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In Vitro Susceptibility Testing of Fluoroquinolone Activity Against *Salmonella*: Recent Changes to CLSI Standards

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Fluoroquinolone (FQ) resistance in *Salmonella enterica* is a significant clinical concern. Recognition of resistance by the clinical laboratory is complicated by the multiple FQ resistance mechanisms found in *Salmonella*. The Clinical Laboratory Standards Institute (CLSI) recently addressed this issue by revising the ciprofloxacin break points for *Salmonella* species. It is critical for clinicians and laboratory workers to be aware of the multiple technical issues surrounding these revised break points. In this article, we review FQ resistance mechanisms in *Salmonella*, their clinical significance, and data supporting the revised ciprofloxacin break points. We encourage clinical laboratories to adopt the revised CLSI ciprofloxacin break points for all *Salmonella* isolates in which susceptibility testing is indicated and discuss the technical issues for laboratories using commercial antimicrobial susceptibility systems.

Nontyphoidal *Salmonella* is one of the most important foodborne pathogens [1–4]. In most cases, *Salmonella* enteritis is self-limiting, and antimicrobial therapy is not generally recommended because of potential prolongation of the carrier state [5, 6]. However, antimicrobial therapy is indicated for management of severe diarrhea and treatment of patients with enhanced susceptibility to *Salmonella*. Antimicrobial therapy is also essential for extra-intestinal infections and typhoid fever caused by the human-adapted *Salmonella* serovars Typhi and Paratyphi A–C. Although severe infections with nontyphoidal *Salmonella* are relatively rare in Europe and North America, invasive nontyphoidal *Salmonella* infections are endemic in sub-Saharan Africa [7–9]. Typhoid fever is endemic in many developing countries, particularly on the Indian subcontinent [10], where multidrug resistance (MDR) to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole is common [10, 11]. Because of this widespread resistance, ceftriaxone or a fluoroquinolone (FQ) is recommended by the World Health Organization for the treatment of uncomplicated typhoid fever, whether caused by MDR or fully susceptible organisms [12]. The FQs ciprofloxacin (CIP) and ofloxacin (OFO) are often preferred as treatment options, because they are available for oral use and are less expensive than ceftriaxone.

High-level resistance to the FQs, defined historically as a CIP minimum inhibitory concentration (MIC) ≥4 μg/mL (CLSI M100 2011), has started to emerge [13, 14] but remains rare among clinical *Salmonella* isolates worldwide [15–18]. However, over the past decade, strains of *Salmonella* with decreased CIP susceptibility (DCS) have emerged, defined as isolates with CIP MICs of 0.12–1.0 μg/mL. The MICs of strains with DCS are greater than the wild-type *Salmonella* MIC distribution of 0.008–0.06 μg/mL (Table 1) [19, 20] but less than the historical 4 μg/mL resistance break point (Figure 1)

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Table 1. Genotype and Phenotype of Common Fluoroquinolone Resistance Mechanisms

	Phenotype	
Genotype	Nalidixic Acid	Ciprofloxacin MIC (μg/mL)
Wild Type (no resistance)	Usually susceptible	0.008–0.06
Chromosomal *gyrA* (single mutation)	Usually resistant	0.12–2.0
Chromosomal *gyrB* (single mutation)	Usually susceptible	0.12–0.5
Chromosomal *gyrA, gyrB* (multiple mutations)	Resistant	≥4.0
PMQR (such as *qnr* or *aac (6′)-Ib-cr*)	Often susceptible	0.12–2.0

Abbreviations: MIC, minimum inhibitory concentration; PMQR, plasmid-mediated quinolone resistance.

[21–24]. When investigated, most isolates with DCS were found to harbor single mutations in the *gyrA* gene, which encodes a subunit of DNA gyrase and is located in the quinolone resistance–determining region (QRDR) [25]. Mutations in *gyrA* also confer high-level resistance (MIC, 128–512 μg/mL) to the non-fluorinated quinolone nalidixic acid (NAL) (Table 1). A second QRDR mutation arising in such isolates may result in high-level FQ resistance (MIC, ≥4 μg/mL).

