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Abstract

An increase of carbapenemase-producing *Bacteroides fragilis* infections is observed. To detect such a resistance in *B. fragilis*, several tests exist that are expensive or show poor sensitivity and specificity. Therefore, we upgraded the Anaerobic Carbapenem Inactivation Method (Ana-CIM) to easily screen for carbapenemase-producing *B. fragilis*. The presence of carbapenemase *cfiA* gene was identified in 50 *B. fragilis* isolates by PCR. We modified the Ana-CIM by (i) increasing the bacterial inoculum and (ii) measuring the differences in diameter between the negative control and the testing disc. We correctly classified the *cfiA*-negative and positive isolates and could define a cut-off of positivity at 2 mm. Our modified Ana-CIM allowed to correctly discriminate the 31 *cfiA*-positive with meropenem MICs ranging from 1 to > 32 µg/mL. We anticipate that our modified Ana-CIM could be used in most clinical laboratories to easily screen for carbapenemase-producing *B. fragilis*, even at low levels.

Keywords: Carbapenem Inactivation Method, *Bacteroides fragilis*, carbapenemase, *cfiA*

1. Introduction

Bacteroides spp are the most predominant anaerobic gram-negative bacteria in the gut. *Bacteroides fragilis* accounts for only 0.5% of the gut microbiota, however it is the most commonly isolated anaerobic pathogen in intra-abdominal infections and bacteremia with high mortality rate (more than 19%) [1]. Although often susceptible to metronidazole, *B. fragilis* infections could be challenging for treatment because physicians must face increasing resistance to antibiotics. For several years, an increase of resistance to β-lactams agents such as amoxicillin-clavulanic acid but also carbapenems has been observed [2]. For the latter, the resistance is mainly due to a carbapenemase enzyme encoded by the *cfiA* gene and belonging to the metallo-β-lactamase (MBL) class [3]. Once activated by an insertion sequence, this gene is able to confer high-level resistance to the carbapenems as well as to the penicillins, cephalosporins and to most β -lactamase inhibitors [4].

To tackle such antibiotic resistance in *B. fragilis*, an imipenem double-ended Etest ± EDTA is commonly used to rapidly detect MBL production [5]. This phenotypic test compares the resistance of strains to imipenem with and without EDTA. Since MBL can be experimentally inhibited with metal chelators such as EDTA, the CfiA production can be inferred with a MIC ratio ≥ 8 that indicates a reduction of imipenem MIC by at least 3 twofold dilutions in the presence of EDTA [5].

A major issue with the imipenem ± EDTA Etest is the only detection of isolates with high-level resistance [6]. This is a significant drawback for clinical microbiology diagnosis because it may clearly underestimate the CfiA prevalence in *B. fragilis* [6]. Therefore, Schwensen *et al.* proposed to solve this problem by using preferentially the meropenem-EDTA double-ended Etest or the ROSCO KPC/MBL Confirm Kit [7]. This latter assay allows to compare the sensitivity to meropenem ± dipicolinic acid, ± boronic acid and ± cloxacillin in order to confirm the presence of MBL. The presence of CfiA is then evidenced by a restoration of

inhibition diameter of the meropenem + dipicolinic acid disc only. However, the meropenem double-ended Etest is expensive and the ROSCO KPC/MBL Confirm kit could misclassify *cfiA*-negative and *cfiA*-positive isolates in case of small diameters of inhibition.

An alternative way to detect MBL production is to use carbapenem inactivation methods (CIM), based on the enzymatic hydrolysis of meropenem susceptibility-testing disc after its exposure to a carbapenemase producing strain, which allows subsequent uninhibited growth of a full susceptible indicator strain [8]. These phenotypic methods are well known for detecting carbapenemase production, mainly in *Enterobacterales* [9] but have also been proposed for the detection of carbapenemase-producing *Acinetobacter* species [11] and *Pseudomonas aeruginosa* [11]. Recently, an anaerobic CIM (Ana-CIM) has been described to improve and facilitate the detection of *B. fragilis* carbapenemase [12].

The aim of this study was to evaluate the practicability in everyday practice and to improve the performance of the Ana-CIM to detect *cfiA* carbapenemase production in *B. fragilis* group.

2. Materials and methods

Fifty isolates of *B. fragilis* were included in this study. The isolates were recovered from several clinical infections, including 23 intra-abdominal infections, 9 bacteriemia, 6 bone and joint infections, 8 skin and soft tissue infections and 4 other types of infections. Isolates were identified by MALDI-TOF mass spectrometry (MALDI Biotyper®, Bruker Daltonics).

