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Clostridium difficile (including epidemiology)

Comparison of *Clostridium difficile* minimum inhibitory concentrations obtained using agar dilution vs broth microdilution methods

Christine J. Hastey [a], Suzanne E. Dale [b], Julia Nary [b], Diane Citron [c], Jennifer H. Law [a], Darcie E. Roe-Carpenter [a, *], Laurent Chesnel [d]

[a] Beckman Coulter Diagnostics, Inc., West Sacramento, CA, United States
[b] ACM Global Laboratories, Rochester, NY, United States
[c] R.M. Alden Research Laboratory, Culver City, CA, United States
[d] Merck Sharp & Dohme Corp, Kenilworth, NJ, United States

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ABSTRACT

Due to increasing antibiotic resistance among anaerobic bacteria, routine antimicrobial susceptibility testing is recommended by the Clinical and Laboratory Standards Institute (CLSI). This study compared the minimum inhibitory concentrations (MICs) from 920 *Clostridium difficile* isolates tested against seven antimicrobial agents using the two current CLSI reference methodologies, agar dilution method, vs broth microdilution method. A subset of isolate testing was performed independently by two laboratories to evaluate reproducibility. A negative bias was noted for MICs generated from broth microdilution compared to agar dilution and the reproducibility was variable and drug dependent. Therefore, broth microdilution is not recommended as an alternative to agar dilution for *C. difficile* antimicrobial susceptibility testing.

1. Introduction

Increasing development and prevalence of antimicrobial resistance in bacteria is well known. This increasing trend is also observed with anaerobic bacteria; however, with anaerobic bacteria antibiotic resistance patterns also have been shown to be variable based on geographic location [1]. This has led to the Clinical and Laboratory Standards Institute (CLSI) recommendation a minimum of annual surveillance assessment of antimicrobial resistance in select anaerobes [2]. Currently, additional susceptibility testing is recommended for patient isolates with particular situations (e.g. brain abscess, endocarditis, and bacteremia) [2]. Agar dilution is the current methodology recommended by CLSI for testing the antimicrobial susceptibility of anaerobic species; however, it does not lend itself to timely testing of an individual isolate. Agar dilution method is cumbersome and labor intensive to perform routinely, making the anaerobic collection and susceptibility testing time-consuming and impractical for routine clinical laboratory testing of anaerobic isolates [3]. Therefore, there is a need for a less labor intensive method for hospital laboratories and reference centers. At this time, only the *Bacteroides* species are recommended for testing by broth microdilution [2]. In this study, we compare agar dilution and broth microdilution testing of *Clostridium difficile* using selected antimicrobials. In an attempt to extend the number of anaerobic species that could be tested by broth microdilution, we compared the intra and inter laboratory reproducibility of *C. difficile* susceptibility data generated by broth microdilution and agar dilution methods using two testing facilities.

2. Materials and methods

2.1. Culture and identification

Clostridium difficile toxin positive stool samples tested by PCR or EIA were collected from worldwide clinical trials conducted from 2012 to 2015. Specimens were frozen and sent to the central microbiology laboratory (ACM Global Laboratory) on dry ice for culture, identification and susceptibility. Upon receipt at the central microbiology laboratory, specimens were stored at −80 °C until testing, at which time they were thawed in an ambient air

* Corresponding author. Beckman Coulter Diagnostics, Inc., 1584 Enterprise Blvd., West Sacramento, CA 95691, United States.
E-mail address: decarpenter@beckman.com (D.E. Roe-Carpenter).

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environment for 16–20 h at 2–8 °C before processing. To optimize recovery of *C. difficile,* broth enrichment and alcohol-shock methods were used. Briefly, a 0.2–0.5 g or 0.2–0.5 mL stool sample was directly inoculated into pre-reduced Cycloserine-Cefoxitin Mannitol Broth with taurocholic acid, lysozyme and cysteine (CCMB-TAL, Anaerobe Systems, Morgan Hill, CA) in anaerobic condition. In addition, 1 g or 1 mL of the sample was mixed 1:1 with ethanol and held at room temperature for 60 min prior to centrifugation to pellet the stool. The r stool pellet was re-suspended 1:10 in 1X PBS and plated onto a Cycloserine Cefoxitin Fructose Agar plate with horse blood and taurocholate (CCFA-HT, Anaerobe Systems). The plates and tubes were incubated anaerobically for up to 48 h at 35–37 °C. CCMB-TAL and CCFA-HT was inspected at 24 and 48 h for positive mannitol fermentation and colony morphologies consistent with *C. difficile*, respectively. *C.* difficile were identified from spreading colonies that were Gram-positive rods and L-proline aminopeptidase-positive (Key Scientific), and displayed the characteristic *p*-cresol odor and chartreuse fluorescence upon exposure to UV light (365 nm). One representative isolate from each stool sample identified as *C. difficile* using the above criteria were frozen at −80 °C in nutrient cryovials with beads (ProLab, Toronto, Canada) for susceptibility testing and long term storage.

