone, clindamycin, erythromycin, and ciprofloxacin were 8.75%, 51.25%, 96.25%, 81.25%, and 96.25%, respectively; 81.25% (65/80) of isolates were multidrug-resistant. Amino acid mutations in *GyrA* and/or *GyrB* conferred quinolone resistance. One novel amino acid substitution, F86Y in *GyrA,* was found in 1 CIP-intermediate strain. The *erm(B)* gene played a key role in mediating macrolide-lincosamide-streptogramin B (MLSB) resistance. *Erm(G)* was also found in *erm(B)*-negative strains that were resistant to both erythromycin and clindamycin. *RpoB* mutations were associated with rifampin resistance, and 2 new amino mutations were identified in 1 intermediate strain (E573A and E603N).

Regional diversity and gene heterogeneity exist in both the ST type and resistant patterns of clinical *C difficile* isolates in Northern China.

Abbreviations: CDI = *Clostridium difficile* infection, CLSI = Clinical and Laboratory Standards Institute (CLSI), MIC = minimum inhibitory concentration, MLSB = macrolide-lincosamide-streptogramin B, MLST = multilocus sequence typing, PBPs = penicillin-binding proteins, PCR = polymerase chain reaction, ST = sequence type.

Keywords: antibiotic resistance, Beijing, *Clostridium difficile*, epidemiology

1. Introduction

Clostridium difficile is a Gram-positive, spore-forming anaerobic bacillus that causes a range of gastrointestinal syndromes, from mild diarrhea to severe pseudomembranous colitis, lethal toxic megacolon, and sepsis.[1] Due, in part, to the emergence and subsequent spread of the hypervirulent NAP1/BI/027 strain,[1]*C difficile* infection (CDI) has become a major cause of healthcare antibiotic-associated diarrhea, accounting for up to 20% of cases.[2,3] Treatment with antimicrobials, including the macrolide-lincosamide-streptogramin B (MLSB) group of antibiotics (eg, clindamycin and erythromycin), fluoroquinolones, and cephalosporins, is a high risk factor for CDI.[4,5]

The major virulence factors of *C difficile* include enterotoxin A (TcdA) and cytotoxin B (TcdB).[6] On the basis of the *tcdA* and or *tcdB* genes located within the pathogenicity locus (PaLoc) and potentially an additional binary toxin, clinical isolates are classified into several types. Most isolates are positive for both tcdA and tcdB ($tcdA^+B^+$); the toxin variant isolates are only positive for tcdB ($tcdA^-B^+$). Nontoxigenic strains ($tcdA^-B^-$) are also isolated from clinical patients. Moreover, the frequency of hypervirulent strains, known as North American pulsed field electrophoresis type 1 or ribotype027 (NAP1/027) encoding *cdtA* and *cdtB*, has increased in recent years, resulting in nosocomial outbreaks in North America, Canada, and Europe.[7,8]

Analysis of antimicrobial susceptibility and mechanisms of resistance are required for surveillance of the emergence and distribution of C *difficile*, and guiding public health measures. In addition to the classical antimicrobials used for CDI, emodin can inhibit the growth of anaerobic bacteria, including Propoinibacterium, Eubacterium, and Clostridium[9]; however, few isolates were analyzed. Therefore, investigating the minimum inhibitory concentrations (MICs) of 11 antimicrobials for C *difficile* strains and the related mechanism of resistance is important to effectively understand, prevent, and control CDI outbreaks.

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Molecular epidemiology analyses of *C difficile* are crucial for identifying and controlling outbreaks of CDI. Using multilocus sequence typing (MLST) analysis, a nucleotide sequence-based characterization of allelic polymorphisms in 7 housekeeping genes was used to analyze the evolutionary genetics and global epidemiology of *C difficile*.[10] Similar analyses in China indicate that CDI epidemiology is region-specific.[11–14] Specifically, analysis of 432 CDI specimens from Eastern China revealed that the sequence type (ST) ST37 was predominant,[11] whereas ST3 and ST54 were predominant in other studies in Northeast China.[12,13] Moreover, genetic diversity of *C difficile* within a region changes over time.[15] Thus, the present study was undertaken to examine the epidemiology of *C difficile* outbreaks in a single institution in Northern China.

2. Materials and methods

2.1. Clostridium difficile isolate collection and culture

Clostridium difficile isolates were obtained from loose stool specimens of patients with suspected CDI at the Beijing Friendship Hospital of Capital Medical University. In total, 80 nonduplicate isolates were collected from 475 in-patients with diarrhea from March, 2016 to December, 2016. This study was approved by the Institutional Review Board of the Beijing Friendship Hospital of Capital Medical University.

