ORIGINAL RESEARCH

Higher Prevalence of Multi-Antimicrobial Resistant *Bacteroides* spp. Strains Isolated at a Tertiary Teaching Hospital in China

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Purpose: The study investigates the molecular epidemiology of multi-drug resistant (MDR) *Bacteroides* spp. isolates and the clinical characteristics of the patients.

Materials and Methods: *Bacteroides* spp. clinical strains were identified through MALDI-TOF MS and VITEK-2 anaerobes and corynebacterium (ANC) cards. A broth microdilution method was employed to detect the antimicrobial sensitivities of *Bacteroides* spp. isolates. PCR was used to detect the resistance genes, including *cfxA, cepA, cfiA, ermF, nim*, as well as the upstream insertion sequence (IS) element of the *cfiA* gene. The effects of broad-spectrum efflux pump inhibitors (EPIs) on the minimal inhibitory concentration (MICs) of cefoxitin, moxifloxacin, and imipenem for MDR *Bacteroides* spp. were investigated.

Results: The total resistance rates of 115 *Bacteroides* spp. isolates to cefoxitin, moxifloxacin, clindamycin, metronidazole, imipenem and meropenem were 4.3%, 16.5%, 80.0%, 5.2%, 13.9% and 13.9%, respectively. The positive rates of carbapenem resistance gene *cfiA* were 38.9% and 8.6% for *B. fragilis* and non-*B. fragilis* isolates, respectively. The isolation rate of MDR isolates reached up to 18.26% (21/115), and the isolation rate among the gastrointestinal cancer patients was significantly higher when compared to the non-gastrointestinal cancer patients (52.38%/26.08%, *P* = 0.006). Furthermore, MDR isolates were more likely to be isolated from the patients exposed to cephalosporins 3 months before *Bacteroides* spp. isolation (76.19%/31.52%, *P* = 0.000).

Conclusion: The overall resistance rates of *Bacteroides* spp. isolates against multiple antimicrobials were at a high level, especially for *B. fragilis*. The *CfiA* gene carrying rate among *B. fragilis* isolates was as high as 38.9%, and its mediated carbapenem resistance was the major resistance mechanism for *B. fragilis*. The findings of this study imply that the real resistance tendency of *Bacteroides* spp. may be underestimated and need to be given more attention.

Keywords: anaerobe, *Bacteroides fragilis*, carbapenem resistance, clinical characteristics

Introduction

Bacteroides spp. is a common anaerobe that occupies the intestines of humans. It is also the most common anaerobe recovered from various infections, such as intra-abdominal infection, foot ulcer and bloodstream infection.[1–3] In recent years, MDR *Bacteroides fragilis* (*B. fragilis*) has emerged in several areas around the world.[4–6] However, its resistance capability has been significantly varied among different geographical locations.[7]

In treating an infection of *Bacteroides* spp., metronidazole, clindamycin and β-lactam antimicrobials are routinely prescribed. Based on relevant literature, the resistance of

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Bacteroides spp. isolates to these kinds of antimicrobials were related to some specific resistance genes. The *nim* gene was found to be closely correlated with metronidazole,[8,9] while the presence of the *cfxA* gene strongly correlated with cefoxitin resistance.[10] Macrolide-Lincosamide-Streptogramin B resistant determinants, such as *erm* genes, are widely distributed among *Bacteroides* spp. isolates and the *ermF* gene is responsible for *B. fragilis* resistance to clindamycin.[10,11] The existence of imipenem resistant *Bacteroides* spp. isolates were first reported over three decades ago.[12] As reported in recent studies, the primary mechanism of carbapenem resistance of *B. fragilis* was the production of metallo-β-lactamase that is encoded by the *cfiA* gene;[13] the specific upstream insertion sequence (IS) element is required for its expression and has been confirmed by some subsequent investigations.[14] However, owing to a lack of popularization of anaerobic cell culture in Chinese hospitals, not as much is known regarding the actual resistance tendency and resistance mechanism of *Bacteroides* spp. clinical isolates.

In this study, we aim to detect the resistance features and possible resistance mechanism(s) of *Bacteroides* spp. clinical isolates collected at a 3000-bed tertiary teaching hospital in China.

Materials and Methods
Anaerobes Culture, Isolation and Identification

Between March 2017 and February 2019, all of the anaerobic samples were cultured under anaerobic conditions. The anaerobe isolates were collected from clinical samples of patients from the Affiliated hospital of Inner Mongolian Medical University and identified with both Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (microTyper MS, Tianrui, China) and VITEK-2 Compact automated microbiology system (BioMérieux, France) with anaerobes and corynebacterium (ANC) cards (BioMérieux, France). The duplicated isolates found in individuals were deleted.