The DCS/NAL-resistant (DCS/NAL^R) phenotype is now prevalent worldwide among both typhoid and nontyphoid serovars [26–28]. Genotyping has identified at least 15 independent *gyrA* mutations that have occurred within a decade among *Salmonella* Typhi from Asia and Africa, suggestive of rapid evolution of DCS, which is maintained through selective pressure [29, 30]. Of importance, the DCS/NAL^R phenotype is correlated in multiple studies with delayed responses, clinical failures, and increased mortality among patients receiving CIP for *Salmonella* Typhi infection [23, 31–42], even with adequate CIP doses [43, 44] and documented therapeutic drug concentrations [45]. Similarly, reports have documented poor FQ treatment outcomes for systemic infections caused by DCS/NAL^R nontyphoidal serovars of *Salmonella* (Table 3).

Because of the clinical significance of DCS/NAL^R strains in systemic infections, clinical laboratories have been encouraged to identify these isolates during routine susceptibility testing. Because nearly all NAL^R *Salmonella* harbor the DCS phenotype, the CLSI recommended in 2004 that laboratories screen extra-intestinal *Salmonella* with CIP MICs ≤1 μg/mL for NAL resistance as a predictor for DCS. If NAL resistance was identified, the laboratory was instructed to indicate to clinicians that FQ treatment might not be efficacious. Despite the paucity of clinical data at the time for nontyphoidal serovars, this recommendation was made universally for all extra-intestinal isolates of *Salmonella*. This strategy is performed across the globe and was, until recently, also supported by the European Committee for Antimicrobial Susceptibility Testing (EUCAST).

THE PROBLEM: EVOLVING FQ RESISTANCE AMONG *SALMONELLA* SPECIES

Recent data have raised concern that NAL resistance may no longer be a reliable marker for DCS as a result of evolving and diverse *Salmonella* FQ resistance mechanisms. There are now numerous reports of NAL-susceptible isolates with DCS (DCS/NAL^S) [46–51]. This phenotype appears to be mediated by

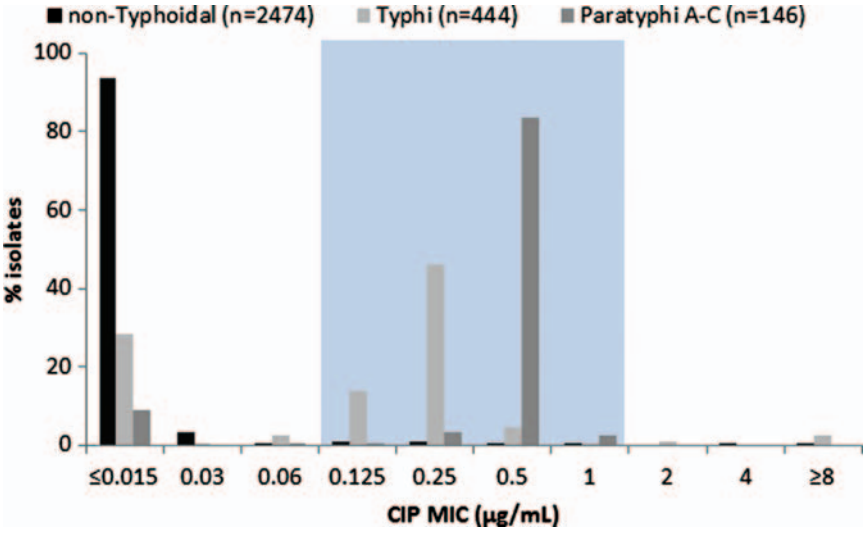

Figure 1. Distribution of 2010 US *Salmonella* ciprofloxacin (CIP) minimum inhibitory concentrations (MICs), measured by broth microdilution. Shaded area indicates the decreased CIP susceptibility MIC zone. Data adapted from [20].

