Inter-Method Comparison of Broth Microdilution and Agar Dilution Testing for *Campylobacter* spp.: Standardization of Broth Methods

JM STREIT, PR RHOMBERG, CC KNAPP, SB KILLIAN, NM HOLLIDAY, RN JONES, TR FRITSCHE
The JONES Group/JMI Laboratories, North Liberty, IA; TREK Diagnostics, Cleveland, OH

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The JONES Group/JMI Laboratories
North Liberty, IA, USA
www.jmilabs.com
319.665.3370, fax 319.665.3371
ronald-jones@jmilabs.com

ABSTRACT

Background: Agar dilution is the only NCCLS recommended method for determining antimicrobial susceptibility (S) of *Campylobacter* spp. We evaluated the results for broth microdilution (BMD) varying conditions for multiple parameters with reference agar dilution (AD) results.

Methods: The method comparison followed the M7-A6 BMD procedure for fastidious organisms (MH broth with 2-5% lysed horse blood [LHB]) and AD methods with 5% sheep blood as recommended by NCCLS guidelines in a microaerophilic atmosphere at 34-36ºC. Two labs performed an AD reproducibility study (3/day times 3 days) using a common 10 isolate set including the NCCLS recommended QC strain (*C. jejuni* ATCC 33560). Inoculum preparation for BMD was varied by 24 hour broth versus 48 direct and 10^5 versus 10^6 concentration in dry-format panels. MICs for AD were determined after 48 hours and BMD after both 24 and 48 hours incubation.

Results: Reproducibility for AD testing was 69/98% for all drugs combined for same/± 1 $\log_2$ dilution results. Between days reproducibility was not acceptable with only 52/89% same/± 1 $\log_2$ dilution results. BMD results demonstrated >95% agreement (± 1 $\log_2$ dilution) between 24 hour and 48 readings for the direct and broth growth inoculums at 10^5 and 10^6, except for the 106 inoculum grown up in broth (88/98%, ± 1 dilution/± 2 dilutions). AD to BMD agreement was 28% identical, 74% within ± 1 dilution, and 93% within ± 2 dilutions. BMD between laboratory agreement was 64% identical results and 97% for ± 1 dilution.

Conclusions: BMD results when tested with a 10^5 direct inoculum from a blood agar plate on MH + LHB panels demonstrated similar results to the NCCLS recommended AD methods for *Campylobacter* spp. isolates. Inter-laboratory MIC results were highly similar and accurately represented the reference methods.

INTRODUCTION

Resistance to fluoroquinolones and macrolides among *Campylobacter* spp., especially *C. jejuni*, is recognized worldwide. In the United States (US), ciprofloxacin resistance has emerged since 1990, and has increased in prevalence since 1997 (13%, increasing to 19% in 2001); resistance to erythromycin has remained unchanged at approximately 2%. Reliable and meaningful susceptibility testing methodologies are needed primarily for epidemiologic studies but would also prove useful for clinical purposes. While a variety of methodologies (agar dilution, Etest [AB BIODISK, Solna, Sweden], disk diffusion and broth microdilution) have been described, only recently (2004) has a standardized agar dilution method been proposed. Quality control ranges using *Campylobacter jejuni* ATCC 33560 have subsequently been established for commonly utilized antimicrobial agents [NCCLS, 2004]. This method is performed using either a 24 hour (42ºC) or 48 hour (36ºC) incubation in a microaerophilic atmosphere. The performance of the agar dilution procedure is generally beyond the abilities of many laboratories, however, and for routine susceptibility testing a broth-based assay system would be preferable.

This report summarizes the initial results of studying a broth microdilution procedure as is currently utilized for fastidious organisms, but with modifications to parameters including inoculum source and concentration, and time of incubation, comparing results with those of the reference agar dilution method, to better define conditions suitable for the development of a reproducible broth microdilution method.

MATERIALS AND METHODS

A 10 isolate set of *Campylobacter* spp. that included the NCCLS recommended QC strain (*C. jejuni* ATCC 33560) were tested by two laboratories with results of broth microdilution compared to those of agar dilution testing. The comparison utilized the NCCLS M7-A6 broth microdilution procedure (dry-form panels; TREK Diagnostics, Cleveland, OH) for fastidious organisms (Mueller-Hinton broth supplemented with 2-5% added lysed horse blood) with inocula prepared from 24 and 48 hour broth growth or growth on blood agar plates, and adjusted to either 10^5 or 10^6 CFU/ml.

Agar dilution MICs were performed using Mueller-Hinton agar with 5% defibrinated sheep blood, with the inoculum prepared by direct colony suspension equivalent to a 0.5 McFarland standard, and with incubation at 36ºC for 48 hours [NCCLS, 2004].