Clinical Characteristics of the Patients Enrolled in This Study

A total of 113 patients were enrolled in this study. The patients were mainly middle-aged and older adults, with a median age of 49 years (range 2 to 82), and male patients accounted for 60.18% (68/113), females accounted for 39.82% (45/113). Intra-abdominal and pelvic infections accounted for the most substantial proportion (92.04%,

104/113), followed by bacteremia (4.42%, 5/113), skin and soft tissue (2.65%, 3/113) and pulmonary infection (0.88%, 1/113).

Antimicrobial Susceptibility Test

The minimal inhibitory concentration (MIC) values of 11 antibiotics against all of the isolates was determined using a broth microdilution method under anaerobic conditions. This was done according to the recommendation of the Clinical and Laboratory Standard Institute (CLSI)[15] and the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[16] The antibiotics were as follows: ampicillin/sulbactam, amoxicillin/clavulanic acid, imipenem, cefoxitin, meropenem, piperacillin, moxifloxacin, piperacillin/tazobactam, metronidazole, clindamycin, and tigecycline. The following concentrations of the antibiotics were tested: ampicillin/Sulbactam (0.25/0.125–32/16μg/mL), amoxicillin/clavulanic acid (0.125/0.06–16/8μg/mL), imipenem (0.25–32μg/mL), cefoxitin (0.5–64μg/mL), meropenem (0.25–32μg/mL), clindamycin (0.125–16μg/mL), piperacillin (0.5–128μg/mL), metronidazole (0.25–32μg/mL), moxifloxacin (0.06–8μg/mL), piperacillin/tazobactam (0.5/4–64/4μg/mL), and tigecycline (0.25–32μg/mL).

After 48 hours incubation at 35°C under anaerobic condition, the antimicrobial MIC values were evaluated based on the breakpoints of CLSI and EUCAST,[16] as seen in Table 1. *B. fragilis* ATCC 25285 was used as the control strain. Production of metallo-β-lactamases (MBLs) was detected by an EDTA inhibition assay for any isolates that had reduced susceptibility or resistance to carbapenem antibiotics.

Detection of Resistance Genes

DNA of the isolates was extracted using a bacterial DNA extraction kit (Qiagen Ltd, Germany). The most common antibiotic resistance genes (*cepA, cfxA, cfiA, ermF, nim*)[4,8] and the upstream insertion sequence (IS)[14] of the *cfiA* gene was amplified. Then, the PCR products were analyzed with 1.2% agarose gel electrophoresis, and the DNA amplicon of the PCR was purified using the QIAquick Gel Extraction Kit (Qiagen Ltd, Germany). The IS elements were further verified by PCR using well-known primers[17] and then sequenced.

Efflux Pump Inhibition Assay

Broad-spectrum efflux pump inhibitors (EPIs), carbonyl cyanide m-chlorophenylhydrazone (CCCP) and verapamil (Sigma, St Louis, MO, US) were selected to test the effects

Table 1 Demographic and Clinical Characteristics of 113 Inpatients Included in the Study

Variables	No. (%) or Mean ± SD for Group		p value
	Cases Infected with MDR Bacteria (21 Cases)	Cases Infected with Non-MDR Bacteria (92 Cases)	
Age	49±21	47±21	0.695
Male gender	15 (71.43%)	53 (57.61%)	0.273
Receiving surgical operation	14 (66.67%)	44 (47.83%)	1.000
Comorbid conditions			
Diabetes	0 (0.00%)	3 (3.26%)	0.942
Gastrointestinal cancer	11 (52.38%)	24 (26.08%)	0.006[a]
Exposure to antibiotics within 3 months before *Bacteroides* spp. isolation			
Penicillins	0 (0.00%)	1 (1.08%)	1.000
Cephalosporins	16 (76.19%)	29 (31.52%)	0.000[a]
Carbapenems	4 (19.05%)	4 (4.35%)	0.053
Fluoroquinolones	4 (19%)	20 (21.74%)	1.000
Metronidazole	6 (28.57%)	15 (16.30%)	0.298
Clindamycin	0 (0.00%)	0 (0.00%)	-

Note: [a]P-values <.05.

of EPIs on the MICs of cefoxitin, moxifloxacin, and imipenem for MDR *Bacteroides* spp. In this study, multi-drug resistant (MDR) isolates were defined as the ones resistant to at least three types of antimicrobials, including penicillins (ampicillin/sulbactam, amoxicillin/clavulanic acid, piperacillin, and piperacillin/tazobactam), cephalosporins (cefoxitin), carbapenems (imipenem and meropenem), clindamycin, nitroimidazoles (metronidazole) and fluoroquinolones. CCCP or verapamil was added to brucella blood agar plates to obtain final concentrations of 25μg/mL and 100μg/mL, respectively.[18] Antimicrobials tested in this study were added to the plates in gradient mode beforehand. Then, the plates were inoculated with the test isolates at the required concentration and incubated anaerobically at 35°C for 48 h, and the MIC of each plate was measured.

