Original Article

Antimicrobial susceptibility testing of *Bacteroides* species by disk diffusion: The NordicAST *Bacteroides* study

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ABSTRACT

Objectives: Antimicrobial susceptibility testing (AST) of anaerobic bacteria has until recently been done by MIC methods. We have carried out a multi-centre evaluation of the newly validated EUCAST disk diffusion method for AST of *Bacteroides* spp.

Methods: A panel of 30 *Bacteroides* strains was assembled based on reference agar dilution MICs, resistance gene detection and quantification of *cfiA* carbapenemase gene expression. Nordic clinical microbiology laboratories (n = 45) performed disk diffusion on Fastidious Anaerobe Agar with 5% mechanically defibrinated horse blood (FAA-HB) for piperacillin-tazobactam, meropenem and metronidazole.

Results: A total of 43/45 (95.6%) laboratories carried out disk diffusion per protocol. Intraclass correlation coefficients were 0.87 (0.80−0.93) for piperacillin-tazobactam, 0.95 (0.91−0.97) for meropenem and 0.89 (0.83−0.94) for metronidazole. For metronidazole, one media lot yielded smaller zones and higher variability than another. Piperacillin-tazobactam and meropenem zone diameters correlated negatively with *cfiA* expression. A meropenem zone diameter of <28 mm in *B. fragilis* indicated presence of *cfiA*. Piperacillin-tazobactam had the most false susceptible results. Categorical errors for this antimicrobial were particularly prevalent in *cfiA*-positive strains, and piperacillin-tazobactam had the highest number of comments describing zone reading difficulties.

Conclusions: Inter-laboratory agreement by disk diffusion was good or very good. The main challenges were media-related variability for metronidazole and categorical disagreement with the reference method for piperacillin-tazobactam in some *cfiA*-positive strains. An area of technical uncertainty specific for such strains may be warranted.

1. Introduction

Bacteroides fragilis is the most prevalent anaerobic bacterial species associated with invasive infections [1]. Increased resistance rates to antibiotics used in empirical treatment of severe infections, such as meropenem, piperacillin-tazobactam and metronidazole, have been reported in *B. fragilis* [2], and varying prevalences of resistance to clinically relevant antibiotics have been observed in different countries [3]. This underlines the importance of routine antimicrobial susceptibility testing (AST) of clinical isolates to support empiric and targeted therapeutic decisions.

The European Committee on Antimicrobial Susceptibility

Testing (EUCAST) publish clinical minimum inhibitory concentration (MIC) breakpoints for anaerobes. Prior to 2022, these breakpoints were not species-specific, and disk diffusion was not part of EUCAST methodology. Recently, EUCAST published a novel method for disk diffusion AST of anaerobic bacteria on Fastidious Anaerobe Agar with 5% mechanically defibrinated horse blood (FAA-HB), with zone diameter breakpoints [4—7].

The main purpose of this study was to evaluate the performance of the EUCAST disk diffusion method for AST of *Bacteroides* spp. in a multi-centre format, mimicking typical use of the method in a diverse group of routine clinical microbiology laboratories in the five Nordic countries.

The chromosomal CfiA carbapenemase encoded by the *cfiA* gene is of particular concern in *B. fragilis*. The *cfiA* gene is present in a subset of *B. fragilis* known as division II, which may be considered a separate genospecies [8,9]. The CfiA carbapenemase can confer resistance or reduced susceptibility to carbapenems and piperacillin-tazobactam [10]. In the absence of a mobile element directly upstream, *cfiA* is usually weakly expressed, conferring only low-level resistance, with MICs potentially in the susceptible range [9]. High-level resistance, typically with MICs >32 mg/L for carbapenems, usually results from an insertion sequence (IS) carrying promoter sequences being inserted upstream of the gene [10—13]. Reduced susceptibility to carbapenems due to other mechanisms such as efflux, altered penicillin-binding proteins or other beta-lactamases is also possible [8], but *cfiA*-mediated resistance appears to be by far the most common mechanism [9]. Imipenem and meropenem MICs correlate with *cfiA* expression [10,11,14,15], but this association has not been explored for other beta-lactams such as piperacillin-tazobactam, which is extensively used as empiric therapy for serious infections involving anaerobes. A secondary aim was therefore to examine whether the presence and/or expression of *cfiA* influenced the accuracy of AST for meropenem and piperacillin-tazobactam.

2. Materials and methods

Bacterial strains. Candidate strains for the study were identified from cryostore records of clinical *Bacteroides* spp. isolates from blood cultures or with unusual resistance phenotypes at the Department of Microbiology, Vestfold Hospital Trust (n = 31), the Department of Clinical Microbiology, Odense University Hospital (n = 14) and the Norwegian National Advisory Unit on Detection of Antimicrobial Resistance (n = 8). After reference analyses (described below), a panel of 27 clinical strains isolated between 2003 and 2017 with variable levels of susceptibility against metronidazole, piperacillin-tazobactam and meropenem was assembled. The panel was supplemented with three copies of the quality control (QC) strain *B. fragilis* ATCC 25285 (Table 1, Fig. 1). Strain characteristics were blinded to the participants.

Species identification and MIC determination. Species identification was performed by MALDI-TOF MS (Maldi Biotyper, Bruker Daltonics, Bremen, Germany). Reference MICs for piperacillin-tazobactam, meropenem and metronidazole were determined by agar dilution as described by the Clinical and Laboratory Standards Institute (CLSI) [16] at the coordinating institution (Department of Microbiology, Vestfold Hospital Trust). Reference MICs were consensus values based on at least duplicate testing; *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741 were used as QC strains for the agar dilution reference analysis [17].

Expression of the *cfiA* gene. *B. fragilis* strains possessing *cfiA* were identified by PCR [13]. For *cfiA* expression analysis, strains were cultured anaerobically (42h) on chocolate agar supplemented with 5 mg/L menadione, inoculated into thioglycolate broth, and incubated anaerobically (18—20h). Total RNA was isolated from

500 μL of broth using RNEasy Protect Bacteria Mini Kit (Qiagen Corporation, Hilden, Germany). Genomic DNA was removed using QIAGEN RNase-Free DNAse Kit (Qiagen Corporation) and Heat&Run gDNA Removal Kit (ArcticZymes, Tromsø, Norway). RNA yields were measured using Qubit RNA BR Assay (Thermo Fisher Scientific, Waltham, Massachusetts, USA). cDNA was synthesised using QuantiNova Reverse Transcription Kit (Qiagen Corporation) with 150 ng RNA. The cDNA synthesis was carried out in three technical replicates for each strain. PCR was performed using QuantiNova Probe PCR Kit (Qiagen Corporation) on a LightCycler 480 II (Roche Diagnostics, Rotkreuz, Switzerland), with 0.5 μM primers, 0.25 μM probe and the program 2 min 95°, 45*(15 sec 95°, 30 sec 60°). Primers and probes used were cfiA F, cfiA R, and cfiA-probe [13] for *cfiA*, and AllBac296f, AllBac412r, and AllBac375Bhqr [18] for 16S rRNA. The *cfiA* positive strain with the lowest meropenem MIC (Strain 4, Table 1) was selected as a calibrator. The expression of *cfiA,* normalised to 16S rRNA, was calculated relative to the calibrator with 95% confidence intervals using the $\Delta\Delta C_t$ method [19]. A standard series of six tenfold dilutions of gDNA, plus two intermediate dilutions, was analysed in three technical replicates to validate the assumption of equal PCR efficiencies for *cfiA* and 16S rRNA.

