

Comparison of Antimicrobial Susceptibility Testing of *Campylobacter* spp. by the Agar Dilution and the Agar Disk Diffusion Methods[∇]

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The correlation and the level of agreement between the standardized agar dilution and the agar disk diffusion methods for antimicrobial susceptibility testing of *Campylobacter* were investigated. A high-level agreement between the two methods was evident for aminoglycosides and fluoroquinolones, while a low-level agreement was observed for other antibiotics.

Campylobacter species, particularly *Campylobacter jejuni*, have been recognized as an important cause of food-borne bacterial diarrhea in humans worldwide (2). As enteric organisms, *Campylobacter* spp. are carried in the intestinal tracts of food animals, especially poultry, and they are often present in food of animal origin through fecal contamination during processing (17). Although most patients with *Campylobacter* infections do not require antibiotic treatment, antimicrobial therapy is necessary for patients with severe or prolonged systemic diseases (2, 4). In this circumstance, macrolides (e.g., erythromycin) and fluoroquinolones (e.g., ciprofloxacin) are considered the drugs of choice (2, 5). However, other antibiotics such as gentamicin, tetracycline, clindamycin, and ampicillin may be listed as alternative drugs for the treatment of systemic *Campylobacter* infections (5). Usually, antimicrobial susceptibility testing prior to treatment of *Campylobacter* infections is unnecessary; however, it may be useful, especially with the increase of resistant *Campylobacter* organisms. Several antimicrobial susceptibility testing methods, including agar dilution, broth microdilution, epsilometer test (E-test), and disk diffusion test, have been used to measure antimicrobial resistance in *Campylobacter* species (1, 9, 10, 11, 12, 13, 14, 15, 16, 18, 19, 20, 24). Recently, the agar dilution method has been considered a standard antimicrobial susceptibility testing method for thermophilic *Campylobacter* species (19, 22). Although the agar dilution method is reliable and highly reproducible and also provides quantitative MICs, it is a labor-intensive, time-consuming, and costly test (6, 20). Alternatively, the agar diffusion test, such as the disk diffusion method, is simple and inexpensive and can provide reproducible results if it is conducted carefully with appropriate standardization and quality controls (6, 25). Over the years, several comparisons of the agreement between different

antimicrobial susceptibility testing methods for *Campylobacter* species have been conducted (1, 9, 10, 11, 12, 13, 15, 16, 18, 24). However, these studies were performed prior to the establishment of a standardized antimicrobial susceptibility test for *Campylobacter*. Since a standardized test is proposed for *Campylobacter* (19), no information has been reported on the agreement between the standardized agar dilution method and the agar disk diffusion method. Hence, the aim of this study was to determine whether the agar disk diffusion test could be used as a reliable alternative method for antimicrobial susceptibility testing of *Campylobacter* species.

Six hundred sixty-eight *Campylobacter* isolates (431 *C. jejuni* and 237 *Campylobacter coli*), obtained from the intestinal tracts of poultry with different histories of antibiotic exposure, were evaluated for resistance to nine antimicrobial agents, including ampicillin, tetracycline, gentamicin, kanamycin, clindamycin, erythromycin, ciprofloxacin, norfloxacin, and nalidixic acid, by both the standardized agar dilution method and the disk diffusion method according to the guideline established by the CLSI (formerly NCCLS) (22). All antimicrobial agents for the agar dilution method were obtained from Sigma Chemical Co., St. Louis, MO, except ciprofloxacin (Serologicals Proteins, Inc., Kankakee, IL), and antibiotic disks for the disk diffusion method were obtained from Becton Dickinson and Company, Sparks, MD. The concentrations of antimicrobial agents tested in this study are shown in Table 1. For the agar dilution method, after *Campylobacter* suspensions were adjusted to a turbidity equivalent to a 0.5 McFarland standard, approximately 10⁴ CFU of these suspensions was inoculated onto Mueller-Hinton agar containing a twofold dilution series of antibiotics and supplemented with 5% defibrinated sheep blood using a multipoint inoculator (a Cathra replicator system) with 1-mm pins (Oxoid, Inc., Ogdensburg, NY). For the disk diffusion method, sterile cotton-tipped swabs were used to transfer the inoculum onto Mueller-Hinton plates to produce a confluent lawn of bacterial growth. After the inoculum on the plates was dried, antibiotic disks were distributed over the inoculated plates using a BBL Sensi-disc dispenser (BBL Becton Dickinson Microbiology Systems, Cockeysville, MD). These plates were then incubated at 42°C for 24 h under

