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Antimicrobial susceptibility testing of *Clostridium difficile* using EUCAST epidemiological cut-off values and disk diffusion correlates

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Abstract

With the emergence of reduced susceptibility of *Clostridium difficile* to metronidazole and vancomycin the value of antimicrobial susceptibility testing has increased. The aim of our study was to evaluate disk diffusion for susceptibility testing of *C. difficile* by comparing disk diffusion results with MICs from gradient tests and to propose zone diameter breakpoint correlates for the EUCAST epidemiological cut-off values (ECOFFs) recently published. We tested 211 clinical isolates of *C. difficile*, from patients with diarrhoea hospitalized at Aarhus and Odense University Hospitals, Denmark. Furthermore, ten clinical isolates of *C. difficile* from the Anaerobe Reference Laboratory, University Hospital of Wales, with known reduced susceptibility to either metronidazole or vancomycin, were included. Isolates were tested with Etest gradient strips and disk diffusion towards metronidazole, vancomycin and moxifloxacin on Brucella Blood Agar supplemented with hemin and vitamin K. We found an excellent agreement between inhibition zone diameter and MICs. For each MIC value, the inhibition zones varied from 0 to 8 mm, with 93% of values within 6 mm for metronidazole, 95% of values within 4 mm for vancomycin, and 98% of values within 4 mm for moxifloxacin. With proposed zone diameter breakpoints for metronidazole, vancomycin and moxifloxacin of WT ≥ 23 mm, WT ≥ 19 and WT ≥ 20 mm, respectively, we found no very major errors and only major errors below 2%. In conclusion, we suggest that disk diffusion is an option for antimicrobial susceptibility testing of *C. difficile*.

Keywords: Breakpoint, *Clostridium difficile*, disk diffusion, gradient test, reduced susceptibility, susceptibility testing
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Introduction

The incidence of *Clostridium difficile* infection (CDI) has been increasing [1,2]. The standard antimicrobial therapy for CDI is oral metronidazole or vancomycin [3,4]. However, emergence of reduced susceptibility, especially towards metronidazole [5–8] but also vancomycin [7,9], has been reported.

This emphasizes the need for antimicrobial susceptibility testing of *C. difficile* and for a simple susceptibility testing method for the routine clinical microbiology laboratory. The Clinical and Laboratory Standards Institute (CLSI) currently recommends the use of the agar dilution method (which is the reference method in CLSI) or one of the gradient methods [10]. The agar dilution method is highly reproducible and it is suitable for surveillance and evaluation of new antimicrobials, but agar dilution is technically demanding and too labour intensive for the routine laboratory.

Gradient tests are convenient in routine laboratories and are suitable for single tests. Several studies have validated the method for susceptibility testing of anaerobes [11,12]. However, the gradient tests are expensive. Disk diffusion is inexpensive and simple to perform and a few studies have

evaluated disk diffusion for antimicrobial susceptibility testing of *C. difficile* [6,13,14].

If routine susceptibility testing of *C. difficile* was to be performed, implementation of a simple and inexpensive method such as the disk diffusion method would be attractive.

Accordingly, the aim of our study was to evaluate disk diffusion for susceptibility testing of *C. difficile* by comparing disk diffusion results with MICs from gradient tests and to propose zone diameter breakpoint correlates for the EUCAST ECOFFs recently published.

Materials and Methods

C. difficile strains

Consecutive clinical isolates of *C. difficile* (n = 211) were collected from patients with diarrhoea hospitalized at Aarhus University Hospital (n = 110) in 2008 and Odense University Hospital (n = 101) in 2010. Furthermore, 10 clinical isolates of *C. difficile* from the Anaerobe Reference Laboratory, University Hospital of Wales, with known reduced susceptibility to metronidazole (n = 4, MIC 1.5, 2, 2, 3 mg/L, ECOFF ≤ 2 mg/L) or vancomycin (n = 6, MIC 1.5, 2, 2, 2, 3, 3 mg/L, ECOFF ≤2 mg/L), were included.