Statistical Analysis

Two-side Student's *t*-tests, Chi-square tests and Fisher's exact tests were used to perform univariate analyses. SPSS software version 23.0 (SPSS, Chicago, IL, USA)

was used for the analysis. *P*-values of <0.05 were considered statistically significant.

Results

Clinical Characteristics of the Patients Infected with MDR Isolates

A total of 21 MDR *Bacteroides* spp. isolates were collected in this study. The isolation rate among the gastrointestinal cancer patients was significantly higher compared to the non-gastrointestinal cancer patients (52.38%/26.08%, *P* = 0.006). Furthermore, MDR isolates were more likely to be isolated from the patients who were exposed to cephalosporins within 3 months before *Bacteroides* spp. isolation (76.19%/31.52%, *P* = 0.000), as shown in Table 1.

Antimicrobial Susceptibility Test results

Among 115 *Bacteroides* spp. isolates, *B. fragilis* accounted for 69.6% (80/115), followed by *Bacteroides thetaiotaomicron* (17.4%, 20/115), *Bacteroides ovatus* (3.5%, 4/115), *Bacteroides uniformis* (2.6%, 3/115), *Bacteroides vulgatus* (2.6%, 3/115), *Bacteroides novobacterioides* (2.6%, 3/115), and *Parabacteroides distasonis* (1.7%, 2/115). The total resistance rates of 115 *Bacteroides* spp. isolates to ampicillin/sulbactam, amoxicillin/clavulanic, cefoxitin, piperacillin, piperacillin/tazobactam, moxifloxacin, clindamycin, metronidazole, imipenem and meropenem were 22.6%, 19.6%, 4.3%, 27.8%, 8.7%, 16.5%, 80.0%, 5.2%, 13.9% and 13.9%, respectively.

When comparing against non-*B. fragilis* isolates, *B. fragilis* isolates showed a higher level of resistance to the majority of the antimicrobials used in this study, as shown in Table 2. The resistance rates of 80 *B. fragilis* isolates to both imipenem and meropenem were 18.8% (15/80). A total of 26 *Bacteroides* spp. isolates had reduced susceptibility or were resistant to imipenem or meropenem, 16 isolates were found to be MBL producers, including 15 isolates of *B. fragilis* and one isolate of *B. thetaiotaomicron*, respectively. Moreover, MIC$_{90}$ values of *B. fragilis* and non-*B. fragilis* isolates against tigecycline were 4 μg/mL and 2 μg/mL, respectively.

Distribution of Resistance Genes

Among 115 *Bacteroides* spp. isolates, the positive rates of resistance genes *cfiA, cfxA, cepA, ermF* and *nim* were 29.6% (34/115), 32.2% (37/115), 65.2% (75/115), 89.6% (103/115) and 30.4% (35/115), respectively. The positive rates of the above resistance genes of *B. fragilis* isolates

Table 2 In vitro Activities of 11 Antimicrobial Agents Against 115 *Bacteroides* spp. Isolates

Antimicrobials	MIC (µg/mL)			No. of Isolates (%)		
	Range	MIC$_{50}$	MIC$_{90}$	S	I	R
Bacteroides fragilis (80)						
Ampicillin/sulbactam	0.25/0.1–32/16	4	≥32	62.5	10.0	27.5
Amoxicillin/clavulanic acid	0.125/0.06–16/8	2	≥16	61.2	5.0	33.8
Cefoxitin	0.5–64	4	32	83.7	12.5	3.8
Piperacillin	0.5–128	8	≥64	67.4	3.8	28.8
Piperacillin/tazobactam	0.5/4–64/4	2	≥64	88.7	0.0	11.3
Imipenem	0.25–32	2	32	74.9	6.3	18.8
Meropenem	0.25–32	0.5	≥32	77.4	3.8	18.8
Clindamycin	0.125–16	≥16	≥16	12.5	0.0	87.5
Metronidazole	0.25–32	0.5	8	92.5	0.0	7.50
Moxifloxacin	0.06–8	1	8	76.2	7.5	16.3
Tigecycline[a]	0.25–32	1	4	-	-	-
Non-*B.fragilis*(35)						
Ampicillin/sulbactam	0.25/0.1–32/16	4	32	77.2	11.4	11.4
Amoxicillin/clavulanic acid	0.125/0.06–16/8	2	16	71.4	8.6	20.0
Cefoxitin	0.5–64	8	32	83.3	11.4	5.7
Piperacillin	0.5–128	32	≥64	74.3	0.0	25.7
Piperacillin/Tazobactam	0.5/4–64/4	4	8	97.1	0.0	2.9
Imipenem	0.25–32	4	8	82.8	14.3	2.9
Meropenem	0.25–32	0.5	1	97.1	0.0	2.9
Clindamycin	0.125–16	≥16	≥16	31.4	0.0	62.9
Metronidazole	0.25–32	0.25	1	100	0.0	0.0
Moxifloxacin	0.06–8	0.5	8	82.9	0.0	17.1
Tigecycline[a]	0.25–32	0.5	2	–	–	–

