Clinical and Experimental Health Sciences

Phenotypic and Genotypic Investigation of the Resistance of Pathogenic *Bacteroides fragilis* Group Bacteria to Carbapenems

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ABSTRACT

Objective: *Bacteroides fragilis*, predominantly found in the intestinal microbiota, is one of the most frequently encountered anaerobic pathogens and exhibits resistance to many antimicrobials. The carbapenem-resistant *B. fragilis* (CR-BF) isolates have been reported in several countries recently with increasing global attention. The high frequency of CR-BF in our hospitalized patients has become an important problem. For this reason, *B. fragilis* isolated in our hospital need to be closely monitored for carbapenem resistance. Therefore, we aimed to determine carbapenem resistance in *B. fragilis* isolated from clinical samples and the presence of the *cfiA* gene, which encodes for carbapenemase.

Methods: *B. fragilis* strains isolated from various clinical samples collected between January 2018 and December 2022 were included in the study. Identification of the isolates was performed using MALDI-TOF MS. The susceptibility testing for meropenem and imipenem was determined by the agar dilution method. The *cfiA* gene was detected by PCR.

Results: A total 89 *B. fragilis* strains were studied, mostly from intra-abdominal abscesses (31%) and blood cultures (27%). Susceptibility rates for meropenem and imipenem were 85.4% and 89%, respectively. Notably, 12 out of 13 *cfiA* gene-positive isolates were resistant, suggesting this gene as a marker for carbapenem resistance. However, resistance in one *cfiA*-negative isolates implies alternative resistance mechanisms.

Conclusion: Routine anaerobic culture, determination of antibiotic susceptibility profiles of isolates, and close monitoring are crucial for managing infections. Regular antimicrobial susceptibility testing helps predict the risk of developing carbapenem resistance, assists clinicians in selecting appropriate antibiotics for empirical therapy, and improves treatment success rates.

Keywords: *Bacteroides fragilis*, carbapenem, *cfiA* gene

1. INTRODUCTION

Bacteroides, a genus of gram-negative, obligate anaerobic bacteria, are important members of the intestinal microbiota. *Bacteroides fragilis* is the most clinically significant species due to its virulence factors and antimicrobial resistance. While a normal resident of the colon, *B. fragilis* can also opportunistically cause serious mixed infections. Typically, this bacterium can spread during intra-abdominal surgery, abdominal trauma, intestinal perforation, or in immunocompromised individuals, leading to serious and potentially life-threatening infections (1).

Bacteroides fragilis species is one of the most resistant anaerobic bacteria. This speces is only susceptible to a few antibiotics, such as beta-lactam/beta-lactamase inhibitors, carbapenems, and metronidazole. However, recent studies show increasing resistance to even these antibiotics (2,3).

Bacteroides fragilis species employs various mechanisms to resist carbapenems. The most common mechanism involves an enzyme called a carbapenemase, encoded by the *cfiA* gene integrated into the bacterial DNA. This enzyme, a metallo-beta-lactamase containing zinc (Zn+2) in its active site, breaks down carbapenems. Metallo-beta-lactamases are not inhibited by classical beta-lactamase inhibitors, but are inactivated by the presence of ethylenediaminetetraacetic acid (EDTA) (4). Interestingly, the cfiA gene can be present silently in B.fragilis bacteria, meaning it doesn't produce the enzyme yet. This silent gene can be activated by a mobile DNA element (insertion sequence: IS element) that inserts itself in front of *cfiA* gene, triggering enzyme production (5,6).

Studies worldwide report that 2-7% of *B. fragilis* isolates carry the *cfiA* gene, but only about 1% are truly carbapenem-resistant. The reported resistance rates for imipenem and meropenem vary geographically: 0.2-1% in the US, Canada, and Europe, 2-4% in Japan, 7-12% in Taiwan and 18.2-29.5% in China (3,7,8). The high incidence of carbapenem-resistant *B. fragilis*

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(CR-BF), isolated from our hospitalized patients, has become an important problem. The first imipenem-resistant *B. fragilis* strain was isolated at Marmara University Hospital's laboratory in 1999 (9). Over the past decade, our carbapenem resistance rate climbed to 8%. Alarmingly, molecular studies revealed that 27% of our isolates carried the *cfiA* gene (10,11).

In this study, we aimed to determine carbapenem resistance in *B. fragilis* isolated from clinical samples and the presence of the *cfiA* gene, which encodes for **carbapenemase.**

2. METHODS

In this study, *B: fragilis* strains isolated from clinical materials in a 650-bed tertiary university hospital were studied.