Whole-genome analysis. Whole genome sequencing has previously been carried out for strains 18 (BFO85), 21 (BFO17), 23 (BFO18), and 28 (BFO42) [20]. The remaining strains were sequenced at Vestfold Hospital Trust using Ion S5 XL (Thermo Fisher Scientific). *De novo* assembly was done using SPAdes version 3.1.0 [21], with k-values 21, 33, 55, 77, 99, and 127, and IonHammer read correction enabled.

The presence of the *cfiA* carbapenemase gene or *nim* nitro-imidazole reductase genes was determined for all strains by BLAST against the ResFinder [22] and NCBI AMRFinderPlus [23] databases (both accessed April 15, 2021) using ABRicate v. 1.0.1.

To overcome contig breaks associated with IS elements [24], the presence of an IS upstream of *cfiA* was determined by PCR and electrophoresis as described [25]. An amplicon size of approximately 350 bp indicated no IS. Longer amplicons were excised, extracted using ZymoClean Gel DNA Recovery Kit (Zymo Research, Irvine, California, USA), and Sanger sequenced (Eurofins GATC, Cologne, Germany). IS elements were identified by BLAST against the ISfinder database [26]. Strains were classified according to whether a mobile element was present upstream of *cfiA* or not.

Multi-centre AST study. Nordic clinical microbiology laboratories were invited to participate through the Nordic Committee on Antimicrobial Susceptibility Testing (NordicAST, www.nordicast.org). Forty-five laboratories enrolled in the study (Denmark n = 6; Finland n = 4; Iceland n = 1; Norway n = 17; Sweden n = 17). One laboratory was a specialized reference laboratory, whereas the rest were general clinical microbiology laboratories that routinely use the EUCAST disk diffusion method for aerobic bacteria. The participants received bacterial strains and disks (Oxoid Ltd., Basingstoke, UK): piperacillin-tazobactam (30-6 μg), meropenem (10 μg), and metronidazole (5 μg). FAA plates with 5% mechanically defibrinated horse blood (FAA-HB, agar depth 4 mm) were prepared by SSI Diagnostica, Hillerød, Denmark, and distributed directly to the participants. For logistic reasons, laboratories were divided into two equal groups performing the study two weeks apart (weeks 2 and 4 of 2019), using different lots of agar plates. One laboratory entered the trial later and received a third lot. Participants were instructed to suspend each strain in sterile saline to 1.0 McFarland turbidity, streak the suspension onto two FAA-HB plates, apply two disks per plate and incubate the plates anaerobically within 15 min of disk application (35—37 °C, 16—20h) before reading inhibition zone diameters. Furthermore, the participants were instructed to read the "obvious" zone edge and ignore haze,

Table 1
Relevant characteristics of the bacterial strains included in the study.

No.	Species	Reference MIC (mg/L)[a]			Resistance genes[b]		*cfiA* expression (95% CI)[c]	Mobile element upstream of *cfiA*[c]	Strain name and GenBank accession no.
		PT	MP	MZ	*cfiA*	*nim*			
1	*B. fragilis* division II	4	32	0.5	*cfiA*	–	23 (16–50)	none	Tbg-23, JAPPUG000000000
2	*B. fragilis* division II	>128	128	64	*cfiA*	*nimE*	310 (210–460)	IS*614B*	Tbg-26, JAPPUE000000000
3	*B. fragilis* division I	0.125	0.125	8	–	*nimE*	NA	NA	Tbg-20, JAPNXN000000000
4	*B. fragilis* division II	0.25	1	0.5	*cfiA*	–	1 (calibrator)	none	Tbg-12, JAQFWE000000000
5	*B. fragilis* division II	64	64	0.5	*cfiA*	–	23 (16–33)	IS*1187*	Tbg-11, JAPMNF000000000
6	*B. fragilis* division II	>128	>128	4	*cfiA*	–	45 (32–65)	IS*614B*	Tbg-43, JAPPTV000000000
7	*B. thetaiotaomicron*[f]	8	1	1	–	–	NA	NA	Tbg-2, JAPPUI000000000
8	*B. fragilis* division I	0.125	0.125	16	–	*nimE*	NA	NA	Tbg-36, JAPPTY000000000
9	*B. fragilis* division I	0.25	0.125	0.5	–	–	NA	NA	Tbg-45, JAPPTT000000000
10	*B. faecis*[f]	8	0.25	1	–	–	NA	NA	Tbg-41, JAPPTW000000000
11	*B. uniformis*	0.5	0.125	1	–	–	NA	NA	Tbg-38, JAPPTX000000000
12	*B. ovatus*	8	2	1	–	–	NA	NA	Tbg-4, JAPPUH000000000
13	*B. fragilis* division I[d]	0.25	0.125	0.5	–	–	NA	NA	NCTC 9343, UFTH00000000
14	*B. fragilis* division II	≤0.06	4	0.5	*cfiA*	–	1.2 (0.9–1.8)	none	Tbg-29, JAPPUB010000000
15	*B. fragilis* division II	>128	128	1	*cfiA*	–	180 (120–260)	Tn*7563*[e]	Tbg-22, JAPONC000000000
16	*B. fragilis* division II	0.25	4	1	*cfiA*	–	8.2 (5.5–12.2)	none	Tbg-27, JAPPUD000000000
17	*B. fragilis* division II	1	4	64	*cfiA*	*nimE*	6.5 (4.5–9.4)	none	Tbg-25, JAPPUF000000000
18	*B. fragilis* division II	2	8	0.5	*cfiA*	–	23 (17–31)	none	DCMOUH0085B, CP037440
19	*replicate of specimen 13*[d]								
20	*B. fragilis* division I	0.25	0.125	8	–	*nimE*	NA	NA	Tbg-32, JAPPTZ000000000
21	*B. fragilis* division II	>128	>128	64	*cfiA*	*nimJ*	110 (77–170)	IS*614B*	DCMOUH0017B, CP036539-CP036541
22	*B. fragilis* division II	0.25	4	0.5	*cfiA*	–	9.8 (6.5–15)	none	Tbg-31, JAPPUA000000000
23	*B. fragilis* division II	64	128	16	*cfiA*	*nimD*	240 (160–350)	IS*Bf12*	DCMOUH0018B, CP036542-CP036545
24	*B. caccae*	4	0.125	16	–	*nimE*	NA	NA	BC_BC_ODE_DK_2015, JAPUBL000000000
25	*B. fragilis* division I	0.25	0.25	8	–	*nimE*	NA	NA	Tbg-44, JAPPTU000000000
26	*replicate of specimen 13*[d]								
27	*B. fragilis* division II	0.25	4	0.5	*cfiA*	–	2.4 (1.6–3.6)	none	Tbg-28, JAPPUC000000000
28	*B. fragilis* division I	0.25	0.125	4	–	*nimA*	NA	NA	DCMOUH0042B, CP036550-CP036552
29	*B. ovatus*	4	0.25	32	–	*nimE*	NA	NA	BO_FA_ODE_DK_2015_87_3, JAPUBM000000000
30	*B. fragilis* division II	1	16	0.5	*cfiA*	–	34 (23–50)	none	Tbg-21, JAPNXO000000000