Note: [a]No breakpoint is available.
Abbreviations: MIC, minimal inhibitory concentration; MIC50, MIC for inhibition of 50% of the isolates tested in this study; MIC90, MIC for inhibition of 90% of the isolates tested in this study.

were all significantly higher than those of non-*B. fragilis* isolates, as shown in Figure 1. A total of 31 *B. fragilis* and four non-*B.fragilis* isolates were positive when testing for the *nim* gene; however, only 6 *B. fragilis* isolates showed resistance to metronidazole.

For 15 imipenem resistant *B. fragilis* isolates, tests for the *cfiA* gene were all positive, and 13 isolates harbored the IS element, which belonged to IS1187 (11 isolates),
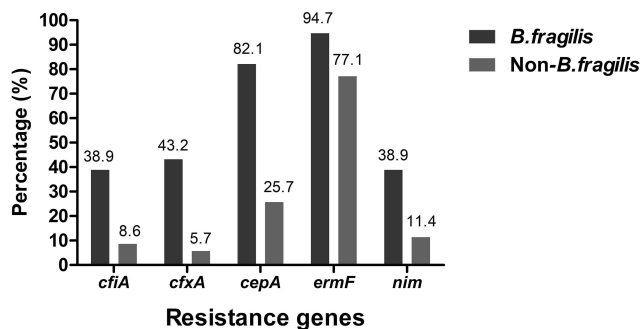
IS942 (1 isolate) and IS1169 (1 isolate), as shown in Table 3. Only 1 non-*B. fragilis* isolate was found to be carbapenem-resistant, which was positive when testing for the *cfiA* gene; however, no IS element was detected (Figure 2). Among the 13 isolates with both a *cfiA* gene and an IS element, the BF-18 isolate had a significantly longer IS element (3290 bp) than any other IS elements of the other 12 isolates. Moreover, the IS sequence was an inverted repeat segment. Nonetheless, there were also two inverted repeats in another typical isolate (BF-15) that belonged to IS1187. The positional relations between the *cfiA* gene and the IS sequence within BF-18 and BF-15 isolates are shown in Figure 3.

Effects of EPIs on MICs of Antimicrobials Tested

Two kinds of EPIs, CCCP and verapamil, were selected in this study as they can inhibit the RND-family and non-RND-family efflux pumps, respectively. A total of

Figure 1 Prevalence of resistance genes among *B. fragilis* and non-*B. fragilis* isolates.

