Original

Isolation frequency of carbapenem-nonsusceptible *Bacteroides* spp. and detection methods for *cfiA*-positive *Bacteroides fragilis*

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ABSTRACT

Aims: *Bacteroides fragilis* is the most common anaerobic bacteria causing infectious diseases in humans. A *cfiA* gene encoding metallo-β-lactamase, which degrades carbapenem, has been reported as a resistance mechanism against carbapenem. Therefore, the detection of *cfiA* gene is important for determining appropriate antibiotic therapy. Although *cfiA* gene is detected via polymerase chain reaction (PCR), simpler and easier methods are required in clinical settings. We compared the detectability of *cfiA*-positive *B. fragilis* using multiple methods. Moreover, the isolation frequency of carbapenem-nonsusceptible *Bacteroides* spp. was evaluated.
Methods: *Bacteroides* spp. was isolated from clinical specimens at the Kanazawa University Hospital. Antimicrobial susceptibility testing was performed via broth microdilution. We explored whether matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) and modified carbapenem inactivation method (mCIM) can show *cfiA* gene positivity.
Results: Around 20-50 *Bacteroides* spp. strains/year were isolated from June 2015 to December 2020. Among these, 0-8 strains exhibited meropenem and/or imipenem nonsusceptibility (intermediate and resistant) in each year. An increased rate of carbapenem resistance, including meropenem- and imipenem-nonsusceptible *Bacteroides* spp., was observed. All carbapenem-nonsusceptible *Bacteroides* spp. were susceptible to metronidazole. Four (66.7%) of 6 strains showed *cfiA* gene positivity based on PCR.
Conclusions: MALDI-TOF MS and mCIM detected *cfiA*-positive strains. MALDI-TOF MS and mCIM are useful for detecting *cfiA*-positive *B. fragilis*. Because the isolation frequency of carbapenem-nonsusceptible *Bacteroides* spp. is increasing, determining their susceptibility is important for appropriate antimicrobial therapy.
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Key Words
Bacteroides spp., carbapenem resistance, *cfiA* gene

I. Introduction

Bacteroides spp. are anaerobic bacteria that form normal microbiota in the intestinal tract. They are important pathogenic bacteria for humans in diseased conditions. In particular, antimicrobial-resistant *Bacteroides* spp. infection reportedly shows poor prognosis and prolonged hospitalization[1]. *Bacteroides fragilis* is the most common

anaerobic bacteria causing infectious disease in humans [2]. Carbapenem resistance in *B. fragilis* was first reported in 1983 in Japan[3] and in 1986 in the United States[4]. Although their resistance mechanism to carbapenem remains unclear, the involvement of *cfiA* gene encoding metallo-β-lactamase (MBL), which degradates carbapenem, has been reported [5]. Considering the poor prognosis of carbapenem-resistant *B. fragilis* infection, the detection of *cfiA* gene is important. While *cfiA* gene is detected via polymerase chain reaction (PCR), this method is available only in some hospitals. Therefore, simpler and easier methods are required in clinical settings. In this study, the detectability of *cfiA*-positive *B. fragilis* was compared using multiple methods. Moreover, the isolation frequency of carbapenem-nonsusceptible *Bacteroides* spp., especially *cfiA*-positive strains, was evaluated.

II. Materials and methods

Strains and identification

Bacteroides spp. were isolated from clinical specimens at the Kanazawa University Hospital between June 2015 and December 2020. The specimen was applied and cultured on brucella agar medium (Kyokuto, Tokyo, Japan) for 2 days under anaerobic conditions at 35℃ . The isolated strains were identified using a Bacteroides Bile Esculin agar (BBE Agar, Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) and AccuDia™ Bacteroides Agar (BA, Shimadzu Diagnostics Corporation, Tokyo, Japan) according to the each directions. *B. fragilis* group were identified by the change of only BBE Agar color from dark brown to black that appears as zones around the colonies. Non-*B. fragilis* group were identified by the growth on BA and no color change in BBE Agar. Those that grew under aerobic conditions were excluded. Eight carbapenem-nonsusceptible strains, which were stored at our hospital, were re-identified using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (MALDI Biotyper, Bruker, Billerica, MA, USA). The strain was applied to the MSP 96 target polished steel BC (Bruker). After drying, 1 μL of 70% formic acid (Beckman Coulter, Brea, CA, USA) was added. Then, the sample was loaded into MALDI Biotyper after 1 μL of HCCA portioned (Bruker) was added. The spectra were analyzed using the MALDI Biotyper Compass software (MBT Compass software) (Bruker) following the manufacturer's instructions.

