Phenotypic and genotypic correlation of antimicrobial susceptibility of Bacteroides fragilis: lessons learnt

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Research Article

Keywords: B. fragilis, antimicrobial resistance, metronidazole, meropenem, genotypic markers

Posted Date: February 15th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1322573/v1

Abstract

Background

Bacteroides fragilis are opportunistic pathogens causing severe infections with increasing antimicrobial resistance. Since, phenotypic susceptibility testing is time-consuming and uneconomical, this necessitates a need for genotypic screening for deciding empirical therapy for *B. fragilis*.

Methods

B. fragilis was isolated from clinical samples, exudates, tissue and body fluids collected between November 2018 and January 2020 in a tertiary care hospital. Samples were cultured in anaerobic blood agar and neomycin blood agar using the Anoxomat Mark system and species identified by MALDI-TOF-MS. Brucella agar supplemented with hemin, Vit K_1 and 5% v/v laked sheep blood was used to determine the minimum inhibitory concentration by agar dilution. Genomic DNA was extracted and *nim*, *erm*F and *cfi*A genes were amplified, and visualized in a 2% agarose gel.

Results

Out of 51 non-duplicate *B. fragilis*, majority (74.5%) were recovered from exudates followed by body fluids. Isolates (*n*=51) expressed 45%, 41%, 16% resistance for clindamycin, metronidazole, and meropenem, respectively with least resistance to piperacillin/tazobactam (6%). Among metronidazole resistant isolates, 52% harboured *nim* genes in addition to 76% (23/30) of susceptible isolates. Similarly, *cfiA* was present in all eight meropenem resistant isolates in addition to 22% (9/41) susceptible ones.

Conclusions

All *cfiA* negative were phenotypically susceptible that can be employed to rule out meropenem resistance. Detecting limited genes for *B. fragilis* does not always correlate with phenotypic resistance, due to IS elements, efflux and other genetic determinants. Redundant use of meropenem with metronidazole should be avoided, as recommendation of metronidazole requires prior phenotypic testing due to the reported 41% resistance.

1. Introduction

Anaerobic bacteria are components of the human intestinal flora that also colonize the oral cavity, upper respiratory tracts and the female genital tract [1]. *Bacteroides fragilis*, the most virulent and most common *Bacteroides* species is an obligate gram negative, non-spore-forming anaerobic bacterium associated with intestinal and extra intestinal infections in humans [2, 3]. Routine diagnosis of anaerobic organisms remains cumbersome and time consuming, requiring a special anaerobic culture environment; also species identification is challenging as anaerobic organisms are inert to most biochemical tests [4]. But correct identification is important as AST (Antibiotic susceptibility testing) varies between organisms [5].

The approach to the treatment of anaerobic infections is mostly empirical as routine testing is time-consuming and is not cost-effective [6]. The most frequently used antibiotics include metronidazole, β-lactams - beta lactamase inhibitor combinations, carbapenems and clindamycin [7]. However, the *Bacteroides fragilis* group tends to be resistant to many agents including metronidazole, carbapenems, and beta lactam- beta lactamase inhibitor combinations. An increasing resistance trend has been reported worldwide in the past two decades [8]. The prevalence of antimicrobial resistance varies both between different geographical areas and also among the different medical centers within the same country [9, 10]. Failure to direct appropriate therapy against anaerobic organisms often leads to clinical failure, therefore susceptibility testing is necessary to provide data for appropriate empirical antimicrobial therapy and to establish national and regional guidelines [11].

The aim of the study is to detect the prevalence of *B. fragilis* infections, determine the minimum-inhibitory concentrations (MIC) for metronidazole, clindamycin, meropenem and piperacillin-tazobactam, and to compare these with genetic markers for antimicrobial resistance: *nim* (metronidazole), *ermF* (clindamycin) and *cfiA* (meropenem).