All incubations were performed in a microaerophilic atmosphere (5% O_2, 10% CO_2, and 85% N_2) as produced by use of Pak-Microaero sachets (Mitsubishi Gas Chemical America, Inc., New York) or its equiavalent in a 2.5 liter jar system. Two sites performed an agar dilution reproducibility study (3 replicates per day for 3 days) using the common 10 isolate set tested against 8 antimicrobial agents.

RESULTS

- The agar dilution reference method was acceptably reproducible within each test day with 89.9% identical MIC results, and 97.9% of results ± one $\log_2$ dilution step (Table 1).
- Agar dilution performance was generally less acceptable, however, due to poor between day reproducibility with 72.5% of results identical and < 90% ± one $\log_2$ dilution step (target being ≥ 90% ± one $\log_2$ dilution step). Greatest variation from the all test median values (12.2 to 14.4% of results being ≥ two $\log_2$ dilution steps) occurred with the two macrolides, ciprofloxacin, gentamicin and tetracycline.
- Broth microdilution results (Table 2) demonstrated > 95% agreement (± one $\log_2$ dilution step) between 24 and 48 hour readings for both the direct and broth growth inoculums at 10^5 and 10^6 CFU/ml. Only the 10^6 CFU/ml broth inoculum was less reproducible (88.0% ± one $\log_2$ dilution step).
- While 24 and 48 hour readings provided similar endpoints, greater growth failure occurred at 24 hours and was primarily due to incubation at 35ºC; incubation at 36-37ºC resulted in interpretable growth in all wells.
- Agreement between agar dilution and broth microdilution results, regardless of inoculum used, was: 27.7% identical, 73.6-76.4% ± one $\log_2$ dilution step, and 93.0-94.4% ± two $\log_2$ dilution steps (Table 3). Agar dilution MIC values tended to be higher overall, with particularly elevated MICs for the macrolide compounds being responsible for the greatest differences in agreement.
- Increased pH effects on macrolides are characteristically observed with agar dilution testing in a CO_2 -enriched atmosphere, resulting in falsely elevated MIC values for pH sensitive agents. Removal of these agents from the analysis produced acceptable agreement (87.1% ± one $\log_2$ dilution step and 96.2% ± two $\log_2$ dilution steps) between agar- and broth-based dilution methods.
- Interlaboratory agreement with broth microdilution testing was highly congruent, yielding 63.9% identical results and 97.2% ± one $\log_2$ dilution step (Table 4), whereas agreement between laboratories using agar dilution tests was not acceptable (only 38.0% identical results and 90.3% ± one $\log_2$ dilution step; Table 5).

Table 1. Reproducibility of the reference agar dilution method for *Campylobacter* spp. testing three replicates daily for three days (10 organisms).

	Occurrences at each variation in $\log_2$ dilutions:									
	Within day					Between days				
Antimicrobial agent	≥-2	-1	Same	+1	≥+2	≥-2	-1	Same	+1	≥+2
Azithromycin	1	5	79	3	2	4	1	66	11	8
Erythromycin	0	7	77	5	1	2	5	60	12	11
Clindamycin	1	3	82	3	1	0	4	71	11	4
Ciprofloxacin	0	0	88	2	0	0	0	68	10	12
Nalidixic acid	1	4	77	5	3	2	5	70	8	5
Chloramphenicol	1	1	86	2	0	3	5	73	6	3
Gentamicin	2	8	76	4	0	10	10	59	10	1
Tetracycline	1	2	82	4	1	7	14	55	8	6
All drugs (720)	7	30[a]	647[a,b]	28[a]	8	28	44[c]	522[c,d]	76[c]	50

a. 97.9%.
b. 89.9%.
c. 89.2%.
d. 72.5%.

Table 2. Effects of varying test conditions when performing the microdilution broth MIC assay for *Campylobacter* spp. isolates.[a]

	Variations in $\log_2$ dilutions for 24 and 48 hour MIC results:						
Inoculum source/concentration (CFU/ml) (no. tested)	-2	-1	0	+1	+2	+3	% agreement ($\log_2$ scale)
BAP[b]/10^5 (n=64)	1.6	7.9	49.2	38.1	3.2	0.0	95.2 ± one dilution
Broth[c]/10^5 (n=61)	0.0	3.3	60.6	32.8	3.3	0.0	96.7 ± one dilution
BAP[b]/10^6 (n=67)	1.5	23.9	55.2	17.9	1.5	0.0	97.0 ± one dilution
Broth[c]/10^6 (n=67)	6.0	10.4	41.8	35.8	4.5	1.5	88.0 ± one dilution 98.5 ± two dilutions

a. Test conditions: MH broth supplemented with 2 - 5% lysed horse blood; incubation in a microaerophilic atmosphere at 36 - 37ºC.
b. BAP = blood agar plate, 24 hour growth in a microaerophilic atmosphere.
c. Cation-adjusted Mueller-Hinton broth, 24 hour growth.