[a] PT: piperacillin-tazobactam, MZ: metronidazole; MP: meropenem.
[b] –: not detected.
[c] NA: Not applicable (*cfiA* not present).
[d] Specimen 13 is the reference isolate *B. fragilis* ATCC 25285.
[e] Strain 15 (Tbg-22) had a promoter carried by Tn*7563*, a novel integrative and conjugative element that was present upstream of *cfiA*.
[f] *B. thetaiotaomicron* and *B. faecis* are closely related, were not reliably distinguished by the participants (data not shown), and were both classified according to breakpoints for *B. thetaiotaomicron*.

but take into account isolated colonies within the zone. The anaerobic incubation system was optional, e.g. an anaerobic workstation, gas-generating envelopes or an automatic jar-filling device.

Participants tested each strain once and submitted zone diameters for each strain/drug combination, as well as an optional free-form comment for each strain, through a web form.

The study protocol and the technical manual distributed to participants are available as supplementary materials.

Analysis of AST results. Only laboratories that submitted
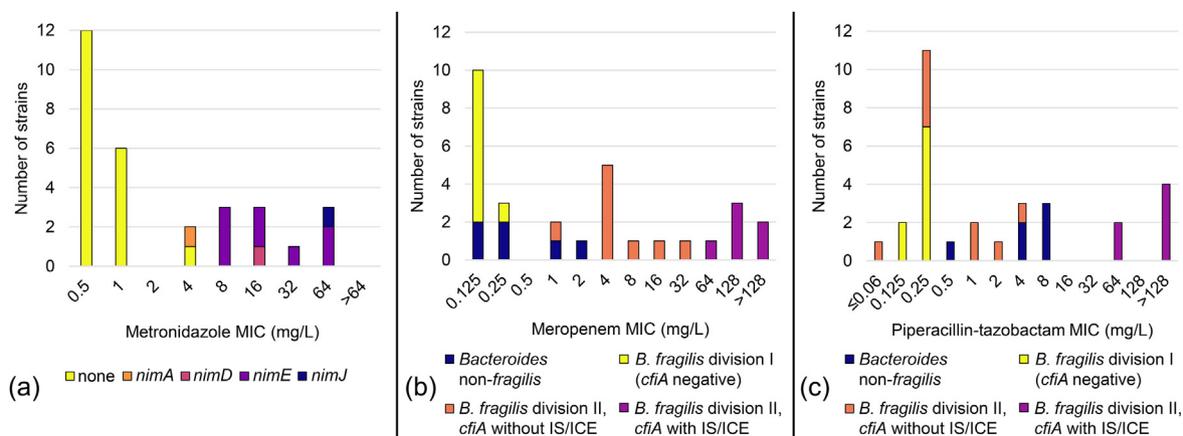
Fig. 1. Reference MIC distributions of the study strains for (a) piperacillin-tazobactam, related to the presence of *cfiA* with or without a strong promoter; (b) meropenem, related to the presence of *cfiA* with or without a strong promoter; (c) metronidazole, related to the presence of *nim* genes.

complete responses were included in the analyses of disk diffusion results.

To assess accuracy, each laboratory's readings for the three replicates of *B. fragilis* ATCC 25285 were compared with accepted QC ranges as recommended by EUCAST [5]. The difference between the highest and lowest zone diameter values for the three replicates was also calculated to assess reproducibility.

Since the reported observations were not independent (clustered by strain and laboratory), analyses of AST results were carried out with strains, rather than individual observations, as unit of analysis, except for the subanalyses of factors influencing laboratories' proportions of correctly categorized strains, for which laboratories were used as unit of analysis. The intraclass correlation coefficient (ICC) was used to assess inter-laboratory agreement for each of the included antimicrobial disks. The ICC is an ANOVA-based measure comparing variance *between* subjects (in this case strains) with variance *within* the same subject, ranging from 0.00 to 1.00 [27]. ICCs were calculated using SPSS Statistics 25 (IBM Corporation, Armonk, New York, USA) with a single-rater, absolute-agreement, two-way random effects model [28]. ICCs >0.75 were considered good, and >0.9 very good [27,28].

Interpretation of MIC values and zone diameters with categorization as susceptible (S) or resistant (R) was done according to EUCAST clinical breakpoints for *Bacteroides* spp. (version 13.0) [6]. The error rate for a particular strain/drug combination was considered high if a categorical error was observed for at least ten percent of the participants. The zone diameter distributions were visualized as boxplots with reference MIC, median and quartile zone diameters, overlaid with each single observation as a point.

The participants' free-form comments were categorized as to which antimicrobial they concerned, or as general (not related to one specific antimicrobial), and into the following themes: "difficulty identifying the zone edge", "irregular zone shape", "double zone", "haze", "colonies in zone" or "poor growth". Comments unrelated to the AST process (e.g. about species identification) were ignored.

Statistics. Correlations were assessed with Spearman's ρ. Comparisons between groups were done with nonparametric tests. Analyses were done with SPSS Statistics 25 (IBM Corporation, Armonk, New York, USA).

3. Results

Species and reference MICs. Species distribution and reference MIC values are shown in Table 1. The clinical strains included in the study panel were *B. fragilis* division II (n = 15), *B. fragilis* division I

(n = 6), *B. ovatus* (n = 2), *B. caccae* (n = 1), *B. faecis* (n = 1), *B. thetaiotaomicron* (n = 1), or *B. uniformis* (n = 1). MIC_{50} values and MIC ranges (mg/L) were piperacillin-tazobactam 1 ($\leq 0.06 - >128$), metronidazole 1 (0.5−64), and meropenem 4 (0.125 − >128).

Resistance genes. A *cfiA* carbapenemase gene was present in 15/27 clinical strains (56%), while a nitroimidazole reductase (*nim*) gene was present in 11/27 (41%) (Table 1). Fig. 1 shows reference MIC distribution versus resistance genes.

Promoter region and expression of the *cfiA* gene. Nine of 15 *B. fragilis* division II strains did not have any detectable mobile genetic element upstream of *cfiA*, five had an upstream IS element, and one (strain 15) had promoters carried by a novel integrative and conjugative element, which will be further characterized in a separate study (registered as Tn*7563* in the Transposon Registry, manuscript under preparation) (Table 1). Reference MICs were positively correlated with *cfiA* expression for meropenem (ρ = 0.93; p < 0.01) and piperacillin-tazobactam (ρ = 0.87; p < 0.01). The six strains with upstream promoters (strains 2, 5, 6, 15, 21, and 23) had the highest MICs (≥ 64 mg/L) for both meropenem and piperacillin-tazobactam.