2.1. Bacterial Isolates

A total of 89 non-duplicate *B. fragilis* strains isolated from various clinical samples collected from different clinics; general surgery (36%), gynecolog/urology (14.6%), emergency unit (14.6%), internal medicine (10%) and intensive care unit care (9%), between January 2018 and December 2022 were studied. The strains were mainly isolated from intra-abdominal abscesses (34.8%), blood culture (27%), abscesses (14.6%), and tissue biopsies (13.5%).

The clinical samples were inoculated on Brucella Blood Agar (BBA) (Becton Dickinson, USA), supplemented with hemin and vitamin K. After incubation at 36°C for 2–4 days in an anaerobic chamber (Bactron-I, SHELLAB, USA), several colonies from each plate were tested for aerotolerance. Strict anaerobic colonies were chosen for identification using MALDI-TOF MS (Vitek MS, bioMérieux, France) automated system (2).

2.2. Identification by VITEK MS

All isolates, grown on BBA at 36 °C for 48 h in an anaerobic chamber, were identified following the manufacturer's instructions. Briefly, a single colony was spotted as a homogeneous smear on the target slide using a 1 µL loop. The bacteria were subsequently covered with 1 µL of α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution (bioMérieux, France). After drying at room temperature, the target slide was loaded into the VITEK MS machine (bioMérieux, France). Microbial identification relied on comparing the generated spectra of the bacterial strains to the reference spectra stored in the VITEK MS version 3.0 database

Viable strains were stored at −80 °C in 10% skimmed milk until use.

2.3. Susceptibility Tests

Antimicrobial susceptibility tests against imipenem and meropenem were performed by agar dilution based on recommendations of the CSLI (2007) (12). Meropenem and imipenem powders used were obtained from Sigma (St. Louis, MO, USA). The Minimal Inhibitory Concentration (MIC) was defined as the lowest concentration that prevented visible growth of the tested microorganism; MIC: ≤ 1 µg/mL was accepted as meropenem or imipenem susceptible, and MIC: >1 µg/mL as resistant according to EUCAST breakpoints (v13.1) (13). A standardized bacterial inoculum (10^5 CFU/mL) was efficiently transferred onto BBA containing half-diluted antibiotics and incubated at 36°C for 48 h in anaerobic chamber. The *Bacteroides fragilis* ATCC-25285 (American Type Culture Collection, Rockville, MD, USA) was included as controls in all assays to assess the reliability of the methods.

2.4. DNA extraction and cfiA Carbapenemase Gene Amplification

Bacteroides fragilis colonies were picked from culture plates and bacterial DNA was extracted by heating. The *cfiA* gene was detected by polymerase chain reactions (PCR) using specific primers; *cfiA*1: 5'-CCA TGC TTT TCC CTG TCG CAG-3' and *cfiA*2: 5'-GGG CTA TGG CTT TGA AGT GC-3' (5). PCR conditions comprised 94°C for 2 min, followed by 35 cycles of 45 s at 94°C, 45 s at 51°C, and 45 s at 72°C, followed by 2 min at 72°C (14). The *cfiA* gene positive *B. fragilis* strain D-5, previously identified in our laboratory, was used as a positive control, and ATCC 25285 strain as negative control.

Amplification products were separated by agarose gel electrophoresis, visualised by UV illumination following staining with ethidium bromide, and identified by comparison with reference markers.

2.4. Statistical Analysis

Statistical analysis of the study data was conducted using the SPSS 20.0 software program.

3. RESULTS

Our analysis of antibiotic susceptibility, following the latest EUCAST guidelines (v13.1), revealed resistance in 10.1% (9 isolates) of *B. fragilis* strains to imipenem and 14.6% (13 isolates) to meropenem. Imipenem MIC_{50} and MIC_{90} were 0.25 and 4 µg/mL, respectively. Meropenem MIC_{50} and MIC_{90} were 0.25 and 32 µg/mL, respectively.

The cfiA gene was identified in 13 B. fragilis isolates (14%). All but one of the meropenem-resistant and imipenem-resistant isolates harbored the cfiA gene. However, one cfiA-positive isolate was susceptible to meropenem, while five were susceptible to imipenem, with a MIC of 1 µg/mL for these particular isolates. This suggests that meropenem may be more sensitive for detecting carbapenem resistance in phenotypic testing. Additionally, a bacteremia isolate of *B. fragilis* from a 54-year-old patient who underwent intra-abdominal surgery displayed resistance to both imipenem and meropenem without harboring the *cfiA* gene. Details of antibiotic susceptibility testing for all resistant and *cfiA*-positive isolates are provided in Table 1. Figures 1 and 2 depict

the correlation between minimum inhibitory concentrations (MICs) of imipenem and meropenem, and the presence of the *cfiA* gene in *B. fragilis* isolates.