2. Methods

2.1. Sample collection

Bacteroides fragilis was isolated from various clinical samples including exudates, tissue and body fluids. Isolates collected between November 2018 and January 2020 in the Department of Clinical Microbiology, CMC Vellore were included in this study. Samples were cultured in anaerobic blood agar and neomycin blood agar and incubated at 37 ˚C for 48-72 hours in anaerobic condition using the Anoxomat Mark system (Mart Microbioogy BV, Lichtenvoorde, Netherlands). Species identification was done by Matrix Assisted Laser Desorption Ionization time of flight mass spectrometry (MALDI TOF) (Vitek MS-DS; bioMérieux, Etoile, France) according to the manufacturer's instructions.

2.2. Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) to metronidazole, clindamycin, meropenem and piperacillin-tazobactam was determined by agar dilution method according to Clinical & Laboratory Standards Institute (CLSI) 2019 guidelines. Brucella agar supplemented with hemin, Vit K_1 and 5% v/v laked sheep blood (Sigma –Aldrich, Darmstadt, Germany) was inoculated with 10^5 CFU / ml per spot of *Bacteroides fragilis* strain (0.5 McFarland). Serial two-fold dilutions of antibiotics were incorporated into media. ATCC 25285 *Bacteroides fragilis* was used as the control strain. Plates were incubated at 37°C for 48 hours in anaerobic condition using the Anoxomat Mark system. The lowest concentration of antibiotics that inhibit bacterial growth was considered as the MIC.

2.3. Identification of genetic markers for antimicrobial resistance

Genomic DNA was extracted using QIAamp DNA mini kit (QIAGEN GmbH, Hilden, Germany) from 48 hours pure growth from anaerobic blood agar culture plate. Extraction was done as per the manufacturer's instruction. The 458 bp region of *nim* gene, 466 bp of *ermF* gene, 353 bp of *cfiA* gene were amplified in a Veriti^{TM} Thermal Cycler (Applied Biosystem, Foster city, CA, USA) as described by Boente et al [1], Nakano et al [11] using the following primer sequence : nimF (5′-ATGTTCAGAGAAATGCGGCGTAAGCG-3′); and nimR (5′- GCTTCCTTGCCTGTCATGTGCTC-3′), for clindamycin :ermF F (5′ -CGGGTCAGCACTTTACTATTG-3′); and ermF R (5′ -GGACCTACCTCATAGACAAG-3′), for meropenem: cfiA F(5′ -ATGGTACCTTCCAACGGG-3′); and cfiA R(5′ -CACGATATTGTCGGTCGC-3′). Thermal profiles used for *nim* gene was 94°C for 60 sec, 55°C for 60 sec, 72°C for 30 sec for 30 cycles; for *ermF* gene 94°C for 30 sec, 50°C for 30 sec, 72°C for 2 min for 35 cycles; for *cfiA* gene 94°C for 60 sec, 56°C for 60 sec, 72°C for 60sec for 35 cycles with a final extension of 72°C for 7 min. The PCR products were visualized in a 2% agarose gel electrophoresis with a 100 bp ladder.

3. Results

A total of 51 non-duplicate *B. fragilis* group isolates were obtained during the study period. The majority of these isolates (74.5%) were recovered from exudates followed by body fluids. In this study piperacillin/tazobactam had the least resistance (6%), followed by meropenem (15%), metronidazole (41%) and clindamycin (45%). Table 1 lists the interpretation criteria and the observed MICs for the tested antimicrobials against *B. fragilis* isolates.

Table 1
MIC distribution of metronidazole, clindamycin, meropenem and piperacillin/ tazobactam for *B. fragilis* isolates

Drug	Interpretation			MIC range tested for	MIC distribution (*n* = 51)		
	S	I	R	(µg/mL)	S	I	R
Metronidazole	≤ 8	16	≥32	0.25-64	30	0	21
Clindamycin	≤ 2	4	≥8	0.125-16	25	3	23
Meropenem	≤ 4	8	≥16	0.031-16	41	2	8
Piperacillin/ tazobactam	≤ 16/4	32/4-64/4	≥128/4	0.062-256	46	3	2

3.1. nim gene and metronidazole

Out of total 51 *B. fragilis* isolates, 21(41%) were resistant (≥32 µg/mL) for metronidazole and 11(52%) of which contained the *nim* gene. Interestingly, 23 susceptible isolates were also harbouring *nim* gene (Figure 1A).

