

# Antibiotic Resistance Pattern of *Bacteroides Fragilis* Isolated From Clinical and Gastrointestinal Specimens

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## Research

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# Abstract

**Background:** *Bacteroides fragilis* is a part of the normal gastrointestinal flora and the most prevalent anaerobic bacteria causes' infection. It is highly resistant to antibiotics and contains abundant antibiotic resistance mechanisms.

**Methods:** The antibiotic resistance pattern of 78 isolates of *B. fragilis* (56 strains from the gastrointestinal [GI] tract and 22 strains from clinical samples) was investigated using agar dilution method. The gene encoding *Bacteroides fargilis* toxin *bft*, and antibiotic resistance genes were targeted by PCR assay.

**Results:** The highest rate of resistance was observed for penicillin G (100%) followed by tetracycline (74.4%), clindamycin (41%) and ceftiofur (38.5%). Only a single isolate showed resistance to imipenem which contained *cfiA* and *IS1186* genes. All isolates were susceptible to metronidazole. Accordingly, *tetQ* (87.2%), *cepA* (73.1%) and *ermF* (64.1%) were the most abundant antibiotic-resistant genes identified in this study. MIC values for penicillin, ceftiofur and clindamycin were significantly different among isolates with the *cepA*, *cfxA* and *ermF* in compare with those lacking such genes. In addition, 22.7% and 17.8% of clinical and GI tract isolates had the *bft* gene, respectively.

**Conclusions:** Therefore, it is of utmost importance to determine the antibiotic resistance patterns of *B. fragilis* periodically in different geographical areas to provide a suitable treatment profile for patients and to prevent improper antibiotic prescriptions.

## Background

*Bacteroides fragilis* is an anaerobic, Gram-negative bacteria and a part of the human gastrointestinal microbiota but can cause severe infections in human opportunistically. Genus *Bacteroides* accounted for about 25% of gastrointestinal (GI) tract flora [(1, 2)]. Among various species of this genus, *Bacteroides fragilis* (*B. fragilis*) has been also introduced as the most abundant opportunistic anaerobic bacterium isolated from clinical specimens (3). The bacteria form 1–2% of the normal flora of the gastrointestinal tract and, if dislocated into other anatomical sites, develop various infections such as abdominal infections, abscesses, and bacteremia with a mortality rate of about 19% (1, 4).

Relevant studies have further revealed that *B. fragilis* exhibits the highest antibiotic resistance and the most abundant antibiotic resistance mechanisms compared with other anaerobic bacteria in the GI tract (5). This not only makes it difficult to treat infections caused by *B. fragilis*, but also has the potential to act as a reservoir of antibiotic-resistant genes (6), leading to the transfer of resistance genes to other normal bacterial flora through integrated transposons, integrated genetic elements, as well as conjugative plasmids (7). In this respect, different resistance patterns of this bacterium have been so far reported from different parts of the world. There have been reports of increased resistance to carbapenems and beta-lactams among *B. fragilis* isolates worldwide (8–12). Of note, the rate of resistance to metronidazole, as an effective antibiotic against anaerobic bacteria, is about 1%, but some reference

laboratories have reported a resistance rate of up to 7.5% (13–15). Also, the number of multidrug-resistant *B. fragilis* isolates has augmented over the last decade (16–18).

Bacterial virulence factors have important roles in the pathogenicity of *B. fragilis*. Enterotoxigenic *B. fragilis* (ETBF) also produces a 20 kDa metalloprotease toxin, mainly known as *B. fragilis* toxin (BFT) (19, 20). Studies in this line have further established that the ETBF strains are more pathogenic than non-toxigenic ones and they are associated with various diseases such as septicaemia, diarrhoea, irritable bowel syndrome (IBS), and colorectal cancer (CRC) (10, 21).

However, due to the costly and time-consuming process of isolation and identification of *B. fragilis*, antibiotic susceptibility testing is not routinely performed in laboratories (22, 23). Therefore, in this study antibiotic resistance profiles of *B. fragilis* isolated from the GI tract and clinical samples were evaluated using phenotypic and genotypic methods.

## Material And Methods

### Study population

The current cross-sectional study examined two populations, the patients, and the healthy controls. This study was approved by the Ethics Committee of National Institute for Medical Research development in Iran (NO. 971329). Informed consent was obtained from all individual participants.

The patient population included people suspected of having anaerobic infection hospitalized in different wards of Imam Khomeini Hospital of Tehran, and the healthy population included people with no history of GI disease or antibiotic consumption for the past three months.