Stool samples were cultured on *C difficile* agar selective D-cycloserine–cefoxitin–fructose agar plates (CM0601, Oxoid, Basingstoke, UK) in an anaerobic atmosphere at 37°C for 48 hours. Presumptive *C difficile* isolates were identified by Gram stain, odor, and typical colony morphology, and confirmed by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight mass spectrometry (MALDI-TOF-MS) (VITEK MS, bioMerieux, Lyon, France). Isolates were maintained in 20% skimmed milk at −80°C for further studies.

2.2. MLST analysis and detection of toxin genes by PCR

Bacterial genomic DNA was extracted using a Bacterial Genomic DNA Extraction Kit (D1600, Solarbio, Beijing, China), and the endotoxin genes, *cdtA* and *cdtB*, were detected as described previously.[16] Seven housekeeping loci (*adk, atpA, dxr, glyA, recA, soda*, and *tpi*) were selected for MLST analysis.[10] PCR was performed, and amplification products were sequenced with a forward primer. DNA sequences were submitted to PubMLST (http://pubmlst.org/clostridium difficile) to obtain the ST; new ST or alleles were given a novel number by *C difficile* database curators.

Genetic diversity was analyzed using MEGA version 7 software. For MLST homology analysis, forward sequences of all the stains were trimmed to the same length, aligned, and analyzed using the MEGA 7.0 software package. The phylogenetic trees from the concatenated sequences were constructed by the neighbor joining method with a Kimura 2-parameter distance model using MEGA 7.0 software, and Bootstrap analysis with 1000 replicates was performed. The evolutionary distance was computed using the maximum composite likelihood method, according to the units of the number of base substitutions per site.[17]

2.3. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the reference agar dilution method according to Clinical and Laboratory Standards Institute (CLSI) 2017 guidelines.[18] Briefly, frozen *C difficile* isolates were thawed and subcultured on Brucella agar plates supplemented with vitamin K_1 (1 μg/mL), hemin (5 μg/mL), and 5% laked sheep red blood twice. Individual colonies were subsequently suspended to an equivalent of 0.5 McFarland, and 1 μL of the suspension was inoculated on 100-mm Brucella blood agar plates with a gradient antimicrobial concentration for 48 hours at 37°C in anaerobic conditions. The following 11 antibiotics were tested: metronidazole, vancomycin, fidaxomicin, piperacillin/tazobactam, meropenem, rifaximin, ceftriaxone, clindamycin, erythromycin, ciprofloxacin, and emodin. MICs of the antimicrobials were determined using the broth micro dilution method and were interpreted according to the 2017 CLSI recommendations. For agents without referenced standards, the breakpoints were as follows: rifaximin, ≥32 μg/mL; vancomycin, ≥32 μg/mL; erythromycin, ≥8 μg/mL; and ciprofloxacin, ≥8 μg/mL.[19,20] There was no previously published reference standard for emodin. *C difficile* ATCC700057 and *Bacteroides fragilis* ATCC 25285 were used as quality control strains.

2.4. Detection of resistance genes and sequencing

The fluoroquinolone resistance-determining regions (QRDRs) of *GyrA* and *GyrB*, *RpoB*, and *erm(B)*, and also the *23S rRNA* gene, were amplified using the related primers as previously described and shown in Table 1.[21–24]

Erythromycin resistance determinants were identified via a set of primers by overlapping PCR.[25] Other classes of *erm* genes (D, F, G, and Q) were also detected.[26–29] For analysis of erm(B)-negative, clindamycin and/or erythromycin-resistant isolates, analysis of *23S* genes were analyzed by PCR mapping using 6 primer pairs as described by Schmidt et al.[21] The DNA products were purified and sequenced on an ABI 3700 sequencer using a forward PCR primer. Mutations in the resistance genes were aligned with the reference sequence of *C difficile* 630 (GenBank accession no. NC 009089.1) using ORF Finder and DNAMAN alignment software.

2.5. Statistical analysis

Data analysis was performed using SPSS 20.0 Version 20.0 (IBM. Armonk, NY). The resistance rates (R%) were summarized as toxigenic and nontoxigenic isolate strains. Differences in the resistance rates between toxigenic and nontoxigenic isolate strains were compared using the Person chi-square test or Fisher

Table 1

Primers for analysis of resistance genes used in this study.

