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Antimicrobial susceptibility of anaerobic bacteria

False amoxicillin/clavulanic acid susceptibility in *Bacteroides fragilis* using gradient strip tests

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ABSTRACT

Background: Repeatedly, too low MIC results were obtained in *Bacteroides fragilis* quality assessment strains, using gradient strip tests with a ratio of amoxicillin:clavulanic acid of 2:1. We aimed to find the most accurate available gradient strip tests for susceptibility testing of amoxicillin/clavulanic acid in *B. fragilis* in comparison with agar dilution with EUCAST methodology and breakpoints.
Methods: Twenty-seven clinical *B. fragilis* isolates were investigated using gold standard EUCAST amoxicillin/clavulanic acid agar dilution (fixed clavulanic acid concentration at 2 mg/L, with increasing amoxicillin concentrations) as well as three commercial gradient strip tests: XL (ratio), AUG (ratio) or AMC (fixed concentration).
Results: Using agar dilution (fixed concentration), 19 isolates were susceptible, 1 isolate was susceptible increased exposure (I) and 7 isolates were resistant. Categorical agreement of the gradient strip tests with agar dilution (fixed concentration) was 70% for XL (ratio), 71% for AUG (ratio) and 89% for AMC (fixed concentration). Very major error rates in comparison with agar dilution (fixed concentration) were 100%, 0%, and 0%, respectively.
Conclusions: EUCAST breakpoint usage in amoxicillin/clavulanic acid susceptibility tests for *B. fragilis* should be accompanied by EUCAST methodology. When using alternative methods such as gradient strip tests, a higher degree of alignment with EUCAST methodology, such as using fixed clavulanic acid concentrations, improves precision.

1. Background

Amoxicillin/clavulanic acid may be used for the treatment of human anaerobic infections. Generally, beta-lactam resistance in *Bacteroides fragilis* is mediated by beta-lactamases. Occasionally, *B. fragilis* may also demonstrate beta-lactam resistance mediated by increased antibiotic efflux, mutations in PBP1 or PBP2Bfr or porin loss [1,2].

Clinical & Laboratory Standards Institute (CLSI), ISO standards and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) all provide recommendations for amoxicillin/clavulanic acid susceptibility testing in *B. fragilis,* with differing degrees of detail. CLSI recommends an amoxicillin:clavulanic acid ratio of 2:1 (ratio). EUCAST recommends a fixed clavulanic acid concentration at 2 mg/L with increasing amoxicillin concentrations for MIC testing (fixed concentration). Many clinical microbiology laboratories use gradient strip tests for susceptibility testing in Gram-negative anaerobes [1,3–5].

Two amoxicillin/clavulanic acid-resistant *B. fragilis* NEQAS Quality Assessment (QA) isolates (NEQAS 2018 4373-4659 and NEQAS 2019 4529-5147) were investigated using “ratio” gradient strip tests (Etest Biomérieux, XL, 412241) in the University Medical Center Utrecht, the Netherlands (UMCU) with repeated MIC results of 8 mg/L (amoxicillin component, “susceptible increased exposure” (I) category according to EUCAST 2020 breakpoints) [6]. NEQAS reported that the amoxicillin/clavulanic acid MIC for these two isolates was ≥256 mg/L (R) (amoxicillin component, EUCAST breakpoints). In comparison with the organisers’ SIR interpretation (R), these at least five doubling dilutions differences in MICs resulted in minor category discrepancies only (“I” versus “R”). Nevertheless, these at least five doubling dilutions differences in

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measured MICs in two independent QA isolates were identified as a possible risk for patient care and prompted further investigation.

In the Netherlands, three different amoxicillin/clavulanic acid CE marked gradient strip tests are available, with differences in clavulanic acid contents. We intended to assess whether low MIC measurements of amoxicillin/clavulanic acid MICs using XL (ratio) in comparison with AUG (ratio) or AMC (fixed concentration) also existed among clinical *B. fragilis* isolates. We aimed to find the most precise gradient strip test for amoxicillin/clavulanic acid MIC determination and SIR categorization in *B. fragilis*, in comparison with gold standard EUCAST agar dilution.

