

Comparison of Agar Dilution, Disk Diffusion, MicroScan, and Vitek Antimicrobial Susceptibility Testing Methods to Broth Microdilution for Detection of Fluoroquinolone-Resistant Isolates of the Family *Enterobacteriaceae*

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Fluoroquinolone resistance appears to be increasing in many species of bacteria, particularly in those causing nosocomial infections. However, the accuracy of some antimicrobial susceptibility testing methods for detecting fluoroquinolone resistance remains uncertain. Therefore, we compared the accuracy of the results of agar dilution, disk diffusion, MicroScan Walk Away Neg Combo 15 conventional panels, and Vitek GNS-F7 cards to the accuracy of the results of the broth microdilution reference method for detection of ciprofloxacin and ofloxacin resistance in 195 clinical isolates of the family *Enterobacteriaceae* collected from six U.S. hospitals for a national surveillance project (Project ICARE [Intensive Care Antimicrobial Resistance Epidemiology]). For ciprofloxacin, very major error rates were 0% (disk diffusion and MicroScan), 0.9% (agar dilution), and 2.7% (Vitek), while major error rates ranged from 0% (agar dilution) to 3.7% (MicroScan and Vitek). Minor error rates ranged from 12.3% (agar dilution) to 20.5% (MicroScan). For ofloxacin, no very major errors were observed, and major errors were noted only with MicroScan (3.7% major error rate). Minor error rates ranged from 8.2% (agar dilution) to 18.5% (Vitek). Minor errors for all methods were substantially reduced when results with MICs within ± 1 dilution of the broth microdilution reference MIC were excluded from analysis. However, the high number of minor errors by all test systems remains a concern.

Fluoroquinolones, such as ciprofloxacin, ofloxacin, and levofloxacin, are commonly used for treatment of a variety of infectious illnesses (2, 14). Newer fluoroquinolones, including sparfloxacin, trovafloxacin, and clinafloxacin, promise even greater activity against a wide variety of pathogens (5, 24, 25). However, resistance to fluoroquinolones appears to be increasing in many species of clinically important bacteria, which may limit the utility of these drugs (1, 3, 8, 18, 26). Mutations in several genetic loci in gram-negative bacteria, including *gyrA*, *gyrB*, *parC*, and *parE*, all are associated with fluoroquinolone resistance (7, 9, 12, 13, 15, 22). Thus, detecting phenotypic resistance to this class of antimicrobial agent is important for guiding therapy. Doern and colleagues reported problems detecting ciprofloxacin resistance with Vitek panels, although corrective action by the manufacturer appears to have resolved the problem (6). However, the accuracy of other methods of antimicrobial susceptibility testing, such as disk diffusion, for detecting fluoroquinolone resistance has not been assessed recently.

From June 1994 to October 1995, 195 clinical isolates of the family *Enterobacteriaceae* assessed as intermediate or resistant to ciprofloxacin and/or ceftazidime were collected from six hospitals in the United States for phase I of Project ICARE (Intensive Care Antimicrobial Resistance Epidemiology) (19). The majority of these isolates were tested at the hospitals by automated methods. To assess testing accuracy, the 195 pooled

isolates from the family *Enterobacteriaceae* were used as a challenge panel to compare fluoroquinolone susceptibilities by agar dilution, disk diffusion, MicroScan conventional panels, and Vitek cards versus susceptibility by the broth microdilution reference method. The use of this predominantly resistant pool of isolates to assess the accuracy of testing methodologies is advantageous in that it uncovers difficulties that might go unnoticed with the testing of a large number of highly susceptible isolates.

MATERIALS AND METHODS

Bacterial strains. A total of 195 isolates from the family *Enterobacteriaceae* collected from six U.S. hospitals were tested for fluoroquinolone resistance (Table 1). Identifications for common organisms (e.g., *Escherichia coli* and *Klebsiella pneumoniae*) were confirmed by colonial morphology, odor, and spot tests (11). Identifications for unusual isolates were confirmed by using reference biochemical tests as performed at the Centers for Disease Control and Prevention (4, 10).