Table 1. *Characterization of isolates phenotypically resistant to imipenem and meropenem and presence of cfiA gene.*

N	ID	Age	Clinical samples	Hospital ward	IMP MIC (µg/mL)	MER MIC (µg/mL)	Presence of cfiA gene
1	18094	43	Tissue biopsy	Infectious diseases	2	64	+
2	18185	79	Intraabdominal abscess	Emergency department	2	16	+
3	18207	55	Intraabdominal abscess	General surgery	4	32	+
4	18230	7	Intraabdominal abscess	General surgery	64	256	+
5	18233	73	Wound/Abscess	Urology	1	1	+
6	18595	58	Intraabdominal abscess	General surgery	1	4	+
7	19232	33	Blood	General surgery	32	32	+
8	20079	66	Intraabdominal abscess	General surgery	1	4	+
9	20577	65	Tissue biopsy	Urology	1	4	+
10	21120	11	Cerebrospinal fluid	General surgery	1	4	+
11	21497	53	Tissue biopsy	Internal medicine clinic	16	128	+
12	22179	71	Blood	Emergency department	2	8	+
13	22379	71	Blood	Intensive Care Units	128	256	+
14	22488	54	Blood	General surgery	2	8	(-)

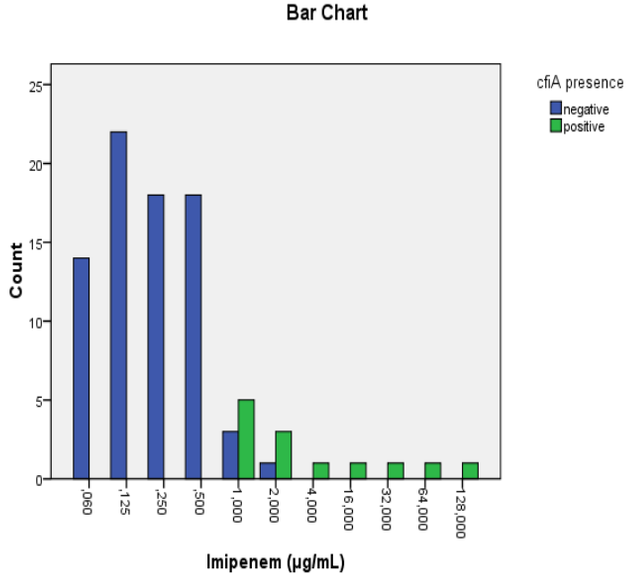

Figure 1. *Correlation between imipenem MIC values and the presence of the cfiA gene in Bacteroides fragilis isolates*

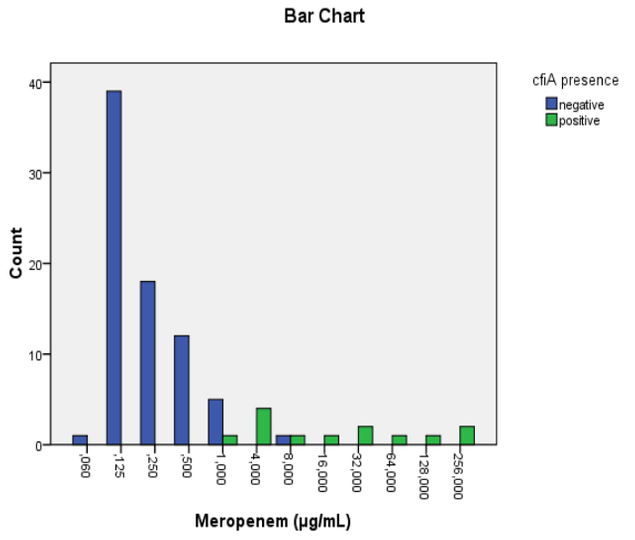

Figure 2. *Correlation between meropenem MIC values and the presence of the cfiA gene in Bacteroides fragilis isolates*

4. DISCUSSION

In this study, carbapenem resistance and the prevalence of the *cfiA* gene were investigated in *B. fragilis* isolates obtained from a tertiary hospital in Türkiye. The findings reveal a worrying increase in carbapenem resistance compared to our previous results and highlight the increasing threat of multidrug-resistant *B. fragilis* (9-11). A significant proportion of *B. fragilis* isolates displayed resistance to imipenem (10.1%) and meropenem (14.6%), exceeding national estimates.