Antimicrobial susceptibility testing

The strain was suspended in ABCM broth (Eiken Chemical Co., Ltd., Tokyo, Japan) and adjusted with McFarland 2 standards. Then, 25 μL of sample were applied to Brucella Broth Eiken (Eiken Chemical Co., Ltd.). After 100 μL of the bacterial solution was added to Dry Plate Eiken (Eiken Chemical Co., Ltd.), the plate was incubated under anaerobic conditions at 35℃ for 48 h. After 48 h, the growth of the strain was visually observed. The minimum inhibitory concentration (MIC) and interpretive category were determined according to the Clinical and Laboratory Standards Institute M100-ED32 [6]. The additional MIC measurement of metronidazole (MNZ) was performed on 8 carbapenem-nonsusceptible strains, which were stored at our hospital.

cfiA gene detection

cfiA gene was detected via PCR. DNA extraction was prepared as follows. The strains were suspended in 200 μL of sterilized water. Then, 5 μL of proteinase K (350 U/mL, Takara Bio Inc., Shiga, Japan) was added, followed by incubation at 50℃ for 5 min. After 7 min of incubation in a boiling water bath, the lysate was centrifuged at 10,000 rpm for 5 min. Then, the supernatant was used as a template. The primers were 5'-CCCAACTCTCGGACAAAGTG-3' and 5'-ACGATCTGCTTGGTATGCTC-3' [7]. PCR was performed using 1 μL of template DNA in a total reaction volume of 25 μL consisting of EmeraldAmp PCR Master Mix (Takara Bio Inc., Shiga, Japan) and 25 pmol of each primer. Reactions were run in a thermal cycler (Biometra, Gottingen, Germany). The PCR program was as follows: 98℃ for 60 s; 30 cycles of 98℃ for 10 s, 56℃ for 30 s, and 72℃ for 60 s; and 72℃ for 60 s. Then, 5 μL of amplicon was analyzed using 2% agarose gel. Strains in which an amplicon was observed at 625 bp were determined to have *cfiA* gene.

Detection of cfiA-positive B. fragilis by MALDI-TOF MS

MALDI Biotyper was used in the same way as for bacterial identification. The MALDI Biotyper Subtyping Module with the MBT Compass software automatically identifies not only bacterial species but also possession of *cfiA* gene. Peaks shifts were reported from 4711 Da and 4817 Da (*cfiA*-negative) to 4688 Da and 4826 Da (*cfiA*-positive), respectively [8]. Strains that matched with the spectra of *cfiA*-positive *B. fragilis* were considered "presumptive *cfiA*-positive".

Detection of cfiA-positive B. fragilis using modified carbapenem inactivation method

We identified carbapenemase-producing *B. fragilis* using modified carbapenem inactivation method (mCIM) [6] with a few changes. Briefly, 1 μL of inoculation loop of

anaerobically cultured strain was firmly suspended in 2 mL of BD BBL™ Trypticase™ Soy Broth (Becton, Dickinson and Company, New Jersey, USA). A meropenem (MEPM) or imipenem (IPM) disk (Eiken Chemical Co., Ltd.), each containing 10 μg of antibiotic, was added and suspended. After incubation under anaerobic conditions at 35℃ for 4 h, the disk was removed from the suspension and placed on a BD BBL™ Muller–Hinton II agar medium (Becton, Dickinson and Company, New Jersey, USA) inoculated with a susceptible *Escherichia coli* indicator (ATCC25922). After incubation under aerobic conditions at 35℃ for 24 h, the diameter of growth inhibition zones was measured. MBL-producing *Klebsiella pneumoniae* was used as a positive control in mCIM analysis. The negative control was a culture medium only.

