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Performance of Etest and Disk Diffusion for Detection of Ciprofloxacin and Levofloxacin Resistance in *Salmonella enterica*

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We compared Etest and disk diffusion to broth microdilution for the detection of fluoroquinolone resistance in 135 typhoidal and nontyphoidal serovars of *Salmonella*. Categorical agreements for the ciprofloxacin and levofloxacin Etests were 89.6 and 83.7%, respectively. Disk diffusion categorical agreements were 88.2 and 93.3%, respectively. Only minor errors were observed.

Salmonella gastroenteritis is normally a self-limiting disease, and antimicrobial therapy is not generally recommended (1, 2). However, antimicrobial therapy is indicated for the management of severe diarrhea or extraintestinal infection caused by *Salmonella* spp. and for the treatment of enteric fever, which is caused by *Salmonella enterica* serovars Typhi and Paratyphi A, B, and C. The fluoroquinolones ciprofloxacin and levofloxacin are important agents for the treatment of these infections (3). Fluoroquinolone resistance in *Salmonella* is a significant clinical concern, particularly among typhoid serovars of *Salmonella* isolated in Asia. The most common fluoroquinolone resistance mechanism among these isolates is mutation to the quinolone resistance-determining region (QRDR) of the topoisomerase gene *gyrA*, resulting in elevated ciprofloxacin and levofloxacin MICs (0.12 to 0.5 μg/ml) and full resistance to the quinolone nalidixic acid (MIC, ≥32 μg/ml) (4). Patients infected with such isolates have delayed responses to fluoroquinolone therapy and, in many cases, clinical failures (4). As such, the Clinical and Laboratory Standards Institute (CLSI) has historically instructed laboratories to perform a nalidixic acid disk diffusion or MIC test in order to screen for this resistance mechanism among *Salmonella* isolates for which susceptibility testing was warranted (4). However, fluoroquinolone resistance in *Salmonella* can also be attributed to mutation to the QRDR of other topoisomerase genes (*gyrB*, *parC/E*) or by the acquisition of plasmid-mediated quinolone resistance determinants, such as the *qnr* and *aac(6′)-Ib-cr* genes. In contrast to QRDR topoisomerase mutations, isolates with fluoroquinolone resistance associated with plasmid-mediated mechanisms are not necessarily resistant to nalidixic acid (5).

In 2013, the CLSI revised the ciprofloxacin, levofloxacin, and ofloxacin MIC interpretive criteria (breakpoints) for *Salmonella* and the disk diffusion breakpoints for ciprofloxacin, in part to better detect these fluoroquinolone resistance mechanisms in *Salmonella* spp. (Table 1) (6). At present, no levofloxacin or ofloxacin disk diffusion breakpoints for *Salmonella* have been established by the CLSI. The U.S. Food and Drug Administration (FDA) has accordingly revised the ciprofloxacin MIC interpretive criteria for *S. enterica* serovar Typhi but not for other *Salmonella* serovars or for levofloxacin or ofloxacin. Furthermore, no commercial MIC susceptibility test panels produced in the United States contain fluoroquinolone concentrations low enough to allow use of the current CLSI breakpoints. Thus, laboratories in the United States have the option of performing a ciprofloxacin disk diffusion test for *Salmonella*. However, it has not been demonstrated that susceptibility to levofloxacin, which is commonly the formulary fluoroquinolone in U.S. hospitals, can be inferred from ciprofloxacin results (7). In addition, some laboratories in resource-limited countries where typhoid fever is endemic and fluoroquinolone resistance is prevalent among *S. enterica* serovar Typhi isolates have anecdotally reported difficulties interpreting ciprofloxacin disk diffusion results. This finding has not been noted among U.S. laboratories.

