Original Article

Antimicrobial Resistance Profile and *Nim* Gene Detection among *Bacteroides fragilis* Group Isolates in a University Hospital in South India

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Abstract

Introduction: Members of *Bacteroides fragilis* group are the most frequently isolated anaerobic pathogens in the clinical laboratory from diverse infection sites. The objective of this study was to characterize *B. fragilis* isolates from various clinical specimens, to analyze their susceptibility profile toward most common anti-anaerobic antimicrobials, and to study the frequency of *nim* gene determining resistance to nitroimidazoles. **Methods:** Specimens processed for anaerobic culture between January 2013 and December 2015 were analyzed. Isolates of *B. fragilis* group were identified and speciated by mass spectrometry. β-lactamase production was detected using nitrocefin disks. Agar dilution and antimicrobial gradient diffusion methods were performed to study their susceptibility profile. The isolates were screened for *nim* gene by conventional gel-based polymerase chain reaction. **Results:** A total of 57 isolates of *B. fragilis* group were studied. The commonly isolated species was *B. fragilis* (73.7%), followed by *Bacteroides thetaiotaomicron* (8.8%), *Bacteroides vulgatus* (8.8%), and others. Most of the isolates were recovered from deep-seated abscesses (47.4%). All isolates were found to be β-lactamase producers. Metronidazole (Mtz) resistance was observed in 4 (7%) isolates. Higher rate of resistance was observed toward clindamycin (31.6%). None of the isolates tested were found resistant to chloramphenicol, piperacillin-tazobactam, and meropenem. *nim* genes were present in 4 (11.4%) *B. fragilis* isolates (n = 35). **Conclusions:** Resistance to the most commonly used empirical anti-anaerobic drugs including Mtz was noted in the isolates of *B. fragilis* group. Routine anaerobic cultures when indicated and continual surveillance of antimicrobial resistance among the anaerobic bacterial pathogens is essential.

Keywords: Anaerobes, antimicrobial resistance, *Bacteroides fragilis* group, clindamycin, metronidazole

Introduction

Bacteroides fragilis group species are the most frequently isolated anaerobes in the clinical Microbiology laboratory. This group consists of >20 species, including *B. fragilis*, *Bacteroides vulgatus*, *Bacteroides ovatus*, *Bacteroides thetaiotaomicron*, *Bacteroides uniformis*, *Bacteroides caccae*, and others.[1] They are associated with a wide spectrum of clinical infections such as intra-abdominal infections, obstetric-gynecologic infections, postoperative wound infections, skin and soft-tissue infections, bacteremia, and others.[2,3] The members of this group can develop resistance to several antimicrobial agents and are responsible for high morbidity and mortality.[3,4] The antimicrobial resistance, particularly within the *B. fragilis* group among the anaerobic bacterial pathogens has been reported from across the globe with reports of resistance to metronidazole (Mtz), β-lactam–β-lactamase inhibitor combinations, and carbapenems.[5] The antimicrobial resistance rates among the *B. fragilis* group have been reported to vary among the various species.[4]

Mtz is utilized clinically for treating various anaerobic infections and also as prophylaxis before certain surgical procedures.[6] However, the increasing frequency of *B. fragilis* group strains resistant to Mtz has resulted in adverse clinical outcomes.[7]

Multiple resistance mechanisms to Mtz have been reported in the *B. fragilis* group. Some of these include, reduced activity

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or complete disruption of electron transport chain components, increased expression of multidrug efflux pumps, overexpression of the DNA repair protein (Rec A) and the expression of 5-nitroimidazole nitroreductases, encoded by *nim* gene types A-G that convert Mtz to non-toxic amino derivatives.[8-10] These *nim* genes can be either found on mobilizable plasmids or be chromosomally encoded.[7,10] However, the presence of *nim* gene may be associated with lower Mtz minimum inhibitory concentrations (MICs) either due to non-expression or low-level expression of the gene. Long-term exposure to Mtz can lead to expression of therapeutic resistance in these strains with silent *nim* genes.[10] Strains which are nim-negative but showing high-level Mtz resistance are also reported, indicative of alternative mechanisms of Mtz resistance in these strains.[6]

Periodic local surveillance of antibiotic resistance rates and the underlying resistance mechanisms among the anaerobic bacteria will help in providing appropriate therapeutic measures. A study was conducted to analyze the antimicrobial susceptibility pattern and detect *nim* gene among the clinical isolates of *B. fragilis* group species.

