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Clinico-microbiological profile of *Bacteroides fragilis* with focus on molecular detection of emerging resistance

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***Background & objective*: *Bacteroides fragilis* is a Gram-negative anaerobic opportunistic pathogen which is managed by empirical anaerobic coverage as a hospital norm. However, with rising reports of resistance among *B. fragilis* strains, antibiotic susceptibility testing for this pathogen may be the only way to understand the magnitude of the problem. This study aimed to characterize resistance patterns among clinical isolates and identify resistance genes.**

***Methods*: A prospective observational study was conducted which included all samples requesting anaerobic cultures within the study period. Minimum inhibitory concentration (MIC) was detected for metronidazole, clindamycin and chloramphenicol by agar dilution. E-test strips were used for imipenem and piperacillin, followed by polymerase chain reaction to detect *nim* and *cfiA* genes.**

***Results*: Among a total of 50 isolates, 94 per cent (47/50) were susceptible and six per cent (3/50) showed intermediate resistance to metronidazole. Susceptibility to clindamycin and piperacillin was noted in 70 and 50 per cent of strains; intermediate resistance in 14 and 2 per cent and resistance in 16 and 48 per cent, respectively. No resistance was observed for chloramphenicol and imipenem. *Nim* gene was found in 26 per cent (13/50) and *cfiA* gene was found in 52 per cent (26/50) of isolates. Isolates with high metronidazole MIC of 8-16 µg/ml were found to carry *nim* gene (χ^2 test, $P<0.001$).**

***Interpretation & conclusions*: Rising resistance among *B. fragilis* is evident and there is a significant association between *nim* gene and metronidazole resistance. Improving awareness among clinicians is paramount in tackling AMR among these pathogens, as empirical anaerobic coverage may not be effective in all cases.**

Key words Agar dilution - AMR - anaerobic infections - antimicrobial susceptibility testing - *Bacteroides fragilis* - carbapenem - metronidazole resistance - MIC

Bacteroides fragilis, an opportunistic anaerobic pathogen, is frequently involved in intra-abdominal infections, intracavitary abscesses and complicated skin/soft tissue wounds[1]. *B. fragilis* group are known to be useful commensals, facilitating host metabolism and shaping host immune responses[1]. However, *B. fragilis* is one of the most frequently isolated anaerobic pathogens associated with maximum virulence and resistance mechanisms among all pathogenic anaerobes[2].

The rise of antibiotic resistance in *B. fragilis* group over the past decade highlight the need for antibiotic susceptibility testing (AST) as a part of routine microbiological procedure. However, isolation of anaerobic pathogens is limited due to difficult culture techniques, maintenance of anaerobiosis and infrequent culture requests[3]. The need for simpler methods of antimicrobial susceptibility testing is rapidly coming to light[4]. *Bacteroides* genus is inherently resistant to aminoglycosides along with first- and second-generation quinolones, making selection of management difficult. Resistance to metronidazole, a commonly used empirical drug, has been observed due to a nitroimidazole reductase enzyme encoded by a '*nim'* gene[5-7]. Currently, nine *nim* genes leading to the emergence of multidrug-resistant *Bacteroides* isolates have been described[8]. Another gene gaining notice is the *cfiA* gene that is associated with metallo-β-lactamase (MBL)-producing, carbapenem-resistant *B. fragilis* isolates causing fatal sepsis[9].

Clinical spectrum of anaerobes is relatively unexplored, and limited regional studies have shown a prevalence of almost 20 per cent in the Southern Karnataka region[10]. Studying the resistance determinants can help provide valuable information to benefit the health and safety of patients. Hence, this study aims to detect phenotypic resistance to metronidazole, clindamycin, imipenem, piperacillin and chloramphenicol by agar dilution method in *B. fragilis* isolates from a tertiary care setting, furthermore to detect the presence of *nim* and *cfiA* resistance genes and to correlate the presence of genotypic determinants with phenotypic findings.

Material & Methods

Study type and setting: This was a prospective observational study conducted at the department of Microbiology, Kasturba Medical College, Manipal Academy of Higher Education (Karnataka, India), as per the STROBE statement for cross-sectional studies, after Institutional Ethics Committee clearance.

Sample collection: All *Bacteroides* spp. isolates from various clinical samples such as soft tissue specimens, pus aspirates, body fluids and wound swabs sent in Robertson's Cooked Meat Media (RCM) were included in study from September 2017 to April 2019 at the study site. Only first isolation of *B. fragilis* among enrolled patients was included, repeat isolates were excluded from analysis. Stool specimens were excluded from the study.

