D-1708

Wild-type MIC Distributions for *Campylobacter* spp. Testing Against Nine Antimicrobials Using Recently Approved CLSI Broth Microdilution (BMD) Methods (2005)

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ICAAC 2005
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ABSTRACT

Background: The lack of standardized MIC methodologies for testing *Campylobacter* spp. has limited the comparison of published reports and prevented laboratories from routinely testing fluoroquinolones (FQ) and macrolides (MAC). Recently, the CLSI (Subcommittees on veterinary and human AST) adopted agar dilution and BMD methods, and established QC ranges for select agents. Using BMD, wild-type (WT) *Campylobacter* spp. isolates were tested to determine MIC frequency distributions for clinically important antimicrobial agents.
Methods: MIC values from a diverse collection of human and animal *C. jejuni* (127) and *C. coli* (26) were determined. Cation-adjusted Mueller-Hinton broth with 2.5% lysed horse blood was used; inoculum was from a direct colony suspension equivalent to a 0.5 McFarland standard; and incubation was 36-37° C for 48 hours in 10% CO_2, 5% O_2, and 85% N_2. Antimicrobials tested: ciprofloxacin (CIP), nalidixic acid (NAL), erythromycin (ERY), azithromycin (AZI), clarithromycin (CLA), clindamycin (CLI), doxycycline (DOX), tetracycline (TET) and gentamicin (GEN).
Results:

Antimicrobial	Mode (Range) MIC in µg/ml: WT population	R population	Epidemiologic breakpoints
CIP	0.06 (≤0.03-0.25)	8 (4 - 64)	0.5 or 1
NAL	4 (4 - 16)	>64 (64 - >64)	32
ERY	0.12 (0.12 - 2)	>64 (>64)	2
AZI	≤0.03 (≤0.03 - 0.25)	2 (1 - >64)	0.5
CLA	0.25 (0.12 - 4)	>64 (64 - >64)	4 or 8
CLI	0.06 (0.06 - 1)	16 (4 - 32)	2
DOX	0.12 (0.12 - 1)	16 (4 - 64)	1 or 2
TET	0.5 (0.5 - 4)	>64 (16 - >64)	4 or 8
GEN	0.25 (0.25 - 1)	>64 (>64)	1 or 2

All agents with the exception of GEN displaed distinct bimodal distributions, allowing for tentative epidemiologic breakpoints.
Conclusions: The presence of distinct R subpopulations when testing FQs and MACs, emphasizes the need for standardized AST methods for R epidemiologic purposes, as well as for monitoring of seriously ill patients who fail therapy.

INTRODUCTION

Campylobacter spp. is a leading cause of human bacterial gastroenteritis with *C. jejuni* and *C. coli* being the most frequently isolated agents of infection. Handling, or ingesting contaminated raw or under-cooked poultry is frequently responsible for intestinal gastroenteritis, and this clinical syndrome is primarily caused by *C. jejuni*. While the vast majority of intestinal *Campylobacter* infections are self-limiting and may not require antimicrobial therapy, early therapy with a macrolide or fluoroquinolone is effective in eradicating the organism, and also may reduce the duration of symptoms and likelihood of complications. Fluoroquinolone resistance has, however, been recognized for many years and varies widely from country to country; resistance rates are reported to exceed 70% in some locales. Likewise, erythromycin resistance is also known to occur and varies from 0 - 11% in published studies, but averages < 5%. Complicating the ongoing surveillance of resistance in these organisms is the lack of standardized susceptibility test methodologies and comparable susceptibility data.

Currently the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards [NCCLS]) recognizes standardized susceptibility methods for agar dilution only (NCCLS M7-A6, M100-S15). Recently (June, 2005) the CLSI/NCCLS Subcommittees on Antimicrobial Susceptibility Testing and Veterinary Antimicrobial Susceptibility Testing developed and approved standardized broth microdilution methodologies for *Campylobacter* spp. and established quality control (QC) ranges for *C. jejuni* ATCC 33560 (12 agents). This method and the QC limits will be published in the upcoming CLSI document M100-S16 in January 2006.

