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ORIGINAL RESEARCH

High-Level Resistance of Toxigenic *Clostridioides difficile* Genotype to Macrolide-Lincosamide-Streptogramin B in Community Acquired Patients in Eastern China

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Background: *Clostridioides difficile* resistant to macrolide-lincosamide-streptogramin B (MLS_B) has not been reported in China.

Methods: In a cross-sectional study in two tertiary hospitals, *C. difficile* isolates from stool specimens from community-onset, hospital-associated diarrheal patients were analyzed for toxin genes, genotype, and antibiotic resistance, and the patients' clinical charts were reviewed.

Results: A total of 190 (15.2%) isolates (102 A^+B^+ and 88 A^-B^+) from 1250 community acquired (CA) patients were recovered and all were susceptible to vancomycin and metronidazole. High-level resistance (minimum inhibitory concentration > 128 mg/L) to erythromycin and clindamycin was recorded in 77.9% and 88.4% of the tested isolates, respectively. Furthermore, 89.3% (159/178) of the isolates resistant to MLS_B carried the erythromycin resistance methylase gene (*ermB*). The statistically significant factors associated with *C. difficile* infection (CDI) induced by A^-B^+ isolates with MLS_B resistance included a severity score of >2 (odds ratio [95% confidence interval], 7.43 [2.31–23.87]) and platelet count (cells × 10^9 cells/L) < 100 [5.19 (1.58–17.04)]. The proportion of A^-B^+ increased with enhanced CDI severity ($x^2 = 21.62$, $P < 0.001$), which was significantly higher than that of *ermB*-positive A^+B^+ in severity score of 4 ($x^2 = 8.61$, $P = 0.003$). The average severity score of *ermB*-positive isolates was significantly higher than that of *ermB*-negative isolates in A^-B^+ ($Z = -2.41$, $P = 0.016$).

Conclusion: The *ermB*-positive A^-B^+ *C. difficile* with MLS_B resistance is described for the first time as a potential epidemic clone inducing severe CDI in CA diarrheal patients in Eastern China.

Keywords: *Clostridioides difficile*, molecular characteristic, macrolide-lincosamide-streptogramin B resistance

Introduction

Clostridioides difficile, an anaerobic, gram-positive, spore-forming bacterium, causes severe infectious colitis, toxic megacolon, and sepsis, which are life threatening, and it is also responsible for antibiotic-associated diarrhea exhibiting asymptomatic carriage or mild manifestations.[1] *Clostridioides difficile* infection (CDI) affects approximately more than 300,000 hospitalized patients each year[2–6] and has become the leading cause of hospital-acquired diarrhea worldwide.[7,8]

Exposure to antibiotics is considered the most important modifiable risk factor for the development of CDI.[9] Antibiotic resistance of *C. difficile* also plays a key

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role in driving the emergence of new strain types. *C. difficile* PCR ribotype 027 has a high correlation with fluoroquinolone resistance, leading to its widespread distribution in North America and Europe.[10] Metronidazole and vancomycin remain the first-line therapeutics for CDI despite the emergence of sporadic metronidazole- and vancomycin-resistant *C. difficile*.[10] Resistance to macrolide-lincosamide-streptogramin B (MLS_B) is considered an important factor not only driving the persistence and transmission of *C. difficile* but also increasing the risk for CDI in patients.[11]

Recently, the rate of MLS_B resistance has been significantly increasing worldwide. The antibiotic resistance profile of *C. difficile* in 2012–2015 revealed that the rate of erythromycin (ERY) resistance ranged from 13% to 100% and that of clindamycin (CLI) resistance ranged from 8.3% to 100%.[10] The emergence of CLI-resistant 027 has been found to be a new strain type increasing the risk of CDI and accelerating its spread in Europe.[11,12] In 2001, a CLI-resistant, toxin A-negative, and toxin B-positive (A^-B^+) *C. difficile* clone with the *ermB* gene has been reported be associated with a nosocomial outbreak in the Netherlands. This clone was also a potential predictor of enhanced CDI in Poland.[13] A systematic review and meta-analysis from 2010 to 2016 in China showed that the rate of ERY and CLI resistance was 80.2% and 81.7%, respectively.[14] A cross-sectional study in Eastern China in 2012–2015 revealed that resistance to ERY and CLI was 64.7% and 62.5%, respectively, with significantly different distribution in different genotypes.[15]

MLS_B-type antibiotic resistance is due to post-transcriptional methylation of 23S ribosomal rRNA.[16] The resistance of *C. difficile* to ERY and CLI has been described to be transferred with no involvement of plasmid DNA. The resistance of *C. difficile* to ERY and CLI is encoded by the erythromycin resistance methylase gene (*ermB*) located on the mobilizable non-conjugative transposon Tn 5398.[16,17] However, *ermB* genes have not been identified in all clinical *C. difficile* isolates expressing high resistance to ERY and CLI, which might be conferred by other mechanisms. Moreover, the data on MLS_B resistance of *C. difficile* in China are limited. The rate of MLS_B resistance and the correlation between genotypes and MLS_B resistance and between *C. difficile* carrying *ermB* genes and virulence properties are still unclear.

The present cross-sectional study in community-onset, hospital-associated diarrheal patients from two hospitals in Eastern China was conducted to reveal the correlations among toxin genes, MLS_B resistance, and *ermB* genes. We also analyzed the difference in CDI severity induced by different genotypes with MLS_B resistance.