In the sampling process from the patients, 130 different clinical samples were collected from hospitalized patients in different wards of Imam Khomeini Hospital during 1 year from August 2018 to August 2019. Sampling, culture and isolation of anaerobic bacteria were performed (24).

In the sampling process from healthy individuals, 40 biopsies of the rectum were collected by a physician during colonoscopy. To isolate *B. fragilis*, the biopsy sample was homogenized by mortar and pestle, and then 2-3 drops were inoculated on a plate containing Bacteroides Bile Esculin Agar (BBE) and Brucella Blood Agar (BBA) containing 5% sheep blood, vitamin K1 (0.5 mg/L) and hemin (5 mg/L) and cultured by isolation method. The cultivated plates were incubated for 48-72 hours at 37°C under anaerobic conditions. The black-colored colonies on the BBE medium and the grown ones on the BBA medium (5-10 colonies) were subcultured on the BBA medium. Ultimately, after observing the obligate anaerobic, gram-negative, bile esculin-positive and catalase-positive coccobacilli, the isolated strains were preserved at 80°C using 5% glycerol (25).

### Identification of anaerobic bacteria

The anaerobic bacteria were phenotypically identified based on colony morphology, gram staining, and differential tests such as catalase, indole, bile disc, and finally Vitek 2 system (Biomérieux, France). Two polymerase chain reactions (PCR) were also performed to amplify the 16S rRNA gene fragment; the first reaction to confirm the *B. fragilis* group and the second reaction to approve the *B. fragilis* species (26, 27). The 16S rRNA gene was sequenced for *B. fragilis* strains and then submitted to the GenBank sequence database.

### **Antibiotic susceptibility of *B. fragilis* isolates**

The antibiotic susceptibility testing of *B. fragilis* isolates was conducted using agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (28). The tested antibiotics included ampicillin/sulbactam, piperacillin/tazobactam, penicillin G, tetracycline, imipenem, meropenem, clindamycin, cefoxitin, and metronidazole. Different concentrations of the antibiotics were also prepared on the BBA medium containing vitamin K1 (0.5 mg/l) and hemin (5 mg/l).

Moreover, 10 µl of microbial suspension with a density of 10<sup>7</sup> colony-forming unit (CFU) ml<sup>-1</sup> was added to the plates containing antibiotics and a negative control plate to achieve a final dilution of 10<sup>5</sup> CFU per spot. The plates were also incubated for 48 hours at 36°C under anaerobic conditions. After the incubation period, the Minimum Inhibitory Concentration (MIC) were calculated according to the CLSI guideline.

### **Identification of resistance genes**

The presence of IS1186 and *cfiA* genes (associated with resistance to carbapenems), the *cepA* and *cfxA* genes (associated with resistance to beta-lactams), the *ermF*, *ermB*, and *mefA* genes (associated with resistance to clindamycin), the *tetQ* gene (associated with resistance to tetracycline) and the *nim* gene (associated with resistance to metronidazole) were determined by the PCR in *B. fragilis* isolates (29). In order to detect the *bft* gene using PCR, parts of this gene were amplified (30).

### **Statistical analysis**

Data were analysed using the SPSS ver. 18.0 (SPSS Inc., Chicago, IL). The Chi-square test was performed to calculate significant differences between presences of antibiotic resistance genes among resistant strains in comparison to non-resistant strains. Also, Mann-Whitney test was employed to examine significant differences of MIC value for each antibiotic class among isolates with resistance genes in compare with isolates lacking these genes. A *p*-value less than 0.05 was considered as statistically significant.

## **Results**

In this study, 130 clinical samples were collected from 68 cases of abdominal infections (52.3%), 25 cases from ulcers (19.3%), 16 cases from blood (12.3%), 6 cases from surgical infections (4.6%), 5 cases

from pleural effusion (3.8%), 5 cases from joint infection (3.8%) and other infections in the remaining 5 cases (3.8%).

Cultivation results in 28 clinical samples (21.5%) were positive for anaerobic bacteria. The GenBank accession numbers of the 16S rRNA gene for these bacteria were MN982885.1, MN955695.1, MN955694.1, MN955585.1, MN955548.1, MN955546.1, MN94720209.1, MN949555 M55.1, MN955544.1, MN954671.1, MN954561.1, MN954557.1, MN937266.1, MN937239.1, MN933933.1, and MN933926.1. Table 1 shows the frequency of anaerobic bacteria isolated from clinical specimens.

