Universiteit Leiden
The Netherlands

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Original article

Antimicrobial susceptibility testing of *Clostridioides difficile*: a dual-site study of three different media and three therapeutic antimicrobials

Jane Freeman [1, 2, *], Ingrid M.J.G. Sanders [3], Céline Harmanus [2, 3], Emma V. Clark [1], Andrea M. Berry [1], Wiep Klaas Smits [2, 3, **]

[1] *Healthcare Associated Infections Research Group, University of Leeds and Leeds Teaching Hospitals NHS Trust, Leeds, United Kingdom*
[2] *European Society of Clinical Microbiology and Infectious Diseases Study Group on Clostridioides difficile, Basel, Switzerland*
[3] *Experimental Bacteriology Research Group, Leiden University Center for Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands*

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ABSTRACT

Objectives: Increasing resistance to antimicrobials used for the treatment of *Clostridioides difficile* infections necessitates reproducible antimicrobial susceptibility testing. Current guidelines take a one-size-fits-all approach and/or offer limited guidance. We investigated how the choice of medium affects measured MIC values across two sites.

Methods: We determined MIC values for the antimicrobials fidaxomicin, metronidazole, and vancomycin for a representative collection of European *C. difficile* strains (n = 235) using agar dilution on three different media: Brucella Blood Agar (BBA), Fastidious Anaerobe Agar supplemented with horse blood (FAA-HB), and Wilkins-Chalgren (WC) agar. The study was conducted at two sites to compare reproducibility. Usability (ease of preparation of the media as well as read-out of the assay) was assessed through a survey.

Results: We found that all media result in highly consistent aggregated MIC data for all antibiotics, with MIC_{50} and MIC_{90} within two-fold of each other across sites. For fidaxomin, MIC values on WC were lower than on the other media (MIC_{90}: WC = 0.125–0.25 mg/L; BBA and FAA-HB = 0.5 mg/L). Metronidazole showed the lowest MIC on BBA and the highest on WC (MIC_{90}: WC = 2 mg/L; BBA = 0.5–1 mg/L; FAA-HB: 1–2 mg/L). For vancomycin, MIC values were similar across media (MIC_{90}: all media = 1–2 mg/L). Though absolute values for individual isolates differed between sites, identified resistant isolates were similar. Results obtained on FAA-HB were most consistent between sites and results obtained on WC showed the most divergence. FAA-HB was positively evaluated in the usability survey.

Discussion: This study shows medium-dependent differences in *C. difficile* MICs for at least two antimicrobials across two sites. We suggest the use of FAA-HB to align with general European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations for susceptibility testing of anaerobic bacteria and deposited reference strains for standard susceptibility testing of *C. difficile* to increase interlaboratory reproducibility. **Jane Freeman, Clin Microbiol Infect 2025;31:1011**

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Introduction

Clostridioides difficile is a clinically important bacterium that can cause potentially fatal gastro-intestinal disease [1]. The disease is associated with a high burden on healthcare systems, society, and the economy [2–5]. At present, three antimicrobial treatments are indicated by international guidelines: fidaxomicin (FDX) and vancomycin (VAN) are first-line treatments [6,7] except in the United Kingdom, where only VAN is recommended as first-line [8]. Metronidazole (MTZ) is no longer recommended because of clinical inefficacy unless first-line therapeutics are not available or contra-indicated [6,7]. However, despite clinical inferiority, MTZ is still widely used [8].

Reduced susceptibility or resistance of *C. difficile* has been reported for all three antimicrobials [9]. However, reported rates of resistance, particularly for MTZ, vary widely and the reasons for this

* Corresponding author: Jane Freeman, Healthcare Associated Infections Research Group, University of Leeds and Leeds Teaching Hospitals NHS Trust, Leeds, United Kingdom.
** Corresponding author: Wiep Klaas Smits, Experimental Bacteriology Research Group, Leiden University Center for Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands.
E-mail addresses: jane.freeman4@nhs.net (J. Freeman), w.k.smits@lumc.nl (W.K. Smits).