Susceptibility testing methods. Agar dilution, broth microdilution, and disk diffusion were performed as described by the National Committee for Clinical Laboratory Standards (20, 21). MicroScan (Dade Behring, Inc., W. Sacramento, Calif.) and Vitek (bioMérieux Vitek, Inc., Hazelwood, Mo.) susceptibility tests were performed according to the manufacturers' directions. Quality control was performed each day of testing for broth microdilution and agar dilution with *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, and *Pseudomonas aeruginosa* ATCC 27853. Quality control for disk diffusion testing was also performed each day with *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, and *E. coli* ATCC 25922. Quality control for testing by Vitek and MicroScan was performed before each new lot of cards or panels was used. *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were used for quality control testing for both Vitek and MicroScan. In addition, *E. faecalis* ATCC 29212 and *E. coli* ATCC 35218 were used for quality control testing for the MicroScan.

Ciprofloxacin was obtained from Bayer Corporation, Pharmaceutical Division (West Haven, Conn.), and ofloxacin was obtained from Sigma Chemical Co. (St. Louis, Mo.) for use in the agar dilution plates and broth microdilution panels. Stock concentrations of each antimicrobial agent were prepared and frozen in

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TABLE 1. One hundred ninety-five isolates of the family *Enterobacteriaceae* from phase I of Project ICARE (19)

Organism identification	No. (%)	No. of isolates intermediate or resistant to indicated drug by broth microdilution	
		Ciprofloxacin	Ofloxacin
<i>Klebsiella pneumoniae</i>	65 (33.3)	30	27
<i>Escherichia coli</i>	32 (16.4)	28	29
<i>Enterobacter cloacae</i>	18 (9.2)	15	16
<i>Proteus mirabilis</i>	18 (9.2)	17	17
<i>Citrobacter freundii</i>	15 (7.7)	13	13
<i>Serratia marcescens</i>	15 (7.7)	12	12
<i>Enterobacter aerogenes</i>	11 (5.7)	9	9
<i>Providencia stuartii</i>	10 (5.1)	9	10
<i>Klebsiella oxytoca</i>	3 (1.6)	3	3
<i>Morganella morganii</i>	3 (1.6)	3	3
<i>Citrobacter koseri</i>	1 (0.5)	0	0
<i>Klebsiella species</i>	1 (0.5)	0	0
<i>Providencia rettgeri</i>	1 (0.5)	0	0
<i>Proteus penneri</i>	1 (0.5)	1	1
<i>Serratia species</i>	1 (0.5)	1	1

aliquots at -70°C before the study began. The agar dilution plates were made in-house daily with previously prepared antimicrobial agent stocks and Mueller-Hinton II powder (Becton Dickinson Microbiological Systems [BDMS], Cockeysville, Md.). The broth microdilution plates were prepared in-house and kept frozen at -70°C until the day of use. Commercially prepared 150-mm-diameter plates with Mueller-Hinton Agar II (BDMS) and 5- μg ciprofloxacin and 5- μg ofloxacin disks (BDMS) were used for disk diffusion testing. MicroScan Neg Combo 15 panels and Vitek GNS-F7 cards were selected for this study because they contain both ciprofloxacin and ofloxacin and are widely used in clinical laboratories.

On each day of testing, 18- to 24-h-old colonies from a plate inoculated from a single colony were used to prepare the inoculum for all systems. Broth microdilution plates were inoculated with MIC-2000 disposable inoculators (Dynatech Laboratories, Inc., Chantilly, Va.). MicroScan software DMS version 20.3 and Vitek software R04.01 and R05.01 were used during this study. The use of two versions of Vitek software did not affect the interpretation of fluoroquinolone results. In addition to the Walk Away automated reading, manual readings were performed on all MicroScan panels. All tests showing very major errors and major errors were repeated in duplicate by the test method and the broth microdilution reference method.

Data analysis. All data analysis was performed by using The SAS System for Windows, release 6.12 (SAS Institute, Cary, N.C.). The resistance breakpoints used in this study were those defined by National Committee for Clinical Laboratory Standards (Table 2). These breakpoints were used to calculate very major, major, and minor errors between the broth microdilution reference method and agar dilution, disk diffusion, MicroScan, and Vitek results. Very major errors occurred with organisms for which MICs indicated resistance by broth microdilution and susceptibility by the test method. Major errors occurred with organisms for which MICs indicated susceptibility by broth microdilution and resistance by the test method. Minor errors occurred with organisms for which MICs indicated intermediate resistance by broth microdilution or another test method and susceptibility or resistance by the other method (either broth microdilution or another test method). Denominators for calculating error rates, based on broth microdilution results, were as follows: the number of resistant isolates (very major error rate), the number of susceptible isolates (major error rate), and the total number of isolates tested (minor error rate).