Table 3 Characteristics of 16 Carbapanem Resistant *Bacteroides*spp. Isolates

Isolates	MIC(μg/mL)											EDTA Inhibition Assay	Resistance Genes					
	IMI	MEM	SAM	AMC	FOX	CLI	MTZ	TZP	PRL	MOX	TGC		cfiA	IS Element	cepA	ermF	cfxA	nim
BF-09	16	16	32/16	≥16/8	16	≥16	16	32/4	≥128	4	1	+	+	IS1187	+	+	+	-
BF-15	32	≥32	≥32/16	≥16/8	16	≥16	16	≥64/4	≥128	2	4	+	+	IS1187	-	+	+	+
BF-18	≥32	≥32	≥32/16	≥16/8	32	≥16	4	≥64/4	≥128	0.12	0.5	+	+	IS942	+	+	+	+
BF-20	16	≥32	32/16	16/8	16	≥16	4	≥64/4	≥128	0.25	1	+	+	IS1187	-	-	-	-
BF-31	≥32	≥32	32/16	16/8	32	16	0.25	32/4	≥128	1	1	+	+	IS1187	-	+	+	-
BF-36	≥32	≥32	≥32/16	≥16/8	32	≥16	4	≥64/4	≥128	0.5	1	+	+	IS1169	-	+	+	+
BF-37	16	16	≥32/16	≥16/8	32	≥16	1	≥64/4	≥128	1	1	+	+	IS1187	-	+	+	+
BF-41	16	32	16/8	16/8	16	≥16	1	32/4	≥128	0.5	1	+	+	IS1187	+	+	-	+
BF-43	16	32	16/8	16/8	16	≥16	1	32/4	≥128	0.5	1	+	+	IS1187	-	+	-	-
BF-49	16	32	16/8	16/8	16	≥16	0.25	32/4	≥128	0.5	1	+	+	IS1187	-	+	+	-
BF-58	≥32	≥32	≥32/16	≥16/8	64	≥16	0.5	≥64/4	≥128	8	2	+	+	None	-	+	-	+
BF-60	32	≥32	≥32/16	≥16/8	64	≥16	1	≥64/4	≥128	≥8	1	+	+	None	+	+	-	+
BF-67	32	≥32	≥32/16	≥16/8	32	≥16	4	≥64/4	≥128	4	4	+	+	IS1187	-	+	-	+
BF-69	16	≥32	≥32/16	≥16/8	32	≥16	1	≥64/4	≥128	0.5	1	+	+	IS1187	-	+	-	+
BF-76	16	32	≥32/16	≥16/8	32	8	0.25	32/4	≥128	0.12	1	+	+	IS1187	-	+	-	-
BT-10	16	≥32	≥32/16	≥16/8	64	≥16	0.25	32/4	≥128	0.5	4	+	+	None	-	+	-	-

Abbreviations: BF, *Bacteroides fragilis*; BT, *Bacteroides thetaiotaomicron*; IPM, imipenem; MEM, meropenem; SAM, ampicillin/sulbactam; AMC, amoxicillin/clavulanic acid; FOX, cefoxitin; CLI, clindamycin; MTZ, metronidazole; TZP, piperacillin/tazobactam; PRL, piperacillin; MOX, moxifloxacin; TGC, tigecycline.
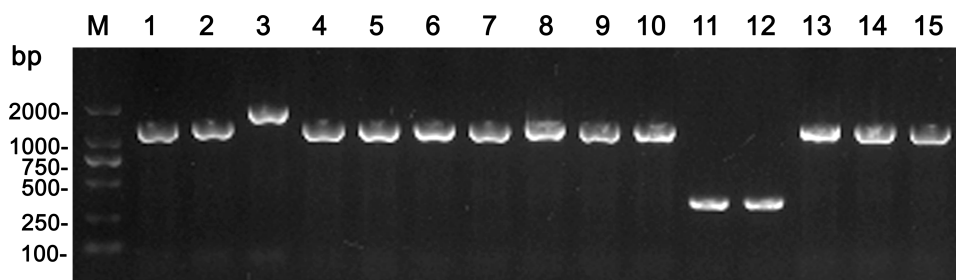
Figure 2 PCR results of *cfiA* and IS element of 15 carbapenem resistant *B. fragilis* isolates.
Notes: 1, BF-09; 2, BF-15; 3, BF-18; 4, BF-20; 5, BF-31; 6, BF-36; 7, BF-37; 8, BF-41; 9, BF-43; 10, BF-49; 11, BF-58; 12, BF-60; 13, BF-67; 14, BF-69; 15, BF-76
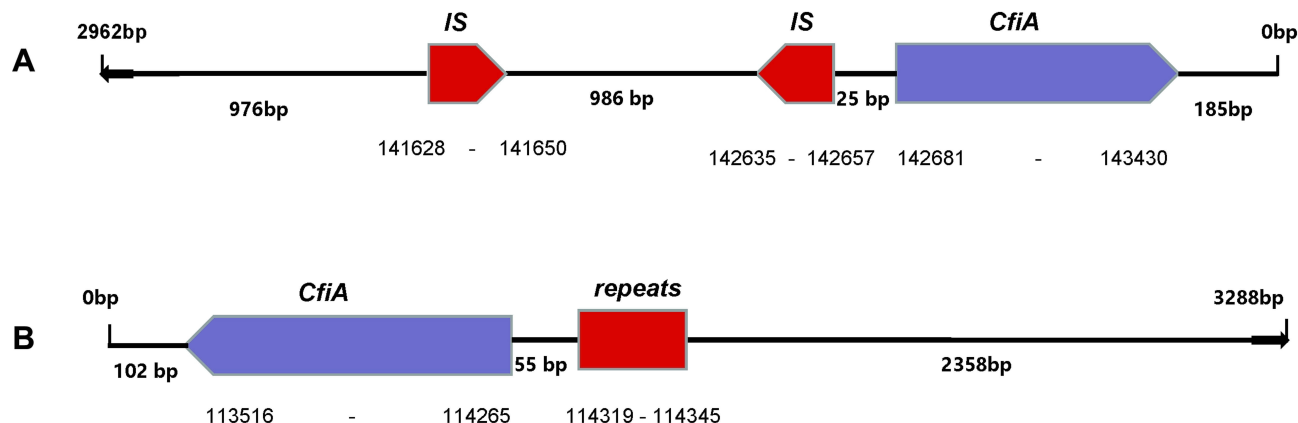