III. Results

Patient characteristics

The study period is from June 2015 to December 2020. Carbapenem-nonsusceptible *Bacteroides* spp. were detected in 17 patients. The mean age of patients was 65.0 years, and 10 patients (59.0%) were female. Their underlying diseases were malignant tumors (11 patients, 64.7%), gastrointestinal disorders (6 patients, 35.3%), and hepatobiliary diseases (4 patients, 23.5%). The strains were detected from pus (9 patients, 52.9%), abdominal cavity and subdiaphragmatic drains (4 patients, 23.5%), bile (3 patients, 17.6%), and blood (1 patient, 5.9%). Fifteen specimens (88.2%) were of abdominal origin. Fourteen patients were treated, and 11 patients (78.5%) received antimicrobials. Especially, 7 (63.6%) of

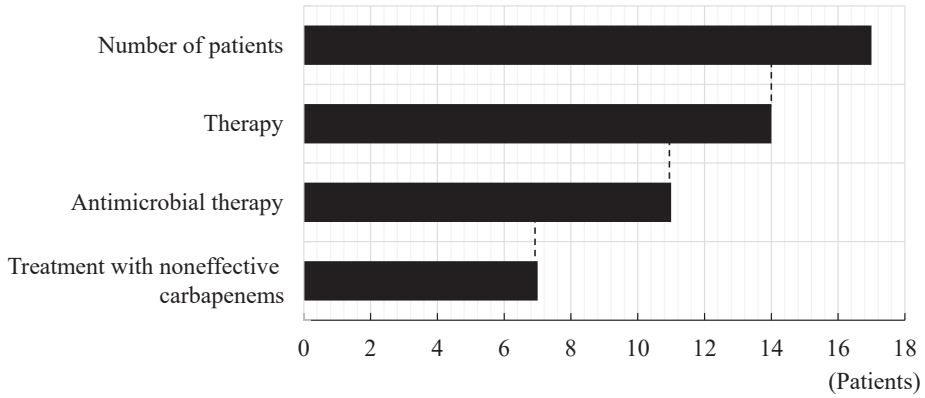
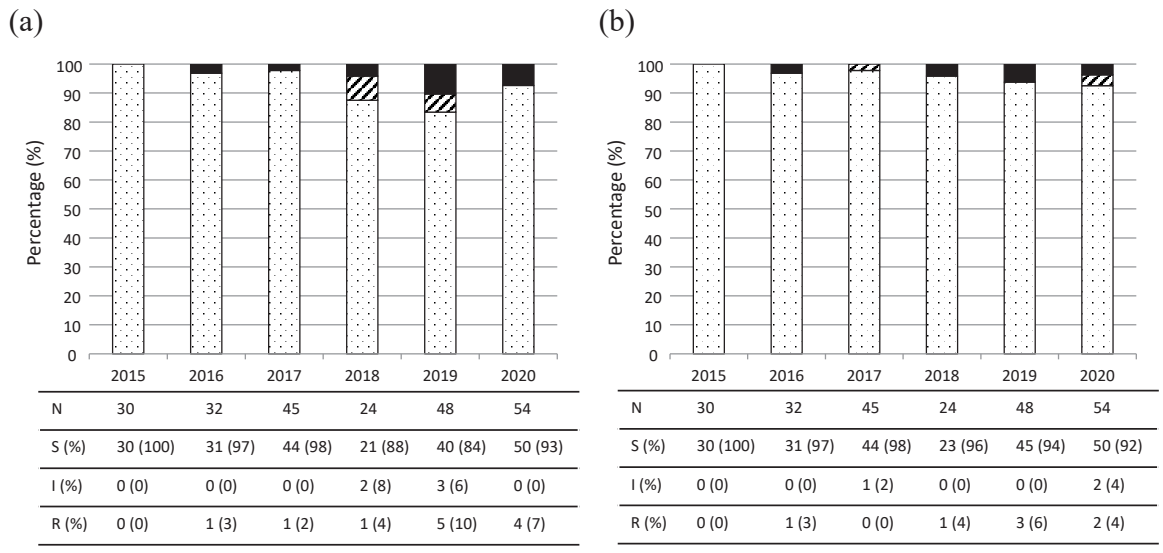


Figure 1 Treatment of patients infected with carbapenem-nonsusceptible *Bacteroides* spp.
Here, 63.6% of patients were treated with noneffective carbapenems.