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are poorly understood. Though geography and lineage of *C. difficile* might contribute to different rates of resistance, it may also be attributable to differences in testing methodology [10]. Current Clinical & Laboratory Standards Institute (CLSI) (as well as past European Committee on Antimicrobial Susceptibility Testing (EUCAST)) guidelines advocate the use of Brucella Blood Agar (BBA) for the determination of antibiotic MICs for all anaerobes, including *C. difficile*, but differ in the breakpoints defined [11,12]. Current EUCAST guidelines (v15.0) indicate the use of Fastidious Anaerobe Agar supplemented with 5% horse blood [13].

Recent studies on MTZ resistance in *C. difficile* underscore the issues outlined above. Whereas most studies report limited MTZ resistance [10], rates up to 44.6% have been reported in Israel [11]. MTZ resistance can be plasmid-mediated [12], but a subset of isolates demonstrates plasmid-independent resistance [14]. Importantly, the latter group demonstrates strong medium dependence [14,15]. This is suggested to be because of a mutation in the promoter of gene *nimB*, resulting in constitutive transcription [16]. Finally, it has been suggested that reduced susceptibility (MIC $\geq$1 mg/L when tested on non-consensus medium) is associated with treatment failure for both MTZ and VAN [17,18]. VAN and FDX achieve gut concentrations several orders of magnitude above the MIC of *C. difficile* isolates classified as resistant to these drugs, and therefore the clinical significance of resistance against these drugs is not yet clear. Data are lacking on whether medium composition affects VAN and FDX susceptibility.

Together these data clearly show the importance or routine screening of *C. difficile* using standardized conditions that might benefit from a re-evaluation of resistance breakpoints.

Methods

As minor differences were inevitable between sites, the catalogue numbers of individual chemicals are provided in Table S1. A standard operating procedure is available as Supplementary Text.

Strain selection

We selected $n = 250$ isolates that were collected during the COMBACTE-CDI (2018) and ClosER studies (2011–2016) [19,20]. The isolates were selected to comprise at least 10 isolates of the top 10 most prevalent European PCR ribotypes, as well as recently emerging ribotypes or ribotypes associated with multi-drug resistance (resistance to >3 antimicrobial classes) (Table S2). We included isolates that were previously identified as showing reduced susceptibility towards MTZ ($n = 39$), VAN ($n = 31$) or FDX ($n = 1$) [19,20]. FDX-resistant strains are very rare to date, and the isolate included in the present study has been extensively characterized [21].

To reduce differences arising from repeated sub-culturing, *C. difficile* isolates were subcultured on cycloserine-cefoxitin egg yolk agar (E&O laboratories, Bonnybridge, Scotland, United Kingdom) for 48 hours in Leeds. Duplicate sets of glycerol stocks were prepared by resuspending the growth in glycerol broths, dividing into two aliquots and freezing at −80°C; one aliquot was sent on dry ice to Leiden. Control strains (*C. difficile* ATCC 700057, *C. difficile* E4 [22], *Bacteroides fragilis* ATCC 25285, and *Enterococcus faecalis* ATCC 29212) were similarly shared between both sites.

Antimicrobial susceptibility testing

C. difficile isolates were removed from −80°C storage and subcultured anaerobically to ensure purity, before inoculation of pre-reduced Schaedler's anaerobic broth for 24 hours [12]. Isolates were transferred to pre-reduced sterile saline and adjusted to McFarland standard 1.0. Non-antibiotic-containing plates were incubated aerobically and anaerobically.

Antibiotic-containing agar plates were prepared by mixing 2 mL dilution of the antimicrobial with 18 mL molten agar and distributing into Petri dishes. Blood, haemin, and vitamin K were added after autoclaving and before distribution into Petri dishes (Supplementary Text). FDX was dissolved in 100% dimethyl sulfoxide (DMSO) as a solvent, and further diluted into 10% DMSO as a diluent. MTZ was dissolved in 100% DMSO as a solvent, and further diluted into water as a diluent. VAN was dissolved in water as a solvent, and further diluted into water as a diluent. The final concentrations of the antimicrobials in the agar dilution experiments were 0.015–16 mg/L for FDX, 0.125–32 mg/L for MTZ, and 0.125–32 mg/L for VAN.