Figure 3 IS element analysis of BF-15 and BF-18 isolates.
Notes: (**A**) BF-15 isolate; (**B**) BF-18 isolate.

21 MDR isolates with six different resistance patterns (a-h) were selected in this study. The antimicrobial MIC values of 10 *Bacteroides* spp. isolates (47.62%%, 10/21) showed a significant decrease by 4- to 64-fold, whereas the remaining isolates were not affected by EPIs. The MICs of four carbapenem-resistant isolates decreased significantly when EPIs were added. The same inhibition effects of CCCP and verapamil were also observed (Table 4). For three cefoxitin resistant *B.fragilis* isolates (BF-10, BF-58 and BF-60), neither CCCP nor verapamil showed an inhibitory effect on their MIC values. When 25 µg/mL of CCCP was added, the MICs of cefoxitin, moxifloxacin and imipenem for one *B. fragilis* isolate (BF-17) decreased simultaneously, as shown in Table 4. However, the isolate was not resistant to cefoxitin, moxifloxacin or imipenem. The MICs of moxifloxacin for four isolates decreased significantly when EPIs were added in eight moxifloxacin resistant isolates enrolled in this study. Lastly, the inhibition effects of CCCP and verapamil were not consistent among different isolates.

Discussion

In recent years, reports of resistance features in anaerobes have increased. However, only a few reports have come from China. In this study, 115 *Bacteroides* spp. clinical isolates were isolated from a 3000-bed tertiary teaching hospital in China that showed higher resistance rates to multiple commonly used antibiotics, including several β-lactams and metronidazole. The resistance rates of *Bacteroides* spp. isolates to carbapenem and metronidazole were at a higher level, and the isolates were mainly *B. fragilis*. It is well known that the mechanism of carbapenem resistance within *B. fragilis* is mainly mediated by the *cfiA* gene, and the upstream IS element is also required. In this study, the positive rate of the *cfiA* gene of carbapenem-resistant *B. fragilis* was as high as 38.9%, which is higher than other recently published reports.[10,18,19] Notably, *B. fragilis* isolates with a *cfiA* gene can easily be converted to resistant genes by the effects of its upstream IS element,[20] which implies that a larger group of patients may act as a potential reservoir of carbapenem-resistant *B. fragilis* producers who are colonized or

Table 4 Effects of EPIs on MICs of Cefoxitin, Moxifloxacin and Imipenem

Isolates	Cefoxitin (0.25–256) µg/mL			Moxifloxacin (0.12–128) µg/mL			Imipenem (0.12–128) µg/mL		
	Blank	+CCCP	+Verapamil	Blank	+CCCP	+Verapamil	Blank	+CCCP	+Verapamil
BF-10[a]	64	32	32	1	1	1	8	2[i]	0.5 [i]
BF-59[b]	4	4	4	32	8 [i]	8 [i]	8	8	8
BF-58[c]	64	64	64	8	1 [i]	4	64	64	64
BF-60[c]	64	64	64	32	16	8 [i]	32	32	32
BF-38[d]	16	8	16	8	4	4	0.12	0.12	0.12
BF-75[d]	16	16	16	8	8	8	2	2	2
BT-12[d]	8	4	4	16	16	8	0.5	0.5	0.5
BT-18[d]	16	4 [i]	4 [i]	16	1 [i]	4 [i]	2	1	1
BF-69[e]	32	32	32	0.5	0.5	0.5	16	16	16
BF-67[e]	32	32	16	4	4	2	32	32	32
BF-18[e]	32	32	32	0.12	0.12	0.12	64	16 [i]	16 [i]
BF-37[e]	32	32	32	1	1	1	16	16	16
BF-20[e]	16	16	16	0.25	0.25	0.25	16	16	16
BF-31[e]	32	32	32	1	0.5	0.5	64	64	64
BF-36[e]	32	8 [i]	16	0.5	0.25	0.5	64	2 [i]	1 [i]
BF-43[e]	16	16	16	0.5	0.5	0.25	16	2 [i]	1 [i]
BF-41[e]	16	16	16	0.5	0.5	0.5	16	16	16
BF-49[e]	16	4 [i]	4 [i]	0.5	0.5	0.5	16	2 [i]	1 [i]
BF-17[f]	8	2 [i]	8	2	0.25 [i]	0.5 [i]	4	0.25 [i]	0.25 [i]
BF-76[g]	32	32	32	8	4	4	16	16	16
BF-15[h]	8	8	8	2	2	2	32	32	32

