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Short Communication

Emergence of carbapenem resistance in *Bacteroides fragilis* in China

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ABSTRACT

The antimicrobial resistance crisis makes it critically important for laboratories to closely monitor trends and mechanisms of emerging antimicrobial resistance in clinical isolates. *Bacteroides fragilis* is an anaerobic pathogen that causes several serious infections and is increasingly resistant to antimicrobial agents. However, data from China regarding antimicrobial resistance in *B. fragilis* are limited. In this work, the mechanism underlying carbapenem resistance in 44 *B. fragilis* isolates collected from a Chinese hospital was investigated. Antimicrobial susceptibility testing for 13 antimicrobial agents was performed by the agar dilution method, and the contribution of efflux pumps to carbapenem resistance was analysed. Genetic relatedness of the isolates was determined by PFGE. Outer membrane porins were analysed in isolates with reduced carbapenem susceptibility. Potential carbapenemase-encoding genes were identified, and the location and environment of the *cfiA* gene was analysed. Among the 44 isolates, 18.2%, 29.5%, 22.7%, 100%, 100%, 29.5%, 15.9%, 81.8%, 88.6% and 47.7% were resistant to imipenem, meropenem, ertapenem, penicillin, ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, clindamycin, tetracycline and moxifloxacin, respectively. None of the isolates were resistant to metronidazole, cefoxitin or chloramphenicol. A chromosomally located gene (*cfiA*) encoding a metallo-β-lactamase was identified in 16 isolates (36.4%). A conserved insertion sequence of IS*1187* or IS*613* was upstream of *cfiA* in eight isolates with high-level carbapenem resistance. The insertion sequences were associated with increased carbapenem resistance in *B. fragilis* by upregulating the expression of *cfiA* as shown by RT-qPCR. This is the first study to describe a mechanism of carbapenem resistance in *B. fragilis* in mainland China.

1. Introduction

Bacteroides fragilis is a Gram-negative, rod-shaped, obligate anaerobic bacterium. It is one of the most common opportunistic pathogenic anaerobes and can cause intra-abdominal and pelvic infections, bloodstream infections, skin and soft-tissue infections and osteomyelitis [1]. Carbapenems are one of the most active antimicrobial agents against *B. fragilis*. However, in recent years *B. fragilis* isolates showing increasing resistance to carbapenems have been observed. A study in 2011 reported that the prevalence of imipenem-resistant *B. fragilis* increased from 0% to 1.2% in a 20-year period in Europe [2], which was similar to the increase in the USA [3]. A study in 2012 reported that 4% and 6% of 83 *B. fragilis* isolates from four Korean hospitals were resistant to imipenem and meropenem, respectively [4]. Furthermore, a report in 2014 showed that 13.5%, 8.5% and 9.9% of 141 *B. fragilis* isolates from Taiwan were resistant to ertapenem, imipenem and meropenem, respectively [5]. However, there is a lack reports of confirmed *B. fragilis*-associated infections in mainland China. More data are still needed to understand the resistance profile of *B. fragilis*.

In the current study, 44 *B. fragilis* isolates collected from Huashan Hospital (Shanghai, China) were investigated to determine the antimicrobial resistance spectrum and the emergence of carbapenem resistance in *B. fragilis* in mainland China.

2. Materials and methods

2.1. Bacterial isolates

A total of 44 non-duplicate *B. fragilis* isolates were collected at Huashan Hospital of Fudan University from January 2009 to December 2015, including 32 isolates from abdominal infections and 12 from faecal specimens of patients with nosocomial diarrhoea. *Bacteroides fragilis* ATCC 25285 was used as a control

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strain. All isolates were identified by 16S rRNA gene sequencing [6].

2.2. Antimicrobial susceptibility testing

Minimum inhibitory concentrations (MICs) were determined by the agar dilution method for 13 antimicrobial agents, including metronidazole, imipenem, meropenem, ertapenem, penicillin, ampicillin, amoxicillin/clavulanic acid (AMC), piperacillin/tazobactam (TZP), cefoxitin, clindamycin, tetracycline, moxifloxacin and chloramphenicol, following the recommendations of the Clinical and Laboratory Standards Institute (CLSI) [7]. Meanwhile, the efflux pump inhibitors carbonyl cyanide *m*-chlorophenyl hydrazine (CCCP) (10 μg/mL), reserpine (25 μg/mL) and phenylalanine-arginine β-naphthylamide (PAβN) (25 μg/mL) (all from Sigma, St Louis, MO) were added to the corresponding carbapenem-containing plates (ertapenem, meropenem or imipenem) to compare MIC changes before and after addition of the inhibitors. The contribution of efflux pump inhibitors to carbapenem resistance was confirmed if the reduction in the MIC was >2-fold [8].