From 40 colorectal tissue biopsies in healthy individuals, 56 *B. fragilis* isolates were identified in 24 specimens (60%). The antibiotic resistance of 78 *B. fragilis* isolates (22 isolates from clinical samples and 56 isolates from the GI tract of healthy individuals) was determined using the agar dilution method. Table 2 shows the antibiotic resistance pattern of *B. fragilis* with the MIC 50 and MIC 90 values ( $\mu\text{g/mL}$ ). The *B. fragilis* isolates also had the highest resistance to penicillin (100%), tetracycline (74.4 %), clindamycin (41%) and ceftiofur (38.5%).

The *tetQ*, *ermF*, *ermB*, *cfiA*, *cepA*, *cfxA*, *mefA*, *nim* genes and the insertion sequence IS1186 were further searched to evaluate antibiotic resistance by the PCR. Absolute and relative frequencies of resistance and insertion sequences genes are presented in Table 3.

In this study, the *tetQ* (87.2%), *cepA* (73.1%) and *ermF* (64.1%) were the most abundant antibiotic-resistant genes. The *nim* and *ermB* genes were not detected in any of the isolates. The IS1186 sequence in the upstream region of the *cfiA* gene was detected in one isolate (1.3%); this isolate was also resistant to imipenem.

The presence of the *cfxA* and *ermF* genes were significantly higher in ceftiofur and clindamycin resistant isolates in compare with ceftiofur and clindamycin susceptible isolates ( $p=0.001$ ,  $0.000$ ).

In addition, MIC value of penicillin, ceftiofur and clindamycin were significantly difference among isolate with the *cepA*, *cfxA* and *ermF* genes in compare with isolates lacking these genes ( $p=0.002$ ,  $0.000$ ,  $0.001$ ) (Fig. 1).

The *bft* gene was observed in 22.7% and 17.8% of the clinical and GI isolates, respectively (Table 3).

## Discussion

*Bacteroidetes* as a large community of gut microbiota can be isolated from human clinical specimens and lead to mixed anaerobic bacterial infections (3). Antibiotic-resistant genes also play important roles in the antibiotic resistance of *B. fragilis* and cause unsuccessful antibacterial therapy. In addition, the transmission of resistance genes through horizontal gene transfer, as the most common mean of acquiring resistance genes among bacteria, is another major problem. In this study, we have evaluated the prevalence of resistance genes and antibiotic resistance profile of *B. fragilis* using phenotypic approaches and amplification of genes of interest.

In this study, *B. fragilis* accounted for 57.4% of anaerobic bacteria isolated from clinical samples.

The MIC 50 and MIC 90 values for ampicillin/sulbactam, piperacillin/tazobactam, metronidazole and clindamycin in clinical isolates were at least twice higher than GI isolates. One possible reason for this might be the use of antibiotics in these patients.

Although carbapenems have been considered as highly effective antibiotics in the prevention of anaerobic infections, bacterial resistance to these antibiotics has increased (6, 11, 15, 31). In this study, 1.3% of isolates (n = 1) were resistant to imipenem and 1.3% of isolates (n = 1) were resistant to meropenem, these isolates were collected from the GI tract of healthy individuals which could be considered as a serious risk. The emergence of carbapenem resistance has also been reported in different studies. For instance, meropenem resistance was found to be 0.5% in the United States and 2% in Europe (6, 12, 32). In a study conducted by Kohsari et al. in Iran, the resistance of *B. fragilis* to meropenem was 13.9% (33). Discrepancies observed in different studies regarding antibiotic resistance profile of *B. fragilis* may be due to different reasons including geographical features, population study, and differences in laboratory techniques.

Resistance to carbapenems in *B. fragilis* is usually caused by the expression of the class B metallo-beta-lactamase encoded by the *cfiA* gene, located on the chromosome. Accordingly, if an insertion sequence is located in its upstream region, it will be expressed and will cause carbapenem resistance (4, 34). In a study conducted by Soki et al., *B. fragilis* isolates (n = 10) contained the *cfiA* gene, of which seven isolates were resistant to imipenem (35). In the present study, 18.1% and 12.5% of the clinical and GI samples had the *cfiA* gene respectively. Moreover, the imipenem-resistant isolates had the *cfiA* gene and the IS1186 insertion sequence in the upstream region of the gene whereas the meropenem-resistant strain had this gene but lacked the IS1186 insertion sequence. The resistance was possibly due to expression of the silent carbapenemase gene (36), the presence of other insertion sequences in the upstream region of this gene (IS1187, IS1188, IS942) (32), or other resistance mechanisms such as membrane permeability or penicillin-binding protein (PBP) affinity (37). In addition, some isolates had the *cfiA* gene but were phenotypically sensitive to carbapenem which demonstrate the antibiotic resistance gene may not be expressed. In a study performed by Rashidian et al. in Iran, 31.5% and 20% in *B. fragilis* group isolate from the patients and control groups harbored *cfiA* gene, respectively (38).