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TABLE 1. Breakpoints of the agar dilution and disk diffusion methods used to determine antimicrobial susceptibility of *Campylobacter* isolates

Antimicrobial agent	Result for method:							
	Agar dilution				Disk diffusion			
	Test range ($\mu\text{g/ml}$)	MIC breakpoint ($\mu\text{g/ml}$) ^a			Disk concn (μg)	Zone diam breakpoint (mm) ^b		
		S	I	R		S	I	R
Ampicillin	0.06–128	≤8	16	≥32	10	≥17	14–16	≤13
Tetracycline	0.06–128	≤4	8	≥16	30	≥19	15–18	≤14
Gentamicin	0.06–128	≤4	8	≥16	10	≥15	13–14	≤12
Kanamycin	0.25–128	≤16	32	≥64	30	≥18	14–17	≤13
Clindamycin	0.06–128	≤0.5	1–2	≥4	2	≥21	15–20	≤14
Erythromycin	0.06–128	≤0.5	1–4	≥8	15	≥23	14–22	≤13
Ciprofloxacin	0.008–128	≤1	2	≥4	5	≥21	16–20	≤15
Norfloxacin	0.06–128	≤4	8	≥16	10	≥17	13–16	≤12
Nalidixic acid	0.25–128	≤16		≥32	30	≥19	14–18	≤13

^a MIC breakpoints for enteric bacteria from the NARMS were used for all antimicrobial agents except norfloxacin. MIC breakpoints for *Enterobacteriaceae* for norfloxacin were recommended by the CLSI (formerly NCCLS). S, susceptible; I, intermediate; R, resistant.

^b Zone diameter breakpoints of ampicillin, tetracycline, gentamicin, kanamycin, clindamycin, and erythromycin for bacteria isolated from animals were recommended by the CLSI. Zone diameter breakpoints of ciprofloxacin, norfloxacin, and nalidixic acid for *Enterobacteriaceae* were recommended by the CLSI. S, susceptible; I, intermediate; R, resistant.

microaerobic conditions (5% O₂, 10% CO₂, and 85% N₂). *C. jejuni* ATCC 33560 was used as a quality control organism in this study. The MIC breakpoints and the zone diameter breakpoints of each antimicrobial agent were determined according to the breakpoints used by the National Antimicrobial Resistance Monitoring System (NARMS) and the CLSI-established guideline for bacteria isolated from animals (7, 22, 23) (Table 1). To measure the correlation and the level of agreement between the standardized agar dilution method and the agar disk diffusion method, the scatter plot, the correlation coefficient, the percent agreement, and the kappa statistic were calculated as previously described (8, 18).

Since there are no antimicrobial resistance breakpoints specific for *Campylobacter* currently available, the resistance breakpoints of enteric bacteria in the family *Enterobacteriaceae* have been used to determine antimicrobial resistance of *Campylobacter* spp. (13, 18, 21). According to these resistance breakpoints, a majority of *Campylobacter* isolates were classified as either susceptible or resistant to ciprofloxacin, norfloxacin, nalidixic acid, gentamicin, and kanamycin by both the agar dilution and the agar disk diffusion methods (Table 2). For erythromycin, clindamycin, and ampicillin, a large number of *Campylobacter* isolates were classified as intermediate to these antimicrobial agents when the current NARMS resistance breakpoints were used (Table 2). The agar dilution method and the disk diffusion method agreed well in identifying aminoglycoside and quinolone/fluoroquinolone resistance in *Campylobacter*. The percent agreements between these methods for gentamicin, kanamycin, ciprofloxacin, norfloxacin, and nalidixic acid were 99.85%, 97.46%, 94.46%, 95.81%, and 91.02%, respectively (Table 3). In terms of the kappa, an almost perfect agreement (kappa > 0.8) was also observed between the agar dilution and the disk diffusion methods for aminoglycosides and quinolone/fluoroquinolones (Table 3). In addition, the correlation coefficient and the scatter plot of the MICs and the zone diameters of

TABLE 2. Antimicrobial susceptibility patterns of *Campylobacter* spp. identified by the agar dilution and the disk diffusion methods^a

Antimicrobial agent	Agar dilution method				Disk diffusion method			
	No. of <i>Campylobacter</i> isolates ^b			% of resistant isolates	No. of <i>Campylobacter</i> isolates ^b			% of resistant isolates
	S	I	R		S	I	R	
Ampicillin	434	154	75	11.31	416	138	109	16.44
Tetracycline	158	4	505	75.71	195	49	423	63.42
Gentamicin	668	0	0	0	667	0	1	0.15
Kanamycin	418	2	248	37.13	415	10	243	36.38
Clindamycin	159	366	143	21.41	329	178	161	24.10
Erythromycin	76	413	179	26.80	449	62	157	23.50
Ciprofloxacin	444	10	214	32.04	437	2	229	34.28
Norfloxacin	451	4	213	31.89	439	0	229	34.28
Nalidixic acid	454	0	214	32.04	434	31	203	30.39

^a The total number of *Campylobacter* isolates tested for antimicrobial resistance was 668 for all antimicrobials except ampicillin (663 isolates) and tetracycline (667 isolates).