This newly described broth microdilution method was used in the current study against > 150 wild-type *Campylobacter* spp. strains (primarily *C. jejuni*) isolated from human and animal sources. The MIC frequency distributions and modes were examined to differentiate between wild-type and antimicrobial-resistant sub-populations, to assist in determining tentative epidemiological breakpoints for these medically important agents.

MATERIALS AND METHODS

The MIC results were generated from three independent laboratories using the recently approved CLSI/NCCLS broth microdilution method described below. Each laboratory tested a collection of approximately 50 unique wild-type isolates of *Campylobacter* spp. from human/animal origins and diverse geographical regions. Quality Control was performed at each site using the CLSI recommended QC strain *C. jejuni* ATCC 33560. MIC results from a total of 127 strains of *C. jejuni* and 26 strains of *C. coli* were determined for the following drugs: ciprofloxacin, nalidixic acid, erythromycin, azithromycin, clarithromycin, clindamycin, doxycycline, tetracycline and gentamicin.

All isolates were stored at –70°C in appropriate media and subsequently sub-cultured on tryptic soy agar with 5% defibrinated sheep blood. Testing was performed on commercially prepared frozen panels containing serial two-fold antimicrobial drug dilutions in cation-adjusted Mueller-Hinton broth with 2 - 5% added lysed horse blood. Inocula were adjusted to a 0.5 McFarland suspension in saline or cation adjusted Mueller-Hinton broth, then further diluted in Mueller-Hinton broth with lysed horse blood to achieve a final in-well concentration of 10^5-10^6 CFU/ml. The 96 well panels were inoculated using Sensititre® auto-inoculators and sealed with perforated gas-permeable covers. All testing supplies were manufactured by Trek Diagnostics (Cleveland, OH, USA). Isolates were incubated at 36-37° C for 48 hours under microaerobic conditions (5% O_2, 10% CO_2, and 85% N_2) using compressed gas incubators, Pack-MicroAero Sachets (Mitsubitshi Gas Chemical America, Inc., NY, USA) with closed-lid containers or equivalent products.

Colony counts were performed on all trays to ensure proper inoculum concentrations were achieved.

RESULTS

- Both quinolone agents clearly displayed bimodal MIC populations with the wild-type and resistant populations, respectively, consisting of 67.3% (MIC, ≤ 0.03 to 0.25 µg/ml) and 32.7% (4 to 64 µg/ml) for ciprofloxacin, and 51.9% (MIC, 4 to 16 µg/ml) and 48.1% (64 to ≥ 64 µg/ml) for nalidixic acid (Table 1; Figures 1 and 2).
- The macrolides erythromycin, azithromycin and clarithromycin also displayed bimodal MIC populations with the susceptible isolates all being ≤ 4 µg/ml and resistant isolates (4.6, 4.7 and 5.2%, respectively) at MICs of ≥ 64 µg/ml (Table 1 and Figure 3).
- The clindamycin frequency distribution was also bimodal but truncated compared to that of the macrolides, with the resistant MIC distribution (5.2%, comparable to the macrolides) spanning the range of 4 to 32 µg/ml (Figure 4).
- Tetracycline and doxycycline shared two (wild-type and resistant) MIC distributions with doxycycline being 4-fold more potent (MIC_{50}, 4 and 16 µg/ml, respectively; Table 1 and Figure 5).
- Over 99% of gentamicin MIC values were ≤ 1 µg/ml (one strain was recovered with a MIC of ≥ 64 µg/ml), suggesting an almost complete lack of resistance to this agent or class (Figure 6).
- Given the results presented here, tentative population-based (epidemiologic) breakpoints directed by the presence of both wild-type and resistant organisms may prove useful when using this CLSI methodology to perform susceptibility testing and to monitor emerging resistances (Table 2).
- Use of the recently approved CLSI broth microdilution method 1) was found to be readily adaptable to the clinical laboratory setting; 2) did not require additional material or reagents; 3) provided reproducible MIC results; and 4) was cost effective and timely.
- Only the addition of microaerobic gas-generating sachets or use of a compressed gas incubator was required; sealed plastic bags or pouches as used for primary recovery of campylobacters are not recommended, due to growth failures (data not shown).
- While the results presented here were performed at 36-37°C with incubation for 48 hours, the approved CLSI method was also approved for use at 42ºC with incubation for 24 hours (QC limits minimally vary from those published for 36-37°C at 48 hours).