B. fragilis isolates were cultivated on Schaedler agar (Thermo Fisher Scientific) and incubated for 48h in anaerobic atmosphere (GenBag anaer®, bioMérieux).

All isolates were evaluated for *cfiA* gene by PCR, and MIC values of meropenem were evaluated by Etest as previously described [13,14]. Isolates were categorized as "susceptible" (MIC ≤ 2 µg/mL), "susceptible, increased exposure" (MIC > 2 and ≤ 8 µg/mL) or "resistant" (MIC > 8 µg/mL) to meropenem based on breakpoints established in 2021 by the antibiogram committee of the French Society for Microbiology (CA-SFM)/European Committee for Antibiotic Susceptibility Testing (EUCAST) [15].

We set up a modified CIM based on the method described by Van der Zwaluw *et al.* [8]. From the pure subculture performed alongside of the antimicrobial susceptibility testing of a suspected carbapenemase-producing *B. fragilis* isolate (i.e. phenotype of carbapenem resistance), a bacterial suspension of *B. fragilis* were made in 1.5 mL of NaCl 0.85%: either with a regular inoculum (picking one colony) or a high inoculum (scrapping all colonies, optical density at 600 nm of the final inoculum ≈ 6]). A meropenem susceptibility-testing disc (10 µg, Biorad) was immersed in these suspensions. A KPC-3-producing strain of *Enterobacter cloacae* (U2A2242) was used as positive control. A negative control was performed, by immersing a disc in NaCl 0.85%. After 2 hours of incubation in aerobic atmosphere at 37°C, the discs were removed and placed on a Mueller-Hinton agar plate (MH, Thermo Scientific) previously inoculated with a 0.5 McFarland suspension of a susceptible *Escherichia coli* strain (ATCC 25922). Inhibition zone diameters around the meropenem discs were read after overnight incubation in aerobic atmosphere at 37°C [Figure 1]. A carbapenemase-producing isolate can hydrolyze the antibiotic within the disc with the consequence to allow the susceptible *E. coli* strain to grow around this disc leading to a small inhibition diameter. Because of the important variations observed in diameters of the negative controls between experiments (ranging from 25mm to 33mm, median = 29mm), we calculated the difference of diameter between the negative control and the *B. fragilis* isolates to normalize the results. Then, the smaller is the diameter of the tested *B. fragilis*, the greater is

the diameters difference with the negative control. The experiments were independently replicated three times and the medians of the differences in diameter were calculated.

3. Results

Of the 50 isolates of *B. fragilis*, 31 (62%) isolates were positive for *cfiA* by PCR. All *cfiA*-negative isolates (n=19) were susceptible to meropenem, with MICs values ranging from 0.06 to 2.0 µg/mL. The *cfiA*-positive isolates showed meropenem MICs ranging from 1 to > 32 µg/mL (median = 4), with 16 (52%) isolates classified as susceptible, increased exposure category, and 10 (32%) as resistant [Figure 2].

First, we performed the CIM on *B. fragilis* isolates as described for *Enterobacterales*, i.e. with a regular inoculum made from 1 CFU/1.5 mL of bacteria. We found diameter differences with the negative control ranging from 0 to 1mm for *cfiA*- isolates and from 0 to 22mm for *cfiA*+ isolates. Therefore, we observed overlaps that did not allow us to define a clear cut-off value for a positive test [Figure 3A].

Second, to circumvent this issue, we improved the method by increasing the inoculum of *B. fragilis* used in the first step of the CIM procedure. This higher inoculum, reached by picking all the CFUs present on the subculture agar plate, allowed to correctly discriminate the *cfIA*- isolates (which displayed a zone diameter difference < 2mm) from the *cfiA*+ isolates (which displayed a zone diameter difference ≥ 2mm) [Figure 3B].

Eventually, we correlated our results of differences of diameters, determined using the high inoculum, with the resistance phenotype. We did not find a good correlation (R^2 = 0.4707) between meropenem MICs and CIM results. For instance, a MIC value of 32 µg/mL could correspond to diameter differences with the negative control ranging from 2 to 27 mm [Supplemental data, Figure S1].

4. Discussion

Carbapenems remain an effective therapeutic option for multidrug-resistant *B. fragilis*, with most isolates being susceptible. Despite the presence of the carbapenemase enzyme CfiA in 31 isolates of the study, only 26 were classified as not susceptible to meropenem according to their MIC values. In the remaining fives isolates, meropenem susceptibility may be due to a low expression of the enzyme, probably due to the absence of insertion sequences (IS) upstream of the *cfiA* promoter [16] and could lead to underestimate detection of this enzyme in clinical practice. This is clinically relevant since it has been previously shown that *cfiA*-positive *B. fragilis* isolates could convert from carbapenem susceptible to carbapenem resistant during carbapenem therapy [13].