Mantel-Haenszel chi-square and Fisher's exact test (FET) *P* values were used to determine if errors were associated with specific species (16, 23). The Wil-

coxon signed-rank test was performed with MIC distributions determined by agar dilution versus broth microdilution to assess any trends in MIC disagreement (17). A *P* value of ≤ 0.05 defined significant associations.

RESULTS

One hundred ninety-five isolates of the family *Enterobacteriaceae* were tested for resistance to ciprofloxacin and ofloxacin by broth microdilution, agar dilution, disk diffusion, MicroScan conventional panels, and Vitek cards (Table 1). The ranges of MICs tested in each susceptibility category by the various methods are shown in Table 2.

Ciprofloxacin testing. The error rates for the various test methods for determining susceptibility to ciprofloxacin compared to the results for broth microdilution testing are shown in Table 3. The major error rates for agar dilution, disk diffusion, MicroScan, and Vitek were 0, 1.9, 3.7, and 3.7%, respectively. Upon repeat testing, the disk diffusion and the two MicroScan major errors resolved; neither Vitek major error resolved.

Only agar dilution (0.9%) and Vitek (2.7%) produced very major errors. Upon repeat testing, the agar dilution very major error did not resolve, but one of the three Vitek very major errors resolved, lowering the percent error to 1.8%. The two remaining very major errors associated with Vitek testing involved *Enterobacter cloacae* isolates from two different hospitals. For these results, MICs were consistently ≤ 0.5 $\mu\text{g}/\text{ml}$ by Vitek, while broth microdilution showed MICs of 4 $\mu\text{g}/\text{ml}$. One of these isolates also accounted for the very major error associated with agar dilution testing, with MICs of 0.5, 1, and 2 $\mu\text{g}/\text{ml}$ upon repeat agar dilution testing.

The MICs for 134 (68.7%) of the 195 isolates tested for susceptibility to ciprofloxacin were the same by agar dilution and broth microdilution. Discordant results were typically 1 dilution lower by agar dilution than by broth microdilution (46 of 195; 23.6%). A Wilcoxon signed-rank test performed on the distribution of MICs by agar dilution compared to those by broth microdilution showed that the distribution of ciprofloxacin MICs by agar dilution was significantly lower than that by broth microdilution (one-tailed *P* value = 0.0001). Because limited dilutions were tested by Vitek and MicroScan, similar comparisons could not be determined for these methods.

Ofloxacin testing. The errors for ofloxacin testing are shown in Table 3. There were no very major errors noted in the study. In fact, with two exceptions, only minor errors were observed. The two major errors involved the testing of two *K. pneumoniae* isolates from the same hospital. Both errors were associated with MicroScan testing, and both were resolved upon repeat testing. Neither isolate produced a major error when tested against ciprofloxacin by MicroScan.

The MICs for 146 (74.9%) of the 195 isolates tested for susceptibility to ofloxacin were the same by agar dilution and broth microdilution. A Wilcoxon signed-rank test comparing the distribution of MICs by agar dilution to that of MICs by

TABLE 2. Ranges of MICs tested, by susceptibility test category and method, for 195 isolates of the family *Enterobacteriaceae*