Study definitions: Empiric antibiotic therapy was defined as the antibiotic selected by the managing clinician before AST report was released. Definitive therapy was defined as antibiotic that the patient received after AST were available. Sepsis as per third international consensus definitions for sepsis and septic shock (Sepsis-3)[11]. Faecal contamination of infection site was recorded as per the surgical assessment of wound documented in medical case records. Improved clinical outcomes were based on the resolution of presenting symptoms and discharge from the hospital in a good state of health.

Sample processing: Clinical samples were collected and processed uniformly according to the standard operating procedure. Gram stain was performed for all specimens and was inoculated in RCM broth. After 48 h of incubation, the samples were inoculated in five per cent sheep blood agar, phenylethyl alcohol agar and neomycin blood agar each along with metronidazole (5U) disc in the anaerobic workstation (Whitley A35 Anaerobic Workstation, Don Whitley Scientific, Shipley, UK). Isolates identified as *Bacteroides* spp. on colony morphology and special potency discs were confirmed by matrix-assisted laser desorption/ ionization time-of-flight (MALDITOF/MS) (Vitek MS, BioMerieux Inc., Marcy L'Etoile, France).

β-lactamase production was detected using nitrocefin impregnated paper discs (BD BBL Cefinase paper discs, Becton Dickinson and Co, Sparks, USA). Those isolates identified as *B. fragilis* were stored in Skim milk broth at −70°C till further processing for molecular and phenotypic testing.

Phenotypic detection of antimicrobial resistance of B. fragilis group strains: Agar dilution method was used to test metronidazole, clindamycin and chloramphenicol (Sigma Aldrich Ltd, St. Louis, USA) resistance among *Bacteroides* spp. isolates on Wilkins-Chalgren agar media (HiMedia Labs, Mumbai, India) with Gram-negative anaerobic supplement (HiMedia Labs, Mumbai, India). The susceptibility to piperacillin and imipenem was evaluated by antimicrobial gradient diffusion method using the E-test strips (E-test, BioMerieux Inc., Marcy L'Etoile, France) on five per cent sheep blood agar. The plates were incubated in anaerobic environment for 48 h. Quality control for susceptibility was performed by using *B. fragilis*

ATCC 25285 as the reference strain. The results were interpreted as per the Clinical Laboratory Standards Institute (CLSI, 2019) guidelines[12].

Molecular detection of antibiotic resistance by conventional polymerase chain reaction (PCR) method: DNA extraction was done by QIAamp DNA Mini Kit (QIAGEN, Valencia, California, USA) according to the manufacturer's instructions. PCR primers and protocol followed were similar to as described previously[13]. The PCR end products were subjected to 1.5 per cent agar gel electrophoresis in tris-acetic acid-ethylenediaminetetraacetic acid buffer with 100 bp ladder. The relationship of strains harbouring *nim* and *cfiA* genes and their susceptibility to metronidazole and imipenem, respectively, was analyzed.

Statistical analysis: A structured study proforma was used to document clinical and laboratory data of study subjects on a real-time basis. The data were analyzed using SPSS version 16 (IBM Corp., Armonk, New York, USA). Chi-square test was used to assess significant association between presence of *nim* gene, *cfiA* gene and phenotypic resistance. The level of significance for all the statistical tests was fixed at five per cent and the results were reported with 95 per cent confidence interval (CI).

Results

A total of 673 samples were received for anaerobic culture during the study period, among which 125 were cultures growing anaerobic bacteria of which 49/125 (39.2%) samples detected 50 clinically significant *B. fragilis* isolates which were in support of the clinical presentation along with final diagnosis and were not amounted to as contaminants. A total of 50 *Bacteroides* spp. isolates were identified by MALDI-TOF comprising *B. fragilis* 31 (62%), *Bacteroides thetaiotaomicron* eight (16%), *Bacteroides ovatus* five (10%), *Bacteroides vulgatus* five (10%) and *Bacteroides uniformis* one (2%). Majority of patients presented with mixed aerobic and anaerobic polymicrobial infections in the study population 41/49 (83.6%). Monomicrobial anaerobic infection were observed in only three (6%) of the patients and polymicrobial anaerobic infections in five (10%) patients. The most commonly isolated aerobe alongside *B. fragilis* was from *Enterobacteriaceae* family 35/50 (70%) – *Escherichia coli* and *Klebsiella pneumoniae*, followed by Gram-positive cocci such as *Staphylococcus aureus* and *Streptococcus* spp. 11/50 (22%).