Genes /Primer	Sequences (5' → 3')	Source
gyrA	AATGAGTGTTATAGCTGGACG TCTTTTAACGACTCATCAAAGTT	Dridi et al, 2002
gyrB	AGTTGATGAACTGGGGTCTT TCAAAATCTTCTCCAATACCA	Dridi et al, 2002
RpoB	ATGGAAGCTATAACGCCTCAA ACAGCACCATTTACAGTTCTA	Curry et al, 2009
erm(B)	GAAAAAGTACTCAACCAAATA AGTAACGGTACTTAAATTGTTTAC	Sebaihia et al, 2006
erm(D)	GCTTTGACAACTGTGCTAAGTCAAAA GGCCATTTGTGATGCATTACATA	Kim et al, 1993
erm(F)	TCGAATTCTCGTTTTACGGGTCAGCACT TCAAGCTTCAGGGACAACTTCCAGCATT	Comstock et al, 1999
erm(G)	TCACATAGAAAAAATAATGAATTGCATAAG CGATACAAATTGTTCGAAACTAATATTGT	Cooper et al, 1996

exact test if any number of strains was less than 5. The statistical assessments were 2-tailed, and a value of $P < .05$ was considered statistically significant.

3. Results

3.1. Toxin typing and molecular epidemiology of the C difficile isolates

On the basis of the toxin genes patterns identified by PCR, the 80 isolates were divided into 65 toxigenic strains (81.25%) and 15 nontoxigenic strains (18.75%). The 65 toxigenic strains were classified into 2 toxin types, including 22 tcdA$^-$B$^+$CDT$^-$ strains (27.5%) and 43 tcdA$^+$B$^+$CDT$^-$ strains (53.75%). The A$^+$B$^+$CDT$^+$ strain was not detected.

The MLST analysis identified 21 different ST types, among which ST409 and ST416 were novel (Table 2). ST409 was a new combination of 7 existing allelic genes, and ST416 contained 5 new alleles (*adk33, atpA47, glyA65, recA36*, and *tpi62*). ST81 was the prevalent type identified in 20 isolates (25%), followed by ST8 (13 isolates, 16.25%), ST42 (9 isolates, 11.25%), and ST39, ST3, and ST2 (6 isolates for each ST type, 7.54% individually). Eleven ST types were found in 1 single isolate.

There was an interesting correlation between ST types and toxin types. All ST42, ST8, and ST2 isolates were toxin type A$^+$B$^+$CDT$^-$ strains, and all ST39 isolates were nontoxigenic strains. Moreover, ST81 was the most dominant type, all of which were A$^-$B$^+$CDT$^-$ strains. Thus, it is possible that the CDI outbreaks occurring during the study period at our institution was caused by ST81.

3.2. Phylogenetic analysis

The genetic diversity and phylogenetic relationships of the 80 isolates were analyzed using MEGA software. As shown in Fig. 1, a neighbor-joining tree was generated from concatenated sequences of 7 loci. Most ST types were classified into clade 1; ST81, ST37, ST39, and ST327 belonged to clade 4. ST416 was not closely related to any of the other isolates.

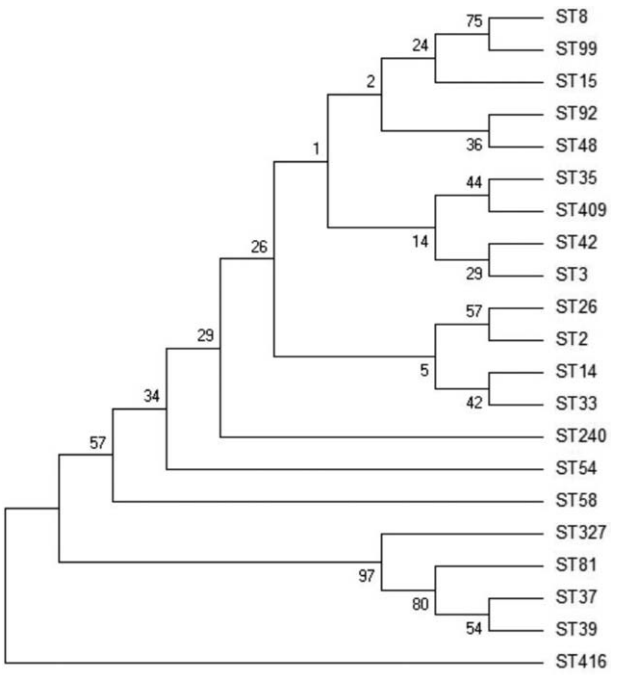

Figure 1. Phylogenetic tree reconstructed using the neighbor-joining method based on composite sequences of seven housekeeping gene fragments.

3.3. Antimicrobial susceptibility analysis

As shown in Table 3, all 80 isolates were susceptible to metronidazole, vancomycin, and fidaxomicin with MIC_{90} values

Table 2

Phenotypic and genotypic characteristics of *Clostridium difficile* isolates.