2.2. MIC testing

Minimum inhibitory concentration (MIC) testing was performed by ACM Global Laboratory on 920 isolates with the following antimicrobials using agar dilution and custom prepared frozen broth microdilution panels. A subset of 210 isolates were also tested at RM Alden Research Laboratory (Culver City, CA). The following antimicrobial agents were tested: ceftriaxone, clindamycin, metronidazole, moxifloxacin, rifaximin, tigecycline and vancomycin. *Bacteroides fragilis* ATCC 25285, *Clostridium difficile* ATCC 700057 and *Eggerthella lenta* ATCC 43055 were used as QC strains per CLSI recommendations [4]. Frozen *C. difficile* isolates were subcultured to Brucella agar twice prior to MIC testing and a fresh 24 h culture was used for inoculation.

Agar dilution testing was performed in accordance with CLSI M11-A8 [2]. Briefly, doubling dilutions of the antimicrobials were prepared from a 1280 μg/mL stock solution (Remel) that was supplemented with vitamin K, hemin and laked sheep blood (Hemostat, Dixon, CA) when added to Brucella agar (Remel). Plates with doubling concentrations of 8–128 μg/mL of ceftriaxone, 0.03–64 μg/mL of clindamycin, 0.12–32 μg/mL of metronidazole, 0.03–16 μg/mL of moxifloxacin, 0.004–8 μg/mL of rifaximin, 0.015–16 μg/mL of tigecycline, and 0.06–8 μg/mL of vancomycin was made. Organism suspension equivalent to a 0.5 McFarland was prepared in Brucella Broth (Anaerobe Systems) and 0.5 mL was added to each well of a Steer's replicator. Once inoculated, the agar dilution plates had a final approximate inoculum of 10^5 CFU/spot. The plates were inoculated with the replicator in ambient air and placed into the 35–37 °C anaerobic chamber within 10 min of inoculation and incubated for 44–48 h under anaerobic conditions. MICs were determined when there was complete or marked inhibition of growth as compared to the antimicrobial free control plate following CLSI guidance.

Broth microdilution testing was performed using the same 0.5 McFarland suspension created for the agar dilution testing and with the same ranges for all antibiotics. Frozen broth microdilution panels (Remel) were thawed for 2–3 h at room temperature prior to testing. The remaining 0.5 McFarland suspension was diluted 1:15 with demineralized water (Remel) in the anaerobic chamber with the rest of the set-up performed in ambient air. An inoculator tray and pin delivery system (Remel) was used to inoculate the broth microdilution panels, delivering 10 μl/well. This resulted in a final inoculum concentration of approximately 10^6 CFU/mL or ~10^5 CFU/well. The panels were covered with an anaerobic seal and incubated in the anaerobic chamber for 44–48 h at 35–37 °C. Minimum inhibitory concentrations (MICs) were determined when there was complete or marked inhibition of growth following CLSI guidance.

RM Alden used the same methodology described above. In addition, the lot numbers of reagents, antimicrobials and BMD panels were the same. The duplicate susceptibility testing of a subset of isolates was done to evaluate inter-laboratory variability.

2.3. Statistical analysis

The intra-laboratory reproducibility was assessed calculating the essential agreement (EA) between broth microdilution and agar dilution using the percentage of isolates that yielded identical or a single 2-fold dilution difference for each agent tested. The inter-laboratory reproducibility was assessed calculating the essential agreements for each method using a subset of 210 isolates. In addition, $MIC_{50,}$ MIC_{90} and the geometric means for each antimicrobial were calculated. Wilcoxon signed-rank test was used to compare the log means between the two sites and also between the two mediums.

3. Results

To determine the intra-laboratory reproducibility of the agar and broth microdilution methods, MICs of seven antimicrobials were compared between agar dilution and broth microdilution for 920 isolates of *Clostridium difficile*. The essential agreement (EA) was determined for each antimicrobial tested when the broth microdilution MIC value was ±1 dilution of the agar dilution (within a single 2-fold dilution (Table 1)). The essential agreement was greater than 95% for broth microdilution within one 2-fold dilution with agar dilution for ceftriaxone and moxifloxacin, between 80 and 90% for vancomycin and metronidazole, and 71.7%, 41.4% and 29.3% rifaximin, clindamycin and tigecycline. The MIC ranges, MIC_{50}, MIC_{90}, and geometric means for the 920 *C. difficile* isolates are summarized for the two methods (Table 2). In most cases, there was a negative bias for MICs generated from broth microdilution as broth microdilution MIC values were shifted at least one doubling dilution lower compared to agar dilution MIC values, particularly noted in the geometric means.

To determine the inter-laboratory reproducibility of the 2 methods, 210 *C. difficile* isolates were tested and the EA was calculated for each method between the two sites (Table 3). The agar dilution method consistently demonstrated greater than 94.3% EA across the two sites for all seven antimicrobials. The EA was lower than 90% for broth microdilution for vancomycin, clindamycin, metronidazole, rifaximin and tigecycline. In contrast, moxifloxacin and ceftriaxone were 97.5% and 98.8% in agreement respectively. Together these data indicate a lack of reproducibility

Table 1
Essential agreement (EA) of broth microdilution MICs compared to agar dilution MICs of selected antimicrobials for 920 isolates of *Clostridium difficile*.

	EA (%)
Clindamycin	41.4
Ceftriaxone	98.8
Metronidazole	81.8
Moxifloxacin	97.5
Rifaximin	71.7
Tigecycline	29.3
Vancomycin	88.5