Materials and Methods

Sample Collection

Clinical samples were consecutively collected from December 2015 to April 2016 from two hospitals, the Affiliated Hangzhou First People's Hospital (HFPH), Zhejiang University school of Medicine and the Lishui Second People's Hospital (LSPH), which are located in Zhejiang Province, China. The HFPH is a general hospital with 2613 beds in 86 units. The LSPH is a general hospital with 500 beds. Clinical stool specimens from patients with suspected CDI were transported to the Zhejiang Provincial Center for Disease Control and Prevention (ZJCDC) within 72 h for further testing. This study was approved by the Institutional Review Board of the ZJCDC, LSPH, and HFPH.

According to the guidelines of the Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America (SHEA/IDSA),[9] inclusion criteria were presented as follows. We collected stool specimens from patients who were admitted within less than 48 h with diarrhea having loose, watery, or unformed stools more than three times within 24 h. Exclusion criteria were detection of other diarrhea-causing pathogens in the stool specimens, neonates or infants under 12 months of age, and patients with diarrhea who have been admitted over 48 h. Duplicated stool specimens from the same patients were removed. A standardized questionnaire including basic information and clinical data was prepared for each patient. All clinical parameters as part of routine care were recorded with biochemical and immunological examinations, and the cutoff values of stool-red blood cells (S-RBCs), stool-white blood cells (S-WBCs), occult blood in stool (OB), C-reactive protein (CRP), white-blood cells (WBCs), neutrophils, lymphocytes, monocytes, eosinophils, basophils, hemoglobin (Hb), platelets (PLT), creatinine, glucose, and kalium were obtained from the clinical standards.[18,19] CDI was diagnosed, clinical cases were classified into category of CDI based on the clinical and laboratory results, and hospital acquired and community acquired (CA) CDI as reported by the SHEA/IDSA.[9] Six categories of CDI severity were evaluated mainly based on clinical manifestations of the patients and the laboratory results. Each patient was classified into one of six severity scores, which was 1 (no clinical CDI), 2 (mild), 3 (mild to moderate), 4 (moderate), 5 (moderate to severe), and 6 (severe) as reported.[15,20,21]

Clostridioides difficile Culture

Stool specimens were treated with alcohol, and then the mixture was inoculated onto cycloserine-cefoxitin fructose agar with selective supplement (Oxoid, Basingstoke, UK) as previously reported.[15] The isolates were identified by morphological characteristics of flat, yellow, ground-glass appearance, and special odor on the CCFA.[22] *C. difficile* was further confirmed using the latex agglutination test (Oxoid, Ltd., Basingstoke, UK). All isolates were stored at −70°C in the brain–heart infusion broth with 10% glycerol until subsequent analyses.

Detection of *C. difficile* Toxin and *ermB* Genes

Genomic DNA of *C. difficile* was extracted using the QIAamp DNA Blood Mini Kit (Valencia, CA, USA), according to the manufacturer's instructions. The toxin genes *tcdA* and *tcdB* and the housekeeping gene *tpi* were detected using a conventional polymerase chain reaction (PCR) assays with the primer sequences reported previously.[15] The *ermB* gene was detected by the conventional PCR assay as previously reported.[13] After PCR amplification, the PCR products were analyzed by agarose gel electrophoresis. The *tcdA*-F and *tcdA*-R primers were used to detect the *tcd*A gene, yielding a 369 bp amplicon for *tcdA*-positive strains or a 110 bp amplicon for *tcdA*-negative strains. The PCR product of *ermB* gene was 688 bp. Two standard *C. difficile* strains (ATCC 43255 and 700057) obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) were used as the positive and negative controls for both *tcdA* and *tcdB*, respectively. The blank, positive, and negative controls were examined in each test simultaneously.

Multilocus Sequence Typing

Six reference *C. difficile* strains, namely, ATCC 43255, 43598, BAA-1870, BAA-1803, BAA-1801, and 700057, were used as the controls. All *C. difficile* isolates were genotyped by multilocus sequence typing (MLST) as described previously.[23] In brief, seven loci, namely, *adk, atpA, dxr, glyA, recA, sodA*, and *tpi*, widely distributed on the chromosome, were amplified by the PCR. The PCR products were sequenced using the 3730 XL DNA Analyzer (Applied Biosystems). All the sequences according to the seven loci were input into the *C. difficile* MLST database (http://pubmlst.org/cdifficile) for determining the sequence types (STs). A minimal spanning tree was constructed using Bionumerics software version 5.1 (Applied Math, Austin, TX, USA).

Antibiotic Susceptibility Testing

The isolates of *C. difficile* were tested for resistance to metronidazole, vancomycin, clindamycin, and erythromycin by agar dilution as previously reported.[24] *Bacteroides fragilis* (ATCC 25285) and *C. difficile* (ATCC 700057) were included in each run as the controls. The minimal inhibitory concentration (MIC) results were interpreted following the guidelines of the Clinical and Laboratory Standards Institute (CLSI) published in 2017 for metronidazole and clindamycin.[24] The breakpoints for vancomycin and erythromycin were determined according to a previous study.[25]

Data Analysis

The data were analyzed using Statistical Package for Social Sciences (SPSS, Chicago, IL, USA) version 19.0 and Epi Info version 3.5.1. The difference in CDI severity score among different genotypes was analyzed using the nonparametric test. Logistic regression analysis was used to identify independent risk factors. The odds ratio (OR), 95% confidence interval (CI), and *P*-values were calculated to assess the differences in clinical characteristics between A^+B^+ and A^-B^+ isolates resistant to CRY and CLI. The results with *P*-values < 0.05 were considered statistically significant.