Anaerobic bacterial culture is performed in very few laboratories worldwide and in our country due to the difficulties, time, and cost associated with isolation and identification. When an anaerobic infection is suspected, empirical treatment with antibiotics thought to have an antianaerobic effect is applied (2). However, the increasing prevalence of antibiotic resistance in recent years has made the susceptibility of anaerobic bacteria, especially *Bacteroides* isolates, unpredictable. Studies have shown that resistance can develop even to the most effective antibiotics, including carbapenems, piperacillin/tazobactam, amoxicillin/clavulanic acid, and metronidazole, although the rates vary between countries, cities, hospitals, and even different clinics within the same hospital. The significant rise in case reports of multidrug-resistant *B. fragilis* isolates is noteworthy (3). In contrast to previous studies in our country that reported no carbapenem resistance in a small number of *Bacteroides* isolates, our study found a high resistance rate (15-17). Reported resistance rates for imipenem and meropenem vary in various world countries; 0.2-1% in the USA, Canada and Europe, 2-4% in Japan, 7-12% in Taiwan and18.2-29.5% in China (3,7,8). This high rate compared to both national and international data may be due to our hospital's frequent use of carbapenems in empirical treatment (18).

The *cfiA* gene, encoding a carbapenemase enzyme, was detected in 14% of isolates and strongly correlated with carbapenem resistance in most cases. Interestingly, some *cfiA*-positive isolates remained susceptible to carbapenems, it may be due to the absence of IS elements in front of the *cfiA* gene, while the presence of these mobile DNA elements could potentially integrate into the cfiA gene and activate carbapenemase production, leading to resistance (5).

Notably, one carbapenem-resistant isolate lacking the *cfiA* gene, indicating the existence of additional mechanisms not investigated in this study. These mechanisms may include porin-mediated resistance, which reduces carbapenem uptake, efflux pumps that remove the antibiotic from the cell, or the acquisition of alternative carbapenemase genes (2,4).

The increase in CR-BF poses a significant problem; because carbapenems are often the last-line treatment for serious *B. fragilis* infections. The *cfiA* gene appears to be a significant contributor to carbapenem resistance in this hospital setting. However, the presence of *cfiA* does not always guarantee resistance and other mechanisms may also be involved (4,6,7). The identification of CR-BF strains lacking the *cfiA* gene highlights the need for further investigation of alternative resistance pathways.

There are some limitations in this study: First, this single-center study limits the generalizability of the findings to other hospitals. Second. The specific mechanisms responsible for carbapenem resistance in *cfiA*-negative isolates were not discussed in detail in the study. Third, the presence of IS elements was not investigated.

The results of our study indicate that continuous surveillance of carbapenem resistance in *B. fragilis* is crucial to monitor trends and guide antibiotic stewardship programs. Infection control measures must be strictly implemented to prevent the spread of multidrug-resistant *B. fragilis.* Clinicians should be aware of the increasing prevalence of carbapenem-resistant *B. fragilis*, especially considering that more than half of our study population was over 50 years of age and one-third was over 65 years of age, (mean: 52.72) (Table1). This highlights the importance of considering alternative treatment options when necessary for this age group with a higher risk of infections caused by carbapenem resistant *B. fragilis.* More research is needed to develop effective strategies to combat multidrug-resistant *B. fragilis* infections. Further research is also required to elucidate the mechanisms of carbapenem resistance in *cfiA*-negative isolates. Investigation of the potential impact of mobile DNA elements on *cfiA* gene activation deserves further study.

5.CONCLUSION

This study highlights the alarming trend of increasing carbapenem resistance among *B. fragilis* isolates, with a prevalence of 10.1% for imipenem and 14.6% for meropenem. The *cfiA* gene, which encodes a carbapenemase enzyme, was identified in 14% of the isolates and showed a strong correlation with carbapenem resistance in most cases. However, some inconsistencies suggest that alternative resistance mechanisms or limitations in *cfiA* detection are involved. Additionally, the absence of the *cfiA* gene in a carbapenem-resistant isolate indicates the existence of additional mechanisms not investigated in this study.

Overall, this study adds valuable data to the growing concern regarding carbapenem-resistant *B. fragilis*. The findings highlight the need for continuous monitoring of carbapenem resistance, investigation of alternative resistance mechanisms, and implementation of effective infection control strategies.

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Acquisition of data for the study: N.U.T, S.O and A.O
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