Table 1. Summary of MIC results from three laboratories each testing approximately 50 isolates of wild-type *Campylobacter* spp. against nine antimicrobial agents using the newly approved CLSI standard broth microdilution method.[a]

Antimicrobial agent (no. tested)	No. of occurrences at MIC (µg/ml): ≤0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	>64
Ciprofloxacin (153)	7	49	18	29	0	0	0	2	24	9	12	3	0
Nalidixic Acid (152)	-[b]	-	-	-	-	-	-	46	27	6	0	4	69
Erythromycin (153)	-	-	51	50	30	9	6	0	0	0	0	0	7
Azithromycin (150)	72	34	13	3	0	2	21	1	0	0	0	0	7
Clarithromycin (153)	-	-	1	62	45	25	7	5	0	0	0	1	7
Clindamycin (153)	-	65	36	18	24	2	0	1	1	5	1	0	0
Doxycycline (153)	-	-	50	20	2	2	1	1	16	30	29	2	0
Tetracycline (152)	-	-	-	-	69	3	1	1	0	5	8	0	65
Gentamicin (152)	-	-	27	79	38	8	0	0	0	0	0	0	1

a. Mueller-Hinton broth with 2.5 - 5% lysed horse blood; inoculum a direct colony suspension equivalent to a 0.5 McFarland Standard; incubation at 36 - 37°C for 48 hours in a microaerobic atmosphere (10% CO_2, 5% O_2 and 85% N_2).
b. - = not tested.

Table 2. Tentative population-based (epidemiologic) breakpoints when testing *C. jejuni* and *C. coli* using the CLSI broth microdilution method (CLSI, 2006).

Antimicrobial agent	MIC (µg/ml): Susceptible	Intermediate	Resistant
Macrolide			
Erythromycin	≤8	16	≥32
Fluoroquinolone			
Ciprofloxacin	≤1	2	≥4
Tetracyclines			
Tetracycline	≤4	8	≥16
Doxycycline	≤2	4	≥8

Figure 1. Distribution of Ciprofloxacin MIC Results Against *Campylobacter* spp. (153 isolates).

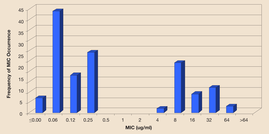


Figure 2. Distribution of Nalidixic Acid MIC Results Against *Campylobacter* spp. (153 isolates).

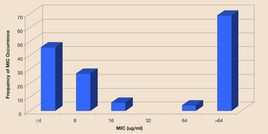


Figure 3. Distribution of Erythromycin MIC Results Against *Campylobacter* spp. (153 isolates).

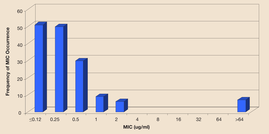


Figure 4. Distribution of Clindamycin MIC Results Against *Campylobacter* spp. (153 isolates).

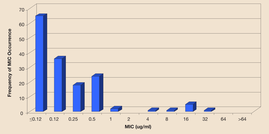


Figure 5. Distribution of Doxycycline MIC Results Against *Campylobacter* spp. (153 isolates).

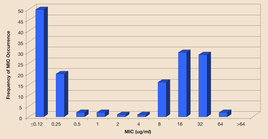
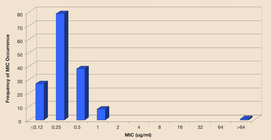


Figure 6. Distribution of Gentamicin MIC Results Against *Campylobacter* spp. (153 isolates).



CONCLUSIONS

- Results for all agents (except gentamicin) using the recently approved CLSI broth microdilution method when testing *C. jejuni* and *C. coli* revealed distinct bimodal MIC populations, allowing for the setting of tentative epidemiologic breakpoints (Table 2).
- Use of this method was readily adaptable to the routine clinical microbiology laboratory and did not require new technology, reagents (except for a microaerobic gas-generating system) or expertise.
- The presence of distinct resistant populations among *C. jejuni* and *C. coli*, especially for the commonly used fluoroquinolones and macrolides, emphasizes the need for standardized antimicrobial susceptibility testing methods for resistance epidemiologic purposes, as well as for monitoring of seriously ill patients or those patients that fail therapy.

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