Isolates were cultured on CCFA (cycloserine-cefoxitin-fructose agar) (Statens Serum Institute (SSI) Diagnostica, Hillerød, Denmark) and incubated in an anaerobic chamber (Aarhus University Hospital, Concept 400, Ruskinn Technology, Bridgend, UK; Odense University Hospital, MiniMACS Anaerobic Workstation, Don Whitley Scientific, West Yorkshire, UK) in an anaerobic atmosphere (10% H2, 10% CO2, 80% N2) at 37°C for 48 h.

Characteristic colonies (morphology, colour and odour) were identified further using a Prolin test (Amino-peptidase Reagent, CH3COOH 2.5%, CH3CH2OH 60%) (Rosco Diagnostica, Taastrup, Denmark).

After identification the strains were swabbed on 5% blood agar plates (SSI Diagnostica, Hillerød, Denmark) and incubated in anaerobic atmosphere for 24 h before freezing. The strains were stored in preservation broth (meat bouillon with 10 % glycerol) at −80°C.

Stored isolates were thawed and cultured on 5% blood agar plates and incubated in an anaerobic atmosphere for 24 h before susceptibility testing was performed. The reference *C. difficile* strain ATCC 700057 was included for quality control.

At Aarhus University Hospital real-time PCR was used for the detection of *C. difficile* toxin genes [15]. At Odense University Hospital toxin production was verified with Immuno-Card (Meridian, Cincinnati, USA).

Selected isolates were further characterized with PCR ribotyping. PCR ribotyping was performed with minor modifications according to O'Neill *et al.* [16] and Stubbs *et al.* [17]. The resulting band patterns were compared and named according to the PCR ribotype of the reference strains.

Antimicrobial susceptibility testing

The antimicrobial agents tested were vancomycin, metronidazole and moxifloxacin. Vancomycin and metronidazole were chosen because of emergence of reduced susceptibility. Moxifloxacin was chosen because it can be used for screening of *C. difficile* PCR ribotype 027.

Cultured isolates were suspended in thioglycollate bouillon (SSI Diagnostica, Hillerød, Denmark) to a density of 1.0 McFarland. A sterile cotton swab was placed in the suspension. The inoculum was spread evenly over the entire surface of the plate. All susceptibility tests were performed on Brucella Blood Agar (9 cm in diameter) supplemented with haemin and vitamin K (Becton Dickinson, Heidelberg, Germany). CLSI [10] recommend this singular medium for susceptibility testing of anaerobes.

To optimize growth of *C. difficile*, plates were reduced for 18–24 h in an anaerobic atmosphere before use. For the preparation of inoculum, inoculation and incubation we followed the 15-15-15-minute rule as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (http://www.eucast.org).

MIC determination was performed by gradient test. Etest strips (bioMérieux, Craponne, France) with metronidazole, vancomycin and moxifloxacin were placed on Brucella Blood Agar supplemented with haemin and vitamin K. Disk diffusion was performed with Oxoid disks (Oxoid, Basingstoke, UK) with vancomycin (5 μg), metronidazole (5 μg) and moxifloxacin (5 μg) on Brucella Blood Agar supplemented with haemin and vitamin K. Plates were incubated in anaerobic atmosphere (as described above) for 24 h. The zone diameters were read at 100% inhibition.

Statistical analysis

Results were analysed using STATA/IC 11.2 (Statacorp, Texas, USA). Bivariable regression analysis was applied to the paired log-transformed MIC vs. the untransformed zone diameter. The error-rate bounded method developed by Metzler and DeHaan [18] was used to describe discrepancy between Etest and disk diffusion. Very major error (VME) was recorded when isolates were susceptible by disk diffusion and resistant by Etest (falsely susceptible) and major error (ME) was recorded when isolates were susceptible by Etest but resistant by disk diffusion (falsely resistant). According to CLSI document M23-A3 [19], VME should be

<1.5% and ME less than 3% for a large collection of unselected clinical isolates.

Results

A total of 221 clinical isolates of *C. difficile* were tested. The distribution of inhibition zone diameters and MICs for *C. difficile* isolates towards metronidazole, vancomycin and moxifloxacin are illustrated in the histograms (Fig. 1) as used by EUCAST for demonstrating the relationship between MIC and zone diameter.