ST (n)	Clade	Toxin genotype			Prevalence (%)
		tcdA	tcdB	cdtA/cdtB	
ST2 (6)	1	+	+	−/−	7.5
ST3 (3)	1	−	−	−/−	3.75
ST3 (3)	1	+	+	−/−	3.75
ST8 (13)	1	+	+	−/−	16.25
ST14 (1)	1	+	+	−/−	1.25
ST15 (2)	1	−	−	−/−	2.5
ST26 (2)	1	+	−	−/−	2.5
ST33 (1)	1	+	+	−/−	1.25
ST35 (1)	1	+	+	−/−	1.25
ST37 (2)	4	−	+	−/−	2.5
ST39 (6)	4	−	−	−/−	7.5
ST42 (9)	1	+	+	−/−	11.25
ST48 (1)	1	−	−	−/−	1.25
ST54 (3)	1	+	+	−/−	3.75
ST58 (1)	1	+	+	−/−	1.25
ST81 (20)	4	−	+	−/−	25
ST92 (1)	1	+	+	−/−	1.25
ST99 (1)	1	+	+	−/−	1.25
ST240 (1)	1	−	−	−/−	1.25
ST327 (1)	4	+	+	−/−	1.25
ST416 (1)		−	−	−/−	1.25
ST409 (1)	**1**	+	+	−/−	1.25

Bold values mean novel identified sequence types (STs); ST416 was not classified in MLST databases.

Table 3

Toxin genotypes and antimicrobial susceptibilities of *Clostridium difficile* isolates.

Antimicrobial agent	All isolates				Toxigenic isolates (n = 65)				Non-toxigenic isolates (n = 15)				
	MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	R%	MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	R%	MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	R%	*P*[*]
Metronidazole	0.125–1	0.5	0.5	0	0.125–2	0.5	0.5	0	0.125–0.5	0.25	0.5	0	NA[†]
Vancomycin	0.125–0.5	0.25	0.25	0	0.125–0.5	0.125	0.25	0	0.06–0.25	0.125	0.25	0	NA
Fidaxomicin	0.03–0.5	0.125	0.25	0	0.03–0.5	0.125	0.25	0	0.03–0.5	0.125	0.125	0	NA
Rifaximin	≤0.0078-512	≤0.0078	0.5	8.75	0.0078 -256	0.0078	0.0078	3.07	0.0078-512	0.0078	256	26.67	<.05
Meropenem	0.5–4	1	1	0	0.5–4	1	1	0	0.5–2	1	1	0	NA
Clindamycin	2-512	256	256	96.25	2-512	128	512	96.92	4-256	128	256	93.33	NS[‡]
Ciprofloxacin	2-128	64	64	96.25	2-128	64	64	96.92	4-64	16	64	93.33	NS
Ceftriaxone	8-512	64	128	51.25	8-256	32	128	58.46	8-512	32	512	46.67	NS
Erythromycin	1-512	512	512	81.25	1-512	256	512	80	4-512	512	512	86.67	NS
Piperacillin/ Tazobactam	1/4–16/4	8/4	16/4	0	1/4- 16/4	8/4	16/4	0	1/4- 16/4	8/4	16/4	0	NA
Emodin	4–8	8	8	0	4–8	8	8	0	4–8	8	8	0	NA

[*] Toxigenic strains %R versus nontoxigenic strains %R.

[†] Not assessed because the strains were susceptible: %R = 0 for both groups.

[‡] No statistical significance.

of 0.5, 0.25, and 0.25 μg/mL, respectively. All isolates were also susceptible to meropenem and piperacillin/tazobactam. Emodin also exhibited effective in vitro antibacterial activities against the *C difficile* isolates with MICs ranging from 4 to 8 μg/mL. Seventy (87.5%) isolates were susceptible to rifaximin (MIC ≤0.0078 μg/mL), 7 (8.75%) isolates were resistant (MIC ≥32 μg/mL), and 3 (3.75%) strains showed intermediate (MIC range 0.25–2 μg/mL) susceptibility to rifaximin. In contrast, 96.25% of the isolates were equally resistant to clindamycin and ciprofloxacin, with MIC_{90} of 256 and 64 μg/mL, respectively (Table 3). The resistance rates to erythromycin and ceftriaxone were 81.25% and 51.25%, respectively.

There was no significant difference in the resistance rates to ceftriaxone, clindamycin, ciprofloxacin, and erythromycin between the toxigenic and nontoxigenic isolates (Table 3). Interestingly, the nontoxigenic strains showed higher resistance to rifaximin as compared with the toxigenic strains ($P < .05$).

Analysis of multidrug-resistant strains defined as those resistant to at least 3 antibiotics revealed that 81.25% (65/80) of isolates were multidrug-resistant strains in this study. Of note, all ST81 strains were multidrug-resistant strains with high resistance to erythromycin (MIC ≥256 μg/mL), clindamycin (MIC ≥32 μg/mL), ciprofloxacin (MIC ≥32 μg/mL), and ceftriaxone (MIC ≥128 μg/mL) (data not shown). All control results were in standard reference ranges.

