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1 **An upgraded version of Carbapenem Inactivation Method to detect**
2 ***Bacteroides fragilis* carbapenemase**

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25
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32 **Abstract**

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

45

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

47

48 1. Introduction

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

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

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

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

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

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

87

88 **2. Materials and methods**

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

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

95 All isolates were evaluated for *cfiA* gene by PCR, and MIC values of meropenem were
96 evaluated by Etest as previously described [13,14]. Isolates were categorized as “susceptible”
97 (MIC \leq 2 μ g/mL), “susceptible, increased exposure” (MIC $>$ 2 and \leq 8 μ g/mL) or “resistant”
98 (MIC $>$ 8 μ g/mL) to meropenem based on breakpoints established in 2021 by the antibiogram
99 committee of the French Society for Microbiology (CA-SFM)/European Committee for
100 Antibiotic Susceptibility Testing (EUCAST) [15].

101 We set up a modified CIM based on the method described by Van der Zwaluw *et al.* [8].
102 From the pure subculture performed alongside of the antimicrobial susceptibility testing of a
103 suspected carbapenemase-producing *B. fragilis* isolate (i.e. phenotype of carbapenem
104 resistance), a bacterial suspension of *B. fragilis* were made in 1.5 mL of NaCl 0.85%: either
105 with a regular inoculum (picking one colony) or a high inoculum (scrapping all colonies,
106 optical density at 600 nm of the final inoculum \approx 6]). A meropenem susceptibility-testing disc
107 (10 μ g, Biorad) was immersed in these suspensions. A KPC-3-producing strain of
108 *Enterobacter cloacae* (U2A2242) was used as positive control. A negative control was
109 performed, by immersing a disc in NaCl 0.85%. After 2 hours of incubation in aerobic
110 atmosphere at 37°C, the discs were removed and placed on a Mueller-Hinton agar plate (MH,
111 Thermo Scientific) previously inoculated with a 0.5 McFarland suspension of a susceptible
112 *Escherichia coli* strain (ATCC 25922). Inhibition zone diameters around the meropenem discs
113 were read after overnight incubation in aerobic atmosphere at 37°C [Figure 1]. A
114 carbapenemase-producing isolate can hydrolyze the antibiotic within the disc with the
115 consequence to allow the susceptible *E. coli* strain to grow around this disc leading to a small
116 inhibition diameter. Because of the important variations observed in diameters of the negative
117 controls between experiments (ranging from 25mm to 33mm, median = 29mm), we calculated
118 the difference of diameter between the negative control and the *B. fragilis* isolates to
119 normalize the results. Then, the smaller is the diameter of the tested *B. fragilis*, the greater is

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

122

123 3. Results

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

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

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

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

145 4. Discussion

146 Carbapenems remain an effective therapeutic option for multidrug-resistant *B. fragilis*, with
147 most isolates being susceptible. Despite the presence of the carbapenemase enzyme CfiA in
148 31 isolates of the study, only 26 were classified as not susceptible to meropenem according to
149 their MIC values. In the remaining five isolates, meropenem susceptibility may be due to a
150 low expression of the enzyme, probably due to the absence of insertion sequences (IS)
151 upstream of the *cfiA* promoter [16] and could lead to underestimate detection of this enzyme
152 in clinical practice. This is clinically relevant since it has been previously shown that *cfiA*-
153 positive *B. fragilis* isolates could convert from carbapenem susceptible to carbapenem
154 resistant during carbapenem therapy [13].

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

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

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

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

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

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

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

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

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264

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267

268 **Competing Interests**

269 None to declare.

270

271 **Author Contributions**

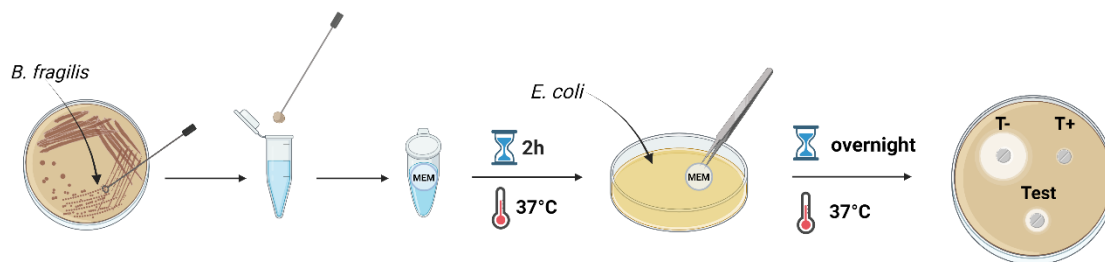
272 All authors contributed to the study conception and design. Material preparation, data
273 collection and analysis were performed by Nathan Nicolau-Guillaumet, Anaëlle Muggeo and
274 Corentine Alauzet. The first draft of the manuscript was written by Nathan Nicolau-
275 Guillaumet and all authors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