2. Methods

2.1. Isolates

Quality control (QC) isolates: *B. fragilis* ATCC 25282 and *Bacteroides thetaiotaomicron* ATCC 29741; QA isolates: NEQAS 2015 N3675-2598 (research nr. 11), NEQAS 2017 N4010-3511 (research nr. 15), NEQAS 2018 4373-4659 (research nr. 25) and NEQAS 2019 4529-5147 (research nr. 28)(Supplementary table 1). Twenty-seven clinical isolates were selected, obtained from 2010 to 2020, from blood (n = 16), tissue (n = 9), wound swab or prosthesis (one each). Isolates were selected from the UMCU Department of Clinical Microbiology, serving a university affiliated tertiary care teaching hospital, a university affiliated children's hospital and a centralized children's cancer referral centre. Isolate selection in the laboratory information system was based on high amoxicillin/clavulanic acid MICs, within the susceptible range, measured in routine clinical practise, using XL (ratio). Additional selection criteria were 1 isolate per patient (isolate with highest amoxicillin/clavulanic acid MIC per patient) and availability. This selection contained 5 clinical *cfiA* positive, division II isolates (research nrs. 4, 13, 20, 24, and 33, Supplementary table 2, data not shown). One NEQAS QA isolate was also *cfiA* positive, division II (NEQAS 2019 4529-5147 research nr. 28).

2.2. Gradient strip tests

Three different amoxicillin/clavulanic acid gradient strip tests were used: Liofilchem MTS 92180 (Liofilchem, Roseto degli Abruzzi, Italy) with a fixed clavulanic acid concentration at 2 mg/L further designated: AMC (fixed concentration), Liofilchem MTS 92024 (Liofilchem, Roseto degli Abruzzi, Italy) with an amoxicillin:clavulanic acid ratio of 2:1, further designated: AUG (ratio), and bioMérieux Etest 412241 (bioMérieux Benelux, Amersfoort, the Netherlands) with a amoxicillin:clavulanic acid ratio of 2:1, further designated: XL (ratio).

2.3. Procedures at the UMCU

Isolates were thawed, sub-cultured, (anaerobic incubation, using Brucella blood agar with hemin and vitamin K (BBA, BD 255509 BD, Erembodegem, Belgium) inspected for purity and re-identified using MALDI-TOF MS (Bruker MBT Sirius or Bruker MBT Smart, using Bruker Compass software and Bruker MBT Compass Library, Revision F, MBT 8468 MSP Library, all from Bruker Bremen, Germany). Classification of *B. fragilis* isolates into division I (*cfiA* negative) versus division II (*cfiA* positive) was according to Johansson et al. [7].

One McFarland suspensions were prepared in Phoenix inoculum broth (BD 246005, BD) from colonies incubated for 48 h. A cotton swab was dipped into this suspension and applied in 3 directions onto the BBA plates. Gradient strip tests were applied and anaerobiosis was immediately reinstalled in anaerobic jars with a final gas mixture of 5% H_2, 10% CO_2 and 85% N_2, using an Axonomat system (Mart BV, the Netherlands). Reusable palladium coated aluminium catalysts were used to remove residual oxygen, and *Pseudomonas aeruginosa* ATCC 27853 was used as indicator to control for anaerobiosis. Jars were opened after 48 h incubation at 35°C and MICs were read at the intersection of growth of the isolate with the strip test. Ingrowth within an ellipse of no growth, not representing contamination, was counted by extrapolation of the ingrowth toward the strip. For convenience, MICs >256 mg/L, obtained by XL (ratio), AUG (ratio) or AMC (fixed concentration) were reduced to 512 mg/L and agar dilution MICs >128 mg/L were reduced to 256 mg/L.

The amoxicillin component was used for SIR interpretations of amoxicillin/clavulanic acid MICs (S $\leq$ 4 mg/L, R > 8 mg/L, the amoxicillin component levels are identical among breakpoints from CLSI and EUCAST 2020 guidelines, but the clavulanic acid component levels differ between breakpoints from these organizations). MICs measured at PHW with agar dilution (fixed concentration) were used as the gold standard comparator MICs. Calculations of category agreement (CA), essential agreement (EA), major discrepancy (ME), minor discrepancy (MiE) and very major discrepancy (VME) were calculated according to NEN-EN-ISO 20776-2 definitions [8].

Agar dilution was not available at the UMCU, and therefore performed only at PHW.