The metronidazole distribution showed that for each MIC value, the inhibition zones varied from 0 to 8 mm, with 93% of the values within 6 mm. Only one of the four isolates from Wales with known reduced susceptibility to metronidazole had an MIC above ECOFF but all four isolates had zone diameters below 14 mm, compared with the consecutive clinical isolates from Denmark, which all had zone diameters above 23 mm. The four isolates with reduced susceptibility towards metronidazole were typed with PCR ribotyping. They belonged to PCR ribotypes 001 (n = 1), 106 (n = 1) and 027 (n = 2).

The vancomycin distribution showed that for each MIC value, the inhibition zones varied from 0 to 6 mm, with 95% of values within 4 mm. The six isolates with known reduced susceptibility to vancomycin had zone diameters between 15 and 19 mm, compared with the consecutive clinical isolates from Denmark, which all had zone diameters above 18 mm. Two of these six isolates had an MIC above the ECOFF as defined by EUCAST and these two isolates had zone diameters of 15 and 18 mm, respectively. The isolates with reduced susceptibility towards vancomycin were also typed with PCR ribotyping. They belonged to the same three PCR ribotypes as found for the isolates with reduced susceptibility to metronidazole: PCR ribotypes 001 (n = 2), 106 (n = 1) and 027 (n = 3).

The moxifloxacin distribution showed that for each MIC value, the inhibition zones varied from 0 to 5 mm, with 98% of the values within 4 mm. We observed a group of eight isolates between the WT and the resistant population. These intermediate isolates all came from patients hospitalized at the same department at Aarhus University Hospital. With PCR ribotyping we found that they belonged to two PCR ribotypes: PCR ribotype 066 (n = 6) or PCR ribotype 014/020/077 (n = 2) (the collective name for PCR ribotype 014/

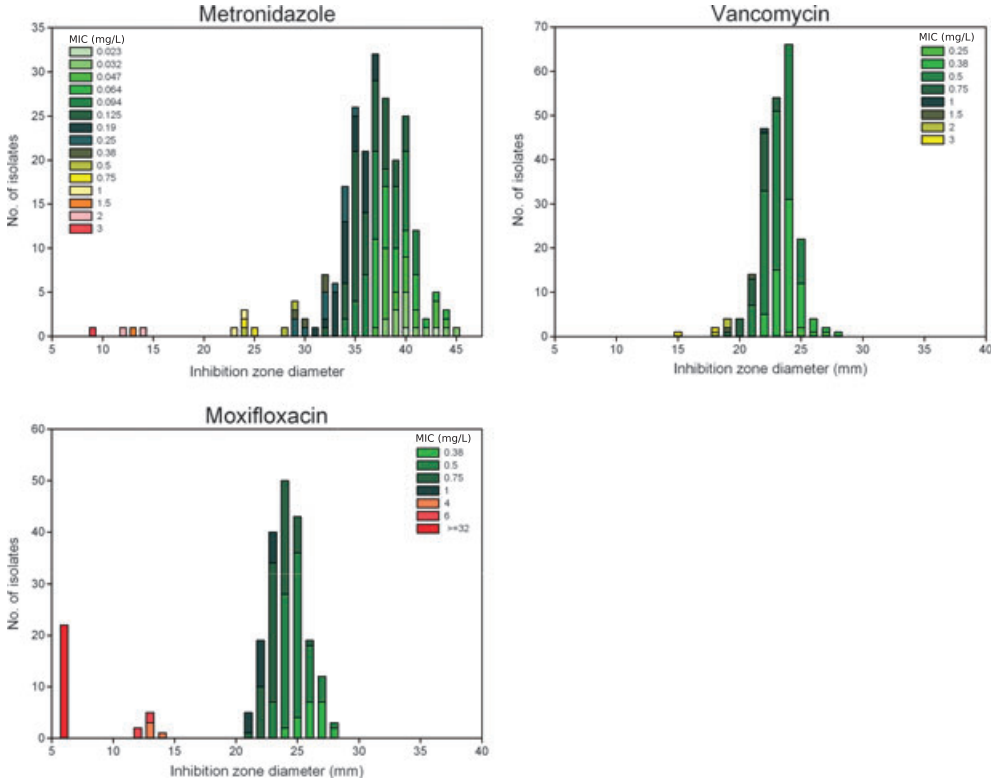

FIG. 1. Distribution of inhibition zone diameters (disk diffusion method) and MICs (gradient test) for 221 isolates of *C. difficile* tested against metronidazole (5 μg disk), vancomycin (5 μg disk) and moxifloxacin (5 μg disk). Each isolate is represented in the zone diameter histogram in a colour representing its MIC value.