277

278 **Data availability**

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

281

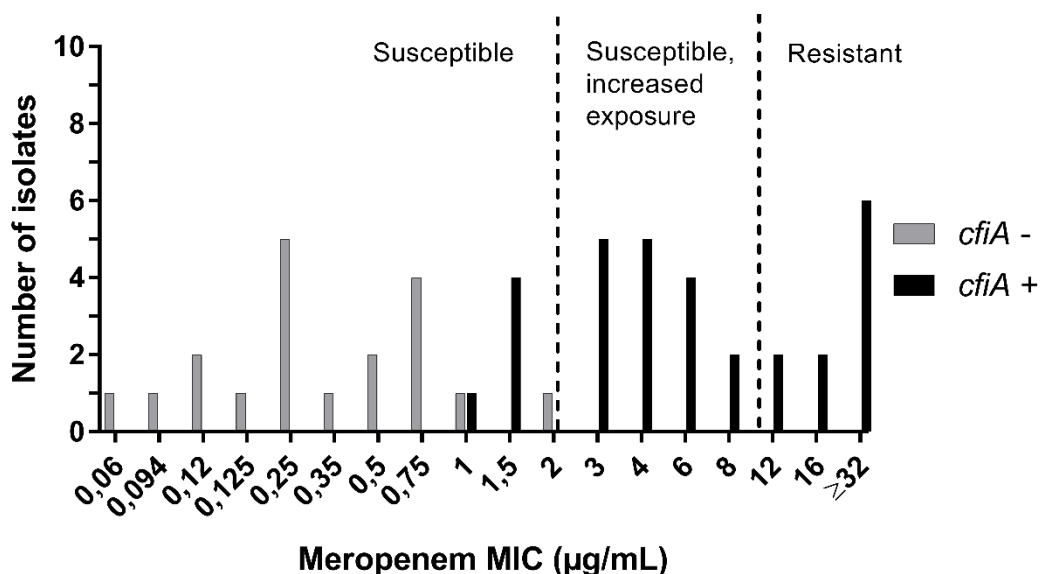


282

283 **Figure 1**

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

288



289

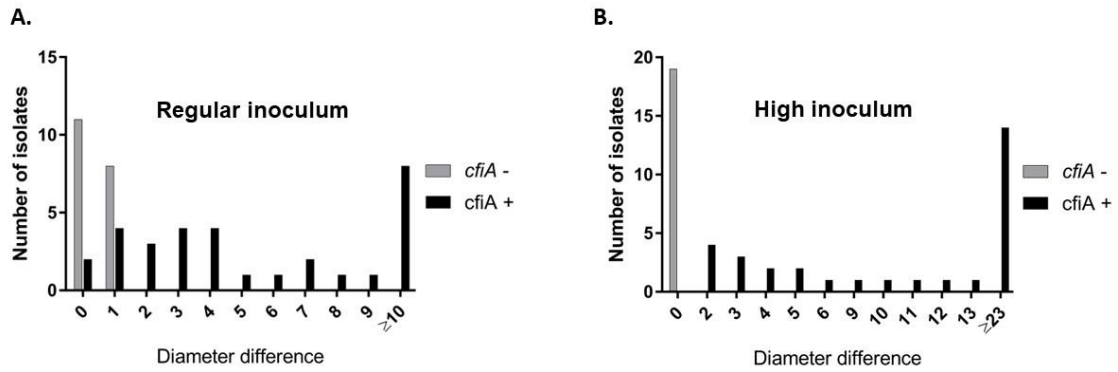
290 **Figure 2**

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

294

295

296



297

298 **Figure 3**

299 **A.** Distribution of *cfiA*+ and *cfiA*- strains according to diameter differences with a regular
300 inoculum. **B.** Distribution of *cfiA*+ and *cfiA*- strains according to diameter differences with a
301 high inoculum. Source data are provided as a supplementary excel file.

302 (<https://doi.org/10.6084/m9.figshare.19877845.v1>).

303

304