	2015	2016	2017	2018	2019	2020
N	30	32	45	24	48	54
S (%)	30 (100)	31 (97)	44 (98)	21 (88)	40 (84)	50 (93)
I (%)	0 (0)	0 (0)	0 (0)	2 (8)	3 (6)	0 (0)
R (%)	0 (0)	1 (3)	1 (2)	1 (4)	5 (10)	4 (7)

	2015	2016	2017	2018	2019	2020
N	30	32	45	24	48	54
S (%)	30 (100)	31 (97)	44 (98)	23 (96)	45 (94)	50 (92)
I (%)	0 (0)	0 (0)	1 (2)	0 (0)	0 (0)	2 (4)
R (%)	0 (0)	1 (3)	0 (0)	1 (4)	3 (6)	2 (4)

Figure 2 Changes in carbapenem sensitivity from 2015 to 2020
Sensitivity to meropenem (a) and imipenem (b).
An increased rate of carbapenem-nonsusceptible *Bacteroides* spp. was observed in meropenem and imipenem.
dot: susceptible, oblique line: intermediate, black: resistant, S: susceptible, I: intermediate, R: resistant.

11 patients were treated with noneffective carbapenems (**Fig. 1**).

Isolation frequency of carbapenem-nonsusceptible Bacteroides spp.

The characters of isolated 233 *Bacteroides* spp. were described in **Fig. 2**. Two hundred strains were *B. fragilis* group, and 33 strains were non-*B. fragilis* group. Around 20-50 strains/year were isolated in Kanazawa University Hospital. Among these, 0-8 strains exhibited MEPM and/or IMP nonsusceptibility (intermediate and resistant) in each year. Although no MEPM-nonsusceptible *Bacteroides* spp. was detected in 2015, its isolation rate peaked at 16% in 2019 and decreased to 7% in 2020 (**Fig. 2a**). In contrast, the frequency of IPM-nonsusceptible *Bacteroides* spp. increased from 0% in 2015 to 8% in 2020 (**Fig. 2b**). An increased rate of carbapenem resistance, including MEPM- and IPM-nonsusceptible *Bacteroides* spp., was observed during the period.

Antimicrobial susceptibility of carbapenem-nonsusceptible Bacteroides spp.

Nineteen strains of carbapenem-nonsusceptible *Bacteroides* spp. were isolated between 2015 and 2020. Seventeen strains were *B. fragilis* group and 2 were non-*B. fragilis* group. Two patients were positive for both *B. fragilis* group and non-*B. fragilis* group. The antimicrobial susceptibility of these strains is shown in **Table 1**. Eight of 19 strains were available for susceptibility tests for MNZ. All of the 8 strains were *B. fragilis* group and showed susceptibility to MNZ.

Detection of cfiA gene from carbapenem-nonsusceptible B. fragilis

Six of 8 carbapenem-nonsusceptible strains that were stored at our hospital were re-identified as *B. fragilis* via MALDI-TOF MS. The other 2 strains were re-identified as *B. thetaiotaomicron* and *B. uniformis*, respectively. We investigated whether these 6 *B. fragilis* strains possessed *cfiA* gene by performing PCR. Four strains (66.7%) were *cfiA*-positive. Among the preserved strains, a *cfiA*-positive strain was identified in 2016 and 2020 in each and 2 strains were identified in 2019. Then, MALDI-TOF MS was performed to identify strains with *cfiA* gene (**Table 2**) and was compared to mCIM.

MALDI-TOF MS detected strains with cfiA gene

Of 6 *B. fragilis* isolates, 4 strains were detected as presumptive *cfiA*-positive based on MALDI-TOF MS. The other 2 strains were detected as presumptive *cfiA*-negative (**Fig. 3**). The results of *cfiA*-positive *B. fragilis* based on MALDI-TOF MS were consistent with those of *cfiA* gene based on PCR.

mCIM revealed the susceptibility of strains with/without cfiA gene

Neither MEPM nor IPM disks inhibited the growth of *E. coli* in mCIM analysis of 4 *cfiA*-positive *B. fragilis*. In the analysis of the other 2 *cfiA*-negative *B. fragilis*, *E. coli* formed growth inhibition zones around MEPM disks, with a diameter larger than 20 mm. IPM disks also inhibited the growth of *E. coli* in the analysis of 2 *cfiA*-negative *B. fragilis*, with growth inhibition zones larger than 18 mm (**Fig. 4**).

Table 1 Antimicrobial susceptibility of carbapenem-nonsusceptible *Bacteroides* spp.