Ciprofloxacin and levofloxacin Etests (bioMérieux, Durham, NC) are a potential alternative; however, these strips are not cleared by the FDA for use with the current CLSI breakpoints for *Salmonella* spp. Laboratories that choose to use the Etest for *Salmonella* and interpret the results using the current CLSI breakpoints must perform a verification study to ensure accurate per-

TABLE 1 Fluoroquinolone interpretive criteria for *Salmonella* used in this study

Antimicrobial and test	Interpretive criteria			Reference
	Susceptible	Intermediate	Resistant	
Ciprofloxacin				
MIC, μg/ml	≤0.06	0.12–0.5	≥1.0	6
Disk diffusion, mm	≥31	21–30	≤20	6
Levofloxacin				
MIC, μg/ml	≤0.12	0.25–1.0	≥2.0	6
Disk diffusion, mm	≥28	19–27	≤18	5

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TABLE 2 Resistance mechanisms and fluoroquinolone MIC ranges for *Salmonella* isolates ($n = 135$) included in the study

Resistance mechanism	No. of isolates (% typhoidal)	BMD[a] MIC range (μg/ml)		
		Ciprofloxacin	Levofloxacin	Nalidixic acid
aac(6′)-Ib-cr	1 (0)	1.0	1.0	32
qnr	36 (0)	0.12 to 1.0	0.25 to 1.0	4.0 to 32
QRDR mutation	45 (0)	0.06 to 0.5	0.25 to 2.0	>128
Not characterized	29 (90)	0.12 to 16	0.25 to 16	128 to >128
None	24 (25)	≤0.08 to 0.06	0.015 to 0.12	2 to 16

[a] BMD, broth microdilution.

formance. In the present study, we evaluated the performances of the Etest and disk diffusion compared to that of the CLSI reference broth microdilution (BMD) method for the determination of ciprofloxacin and levofloxacin susceptibility for a collection of *Salmonella* isolates with a variety of fluoroquinolone resistance mechanisms.

The *Salmonella* isolates tested in this study are listed in Table 2. These included 29 *S. enterica* serovar Typhi, 2 *S. enterica* serovar Paratyphi A, and 1 *S. enterica* serovar Paratyphi B isolates and 103 *Salmonella* isolates of nontyphoid serovars. Based on molecular analysis, 24 isolates had no fluoroquinolone resistance genes, 36 isolates had a *qnr* gene, 1 had an *aac(6′)-Ib-cr* gene, and 45 had a mutation to the QRDR of the *gyrA* topoisomerase gene. An additional 29 isolates with ciprofloxacin-intermediate nalidixic acid-resistant phenotypes associated with mutation to the QRDR were included in the study but were not subjected to molecular analysis.

Antimicrobial susceptibility testing was performed for ciprofloxacin, levofloxacin, and nalidixic acid using the CLSI reference broth microdilution (BMD) method in cation-adjusted Mueller-Hinton broth (6, 8) on panels prepared in-house at UCLA. Incubation was performed at 35°C in ambient air for 18 to 20 h. The ciprofloxacin and levofloxacin Etests were performed on Mueller-Hinton agar (BD, Sparks, MD) following the manufacturer's instructions. Disk diffusion was performed on Mueller-Hinton agar with 5-μg ciprofloxacin and 5-μg levofloxacin disks (BD). All three susceptibility testing methods were performed in parallel, using the same organism suspension, for each isolate. MICs determined by the Etest were rounded up to the nearest $\log_2$ dilution. We used *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213, and *Enterococcus faecalis* ATCC 29212 as quality control strains. The quality control results for all MIC and disk diffusion tests were within acceptable quality control ranges according to CLSI guidelines (6). We compared the Etest and disk diffusion results to those of the BMD method. Essential agreement (EA), categorical agreement (CA), very major errors (VMEs), major errors (MEs), and minor errors (mEs) were calculated as previously described (9). EA was defined as an MIC within a $\log_2$ dilution of the BMD MIC. CA was defined as a susceptible, intermediate, or resistant interpretation that was consistent with the BMD result. A VME was defined as a false-susceptible result, whereas an ME was a false-resistant result; an mE was a result that was intermediate by one method and resistant or susceptible by the other.