Notes: [a]Resistant to penicillins, cephalosporins, clindamycin and nitroimidazoles; [b]resistant to penicillins, clindamycin, nitroimidazoles and fluoroquinolones; [c]resistant to penicillins, cephalosporins, carbapenems, clindamycin and fluoroquinolones; [d]resistant to penicillins, clindamycin and fluoroquinolones; [e]resistant to penicillins, clindamycin and carbapenems; [f]resistant to penicillins, nitroimidazoles and clindamycin; [g]resistant to penicillins, carbapenems, clindamycin and fluoroquinolones; [h]resistant to penicillins, carbapenems, clindamycin, nitroimidazoles and fluoroquinolones; [i]MIC values decreased ≥4 folds.

infected by *B. fragilis* with a silent *cfiA* gene. Since the *B. fragilis* isolates tested in this study were mainly from intra-abdominal samples, intestinal screening of *cfiA* positive *B. fragilis* within a specific group of people appears to be a beneficial strategy for better understanding the actual distribution of the *cfiA* gene. So far, only a few reports have shown that an IS-less activation mechanism was found to exist in *B. fragilis* isolates with elevated imipenem MICs or imipenem resistant isolates.[17] In this study, two *B. fragilis* isolates and one non-*B. fragilis* isolate resistant to carbapenem was found to be *cfiA* positive; however, the IS element was deficient. The potential non-IS resistance mechanism needs to be further investigated. In addition, a longer IS element (3290 bp) was detected in a carbapenem-resistant *B. fragilis* isolate, which was significantly different from other IS elements observed in this study. For this isolate, the MIC value of imipenem and meropenem were both higher than 32 µg/mL, which may possess a unique regulating mechanism in mediating carbapenem resistance. Furthermore, one *B. fragilis* isolate with an elevated imipenem MIC showed a reduction in their imipenem MIC value after exposure to the EPIs,

CCCP and verapamil. Interestingly, the *cfiA* gene was not detected in this isolate. Moreover, the *cfiA* mediated resistance mechanism and the overexpression of the efflux pump may be another important mechanism in mediating carbapenem resistance in *B. fragilis*, especially for those with lower resistance to carbapenem.

Apart from carbapenem antibiotics, the resistance rates of *B. fragilis* isolates to several other β-lactam antibiotics were all higher than those of non-*B. fragilis* isolates, including ampicillin/sulbactam, amoxicillin/clavulanic acid, piperacillin and piperacillin/tazobactam. Consistent with a resistance phenotype, the prevalence of *cepA* and *cfxA* genes among *B. fragilis* isolates were significantly higher than those of non-*B. fragilis* isolates. Veloo et al[5] reported that *cfiA* and *cepA* genes were collectively responsible for amoxicillin resistance, yet none of the *B. fragilis* isolates harbored both genes in this study. However, the resistance of amoxicillin was not detected, and a total of 20 *B. fragilis* isolates harbored both *cfiA* and *cepA* in this study. Notably, nine non-*B. fragilis* isolates harbored *cepA*, which was considerably different from what has been reported in previous reports.[5,21,22]

Kierzkowska et al[10] reported that in *B. fragilis*, phenotypic cefoxitin resistance was strongly correlated with *cfxA* gene expression. However, only two *B. fragilis* and two non-*B. fragilis* isolates were resistant to cefoxitin, and none of the isolates harbored the *cfxA* gene in this study. Owing to the limitation of the number of isolates, the correlation between cefoxitin resistance and expression of *cfxA* gene cannot be confirmed.

The resistance rate of *B. fragilis* to metronidazole in this study was 7.5%, which is notably higher than previous reports.[5–7,10,23,24] No metronidazole resistant non-*B. fragilis* isolate was detected either. To date, the mechanism of metronidazole resistance is still not well-known, and it is reported that *nim* genes closely correlate with metronidazole resistance.[8,9,25] The positive rate of *nim* gene detection in this study was 38.9% and 11.4% for *B. fragilis* and non-*B. fragilis* isolates, respectively. However, the correlation between metronidazole resistance and expression of the *nim* gene was not observed. For six metronidazole resistant *B. fragilis* isolates, only three isolates were positive with the *nim* gene. The primers selected to amplify *nim* genes was a pair of universal primers, which could simultaneously detect *nim A, nimB, nim C* and *nim D* genes. Despite the sequence divergence of the *nim* genes, the primers were used for epidemiological investigations of *nim* genes in some previous studies.[4,10] So, the *nim* genes reported in this study could not be assigned to a specific type of *nim* gene.