Table 2. Interpretive Criteria for Ciprofloxacin and *Salmonella*

	CIP MIC (μg/mL) Interpretive Criteria *Salmonella*		
Criterium	Susceptible	Intermediate	Resistant
CLSI (M100 S21; all *Salmonella*)	≤1.0	2.0	≥4.0
CLSI (M100 S22; extra-intestinal & *S.* Typhi)	≤0.06	0.12–1.0	≥2.0
CLSI (M100 S22; intestinal *Salmonella*)	≤1.0	2.0	≥4.0
CLSI (proposed M100 S23, all *Salmonella*)	≤0.06	0.12–1.0	≥2.0
EUCAST[a]	≤0.5	1.0	≥2.0
FDA	≤1.0	2.0	≥4.0

[a] The EUCAST break point is for all Enterobacteriaceae, with a footnote to indicate *Salmonella* species with low-level fluoroquinolone resistance (MIC, >0.06 mg/L) respond poorly to CIP treatment.

Abbreviations: CLSI, Clinical Laboratory Standards Institute; CIP, ciprofloxacin; EUCAST, European Committee for Antimicrobial Susceptibility Testing; FDA, US Food and Drug Administration; MIC, minimum inhibitory concentration.

resistance mechanisms outside the *gyrA* gene [52]. Mutations in *gyrB* occur among 1%–11.6% of *S.* Typhi in the United States and United Kingdom [22, 53] and result in CIP MICs of 0.125–0.5 μg/mL and NAL MICs of 2–16 μg/mL, both within the susceptible range [54, 55]. Similarly, plasmid-mediated quinolone resistance (PMQR) determinants, such as the *qnr* and *aac-6′-Ib-cr* genes [56], are associated with DCS [57–60] but result in only modest NAL MIC elevations (8–32 μg/mL) (Table 1) [61, 62]. PMQR appears to be uncommon among nontyphoidal *Salmonella* strains in the United States at present [51, 63] but are commonly found in Europe and Asia [54, 57, 64]. Occurrence among nontyphoidal *Salmonella* in African countries is less well known but has been reported [65].

The clinical impact of the DCS-NAL^S phenotype on FQ treatment of salmonellosis is unknown, because there have been no studies that document outcomes for such infections when treated with FQs. However, the DCS phenotype is likely to be the most important determinant of the clinical response to therapy [23], regardless of the resistance mechanism, whether NAL^R or NAL^S [66, 67]. Furthermore, significant concern exists that under-reporting of DCS by a failure to detect DCS-NAL^S isolates may facilitate the subsequent emergence of high-level FQ resistance [56, 62]. DCS-NAL^S isolates require higher concentrations of FQ to prevent in vivo selection of additional mutations that result in high-level FQ resistance [68].

RE-EVALUATING THE FQ BREAK POINTS

Given the wild-type *Salmonella* CIP MIC distribution of 0.008–0.06 μg/mL, many have suggested lowering the susceptible break point for CIP to ≤0.06 ug/mL. This change is supported by accumulating clinical, microbiological, and pharmacokinetic-pharmacodynamic (PK-PD) studies that indicate that such a revised break point is more appropriate for determining CIP susceptibility among contemporary *Salmonella* isolates causing systemic infection. EUCAST revised their CIP susceptible break point to ≤0.5 ug/mL for all *Enterobacteriaceae*, with a comment that MICs>0.06 μg/mL predict a poor response for systemic *Salmonella* infection. The CLSI approved a ≤0.06 ug/mL susceptibility break point for *Salmonella* Typhi and extraintestinal isolates of *Salmonella* and decided to eliminate the NAL screen at the January 2011 Antimicrobial Susceptibility Testing subcommittee meeting (Table 2). However, in June 2011, after appeals from individuals in countries where typhoid fever is endemic, the NAL screen recommendation was reinstated. CLSI currently suggests that NAL may be used to test for reduced FQ susceptibility in *Salmonella* Typhi or extraintestinal *Salmonella* isolates. However, laboratory workers and clinicians should be aware that NAL screening does not detect all mechanisms of FQ resistance, and thus, CIP should also be tested and interpreted using the new susceptible MIC break point of ≤0.06 μg/mL or zone measurement of ≥31 mm with disk diffusion (DD) testing.

