C-177

Disk Diffusion Susceptibility Testing of *Campylobacter* spp.: Use in Detection of Resistance to Macrolides and Fluoroquinolones

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ABSTRACT

Background: Methods for susceptibility (S) testing of *Campylobacter* spp. (agar and broth microdilution [BMD]) have recently been established by CLSI to aid in patient management and for epidemiologic purposes. Development of a disk-diffusion (DD) method for resistance (R) detection offers a practical testing alternative for clinical laboratories.

Methods: The DD method was that specified in CLSI M100-S15 for testing of streptococci, using Mueller-Hinton agar with added 5% sheep blood, but with incubation in a microaerobic atmosphere (10% CO_2, 5% O_2, 85% N_2) at 36-37ºC for 48hr. Disk contents for ciprofloxacin (CIP; 5ug) and erythromycin (ERY; 15ug) were standard. Testing was performed at two sites with a total of 417 *C. jejuni* and *C. coli* strains. BMD was performed as described (M100-S16 and M45-P) using reference panels provided by TREK Diagnostic Systems (Cleveland, OH). QC was performed using *C. jejuni* ATCC 33560. Zone sizes were plotted against MIC values generated for each strain. Tentative MIC R breakpoints for CIP (≥ 4 µg/ml) and ERY (≥ 32 µg/ml) are those listed in M45-P.

Results: MIC population distributions were bimodal for both agents (CIP, ≤ 0.5 µg/ml and ≥ 2 µg/ml; ERY, ≤ 4 µg/ml and ≥ 64 µg/ml) with 21% (88 strains) being CIP-R and 0.4% (15 strains) ERY-R. Zone diameters to CIP (88 strains, no zone; 329, 20 to 48 mm) and ERY (16 strains, no zone; 401, 14 to 40 mm) were also bimodal. Correlation was 98.9 and 100% between the absence of a disk zone (6mm) and R MIC results for CIP and ERY, respectively; one strain with a 6mm CIP zone had an intermediate (I) MIC of 2 µg/ml. Inter-laboratory variability in zone measurements (recognized previously) prevents recommendation of correlative disk S and I categories at this time.

Conclusions: Development of a practical and economical test for CIP- and ERY-R detection in *Campylobacter* spp. may be of use in clinical laboratories, including those in resource poor regions. The test may serve as a simple epidemiologic screen and for testing isolates from patients who have failed therapy. At this time, the presence of any zone requires further action (MIC determination) to better characterize strain susceptibility.

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 < 2% in the USA.

Currently, the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards [NCCLS]) recognizes standardized susceptibility methods for broth and agar dilution (M100-S16, M31-A2; M45-A) with the recent adoption of quality control (QC) ranges for *C. jejuni* ATCC 33560 (13 agents). This method and the QC limits were recently published in CLSI document M100-S16 (January 2006).

Unfortunately, use of manual broth and agar dilution methodologies are not possible for many smaller clinical laboratories, necessitating development of a simplified method that could be widely adapted. In this study, we report on the development of a disk diffusion test for resistance screening purposes (epidemiologic and clinical), in a format that could be utilized in clinical laboratories using existing supplies and equipment, but also in resource-poor regions where MIC broth or agar testing by the CLSI standard methods would not be possible, and where resistance rates to fluoroquinolones and macrolides may be increasing.

MATERIALS AND METHODS

Specimen Collection

Results were generated from two laboratories using the recently approved CLSI broth microdilution method and a developmental disk diffusion method described below. A total of 417 unique wild-type isolates of *C. jejuni* and *C. coli* from human/animal origins and diverse geographical regions were tested. Quality Control was performed at each site using the CLSI recommended QC strain *C. jejuni* ATCC 33560. All isolates were stored at –70°C in appropriate media and subsequently sub-cultured on tryptic soy agar with 5% defibrinated sheep blood.

MIC Broth Microdilution Testing

MIC testing was performed on commercially prepared frozen reference panels (TREK Diagnostic Systems, Cleveland, OH) containing serial two-fold dilutions of ciprofloxacin and erythromycin in cation-adjusted Mueller-Hinton broth with 2 - 5% added lysed horse blood (per M100-S16 and M45-A). 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. 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 or Pack-MicroAero Sachets (Mitsubitshi Gas Chemical America, Inc., NY, USA) with closed-lid containers. Sealed plastic bags or pouches as used for primary recovery of campylobacters are not recommended, due to recognized growth failures. Colony counts were performed on all trays to ensure proper inoculum concentrations were achieved.

