

Phenotypic and genotypic antimicrobial resistance in clinical anaerobic isolates from India

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Background: Antimicrobial resistance (AMR) in anaerobes remains a neglected field. The laborious procedures, non-compliance with the standard methodology and differences in interpretive breakpoints add variation in resistance data.

Objectives: To assess the phenotypic and genotypic resistance among clinically important anaerobes to six antibiotics frequently used as empirical therapy for anaerobic infections.

Methods: A total of 150 anaerobic isolates were recovered from clinical specimens. The antimicrobial susceptibility was determined by the breakpoint agar dilution method as per CLSI guidelines. The presence of genes encoding resistance to metronidazole (*nim* gene), imipenem (*cfiA* gene) and mobilizable insertion sequence (IS) elements was detected to comprehend their association with phenotypic resistance.

Results: This is a first study of its kind from the Indian subcontinent looking at the AMR and associated genes in anaerobes. Resistance to metronidazole, clindamycin, imipenem, piperacillin/tazobactam and ceftiofuran was 32.6%, 42.6%, 0.6%, 38% and 35.3%, respectively. No resistance was observed to chloramphenicol. The *nim* gene was detected in 24.6% of isolates, of which 70.2% were resistant by phenotype. On sequencing, the PCR products of six random *nim* genes showed a close similarity to *nimE* of *Bacteroides fragilis* with 99% nucleotide and 100% amino acid sequence similarity. The *cfiA* gene, associated with imipenem resistance, was detected in 16% of isolates.

Conclusions: The possibility of isolates carrying AMR genes to become resistant to antibiotics by acquisition of IS elements mandates attention to periodically monitor the resistance patterns and geographic distribution of these genes and IS elements to understand the trends of AMR in anaerobes.

Introduction

Antimicrobial resistance (AMR) remains a neglected concern in anaerobes. Anaerobes are overlooked and infrequently reported due to the difficulties involved in their isolation and identification¹ but their role in human health and disease should not be underestimated. They constitute the majority of commensal flora colonizing the skin, oral cavity and human gut. They are likely to cause bacterial infections of endogenous origin if translocated or displaced from their natural habitat² and have been found associated with other serious, life-threatening infections such as CNS infections,³ bacteraemia⁴ endocarditis and infections in compromised patients.² The data on AMR in anaerobes is scarce because antimicrobial susceptibility testing (AST) in anaerobes is a laborious process, and majority of the existing literature does not comply

with CLSI and EUCAST guidelines.⁵ Differences in the methodology and interpretive breakpoints between CLSI and EUCAST add to the variation of resistance data in anaerobes.⁶ The dearth of surveillance systems and the conviction that resistance in anaerobes is fairly predictable often makes treatment empirical. However, emerging resistance is being recognized among anaerobes that were earlier believed to be highly susceptible.⁷ Therefore, there is an urgent need to periodically monitor the resistance patterns of clinically significant anaerobes to help the microbiologist as well as the clinician understand the trends of AMR in anaerobes and devise better therapeutic strategies to combat resistance and improve outcomes.

AMR among anaerobes has been growing at a steady pace since the 1970s and poses a serious threat to global healthcare.⁷

To date, most of the available studies have addressed AMR in *Bacteroides* spp., which is also the most frequently isolated antibiotic-resistant anaerobic bacteria.⁵ AMR and the role of the mobile genetic elements (MGE) have been studied for a limited number of antibiotics. Therefore, we designed our study to target the resistance pattern among various clinically important anaerobic bacteria against six antimicrobial agents, namely, metronidazole, imipenem, piperacillin/tazobactam, clindamycin, chloramphenicol and ceftioxin, which are among the CLSI-recommended antimicrobial agents to be considered for anaerobic infections. For decades, these antimicrobial agents have been routinely used as an empirical therapy in our clinical setup and also in the United States, European and Asian countries.^{8–10} The AST was performed using the gold standard agar dilution method according to the CLSI guidelines. To comprehend the relationship between phenotypic and genotypic resistance, the strains were screened for the presence of two genes encoding resistance to metronidazole (*nim* gene) and imipenem (*cfiA* gene). The susceptible strains with 'silent' *cfiA* genes may become resistant by the presence and expression of insertion sequence (IS) elements at a region upstream of the *cfiA* genes via a one-step mutation. Also, these IS elements carry regulatory signals that are not only associated with the *cfiA* expression but also induce *nim* gene-mediated metronidazole resistance.^{11,12} Thus, the presence of these IS elements particularly (IS1186) was also investigated in this study.