Penicillins and second-generation cephalosporin resistance have also been observed in *B. fragilis*.

The most important mechanisms contributing to this resistance is the expression of beta-lactamases which are encoded by the *cepA* gene (resistance to penicillin and cephalosporins other than cefoxitin) and *cfxA* gene (resistance to cefoxitin) (39, 40).

In this study, all the isolates (100%) were resistant to penicillin, of which 73.1% had the *cepA* gene. There was also meaningful difference in penicillin MIC value of isolates with *cepA* gene compared to isolates without *cepA* gene indicating the importance of this gene in resistance to penicillin. In addition, 45.5% and 35.7% of the clinical and GI isolates were respectively resistant to cefoxitin, and 22.7% and 26.8% of

these isolates had the *cfxA* gene, respectively. The presence of the *cfxA* gene was significantly higher in ceftiofur-resistant isolates compared to ceftiofur-susceptible isolates, which was also statistically significant.

The rate of *B. fragilis* resistance to ceftiofur in recent years has been 6.8–33.3% in Europe, 12.6% in Canada, and 23% in Brazil (6, 41, 42). In a study conducted by Kangaba et al. in Turkey, 28% of *B. fragilis* isolates and 32% of isolates from the GI tract had been found to be resistant to ceftiofur. In this study, resistance to ampicillin/sulbactam and piperacillin/tazobactam were 6.4% and 2.6%, respectively (10). In another investigation, 5.4% of *B. fragilis* isolates were resistant to piperacillin/tazobactam which was relatively consistent with the findings reported by Maraki et al. (5.4%) and Yunoki et al. studies (2.8%) (15, 43).

The *ermB* and *mefA* genes were also involved in the development of macrolide resistance in *B. fragilis* (44). The prevalence of clindamycin resistance had been further reported by 54.5% in clinical isolates and 42.9% in the GI isolates which were mainly associated with the presence of the *ermF* gene (40). Clindamycin resistance among *B. fragilis* have been reported in several countries (8, 45–47).

In the present study, all clindamycin-resistant isolates had the *ermF* genes. In addition, five isolates had the *mefA* gene and three of which were clindamycin-resistant strains. The presence of the *ermF* gene also was higher in clindamycin-resistant isolates than clindamycin-susceptible-isolates respectively, which was statistically significant. None of the isolates in this study had *ermB* gene.

The presence of *tetQ* gene associated with tetracycline resistance has been further reported in clinical isolates (43, 48). In the present study, 81.8% and 71.4% of the clinical and GI isolates had tetracycline resistance, and 90.9% and 85.7% of these isolates had the *tetQ* genes, respectively.

In a study conducted by Narimani et al., 86% of the GI isolates were resistant to tetracycline, and the *tetQ* gene was found in 85% of the isolates (48). In the investigation by Kangaba et al. study, 72% of clinical isolates and 92% of GI isolates were resistant to tetracycline, 64% and 92% of them had the *tetQ* gene, respectively (10).

The metronidazole resistance rate was found to be 0–3% in different parts of the world (6, 10, 38, 49). There were no isolates resistant to metronidazole in this study and the *nim* gene was not detected in any isolates.

Based on previous studies, the prevalence of the *bft* gene was reported to be 6.2–20% in the GI isolates (37, 50–53) and 18.5–38.2% in clinical isolates (53–55) which was consistent with the findings in the present study.

Although phenotypic findings indicated resistance to some antibiotics in this study, the PCR findings did not confirm the presence of corresponding resistance genes in the isolates. This fact may suggest the role of other resistance mechanisms such as efflux pumps, changes in the cell wall structure, and catalytic enzymes in *B. fragilis* isolates (40, 56).

## Conclusion

In conclusion, metronidazole, imipenem and meropenem were the most active agents against *B. fragilis* isolates. It was concluded that continuous monitoring of antibiotic resistance patterns of *B. fragilis* in different geographical areas was vital to provide a suitable treatment profile and to prevent infection more accurately. In other words, with regard to the presence of antibiotic-resistant genes and the high risk of antibiotic-resistant strains in the GI tract of healthy people, proper prescription of antibiotics and avoidance of its arbitrary use can help prevent infection and transmission of resistant isolates.