Saline suspensions of *C. difficile* isolates were inoculated onto agar plates using a multipoint inoculator and incubated anaerobically for 48 hours. The minimum inhibitor concentration is defined as the lowest dilution at which growth is completely inhibited or where only a single colony remains [23].

Analysis and visualization

Plates were read by two technicians and results were logged only when control strains demonstrated MIC values within a predefined range (Table 1). For FDX, no breakpoints were defined at the time of testing (2019). We therefore defined breakpoints for this study as $\leq$1 mg/L: susceptible; >1 mg/L: resistant, in line with those used in the ClosER study [19]. We note that this is two-fold higher than the current (v15.0) EUCAST epidemiological cut-off (ECOFF) of 0.5 mg/L. For MTZ and VAN, EUCAST breakpoints (ECOFF) at the time of testing were used ($\leq$2 mg/L: susceptible, >2 mg/L: resistant. MIC_{50} and MIC_{90} were determined on the basis of ranked MIC values.

Data were collected in Microsoft Excel and converted into a tidy format (Table S2). Graphs were generated in SuperPlotsOfData [24] and Eulerr [25], and further compiled in Adobe Illustrator 2022 (26.3.1).

Results

Agar dilution provides highly similar overall susceptibility data between sites

Of the $n = 250$ strains that were initially selected, some could not be revived from the original stocks. As a result, $n = 235$ isolates were shared between laboratories in Leeds and Leiden. During the experiments in the Leeds laboratory, three additional isolates we judged not to contain *C. difficile* on subculture were excluded from the subsequent analysis.

MIC_{50} and MIC_{90} values were generally within two-fold of each other between sites; only for the VAN MIC_{50}, a four-fold difference was observed on BBA medium. When differences were observed, MIC values were higher at the Leiden University Medical Center (LUMC) site in comparison to the Leeds site. This suggests that

Table 1
Expected MIC ranges for control strains.

Strain	Metronidazole (mg/L)	Vancomycin (mg/L)	Fidaxomicin (mg/L)
B. fragilis ATCC 25285	0.025–1	N/A	N/A
C. difficile E4	4–16	0.5–4	0.03–0.125
C difficile ATCC 700057	0.125–0.5	0.5–4	0.03–0.125
E. faecalis ATCC 29212	>32	2–8	2–8

N/A, not assessed.