^b Number of susceptible (S), intermediate (I), and resistant (R) *Campylobacter* isolates identified by the agar dilution method or by the disk diffusion method.

each antimicrobial agent evaluated in this study also demonstrated a correlation between the standardized agar dilution and the agar disk diffusion methods for aminoglycosides and quinolone/fluoroquinolones as well as for erythromycin, clindamycin, and tetracycline. The correlation coefficients of kanamycin, ciprofloxacin, erythromycin, clindamycin, and tetracycline were 0.937, -0.86, -0.885, -0.8, and -0.863, respectively, whereas the correlation coefficient between the MICs and the zone diameters of ampicillin was -0.588. When the numbers of falsely susceptible and falsely resistant *Campylobacter* isolates were investigated, the numbers of isolates that were classified as resistant by the agar dilution method but susceptible by the disk diffusion method (falsely susceptible) were less than 1.5% of the isolates tested for resistance to every antimicrobial agent except ampicillin and tetracycline (Table 3). Likewise, the numbers of *Campylobacter* isolates that were classified as susceptible by the agar dilution method but resistant by the disk diffusion method (falsely resistant) were less than 3% of the isolates tested for resistance to every antimicrobial agent except ampicillin (Table 3).

In this study, *Campylobacter* strains that were resistant to fluoroquinolones and aminoglycosides by the standardized agar dilution method were also resistant to these antimicrobial agents by the disk diffusion method. No zones of inhibition around these antibiotic disks were observed among the resistant *Campylobacter* strains, while large clear zones of inhibition averaging more than 37 mm in diameter were observed around the disks of the susceptible *Campylobacter* strains. The drastic difference in the zone of inhibition diameters, a high correlation coefficient, a high percent agreement, and a high kappa value between the standardized agar dilution and the agar disk diffusion methods indicate that the disk diffusion test is a reliable screening method for antimicrobial susceptibility testing of thermophilic *Campylobacter* to quinolone/fluoroquinolone and aminoglycoside antibiotics. This finding is correlated well with the previous study by Gaudreau and Gilbert, who reported a complete agreement between the agar dilution method and the disk diffusion method for susceptibility testing of *C. jejuni* and *C. coli* to ciprofloxacin (12). In addition,

TABLE 3. Agreement between the agar dilution and disk diffusion methods for antimicrobial susceptibility testing of *Campylobacter* species^a

Antimicrobial agent	No. of <i>Campylobacter</i> isolates				Total no. of isolates tested by both AD and DD	Total no. of isolates with agreement of results for both AD and DD	% agreement between AD and DD	Kappa ^b
	Susceptible by both AD and DD	Susceptible by AD but resistant by DD	Susceptible by DD but resistant by AD	Resistant by both AD and DD				
Ampicillin	336	39	14	45	663	444	66.97	0.37
Tetracycline	143	14	50	408	667	552	82.76	0.62
Gentamicin	667	1	0	0	668	667	99.85	NA ^c
Kanamycin	408	1	6	243	668	651	97.46	0.95
Clindamycin	127	0	7	129	668	397	59.43	0.41
Erythromycin	74	0	9	150	668	264	39.52	0.25
Ciprofloxacin	422	20	5	209	668	631	94.46	0.88
Norfloxacin	431	20	4	209	668	640	95.81	0.91
Nalidixic acid	425	20	9	183	668	608	91.02	0.81

^a AD, agar dilution; DD, disk diffusion.

^b The magnitude of kappa indicates the level of agreement between the two tests as follows: <0.2, slight agreement; 0.2 to 0.4, fair agreement; 0.4 to 0.6, moderate agreement; 0.6 to 0.8, substantial agreement; >0.8, almost perfect agreement.

^c The kappa value for gentamicin could not be calculated because none of the isolates was classified as resistant to this antibiotic by both methods.

Frediani-Wolf and Stephan also suggested that the disk diffusion method can be used as a reliable and easy tool for monitoring the prevalence of ciprofloxacin-resistant *C. jejuni* strains, although they found a weak correlation between the MIC and zone diameter results for ciprofloxacin-susceptible strains (11).