Materials and methods

Sample collection and processing

In this study, 150 anaerobic bacteria were prospectively isolated over a period of 1 year (2018) from routine samples submitted to the Clinical Bacteriology Laboratory of the department of Medical Microbiology, Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. PGIMER is a 2300 bed tertiary care hospital that caters to a population of around 367 million people, primarily of North-West Indian states, and includes referrals from rest of the country. The clinical samples were obtained from a wide range of sources and infection sites from patients of all age groups (Table S1, available as [Supplementary data](#) at [JAC-AMR Online](#)). A total of 11 088 samples were processed anaerobically in the study period of 1 year and 762 anaerobic bacteria were recovered from 554 samples (Figure S1). One-hundred and fifty consecutive isolates identified correctly at species level and belonging to patients with complete clinical and demographic data were included in the study. Isolates were cultured on brucella agar supplemented with 5% laked sheep blood, vitamin K (1 mg/L), hemin (5 mg/L) and metronidazole disc (5 µg) placed at the centre of the plate for presumptive testing of anaerobes. An automated anaerobic gas generation system (Anoxomat Mart II, Mart Microbiology BV, Lichtenvoorde, Netherlands) and anaerobic jars were used to create anaerobiosis from the gas mixture (80% N₂, 10% CO₂, 10% H₂). In each run, the anaerobiosis was validated by one obligate aerobic and anaerobic strain. Following incubation at 37°C for 48 h, the anaerobic plates were examined for a zone of inhibition around the metronidazole disc. Presence of a zone, irrespective of the zone size, was suggestive of anaerobic growth; whereas in case of no zone of inhibition, the anaerobic plates were correlated with their aerobic plates to rule out metronidazole-resistant anaerobic growth. A metronidazole disc was only used to aid in the presumptive recognition of anaerobes and has no bearing on therapeutic testing. Identification of isolates to species level was done using MALDI-TOF MS; Biotyper 2.0 database (Bruker Daltonik GmbH, Bremen, Germany) following the standard Bruker interpretative criteria. Scores of ≥ 2.0 were considered as an accurate identification to species level; ≥ 1.7 but < 2.0 , an identification to genus level and

scores < 1.7 were considered unreliable.¹³ All isolates included in our study were correctly identified to the species level with a MALDI score value of ≥ 2.0 (Table S2).

Antimicrobial susceptibility testing

Susceptibility of these isolates was determined by breakpoint agar dilution method as per CLSI (M11-A8) protocol. A serial two-fold dilution of each antibiotic was added to the medium. An inoculum of turbidity matching 0.5 McFarland standard was prepared and 10 µL of each isolate was spot inoculated on the surface of the agar plate. Approximately 25 isolates were tested on each plate (Figure S2). Following incubation, the inoculated spots were examined visually for bacterial growth. The lowest concentration of antibiotics that prevented bacterial growth was considered to be the MIC. The MIC was interpreted as per the CLSI breakpoints (Table S3). In each run *B. fragilis* ATCC 25285 was used as a quality control of susceptibility testing.

Detection of AMR genes and IS element

DNA was extracted by the boiling method¹⁴ and the presence of *nim* gene, *cfiA* gene and IS1186 was detected using PCR primers and the thermal cycling parameters mentioned in Table S4^{15–17} with the reaction setup being summarized in Table S5.

Sequencing and phylogenetic analysis

Amplified PCR products were sequenced in house and by commercially available sequencing services (AgriGenome Labs India). Using BLAST, the nucleotide sequences were compared in National Center for Biotechnology Information (NCBI). The GenBank accession number for *nimE* gene of *B. fragilis* is MH341532.

Results

A total of 150 clinical isolates of anaerobic bacteria representing 12 genera and 29 species were isolated from various clinical specimens comprising varied infections with an almost equal distribution of polymicrobial and monomicrobial growth as represented in Table S1 and Figure S1, respectively. *Escherichia coli* was the commonest facultative anaerobic species (20.2%) associated with the polymicrobial growth; the distribution of anaerobic pathogens isolated in this study is given in Table 1.

Antimicrobial susceptibility

The study evaluated AMR to six antibiotics. A representative picture for AST by agar dilution method and the interpretation of the MIC is shown in Figure S3. The AST and MICs of antimicrobial agents as per CLSI range is summarized in Figure 1 and the data is interpreted by incorporating intermediate in the resistant category.