The CIM is a simple way to detect carbapenemase with a high accuracy. This method has multiple advantages: (i) it is an easy-to-perform and easy-to-interpret test; (ii) only control strains and a 10 µg meropenem susceptibility-testing disc are necessary; (iii) several isolates can be tested on the same MH agar plate (standard size). The main disadvantage is that it requires an overnight incubation of the plates to obtain results, compared to some other phenotypic carbapenemase tests [8,17].

Although Ana-CIM offers interesting results, it remains up to 7% of major errors (percentage of isolates susceptible by Etest but interpreted as resistant by the Ana-CIM) and 11% of very major errors (percentage of isolates testing ertapenem resistant by Etest that tested Ana-CIM susceptible) [12]. We observed similar results with overlaps between the differences of diameters of *cfiA*-negative and positives isolates by performing the CIM as initially described for *Enterobacterales.* Since the *cfiA* gene can be expressed at different levels [16], we

assumed that a weak bacterial inoculum might not be sufficient to correctly hydrolyze meropenem.

In *Enterobacterales*, the absence of carbapenemase enzyme was considered in case of an inhibition zone diameters of ≥ 20mm [9,17]. More recently, some authors proposed to consider the presence of carbapenemase enzyme with inhibition diameters < 10 mm [18]. Ana-CIM proposed interpretive criteria adapted to *B. fragilis* based on test diameter: positive for zone size ≤ 8 mm and negative for zone size ≥ 15 mm. However, the use of this only criterium leaves an indeterminate zone which does not allow to determine the sensitivity of *B. fragilis* isolates exhibiting zone sizes ranging from 9 to 14 mm.

In order to complete the Ana-CIM, we decided to increase the bacterial inoculum, which increases the amount of produced enzymes, allowing the detection of low levels of MBL. Furthermore, as we observed important variations in the diameter of the negative control between replicates, we chose to measure the differences in diameter between the negative control and the testing disc and not the diameter of the inhibition zone of the testing disc. With these two modifications, we obtained a correct classification of the *cfiA*-negative and positive isolates and could define a cut-off of positivity at 2 mm. Indeed, all the *cfiA*-negative isolates generated differences in diameter < 2 mm while all the *cfiA*-positive isolates exhibited differences in diameter ≥ 2 mm. Thus, in our study, this test permitted to reach a sensitivity and a specificity of 100%.

Since January 2022, the EUCAST decided to categorize as susceptible *B. fragilis* isolates with MICs ≤1 µg/mL to detect all *cfiA*-positive isolates (https://www.eucast.org). This might reduce the underestimation of the *cfiA*-positive isolates. However, using this breakpoint may still lead to misclassification. Indeed, in our study, one *cfiA*-positive isolate would have been classified as susceptible (MIC = 1 µg/ml, Figure 2) while one *cfiA*-negative isolate would

have been classified as resistant. This could badly affect the antibiotic stewardship. Our modified Ana-CIM may permit to avoid such errors and correctly classify the different isolates regardless of the guidelines used.

In spite of the small number isolates, which is a limitation to be considered for our study, we improved the Ana-CIM and defined a positive cutoff at 2 mm. Our modified Ana-CIM could be appliable in most of clinical laboratories to easily screen for carbapenemase-producing *B. fragilis*, even at low levels conversely to the usual Etest with imipenem [6]. Although the Etest with meropenem and the ROSCO KPC/MBL Confirm kit are good alternatives, this modified Ana-CIM has a better sensitivity and specificity and is much more cost-effective.

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Competing Interests

None to declare.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Nathan Nicolau-Guilllaumet, Anaëlle Muggeo and Corentine Alauzet. The first draft of the manuscript was written by Nathan Nicolau-Guillaumet and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data availability

The datasets generated and analysed during the current study are available in the Figshare repository, https://doi.org/10.6084/m9.figshare.19877845.v1

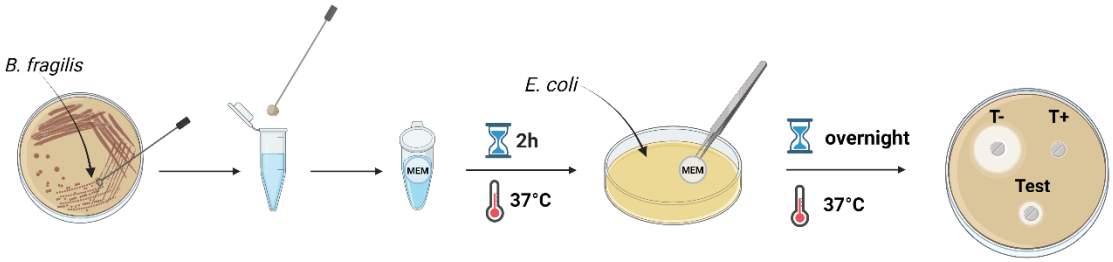

Figure 1

A susceptibility-testing disc containing 10 µg of meropenem was immersed in a suspension containing *B. fragilis* and incubated for two hours at 37°C. If the bacteria produced a carbapenemase, the antibiotic in the disc was hydrolyzed, allowing a susceptible strain of *E. coli* to grow around this disc placed on the agar plate.