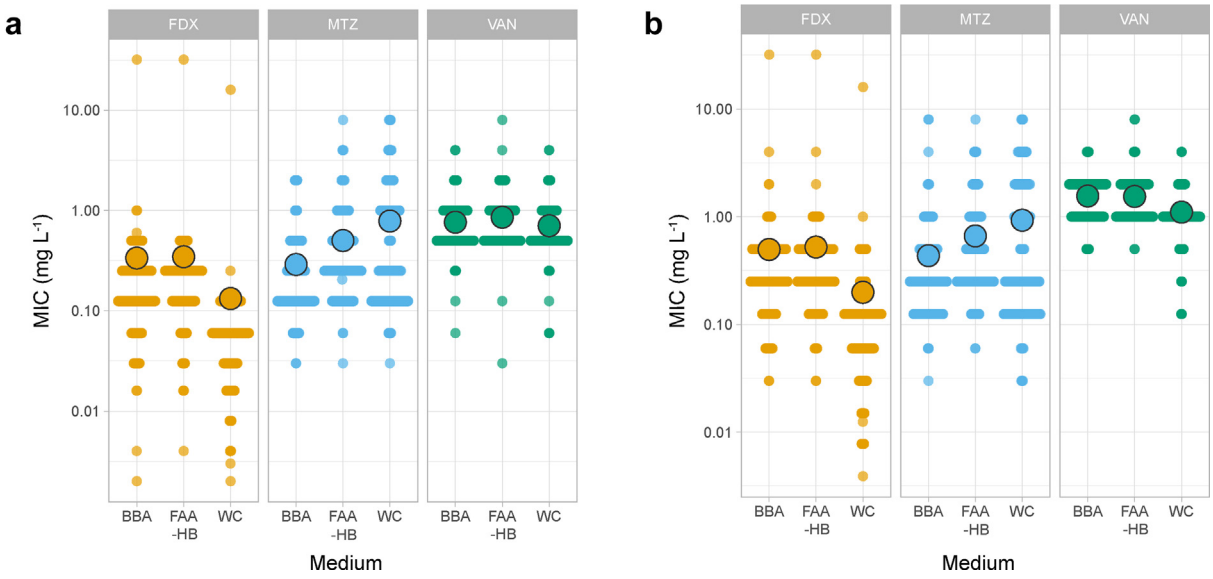

Fig. 1. Comparison of MIC values for Leeds (a) and Leiden (b). Individual datapoints are shown, with the mean indicated with the largest circle. Data are shown for fidaxomicin (FDX, ochre), metronidazole (MTZ, blue), and vancomycin (VAN, green) on three different media: Brucella Blood Agar (BBA), Fastidious Anaerobe Agar supplemented with horse blood (FAA-HB), and Wilkins-Chalgren (WC). For details, see Methods section.

subtle differences in experimental procedures may lead to a systematic difference in MIC values measured.

MIC ranges show a similar trend; when differences are observed, these are mostly at the lower end of the MIC range and maximum MIC values are within two-fold of each other. Any differences are therefore unlikely to affect the qualification of strains as resistant.

FDX and MTZ show medium-dependent differences in antimicrobial susceptibility

Next, we compared the distribution in MIC values observed for each combination of medium and antibiotic. Overall, we observed a strikingly similar pattern for values determined in Leeds (Fig. 1(a)) and Leiden (Fig. 1(b)).

As also noted above, mean values determined at Leiden appear to be slightly higher than those determined in Leeds. However, within sites, the different antimicrobial-medium combinations show similar trends. For FDX, we find that mean MICs are similar for FDX and MTZ, but are markedly lower for Wilkins-Chalgren (WC) medium. This suggests that the use of WC in agar dilution experiments may lead to a systematic underestimation of potentially reduced susceptible isolates, compared with other media. An opposite pattern is observed for MTZ: mean MIC values progressively increase from BBA, through FAA-HB to WC medium. Thus, for both FDX and MTZ, there is a strong medium-dependent effect on susceptibility. For VAN, results indicate a lesser medium dependence with mean values being similar for all media in the Leeds dataset, and only WC resulting in slightly lower mean MIC values in the Leiden dataset.

Interlaboratory differences differ per medium used

Though aggregated data show highly similar trends (Table 2 and Fig. 1), this analysis could potentially mask differences between the MIC values for individual isolates obtained in the two laboratories. To assess this, we calculated the ratio of the MIC values for each isolate; if data were 100% congruent, this should result in a ratio of 1 (the same MIC value). We find that the mean value indeed approaches this (Fig. 2(a)), in particular for FDX on all media.

Table 2
Aggregated MIC values.

Site	Antimicrobial	Medium	MIC_{50} (mg/L)	MIC_{90} (mg/L)	MIC range (mg/L)
Leeds	FDX	BBA	0.125	0.5	0.002–32
		FAA-HB	0.25	0.5	0.004–32
		WC	0.06	0.125	0.002–16
	MTZ	BBA	0.25	0.5	0.03–2
		FAA-HB	0.25	1	0.03–8
		WC	0.25	2	0.03–8
	VAN	BBA	0.5	1	0.06–4
		FAA-HB	0.5	1	0.03–8
		WC	0.5	1	0.06–4
Leiden	FDX	BBA	0.25	0.5	0.03–32
		FAA-HB	0.25	0.5	0.03–32
		WC	0.125	0.25	0.004–16
	MTZ	BBA	0.25	1	0.03–8
		FAA-HB	0.25	2	0.06–8
		WC	0.25	2	0.03–8
	VAN	BBA	2	2	0.5–4
		FAA-HB	1	2	0.5–8
		WC	1	2	0.125–4

MIC values for individual isolates were aggregated to the concentration that inhibits the growth of >50% of all isolates (MIC50) or >90% of all isolates (MIC90).
Data are shown for fidaxomicin (FDX), metronidazole (MTZ), and vancomycin (VAN) on three different media: Brucella Blood Agar (BBA), Fastidious Anaerobe Agar supplemented with horse blood (FAA-HB), and Wilkins-Chalgren (WC). For details, see Methods section.