Majority of the patients were male (53, 71.4%) and the average affected age group was 51.3±15.4 yr. Improved clinical outcome was noted in 42 (85.7%) of the patients (Table I). Death during hospital stay was noted in two (4%) and five (10.2%) patients were lost to follow up as they were progressively worsening and were discharged against medical advice. Majority of the patients presented with intra-abdominal (13, 24.5%) source of infection which included intra-abdominal abscess (6/12, 50%), appendicular abscess (1/12, 83.3%), perforation peritonitis (2/12, 16.6%) and post-surgical peritonitis (3/12, 25%). Fournier's gangrene was significantly associated with *B. vulgatus* (2/3, 66.6%) and *B. ovatus* (1/3, 33.3%), this association was found to be statistically significant with P=0.05, odds ratio (OR): 2.875 (CI: 1.935-4.27). There were no cases of bloodstream infection with *B. fragilis* during the study period. *In vitro* activities of antimicrobial agents against clinical *B. fragilis* isolates are tabulated in Table II. All isolates were found positive for β-lactamase activity.

Among total isolates, 47/50 (94%) were susceptible and 3/50 (6%) showed intermediate resistance [minimum inhibitory concentration (MIC)=16 µg/ml] to metronidazole. No phenotypic resistance to metronidazole was however, detected. Sensitivity to clindamycin and piperacillin was noted in 35/50 (70%) and 25/50 (50%) of strains; intermediate resistance in 7/50 (14%) and 1/50 (2%) and resistance in 8/50 (16%) and 24/50 (48%), respectively. No resistance was observed for chloramphenicol and imipenem as per the CLSI guidelines. Highest MIC_{50} was found in piperacillin 48 µg/ml, followed by clindamycin 2 µg/ml, metronidazole 0.5 µg/ml, chloramphenicol 4 µg/mL and imipenem 0.19 µg/ml.

On genotypic analysis of the study isolates (Figure), *nim* gene was found in 13 (26%) and *cfiA* gene was found in 26 (52%) of the isolates. Among the 13 *nim*-positive isolates, five showed high metronidazole MICs (8-16 µg/ml) and eight showed low metronidazole MICs (<0.25-8 µg/ml). High metronidazole MIC was found to be significantly associated with presence of *nim* gene with [P<0.001, OR – 5.626 (CI: 3-10.5); (Table III)]. Among the 26 *cfiA*-positive isolates, none showed high imipenem MICs (>4 µg/ml) and 26 showed low imipenem MICs (<0.25-2 µg/ml). Of the 24 *cfiA*-negative isolates, two isolates had high imipenem MIC (4 µg/ml)

Table I. Demographics, comorbidities and other clinical characteristics of the study population (n=49)

Characteristics	n (%)
Demographic data	
Age (yr), mean±SD	51.3±15.4
Male gender, n (%)	35 (71.4)
Charlson Comorbidity Index, median (IQR)	3 (2-5)
Comorbidities	
Diabetes mellitus type 2, n (%)	31 (62)
Other aerobic infections, n (%)	14 (28.5)
Solid organ tumour, n (%)	5 (10.2)
Immunosuppressive drugs*	4 (8.2)
Chronic renal dysfunction (CKD stage 4/5), n (%)	2 (4)
Clinical characteristics	
History of trauma, n (%)	5 (10.2)
ICU admission during hospital stay (>two days), n (%)	9 (18.4)
Sepsis	10 (20.4)
Surgical intervention prior to isolation	12 (24.4)
Faecal contamination of wound	23 (46.9)
Concomitant aerobic bacteraemia	6 (12.2)
Empirical therapy	25 (51)
Definitive therapy for *Bacteroides fragilis*	36 (73.4)
Improved clinical outcome	42 (85.7)
Clinical spectrum of infection	
Intra-abdominal infection	12 (24.5)
Diabetic foot ulcer	9 (18.4)
Necrotizing fasciitis	9 (18.4)
Deep seated abscess	8 (16.3)
Fournier's gangrene	3 (6.1)
Cellulitis	2 (4.1)
Intracranial abscess	2 (4.1)
Osteomyelitis	2 (4.1)
Empyema	1 (2)
Pyometra	1 (2)
Total	49

*Immunosuppressive drugs included in the study were corticosteroids >six months, methotrexate, cyclosporine, tacrolimus and monoclonal antibodies. SD, standard deviation; IQR, interquartile range; CKD, chronic kidney disease; ICU: intensive care unit

and the other 22 strains had low imipenem MICs (<0.25-2 µg/ml) (Table III). However, all study isolates were in the susceptible range for imipenem.