Of the 150 isolates, excluding four inherently resistant organisms [i.e. *Cutibacterium* spp. ($n = 2$), *Actinomyces* spp. ($n = 1$) and *Streptococcus* spp. ($n = 1$)] metronidazole resistance was seen in 30.8% (45/146) isolates. The highest resistance was seen in Gram-negative anaerobes, among which *B. fragilis* was the most resistant at 57.7% (26/45), whereas resistance was detected in only three Gram-positive isolates [*Clostridium* spp. (2) and *Bifidobacterium* spp. (1)]. A higher rate of resistance was noted for clindamycin, as 42.6% (64/150) of the tested strains were clindamycin resistant with *Bacteroides* spp. [53.6% (30/56)] exhibiting the highest resistance followed by *Clostridium* spp. [47.1% (25/53)]. The overall resistance to β -lactams was as

Table 1. Distribution of anaerobic bacteria isolated during the study

Genus	Species (n = 150)	Revised nomenclature	No.
Anaerobic Gram-positive cocci (n = 7; 4.6%)			
<i>Peptoniphilus</i> spp. (2)	<i>P. harei</i>		2
<i>Peptostreptococcus</i> spp. (2)	<i>P. anaerobius</i>		2
<i>Finegoldia</i> spp. (2)	<i>F. magna</i>		2
<i>Streptococcus</i> spp. (1)	<i>S. constellatus</i>		1
Anaerobic Gram-positive bacilli (n = 57; 38%)			
<i>Actinomyces</i> spp. (1)	<i>A. oris</i>		1
<i>Bifidobacterium</i> spp. (1)	<i>B. longum</i>		1
<i>Clostridium</i> spp. (53)	<i>C. aerotolerans</i>	<i>Lacrimispora aerotolerans</i>	4
	<i>C. bifermentans</i>	<i>Paraclostridium bifermentans</i>	5
	<i>C. butyricum</i>		1
	<i>C. celerecrescens/C. sphenoides</i>	<i>Lacrimispora celerecrescens/Lacrimispora sphenoides</i>	1
	<i>C. cochlearium</i>		1
	<i>C. difficile</i>		6
	<i>C. glycolicum</i>	<i>Terrisporobacter glycolicus</i>	1
	<i>C. paraputrificum</i>		2
	<i>C. perfringens</i>		13
	<i>C. ramosum</i>		1
	<i>C. septicum</i>		2
	<i>C. sordellii</i>	<i>Paeniclostridium sordellii</i>	6
	<i>C. sporogenes</i>		9
	<i>C. subterminale</i>		1
<i>Propionibacterium</i> spp. (2)	<i>P. acnes</i>	<i>Cutibacterium acnes</i>	1
	<i>P. avidum</i>	<i>Cutibacterium avidum</i>	1
Anaerobic Gram-negative bacilli (n = 61; 40.6%)			
<i>Bacteroides</i> spp. (56)	<i>B. fragilis</i>		47
	<i>B. nordii</i>		1
	<i>B. thetaiotaomicron</i>		8
<i>Fusobacterium</i> spp. (4)	<i>F. varium</i>		4
<i>Prevotella</i> spp. (1)	<i>P. bivia</i>		1
Anaerobic Gram-negative cocci (n = 25; 16.6%)			
<i>Veillonella</i> spp. (25)	<i>V. dispar</i>		2
	<i>V. parvula</i>		23

follows: imipenem (0.6%); piperacillin/tazobactam (38%) and cefoxitin (35.3%). Gram-negative anaerobes displayed the highest resistance to piperacillin/tazobactam whereas only four Gram-positive isolates were resistant to that drug. For cefoxitin, the most common group to show resistance was *Bacteroides* spp. [48.2% (27/56)] followed by *Clostridium* spp. [28.3% (15/53)]. In our study, none of the isolates showed resistance to chloramphenicol. Results of AST of clinical isolates to various antibiotics is depicted in Figure 2.

Distribution of resistance genes

The genotypic identification of AMR genes and their association with phenotypic resistance is summarized in Table 2 and Figure 3. The *nim* gene was detected in 24.6% (37/150) isolates, of which 70.2% (26/37) isolates were resistant phenotypes. The remaining 29.7% (11/37) isolates carried *nim* genes, but they were phenotypically susceptible. The PCR products for six random *nim* genes (Figure S4) were further sequenced and showed a close similarity

to *nimE* of *B. fragilis*, with 99% nucleotide and 100% amino acid sequence similarity. The *cfiA* gene, associated with imipenem resistance, was detected in 16% of isolates; however, only one resistant phenotype was detected corresponding to a very low agreement rate among imipenem-resistant genotypes and phenotypes. None of the isolates showed the presence of IS1186.

