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

L. T. Erikstrup[1,2], T. K. L. Danielsen[3], V. Hall[4], K. E. P. Olsen[5], B. Kristensen[6], G. Kahlmeter[7], K. Fuursted[1] and U. S. Justesen[3]

1) *Department of Clinical Microbiology, Aarhus University Hospital, Aarhus,* 2) *Institute of Clinical Medicine, Aarhus University, Aarhus,* 3) *Department of Clinical Microbiology, Odense University Hospital, Odense, Denmark,* 4) *Anaerobe Reference Unit, University Hospital of Wales, Cardiff, Wales, UK,* 5) *Microbiological Surveillance and Research, Statens Serum Institut, Copenhagen,* 6) *National Center for Infection Control, Statens Serum Institut, Copenhagen, Denmark and* 7) *Department of Clinical Microbiology, Central Hospital, Växjö, Sweden*

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 $\geq$ 23 mm, WT $\geq$ 19 and WT $\geq$ 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
Original Submission: 27 February 2012; **Revised Submission:** 22 April 2012; **Accepted:** 27 April 2012
Editor: S. Cutler
Article published online: 3 May 2012
Clin Microbiol Infect 2012; **18:** E266–E272
10.1111/j.1469-0691.2012.03907.x

Corresponding author: L. T. Erikstrup, MD, Department of Clinical Microbiology, Aarhus University Hospital, Brendstrupgaardsvej 100, DK-8200 Aarhus N, Denmark
E-mail: liseerik@rm.dk

Preliminary results from this study will be presented as a poster at ECCMID 2012 (poster number P 681, The 22nd ECCMID, 31 March to 3 April 2012, London, UK).

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