Discussion

Bacteroides spp. are key members of the normal intestinal flora of a healthy adult. Infections due to *Bacteroides* spp. have been observed in patient samples ranging from 19.9 to 64 per cent in India and from seven to 26 per cent internationally[1,5,10,14]. Among clinically significant anaerobic infections by the various subspecies, *B. fragilis* is the most commonly 31/50 (62%) isolated anaerobic pathogen[8,15]. Monomicrobial anaerobic infections in the study region were previously reported as 21.9 per cent; however, our study observed these infections in only three (6%) patients due to the widespread use of empirical therapy (25, 51%)[16]. On demographic analysis, type 2 diabetes was noted in 31 (62%) patients in the study population, suggesting diabetes-associated complications as one of the main risk factors for patients with *B. fragilis* infection. Surgical manipulation of the gastrointestinal tract was another important risk factor due to the abundance of *Bacteroides* spp. in the gut flora.

In the present study, no isolates were found resistant to metronidazole, imipenem or chloramphenicol. A MIC of 16 µg/ml was observed for metronidazole in 3/50 (6%) of the isolates, suggesting intermediate susceptibility. Previous CLSI guidelines identified MIC >16 µg/ml as 'resistant' *B. fragilis* strains, however, with updated cut-offs these isolates are now reported as 'intermediate'[12]. This creates difficulties in the comparison of reported resistance over periods of time and across geographical areas. Majority of isolates were resistant to piperacillin, 24 (48%), followed by clindamycin, eight (16%). A decrease in MIC of clindamycin and metronidazole was observed, possibly due to the increased awareness among clinicians, antibiotic stewardship policies and benefits of local antibiograms[17]. In any case, global metronidazole resistance is still low (<1%), but studies from India have shown significantly higher resistance to metronidazole (30%-31%)[14]. This variation in reported resistance may be due to the lack of routine anaerobic AST in most clinical laboratories and reliance upon disc-diffusion test leading to inaccurate reporting of resistant strains[5,14]. Even in developed countries like the United States of America, only 21 per cent of the laboratories conduct routine AST for anaerobic infections[18]. Studies have reported carbapenem resistance from south America (1.1%), Taiwan (7%), Japan (3.8%) and Europe (<1.2%), but this has not yet been reported from India[15,19-21].

In the present study, majority, 52 per cent of isolates, were found to carry *cfiA* gene; however, none were found

Table II. Overview of *in vitro* activities of antimicrobial agents via agar dilution method against clinical *Bacteroides fragilis* isolates (n=50)

Strain	Agents	MIC (µg/ml) (%)												MIC
		<0.25	0.25	0.5	1	2	4	8	16	32	64	128	256	50
Bacteroides	MTZ	6 (19.4)		20 (64.5)		1 (3.2)	2 (6.5)	1 (3.2)	1 (3.2)					0.5
fragilis (n=31;	CLI	7 (22.6)		4 (12.9)	4 (12.9)	7 (22.6)	4 (12.9)					5 (16.1)		2
62%), n (%)	CPL					1 (3.2)	29 (93.5)	1 (3.2)						4
	IPM		23 (74.2)	3 (9.7)	1 (3.2)	1 (3.2)	3 (9.7)							0.25
	PIP			1 (3.2)	1 (3.2)	9 (29.1)	3 (9.7)			2 (6.5)	1 (3.2)		14 (45.2)	32
Bacteroides	MTZ	3 (60)		2 (40)										0.25
ovatus (n=5;	CLI				2 (40)	2 (40)						1 (20)		2
10%), n (%)	CPL					1 (20)	4 (80)							4
	IPM		3 (60)	1 (20)	1 (20)									0.25
	PIP					1 (20)	1 (20)		1 (20)				2 (40)	16
Bacteroides	MTZ			6 (75)					2 (25)					0.5
thetaiotaomicron	CLI	1 (12.5)			1 (12.5)	2 (25)	3 (37.5)					1 (12.5)		4
(n=8; 16%),	CPL					1 (12.5)	7 (87.5)							4
n (%)	IPM		5 (62.5)	1 (12.5)	1 (12.5)		1 (12.5)							0.25
	PIP			1 (12.5)		1 (12.5)			1 (12.5)	1 (12.5)			4 (50)	256
Bacteroides	MTZ	1 (20)		3 (60)				1 (20)						0.5
vulgatus (n=5;	CLI	1 (20)		1 (20)		2 (40)						1 (20)		2
10%), n (%)	CPL					1 (20)	4 (80)							4
	IPM		3 (60)		1 (20)	1 (20)								0.25
	PIP						1 (20)	1 (20)					3 (60)	256
Bacteroides	MTZ						1 (100)							NA
uniformis (n=1;	CLI				1 (100)									NA
2%), n (%)	CPL						1 (100)							NA
	IPM		1 (100)											NA
	PIP												1 (100)	NA