	N	S (%)	I (%)	R (%)
S/A	19	3 (15.8)	4 (21.1)	12 (63.2)
T/P	19	9 (47.4)	1 (5.3)	9 (47.4)
MEPM	19	2 (10.5)	6 (31.6)	11 (57.9)
IPM	19	9 (47.4)	3 (15.8)	7 (36.8)
CLDM	19	11 (57.9)	0 (0.0)	8 (42.1)
MNZ	8	8 (100.0)	0 (0.0)	0 (0.0)

S/A: sulbactam-ampicillin, T/P: tazobactam-piperacillin, MEPM: meropenem, IPM: imipenem, CLDM: clindamycin, MNZ: metronidazole, S: susceptible, I: intermediate, R: resistant.

Table 2 Detection results of *cfiA*-positive *B. fragilis* via MALDI-TOF MS and mCIM

cfiA gene by PCR	MALDI-TOF MS	mCIM		N
		MEPM (mm)	IPM (mm)	
Detected	Detected	0	0	4
Not detected	Not detected	≥ 20	≥ 18	2

PCR: polymerase chain reaction, MALDI-TOF MS: matrix-assisted laser desorption ionization–time of flight mass spectrometry, mCIM: modified carbapenem inactivation method, MEPM: meropenem, IPM: imipenem.

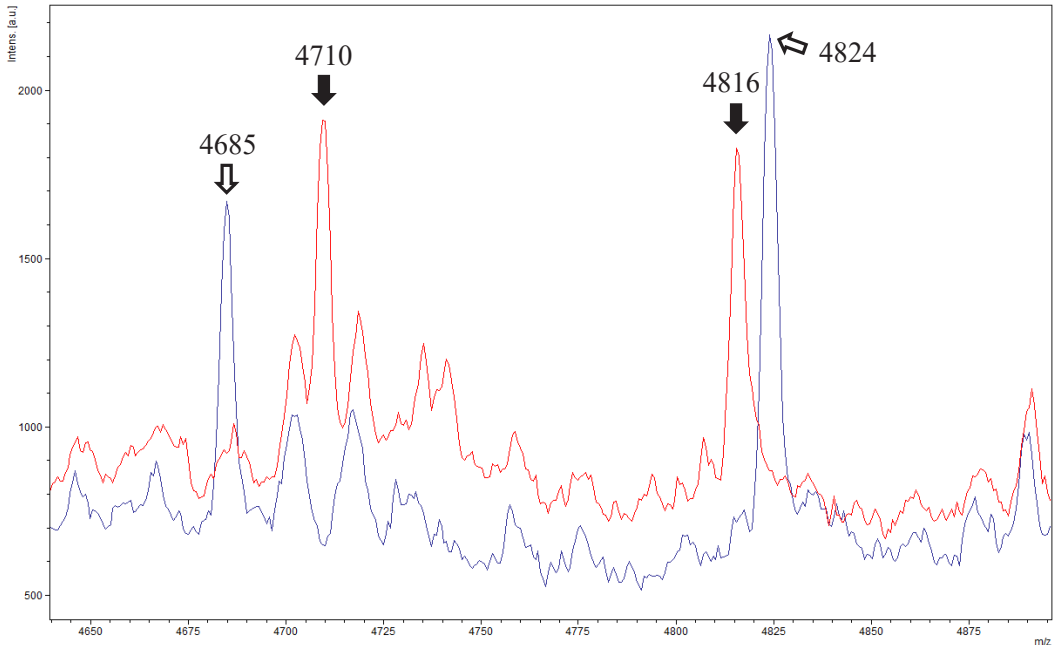


Figure 3 MALDI-TOF MS peaks of *cfiA*-negative and *cfiA*-positive *B. fragilis*.
White arrows indicate peaks of *cfiA*-positive *B. fragilis*. Black arrows indicate peaks of *cfiA*-negative *B. fragilis*.