A high reproducibility should result in a tight clustering of datapoints. We note that data obtained on the FAA-HB medium shows a narrower distribution of ratios, compared with BBA and WC (Fig. 1(a)).

Next, we assessed how medium-antimicrobial combinations affect the identification of resistant isolates, which we deem to be the clinically most relevant outcome parameter. We identified all isolates with a MIC >1 mg/L (FDX) or MIC >2 mg/L (MTZ or VAN) (Table S2) on one or more medium-antimicrobial combinations. As expected, the number of isolates identified in this way was higher

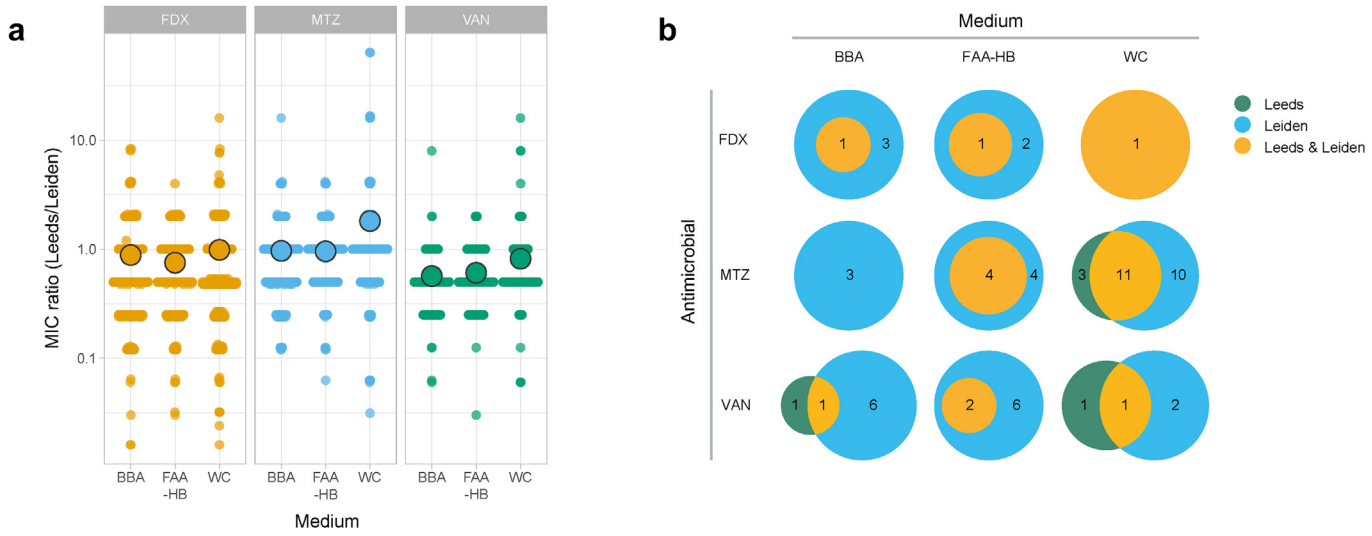

Fig. 2. Inter-site reproducibility of antimicrobial susceptibility testing. (a) Ratios of MIC values obtained at the two sites were calculated for each isolate. Individual datapoints are shown, with the mean indicated with the largest circle. Data are shown for fidaxomicin (FDX, ochre), metronidazole (MTZ, blue), and vancomycin (VAN, green) on three different media: Brucella Blood Agar (BBA), Fastidious Anaerobe Agar supplemented with horse blood (FAA-HB), and Wilkins-Chalgren (WC). (b) The number of isolates with MIC≥1 mg/L (FDX) or MIC≥4 mg/L (MTZ and VAN) under one or more conditions was compared. A value of 1 indicates that 1 isolate met this criterion on one medium. For details, see Methods section.

for the Leiden site than the Leeds site, consistent with the higher mean MIC values. However, in general, those isolates identified in Leeds were also identified in Leiden (Fig. 2(b)). Notably, however, identifications of VAN-resistant isolates and/or identifications on WC medium showed a significant laboratory-specific effect. The most consistent results were observed for the FAA-HB medium, where all isolates identified as resistant in Leeds were also identified in Leiden.