3.4. Molecular analysis of the mechanisms of resistance

The quinolone resistance-determining regions of *GyrA* and *GyrB* were individually sequenced from the nucleotide codons 48 to 152 and 371 to 479. The results indicated that 18.75% (15/80) of the *C difficile* isolates had 1 single amino acid substitution in *GyrA*, including T82I in 14 strains and T82V in 1 strain. In addition, 36.25% (29/80) had 1 amino acid substitution in both *GyrA* and *GyrB*, and 5% (4/80) had a single amino acid change in *GyrB* D426N (Table 4). Moreover, 1 intermediate nontoxigenic isolate, ST416 (MIC 4 μg/mL), showed 1 novel amino acid substitution, F86Y, in *GyrA*. No amino acid substitutions in *GyrA* and *GyrB* were found in the *C difficile*-susceptible strains (data not shown). T82I was the most frequent amino acid change in the highly resistant *C difficile* isolates (ie, those with a MIC ≥32 μg/mL) that was found in various ST types. *C difficile* isolates with amino acid substitutions in both *GyrA* (T82I) and *GyrB* (D426V) belonged to the ST81 type. In addition, strains with amino acid substitutions in both *GyrA* (T82I) and *GyrB* (D426N) were the ST8 type.

We next analyzed the *RpoB* gene (nucleotide codons 480–616) in all isolates for possible amino acid substitutions. As shown in Table 5, there were 5 different *RpoB* polymorphisms identified in the rifaximin-intermediate and rifaximin-resistant *C difficile* isolates. Three amino acid substitutions had been previously reported.[20,30] However, the amino acid substitutions E573A combined with G603D in the ST416 strain were both novel. The amino acid substitutions H502N and R505K in *RpoB* were the most frequent mutations in rifaximin-resistant strains (Table 5). However, there was no relationship between ST type and the mechanism of resistance with respect to the *RpoB* gene. All 60 *C difficile*-susceptible strains had no amino acid substitution in *RpoB* (data not shown).

Among all 80 *C difficile* isolates, 60 strains (75%) were *erm(B)*-positive, and these isolates were resistant to both erythromycin

Table 4

Amino acid substitution in *gryA* and *gryB* associated with resistance to quinolones in the *Clostridium difficile* isolates.

ST (n)	MIC range	Amino acid substitutions	
	Ciprofloxacin	*GyrA*	*GyrB*
ST81 (20)	≥32	T82I	D426V
ST39 (4)	≥32	T82I	
ST39 (1)	≥64	T82I	
ST42 (3)	≥32	T82I	
ST37 (2)	≥32	T82I	
ST3 (2)	≥32	T82I	
ST3 (2)	≥32		D426N
ST26 (1)	≥32	T82I	
ST1 (1)	≥32	T82I	
ST240 (1)	≥32	T82I	
ST8 (9)	≥64	T82I	D426N
ST8 (2)	≥16		D426N
ST416 (1)	4	F86Y [*]	S416A

MIC = minimum inhibitory concentration, ST = sequence type.

[*] Novel amino acid substitutions in *GyrB* identified in this study.

Table 5

Amino acid substitutions in *RpoB* associated with rifaximin resistance in *Clostridium difficile isolates.*

ST (n)	MIC range	Amino acid substitutions	
	Rifaximin	*RpoB*	
ST39 (4)	≥256	H502N	R505K
ST37 (2)	0.25–128	H502N	R505K
ST48 (1)	256	H502N	R505K
ST240 (1)	2	H502N	
ST3 (1)	256		R505K
ST416 (1)	0.5	E573A*	E603D*

MIC = minimum inhibitory concentration, ST = sequence type.
* Novel amino acid substitutions in *RpoB* identified in this study.

and clindamycin. However, the other 20 *erm(B)*-negative isolates contained 12 clindamycin-resistant strains (≥8 μg/mL), and 6 were resistant to both erythromycin and clindamycin, including some that were highly resistant (erythromycin, MIC ≥64 μg/mL and clindamycin, MIC ≥64 μg/mL).

We further analyzed the genetic organizations of the *Erm(B)* determinant region of 66 isolates resistant to both erythromycin and clindamycin, including 60 *erm(B)*-positive isolates and 6 *erm (B)*-negative isolates. In total, 13 different genetic organizations were identified, and 7 arrangements (designated Eyy1–Eyy7) were new (Table 6). E4, E7, E13, Erj1, Erj2, and Erj4 were reported previously.[19,25] The majority of isolates were Erj2 (36.36%), followed equally by Erj4 and E7 (both 16.67%).