2.3. Analysis of genetic relatedness and porin expression

The genetic relatedness of the 44 *B. fragilis* isolates was analysed by pulsed-field gel electrophoresis (PFGE) according to a previously described method [9]. *Salmonella enterica* serovar Braenderup H9812 was used as a molecular weight marker. Data were analysed using BioNumerics 4.0 software (Applied Maths, Sint-Martens-Latem, Belgium) to generate dendrograms to compare PFGE fingerprint similarities. Outer membrane proteins (OMPs) of the isolates with reduced carbapenem susceptibility (MIC > 4 μg/mL) were analysed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) [10].

2.4. Phenotypic identification of carbapenemases

The 3-aminobenzeneboronic acid inhibition assay and ethylene diamine tetra-acetic acid (EDTA) disk synergy assay were carried out to identify class A and class B carbapenemases. A carbapenemase-positive isolate was identified if the diameter of the bacterial inhibition zone was ≥5 mm larger than that around a disk containing the carbapenem alone [11].

2.5. Genotypic assay

PCR was conducted for the 44 *B. fragilis* isolates to identify their potential carbapenemase-encoding genes, including bla_{IMP}, bla_{SPM}, bla_{AIM}, bla_{VIM}, bla_{OXA}, bla_{GIM}, bla_{BIC}, bla_{SIM}, bla_{NDM}, bla_{DIM}, bla_{KPC} [12] and *cfiA* [13]. Positive amplicons were further analysed by DNA sequencing [6].

2.6. Plasmid transformation and Southern blotting

The chromosomal or plasmidic location of the *cfiA* gene was identified by *S1* nuclease PFGE (S1-PFGE) [14] and Southern blotting using a DIG High Prime DNA Labeling and Detection Starter Kit I (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. The primers used are given in Supplementary Table S1. If the result of S1-PFGE was negative, the genome of *cfiA*-containing isolates was extracted as a template for PCR amplification. Amplicons were subjected to Southern blotting following electrophoretic separation. The distribution of *cfiA* in plasmids was further confirmed by a plasmid transformation assay. Plasmids of *cfiA*-containing isolates were extracted using a Plasmid Miniprep Kit (Tiangen Biotech Co., Beijing, China). *Escherichia coli* DH5α competent cells were used to conduct the transformation of plasmids following the manufacturer's instructions (TaKaRa, Beijing, China). A plate containing ertapenem (0.5 μg/mL) was used to screen for transformants.

2.7. Quantitative reverse transcription PCR (RT-qPCR) analysis of cfiA transcription

RNA was extracted from *cfiA*-containing isolates, including insertion sequence (IS)-negative and IS-positive isolates, as well as *B. fragilis* ATCC 25285 using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). Genomic DNA was removed following the instructions for the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Shiga, Japan) for reverse transcription. The resulting cDNA were used as template for two-step RT-qPCR according to the instructions for the SYBR® Premix Ex Taq™ Kit (Tli RNaseH Plus) (TaKaRa). The *B. fragilis* 16S rRNA gene was used as an internal reference. One *cfiA*-containing and IS-negative isolate was used as a positive control. *Bacteroides fragilis* ATCC 25285 was used as a negative control. The primers for *cfiA* and the internal reference 16S rRNA gene are listed in Supplementary Table S1. All experiments were performed with at least three independent replicates. Three technical replicates were included for each experiment. Relative gene expression was quantified by the $2^{-\Delta\Delta CT}$ method [15]. Data were analysed by an independent samples *t*-test, and a *P*-value of <0.05 was considered significant. Expression changes were presented as the mean ± standard deviation.

2.8. Analysis of cfiA and its upstream insertion sequence

As previously described [16], *cfiA* and the upstream IS of all *cfiA*-containing *B. fragilis* isolates were characterised by PCR amplification and bidirectional sequencing (see Supplementary Table S1 for details of primers). DNA sequencing data were analysed using DNASTAR Lasergene 14.1 and ApE–A plasmid Editor v.2.0.52 software and were aligned with the sequences in the National Center for Biotechnology Information (NCBI) database (https://www.ncbi.nlm.nih.gov/) and ISfinder (https://www-is.biotoul.fr/), respectively. Potential promoter sequences were predicted using the BPROM database (http://www.softberry.com/).