2.4. Procedures at the PHW

AUG (ratio) was not available at PHW and was performed at the UMCU only. MICs for all isolates were determined by agar dilution using a fixed concentration of 2 mg/L clavulanic acid with various amoxicillin concentrations and also via AMC (fixed concentration) gradient strip tests. Agar dilution was performed according to CLSI guidelines using supplemented Brucella agar (5 μg/ml hemin, 1 μg/ml vitamin K, 5% (v/v) laked sheep blood) and amoxicillin (Merck, Poole, UK) from 0.008 mg/L to 128 mg/L but fixed 2 mg/L clavulanic acid (Merck, Poole UK). Inocula were prepared using Brucella broth to 0.5 McFarland density and 1 μl spotted to the agar surface. Control isolates *B. fragilis* ATCC 25285 and *B. thetaiotamicron* ATCC 29741 were also tested. Plates were allowed to dry (approx. 10 mins) then inverted and incubated in an anaerobic workstation (Don Whitley Ltd, UK) at 35–37°C for 42–48 h. The MICs were taken as the concentration where there was a marked decrease in growth when compared to the control plate.

Gradient strip tests (AMC, fixed concentration and XL, ratio) were performed according to manufacturer's instructions on Brucella agar (BBA plates) and inocula prepared to 1 McFarland in Brucella broth. A cotton swab was dipped into the inocula and applied in 3 directions onto the BBA plates. Plates were allowed to dry then gradient strip tests applied. Plates were incubated in anaerobic conditions as stated above for 48 h. MICs were read as stated above.

CLSI recommended QC isolates and CLSI derived target ranges for QC were chosen even though these QC ranges may be inadequate for the purpose of fixed concentrations of clavulanic acid at 2 mg/L, since QC ranges are intended for use of CLSI methodology (ratio).

3. Results

3.1. Quality control isolates (QC)

All five measured amoxicillin/clavulanic acid gradient strip test MICs for *B. thetaiotaomicron* ATCC 29741 were within CLSI target range, except for AMC (fixed concentration) at PHW

(Supplementary table 1). For *B. fragilis* ATCC 25285, amoxicillin/clavulanic acid MIC's were within target range using XL (ratio) and AUG (ratio). In contrast, for *B. fragilis* ATCC 25285, the amoxicillin/clavulanic acid MIC using the gold standard agar dilution (fixed concentration) and AMC (fixed concentration) was 0.125 mg/L and therefore below the CLSI MIC target range (0.25–1 mg/L).

3.2. NEQAS QA isolates with a known amoxicillin/clavulanic acid MIC of ≥256 mg/L

Of two NEQAS QA isolates with a known amoxicillin/clavulanic acid MIC of ≥256 mg/L, one isolate displayed an MIC of 64 mg/L using gold standard agar dilution (fixed concentration). In both isolates, a repeatedly too low MIC of 8–16 mg/L was found using XL (ratio) (Supplementary table 1). Using AUG (ratio) MICs were 128 and 64 mg/L (both R). Using AMC (fixed concentration) one isolate demonstrated the known MIC of >256 mg/L and in the other isolate >256 mg/L, and 64 mg/L at the UMCU and PHW respectively. However, at the UMCU, this last isolate was difficult to read because an ellipse was visible but with growth over the whole agar (not representing contamination); this "second ellipse" was at 48 mg/L (with rounding up this would be an MIC of 64 mg/L), whereas growth over the entire plate would result in an MIC of the >256 mg/L, which was taken as the final result at UMCU.

3.3. NEQAS QA isolates without known amoxicillin/clavulanic acid MICs

Using agar dilution (fixed concentration), isolate NEQAS 2015 N3675-2598 (research nr. 11) had an amoxicillin/clavulanic acid MIC of 0.5 mg/L (Supplementary table 2). With all three gradient strip tests MICs were within the range of 0.5–1 mg/L in this isolate (S, according to EUCAST 2020 breakpoints, with essential agreement (EA) and categorical agreement (S) with the gold standard agar dilution MIC (fixed concentration, Supplementary table 1).

NEQAS 2017 N4010-3511 (research nr. 15) had an amoxicillin MIC of >128 mg/L by agar dilution (fixed concentration), but 32 mg/L using XL (ratio), i.e. in CA with agar dilution (fixed concentration) but at least three doubling dilutions discordant with the agar dilution MIC and also highly discordant with the MIC measurements using AUG (ratio): 256 mg/L or AMC (fixed concentration): >256 mg/L (Supplementary table 1).