When the current NARMS resistance breakpoints were

used, a large number of *Campylobacter* isolates were classified as intermediate to erythromycin and clindamycin by the agar dilution method. Since the MICs of erythromycin for *C. jejuni* ATCC 33560, the quality control organism, and the majority of *Campylobacter* isolates tested in this study fell consistently in a range between 1 and 4 µg/ml, this information, plus the findings from other studies (3, 12, 14, 16), warrants a change of the

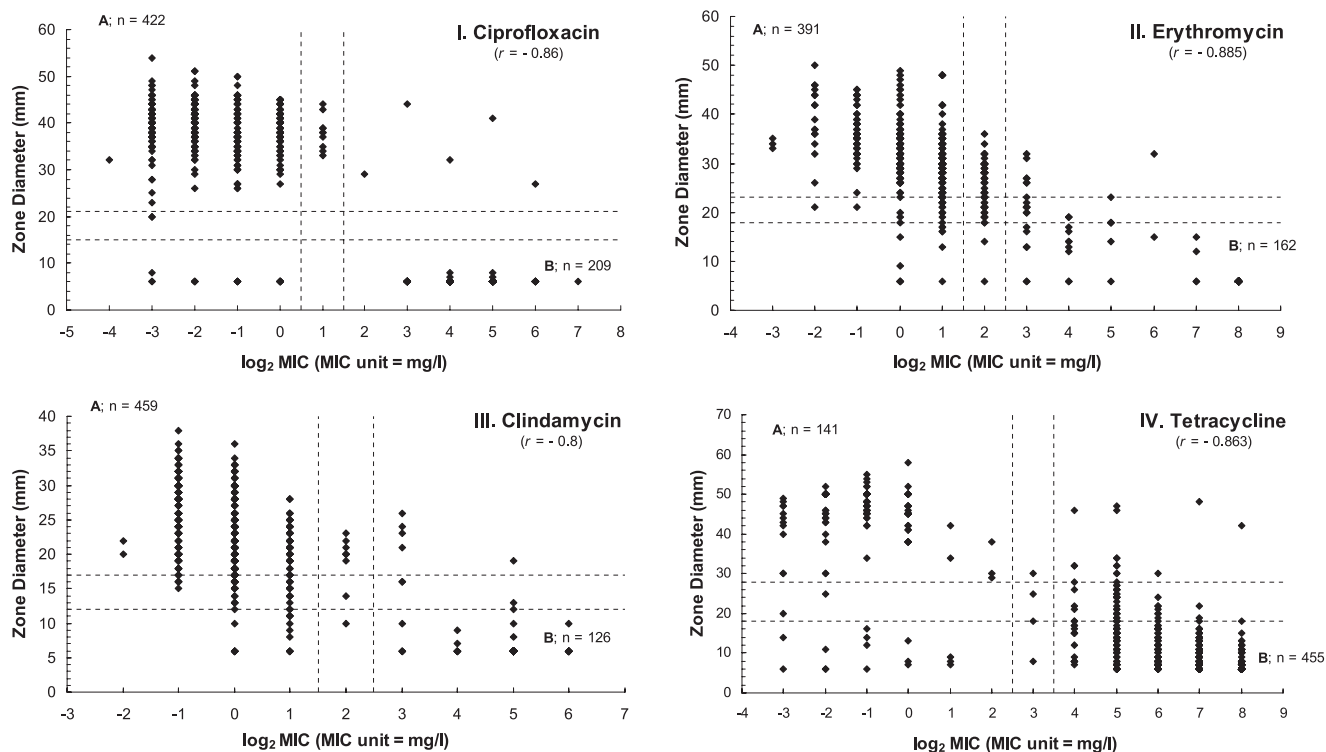


FIG. 1. Scatter plot of the MICs in the agar dilution method and the zone diameters in the disk diffusion method with tentative breakpoints (dashed line) for ciprofloxacin (I), erythromycin (II), clindamycin (III), and tetracycline (IV) against *Campylobacter* species. A, the area where *Campylobacter* isolates were susceptible to the antimicrobial agent by both the agar dilution and the disk diffusion methods. B, the area where *Campylobacter* isolates were resistant to the antimicrobial agent by both the agar dilution and the disk diffusion methods. The values of -4, -3, -2, -1, 0, 1, 2, 3, 4, 5, 6, and 7 on the x axis represents the log₂ MIC of each MIC, which are equivalent to 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 µ/ml, respectively.