EUCAST disk diffusion. In total, 43/45 (95.6%) laboratories returned complete disk diffusion results. One participant failed to return results for three strains (no growth), and one did not report piperacillin-tazobactam results for one strain (inhibition zone difficult to discern).

Median differences between the lowest and highest measurement of the QC strain ATCC 25285 for each laboratory were 2 mm (range, 0−13) for piperacillin-tazobactam, 2 mm (0−8) for meropenem, and 2 mm (0−22) for metronidazole. The proportions of laboratories achieving a difference of ≤ 3 mm between measurements of were 77% (33/43) for piperacillin-tazobactam, 81% (35/43) for meropenem, and 67% (37/43) for metronidazole. The proportions of laboratories with all results within accepted QC ranges were 72% (31/43) for piperacillin-tazobactam, 81% (35/43) for meropenem, and 51% (22/43) for metronidazole. Fig. 2 shows zone diameters by FAA-HB lot for the QC strain. For the metronidazole disk, but not for other agents, there was a difference between the FAA-HB lots, with lot 1 (median, 28 mm; range, 6−35 mm) yielding smaller zones and higher variability than lot 2 (median, 33 mm; range, 27−39 mm). Lot 1 yielded significantly lower median metronidazole zone diameters per isolate than lot 2 also for the clinical strains (p < 0.001, Wilcoxon signed-rank test), and poorer discrimination among the strains with MICs in the 1−8 mg/L range (Fig. 3). No such difference was seen for piperacillin-tazobactam or meropenem.
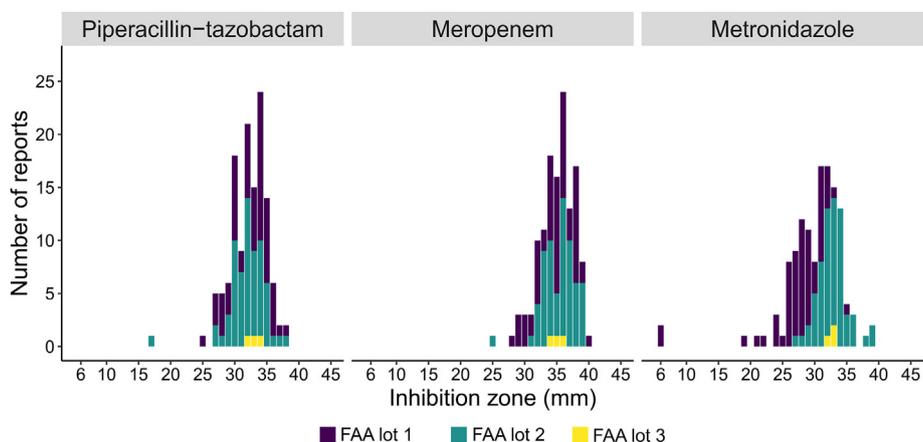
Fig. 2. Distributions of zone diameters reported for the ATCC 25285 QC strain for piperacillin-tazobactam, meropenem and metronidazole, by FAA-HB lot used.
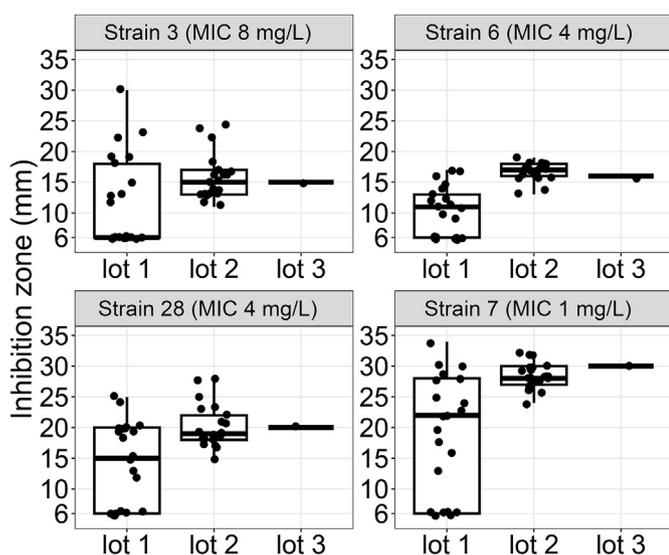
Fig. 3. Example of differences between FAA-HB lots for strains with metronidazole MICs in the 1−8 mg/L range: Boxplots of metronidazole zone diameters for strains 3, 6, 28 and 7 according to FAA-HB lot used.

The proportion of clinical strains correctly categorized for metronidazole was lower (p < 0.01, Mann-Whitney *U* test) for laboratories reporting zones within the QC ranges for all replicates of ATCC 25285 (median 0.90, range 0.73−0.93) than for those who did (median 0.93, range 0.87−0.97). No significant difference was found for meropenem or piperacillin-tazobactam (Supplementary Figs. S1−S3).

Inter-laboratory agreement for the clinical strains assessed by ICCs was very good for meropenem (0.94, 95% CI 0.91−0.97), and good for piperacillin-tazobactam (0.87, 95% CI 0.80−0.93) and metronidazole (0.89, 95% CI 0.83−0.94).

Median zone diameter−reference MIC correlations were $\rho = -0.89$ for piperacillin-tazobactam, $\rho = -0.91$ for metronidazole and $\rho = -0.96$ for meropenem (all p < 0.01).

In *cfiA*-positive *B. fragilis*, a significant negative correlation was seen between *cfiA* expression and median zone diameter for the piperacillin-tazobactam ($\rho = -0.80$) and meropenem ($\rho = -0.86$) disks (both p < 0.001).

Full overviews of zone diameter readings for all strains are shown in Figs. 4−6. For **piperacillin-tazobactam** (Fig. 4), four strains had high error rates: high false susceptibility rates were

seen for the resistant strains no. 5 (33%, 14/43) and 23 (40%, 17/43), both *B. fragilis* with MIC 64 mg/L possessing a strongly expressed *cfiA* gene. Conversely, high false resistance rates were observed for the susceptible non-*B. fragilis* strains 12 (88%, 38/43) with MIC 8 mg/L and 29 (14%, 6/43) with MIC 4 mg/L. Strains 7 and 10 (*B. thetaiotaomicron*) could not be categorized due to *B. thetaiotaomicron* lacking a clinical breakpoint for piperacillin-tazobactam. Several *B. fragilis* division II strains with strong *cfiA* expression and MIC $\geq$64 mg/L had bimodal distributions, with a sizable minority of laboratories reporting zone diameters overlapping those of susceptible strains.

For **meropenem** (Fig. 5), high false resistance rates were observed for the two susceptible strains 7 (98%, 42/43) and 4 (91%, 39/43), both with reference MIC 1 mg/L. Strain 4 was a *B. fragilis* carrying a weakly expressed *cfiA* gene (Table 1). The remaining strains had low error rates. The EUCAST zone diameter breakpoint of S $\geq$ 28/R < 28 mm separated the division I (*cfiA* negative) and division II (*cfiA* positive) *B. fragilis* populations.