Methods

Specimen collection and processing

A cross-sectional study was undertaken in the department of Microbiology attached to a tertiary care teaching hospital including consecutive *B. fragilis* group isolates obtained from January 2013 to December 2015. Specimens, including pus aspirates, body fluids, and tissues from diverse infectious sites with suspected anaerobic etiology were processed for anaerobic culture. The specimens were subjected to Gram stain and were inoculated onto 5% sheep blood agar, neomycin blood agar, and phenyl ethyl alcohol agar as per standard guidelines.[11]

The inoculated plates were incubated in an anaerobic jar (GasPak 100 with GasPak EZ Anaerobe container system sachets, Becton Dickinson and Co., Sparks, USA) or in Whitley A35 Anaerobic workstation (Don Whitley Scientific, Shipley, UK). *B. fragilis* group were identified by colony morphology, Gram stain, resistance to special potency disks, vancomycin (5 μg), kanamycin (1000 μg), and colistin (10 μg) and their ability to grow in the presence of 20% bile. Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (Vitek MS, bioMerieux Inc., France) was used for species identification.

β-lactamase production was detected using nitrocefin impregnated paper disks (BD BBL Cefinase, Becton Dickinson and Co, Sparks, USA). The colonies were smeared on the disks and change in color from yellow to red was considered as a positive result.[11]

Determination of antimicrobial susceptibility

The MICs of *B. fragilis* group isolates were determined by agar dilution and/or antimicrobial gradient diffusion method (E test, bioMerieux Inc., Marcy L'Etoile, France). MIC for Mtz (range, 0.25–64 μg/mL), clindamycin (range, 0.25–256 μg/mL) and chloramphenicol (range, 0.25–64 μg/mL) was determined by agar dilution method[11] on Wilkins-Chalgren agar media with Gram-negative anaerobic supplement (HiMedia Labs, Mumbai, India). The antibiotic powders were procured from Sigma-Aldrich, USA. An inoculum size of 10^5 colony-forming unit was applied and plates were incubated for 48 h in anaerobic environment. The MIC endpoint was defined as the lowest concentration of antimicrobial agent where marked reduction in the appearance of growth is observed on the test plate as compared to that of growth on the anaerobic control plate. The susceptibility to meropenem (range 0.002–32 μg/mL), moxifloxacin (range 0.002–32 μg/mL), and piperacillin-tazobactam (range 0.016–256 μg/mL) was tested by antimicrobial gradient diffusion method (E test, bioMerieux Inc., Marcy L'Etoile, France) on 5% sheep blood agar. The plates were incubated in anaerobic environment for 48 h. The MIC values were read at the point where the elliptical zones intersected with the strips. Quality control for antibiotic susceptibility testing was performed with *B. fragilis* ATCC 25285 as reference strain. The results were interpreted as per the Clinical Laboratory Standards Institute (CLSI) guidelines.[12]

Nim gene detection

DNA extraction

For DNA extraction, *B. fragilis* isolates were grown on 5% sheep blood agar for 48 h. Three to four colonies were inoculated into 100 μL distilled water in a microcentrifuge tube to match 3 McFarland standard. The tubes were heated for 15 min at 95°C, after cooling were centrifuged to remove the debris. The lysates were stored at −20°C, till further use.[13]

The *B. fragilis* group was screened for *nim* gene, as described by Trinh and Reysset[14] The primer pair (Sigma Aldrich) used was NIM-3, (5`-ATGTTCAGAGAAATGCGGCGTAAGCG-3`); and NIM-5, (5-GCTTCCTTGCCTGTCATGTGCTC-3`). Amplification process included, an initial denaturation step at 94°C for 10 min followed by 32 cycles of amplification consisting of denaturation at 94°C for 30 s, annealing at 62°C for 1 min, extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. The end products were analyzed by agar gel electrophoresis. Fragments of approximately 458 bp in any of the isolates were considered as presumptive positive for *nim* gene.[14]