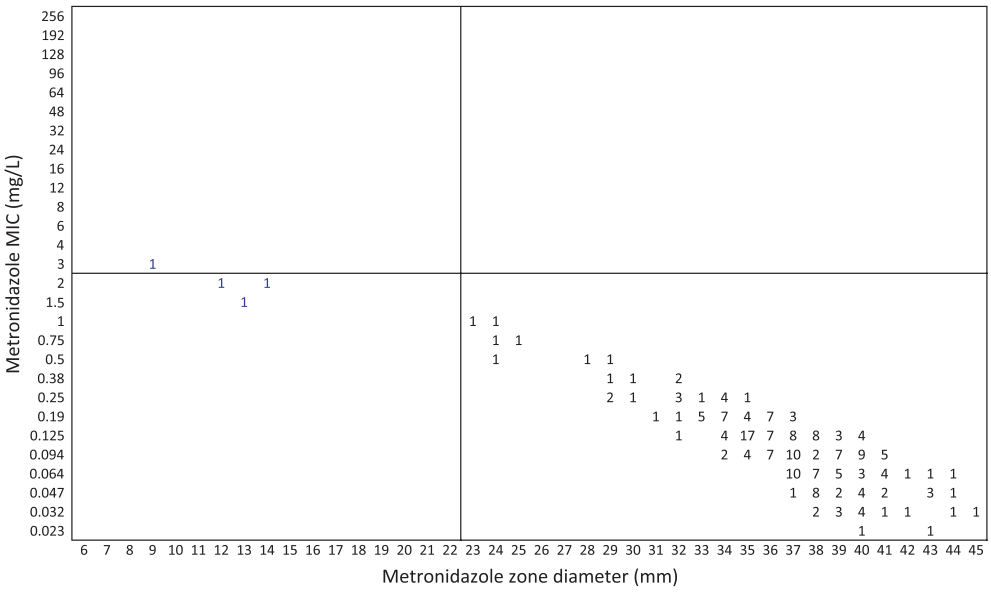

FIG. 2. Scattergram comparing metronidazole (5 μg disk) zone diameter with gradient test MICs tested against 221 clinical isolates of *C. difficile*. The solid horizontal line represents the MIC ECOFF established by EUCAST. The solid vertical line represents the proposed zone diameter breakpoint. The isolates with known reduced susceptibility towards metronidazole (n = 4) are marked with blue.

020/077 is well known, as these three PCR ribotypes cannot always be divided).

Figures 2–4 show the scattergram of MICs and zone diameters for all three antimicrobials. The solid horizontal line in each scattergram represents the MIC ECOFF established by EUCAST. The solid vertical line in each scattergram represents the zone diameter breakpoint, which best correlates to the MIC ECOFFs determined by EUCAST. We calculated VME and ME for the proposed breakpoints. For metronidazole we propose a zone diameter breakpoint of WT ≥ 23 mm (no VME, 1.4% ME). For vancomycin we propose a zone diameter breakpoint of WT ≥ 19 mm (no VME, 0.5% ME). For moxifloxacin we propose a zone diameter breakpoint of WT ≥ 20 mm (no VME, 1.8% ME) (Table 1).

Bivariable regression analysis showed an acceptable to excellent correlation between the log-transformed MIC vs. the untransformed zone diameter, thus the R^2 was 0.75 for metronidazole, 0.55 for vancomycin and 0.97 for moxifloxacin.

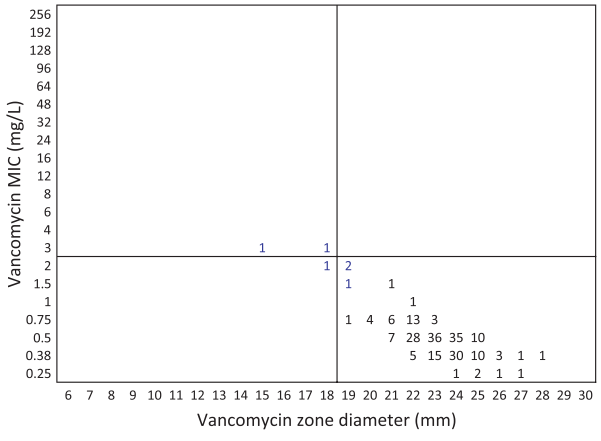

FIG. 3. Scattergram comparing vancomycin (5 μg disk) zone diameter with gradient test MICs tested against 221 clinical isolates of *C. difficile*. The solid horizontal line represents the MIC ECOFF established by EUCAST. The solid vertical line represents the proposed zone diameter breakpoint. The isolates with known reduced susceptibility towards vancomycin (n = 6) are marked with blue.