Test method	Ciprofloxacin			Ofloxacin		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
Agar dilution	0.06–1 $\mu\text{g}/\text{ml}$	2 $\mu\text{g}/\text{ml}$	4–16 $\mu\text{g}/\text{ml}$	0.06–2 $\mu\text{g}/\text{ml}$	4 $\mu\text{g}/\text{ml}$	8–32 $\mu\text{g}/\text{ml}$
Broth microdilution	0.06–1 $\mu\text{g}/\text{ml}$	2 $\mu\text{g}/\text{ml}$	4–16 $\mu\text{g}/\text{ml}$	0.06–2 $\mu\text{g}/\text{ml}$	4 $\mu\text{g}/\text{ml}$	8–32 $\mu\text{g}/\text{ml}$
Disk diffusion	≥ 21 mm	16–20 mm	≤ 15 mm	≥ 16 mm	13–15 mm	≤ 12 mm
MicroScan GNC 15 panel (two wells)	1 $\mu\text{g}/\text{ml}$	2 $\mu\text{g}/\text{ml}$	— ^a	2 $\mu\text{g}/\text{ml}$	4 $\mu\text{g}/\text{ml}$	—
Vitek GNS-F7 card (three wells)	0.5–1 $\mu\text{g}/\text{ml}$	2 $\mu\text{g}/\text{ml}$	—	1–2 $\mu\text{g}/\text{ml}$	4 $\mu\text{g}/\text{ml}$	—

^a No concentration in the resistant range was tested by this method.

TABLE 3. Number of ciprofloxacin and ofloxacin errors by method compared to results by the broth microdilution reference method for 195 isolates of the family *Enterobacteriaceae*

Test method	No. (%) of errors for ciprofloxacin			No. (%) of errors for ofloxacin		
	Minor (<i>n</i> = 195) ^a	Major (<i>n</i> = 54) ^a	Very major (<i>n</i> = 110) ^a	Minor (<i>n</i> = 195) ^a	Major (<i>n</i> = 54) ^a	Very major (<i>n</i> = 116) ^a
Agar dilution	24 (12.3)	0	1 (0.9)	16 (8.2)	0	0
Disk diffusion	33 (16.9)	1 (1.9)	0	32 (16.4)	0	0
MicroScan	40 (20.5)	2 (3.7)	0	27 (13.8)	2 (3.7)	0
Vitek (MICs)	28 (14.4)	2 (3.7)	3 (2.7)	36 (18.5)	0	0

^a Denominator used for calculating error rates.

broth microdilution showed that the distribution of ofloxacin MICs was not significantly lower by agar dilution than by broth microdilution (one-tailed *P* value = 0.098).

Comparison of ciprofloxacin and ofloxacin data. Many of the minor errors observed for both ciprofloxacin and ofloxacin clustered around the intermediate breakpoints, which consist of only a single MIC (Table 2). Thus, a change in the MIC for an organism by ± 1 dilution frequently resulted in a minor error. When errors within ± 1 dilution were excluded from the data shown in Table 3, the number of minor errors dramatically decreased. The adjusted error rates are shown in Table 4.

Disk diffusion testing also produced many minor errors compared to broth microdilution. However, 30 of the 33 minor errors for ciprofloxacin and 32 of the 32 minor errors for ofloxacin were within 3 mm of the broth microdilution interpretive category.

Ciprofloxacin and ofloxacin interpretive results were in agreement for most isolates by the susceptibility test methods examined. By all methods, some isolates tested intermediate to one fluoroquinolone and susceptible or resistant to the other. Only two isolates tested susceptible to one fluoroquinolone and resistant to the other. One, a *K. pneumoniae* isolate, was resistant to ciprofloxacin (MIC = 4 μ g/ml) and susceptible to ofloxacin (MIC = 2 μ g/ml) by agar dilution. The other, a *C. freundii* isolate, was susceptible to ciprofloxacin (zone = 21 mm) and resistant to ofloxacin (zone = 11 mm) by disk diffusion.

Within specific test methods, certain species were significantly associated with errors. For example, *K. pneumoniae* isolates tested against ofloxacin by disk diffusion produced minor error associations (18 of 65 isolates, Mantel-Haenszel chi-square = 9.047, *P* value = 0.003). *Serratia marcescens* isolates tested against ciprofloxacin by Vitek produced minor error associations (5 of 15 isolates; FET upper-tail *P* value = 0.045). In addition, a significant number of *E. cloacae* isolates (7 of 18) tested against ciprofloxacin produced very major, major, and minor errors by Vitek cards (FET upper-tail *P* value = 0.017). Within this group of *E. cloacae* isolates causing Vitek ciprofloxacin errors, 2 of 18 organisms produced two of the three very major errors (FET upper-tail *P* value = 0.023).