Results

Clinical Information of Diarrheal Patients from Two Hospitals

A total of 1250 patients with diarrhea at the HFPH (n = 1053) and LSPH (n = 197) were enrolled in this cross-sectional study conducted over five months. One stool specimen was collected from each patient, with no duplicated specimens, when the patients were admitted within less than 24 h. We recovered a total of 190 (15.2%) isolates (HFPH, n = 141; LSPH, n = 49) with the *tpi* gene from these stool specimens. All the isolates were toxigenic *C. difficile* carrying either or both *tcdA* and *tcdB*; no nontoxigenic isolates were found (Figure 1). All the clinical information, including basic information and immune-biochemical data, was compared between the patients from the HFPH and LSPH (Table 1). All 190 patients had CA CDI. The CDI rate in patients from the HFPH was 13.4%, which was significantly lower than that in patients from the LSPH (24.9%) (χ^2 = 20.40, P < 0.0001). The patients enrolled in this study were mainly from the wards of infectious diseases,

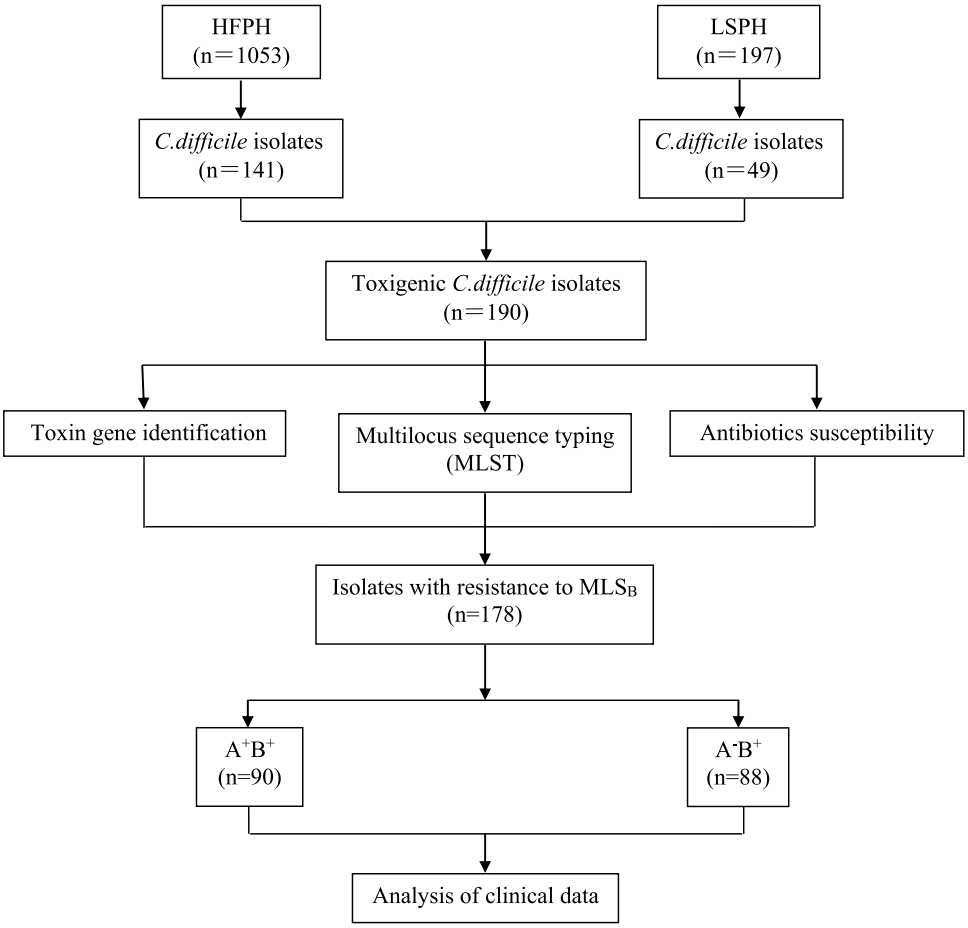

Figure 1 Flow diagram of data collected during the study.

psychiatry and neurology, and blood and cardiology in the LSPH; however, the wards of gastroenterology, infectious disease, and pediatrics were the main sources in the HFPH, which resulted in the difference in ward distribution between the LSPH and HFPH ($\chi^2 = 429.22$, $P < 0.0001$).

CDI Severity

The clinical data of all the enrolled patients were assessed via blinded chart review by physicians as reported previously.[15,20,21] No case was categorized under the CDI severity scores of 1, 5, and 6. Furthermore, 69.5% of CDI was categorized under the severity score 2 and 22.6% was under the severity score 3. Only 15 (7.9%) patients with CDI presented moderate clinical symptoms graded as CDI severity score 4. All the isolates were subjected to further analysis (Figure 1).