3.4. Clinical B. fragilis isolates

In gold standard agar dilution (fixed concentration) 7/27 isolates were resistant to amoxicillin/clavulanic acid (MIC ≥16/2 mg/L) and 1 isolate was in the susceptible, increased exposure category (MIC = 8/2 mg/L, I). In comparison with agar dilution (fixed concentration), in both laboratories using XL (ratio), all isolates had MICs in the susceptible range (MIC ≤ 4/2 mg/L), resulting in 7 VME (VME rate 7/7, 100%) (Table 1, Supplementary table 2 and Fig. 1). AUG (ratio) was tested at the UMCU only and not at PHW. Moreover, in three isolates, AUG (ratio) data were missing. Using AUG (ratio), 4/24 isolates had MICs ≥16/8 mg/L (R) and 6/24 were I (MIC = 8/4 mg/L) with no VMEs (Table 1, Supplementary table 2 and Fig. 1). However, CA in comparison with agar dilution (fixed concentration) remained low at 71%. Using AMC (fixed concentration), 6/27 and 5/27 isolates were amoxicillin/clavulanic acid resistant at the UMCU and PHW, respectively. Two of 27 isolates were "susceptible, increased exposure" (I). In comparison with agar dilution (fixed concentration), no VME and relatively high EA (81% and 85%, respectively) and CA (89%) were found (Table 1, Fig. 1 and Supplementary table 2).

Notably, XL (ratio) demonstrated a small amoxicillin/clavulanic acid MIC range (0.25–4 mg/L, Fig. 1). The MIC range using AUG (ratio) was slightly broader: MIC range 0.5–32 mg/L (Fig. 1). Finally, AMC (fixed concentration) demonstrated the broadest amoxicillin/clavulanic acid MIC range (0.125–256 mg/L, Fig. 1).

3.5. B. fragilis divisions

None of the five *cfiA* positive, division II clinical isolates were categorized as resistant to amoxicillin/clavulanic acid in either agar dilution or gradient strip tests (research nrs. 4, 13, 20, 24, and 33, Supplementary Table 2). In contrast, 2/5 *cfiA* positive, division II isolates were resistant to meropenem and 3/5 were in the "susceptible, increased exposure" (I) category for meropenem (data not shown).

Interlaboratory variability in XL (ratio) and AMC (fixed concentration) MIC measurements.

Both XL (ratio) and AMC (fixed concentration) were used for MIC measurements in both UMCU and PHW. For XL (ratio) the interlaboratory measurements were 100% in EA and CA (Supplementary Tables 2 and 3). Using AMC (fixed concentration), 4/27 isolates displayed essential discrepancies (research nrs. 14, 16, 31 and 34) leading to an EA of 85%, but all these discrepancies were in categorical agreement. Additionally, AMC (fixed concentration) MICs between PHW and UMCU, resulted in minor discrepancies in four different clinical isolates (research nrs. 10, 21, 26 and 30) that were within 1 dilution difference. Since there were no major or very major discrepancies between the two labs the 15% minor discrepancy rate (4/27) led to a CA of 85%.

4. Discussion

Here, we explored accuracy of available gradient strip tests for amoxicillin/clavulanic acid susceptibility testing in *B. fragilis*, prompted by 2 QA rounds of incorrect MIC results using XL (ratio). AMC (fixed concentration) improved MIC results accuracy in these QA isolates.

The main findings are firstly, a high VME rate using XL (ratio) in comparison with gold standard agar dilution (fixed concentration), amongst a small sample of twenty-seven clinical isolates. Secondly, although AMC (fixed concentration) correlated well with the gold standard agar dilution, it displayed a broader MIC range than for XL (ratio). Since all isolates are determined S using XL (ratio) in the UMCU and PHW, the inter-laboratory variability was lower in XL (ratio) in comparison with AMC (fixed concentration). Thirdly, the CLSI target range of *B. fragilis* ATCC 25285 and *B. thetaiotaomicron* ATCC 29741 may not be appropriate for agar dilution or gradient strip testing with a fixed clavulanic acid concentration of 2 mg/L as these target ranges are set for a different methodology. Appropriate QA testing is instrumental in finding errors that may impact treatment decisions in patient care. Lastly, *cfiA* associated meropenem resistance did occur in the presence of amoxicillin/clavulanic acid susceptibility. This is remarkable and unexplained as CfiA does degrade amoxicillin and is not inhibited by clavulanic acid [9,10]. Although infrequently encountered in literature, Trevino et al. previously described this phenotype of carbapenem resistance in combination with low amoxicillin/clavulanic acid MIC's [11]. Since not all promotors in *B. fragilis* are currently known, differences in expression regulation may explain this phenomenon, e.g., constitutive expression versus different levels of induction of expression by different beta-lactam antibiotics [12–14]. In 2017, 15 different *cfiA* alleles were present in public databases, and these alleles might be differentially regulated and/or differ in amoxicillin degrading efficiency [15]. In addition, efflux pump regulation may influence susceptibility to beta-lactam antibiotics [16].