EA between the ciprofloxacin BMD and Etest MICs was excellent at 97% (Table 3). CA between the BMD and Etest for ciprofloxacin was 89.6%. No overall trend was noted for higher or lower MICs by the Etest compared to those of the BMD method. All 14 observed errors were mEs. For one isolate, the BMD MIC was 0.06 μg/ml (susceptible) and the Etest MIC was 0.12 μg/ml (intermediate). For 13 isolates, the BMD MIC was 0.5 μg/ml (intermediate) and the Etest MICs were 0.75 μg/ml or 1.0 μg/ml (resistant, when rounded up to the nearest $\log_2$ dilution). Similarly, only minor errors ($n = 16$) were identified by disk diffusion. For two isolates, the zones of inhibition were interpreted as intermediate (27 and 30 mm), but the MICs were ≤0.06 μg/ml (susceptible). For 2 isolates, the zones of inhibition were 20 mm (resistant), but the MICs were 0.5 μg/ml (intermediate). Twelve isolates had zones of inhibition that were interpreted as intermediate, but the BMD MICs were ≥1 μg/ml (resistant).

Clinically, patients whose *Salmonella* isolates showed a ciprofloxacin MIC of ≥0.12 μg/ml are treated with an alternative agent, such as ceftriaxone or azithromycin, as there are no data to indicate that high-dose ciprofloxacin monotherapy is efficacious for isolates with ciprofloxacin MICs in the intermediate range (0.12 to 0.5 μg/ml) (4). As such, the only error observed by the Etest that would impact a treatment decision was for an *S. enterica* serovar Newport isolate with a BMD MIC of 0.06 μg/ml and an Etest MIC of 0.12 μg/ml. Interestingly, this isolate also had an intermediate

TABLE 3 Performances of ciprofloxacin and levofloxacin Etests and disk diffusion compared to that of BMD for testing *Salmonella* isolates ($n = 135$)

Method and antibiotic	No. of isolates[a]				Performance (no. [%])[b]				
	Total	Susc	Int	Res	EA	CA	mE	ME	VME
Etest									
Ciprofloxacin	135	25	89	21	131 (97.0)	121 (89.6)	14 (10.4)	0	0
Levofloxacin	135	24	98	13	125 (92.6)	112 (83.7)	22 (16.3)	0	0
Disk diffusion									
Ciprofloxacin	135	25	89	21	NA	119 (88.2)	16 (11.8)	0	0
Levofloxacin	135	24	98	13	NA	127 (94.1)	8 (5.9)	0	0

[a] Values determined by BMD method. Susc, susceptible; Int, intermediate; Res, resistant.
[b] EA, essential agreement; CA, categorical agreement; VME, very major error; ME, major error; mE, minor error; NA, not applicable.

ciprofloxacin disk diffusion zone of 27 mm and a mutation in the QRDR of *gyrA*, and it was nalidixic acid resistant (MIC, >128 μg/ml). This isolate was the only one included in the study for which the ciprofloxacin MIC did not correctly identify fluoroquinolone resistance. Disk diffusion yielded ciprofloxacin-intermediate results for only two isolates that were ciprofloxacin susceptible by BMD and were negative for a fluoroquinolone resistance gene by molecular analysis. The first of these was for the *S.* Newport isolate described previously, and the second was for an *S.* Typhi isolate with a ciprofloxacin BMD MIC of 0.03 μg/ml and a nalidixic acid MIC of 8 μg/ml. Together, these data demonstrate that the Etest is an acceptable alternative for laboratories when testing *Salmonella* isolates for ciprofloxacin susceptibility. Disk diffusion is also a good option, although others have noted difficulties in reading ciprofloxacin disk diffusion results, and we have noted that the performance of disk diffusion varies by the brand of Mueller-Hinton agar used (R. Skov, unpublished observations). A previous study that evaluated ciprofloxacin disk diffusion for human and veterinary isolates of *Salmonella* noted 9 of 18 isolates (50%) with zones of inhibition of ≥31 mm but the presence of one amino acid substitution in the QRDR of *gyrA*. These isolates would be expected to have nonsusceptible ciprofloxacin MICs, although in this study, 6 of these isolates had an MIC of 0.06 μg/ml (10). All 10 isolates with *qnr* or *aac(6′)-Ib-cr* and all 5 isolates with 2 mutations in the QRDR of *gyrA* had disk diffusion zones of ≥30 mm, which is similar to our findings.