Distinct media offer easier handling or read-out

We considered several aspects that might contribute to experimental variability in our data. In particular steps necessary to prepare media, as well as the ability to clearly read the MIC data stemming from the agar dilution data appeared to be important sources of variation to us. We therefore queried the teams that performed the experiments about these aspects. Four members of staff were surveyed, with three giving feedback using Likert scales on ease of preparation, readability, and detection of contamination (Table S3). The currently CLSI-recommended BBA consistently scored the least well on all survey questions and was the least preferred option among staff performing the procedure.

Discussion

Here, we determined how the choice of medium affects antimicrobial susceptibility testing for *C. difficile* for therapeutic antimicrobials FDX, MTZ, and VAN. We find that agar dilution offers a reliable assessment of susceptibility with minor systematic differences between sites using a harmonized protocol. There were mechanical differences in the agar preparation method between the two sites (Leeds used an automated agar preparation, whereas Leiden used a hand-poured agar technique) which may account for such minor differences. Nonetheless, we found clear evidence for medium-dependent susceptibility for FDX and MTZ and show that determining susceptibility on FAA-HB medium shows the highest interlaboratory reproducibility. FAA-HB also scored highly for ease of use, ease of reading MICs, and detection of contamination among staff performing the procedure, in contrast to BBA, which was consistently the lowest scoring medium.

Medium-dependent resistance in *C. difficile* has so far only been described for MTZ [14,15], and medium-dependent effects on FDX susceptibility have to the best of our knowledge not been documented before. Though the use of WC could clearly result in lower MIC values for FDX (and possibly VAN), it should be noted that this does not affect the identification of highly resistant strains [21]. FDX-reduced susceptible strains have rarely been identified but the inclusion of the only FDX-resistant *C. difficile* isolate so far identified in large-scale surveillance studies [21,25] gives confidence that FDX resistance would be detected on the recommended FAA-HB medium. For MTZ, haem in the medium appears to be a key determinant for resistance, and it should be noted that when using any fresh blood-based medium for determining MTZ MICs in *C. difficile*, blood should be fresh and shielded from light, to avoid degradation of haemin (which can be noted within 24 hours) [14,15]. The mechanisms behind MTZ resistance are only recently being elucidated: plasmid mediated and *nim* gene expression. Importantly, the use of FAA-HB as recommended on the basis of this study and in current EUCAST guidelines can detect both types of MTZ resistance. The reason for increased susceptibility for FDX (and potentially VAN) on WC medium is at present unknown but may be influenced by the more defined and minimal nature of WC, compared with the blood-supplemented media (FAA-HB and BBA). This may marginally increase growth and accentuate differences at the lower end of concentration ranges seen for FDX.

The strengths of the present study include the well-controlled setup and the inclusion of a large number of representative *C. difficile* isolates. Our data are limited by the fact that we did not perform an in-depth analysis of potentially discrepant results or investigate the mechanism of resistance in our set of strains, and did not perform repeat testing of the same set of isolates.

Our study suggests that the use of BBA medium for agar dilution as recommended in some current susceptibility testing guidelines (e.g. CLSI) might lead to an underestimation of in particular MTZ resistance and could contribute to interlaboratory variation in reported MIC values. Our data support the use of FAA-HB medium for susceptibility testing in *C. difficile*, because it offers a good balance