Discussion

This is the first Indian study of its kind that describes the distribution of resistant phenotypes and genotypes of various clinically significant anaerobic bacteria. AMR was most commonly found in *Bacteroides* spp. Metronidazole resistance among *B. fragilis* group isolates is emerging worldwide. European data from the early 1990s showed no resistance, but over the next few years (2002–09) there was an increase to 0.5% resistance.^{12,18,19} On the contrary, a significantly higher resistance rate of up to 15% has been seen in many Western countries^{20–22} and an even higher rates up to 30% from a few Asian regions.^{15,21–23} In India, metronidazole

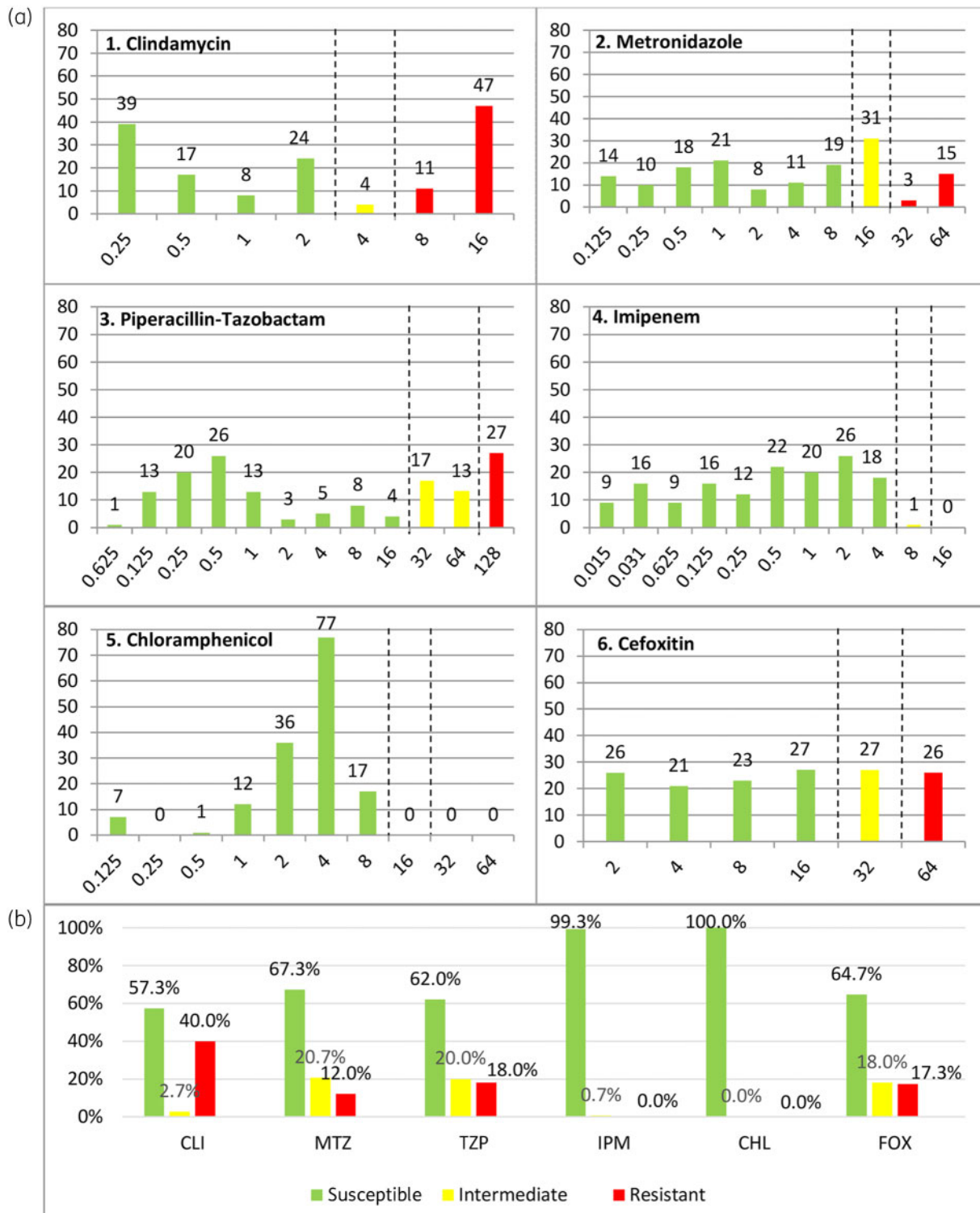
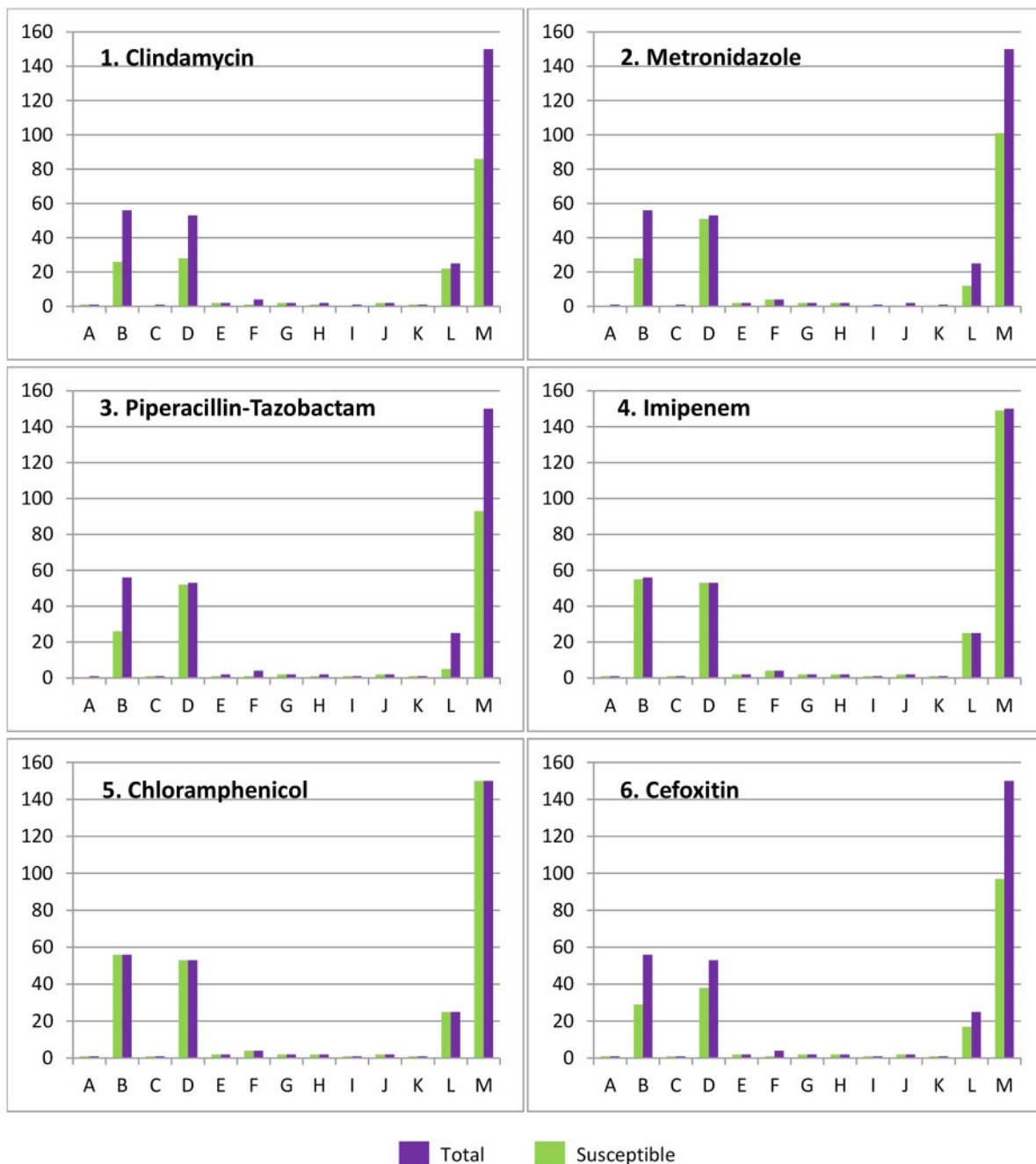