All *erm(B)*-negative strains were also negative for other erm class genes found in anaerobes (eg, D, F, G, and Q). However, *erm(G)* was found in 1 *erm(B)*-positive isolate with resistance to both erythromycin and clindamycin, and also 5 *erm(B)*-negative strains, including 2 isolates with resistance to both erythromycin and clindamycin and 3 clindamycin-resistant isolates. Although *erm(G)* nucleotide sequences were not in alignment with the reference isolate, *C difficile* 630, they had 98% sequence identity with *erm(G)* of *Bacteroides thetaiotaomicron* conjugal transposon Tcr Emr 7853 (GenBank accession no. L42817). This suggests that *erm(G)* may mediate resistance to the macrolide antibiotics in *C difficile* isolates.

We next analyzed the *23S rDNA* gene of 20 *erm(B)*-negative strains and 5 *erm(B)*-positive strains resistant to both erythromycin and clindamycin. No mutation of the nucleotide in position 656 of the *23S rDNA* gene was found in 12 of 20 isolates that were *erm(B)*-negative, including 2 strains resistant to both erythromycin and clindamycin and 10 clindamycin-resistant only strains. In addition, PCR amplification of the *23S rDNA* gene was low in 2 strains; therefore, they were not sequenced successfully. In addition, this mutation was also not identified in 4 *erm(B)*-positive strains resistant to both erythromycin and clindamycin. In contrast, a nucleotide C656T substitution within the *23S rDNA* gene was found in 6 strains, including 4 strains—3 *erm(B)*-negative and 1 *erm(B)*-positive strains—resistant to both erythromycin and clindamycin, 1 strain resistant to only clindamycin, and 1 susceptible strain.

4. Discussion

Clostridium difficile is recognized as a major source of nosocomial antibiotic-associated diarrhea. In recent years, the incidence of CDI has increased in the United States, Canada, and Europe, and a recent study in Eastern China found a 10% prevalence of CDI in hospitalized patients with diarrhea.[11] Because the epidemiology of CDI is region-specific, the present study was undertaken to examine the epidemiology of *C difficile* outbreaks in Northern China. Of the 80 isolates analyzed, 65 were toxigenic strains (81.25%), including 22 $tcdA^-B^+CDT^-$ strains (27.5%) and 43 $tcdA^+B^+CDT^-$ strains (53.75%), and 15 were nontoxigenic strains ($tcdA^-B^-CDT^-$; 18.75%). Twenty-one different STs were detected, including 2 novel types (ST409 and ST416). All isolates were susceptible to metronidazole, vancomycin, fidaxomicin, piperacillin/tazobactam, and meropenem, and all were effectively inhibited by emodin. Resistance to rifaximin, ceftriaxone, clindamycin, erythromycin, and/or ciprofloxacin was detected in some isolates, and 81.25% were multidrug-resistant. Amino acid mutations in *GyrA* and/or *GyrB* conferred quinolone resistance. The *erm(B)* gene played a key role in mediating macrolide-lincosamide-streptogramin B (MLSB) resistance, and *RpoB* mutations were associated with rifampin resistance.

The prevalence of CDI in the present study was 13.7%, which is higher than that (9.8%) reported in another study conducted in China[31] and lower than that reported in a similar epidemiological study in Indonesia.[32] $TcdA^-B^+CDT^-$ strains accounted for a

Table 6

Characterization of *erm(B)* determinant organization of the *Clostridium difficile* strains resistant to macrolide-lincosamide-streptogramin B.

Erm(B) determinant Organization (n)	Length of PCR products (bp)							MICs (μg/mL)		
	1	2	3	4	5	7	8	Erythromycin	Clindamycin	ST (n)
E4 (1)	388	1506	—	—	—	—	—	≥256	≥256	ST37 (1)
E7 (11)	2000	—	—	—	—	—	—	≥8	≥64	ST3 (1), ST8 (1), ST15 (1), ST26 (1), ST39 (1), ST54 (1), ST42 (5)
E13 (1)	2000	—	—	—	—	1166	—	512	512	ST35 (1)
Erj1 (3)	610	—	—	—	—	1166	—	≥64	≥64	ST54 (1), ST2 (1), ST92 (1)
Erj2 (24)	610	—	—	—	—	-	—	≥128	≥256	ST81 (18), ST240 (1), ST8 (2), ST3 (1), ST2 (1), ST42 (1)
Erj4 (11)	—	—	—	—	—	—	—	≥8	≥256	ST8 (8), ST3 (1), ST81 (1), ST58 (1)
Eyy1 (1)	—	—	582	1247	—	—	—	512	256	ST42 (1)
Eyy2 (2)	—	—	-	—	—	1166	—	≥8	≥4	ST48 (1), ST54 (1)
Eyy3 (7)	—	—	582	—	—	—	—	≥128	≥256	ST39 (4), ST37 (1), ST42 (1), ST3 (1)
Eyy4 (1)	2000	-	582	—	—	—	—			ST3 (1)
Eyy5 (1)	2000	-	582	—	—	1166	—	512	512	ST42 (1)
Eyy6 (1)	-	-	582	—	—	1166	—	128	512	ST39 (1)
Eyy7 (2)	610	-	582	1247	—	—	—	≥256	≥256	ST3 (1), ST81 (1)