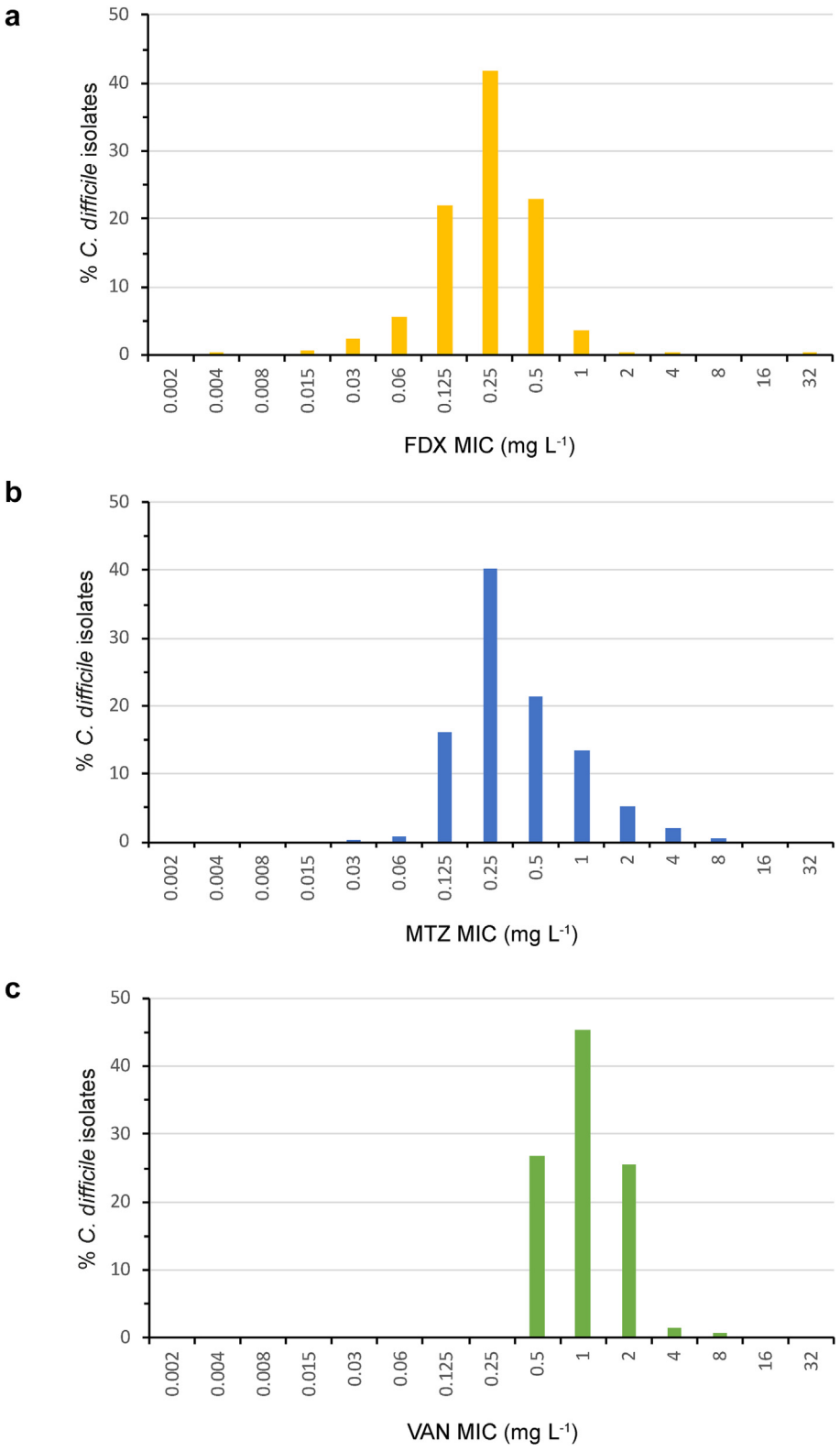

Fig. 3. MIC distributions. Aggregated data for MICs determined by FAA-HB agar dilution method across both sites for (a) fidaxomicin (FDX, orange), (b) metronidazole (MTZ, blue), and (c) vancomycin (VAN, green). FAA-HB, Fastidious Anaerobe Agar supplemented with horse blood. MIC, minimal inhibitory concentration.

of usability, reproducibility, and sensitivity. Notably, this is in line with studies of disk susceptibility in other anaerobes, including *Bacteroides*/*Phocaeicola*/*Parabacteroides*, *Prevotella*, *Fusobacterium*, *Clostridium*, and *Cutibacterium* and now part of EUCAST recommendations [26,27]. MIC distributions on FAA-HB for all three antimicrobials are shown in Fig. 3.