The number of isolates, along with strain percentage, is illustrated as per their corresponding MICs. MTZ, metronidazole; CLI, clindamycin; CPL, chloramphenicol; IPM, imipenem; PIP, piperacillin; MIC, minimum inhibitory concentration; NA, not available

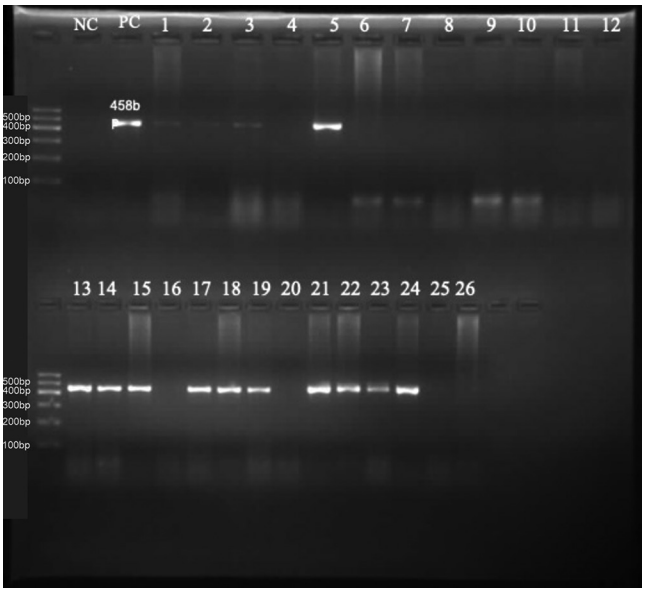

Figure. Identification of *nim* gene as 458 bp amplification products on agarose gel documentation seen on lanes – 1, 3, 5, 13, 14, 15, 17, 18, 19, 21, 22, 23 and 24.

Table III. Relationship among strains with *nim* and *cfiA* genes and their susceptibility to metronidazole and imipenem (n=50)

Metronidazole MIC	*nim* gene present (%)	*nim* gene absent (%)	Total (%)
High (8-16 µg/ml)	5 (100)	0 (0)	5 (10)
Low (<0.25-8 µg/ml)	8 (61.5)	37 (100)	45 (92)
P, odds ratio (CI)	<0.001, 5.626 (3-10.5)		
Imipenem MIC	*cfiA* gene present (%)	*CfiA* gene absent (%)	Total (%)
High (>4 µg/ml)	0 (0)	2 (100)	2 (4)
Low (<0.25-2 µg/ml)	26 (54.1)	22 (45.8)	48 (96)
P		0.225	

resistant on E-test as per the CLSI guidelines (MIC >16 µg/ml)[12]. The clinical implications of low-level MBL production by *B. fragilis* are unknown, and studies looking into *Bacteroides* spp. resistance patterns from India are limited. The discrepancy between phenotypic resistance and presence of genetic determinants has been characterized by other studies. It has been explained that the resistance genes are carried on conjugative and mobilizable plasmids, conjugative transposons and integrated genetic elements[6]. Resistance due to these genes has been associated with upstream insertion sequences (ISs) such as IS1186, IS1187, IS1188 and IS942 and absence of these insertion sequence allows for *in vitro* susceptibility on AST[22,23]. This suggests that *cfiA* gene may not be the only factor at play when conferring resistance among *B. fragilis* strains[24].

The present study is limited by the small sample size and single-centre inclusion criteria. Furthermore, patient follow up and management of anaerobic pathogens were outside the scope of our study. Further studies into the complete antimicrobial susceptibility profile of *B. fragilis* are required to assess growing phenotypic resistance. The present study also lacked insight into other important genes such as *ermF* gene (clindamycin resistance), *cepA* gene (penicillin G resistance) and upstream IS associated with activation of *cfiA*. As India is one of the largest consumers of antibiotics, it is important to study resistance patterns among anaerobic bacteria and to prevent isolating carbapenem resistant *B. fragilis* strains in the future. Therefore, routine AST for anaerobes and the creation of detailed regional antibiograms for *B. fragilis* should be considered in all microbiological laboratories. Furthermore, identifying resistance in patients with multiple comorbidities or intra-abdominal surgical interventions will assist antibiotic selection and adherence to antimicrobial stewardship programmes.

This study highlights the importance of anaerobic infection in a tertiary care setting and gives special focus to the upcoming resistance in *B. fragilis*. Improving awareness among clinicians is paramount in tackling AMR among these pathogens as empirical anaerobic coverage may not be effective in all cases. The study stresses on implementing susceptibility testing of anaerobes so as to improve patient care.

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***Conflicts of Interest*:** None.

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