3. Results

3.1. Genetic relatedness of the Bacteroides fragilis isolates

Using 70% similarity as a cut-off criterion, PFGE patterns showed that the 44 *B. fragilis* isolates were classified into two groups, comprising 30 (68.2%) in group A and 14 (31.8%) in group B (Supplementary Fig. S1).

3.2. Susceptibility profile of Bacteroides fragilis to antimicrobials

Among the 44 *B. fragilis* isolates, 8 (18.2%), 13 (29.5%), 10 (22.7%), 44 (100%), 44 (100%), 13 (29.5%), 7 (15.9%), 36 (81.8%), 39 (88.6%) and 21 (47.7%) were resistant to imipenem, meropenem, ertapenem, penicillin, ampicillin, AMC, TZP, clindamycin, tetracycline and moxifloxacin, respectively. None of the isolates were resistant to metronidazole, cefoxitin or chloramphenicol (Table 1). Sixteen isolates showed reduced carbapenem susceptibility as the MIC of at least one of three carbapenems tested was >4 μg/mL (Table 2). Eight isolates were highly resistant to all three carbapenems (MIC range 16–128 μg/mL) (Table 2).

3.3. Efflux pumps and porins analysis

The MICs of imipenem, meropenem and ertapenem in the eight highly carbapenem-resistant *B. fragilis* isolates were not changed

Table 1
Minimum inhibitory concentrations (MICs) of antimicrobial agents against 44 *Bacteroides fragilis* isolates by using the agar dilution method.

Antimicrobial agent	MIC interpretive criteria (μg/mL)			MIC (μg/mL)			Susceptibility [*n* (%)]		
	S	I	R	MIC_{50}	MIC_{90}	Range	S	I	R
Penicillin	≤0.5	1	≥2	128	>265	4 to >256	0	0	44 (100)
Ampicillin	≤0.5	1	≥2	64	>256	2 to >256	0	0	44 (100)
Amoxicillin/clavulanic acid	≤4/2	8/4	≥16/8	2	16	0.25–32	29 (65.9)	2 (4.5)	13 (29.5)
Piperacillin/tazobactam	≤32/4	64/4	≥128/4	1	128	<0.03 to >256	36 (81.8)	1 (2.3)	7 (15.9)
Cefoxitin	≤16	32	≥64	8	32	4–32	37 (84.1)	7 (15.9)	0
Ertapenem	≤4	8	≥16	>1	1	0.06–64	30 (68.2)	4 (9.1)	10 (22.7)
Imipenem	≤4	8	≥16	1	32	<0.03–64	34 (77.3)	2 (4.5)	8 (18.2)
Meropenem	≤4	8	≥16	1	128	0.06–128	27 (61.4)	4 (9.1)	13 (29.5)
Clindamycin	≤2	4	≥8	>256	>256	0.125 to >256	7 (15.9)	1 (2.3)	36 (81.8)
Tetracycline	≤4	8	≥16	64	128	0.125–128	4 (9.1)	1 (2.3)	39 (88.6)
Moxifloxacin	≤2	4	≥8	4	32	0.25–64	20 (45.5)	3 (6.8)	21 (47.7)
Chloramphenicol	≤8	16	≥32	4	4	4–8	44 (100)	0	0
Metronidazole	≤8	16	≥32	0.5	0.5	0.25–1	44 (100)	0	0

S, suspectable; I, intermediate; R, resistant; $MIC_{50/90}$, MIC, required to inhibit 50% and 90% of the isolates, respectively.