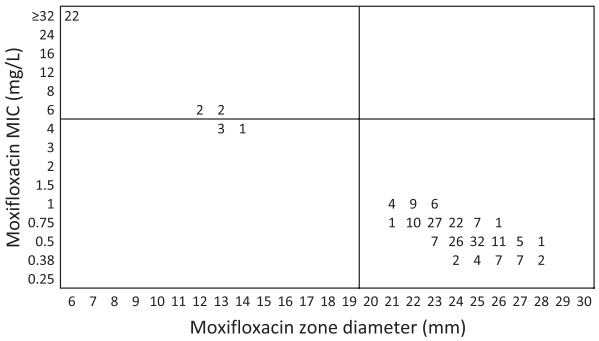

FIG. 4. Scattergram comparing moxifloxacin (5 μg disk) zone diameter with gradient test MICs tested against 221 clinical isolates of *C. difficile*. The solid horizontal line represents the MIC ECOFF established by EUCAST. The solid vertical line represents the proposed zone diameter. The 10 Welsh isolates were all resistant towards moxifloxacin.

TABLE 1. Categorization of *C. difficile* as wild type or non-wild type using a gradient test and the proposed disk diffusion method. Zone diameter correlates to ECOFFs as determined by EUCAST were used and very major errors (VME) and major errors (ME) calculated

Antimicrobial agent	Zone diameter* ECOFF (mm)	EUCAST ECOFF (mg/L)	Errors (%)	
			VME	ME
Metronidazole	WT ≥ 23	WT ≤ 2	0	1.4
Vancomycin	WT ≥ 19	WT ≤ 2	0	0.5
Moxifloxacin	WT ≥ 20	WT ≤ 4	0	1.8

*Tentative zone diameter ECOFF for the proposed disk diffusion method.

Discussion

According to the CLSI procedural documents, susceptibility testing is indicated for any organism that contributes to an infection warranting antimicrobial chemotherapy if susceptibility cannot reliably be predicted from existing antibiograms [10]. *C. difficile* is an organism that can contribute to an infection warranting antimicrobial chemotherapy, and with the changes in antimicrobial susceptibility, especially to metronidazole, susceptibility testing is indicated and a simple susceptibility testing method is needed.

We compared disk diffusion zone diameters with Etest MICs, and using bivariable regression analysis we found an acceptable to excellent agreement between disk diffusion and Etest for all three antimicrobials tested. However, more importantly, the agreement between disk diffusion and Etest can be clearly visualized in the MIC-coloured zone diameter histogram. In 1999 Wong *et al.* conducted a study with 100 clinical isolates of *C. difficile* where they compared disk diffusion with Etest. The method used by Wong *et al.* resembles our method except they used vancomycin disks with a disk content of 30 μg instead of 5 μg, and the incubation time was 48 h instead of 24 h. The authors concluded that the correlation coefficients found for metronidazole (R = 0.574) and vancomycin (R = 0.473) were too low to allow accurate prediction of MIC using the disk diffusion test. This study has often been cited for their conclusion. When the absolute majority of isolates belong to the MIC wild-type population (i.e. there are no or only an occasional resistant organism in the population) a low correlation coefficient is to be expected, hence the low R-value for vancomycin. On the other hand, the R-value becomes more acceptable with the presence of a resistant population. When dealing primarily with organisms without resistance mechanisms, regression analysis and regression lines are of little help or value. Therefore, regression analysis should always be used with caution [18,20,21]. For most reference strains, almost regardless of species and antimicrobial agent, the aggregated inhibition zone values, of repeated testing, form a distribution most often covering 6–8 mm [22]. For the 221 clinical isolates of *C. difficile* tested towards metronidazole, vancomycin and moxifloxacin, we found that for each MIC value, the inhibition zones varied from 0 to 8 mm, with 93% of the values within 6 mm for metronidazole, 95% of values within 4 mm for vancomycin, and 98% of values within 4 mm for moxifloxacin. Therefore, we conclude that there is an excellent agreement between disk diffusion and the MIC as measured by Etest.