The increasing number of reports of *C. difficile* with reduced susceptibility or resistance to the antibiotics tested here

Table 3
Recommended *Clostridioides difficile* strains for antimicrobial susceptibility testing of *C. difficile* on FAA-HB medium with MIC values as determined in this study

Isolate	NCTC accession	Fidaxomicin MIC (mg/L)	Metronidazole MIC (mg/L)	Vancomycin MIC (mg/L)	Description	Reference
15-7365,627	NCTC 15114	0.25	4	0.5-2	RT016. Medium-dependent resistance[a].	This study
E4	NCTC 15085	0.125	4-16	0.5-1	RT010	[30]
L_16.7570132	NCTC 15086	32	0.25	1.0	RT344	[21], this study
L_13.7933412	NCTC 15087	0.125–0.25	0.125–0.25	8	RT356	This study

FAA-HB, Fastidious Anaerobe Agar supplemented with horse blood; NCTC, National Collection of Type Cultures.
[a] For medium-independent metronidazole resistance, the pCD-METRO containing strain IB136 [13] (NCTC 14835) can be used, which has a MIC of 8–16 mg/L.

[12,21,16,28,29] warrants a systematic surveillance of drug resistance for this organism and a re-evaluation of breakpoints. At present, the prevalence of resistant isolates varies strongly between reports and is a subject of dispute [29,30]. A consensus method for susceptibility testing can contribute to interlaboratory reproducibility. This is particularly important in light of documented MTZ resistance in PCR RT027 [19,16] and the closely related RT955 [31]. The lack of an optimized medium could lead to this being underestimated. Both laboratories involved in the present study have confirmed MTZ resistance in at least a subset of RT955 strains using the newly recommended FAA-HB medium (data not shown). These data support the adoption of FAA-HB as the optimal medium for determining *C. difficile* susceptibility to relevant agents for treatment as well as the current EUCAST breakpoints (ECOFF) for MTZ and VAN ($\leq$2 mg/L: susceptible, >2 mg/L: resistant; v15.0) [13], while noting that the panel was selected to include isolates previously showing reduced susceptibility. The panel included only one *C. difficile* isolate known to harbour FDX resistance (16 mg/L), with the remaining isolates demonstrating susceptibility 0.004 and 4 mg/L. More than 99% of *C. difficile* isolates were susceptible at $\leq$1 mg/L FDX (breakpoint used in this study), and >95% at $\leq$0.5 mg/L (current EUCAST ECOFF), on FAA-HB (aggregated data from both sites). The difference between these breakpoints did not affect the qualification of the FDX-resistant isolate as such. We note that MICs on FAA-HB are higher than on WC medium; thus, FDX ECOFFs may need to be further revised to avoid erroneously assigned resistance, as further data are gathered on this medium recommended here.

To further assist laboratories undertaking susceptibility testing of *C. difficile* isolates, we have deposited isolates that were identified as resistant against FDX, MTZ, and VAN in both laboratories at the National Collection of Type Cultures (NCTC) for use as reference strains by other laboratories (Table 3) [13,21,30], in addition to the guideline-recommended *C. difficile* ATCC 70057. Use of these strains in future work will facilitate interlaboratory comparisons of absolute MIC values.

The results from the present study have been communicated to EUCAST and will be recommended for the determination of *C. difficile* MICs by agar dilutions in the next revision of their guidance.

Beyond the relevance for the epidemiological monitoring of the emergence of strains resistant to treatment antimicrobials, the approach proposed here may help to effectively guide healthcare professionals in treating *Clostridioides difficile* infection (CDI) patients.

Author contributions

J.F. contributed to writing, supervision, writing-original draft. I.M.J.G.S., C.H., E.V.C., and A.M.B. contributed to investigation. W.K.S. contributed to formal analysis, data curation, writing-original draft, visualization, and supervision. All authors reviewed and edited the manuscript, and approved the final version.

Transparency declaration

Potential conflict of interest

J.F. has received research funding from Pfizer and Crestone, and speaking honoraria and sponsorship to attend meetings from Tillotts. W.K.S has performed research for Cubist Pharmaceuticals and holds a public–private partnership grant with Acurx Pharmaceuticals. All other authors declare no conflict of interest.

Financial report

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Data availability

All data are contained in the manuscript and the provided Supplemental Materials.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2025.01.028.

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