For **metronidazole** (Fig. 6), high false resistance rates were observed for the three susceptible strains 6 (100%, 43/43), 28 (91%, 39/43), and 7 (35%, 15/43). Strains 6 and 28 (MIC 4 mg/L) were both *B. fragilis*, while strain 7 (MIC 1 mg/L) was *B. thetaiotaomicron*. Only strain 28 had a *nim* gene (*nimA*) (Table 1). The median metronidazole zone diameter per isolate was lower among participants incubating plates in jars versus those using an anaerobic workstation (p < 0.01, Wilcoxon signed-rank test), but the difference was small (ranging from −4 to +1 mm, 12 negative differences, 2 positive differences and 16 ties). Laboratories using jars tended to have a lower proportion of correctly categorized strains (Supplementary Fig. S4), but the difference was not statistically significant (p = 0.17, Mann-Whitney *U* test). We did not find any difference between using gas-generating envelopes or a jar-filling device for making jars anaerobic (data not shown).

Participants' comments. A total of 80 free-form comments about the AST process were entered by 25 laboratories about 24 strains, concerning piperacillin-tazobactam (n = 49), meropenem (n = 17), metronidazole (n = 12), or general comments (n = 9). A summary of the comments is shown in Supplementary Table S1. Some high-frequency comments were identified: for piperacillin-tazobactam, double zone (n = 19), haze (n = 12) and colonies in the zone (n = 14), and for meropenem, double zone (n = 10). Notably, the comments for piperacillin-tazobactam concerned 18 strains, including all of the 15 *B. fragilis* division II (*cfiA* positive) strains, plus two *B. fragilis* division I (*cfiA* negative), and one non-*B. fragilis* species.
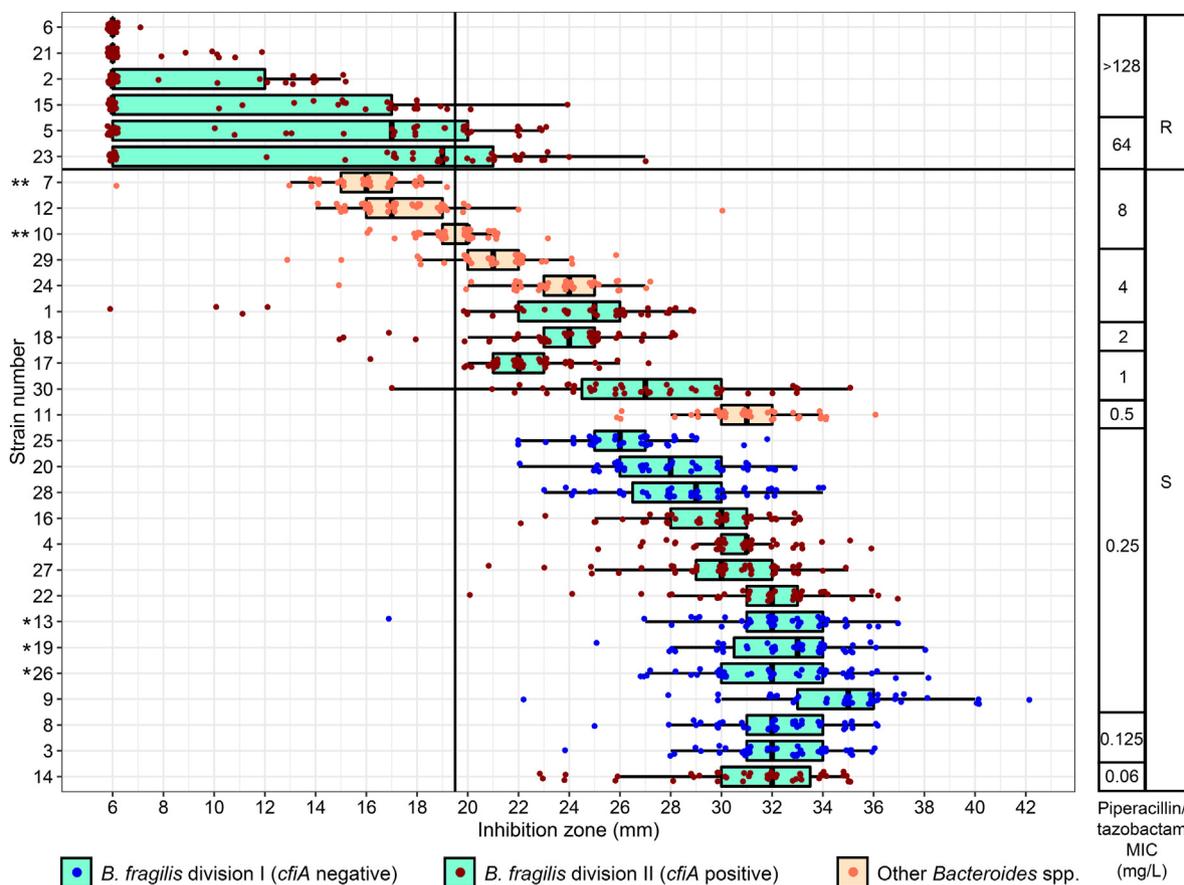
Fig. 4. Plots of all reported piperacillin-tazobactam zone diameters per strain after 16–20 h incubation. Solid black lines indicate EUCAST clinical breakpoints for MICs and zone diameters. *: ATCC 25285 QC strain; **: *B.thetaiotaomicron/faecis* which lack clinical breakpoints.

4. Discussion

Our observations indicate that the newly published EUCAST disk diffusion method for AST of anaerobes generally performs well when applied on *Bacteroides* spp. in a routine setting, providing a simple, accurate and low-cost alternative to MIC determination.

The multi-centre study format, using a challenge panel of well-characterized strains enriched with clinically relevant resistance mechanisms, is suited for assessing real-world feasibility, agreement between laboratories, and the impact of specific resistance mechanisms on AST accuracy. Notably, it is not suited for setting or evaluation of clinical breakpoints; this requires a larger sample of unselected strains [29] and has been carried out separately [4,7,30].

Our results show that a clear majority of the participants managed to carry out disk diffusion per protocol. However, the results for the three replicates of QC strain *B. fragilis* ATCC 25285 revealed some challenges, as a sizable number of labs did not report zone diameters within the QC ranges for all three replicates. It should be noted that the participants used the method for the first time, with no opportunity for calibration, training or optimization, as normally would be done before introducing a new method into routine use, and only carried out the tests once for each strain. Furthermore, in order to make sure that zone readings were un-biased, the study protocol did not disclose information about QC strains or accepted QC ranges, which were still tentative and un-published at the time of the study. Anaerobic disk diffusion has specific reading guidelines on how to identify zone edges [31], which were less clear at the time of the study than the ones now published with photographic examples, and an opportunity to calibrate one's performance and reading by repeated analysis of a QC strain would likely have improved performance, whereas the present study only offered participants one opportunity to test each isolate.