In this study, the total resistance rate of *B. fragilis* isolates against clindamycin was as high as 87.5%, which is significantly higher than that reported by Fernández-Canigia et al[6] and Justesen et al.[26] It is well known that *erm* (A-F) is widely distributed in *Bacteroides* spp. strains and are responsible for macrolide-lincosamide-streptogramin B resistance in *B. fragilis*.[27] In the present study, the positive rate of the *ermF* gene (94.7%) was comparable with the phenotypic resistance rate (87.5%), which suggests that the *ermF* gene may be mainly responsible for *B. fragilis* resistance in this study.

The resistance rate to moxifloxacin was 16.5% and is significantly lower than that observed in another study from China.[7] However, there is no apparent difference between *B. fragilis* isolates and non-*B. fragilis* isolates. It is reported that fluoroquinolone resistance may correlate with multiple mechanisms, including the mutation of the fluoroquinolone resistance determining region of the genes of gyrase and/or topoisomerase IV, as well as increased efflux.[10,28] In this study, the effects of overexpression of the efflux pump on MIC values of moxifloxacin was observed in 50% (4/8) of the isolates investigated in this study, including 3 *B. fragilis* and 1 *B. thetaiotaomicron* isolates.

Moreover, both RND-family and non-RND-family EPIs showed significant inhibition effects on the MIC of the *B. thetaiotaomicron* isolate. The inhibition effects of non-RND-family efflux pump inhibitor on fluoroquinolone resistance of *B. thetaiotaomicron* has been confirmed in a previous study,[28] and a multi-drug and toxic compound extrusion (MATE)-type efflux system has been well described.[28] The specific features of the efflux pump system in this isolate needs to be further investigated.

For tigecycline, the MIC_{50} and MIC_{90} for both *B. fragilis* and non-*B. fragilis* isolates in this study were all higher than those reported in a previous study.[6] Since there is no available breakpoint for tigecycline, it has not been feasible to assess its clinical significance, until now. In addition, the antimicrobial susceptibility testing method used in this study was a broth microdilution method; the results need to be further verified with the agar dilution method in a subsequent investigation.

During this study, a total of 21 multi-drug-resistant isolates were isolated from 21 different patients, 20 (95.24%, 20/21) of which were successfully cured with suitable antimicrobial combinations that were based on antimicrobial susceptibility test results; 19 patients among them were also co-infected with aerobic bacteria. One male patient who was 78 years old and had rectal cancer died during treatment; they had also received surgical intervention before *B. fragilis* isolation. Moreover, this patient developed bacteremia during the process of anti-infection treatment. Significantly, three patients aged 2, 4 and 13 years old, received surgical intervention for the treatment of acute appendicitis or hepatic abscess, and the MDR isolates should have originated from themselves. Notably, it is a stark reminder that resistance tendency of anaerobes among children may be underestimated or not well recognized, and universal monitoring resistance characteristics of anaerobic bacteria among specific groups of people should be given more attention.

Conclusion

The overall resistance rates of *B. fragilis* clinical isolates to several commonly used antimicrobials were at a high level, especially for carbapenem, and the dominant resistance mechanism to carbapenem was mediated by the *cfiA* gene. In addition, the resistance rate of *Bacteroides* spp. to metronidazole is at a high level in this study, and the actual resistance rate should be investigated with a larger quantity of isolates in the

future. The actual resistance tendency of anaerobes may be underestimated in some geographical locations. The MDR *B. fragilis* isolates were more likely to be isolated from patients who had a history of exposure to cephalosporins at some point over a previous 3-month period. Moreover, it should be noted that the emergence of MDR *B. fragilis* among child patients implies that antimicrobial usage should be more strictly restricted. Furthermore, universal screening for the resistance determinants of *B. fragilis* should also be performed among specific groups of people in the districts without any strict regulations against antibiotic prescription.

Ethics Approval and Consent to Participate

This study is exempt from formal ethical approval and informed consent according to the local ethical guidelines, since the isolates identification and in vitro antimicrobial susceptibility test were routinely performed for normal clinical microbiological samples and the data analysis was analyzed anonymously.

Acknowledgments

Disclosure

The authors report no conflicts of interest in this work.

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