Genotype of *C. difficile* Isolates

Of the 190 toxigenic *C. difficile*, 102 (53.7%) isolates were positive for *tcdA* and *tcdB* (A^+B^+), in which ST3, ST35, and ST54 were the dominant genotypes. The 88 (46.3%) isolates were negative for *tcdA* but positive for *tcdB* (A^-B^+), among which three STs were ST37, ST39, and ST81. All the isolates were divided into 18 STs, indicating a high genetic diversity of *C. difficile* in these two hospitals, resulting in the difference in ST distribution between these hospitals ($\chi^2 = 6.02$, $P = 0.049$) (Table 2).

A minimal spanning tree was constructed to reveal the allelic difference among isolates. As shown in Figure 2, two independent clusters (A and B) were obviously divided in the minimal spanning tree. The cluster A presented high diversity within 14 STs, and seven of them were completely resistant to MLS_B. Half of the ST8 and ST233 isolates were susceptible to MLS_B, and the other four STs had lower resistance rates, ranging from 6.5% to 33.3%. ST11 and all the A^-B^+ isolates, including ST37, ST39, and ST81, were included in cluster B with 100% MLS_B resistance. The A^-B^+ isolates with MLS_B resistance were closely related and had single or four different allelic genes in cluster B. The MLS_B-susceptible isolates were distributed

Table 1 Clinical Information of Diarrheal Patients Involved in This Study

Characteristics	Diarrheal Patients at HFPH (n=1053)	Diarrheal Patients at LSPH (n=197)
Male	513 (48.7%)	96 (48.7%)
Age (Median [range])	51 (5–101)	76 (20–102)
Inpatients	536 (50.9%)	149 (75.6%)
CDI	141 (13.4%)	49 (24.9%)
Ward type		
Gastroenterology	439 (41.7%)	0
Psychiatry and neurology	27 (2.6%)	36 (18.3%)
Infectious disease	155 (14.7%)	109 (55.3%)
Blood and cardiology	68 (6.5%)	33 (16.8%)
Pediatrics	181 (17.2%)	0
Surgery	63 (6.0%)	4 (2.0%)
rehabilitation	23 (2.2%)	0
Others [a]	97 (9.2%)	15 (7.6%)

Notes: [a]Other ward types included urology, gynecology, obstetrics, oncology, transplantation, respiratory medicine, bone and joint, and etc.

in cluster A in a disorderly manner, and no genetic relationship between MLS_B-resistant and MLS_B-susceptible *C. difficile* isolates was found. There were no differences on distribution of MLS_B-resistant *C. difficile* isolates between two hospitals ($\chi^2 = 3.13$, $P = 0.077$), indicating that this clone was a common genotype in community associated CDI in Eastern China (Table 2).

Antibiotic Resistance and *ermB* of Isolates

The antibiotic-susceptibility pattern of 190 toxigenic *C. difficile* isolates is presented in Table 2. No isolates were resistant to metronidazole (MIC ≤ 8 mg/L) or vancomycin (MIC ≤ 2 mg/L). However, a high level of CLI resistance (MIC > 128 mg/L) was observed for all the isolates, including all the 49 isolates from the LSPH and the 119 isolates from the HFPH; the 13 isolates from the HFPH had intermediate susceptibility to CLI (MIC = 4 mg/L), and the remaining 9 were susceptible to CLI. The distribution of CLI resistant isolates were found statistically significant between HFPH and LSPH (χ^2=8.65, $P = 0.005$). A total of 148 isolates, including 108 from the HFPH and 40 from the LSPH, were highly resistant to ERY (MIC >128 mg/L). The 88.2% of A^+B^+ (90/102) and 100% of A^-B^+ (88/88) *C. difficile* isolates were resistant to MLS_B. The 12 ERY-susceptible isolates (6.3%, 12/190), including 4 isolates susceptible to CLI and 8 isolates intermediate-susceptible to CLI did not carry the *ermB* gene. The rate of carrying *ermB* in MLS_B-resistant isolates was 89.3% (159/178). The 19 *ermB*-negative isolates (10.7%, 19/178) were simultaneously resistant to ERY and CLI, except one from the HFPH, which was resistant to CLI but susceptible to ERY.

Predictive Factors for Infection with MLS_B-Resistant *C. difficile*

The basic information, CDI severity score, and immunobiochemical data of the patients with CDI were analyzed and compared between A^+B^+ and A^-B^+ *C. difficile* isolates resistant to MLS_B. A bivariate analysis between 90 A^+B^+ and 88 A^-B^+ isolates resistant to MLS_B was performed, and the results are presented in Table 3. The number of patients infected by A^-B^+ *C. difficile* isolates resistant to MLS_B was significantly more than the number of patients with A^+B^+ *C. difficile* isolates resistant to MLS_B in CDI severity score of >2, WBC (cells × 10^9/mL) count of >10, and platelet (cells × 10^9/L) count of <100, respectively. A multivariate analysis including statistically significant parameters from the bivariate analysis was subsequently conducted. The percentages of patients infected by A^-B^+ *C. difficile* isolates resistant to MLS_B was significantly more than those of patients with A^+B^+ *C. difficile* isolates resistant to MLS_B in only two parameters, CDI severity score of >2 and platelet (cells × 10^9/L) count of <100, respectively. There were no significant differences among different ages ranging from >20 to >80 years of age. However, we notably found that CDI induced by MLS_B-resistant A^-B^+ *C. difficile* was more severe than that by MLS_B-resistant A^+B^+ *C. difficile*.