## Abbreviations

*B. fragilis*

*Bacteroides fragilis*; GI tract:gastrointestinal tract; ETBF:Enterotoxigenic *B. fragilis*; BFT:*B. fragilis* toxin; IBS:irritable bowel syndrome; CRC:colorectal cancer; CLSI:Clinical and Laboratory Standards Institute; MIC:Minimum Inhibitory Concentration; CFU:colony-forming unit; BBE:Bacteroides Bile Esculin Agar; BBA:Brucella Blood Agar; PCR:Polymerase Chain Reaction.

## Declarations

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**Author Contributions:** SJ carried out all laboratory experiment, collected data and drafted the manuscript. ZA and MSF are infectious disease specialists and gastroenterologists who provided the specimens from all cases. LS and FH participated in the design of the study. MF and ME supervised all parts of the study. All authors read and approved the final manuscript.

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**Availability of data and materials:** All data relevant to the study are included in the article.

**Ethics approval and consent to participate:** This study was approved by the Ethics Committee of Tehran University of Medical Sciences. Ethics Approval Code: 9421133003.

**Consent for publication:** Informed consent was obtained in all cases.

**Competing interests:** All authors declare that they have no conflict of interest.

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## Tables

**Table 1** Anaerobic bacteria isolated from clinical specimens

<b>Anaerobic bacteria (Genus)</b>	<b>N (%)</b>
<b><i>Bacteroides</i> sp.</b>	
<i>Bacteroides fragilis</i>	22 (46.8)
<i>Bacteroides thetaiotaomicron</i>	3 (6.3)
<i>Bacteroides stercoris</i>	2 (4.3)
<b><i>Clostridium</i> sp.</b>	
<i>Clostridium clostridioforme</i>	2 (4.3)
<i>Clostridium perfringens</i>	2 (4.3)
<i>Clostridium sporogenes</i>	1 (2.1)
<i>Paeniclostridium sordelli</i>	1 (2.1)
<b><i>Prevotella</i> sp.</b>	
<i>Prevotella bivia</i>	2 (4.3)
<i>Prevotella oralis</i>	1 (2.1)
<b><i>Fusobacterium mortiferum</i></b>	1 (2.1)
<b><i>Veillonella</i> sp.</b>	2 (4.3)
<i>Veillonella parvula</i>	2 (4.3)
Other <i>Veillonella</i> spp.	
<b>Gram positive cocci</b>	
<i>Anaerococcus prevotii</i>	1 (2.1)
<i>Finnegoldia magna</i>	2 (4.3)
<i>Peptoniphilus asaccharolyticus</i>	1 (2.1)
<i>Peptostreptococcus</i> spp.	1 (2.1)
<i>Parvimonas micra</i>	1 (2.1)
<b>Total</b>	<b>47 (100)</b>

Due to technical limitations, table 2,3 is only available as a download in the Supplemental Files section.

# Figures

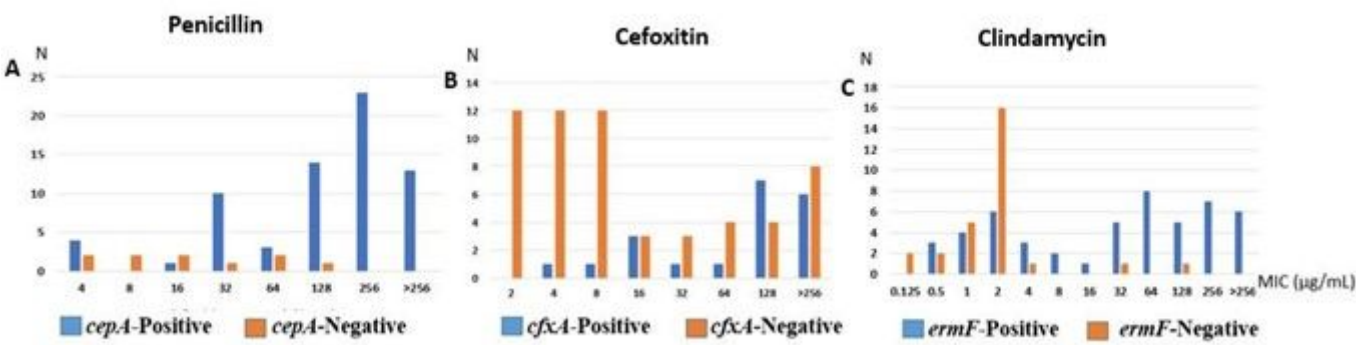


Figure 1

MIC values of (A): Penicillin, (B): Cefoxitin and (C): Clindamycin with the presence of the *cepA* gene, *cfxA* gene and *ermF* genes in *B. fragilis*.

## Supplementary Files

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