The disk diffusion method was able to detect reduced susceptibility towards metronidazole. We found the population of organisms with reduced susceptibility to be clearly separated from the WT by 9 mm. The EUCAST established ECOFF for metronidazole is ≤2 mg/L (http://www.eucast.org; 12 February 2012). This is based on MIC-values in 4435 isolates from 10 studies performed with agar dilution (Brucella agar and Wilkins–Chalgren agar) and gradient tests on Brucella agar. In our study, isolates with MIC above 1 mg/L exhibited a marked decrease of 9 mm in inhibition zone diameter, which explains the major error of 1.4% (Fig. 2). This would indicate that either the ECOFF for metronidazole should be ≤1 mg/L (instead of ≤2 mg/L as currently recommended) or there is method dependency corresponding to a +/−1 dilution step difference in the MIC-value. Several studies have shown that metronidazole MICs obtained by Etest are slightly lower than MICs obtained by agar dilution [11,23]. The broad EUCAST distribution could also be explained by the inclusion of PCR ribotypes that are known to have reduced susceptibility to metronidazole (e.g. PCR ribotype 001) [8]. For epidemiological purposes we propose a zone diameter breakpoint of WT ≥ 23 mm. Only four isolates from the WT had zone diameters below 28 mm. These four isolates had zone diameters between 23 and 25 mm, with corresponding MICs of 0.5–1 mg/L. They were clearly separated from the isolates with reduced susceptibility on the zone diameter and these isolates were therefore included in the WT. However, decreased effectiveness of metronidazole treatment of CDI has been reported [24,25]. This is believed to be due to suboptimal intraluminal concentrations of metronidazole during CDI. This hypothesis is based upon the pharmacokinetics and pharmacodynamics of metronidazole, and the wide variability in metronidazole levels measured in watery stools during acute CDI [26,27]. The changes in susceptibility to metronidazole have not yet been linked to treatment failure [28]. However, it is possible that a slight

increase in MIC of metronidazole for *C. difficile* may contribute to insufficient faecal antimicrobial concentration to inhibit bacterial growth.

For vancomycin the reduced susceptibility was not as clear-cut, either by disk diffusion or Etest. Two of the six isolates with known reduced susceptibility towards vancomycin had an MIC above the EUCAST ECOFF (Fig. 2). With a proposed zone diameter breakpoint of WT ≥ mm 19 these two isolates with an MIC of 3 mg/L were separated from the WT. With a major error of 0.5% we suggest that isolates with zone diameters <19 mm be confirmed with an MIC. The reduced susceptibility to vancomycin, as observed in this study, probably has no clinical consequence as oral vancomycin (125–250 mg every 6 h) generally yields faecal levels >1000 mg/L in patients with CDI [29]. However, it indicates that vancomycin resistance development needs to be closely monitored.

Despite the fact that disk diffusion was able to separate the intermediate and resistant population from the WT for moxifloxacin we still had a major error of 1.8%. This is due to the fact that MICs of the intermediate isolates were very close to the ECOFF (Fig. 2). If the disk diffusion method with moxifloxacin was to be used for screening (e.g. PCR ribotype 027) we propose a zone diameter breakpoint of WT ≥ 20 mm because this would enable us to distinguish between the WT and the intermediate and resistant populations.

With the emergence of reduced susceptibility towards metronidazole and vancomycin, the value of antimicrobial susceptibility testing of *C. difficile* has increased. A simple susceptibility testing method is needed and based on our results the disk diffusion method is an option. We found an excellent agreement between inhibition zone diameters by disk diffusion and MICs by Etest. Disk diffusion was able to distinguish between the WT and resistant and intermediate populations and disk diffusion was able to detect reduced susceptibility towards metronidazole and vancomycin. Therefore, we conclude that the disk diffusion method is a reliable option for antimicrobial susceptibility testing of *C. difficile*.

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Transparency Declaration

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