Table 2
Characteristics of 16 *Bacteroides fragilis* isolates with reduced susceptibility to carbapenems.[a]

Isolate	MIC (μg/mL)			EDTA synergy test	Genotype		PFGE pattern	*cfiA* RQ (mean ± S.D.)
	ETP	IPM	MEM		*cfiA*	IS		
P1071100511	32	16	128	+	+	IS*1187*	A	5.507 ± 0.628
BFR4	64	32	128	+	+	IS*613*	B	5.402 ± 0.246
BFR30	64	64	128	+	+	IS*1187*	B	6.523 ± 0.134
13-w24	32	16	64	+	+	IS*1187*	B	1.371 ± 0.437
13-w25	64	64	128	+	+	IS*1187*	A	5.716 ± 1.960
13-w27	64	32	128	+	+	IS*1187*	B	5.496 ± 0.129
13-w29	64	32	128	+	+	IS*1187*	A	2.743 ± 1.077
13-w31	64	16	128	+	+	IS*613*	A	4.581 ± 2.169
13-w10	32	8	64	+	+	–	A	0.730 ± 0.161
BFR33	32	8	64	+	+	–	A	0.748 ± 0.249
13-w5	8	4	16	+	+	–	A	1.186 ± 0.240
13-w22	8	4	16	+	+	–	A	1.712 ± 0.079
BFR17	8	4	8	+	+	–	B	1.186 ± 0.306
BFR41	8	2	8	+	+	–	A	1.263 ± 0.154
P1058100522	4	1	8	+	+	–	B	1.441 ± 0.588
15-w1[b]	4	0.5	8	+	+	–	B	1

MIC, minimum inhibitory concentration; ETP, ertapenem; IMP, imipenem; MEM, meropenem; EDTA, ethylene diamine tetraacetic acid; IS, insertion sequence; PFGE, pulsed-field gel electrophoresis; RQ, relative quantity; S.D., standard deviation.
[a] Isolates with an MIC > 4 μg/mL for any of the carbapenems were defined as having reduced carbapenem susceptibility.
[b] *Bacteroides fragilis* isolate 15-w1 was used as a control strain to quantify the relative expression levels of *cfiA*.

in the presence of the efflux pump inhibitors CCCP, reserpine and PAβN (Supplementary Table S2). The results of SDS-PAGE suggested that the protein bands in carbapenem-resistant isolates did not decrease compared with carbapenem-susceptible isolates (Supplementary Fig. S2).

3.4. Prevalence of carbapenemases

Metallo-β-lactamase (MBL) activity was detected in all 16 isolates with reduced carbapenem susceptibility by the EDTA disk synergy assay, but not in the 28 carbapenem-susceptible isolates. No *Klebsiella pneumoniae* carbapenemase (KPC) activity, a representative of class A carbapenemase, was observed in the isolates as assayed using the 3-aminobenzeneboronic acid inhibition test. Furthermore, PCR amplification showed that only *cfiA* was found in the 16 isolates (Table 2). None of the isolates harboured genes encoding other common classes of carbapenemases.

3.5. Location of *cfiA*

The results of S1-PFGE and plasmid transformation assay were negative, indicating that *cfiA* was not located on a plasmid. Next, a *cfiA*-specific primer and genomic DNA was used to perform Southern blotting for identification of *cfiA*. As expected, a signal indicating the presence of *cfiA* was observed for all 16 isolates (Supplementary Fig. S3). Thus, the carbapenemase-encoding gene *cfiA* was located on the chromosome.

3.6. Correlation of cfiA upregulation with carbapenem resistance

Similar transcriptional levels of *cfiA* were seen in the eight *cfiA*-containing and IS-negative isolates, as shown in the left panel of Fig. 1. Interestingly, significantly higher transcriptional levels of *cfiA* were observed in the other eight isolates with IS upstream of *cfiA* (Fig. 1). As expected, the MICs of antimicrobials against the isolates with *cfiA* upregulation were higher than those in isolates with low *cfiA* expression (Table 2).

3.7. Sequencing analysis of cfiA and the upstream insertion sequence

In contrast to isolates with lower *cfiA* expression, eight isolates with high *cfiA* mRNA levels contained long ISs upstream of *cfiA*. Bioinformatic analysis identified that the ISs were IS*1187* in six isolates and IS*613* in two isolates (Supplementary Fig. S4). The eight ISs have been deposited in GenBank under accession nos. **MH025893**, **MH025894**, **MH025895**, **MH025896**, **MH025897**, **MH025898**, **MH025899** and **MH025900**. Similar promoters were