Correlation Among CDI Severities, Toxin A/B Types, and *ermB* Gene

CDI severities were assessed as described above. The average ± standard deviation severity score of MLS_B-resistant A^+B^+ and A^-B^+ isolates with or without the *ermB* gene was calculated (Table 4 and Table S1). The average CDI severity scores induced by *ermB*-positive isolates in A^-B^+ *C. difficile* were significantly higher than those induced by the *ermB*-positive isolates in A^+B^+ *C. difficile* isolates ($Z = -4.68$, $P < 0.001$) (Table S1) and *ermB*-negative isolates in A^-B^+ *C. difficile* isolates ($Z = -2.41$, $P = 0.016$) (Table 4), respectively. However, no statistically significant differences were found between the *ermB*-positive and *ermB*-negative A^+B^+ isolates ($Z = -0.21$, $P = 0.836$) (Table 4) and between A^+B^+ and A^-B^+ in the *ermB*-negative isolates ($Z = -1.11$, $P = 0.267$) (Table S1). The above results showed that *ermB*-positive A^-B^+ *C. difficile* isolates with MLS_B-resistance led to more severe CDI cases than others.

Table 2 Molecular Characteristics of Toxigenic *C. difficile* Isolates in HFPH and LSPH

Characteristics	No. (%) of CDI Patients		Analysis Results	
	HFPH (n=141)	LSPH (n=49)	value	*P* value
CDI severity score [a]			χ^2=3.16	0.206
2 (n=126)	92 (65.2%)	34 (69.4%)		
3 (n=49)	35 (24.8%)	14 (28.6%)		
4 (n=15)	14 (9.9%)	1(2.0%)		
Toxin gene pattern				
A^+B^+(n=102)			χ^2=2.72	0.436
MLST type				
ST3 (n=24)	19 (13.5%)	5 (10.2%)		
ST35 (n=15)	9 (6.4%)	6 (12.2%)		
ST54 (n=31)	23 (16.3%)	8 (16.3%)		
Others (n=32)	26 (18.4%)	6 (12.2%)		
A^-B^+(n=88)			χ^2=6.02	0.049
MLST type				
ST37 (n=58)	38 (27.0%)	20 (40.8%)		
ST39 (n=11)	11 (7.8%)	0 (0.0%)		
ST81 (n=19)	15 (10.6%)	4 (8.2%)		
Antibiotic resistance rate [b]				
MLS_B			χ^2=3.13	0.077
S (n=12)	12 (8.5%)	0 (0.0%)		
R (n=178)	129 (91.5%)	49(100%)		
Clindamycin			χ^2=8.65	0.005
S (n=9)	9 (6.4%)	0 (0.0%)		
I (n=13)	13 (9.2%)	0 (0.0%)		
R (n=168)	119 (84.4%)	49 (100%)		
Erythromycin			χ^2=0.54	0.464
S (n=42)	33 (23.4%)	9 (18.4%)		
R (n=148)	108 (76.6%)	40 (81.6%)		
Metronidazole			N/A[c]	
S (n=190)	141 (100.0%)	49 (100.0%)		
Vancomycin			N/A	
S (n=190)	141 (100.0%)	49 (100.0%)		

Notes: [a]CDI Severity: 2, mild CDI; 3, mild to moderate CDI; 4, moderate CDI. [b]S: susceptible; R: resistant; I: intermediate. [c]N/A: not applicable.

Comparison Among CDI Severities, Genotypes, and *ermB* Gene in Isolates Resistant to MLS_B

CDI severities, STs, and *ermB* gene were analyzed as follows in *C. difficile* isolates resistant to MLS_B. In MLS_B-resistant *ermB*-positive isolates, the proportion of A^-B^+ increased with severity score (x^2 = 21.62, $P < 0.001$). The number of A^-B^+ isolates (91.7%, 11/12) was significantly more than the number of A^+B^+ isolates (8.3%, 1/12) with *ermB* in the category of CDI severity score of 4 ($x^2 = 8.61$, $P = 0.003$) (Table 5). There were statistically significant differences in the CDI severity scores among different STs in the *ermB*-positive isolates (x^2 = 36.77, P = 0.001) (Table 5). In patients with a CDI severity score of 4, a total of 9 ST37 genotype isolates (75.0%, 9/12), one of A^-B^+ *C. difficile*, was found, which was more frequently than other STs, and the number of ST37 increased with the CDI severity score (x^2 = 26.38, $P < 0.001$) (Table 5). In *ermB*-negative isolates, only one