A specific issue was identified for metronidazole, as there was considerable variability between media lots, leading to lower overall precision and accuracy for the metronidazole disk compared to the other drugs. Comparing metronidazole to the other agents with regard to results for the QC strain, inter-laboratory agreement and correct categorization of strains, the performance of lot 1 for metronidazole appears aberrant and sig-nals a problem with this lot not present in lot 2. The specific reason has not been conclusively determined; however, insufficient anaerobiosis is a well-known cause of metronidazole AST errors, and factors such as dissolved air or trapped air bubbles in the medium are known to impair anaerobic conditions [32]. A warning about this phenomenon is warranted, underlining the importance of careful quality control of new agar lots and of the anaerobic at-mosphere in each setup. In sum, the laboratories' ability to achieve results within the accepted QC ranges was moderate for metroni-dazole, and good for piperacillin-tazobactam and meropenem.

Overall inter-laboratory agreement, as measured by ICC, was good or very good for the three evaluated disks. Notably, the per-formance of the metronidazole disk was affected by media lot variability and would likely be improved with proper QC. The ICC takes into account intra-rater as well as inter-rater variance and, although little used in microbiology, it is the preferred statistical approach for assessment of overall test reliability (i.e. to which extent measurements can be replicated) in a situation where
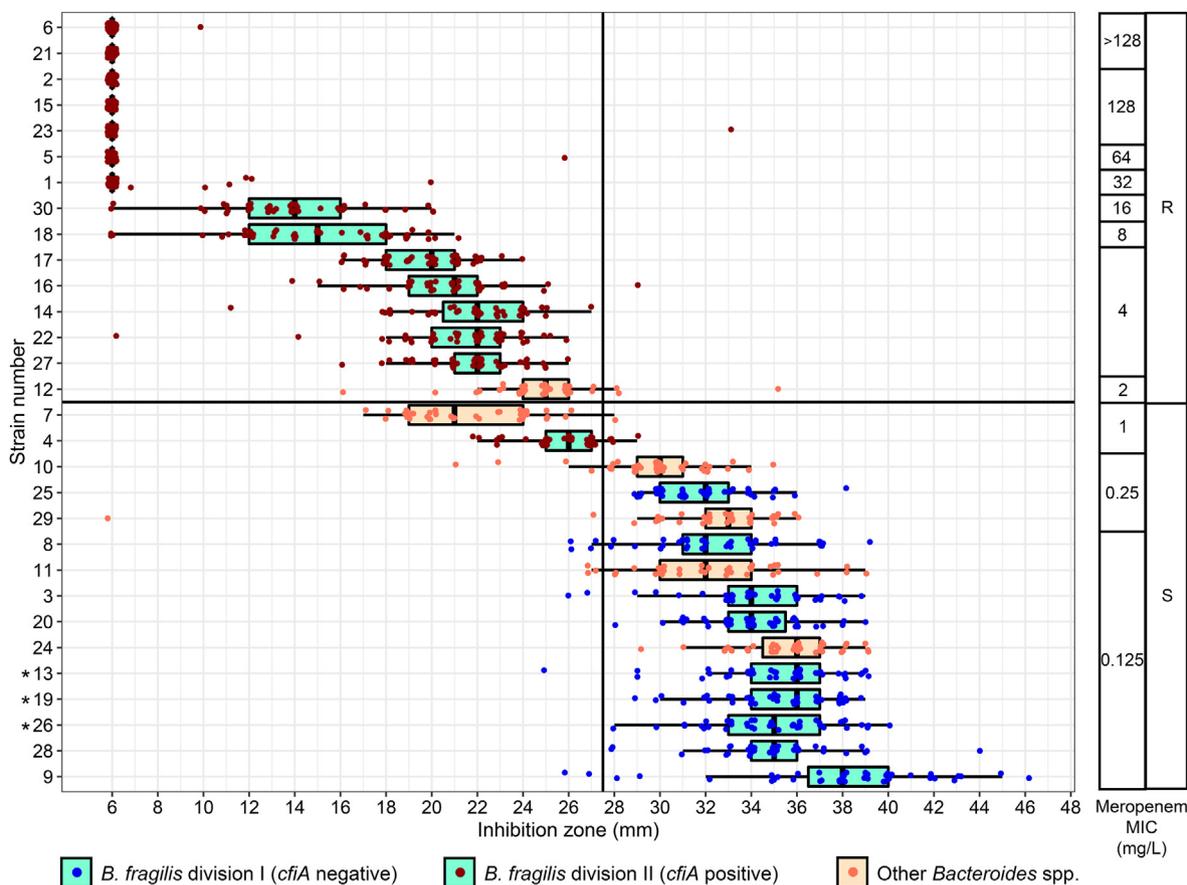
Fig. 5. Plots of all reported meropenem zone diameters per strain after 16–20 h incubation. Solid black lines indicate EUCAST clinical breakpoints for MICs and zone diameters. *: ATCC 25285 QC strain.

multiple observers test the same panel of subjects (in this case strains) [27].

We found strong correlation between median inhibition zone and reference MIC for the three disks. With the strain panel used in this study, the frequency of categorical errors was low for most strain/drug combinations, and the overall number of combinations with high proportions of false resistance was higher than the number of combinations with high proportions of false susceptibility (seven and two, respectively).

For metronidazole and meropenem, false resistance was the predominant type of categorical errors, consistent with the validation carried out by EUCAST [30]. Strains with high proportions of false resistance by disk diffusion had detectable resistance genes (*nimA* or *cfiA*) or MICs similar to such strains. As both *cfiA* and *nim* genes have variable expression patterns, this may indicate that disk diffusion is somewhat more influenced by weakly expressed resistance mechanisms than the reference method. The EUCAST clinical zone diameter breakpoint for meropenem classified all *B. fragilis* division II as resistant, consistent with the findings from EUCAST's validation [4,30].

For piperacillin-tazobactam, the two strains with high proportions of false susceptible results were *B. fragilis* with high expression of *cfiA*; these had high zone variability with bimodal distributions. *B. fragilis* division II strains with low *cfiA* expression generally had low piperacillin-tazobactam error rates, whereas strains with moderate or high *cfiA* expression had greater inter-laboratory variation and higher frequencies of categorical errors in both directions. *B. fragilis* division II strains were also over-represented among strains where participants reported that reading

the piperacillin-tazobactam zone diameter was difficult due to double zones, haze or colonies in the zone. These observations suggest that AST for piperacillin-tazobactam is inherently challenging in *cfiA*-carrying strains. As a consequence, apparent susceptibility to piperacillin-tazobactam in meropenem-resistant *B. fragilis* should be interpreted with caution, and an area of technical uncertainty (ATU) specific for meropenem-resistant strains (potentially with a recommendation discouraging use of piperacillin-tazobactam) may be warranted. The fact that reference MIC determination for piperacillin-tazobactam uses a fixed tazobactam concentration of 4 mg/L, whereas disk diffusion involves a gradient of both piperacillin and tazobactam, may contribute to discrepancies between the two methods. High proportions of false resistant results for piperacillin-tazobactam were only seen in two non-*B. fragilis* strains. The results suggest that species-specific zone breakpoints for *Bacteroides* non-*fragilis* species could be appropriate.