MIC breakpoints of erythromycin for susceptible *Campylobacter* isolates. Our data suggest that the breakpoint for erythromycin-susceptible *Campylobacter* strains may be more appropriately set at ≤ 2 $\mu\text{g/ml}$ instead of ≤ 0.5 $\mu\text{g/ml}$. If the MIC breakpoints of erythromycin for thermophilic *Campylobacter* are changed to ≤ 2 $\mu\text{g/ml}$ for susceptible isolates and ≥ 8 $\mu\text{g/ml}$ for resistant isolates and the zone diameter breakpoints of the disk diffusion method are set at ≥ 23 mm for susceptible isolates and ≤ 18 mm for resistant isolates (Fig. 1), the percent agreement as well as the kappa value between the agar dilution method and the disk diffusion method for erythromycin would significantly improve, while the numbers of falsely susceptible and falsely resistant isolates would still be less than 1.5% and 3%, respectively. Likewise, if the MIC breakpoints for clindamycin are changed to ≤ 2 $\mu\text{g/ml}$ for susceptible isolates and ≥ 8 $\mu\text{g/ml}$ for resistant isolates and the zone diameter breakpoints are changed to ≥ 17 mm for susceptible isolates and ≤ 12 mm for resistant isolates (Fig. 1), the percent agreement and the kappa value between the agar dilution and the disk diffusion methods for clindamycin would increase dramatically. Also, the numbers of falsely susceptible and falsely resistant *Campylobacter* isolates would still be in an acceptable range.

Some *Campylobacter* isolates that were determined to be tetracycline resistant by the agar dilution method were classified as susceptible or intermediate by the disk diffusion method. This may explain why the percent agreement and the kappa between the agar dilution method and the disk diffusion method for tetracycline were not as high as those of the quinolone/fluoroquinolones and aminoglycosides. Although the kappa value for tetracycline demonstrated moderate agreement (kappa = 0.62) between the two methods, the correlation coefficient revealed a strong correlation ($r = -0.863$) between the agar dilution and disk diffusion methods. Likewise, Alfredson et al. also reported that the disk diffusion method correlated well with the agar dilution method when they were used for the screening of tetracycline-resistant *Campylobacter* strains (1). Although the current breakpoints used for tetracycline for enteric bacteria belonging to the family *Enterobacteriaceae* may be used for *Campylobacter* spp., it will be better if the zone diameter breakpoints are modified to ≥ 28 mm for susceptible strains and ≤ 18 mm for resistant strains (Fig. 1). These new zone diameter breakpoints for tetracycline will help reduce the numbers of falsely susceptible *Campylobacter* strains by the agar disk diffusion test from 50 isolates to 16 isolates while increasing the percent agreement between the agar dilution method and the disk diffusion method to 89.51%.

In this study, a weak agreement (kappa = 0.37) and a weak correlation ($r = -0.58$) between the agar dilution and the disk diffusion methods was observed for ampicillin. Similar to our finding, Gaudreau and Gilbert also reported that the correlation coefficient between the two methods for ampicillin was only 0.57 (12). Since the scatter plot of the MICs and the zone diameters for ampicillin were widely distributed in this study and because the correlation between the two methods was quite poor, the tentative breakpoints of ampicillin for thermophilic *Campylobacter* cannot be provided by this study.

Although the disk diffusion method is not as complicated to perform as the agar dilution method and provides reliable results for several classes of antimicrobials, only qualitative

data can be obtained from this method. Moreover, the poor growth of *Campylobacter* isolates on the plates, which was observed sometimes with the disk diffusion method, can also cause difficulty in interpreting the antimicrobial resistance results. Nevertheless, this method is very useful, especially when several antimicrobial agents need to be tested against a few isolates. If the quantitative data are required, other methods, such as the agar dilution method or the E-test, should be used.

In conclusion, this study reveals a high-level correlation between the standardized agar dilution method and the agar disk diffusion method for aminoglycosides, quinolone/fluoroquinolones, erythromycin, clindamycin, and tetracycline in evaluating the resistance of *Campylobacter* spp. to these antimicrobial agents. This study also suggests that the disk diffusion method can be used as a reliable alternative method for susceptibility testing of thermophilic *Campylobacter* to several classes of antimicrobial agents, particularly to quinolone/fluoroquinolones and aminoglycosides. Based on the data obtained in this study, we proposed some changes for the breakpoints of erythromycin, clindamycin, and tetracycline. However, until the standard breakpoints specific for *Campylobacter* are established and validated, the agar dilution method is likely to be a preferable method for determining antimicrobial resistance of *Campylobacter* species.

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