This investigation has many shortcomings. These include deviations from the manufacturer's recommendation on inoculum

Table 1
Agreements and discrepancies (errors) using XL (ratio), AUG (ratio) or AMC (fixed concentration) in comparison with gold standard agar dilution (fixed concentration).

test:	Agar Dilution (fixed concentration 2 mg/L) PHW	XL (ratio) UMCU	XL (ratio) PHW	AUG (ratio) UMCU	AMC (fixed concentration at 2 mg/L) UMCU	AMC (fixed concentration at 2 mg/L) PHW
number	27	27	27	24	27	27
missing	0	0	0	3	0	0
number S	19	27	27	14	19	20
number I	1	0	0	6	2	2
number R	7	0	0	4	6	5
% EA		67%	70%	75%	81%	85%
% CA		70%	70%	71%	89%	89%
VME		7	7	0	0	0
VME rate		100%	100%	0%	0%	0%
ME		0	0	0	0	0
ME rate		0%	0%	0%	0%	0%
MiE		1	1	7	3	3
MiE rate		4%	4%	29%	11%	11%

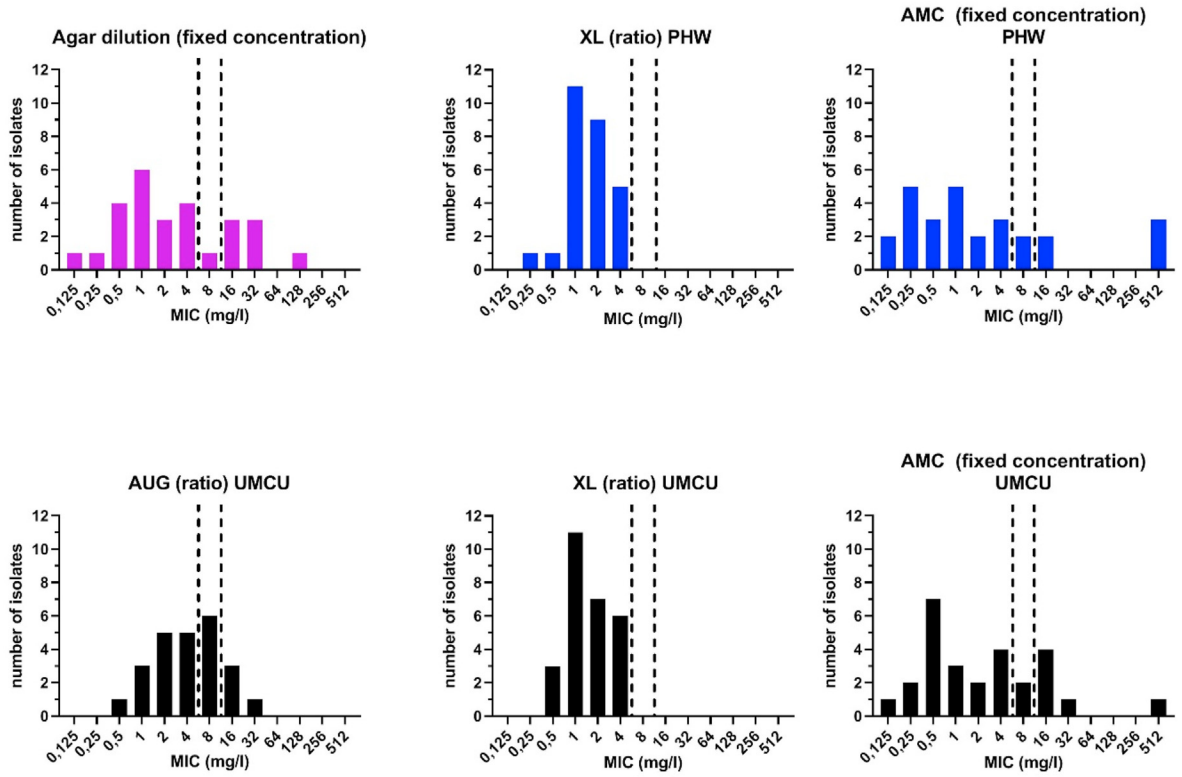

Fig. 1. Amoxicillin/clavulanic acid MIC distributions of 27 *B. fragilis* clinical isolates as measured with gold standard agar dilution (fixed concentration) and different amoxicillin/clavulanic acid gradient strip tests. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

preparation in the UMCU: dilution in Phoenix inoculum broth, whereas it should be Brucella broth, MH broth or Schaedler broth supplemented with vitamin K3. In addition, the inoculum was vortexed, which is against the manufacturer's recommendation. BBA media were not pre-reduced. All these "protocol violations" occurred through all experiments at the UMCU and the same inoculums were used for the different gradient strip tests. Moreover, such violations of manufacturer's recommendations possibly do occur in other clinical microbiology laboratories, in an attempt to decrease complexity by reducing numbers of different methodologies, reagents and protocols.

The number of tested isolates is low and a bias for higher amoxicillin/clavulanic acid MICs was incorporated. Therefore, this set of isolates is very likely not representative for clinical isolates from the Netherlands, but also is not a set of resistant isolates only. Indeed, 5/27 (19%) *B. fragilis* clinical isolates were from division II (*cfiA* positive), an unrepresentatively high proportion (usually ≤10%) [17–19].

Despite these shortcomings, in our opinion, the major finding of this investigation may be important for clinical microbiology laboratories using gradient strip tests for *B. fragilis* susceptibility testing: very major discrepancies in gradient strip testing using ratio may occur in comparison with agar dilution using fixed clavulanic acid concentration at 2 mg/L. Proficiency testing may be