EA between the levofloxacin BMD and Etest MICs was 92.6% (Table 3). Twenty-two mEs were observed, yielding an overall CA of 83.7%. Again, no overall trend was noted for higher or lower MICs by the Etest compared to those of the BMD method. Two of the errors were with isolates that demonstrated levofloxacin-intermediate MICs of 0.25 μg/ml by the Etest and susceptible MICs of 0.12 μg/ml by BMD. The remaining 20 errors resulted from isolates that demonstrated levofloxacin-resistant MICs by the Etest and intermediate MICs by BMD. Half of these errors (n = 10) occurred among isolates for which the Etest MIC was within a $\log_2$ dilution of the BMD MIC. However, the MIC results for 10 isolates were more than 1 $\log_2$ dilution above the BMD MIC. While the CLSI has yet to publish disk diffusion breakpoints for levofloxacin, Sjölund-Karlsson and colleagues (5) have proposed levofloxacin disk diffusion breakpoints (Table 1) for *Salmonella* spp., and these were evaluated in this study. Eight mEs were noted when applying these levofloxacin breakpoints, including 1 isolate that demonstrated a zone diameter of 27 mm (intermediate) but an MIC of 0.12 μg/ml (susceptible) and 1 isolate showing a zone diameter of 28 mm (susceptible) but an MIC of 0.25 μg/ml (intermediate). The remaining 6 isolates had either an intermediate disk diffusion result and a resistant MIC (n = 5) or a resistant disk diffusion result and an intermediate MIC (n = 1).

This study demonstrates that the Etest is a reliable option for testing ciprofloxacin and levofloxacin against *Salmonella* isolates. The Etest has been shown to perform well for the detection of high-level ciprofloxacin resistance (i.e., MICs of ≥4 μg/ml) among *Salmonella* serovar Typhi isolates (7). The present study extends these findings to a collection of both typhoid and nontyphoid *Salmonella* serovars, enriched to include isolates with ciprofloxacin MICs in the intermediate range (0.12 to 0.5 μg/ml), which are among the most challenging to detect. Additionally, we demonstrated that disk diffusion performance using established CLSI interpretive criteria for ciprofloxacin and the interpretive criteria for levofloxacin suggested by Sjölund-Karlsson et al. (5) was good for this collection of *Salmonella* isolates, confirming that disk diffusion is also a reliable option for testing ciprofloxacin and levofloxacin against *Salmonella* spp. A major limitation of this study is the inclusion of only one isolate with the *aac(6′)-Ib-cr* resistance mechanism; while not prevalent in the United States, this resistance mechanism has been described in other areas of the world (11). An important consideration is that isolates with *aac(6′)-Ib-cr* alone are expected to test susceptible to levofloxacin, as the target of this enzyme, the piperazinyl amide side chain of ciprofloxacin, is not present on levofloxacin (12). A second limitation to this study is the relatively small number (n = 24) of wild-type *Salmonella* isolates included. These isolates are anticipated to be much more frequently encountered in U.S. laboratories, and as such, further testing of such isolates will be required to fully evaluate the number of MEs that might be encountered with disk diffusion and the Etest. This has important implications for laboratories in the United States, particularly those without the resources to perform a verification study for the ciprofloxacin or levofloxacin Etest, despite the fact that extraintestinal *Salmonella* infections are infrequently encountered in the United States. The results of this study do not replace those from a clinical verification study but rather provide insight about the performance of disk diffusion and the Etest for those laboratories considering these tests. Furthermore, official levofloxacin disk diffusion breakpoints from the CLSI are needed.

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