Figure 1. MIC distribution of anaerobic isolates to different tested antibiotics. (a) Bars depict the numbers of resistant (red), intermediate (yellow) and susceptible (green) isolates to clindamycin (CLI), metronidazole (MTZ), piperacillin/tazobactam (TZP), imipenem (IPM), chloramphenicol (CHL), cefoxitin (FOX) at different concentration of antibiotics. The broken lines represent clinical breakpoints (mg/L) as per the CLSI guidelines. The x-axis shows drug concentration in (mg/L) and y-axis shows the number of isolates. (b) The overall resistance rate to different antibiotics tested.



*A—*Actinomyces* spp.; B—*Bacteroides* spp.; C—*Bifidobacterium* spp.; D—*Clostridium* spp.; E—*Finexordia* spp.; F—*Fusobacterium* spp.; G—*Peptoniphilus* spp.; H—*Peptostreptococcus* spp.; I—*Prevotella* spp.; *J—*Cutibacterium* spp.; *K—*Streptococcus* spp.; L—*Veillonella* spp.; M—Overall susceptibility

*Organisms intrinsically resistant to metronidazole

Figure 2. Antimicrobial susceptibility of clinical isolates representing different genera to tested antibiotics.

resistance varying from 7% to 31%²⁴ has been reported, which is comparable to that found in our study (32.6%). In our isolates, resistance was not detected in Gram-negative bacteria such as *Fusobacterium* spp., which was similar to various studies

worldwide, with a few exceptions.^{25,26} However, AMR to metronidazole is emerging in the genera *Prevotella*²⁷ and *Veillonella*. A reduced susceptibility of *Veillonella* isolates to metronidazole has been seen in our isolates also, which is in line with the literature

Table 2. Comparison of antimicrobial resistance in various anaerobic isolates according to phenotypic and genotypic testing

Antimicrobial	Association of antimicrobial resistance phenotypes and genotypes							IS1186
	PR	GR	Genes(s) carried	PR+/GR+	PR-/GR-	PR+/GR-	PR-/GR+	
Metronidazole	49	37	<i>nimE</i>	26	90	23	11	0
Imipenem	1	24	<i>cfiA</i>	0	125	1	24	0

PR, number of isolates expressing phenotypic resistance to the indicated antimicrobial agent.

GR, number of isolates carrying the indicated antimicrobial resistance gene.

PR+/GR+, phenotypically resistant isolates carrying antimicrobial resistance genes.

PR-/GR-, phenotypically susceptible isolates carrying no antimicrobial resistance genes.

PR+/GR-, phenotypically resistant isolates carrying no antimicrobial resistance genes.

PR-/GR+, phenotypically susceptible isolates carrying antimicrobial resistance genes.

IS1186, isolates carrying insertion sequence 1186.