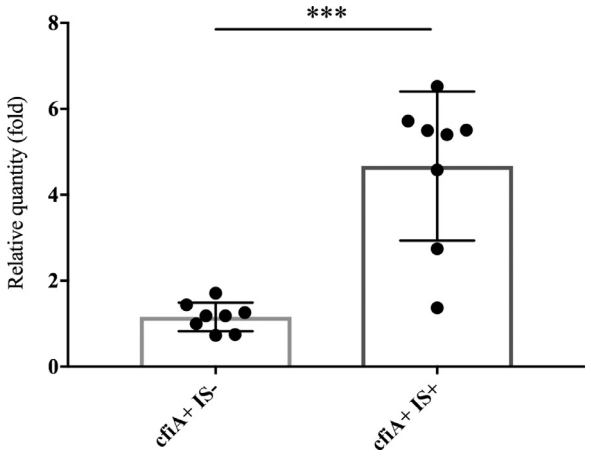

Fig. 1. Relative expression of *cfiA* in *Bacteroides fragilis* isolates. Quantitative reverse transcription PCR (RT-qPCR) was performed to measure the *cfiA* mRNA level in 16 *cfiA*-containing *B. fragilis* isolates, including 8 isolates with upstream insertion sequence (IS) sequences and 8 without an IS. Each dot indicates the mean value of the relative quantity from three independent experiments for each isolate. Error bars represent the standard deviation. *** $P < 0.001$.

predicted in the ISs. The results suggested that the presence of ISs might increase the transcriptional levels of *cfiA*.

4. Discussion

Antimicrobial resistance is a growing problem in Huashan Hospital. The *B. fragilis* isolates in this study showed a higher resistance rate to TZP (15.9%) than isolates in Europe (1.7%) [2], North America (1.3%) [3], Korea (2%) [4] and Taiwan (9.9%) [5]. It is of note that >80% of the 44 *B. fragilis* isolates were resistant to clindamycin and tetracycline, much higher than percentages in North America and Europe (≤60%) [2,3] and higher than other Asian reports (48.9–53.1%) [4,5]. However, in the present study none of the isolates were resistant to metronidazole or cefoxitin. The rate of moxifloxacin resistance (47.7%) was lower than data from the USA (>80%) [3].

It has been reported that <5% of *B. fragilis* isolates in Europe and North America [2,3] and <10% in Asia are resistant to carbapenems [4,5]. In the current study, the resistance rate of *B. fragilis* isolates to carbapenems ranged from 18.2% (imipenem) to 29.5% (meropenem). Carbapenem-resistant *B. fragilis* isolates are more likely to develop resistance to other anti-anaerobic antimicrobials. Almost all (95.5%) of the 44 *B. fragilis* isolates were multidrug-resistant.

In this study, *cfiA* existed in all 16 *B. fragilis* isolates with reduced susceptibility to carbapenems. Furthermore, high-level carbapenem resistance was identified when there was an IS upstream of *cfiA*. The isolates had lower susceptibility or low-level resistance to carbapenems if there was *cfiA* without an IS. In the 44 *B. fragilis* isolates tested, 16 (36.4%) contained the *cfiA* gene, including 8 IS-positive isolates. The prevalence was much higher than the reported prevalences of 8.8% (43/486) in Europe [17], 4.0% (11/276) in Korea [13] and 19.1% (81/424) in Hong Kong, China [18]. It is interesting to note that the *cfiA*-containing *B. fragilis* isolates may become carbapenem-resistant mutants via a one-step mutation [19]. Considering the emergence of *cfiA*-containing *B. fragilis* in this report, it is noteworthy to screen *cfiA* in *B. fragilis* isolates to identify highly resistant clones.

Based on the bioinformatics analysis, we speculate that IS upstream of *cfiA* may be a strong promoter that can upregulate the expression of *cfiA* to mediate high-level resistance to carbapenems. Nevertheless, carbapenem resistance is also reported in the absence of *cfiA* [17], therefore further investigation of other possible mechanisms of reduced carbapenem susceptibility is necessary.

This is the first report to describe carbapenem-resistant *B. fragilis* isolates in mainland China. However, some limitations may affect this study, including the small sample size and the discontinuous collection of isolates from a single centre. In addition, anaerobic cultures in Chinese hospitals are not routinely performed unless clinicians have specific requirements. Therefore, the results may not be appropriate for understanding the prevalence of carbapenem-resistant *B. fragilis* in other hospitals or regions. A multicentre study with a large sample size should be conducted to further understand carbapenem-resistant *B. fragilis* isolates in China. More efforts should be applied to elucidate the interaction of *cfiA* with its upstream IS as well as other potential mechanisms of carbapenem resistance.

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Competing interests

None declared.

Ethical approval

Not required.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2019.02.017.

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