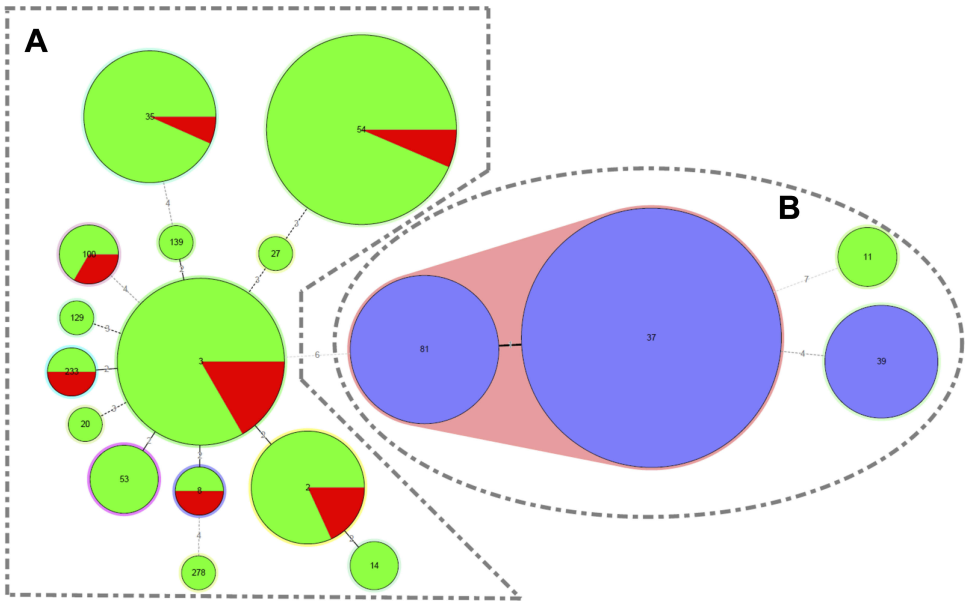

Figure 2 Relationship of toxigenic *C. difficile* isolates susceptible or resistant to MLS_B by minimal spanning tree.

patient had severe CDI and was categorized under the CDI severity score of 4. Most of the *ermB*-negative isolates had mild CDI with a CDI severity score of 2. Obviously, the *ermB*-positive A^-B^+ *C. difficile* isolates with MLS_B-resistance, especially ST37, were the main drivers to induce increased CDI severity.

Table 3 Differences of Clinical Characteristics Between A^+B^+ and A^-B^+ *C. difficile* with Resistance to MLS_B

Characteristics[a]	NO. (%) of Diarrheal Patients		Analysis Results					
	Patients with *C. difficile* Isolates with Resistance to MLS_B		Bivariate			Multivariate Logistic		
	A^+B^+ (n=90)	A^-B^+ (n=88)	OR	95%CI	*P* value	OR	95%CI	*P* value
Gender, male	48(53.3%)	51(58.0%)	1.21	0.67–2.18	0.535			
Years of age, >55	46(51.1%)	48(54.6%)	1.15	0.64–2.07	0.646			
CDI severity score, >2 (n=54)	14(15.6%)	40(45.5%)	4.52	2.23–9.18	<0.001	7.43	2.31–23.87	0.001
Stool								
S-RBC Positive (n=9)	5 (5.6%)	4 (4.6%)	0.81	0.21–3.12	0.759			
S-WBC Positive (n=39)	22 (24.4%)	17 (19.3%)	0.74	0.36–1.51	0.409			
OB Positive (n=43)	20 (22.2%)	23 (26.1%)	1.24	0.62–2.46	0.542			
Blood								
CRP (mg/L) >10 (n=82)	38 (42.2%)	44(50.0%)	0.73	0.41–1.32	0.298			
WBC (cells $\times 10^9$/L) >10 (n=50)	18 (20.0%)	32 (36.4%)	2.29	1.16–4.49	0.016	0.58	0.18–1.89	0.368
Neutrophils > 70% (n=79)	34 (37.8%)	45(48.9%)	0.64	0.35–1.15	0.136			
Lymphocyte > 40% (n=139)	23 (25.6%)	16 (18.2%)	1.55	0.75–3.17	0.236			
Monocyte > 10% (n=20)	10 (11.1%)	10 (11.4%)	0.88	0.34–2.28	0.788			
Eosinophils > 8% (n=6)	2 (2.2%)	4 (4.5%)	2.10	0.37–11.74	0.400			
Basophils > 1% (n=1)	1 (1.1%)	0	N/A[b]	N/A	N/A			
Hb (g/L) < 120 (n=95)	45 (50.00%)	50 (56.8%)	0.76	0.42–1.37	0.362			
PLT (cells $\times 10^9$/L) <100 (n=19)	4 (4.4%)	15 (17.0%)	4.42	1.40–13.90	0.011	5.19	1.58–17.04	0.007
Creatinine (umol/L) > 111 (n=20)	9 (10.0%)	11 (12.5%)	0.78	0.31–1.98	0.598			
Glucose (mmol/L) >6.1 (n=40)	22 (24.4%)	18 (20.5%)	1.26	0.62–2.55	0.524			
Kalium (mmol/L) >5.5 (n=5)	1 (1.1%)	4 (4.5%)	0.47	0.04–5.30	0.543			

Notes: [a]S-RBC: stool-red blood cell; S-WBC: stool-white blood cell; OB: occult blood; CRP: C-reactive protein; Hb: haemoglobin; PLT: platelets. [b]N/A: not applicable.

Table 4 Comparison of CDI Severity Scores in MLS_B Resistant *C. difficile* Between with and Without *ermB* Genes

Toxin Gene Pattern	CDI Severity Score		Nonparametric Tests	
	Positive to *ermB*	Negative to *ermB*	*Z* value	*P* value
A^+B^+	2.17±0.41 (n=78)	2.25±0.62 (n=12)	−0.21	*P*= 0.836
A^-B^+	2.63±0.72 (n=81)	2.00 (n=7)	−2.41	*P*= 0.016

Discussion

CDI is increasingly becoming a public health concern worldwide. During recent years, several studies conducted in China have revealed that CDI might gradually be a leading intestinal infection related to antibiotic usage. Furthermore, more attention should be paid to the antibiotic resistance of *C. difficile* in China, following the clarification of the molecular epidemiology of CDI. To the best of our knowledge, this is the first study to disclose the molecular characteristics of toxigenic *C. difficile* resistant to MLS_B in community acquired CDI patients in Eastern China to address the different genotypes resulting in various CDI severities. In this study, we found that *C. difficile* isolates in this region have a high level of resistance to MLS_B, with 88.4% resistance to CLI and 77.9% resistance to ERY. The A^-B^+ *C. difficile* isolates, especially ST37, led to more severe CDI, with higher severity score than that induced by A^+B^+ isolates in *ermB*-positive MLS_B-resistant isolates, indicating that *ermB*-positive A^-B^+ *C. difficile* resistant to MLS_B has become an epidemic clone inducing severe CDI in Eastern China.