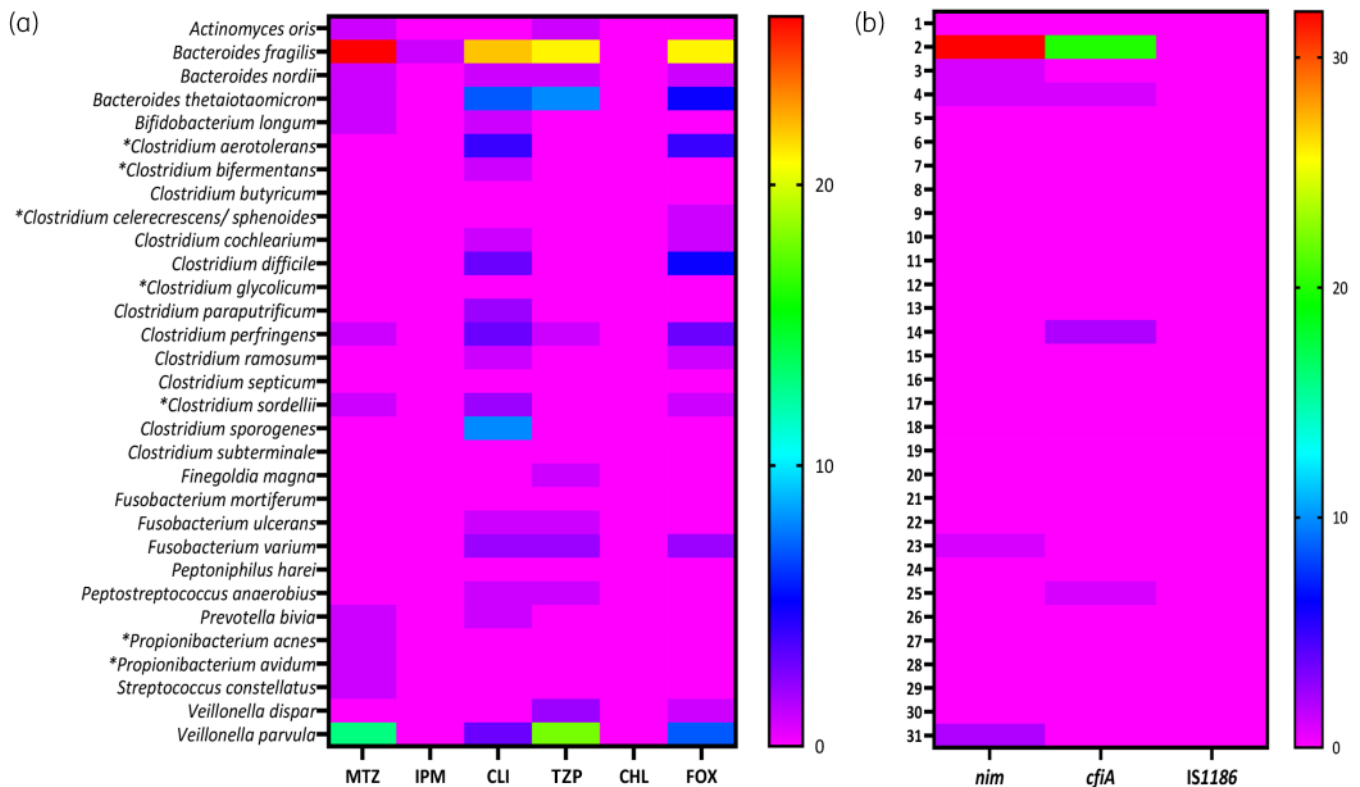


Figure 3. Prevalence of antimicrobial resistance, AMR genes and the association between resistant genotypes and phenotypes. (a) Prevalence of antimicrobial resistance (b) Prevalence of antimicrobial resistance genes. *These species have been reassigned to other genera in recent years. The revised nomenclature is given in Table 1.

from East Asian countries.^{26,28} In contrast a significantly higher resistance of up to 28.6% has been reported in Gram-positive anaerobes^{25,29} as compared with our study [6.8% (4/59)]. In our study both Gram-positive anaerobic cocci (GPAC) and *Clostridium* spp. showed an almost equal number of resistant isolates and results were in agreement with the resistance rate of the Eastern world.³⁰ However, none of the studies have reported resistance patterns of Gram-positive anaerobes from India.

The main mode of metronidazole resistance is nitroimidazole reductase activity encoded by *nim* genes.³⁰ Studies evaluating the

presence of *nim* genes are limited, nevertheless they depict a low prevalence ranging from 0.5% to 2.8% in *Bacteroides* spp.^{25,30}; 0% to 5.3% in *Prevotella* spp.²⁵ and 0% to 5.9% in *Fusobacterium* spp.^{25,30} Yet again, majority of the literature is from European countries³⁰ and the geographic distribution of *nim* genes in the Indian subcontinent is relatively under explored. In our study the *nim* genes were detected in 24.6% (37/150) of isolates and were more prevalent in 60.7% (34/56) *Bacteroides* spp., followed by 8% (2/25) of *Veillonella* spp. The findings were in accordance with an Indian study, where *nim* gene positivity was seen in 53% (20/38)

of *Bacteroides* spp.²⁴ None of the other isolates, including the intrinsically metronidazole-resistant isolates, showed the presence of *nim* genes. Out of the 24.6% (37/150) *nim* gene positive isolates, 29.7% (11/37) isolates carried *nim* genes, yet they were phenotypically susceptible; which is possibly due to the absence of IS elements that regulate the expression of *nim* genes. Of all IS elements reported so far, the isolates were tested for the presence of IS1186, which has been frequently described to be associated with the most prevalent *nimA* and *nimB* genes.³⁰ Unexpectedly, all 150 isolates tested negative for IS1186, which shows that perhaps *nimA* and *nimB* are not circulating in our geographical region or may be less prevalent. To further validate this suggestion, six random *nim* gene-positive PCR amplicons were sequenced and showed a close similarity to *nimE* from *B. fragilis*. In contrast, the *nimE* gene has been found to be associated with another IS element (ISBf6).³⁰ This possibly explains the absence of IS1186 in our study isolates. The detection and study of *nim* genes is of great importance since resistance can be induced not just in *nim*-positive strains but also in *nim*-negative strains on exposure to sub-MICs of metronidazole, whereas this phenomenon could not be induced in a *nimF*-positive strain.¹¹ Although *nim* genes play a major role in metronidazole resistance, but the non-*nim* gene-based mechanisms of metronidazole resistance (such as overexpression of efflux pump; RecA proteins; rhamnase catabolism regulatory protein; activation of antioxidant defence systems and deficiency of ferrous iron transporter FeoAB) have also been described, but the supporting literature is meagre.³⁰ In our study, this non-*nim* gene-mediated resistance was seen in 15.3% (23/150) of phenotypically resistant isolates.