3.2. ermF gene and clindamycin

In this study, 17(74%) out of 23 resistant isolates (16 µg/ml) were positive for the *ermF* gene (Figure 1B). None were present among the susceptible isolates (Figure 1B).

3.3. cfiA gene and resistance to meropenem

The *cfiA* gene was present in all eight meropenem resistant isolates. The two intermediate isolates both harboured the *cfiA* gene. Remarkably, 22% (9/41) of the susceptible isolates also carried the *cfiA* gene. All *cfiA* negative isolates were phenotypically susceptible (Figure 1C).

4. Discussion

Bacteroides fragilis has been reported for being the cause of a wide range of infections [12] and is often associated with drug resistance [13]. Most patients receive empiric treatment as anaerobic susceptibility test results are unavailable or delayed [14]. Determining MICs by the recommended agar dilution method gives information regarding resistance trends over time [15]. Due to recent developments in molecular microbiology, there are reported PCR methods to rapidly identify resistance by genetic determinants.

Though, phenotypic testing is more reliable, phenotypic resistance in anaerobes may not always positively correlate with the genotypic findings. This is mainly due to the multiple resistant markers and the varying mechanisms of resistance, including the involvement of IS elements for gene regulation [16, 17, 18, 19]. This study is the first of its kind in India to correlate phenotypic and genotypic resistance mechanism in *B. fragilis*, in order to define the most plausible way of treating *B. fragilis* infections.

Antimicrobial resistance in *B. fragilis* is mediated by various mechanisms including the production of drug modifying enzymes, efflux of drug or inactivation of drug through the expression of resistance genes [20, 21]. This study utilised *nim* (metronidazole), *ermF* (clindamycin) and *cfiA* (meropenem) genes as molecular markers.

Phenotypic results revealed that 41% of *B. fragilis* were resistant to metronidazole, the commonest drug used for treating anaerobic infections [22]. This is comparable with an Indian study by Sethi et al. [23] which reported 41% resistance, and Sood et al.(2021) who reported 32.6%. In contrast, 100% susceptibility was reported from Korea by Byun et al and Veloo et al from The Netherlands [24, 25]. The increased prevalence of metronidazole resistance (41%) reported in this study could be region specific, as antimicrobial resistance varies among different regions.

In this study, 50% of the metronidazole resistant isolates carried the *nim* gene, which agrees with studies by Vishwanath et al [26] and Gal et al [27]. To date, 11 variants of *nim* genes (*nimA* to *nimK*) have been reported [28]. It has been recorded that both phenotypic metronidazole resistance and *nim* gene positivity were 50% and 48%, respectively [26, 27]. In contrast, Akhi et al (Iran) showed 0% prevalence of the *nim* gene in resistant isolates. This might be due to other mechanisms such as overexpression of the multidrug efflux pump, overexpression of *recA* or deficiency of *feoAB* [29]. Interestingly, in a study from Gal and Brazier (2004), 14% of susceptible isolates were reported to carry the *nim* gene. Silent *nim* genes

might be due to the absence of an IS element promoter region [30]. There is strong evidence that these IS elements carry regulatory signals for the expression of *nim* genes [31]. Studies have shown that silent *nim* genes can be expressed when isolates carrying them are exposed to metronidazole for longer periods of time [16].

The present study reports 45% phenotypic resistance for clindamycin. This high percentage resistance to clindamycin may be due to its widespread use in the treatment of anaerobic infections, in intra-abdominal, pelvic, lower respiratory, bone, and skin and soft tissue infections [32, 33]. The study data is comparable to the observation by Vishwanath et al [34] which reported resistance rate of 38%. Further, 74% phenotypically resistant isolates were positive for *ermF*. The *ermF* gene was present only in phenotypically resistant isolates. Similar findings were reported by Kouhsari et al [35] and Eitel et al [36] which showed prevalence rates of 76% (206/364) and 74% (23/31) for *ermF* in resistant isolates. It has been observed that the *ermF* gene is frequently present on conjugative transposons. In this study, 9% of *B. fragilis* isolates were not carrying *ermF*. Resistance in these isolates might be due to the other mechanisms conferred by *linA*, other *erm* genes such as *ermG*/ *ermS*, *msrA* or efflux pump mechanism [17].