Results

A total of 57 nonduplicate *B. fragilis* group isolates were obtained during the study period. Majority of these isolates (36, 63.1%) were recovered from pus aspirates followed by tissue specimens (16, 28.1%) and body fluids (5, 8.8%). The most commonly affected age group was 41–60 years (24, 42.1%) with male predominance (38, 66.7%). The isolates were more frequently obtained from deep-seated abscesses (27, 47.4%) followed by diabetic foot infections (7, 12.3%) and necrotizing fasciitis (6, 10.5%) [Table 1]. Monomicrobial growth of *B. fragilis* was observed in 42.1% (n = 24) of the

Table 1: Spectrum of infections with *Bacteroides fragilis* group species

Clinical condition	*n* (%)
Deep seated abscess	27 (47.4)
Diabetic foot infection	7 (12.3)
Necrotising fasciitis	6 (10.5)
Osteomyelitis	4 (7)
Suppurative otitis media	3 (5.3)
Pyometra	3 (5.3)
Gangrene	2 (3.5)
Bartholin gland cyst	2 (3.5)
Nonhealing ulcer of leg	2 (3.5)
Lower limb cellulitis	1 (1.8)
Total	57 (100)

infections and polymicrobial growth in the rest 57.9% ($n = 33$) cases. The members of Enterobacteriaceae family were the commonly isolated aerobes in the polymicrobial growth. Among them, the most frequent were *Escherichia coli* (16, 48.5%) and *Klebsiella* spp. (8, 24.2%).

Among the Group, *B. fragilis* (42, 73.7%) was the frequently isolated species followed by *B. thetaiotaomicron* (5, 8.8%), *B. vulgatus* (5, 8.8%), *B. ovatus* (4, 7%) and *B. stercoris* (1, 1.8%). β-lactamase activity was detected in all (57, 100%) isolates of the *B. fragilis* group.

Susceptibility to Mtz, clindamycin and chloramphenicol were carried out on all *B. fragilis* group isolates ($n = 57$) by agar dilution. Susceptibility testing to meropenem, piperacillin-tazobactam, and moxifloxacin by antimicrobial gradient diffusion method (E test, bioMerieux Inc., Marcy L'Etoile, France) and screening for *nim* gene was done for 35 (61.4%) isolates, including, *B. fragilis* ($n = 27$), *B. thetaiotaomicron* ($n = 3$), *B. vulgatus* ($n = 3$), and *B. ovatus* ($n = 2$) due to financial constraints.

Among the 57 *B. fragilis* group isolates, resistance to Mtz was observed in 7% ($n = 4$), of which three were *B. fragilis* and one isolate was of *B. thetaiotaomicron*. All the four Mtz resistant isolates had MICs of 16 μg/mL. Resistance to moxifloxacin was observed in 8.6% ($n = 3$) isolates, of which two were *B. fragilis* and one was *B. vulgatus* and all three had a MIC of ≥32 μg/mL. Maximum resistance was noted toward clindamycin (18, 31.6%) which included *B. fragilis* ($n = 16$), *B. ovatus* ($n = 1$), and *B. thetaiotaomicron* ($n = 1$) isolates.

The *nim* gene was detected in four isolates (11.4%) of *B. fragilis* species tested ($n = 35$) of which two had higher Mtz MIC of 16 μg/mL, and two isolates had lower Mtz MIC values of 0.5 and 1 μg/mL. Furthermore, *nim* gene was not detected in two of the *B. fragilis* isolates with Mtz MIC of 16 μg/mL.