The strength of this study is the large number of participating laboratories from five countries, allowing estimation of inter-laboratory agreement, and evaluation of the method's robustness in a typical-use scenario. There are some limitations. The number of *Bacteroides* non-*fragilis* species was limited, making it difficult to draw any specific conclusions for these species. Only one lot and manufacturer of disks was tested. Further, participants did not have access to QC guidelines, and only tested each strain once. Finally, clindamycin, which is one of only four agents with clinical breakpoints for *Bacteroides* spp. defined by EUCAST, was also tested by the participants, but the results had to be excluded due to a change in disk content from 10 to 2 μg between the study date and the final publication of the EUCAST method.
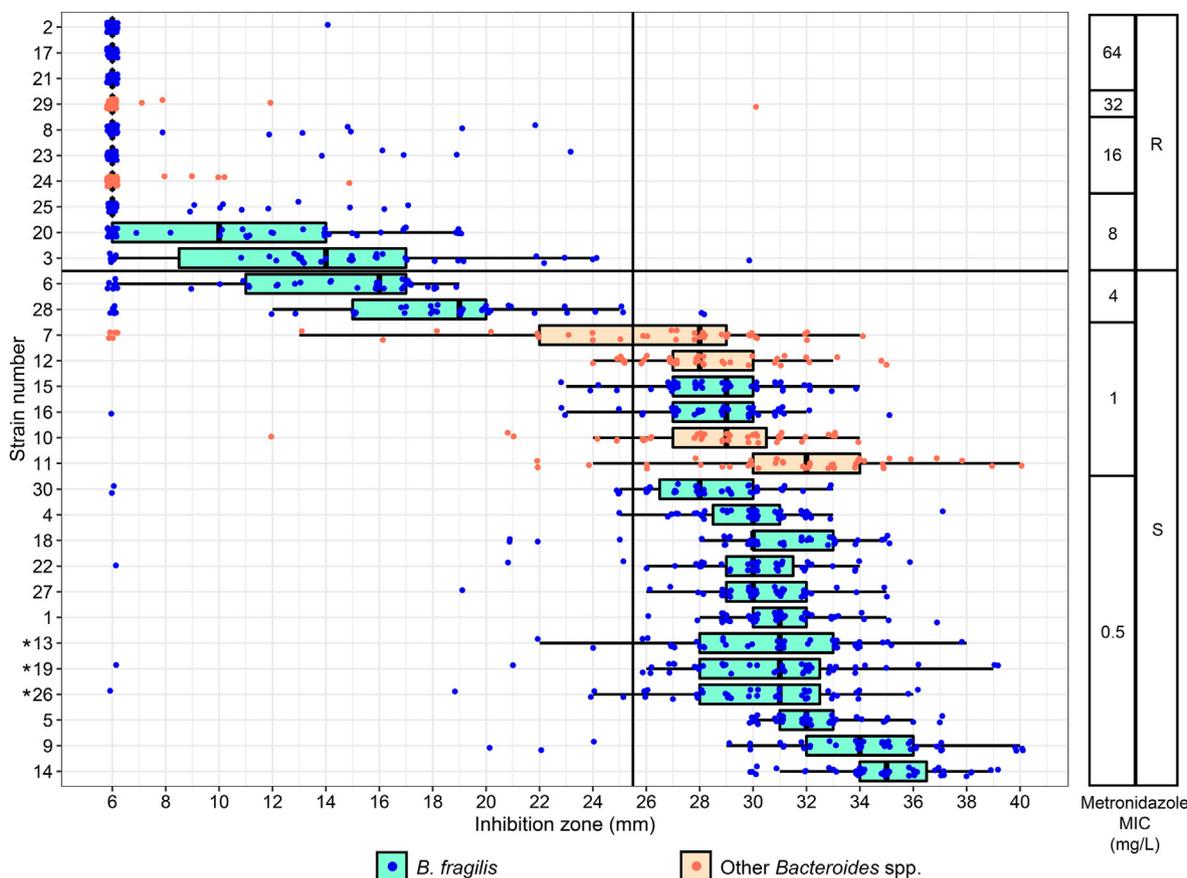
Fig. 6. Plots of all reported metronidazole zone diameters per strain after 16–20 h incubation. Solid black lines indicate EUCAST clinical breakpoints for MICs and zone diameters. *: ATCC 25285 QC strain.

In conclusion, the study showed that the novel EUCAST disk diffusion method is a feasible and accurate option for routine AST of *Bacteroides*, despite participants carrying out the method for the first time with no opportunity to perform standard QC measures. We also detected specific factors adversely impacting accuracy for metronidazole (vulnerability to variation of the FAA-HB media) and piperacillin-tazobactam (difficulty interpreting zones in *cfiA*-carrying *B. fragilis*). Knowledge of these pitfalls will allow laboratories to optimize their reading, carry out the necessary quality control, and minimize categorical errors due to these factors.

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CRediT authorship contribution statement

Tore Taksdal Stubhaug: Conceptualization, Methodology, Software, Investigation, Formal analysis, Writing – original draft, Writing – review & editing, Visualization, Funding acquisition. **Christian G. Giske:** Conceptualization, Methodology, Resources, Formal analysis, Writing – review & editing, Supervision. **Ulrik S. Justesen:** Conceptualization, Formal analysis, Resources, Writing – review & editing, Supervision. **Gunnar Kahlmeter:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision. **Erika Matuschek:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision. **Arnfinn Sundsfjord:** Conceptualization, Methodology, Resources, Formal analysis, Writing – review & editing, Supervision. **Dagfinn Skaare:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Visualization, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

References

[1] H.M. Wexler, Bacteroides: the good, the bad, and the nitty-gritty, Clin. Microbiol. Rev. 20 (2007) 593−621.

[2] J. Soki, M. Hedberg, S. Patrick, B. Balint, R. Herczeg, I. Nagy, et al., Emergence and evolution of an international cluster of MDR Bacteroides fragilis isolates, J. Antimicrob. Chemother. 71 (2016) 2441−2448.

[3] A.C.M. Veloo, H.B. Tokman, H. Jean-Pierre, Y. Dumont, S. Jeverica, R. Lienhard, et al., Antimicrobial susceptibility profiles of anaerobic bacteria, isolated from human clinical specimens, within different European and surrounding countries. A joint ESGAI study, Anaerobe 61 (2019), 102111.

[4] H. Bavelaar, U.S. Justesen, T.E. Morris, B. Anderson, S. Copsey-Mawer, T.T. Stubhaug, et al., Development of a EUCAST disk diffusion method for the susceptibility testing of rapidly growing anaerobic bacteria using Fastidious Anaerobe Agar (FAA): a development study using Bacteroides species, Clin. Microbiol. Infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases 27 (2021), 1695.e1-.e6.