Meropenem has been reported to have good coverage against anaerobes [37]. In this study, 15% of *B. fragilis* were resistant to meropenem. Similar results have been reported by Jamal et al [38] and Wang et al [39] where they found 17% and 19% resistance respectively whilst slightly reduced rates of resistance were reported Wybo et al [40] at 10%. Contrary to this, Vishwanath et al [34] and Sood et al. [41] reported the nearly complete absence of resistance for meropenem.

Overall the prevalence of the *cfiA* gene in this study was 37%. All isolates (n = 8) with high MICs (≥ 16 µg/ml) for meropenem were carrying resistance gene. Similar result was reported by Gao et al in China [42] and Wybo et al [40]. However, 21% of *cfiA* positive isolates displayed lower levels of meropenem MICs. This might be due to the proven fact that *cfiA* without an upstream insertional sequence (IS) will display lower MICs as opposed to high MICs for *cfiA*s with an upstream IS, which provides a strong promoter for *cfiA* expression [18, 19]. All *cfiA* negative isolates were phenotypically susceptible. Based on this observation, it could be interpreted that *cfiA* negative isolates will mostly be phenotypically susceptible.

In most cases, meropenem is used for treating *B. fragilis* along with metronidazole. Though, both the antimicrobials have good coverage for anaerobes, use of redundant antimicrobials will further add to the existing resistance and complicate antimicrobial stewardship. This has been observed from our study centre, where unnecessary double coverage for anaerobes were offered by second antibiotics (19.8%) [43].

Beta-lactam, beta-lactamase inhibitor combinations (BL-BLICs) are frequently used for mixed infections (aerobic-anaerobic) as they have a wide range of activity against the majority of anaerobic bacteria [44]. Interestingly, studies from Scandinavian countries and one report from India have presented increased resistance to piperacillin/tazobactam in comparison to meropenem [45, 46, 41]. Contrary to these reports,

the present study showed *B. fragilis* was highly susceptible to piperacillin/tazobactam as only 2 out of 51 isolates had high MICs. Our result was concordant with other studies where resistance reported for piperacillin/tazobactam varied from 0-5% [8, 12, 47, 45], and was less than for meropenem, for example Snydman et al. [48] studied around 2722 *B. fragilis* isolates over 8 years.

Observations from this study and the corresponding literature reveal that to decide empirical therapy for *B. fragilis*, the local site should generate AMR surveillance data. This aggregated data would help to recommend appropriate anaerobic cover for empiric therapy and would avoid the use of redundant antibiotics thereby saving “watch” antibiotics such as meropenem.

Conclusions

Anaerobic resistance levels worldwide are generally not higher. For the BL-BLI piperacillin/tazobactam there is observed discrepancy where piperacillin/tazobactam susceptibility may be increased or decreased compared to meropenem. This study reports reduced carbapenem susceptibility for *B. fragilis* over piperacillin/tazobactam. Unless all resistance mechanisms are analysed, in addition to the selected genes, evaluating genotypically may not reveal the true correlation with phenotypic results. We have learnt that phenotypic results are more reliable and applicable for the clinical scenario, at least for *B. fragilis*. With varying susceptibility profiles for major drugs, it is imperative to do AMR surveillance and to periodically report and recommend the appropriate antimicrobial choice. To facilitate this improved, less cumbersome, quicker, more economic diagnostic test need to be developed. Moreover, this study highlights that metronidazole requires antimicrobial susceptibility testing before it can be recommended, as the reported resistance of over 40% is deemed unacceptable for empirical choice.

Declarations

Ethics approval and consent to participate

Not applicable

Competing interests

None

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors' contributions

BV, JAJP and BL designed the study. LJ performed the phenotypic and genotypic experiments. LJ and NKDR analysed and interpreted that data and prepared the manuscript. BV, BL and NKDR reviewed the

manuscript. All authors read and approved the final manuscript.