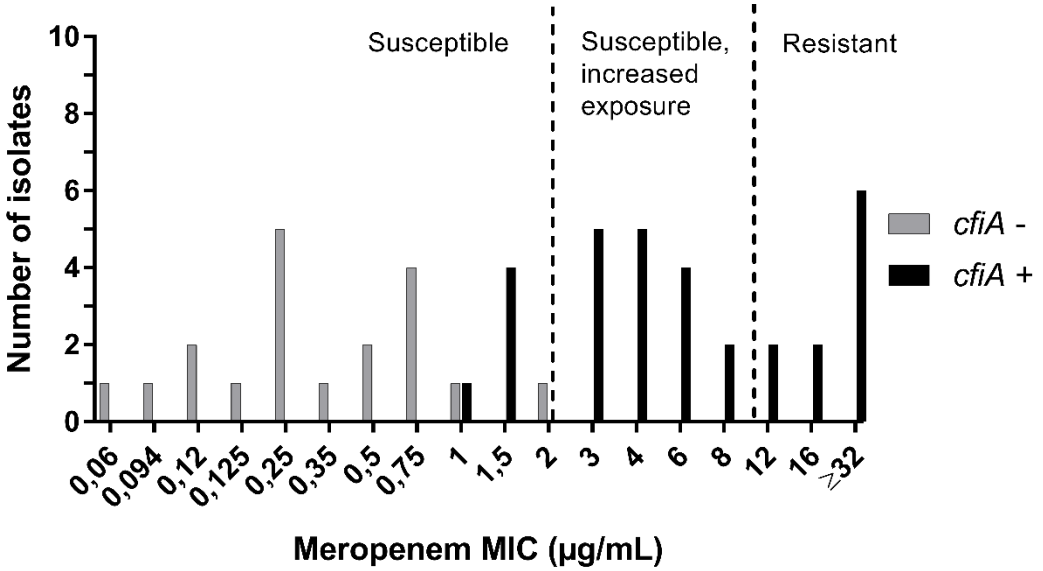

Figure 2

Distribution of *cfiA*+ and *cfiA*- strains according to meropenem MIC values and susceptibility categorization. Source data are provided as a supplementary excel file. (https://doi.org/10.6084/m9.figshare.19877845.v1).

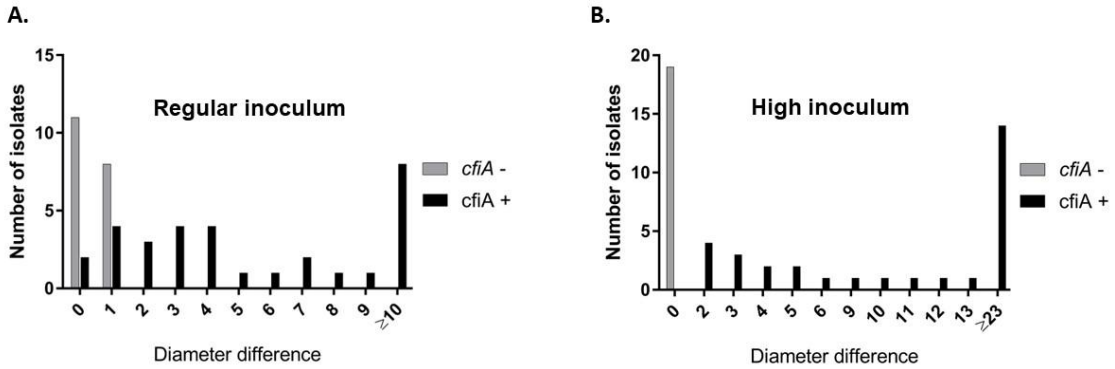

Figure 3

A. Distribution of *cfiA*+ and *cfiA*- strains according to diameter differences with a regular inoculum. **B.** Distribution of *cfiA*+ and *cfiA*- strains according to diameter differences with a high inoculum. Source data are provided as a supplementary excel file. (https://doi.org/10.6084/m9.figshare.19877845.v1).