Emergence of metronidazole resistance has led to use of carbapenems for the treatment of anaerobes, particularly imipenem.²³ The resistance to carbapenems is mediated by the chromosomal *cfiA* gene, which again requires IS elements for its expression.¹² Western data from various studies have reported an overall carbapenem resistance rate varying from 1% to 9.6% and *cfiA* positivity of 5% to 27%.^{31,32} East Asian literature shows 7% imipenem resistance in *B. fragilis*, 4% in *Fusobacterium* spp. and 15% in *Clostridium* spp. over 16 years.¹⁰ In other Asian regions resistance has risen from 0% to 24.1% in 5 years^{23,33} whereas in our isolates it was only 0.6%. None of our isolates was found to have IS elements; however, 16% of isolates were 'silent' *cfiA* strains, as these isolates were phenotypically susceptible despite having the *cfiA* gene. In contrast, the resistant *B. fragilis* reported in our study was *cfiA* negative. Such strains have also been reported previously and it has been hypothesized that resistance in these strains might be due to other mechanisms, such as the upregulation of drug efflux, accumulation of mutations in the outer membrane porin molecules and penicillin binding proteins.¹² Another reason to study the distribution of *cfiA* genes is their association with decreased susceptibility to β -lactam- β -lactamase inhibitor combinations (BL-BLIs), such as piperacillin/tazobactam,³⁴ however, no such association was seen in our isolates.

BL-BLIs are the commonly used choices for mixed aerobic-anaerobic infections. Resistance rates to piperacillin/tazobactam are generally <1% for all *B. fragilis* group species.³⁵ In our isolates, *Veillonella* spp. showed the highest resistance 80% (20/25) followed by *Bacteroides* spp. [53.6% (30/56)]. A significantly low resistance was observed in Gram-positive anaerobes [4.7% (3/64)] with two resistant GPAC and one *Clostridium* isolate. There are few

studies from the Indian subcontinent whereas the Western literature shows 0.6%³⁵ and 2%⁹ resistance in *B. fragilis*; 0.6%³⁵ and 12%⁹ in *B. thetaiotaomicron*; 2% to 8% in *B. fragilis* group;^{9,36,37} 2% in GPAC³⁷ and no resistance in *Clostridium* spp.³⁷

Over the past few years a dramatic increase in resistance has been observed for ceftioxin (17.2%).⁹ Resistance rates of *Bacteroides* spp. in three Europe-wide studies following CLSI breakpoints revealed 3%,¹⁹ 6%¹⁸ and 17.2%⁹ resistance rates among *Bacteroides* strains during the past 20 years. In our study, the overall resistance in ceftioxin was 35.3% with *Bacteroides* spp. exhibiting the highest resistance at 48.2% (27/56) followed by *Clostridium* spp. with 28.3% (15/53), whereas Western data shows a relatively lower resistance of 16% in *Clostridium* spp.³⁷

Over the past 20 years, resistance to clindamycin has increased worldwide (32.4%).⁹ In our isolates, the overall resistance to clindamycin was 42.6% and the highest resistance was seen in 53.6% (30/56) of *Bacteroides* spp. *B. fragilis* showed 46.8% (22/47) resistance, which is equivalent to that shown by the majority of the available literature, which shows resistance up to 40%.^{9,35,38,39} In non-*Bacteroides fragilis* species, the worldwide resistance rate is even higher, up to 52% from the Western world³⁷ and 70% from Eastern regions.³⁸ A considerable amount of resistance was detected among Gram-positive anaerobes, with 45.2% (24/53) resistance in *Clostridium* spp., where three out of six *Clostridioides difficile* isolates were found to be resistant, similar to a statistical analysis of clinical isolates of *C. difficile* from 30 different studies.³⁷ In our study, all organisms showed 100% susceptibility to chloramphenicol and for more than half of these isolates, the clustering of MICs was observed around the breakpoints (12% at 8 mg/L; 52% at 4 mg/L). A similar clustering was observed in another study also,⁴⁰ which may pose a threat in case of MIC creep over time.

Only few studies have investigated AMR in anaerobes following standard methodology; therefore, our study is important. A reasonable amount of literature is available from the West, but data remains scarce from the East, especially the Indian subcontinent. Our study has evaluated a good number of various clinically significant groups such as *Bacteroides* spp., *Clostridium* spp. and isolates from the genus *Veillonella*, which is seldom reported in other studies especially from the Indian subcontinent. However, the study has limitations such as the number of isolates for certain species, since the prevalence of organisms in a clinical setup is reflective of their epidemiology. Our study (and also the available literature depicting the absolute prevalence of resistant phenotypes and genotypes) does not signify the frequency of these attributes in other geographies because of the difference in epidemiology and interpretive breakpoints between CLSI and EUCAST.⁶

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Transparency declarations

None to declare.

Supplementary data

Tables S1 to S5 and Figures S1 to S4 are available as [Supplementary data](#) at JAC-AMR Online.

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