inadequate in some laboratories to detect false susceptibility with their ratio gradient strip tests. However, the frequency of this occurrence is, at present unclear and probably dependent on the frequency of resistance and the resistance mechanisms in the population. These findings might stimulate further research in amoxicillin/clavulanic acid MIC testing in correlation with treatment outcome and meanwhile help select the safest gradient strip tests for *B. fragilis* amoxicillin/clavulanic acid susceptibility testing in clinical microbiology laboratories. Our findings might also be relevant for amoxicillin/clavulanic acid susceptibility testing with gradient strip tests in other Gram-negative anaerobes.

Amoxicillin/clavulanic acid MIC distributions broaden, when a fixed concentration of clavulanic acid is used in the gradient strip tests. In aerobic clinical microbiology, several studies suggest that amoxicillin/clavulanic MICs obtained with fixed concentrations of clavulanic acid more accurately predict clinical outcome of antibiotic treatment with amoxicillin/clavulanic acid [20,21]. One of the mechanisms contributing to this observation may be broadening of MIC distributions, with some susceptible isolates displaying lower MICs, whereas some resistant isolates displaying higher MICs, and, thereby, better discrimination between truly sensitive and resistant isolates. Generally, *B. fragilis* is thought not to be intrinsically susceptible to clavulanic acid, with isolated clavulanic acid MICs usually well above 16 mg/L [22]. Occasionally, *B. fragilis* may be intrinsically susceptible for clavulanic acid at lower MICs [23]. In many but not all other beta-lactam/beta-lactamase inhibitor combination antimicrobial susceptibility tests with MICs, fixed concentrations of the beta-lactamase inhibitor component are used in both EUCAST as well as CLSI methodologies. Nevertheless, awaiting practical, affordable, accurate and reproducible antibiotic susceptibility testing methods for anaerobic bacteria, we recommend clinical microbiology laboratories that use gradient strip tests in anaerobic antimicrobial susceptibility tests to analyse whether their quality assessments for amoxicillin/clavulanic acid gradient strip tests in anaerobes would adequately detect false susceptibility. Laboratories that use gradient strip tests in *B. fragilis* susceptibility testing, in combination with EUCAST breakpoints should be aware that CE marked gradient strip tests may not be as closely aligned with EUCAST advice as possible, with respect to fixed clavulanic acid concentration.

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.

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Appendix A. Supplementary data

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