Table 3. Comparison of *Campylobacter* spp. MIC results when performing the agar dilution (10^5 CFU/ml inoculum) and broth microdilution (10^5 and 10^6 CFU/ml inocula) tests.

	Agar/broth MIC ratio (ratio using a 10^6 inocula for broth MIC):						
Antimicrobial agent	≥8	4	2	1	0.5	0.25	≤0.12
Ciprofloxacin	0(0)	2(1)	6(5)	0(2)	1(1)	0(0)	0(0)
Nalidixic Acid	0(0)	0(0)	2(0)	5(4)	1(4)	1(1)	0(0)
Erythromycin	2(1)	6(5)	1(3)	0(0)	0(0)	0(0)	0(0)
Azithromycin	1(1)	3(3)	4(4)	1(1)	0(0)	0(0)	0(0)
Clindamycin	0(0)	0(0)	3(2)	4(6)	2(0)	0(1)	0(0)
Chloramphenicol	0(0)	1(0)	4(5)	3(3)	1(1)	0(0)	0(0)
Gentamicin	0(0)	0(0)	1(0)	4(2)	4(6)	0(1)	0(0)
Tetracycline	1(1)	0(0)	2(1)	3(2)	1(3)	1(1)	1(1)
Total (n=72)	4(3)	12(9)[c]	23(20)[b,c]	20(20)[a,b,c]	10(15)[b,c]	2(4)[c]	1(1)

a. 27.7 (27.7)% identical results.
b. 73.6 (76.4)% ± one $\log_2$ dilution.
c. 93.0(94.4)% ± two $\log_2$ dilutions.

Table 4. Interlaboratory comparison (JMI Laboratories; TREK Diagnostics) of broth microdilution test results when testing *Campylobacter* spp.[a]

	MIC ratio (JMI/TREK):						
Antimicrobial agent	≥8	4	2	1	0.5	0.25	≤0.12
Ciprofloxacin	0	1	2	3	3	0	0
Nalidixic Acid	0	0	1	4	4	0	0
Erythromycin	0	1	0	7	1	0	0
Azithromycin	0	0	2	4	3	0	0
Clindamycin	0	0	1	8	0	0	0
Chloramphenicol	0	0	0	7	2	0	0
Gentamicin	0	0	0	8	1	0	0
Tetracycline	0	0	0	5	4	0	0
Total (72)	0	2	6[c]	46[b,c]	18[c]	0	0

a. Using cation-adjusted MH broth supplemented with 2-5% lysed horse blood; inoculum prepared from a BAP, 10^5 CFU/ml; interpretation made at 48 hours; microaerophilic incubation at 36-37ºC.
b. 63.9% of results identical.
c. 97.2% ± one $\log_2$ dilution.

Table 5. Interlaboratory comparison (JMI Laboratories; TREK Diagnostics) of agar dilution MIC results when testing *Campylobacter* spp.[a]

	Agar dilution MIC ratio (JMI/TREK)					
Antimicrobial agent	≥8	4	2	1	0.5	0.25
Ciprofloxacin	0	0	3	6	0	0
Nalidixic Acid	1	0	1	4	3	0
Erythromycin	1	1	7	0	0	0
Azithromycin	1	1	3	4	0	0
Clindamycin	0	0	6	2	1	0
Chloramphenicol	1	0	1	5	2	0
Gentamicin	0	0	3	3	3	0
Tetracycline	1	0	4	3	1	0
Total (72)	5	2	28[c]	27[b,c]	10[c]	0

a. Using MH agar supplemented with 5% sheep blood; inoculum prepared from a BAP, 10^5 CFU/ml; interpretation made at 48 hours; microaerophilic incubation at 34-36ºC.
b. 38.9% of results with identical values.
c. 90.3% ± one $\log_2$ dilution.

CONCLUSIONS

- A broth microdilution procedure utilizing Mueller-Hinton broth with 2-5% added lysed horse blood, an inoculum consisting of a direct colony suspension equivalent to a 0.5 McFarland standard, and incubation for 48 hours at 36-37ºC in a microaerophilic atmosphere produced highly congruent results between laboratories when testing *Campylobacter* spp.
- Overall agreement between broth microdilution and agar dilution was reduced, primarily due to elevated macrolide MIC values in the latter assay secondary to pH effects.
- The development of a standardized broth microdilution methodology using the NCCLS M7-A6 procedure for fastidious (streptococci) organisms with slight modifications of incubation temperature, time, and atmosphere provides a practical and economical approach for reference testing of *Campylobacter* spp.

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