The MLS_B resistance rates in different countries ranged from 14.7% to 90.9% between 2002 and 2009.[17] The strains of *C. difficile* carried over 50% of MLS_B resistance rates with high-level resistance in Asia, and the MIC_{90} (mg/L) was equal to or more than 128 mg/L, except in a study in Taiwan.[26] Antibiotic resistance data for *C. difficile* strains are still not enough to reveal the accurate antibiotic resistance profile in China. MLS_B resistance data over the last 10 years showed that the average rate of resistance to ERY and CLI was 80.2% and 81.7% in China mainland, respectively. Our previous study results showed that the MLS_B resistance rates in Zhejiang were lower than those observed in Shanghai.[15,27] Nevertheless, high levels of MLS_B with 88.4% (168/190) resistance to CLI and 77.9% (148/190) resistance to ERY were observed in this study, and 100% (88/88) of the A^-B^+ *C. difficile* isolates were resistant to MLS_B. These data show a sharp increase in MLS_B resistance with statistical differences in comparison with the data on ERY (64.7%, 266/411) and CLI (62.5%, 257/411) resistance obtained in our previous study before 2015 (ERY: $\chi^2 = 10.52$, $P = 0.001$, and CLI: $\chi^2 = 42.06$, $P < 0.001$).[15] Thus, an obvious elevated trend of MLS_B resistance was observed, which is noteworthy.

It has been well documented that high-level resistance against MLS_B requires the *ermB* gene despite the low carrying rate of the *ermB* gene commonly found in MLS_B-resistant *C. difficile* strains.[17] A high proportion, that is, 85% of *ermB*-positive A^-B^+ *C. difficile* isolates presented resistance to MLS_B antibiotics in a previous study.[28] A study on the emergence of an epidemic due to a CLI-resistant *C. difficile* clone among Polish patients also showed that both A^-B^+ and A^+B^+ strains were resistant to MLS_B antibiotics and that it was related with the *ermB* gene.[13] Interestingly, we also found that a remarkably high proportion, that is, 90% (81 of 88) of A^-B^+ and 86.7% (78 of 90) of A^+B^+ *C. difficile* isolates showed resistance to MLS_B associated with the presence of *ermB*. The above results demonstrated that *C. difficile* strains resistant to MLS_B might be mainly driven through the *ermB* gene in Eastern China. We are currently conducting a large-scale molecular epidemiological study to determine whether the *ermB* gene mediates MLS_B resistance.

PCR ribotypes 027 and 078 were hypervirulent *C. difficile* inducing severe CDI. We found that patients infected by the *ermB*-positive A^-B^+ isolates resistant to MLS_B had more severe symptoms, with higher CDI severity scores than those of patients infected by A^+B^+ isolates. Interestingly, the *ermB*-positive ST37 strain, one of the three A^-B^+ genotypes in this study, seemed to be the main genotype driving moderate-to-severe CDI. Moreover, all the CDI patients were community-acquired with high diverse STs, demonstrating that community-associated CDI might be a main pattern of *C. difficile* transmission in China. The above similar conclusions were drawn from our previous studies.[15,29] A high proportion of MLS_B resistance among A^-B^+ isolates, especially ST37, should be underlined in patients with community-acquired CDI in comparison with that of A^+B^+ *C. difficile*. Resistance to MLS_B enhanced the risk of CDI and promoted the persistence and transmission of *C. difficile*. The

Table 5 Correlation Among CDI Severities, Genotypes and *ermB* of *C. difficile* with Resistance to MLS_B

Characteristics[a]	Positive to *ermB*					Negative to *ermB*				
	CDI Severity Score			Trend Chi-square test		CDI Severity Score			Trend Chi-square Test	
	2(n=107)	3(n=40)	4(n=12)	x^2 value	*P* value	2(n=17)	3(n=1)	4(n=1)	x^2 value	*P* value
Toxin gene pattern										
A^+B^+(n=90)	66(61.7%)	11(27.5%)	1(8.3%)[b]	21.62	<0.001	10(58.8%)	1(100.0%)	1(100.0%)	1.10	0.509
A^-B^+(n=88)	41(38.3%)	29(72.5%)	11(91.7%)[b]			7(41.2%)	0(0.0%)	0(0.0%)		
ST types[c]										
3(n=20)	13(2.1%)	4(10.0%)	0(0.0%)	1.35	0.311	2(11.8%)	0(0.0%)	1(100%)	3.67	0.158
35(n=14)	8(7.5%)	4(10.0%)	1(8.3%)	0.13	0.818	1(5.9%)	0(0.0%)	0(0.0%)	0.11	1.000
54(n=29)	21(19.6%)	2(5.0%)	0(0.0%)	6.80	0.010	5(29.4%)	1(100.0%)	0(0.0%)	<0.01	1.000
37(n=58)	21(19.6%)	22(55.0%)	9(75.0%)	26.38	<0.001	6(35.3%)	0(0.0%)	0(0.0%)	0.87	0.772
39(n=11)	7(6.5%)	3(7.5%)	0(0.0%)	0.29	0.632	1(5.9%)	0(0.0%)	0(0.0%)	0.11	1.000
81(n=19)	13(12.1%)	4(10.0%)	2(16.7%)	0.02	1.000	0(0.0%)	0(0.0%)	0(0.0%)	N/A[d]	N/A
Other STs(n=27)	24(22.4%)	1(2.5%)	0(0.0%)	9.90	0.001	2(11.8%)	0(0.0%)	0(0.0%)	0.22	1.000