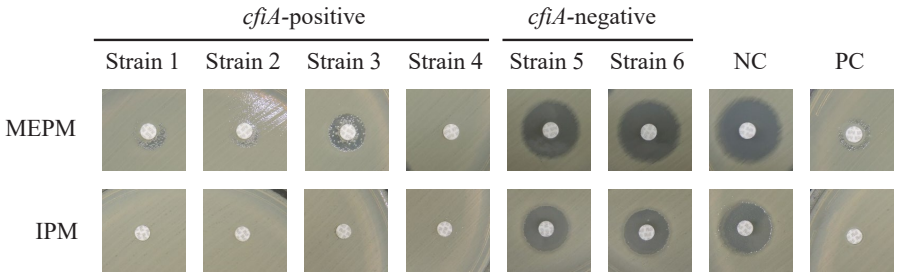


Figure 4 Results of modified carbapenem inactivation method
B. fragilis strains 1, 2, 3, 4 were *cfiA*-positive, whereas strains 5 and 6 were *cfiA*-negative. NC is the result of a bacteria-free test. PC is the result of metallo-β-lactamase-producing *Klebsiella pneumoniae*.
MEPM: meropenem, IPM: imipenem, NC: negative control, PC: positive control.

IV. Discussion

The isolation frequency of carbapenem-nonsusceptible *Bacteroides* spp. was investigated, and methods for detecting *cfiA*-positive *B. fragilis* were examined. This study revealed that the maximum isolation frequency of MEPM- and IPM-nonsusceptible *Bacteroides* spp. was 16% and 8%, respectively. The isolation rate of carbapenem-nonsusceptible *Bacteroides* spp. increased over the past 6 years. Recent reports indicate that carbapenem-nonsusceptible *Bacteroides* spp. have been isolated in some countries with various frequencies[9)-11)]. The increased frequency of carbapenem-resistant *Bacteroides* spp. was also reported in some studies[9),12),13)]. The isolation frequency increased similar to that reported in other studies in Japan[9)]. Therefore, monitoring the isolation frequency of carbapenem-nonsusceptible *Bacteroides* spp. is important in hospitals. Moreover, the detection of carbapenem-nonsusceptible *Bacteroides* spp. is important for determining appropriate antibiotic therapy. We should have investigated the isolation frequency of carbapenem-nonsusceptible *B. fragilis*. Although MALDI-TOF MS analysis identify strains to *B. fragilis*, the equipment was not available during the study period in our facility. Therefore, we investigated the isolation frequency of carbapenem-nonsusceptible strains in the genus *Bacteroides*.

The isolation rate of MEPM-nonsusceptible *Bacteroides* spp. peaked at 16% in 2019 and decreased to 7% in 2020. Although the reason for the reduced MEPM-nonsusceptible *Bacteroides* spp. frequency is not clear, small sample number may affect the change of frequency in this case.

In our study, while 4 strains (66.7%) of carbapenem-nonsusceptible *B. fragilis* were *cfiA*-positive, the other 2 strains did not possess the gene. Other than *cfiA* gene, the mechanisms of carbapenem nonsusceptibility remain unclear[14]. One study showed that functional changes in efflux pumps affect carbapenem sensitivity[15]. Further studies are needed to clarify the resistance mechanisms, especially in 2 carbapenem-nonsusceptible *B. fragilis* without *cfiA* gene.

To date, there is no routine method to detect *cfiA*-positive *B. fragilis*. In this study, MALDI-TOF MS and mCIM were useful in detecting *cfiA*-positive *B. fragilis*. A recent study reported that the sensitivity and specificity of MALDI-TOF MS in detecting *cfiA*-positive *B. fragilis* were 100.0% and 99.7%, respectively[16]. Although MALDI-TOF MS is a simple and quick method, it can be performed only in the facilities with the required equipment. In facilities where MALDI-TOF MS is unavailable, mCIM may be useful. Because mCIM does not require specialized equipment or reagents, it is widely used and can be performed at any facility. Therefore, MALDI-TOF MS and mCIM might provide earlier and more accurate results in determining *cfiA*-positive strains. This will help determine appropriate antimicrobial use for treating carbapenem-nonsusceptible *B. fragilis* infection.

In summary, even if MALDI-TOF MS or PCR is not available, mCIM is useful for detecting *cfiA*-positive *B. fragilis*. Because the isolation frequency of carbapenem-nonsusceptible *Bacteroides* spp. is increasing, determining its susceptibility is important for appropriate antimicrobial therapy.

Declarations

Funding:

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Conflicts of interest:

The authors have declared that no conflict of interest exists.

Ethics approval:

All experiments were performed in accordance with approved guidelines of Kanazawa University. This study was conducted with the approval of the ethics committee of Kanazawa University (approval number: 3596).

Authorship contributions:

YTS and YI designed the study and interpreted the data. YTS performed experiment and wrote the draft of the manuscript together with YI. All authors read the manuscript and commented.

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