[5] European Committee on Antimicrobial Susceptibility Testing, EUCAST Disk Diffusion Methodology and Tentative QC Criteria for Selected Rapidly Growing Anaerobic Bacteria on Fastidious Anaerobe Agar (FAA), 2022 [Available from: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Anaerobic_bacteria/2022_anaerobes/EUCAST_disk_diffusion_methodology_for_rapidly_growing_anaerobic_bacteria_with_QC_v_1.0_2022.pdf].

[6] European Committee on Antimicrobial Susceptibility Testing, Breakpoint tables for interpretation of MICs and zone diameters - bacteria, Version 13.0. 2022. [Available from: https://eucast.org/clinical_breakpoints/].

[7] E. Matuschek, S. Copsey-Mawer, S. Petersson, J. Åhman, T.E. Morris, G. Kahlmeter, The European committee on antimicrobial susceptibility testing disc diffusion susceptibility testing method for frequently isolated anaerobic bacteria, in: Clinical Microbiology and Infection, the official publication of the European Society of Clinical Microbiology and Infectious Diseases, 2023.

[8] S. Jean, M.J. Wallace, G. Dantas, C.D. Burnham, Time for some group therapy: update on identification, antimicrobial resistance, taxonomy, and clinical significance of the Bacteroides fragilis group, J. Clin. Microbiol. 60 (2022), e0236120.

[9] M.J. Wallace, S. Jean, M.A. Wallace, C.D. Burnham, G. Dantas, Comparative genomics of Bacteroides fragilis group isolates reveals species-dependent resistance mechanisms and validates clinical tools for resistance prediction, mBio 13 (2022), e0360321.

[10] J. Soki, R. Edwards, M. Hedberg, H. Fang, E. Nagy, C.E. Nord, Examination of cfiA-mediated carbapenem resistance in Bacteroides fragilis strains from a European antibiotic susceptibility survey, Int. J. Antimicrob. Agents 28 (2006) 497−502.

[11] R. Edwards, C.V. Hawkyard, M.T. Garvey, D. Greenwood, Prevalence and degree of expression of the carbapenemase gene (cfiA) among clinical isolates of Bacteroides fragilis in Nottingham, UK, J. Antimicrob. Chemother. 43 (1999) 273−276.

[12] I. Podglajen, J. Breuil, A. Rohaut, C. Monsempes, E. Collatz, Multiple mobile promoter regions for the rare carbapenem resistance gene of Bacteroides fragilis, J. Bacteriol. 183 (2001) 3531−3535.

[13] K.H. Roh, S. Kim, C.K. Kim, J.H. Yum, M.S. Kim, D. Yong, et al., New cfiA variant and novel insertion sequence elements in carbapenem-resistant Bacteroides fragilis isolates from Korea, Diagn. Microbiol. Infect. Dis. 66 (2010) 343−348.

[14] R. Edwards, P.N. Read, Expression of the carbapenemase gene (cfiA) in Bacteroides fragilis, J. Antimicrob. Chemother. 46 (2000) 1009−1012.

[15] J. Soki, R. Edwards, E. Urban, E. Fodor, Z. Beer, E. Nagy, Screening of isolates from faeces for carbapenem-resistant Bacteroides strains; existence of strains with novel types of resistance mechanisms, Int. J. Antimicrob. Agents 24 (2004) 450−454.

[16] Clinical and Laboratory Standards Institute, M11-A8: Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria, Approved Standard - Eighth Edition, Wayne, PA, 2012.

[17] Clinical and Laboratory Standards Institute, M100-ED33: Performance Standards for Antimicrobial Susceptibility Testing, 33rd Edition, Table 5D MIC QC Ranges for Anaerobes (Agar Dilution Method), Wayne, PA, 2023.

[18] A. Layton, L. McKay, D. Williams, V. Garrett, R. Gentry, G. Sayler, Development of Bacteroides 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water, Appl. Environ. Microbiol. 72 (2006) 4214−4224.

[19] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods 25 (2001) 402−408.

[20] T.V. Sydenham, S. Overballe-Petersen, H. Hasman, H. Wexler, M. Kemp, U.S. Justesen, Complete hybrid genome assembly of clinical multidrug-resistant Bacteroides fragilis isolates enables comprehensive identification of antimicrobial-resistance genes and plasmids, Microb. Genom. 5 (2019).

[21] A. Bankevich, S. Nurk, D. Antipov, A.A. Gurevich, M. Dvorkin, A.S. Kulikov, et al., SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing, J. Comput. Biol. : a journal of computational molecular cell biology 19 (2012) 455−477.

[22] V. Bortolaia, R.S. Kaas, E. Ruppe, M.C. Roberts, S. Schwarz, V. Cattoir, et al., ResFinder 4.0 for predictions of phenotypes from genotypes, J. Antimicrob. Chemother. 75 (2020) 3491−3500.

[23] M. Feldgarden, V. Brover, N. Gonzalez-Escalona, J.G. Frye, J. Haendiges, D.H. Haft, et al., AMRFinderPlus and the Reference Gene Catalog facilitate examination of the genomic links among antimicrobial resistance, stress response, and virulence, Sci. Rep. 11 (2021), 12728.

[24] T.V. Sydenham, J. Soki, H. Hasman, M. Wang, U.S. Justesen, Identification of antimicrobial resistance genes in multidrug-resistant clinical Bacteroides fragilis isolates by whole genome shotgun sequencing, Anaerobe 31 (2015) 59−64.

[25] I. Podglajen, J. Breuil, E. Collatz, Insertion of a novel DNA sequence, IS1186, upstream of the silent carbapenemase gene cfiA, promotes expression of carbapenem resistance in clinical isolates of Bacteroides fragilis, Mol. Microbiol. 12 (1994) 105−114.

[26] P. Siguier, J. Perochon, L. Lestrade, J. Mahillon, M. Chandler, ISfinder: the reference centre for bacterial insertion sequences, Nucleic Acids Res. 34 (2006) D32−D36.

[27] L.G. Portney, M.P. Watkins, Foundations of Clinical Research : Applications to Practice, third ed., Pearson Prentice Hall, Upper Saddle River, 2009.

[28] T.K. Koo, M.Y. Li, A guideline of selecting and reporting intraclass correlation coefficients for reliability Research, Journal of chiropractic medicine 15 (2016) 155−163.

[29] G. Kronvall, C.G. Giske, G. Kahlmeter, Setting interpretive breakpoints for antimicrobial susceptibility testing using disk diffusion, Int. J. Antimicrob. Agents 38 (2011) 281−290.

[30] European Committee on Antimicrobial Susceptibility Testing, Anaerobic Bacteria: Calibration of Zone Diameter Breakpoints to MIC Values, 2022 [Available from: https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_criteria/Validation_2022/Anaerobic_bacteria_v_1.0_January_2022.pdf].

[31] European Committee on Antimicrobial Susceptibility Testing, EUCAST disk diffusion test methodology [Available from: https://www.eucast.org/ast_of_bacteria/disk_diffusion_methodology].

[32] M.G. Cormican, M.E. Erwin, R.N. Jones, False resistance to metronidazole by E-test among anaerobic bacteria: investigations of contributing test conditions and medium quality, Diagn. Microbiol. Infect. Dis. 24 (1996) 117−119.