Vitek expert system results. The Vitek results reported in Tables 3 and 4 were based on the actual instrument-reported MICs. However, the Vitek expert system interpretations showed similar results. Prior to repeat testing, the expert system reported 3 (2.7%) very major errors, 2 (3.7%) major errors, and 26 (13.3%) minor errors for ciprofloxacin. For ofloxacin, the expert system reported no very major or major errors and 37 (19.0%) minor errors.

DISCUSSION

Resistance to ciprofloxacin and ofloxacin is emerging in a variety of genera of the family *Enterobacteriaceae* (1, 3, 18, 26).

The automated susceptibility instruments used by most clinical laboratories, MicroScan and Vitek, did not perform well compared to broth microdilution because of the high level of minor errors, although many of the errors were within 1 dilution of the reference value. Manual readings of MicroScan panels, which were typically within ± 1 dilution from the instrument readings, did not indicate any systematic errors by this method.

The MicroScan panels and Vitek cards included in the study contained only two or three wells for ciprofloxacin and ofloxacin susceptibility determinations. Because of the small number of wells and the fact that the intermediate range for both antimicrobial agents consists of a single dilution, an error in one well could cause the report to be inaccurate. When errors within ± 1 dilution of the broth microdilution reference method were excluded from analysis, most minor errors were eliminated. Many of the minor errors produced by disk diffusion testing were within 3 mm of the broth microdilution interpretive category.

Agar dilution testing performed very well compared to broth microdilution. Fewer errors occurred by agar dilution than by all other susceptibility methods. For both ciprofloxacin and ofloxacin, only eight errors occurred outside ± 1 dilution from the reference broth microdilution MICs. Three of these errors (one very major and two minor) were interpretive category errors. Even though the Wilcoxon signed-rank test showed that ciprofloxacin MICs were significantly lower by agar dilution than by broth microdilution, the only result greater than 1 dilution lower was the very major error. The very major error, for an *E. cloacae* isolate, remained even after repeat testing and may reflect the unique interaction of fluoroquinolones with this isolate.

Within each test method, the agreement between ciprofloxacin and ofloxacin with breakpoint interpretations was very good. Ofloxacin MICs were generally 1 dilution higher than ciprofloxacin MICs for the same organism. However, there was good interpretive agreement because of the different break-

TABLE 4. Adjusted error rates for ciprofloxacin and ofloxacin MIC determinations, excluding errors within ± 1 dilution by broth reference test method for 195 isolates of the family *Enterobacteriaceae*^a

Test method	No. (%) of errors for ciprofloxacin ^b			No. (%) of errors for ofloxacin ^b		
	Minor	Major	Very major	Minor	Major	Very major
Agar dilution	1 (0.5)	0	1 (0.9)	1 (0.5)	0	0
MicroScan	2 (1.0)	0	0	3 (1.5)	0	0
Vitek	3 (1.5)	2 (3.7)	2 (1.8)	2 (1.0)	0	0

^a Error rates after repeat testing.

^b Denominators used for calculating error rates are the same as those in Table 3.

points for the two agents; the ofloxacin breakpoint is 1 dilution higher than the ciprofloxacin breakpoint.

Only two isolates were associated with very major or major errors in more than one system. One, a *K. pneumoniae* isolate, produced a major error by both the disk diffusion and MicroScan test methods with ciprofloxacin. The other, an *E. cloacae* isolate, produced a very major error by both the Vitek and agar dilution test methods with ciprofloxacin. All other very major errors and major errors were noted with different isolates. The mechanisms of resistance in these isolates and in the *E. cloacae* isolate that produced a very major error by agar dilution testing are under investigation.

The error rates observed in this study are due in part to the selection of organisms, which favors resistant strains and includes many organisms for which ciprofloxacin and ofloxacin MICs are close to the intermediate breakpoint. These organisms challenge the susceptibility test methods to detect intermediate and resistant MICs, highlighting problems with various testing methods that may not be uncovered in a largely susceptible isolate population. The MICs for the strains in this study and the prevalence of resistant strains are likely higher than those for strains commonly encountered in clinical microbiology laboratories at this time. This study demonstrates the need to continuously monitor the susceptibility patterns of various species to fluoroquinolones.

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