Bold: Eyy1 to Eyy7 were novel gene organizations in the Erm(B) determinant identified in this study.

large proportion (27.5%) of the collected isolates, which was similar to a previous report in China.[33] However, we did not identify any $tcdA^+B^+CDT^+$ stains, indicating that the hypervirulent strain, NAP1/027, was not prevalent in our hospital. Although ST54 and ST37 were the major types reported in other studies in China,[31,34] ST81 was the dominant type observed in the present study, followed by ST8 and ST42. Moreover, ST81 carried a single allelic variant (atpA). Thus, *C difficile* isolates likely have genetic variances that differ between regions as a result of genetic shift over the years. Interestingly, 90.9% of the $tcdA^-B^+$ strains belonged to the prevalent type ST81, which implied that the CDI outbreaks were caused by ST81 in our hospital during the study period. Both of the toxigenic strains (classified into 14 STs) and nontoxigenic strains (divided into 7 STs) had great genetic diversity, which was in accordance with data published by Kuwata et al,[35] and also other studies conducted in China.[11–14]

Metronidazole is considered the first choice for mild-to-moderate CDI, and vancomycin is the first-line antibiotic for moderate-to-severe CDI. Although the emergence of strains with reduced susceptibility to metronidazole and vancomycin has been reported,[19,36–38] antimicrobial susceptibility testing of both toxigenic strains and nontoxigenic strains did not identify any strain resistant to metronidazole and vancomycin in the present study. Fidaxomicin was also approved by the US Food and Drug Administration for the treatment of CDI in 2011; it has potent antibiotic activity in vitro with minimal impact on intestinal microbiota composition in vivo.[39–41] Although fidaxomicin has not been licensed for the treatment of CDI in China, we analyzed the susceptibility of the *C difficile* isolates to this antibiotic, which revealed a MIC_{50} of 0.125 μg/mL and MIC_{90} of 0.125 μg/mL, which was identical to a previous report in Taiwan.[36] Both meropenem and piperacillin/tazobactam showed activity against all strains.

In contrast to a study conducted in Hebei Province in Northeast China,[13] some of the isolates in the present study were resistant to rifaximin—a gastrointestinal-selective antibiotic that inhibits the gene transcription of bacteria via binding RNA bacteria polymerase target sites. Although it holds promise as an alternative for the treatment of relapsing CDI,[42] sufficient clinical data worldwide are lacking. We observed that the MICs for rifaximin were either very high or very low, which was consistent with previous findings,[20] with a resistance rate of 8.7%, which is lower than the 29.8% resistance rate reported in Shanghai,[43] but similar to another report (10.9%) in Taiwan.[36] In addition, we found that nontoxigenic strains had a higher resistance rate to rifaximin than toxigenic strains, which was likely a result of the high-level resistance observed with 5 isolates (≥256 μg/mL). This result also demonstrated that although nontoxigenic strains were part of the normal intestinal tract flora, they may mediate acquired drug resistance of toxigenic strains by horizontal transmission.

Wang et al[9] first described that emodin inhibited the growth of *Clostridia* in 1990; however, only 5 *C difficile* isolates were analyzed. Using the broth microdilution method to determine the MICs of emodin, we found that all isolates were effectively inhibited. However, the antibacterial action and gastrointestinal selectivity of emodin remain to be examined.

In the present study, 81.25% of the isolates were multidrug-resistant strains, which was higher than the 73.33% reported by Dong et al.[19] ST81 strains showed high-level resistance to erythromycin, ciprofloxacin, and clindamycin, which was identical to another study described in Japan.[35] Amino acid substitutions in both *GyrA* and/or *GyrB* have been associated with fluoroquinolone resistance.[44] In the present study, the T82I mutation was the most frequently observed in high-level ciprofloxacin-resistant strains, which is similar to previous reports, respectively.[39,40,45,46] Liao et al[36] detected 1 isolate (MIC 8 μg/mL) with only amino acid mutation, S416A in *GyrB* or T82I in *GyrA*. In the present study, we identified a ciprofloxacin-intermediate strain ST416 (MIC 4 μg/mL) with a novel amino acid substitution, F86Y, in *GyrA* combined with the S416A mutation in *GyrB*. We also observed different MICs in isolates with identical amino acid substitutions in *GyrB*, suggesting that *GryB* mutations alone or combination with those in *GyrA* result in diverse resistance to CIP.