Acknowledgements

The authors acknowledge the Department of Clinical Microbiology, Christian Medical College, Vellore for the infrastructure support towards the study.

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Figures

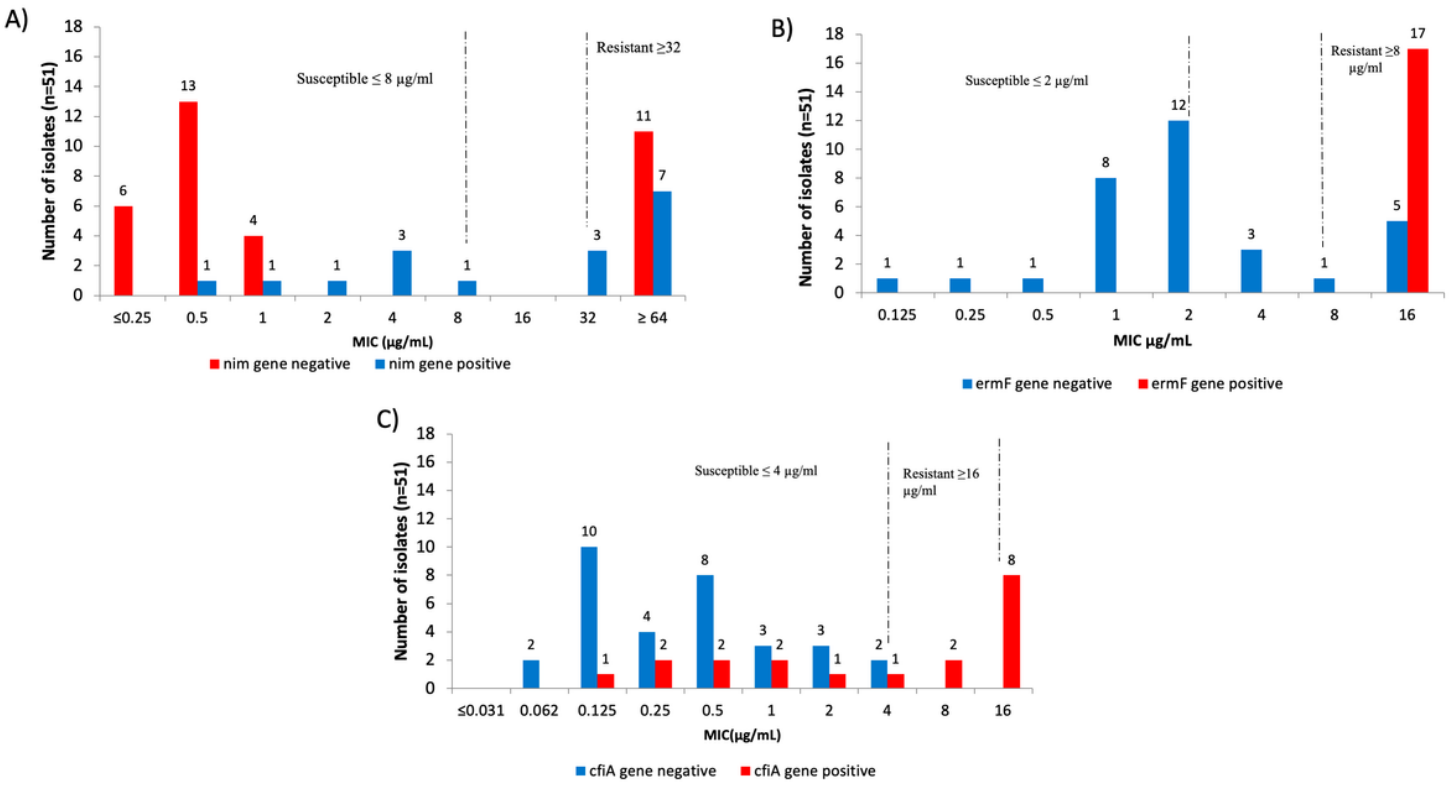

Figure 1

Representation of phenotypic MICs and the genes present for A) metronidazole, B) clindamycin, and C) meropenem resistance.