Discussion

Bacteroides and *Parabacteroides* genera of the order Bacteroidales are the important components of the colonic microflora which also cause diverse polymicrobial opportunistic infections.[3,15] The virulence factors which help *B. fragilis* in the adherence, immune evasion, and tissue destruction include the fimbriae, lipopolysaccharide, polysaccharide capsule, neuraminidase, and histolytic enzymes such as hyaluronidase and chondroitin sulfatase.[16]

Susceptibility testing for anaerobes is not performed routinely in the clinical Microbiology laboratories due to the need for dilution methods which are technically more demanding and have longer turnaround time, widespread use of 5-nitroimidazole drugs for empirical antibiotic therapy and use of broad-spectrum antimicrobials active against both aerobic and anaerobic bacteria in polymicrobial infections. Antimicrobial resistance among anaerobic bacteria is on the rise worldwide and *B. fragilis* group species are known to exhibit a higher degree of antimicrobial resistance in comparison to other anaerobic pathogens.[1]

In this study, the majority of the *B. fragilis* group isolates were recovered from deep-seated abscesses (47.4%). Ulug *et al*.,[17] Al Benwan *et al*.,[18] and Navarro López *et al*.[19] have also reported recovery of *B. fragilis* as the predominant anaerobe from various abscess sites. The chief virulence factor responsible for abscess formation is the capsule of *B. fragilis*.[16] *B. fragilis* (42, 73.7%) was the most frequently isolated species in our analysis. A similar finding has been reported earlier in a Europe-wide study involving 13 countries.[2] *Bacteroides* species are the important constituents of fecal bacterial flora and account for approximately 25% of the anaerobic gut flora.[16,20] *B. thetaiotaomicron* and *B. vulgatus* are the more prevalent species in this flora. Whereas, *B. fragilis* is the most prevalent species seen clinically in various infections.[20] Varying susceptibility pattern toward the anti-anaerobic antimicrobials has been reported among the different species of the *B. fragilis* group with, *B. fragilis* being more susceptible than other species.[1,21,22] It has been reported that high rates of antimicrobial resistance are seen among clinical isolates of *B. thetaiotaomicron* and *P. distasonis* which account for about 13%–23% of all *Bacteroides* isolates.[21] Routine species-level identification and surveillance of species-wise distribution of antimicrobial resistance among the *B. fragilis* group is essential in laboratories reporting higher isolation rates of this clinically significant group of pathogenic species.

Mtz remains the drug of choice for most of the anaerobic infections including those caused by *B. fragilis* group species. Antimicrobial resistance surveys in the past have indicated very low rates of resistance to Mtz.[2,23,24] However, we found 7% ($n = 4$) of isolates resistant to Mtz. Nagy *et al*. have proposed disc diffusion zone diameter breakpoints for Mtz and other antibiotics for testing *B. fragilis* group isolates.[25] There is a need for standardization and adoption of disc diffusion procedure and breakpoints for anaerobic bacteria by the CLSI and European Committee on Antimicrobial Susceptibility Testing.

Four of our *B. fragilis* isolates showed Mtz MIC of 16 μg/mL and *nim* gene was detected in only two of them. This suggests

an alternative resistance mechanism in the other two strains which had susceptible MIC values. Furthermore, the presence of *nim* genes *per se* does not necessarily confer therapeutic Mtz resistance, as *nim* genes have been detected in members of the *Bacteroides* group with MICs in the susceptible range.[10,26] Similar detection of *nim* genes in the absence of phenotypic resistance has been described earlier.[27]

High rates of clindamycin and moxifloxacin resistance among *B. fragilis* group. have been reported in other studies.[4,23,28] Resistance to β-lactam–β-lactamase inhibitor combinations and carbapenems are also reported among *B. fragilis* group species but currently at lower but significant rates.[4,23,24] With significant resistance being noted toward both commonly used and second line antimicrobials among the *B. fragilis* group across the globe, it is necessary that antimicrobial resistance among anaerobic bacterial pathogens be considered in hospital stewardship programs.

Conclusions

The susceptibility among anaerobes to different antimicrobials differs from species to species and also varies among the regions. Performing anaerobic antimicrobial susceptibility testing on a routine basis in Microbiology laboratory will help in detection of the resistant strains, and also aid in monitoring the changing trends of susceptibility among the anaerobic pathogens. Judicious usage of empiric antimicrobials including Mtz has to be considered; else it may result in the development of superbugs similar to their aerobic counterparts.

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Conflicts of interest

There are no conflicts of interest.

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