Notes: [a]Isolates with resistance to MLS_B. [b]The number of A^-B^+ isolates was significantly more than those of A^+B^+ (x^2 = 8.61, *P* = 0.003). [c]There were statistically significant differences in the CDI severity scores among different STs in *ermB*-positive isolates (x^2 = 36.77, *P* = 0.001). [d]N/A: not applicable.

ermB gene is located in a transposon incorporated at a homologous site to the *tcdA* gene, indicating that there is a direct association between the biological characteristics of A^-B^+ isolates and MLS_B resistance.[28] However, it is still not clear why only ST37 rather than ST39 and ST81 caused moderate to severe CDI. We speculate that gene polymorphism, interaction among intestinal bacteria, metabolites, and other factors might have affected the pathogenicity of ST37.

Epidemics of CDI induced by MLS_B-resistant *C. difficile* have been reported in the US and Europe.[11,30,31] PCR ribotype 027 resistant to MLS_B has been considered a significant and alarming emergence, driving the spread of this clone. The multiple locus variable number tandem repeat analysis showed that CLI-resistant ribotype 027 has a distant genetic relationship with CLI-susceptible 027 with a summed tandem repeat difference of 17.[11] In the present study, MLST was used to analyze the genetic relationship among different isolates because of various STs existed. The results revealed that 93.7% of the isolates, including all the A^-B^+ and 88.2% of A^+B^+ isolates, were resistant to MLS_B. Most *C. difficile* strains have obtained the characteristic of MLS_B resistance with the overwhelming trend. The above results strongly revealed that *C. difficile* with MLS_B resistance has been gradually becoming a dominant resistance phenotype in Eastern China. However, our study still has its own limitations as follows. This study enrolled a small scale of *C. difficile* isolates from only two tertiary hospitals, and only CA CDI cases were involved in this study. Moreover, we did not test resistance to other antibiotics, including fluoroquinolones, aminoglycoside, and tetracylines; therefore, the dynamic change of this clone should be underlined in the future in a large-scale study on molecular epidemiology of CDI.

Conclusions

In conclusion, the results of the present study showed the latest MLS_B resistance development of *C. difficile* isolates from CA CDI patients in Eastern China. MLS_B-resistant *C. difficile* isolates have been increasing in CDI cases dynamically. The *ermB*-positive A^-B^+ *C. difficile* resistant to MLS_B has been a potential epidemic clone inducing severe CDI in this region. The epidemic scale of this clone might be widened gradually, and we speculate that MLS_B resistance in A^-B^+ *C. difficile* with the *ermB* gene might be a probable predictor of enhanced CDI. Continuous surveillance by genotyping and antibiotic resistance testing focusing on *ermB*-positive A^-B^+ *C. difficile* resistant to MLS_B should be conducted for monitoring changes in MLS_B resistance and preventing epidemics with severe CDI.

Ethics Approval

The ethics committee of Zhejiang Provincial Center for Disease Control and Prevention has approved this study.

Abbreviations

MLS_B: Macrolide-lincosamide-streptogramin B; *Clostridioides difficile* infection: CDI; ermB: erythromycin resistance methylase gene; CLI: Clindamycin; ERY: erythromycin; HFPH: Affiliated Hangzhou First People's Hospital; LSPH:

Lishui Second People's Hospital; SHEA/IDSA: Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America; ZJCDC: Zhejiang Provincial Center for Disease Control and Prevention; S-RBCs: Stool-red blood cells; S-WBCs: Stool-white blood cells; OB: Occult blood; CRP: C-reactive protein; WBCs: White-blood cells; Hb: hemoglobin; PLT: Platelets; MIC: Minimal inhibitory concentration; CLSI: Clinical and Laboratory Standards Institute; MLST: Multilocus sequence typing; ST: Sequence type.

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Author Contributions

LYZ, YL, and DZJ wrote the manuscript and performed antibiotics resistance testing. QB, YL, YWT and JMJ analyzed the data. LYZ, LQW and XJW collected clinical samples and summarized clinical data. LYZ, YL, JLY and XJS performed isolation, MLST and antibiotics resistance testing. DZJ, YWT, and JMJ conceived the study and designed the experiments. XJW and YWT edited the manuscript. All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work. All authors also read and approved the final manuscript.

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Disclosure

Yi-Wei Tang was an employee of Cephei, Danaher Diagnostic Platform, Shanghai, China during the conduct of the study. All authors declare that they have no other competing interests.

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