Disk Diffusion Testing

The disk diffusion method was that specified in CLSI M100-S16 for testing of streptococci, using Mueller Hinton agar with added 5% sheep blood, but with incubation in a microaerobic atmosphere (10% CO_2, 5% O_2, 85% N_2) at 36-37°C for 48hr. Disk contents for ciprofloxacin (5-µg) and erythromycin (15-µg) were standard. Zone sizes were plotted against MIC values generated for each strain. Approved MIC resistance breakpoints for ciprofloxacin (≥ 4 µg/ml) and erythromycin (≥ 32 µg/ml) are those listed in M45-A.

RESULTS

- MIC population distributions were bimodal for both agents (ciprofloxacin, ≤ 0.5 µg/ml and ≥ 2 µg/ml; erythromycin, ≤ 4 µg/ml and ≥ 64 µg/ml) with 21% (88 strains) being ciprofloxacin-resistant and 0.4% (15 strains) being erythromycin-resistant (Figures 1 and 2).
- Zone diameters for ciprofloxacin (88 strains, no zone [6 mm]; 329 strains, 20 to 48 mm) and erythromycin (16 strains, no zone [6 mm]; 401 strains, 14 to 40 mm) were also bimodal (Figures 3 and 4).
- Correlation was 98.9% and 100% between the absence of a disk zone (6mm) and a resistant MIC result for ciprofloxacin and erythromycin, respectively; one strain with a 6mm ciprofloxacin zone diameter had an intermediate MIC of 2 µg/ml (Figure 3).
- Inter-laboratory variability in zone measurements (recognized previously) prevents recommendation of correlative disk susceptible and intermediate categories.
- While use of the recently approved CLSI broth microdilution method may be somewhat difficult for many clinical laboratories, use of the described disk diffusion method was: 1) found to be readily adaptable to the laboratory setting; 2) did not require additional material or reagents; 3) provided reproducible results; and 4) was cost effective and timely.
- 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 42°C with incubation for 24 hours (QC limits minimally vary from those published for 36-37°C at 48 hours).

Table 1. Current population-based (epidemiologic) breakpoints when testing *C. jejuni* and *C. coli* using the CLSI broth microdilution method (CLSI M45-A, 2006).

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

Figure 1. Distribution of ciprofloxacin MIC results against *Campylobacter* spp. (399 isolates).

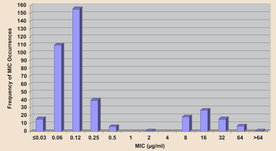


Figure 2. Distribution of erythromycin MIC results against *Campylobacter* spp. (417 isolates)

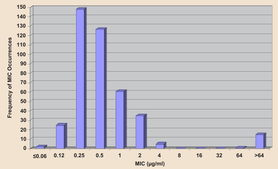


Figure 3. Comparison of ciprofloxacin MIC values and correlate disk zone diameter (mm) measurements when testing 417 isolates of *Campylobacter* spp.

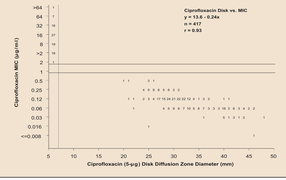
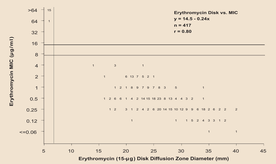


Figure 4. Comparison of erythromycin MIC values and correlate disk zone diameter (mm) measurements when testing 417 isolates of *Campylobacter* spp.



CONCLUSIONS

- Development of a practical and economical test that does not require new technology or expertise for detection of fluoroquinolone- and macrolide-resistant *Campylobacter* spp. may be of use in clinical laboratories, including those in resource poor regions.
- At this time, the presence of any ciprofloxacin or erythromycin disk zone of inhibition requires further action (MIC determination) to better characterize the level of susceptibility of the tested strain.
- 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 to assist resistance epidemiologic investigations, as well as to monitor seriously ill patients or those patients who have failed therapy.

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