We also identified H502N and/or R505K mutations in the *RpoB* gene in all of the rifaximin-resistant and intermediate strains. In addition, we identified 2 previously unreported amino acid substitutions in 1 intermediate strain: E573A and E603N with a MIC of 0.5 μg/mL. Further studies are required to examine how these novel substitutions reduced the susceptibility to rifaximin.

Erm(B) carried by the mobile element Tn5398 plays a major role in resistance to the MLSB group of antibiotics. A detailed analysis of the genetic organization of the *Erm(B)*-determinant region revealed the E4, E7, E13, Erj1, Erj2, and Erj3 genetic arrangements with Erj2 as the most prevalent form, which is consistent with a previous study in China.[19] However, we identified 7 genetic arrangements, Eyy1 to Eyy7, that were novel. PCR fragment 3 with a new product of 582 bp may be a result of a DNA fragment deletion between ORF298 and the *erm(2B)* gene, suggesting that genetic exchange and recombination frequently occur in clinical strains. Therefore, the genetic arrangement of the *Erm(B)*-determinant region diverges between different countries.[19,47] Although other *Erm* classes (ie, D, F, and Q) were also negative in *Erm(B)*-negative strains, we demonstrated the presence of *erm(G)* in ERY-resistant strains, including those that were *erm(B)*-negative and positive. Although the nucleotide sequence of *erm(G)* was not aligned with the reference strain 630, high sequence identity was observed with the *B thetaiotaomicron* conjugal transposon, Tcr Emr 7853. There were only 2 amino acids change (T201M and A238T) in the nucleotide codons of *erm(G),* suggesting that the dissemination of erythromycin resistance genes between *Bacteroides* and *Clostridia* may occur horizontally via conjugative transposons, demonstrating the importance of mobile genetic elements in the spread of resistance in clinical *C difficile* isolates.

We also identified a C656T mutation in the *23S rDNA* gene in both clindamycin-resistant and susceptible strains, suggesting that this mutation does not impact resistance to MLSB antibiotics. Moreover, all ST2 isolates were susceptible to ceftriaxone, indicating a possible relationship between ST type and ceftriaxone resistance. It is possible that the *C difficile* isolates were not consistently resistant to ceftriaxone, and this divergence of resistance to ceftriaxone depended is strain-specific. Further studies are necessary to characterize the mechanisms by which the isolates were resistant to ceftriaxone, including the presence of antibiotic-degrading enzymes, ß-lactamases, and penicillin-binding proteins (PBPs), and also modification of target sites.

In this study, ciprofloxacin was 1 of the antibiotics studied. However, it is known that *C difficile* resistance to ciprofloxacin is relatively common. Because of its low price and its excellent antibacterial effect, ciprofloxacin is regarded as the drug of first choice for treating nosocomial infectious diarrhea caused by pathogenic bacteria, such as *Escherichia coli* and *Salmonella*, in

the Infectious Diseases Department in our hospital. In addition, ciprofloxacin is a drug that is important in the treatment of upper and lower respiratory tract infections, urinary tract infections, and other common infections seen in general practice.[48–51] In this study, the resistant rate of isolated *C difficile* strains to ciprofloxacin was high (96.25%), which suggests that use of ciprofloxacin may be a risk factor for the development of CDI in our hospital. We believe that investigating the resistance of *C difficile* strains to CIP will help clinicians to rationally choose the antibiotics and prevent secondary *C difficile* infections in our hospital.

The present study is limited in the small number of specimens characterized from a single institution. Thus, additional larger studies are required to confirm our results in this specific region, especially given that transmission between healthcare facilities may occur.[52] Furthermore, the mechanisms by which some of the isolates were resistant to specific antimicrobials were not determined in detail. In addition, the mechanism underlying emodin suppression of *C difficile* growth was not analyzed. Finally, the association between the genotypes identified and severity of CDI was not assessed.

In conclusion, this study described the genetic diversity and resistance patterns of toxigenic and nontoxigenic strains of *C difficile* isolates collected from Beijing Friendship Hospital in China. No strain was resistant to metronidazole, vancomycin, and fidaxomicin, and emodin effectively inhibited the growth of all strains in vitro. ST81 was the most prevalent type with high-level resistance to erythromycin, clindamycin, ciprofloxacin, and ceftriaxone. In addition, several novel resistance genes were identified, which may affect the patterns of resistance and increase their dissemination between *C difficile* isolates. Given the high degree of resistance and diversity observed in the present study, it is necessary to continually examine the epidemiological distribution of *C difficile* in China. Further studies analyzing the resistance mechanisms of *C difficile* in detail will be of potential benefit in effectively preventing and controlling CDI in hospitals.

Author contributions

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