CLSI rationale for maintaining the NAL screen arose from consideration of technical challenges faced by clinical laboratories in resource-limited countries. These laboratories have found NAL DD screening to be a reliable method for the detection of DCS to inform typhoid fever treatment. CIP MIC testing is generally not an option in these countries because of cost and limited availability of materials. In addition, laboratories in resource-limited countries have found that CIP DD id difficult to interpret, which may relate to local materials, strains, or other factors. Because the DCS/NAL^S phenotype is currently uncommon in many parts of the world, NAL screening to predict DCS is still associated with a sensitivity of 92.9% and specificity of 98.4% for *Salmonella* Typhi [19, 69] and, thus, continues to be of use in areas where typhoid is endemic. The CLSI is presently investigating alternative tests to replace the NAL screen, including OFO DD, which is associated with a sensitivity of 97.3% and specificity of 99.3% for the prediction of DCS [69]. Using disks with a lower content (eg, 1 mg CIP rather than 5 mg) can also improve both sensitivity and specificity [19].

Along with the revised CIP break point for *Salmonella* Typhi and extraintestinal *Salmonella* isolates, the CLSI in 2012 indicated that clinicians may consider maximal oral or parenteral CIP dosage regimens for those *Salmonella* isolates with CIP MICs or DD zone diameters in the intermediate range. The CLSI voted to remove this comment in M100-S23, because isolates that test in the intermediate range of the revised CIP break point include those that harbor PMQR

Table 3. Documented Cases of Ciprofloxacin (CIP) Treatment Failures in Patients Infected with Decreased CIP Susceptibility Nontyphoidal *Salmonella*

Study	Underlying Condition	*Salmonella* Serovar	Infection	MIC Following CIP Treatment (μg/mL)		Ciprofloxacin Treatment Dose	Outcome
				CIP	NAL		
Boswell et al [36]	Spherocytosis	S. Virchow	Gastroenteritis	0.75	Resistant	500 mg p.o. b.i.d., 14 d	*Salmonella* eradicated by 7 d course p.o. trimethoprim
Vasallo et al [37]	Diabetes	S. Enteritidis	Bacteremia	1.0	NA	200 mg i.v. b.i.d., 12 d	*Salmonella* eradicated by imipenem therapy
Vasallo et al [37]	AIDS	S. Enteritidis	Bacteremia + Septic Arthritis	0.5	NA	750 mg p.o. b.i.d., 12 d	Patient expired
Piddock et al [38, 39]	NA	S. Typhimurium	Upper Urinary Tract Infection	2.0	256	500 mg b.i.d., 14 d; formulation not reported	NA
Piddock et al [38, 39]	Aortic Aneurysm Surgery	S. Typhimurium	Bacteremia + Wound Infection	0.25	256	200 mg i.v. b.i.d., 10 d	Patient recovered following 12 w i.v. aztreonam 2 g b.i.d.
Chang et al [40]	Chronic Liver Disease	S. Choleraesuis	Bacteremia + Vertebral Osteomyelitis	0.19	>256	300 mg i.v. b.i.d., 14 d, followed by 750 mg p.o. b.i.d for 7 d	Patient recovered following CIP + cefotaxime therapy
de Toro et al [68]	NA	S. Typhimurium	Gastroenteritis	0.5	16	7 d (no dosage provided)	NA

Abbreviations: b.i.d., twice daily; CIP, ciprofloxacin; IV, intravenous; MIC, minimum inhibitory concentration; NA, not available; NAL, nalidixic acid; p.o., per os.

determinants that may be associated with in vivo selection of high-level FQ resistance, as detailed above. Furthermore, because no data document favorable treatment outcomes using high dose CIP monotherapy for such isolates and high-dose CIP may be associated with an increased risk of toxicity, it is the opinion of the authors that treatment with an alternative agent, such as ceftriaxone, may be preferable for such cases. Susceptibility to ceftriaxone should be confirmed, because resistance mediated by extended-spectrum β-lactamases and plasmid-mediated cephalosporinases has been reported worldwide [70]. In 2010, 70 (2.8%) of 2474 nontyphoidal *Salmonella* isolates but none of the *Salmonella* Typhi or Paratyphi isolates included in the Centers for Disease Control and Prevention National Antimicrobial Resistance Monitoring System report were resistant to ceftriaxone (MIC, ≥4 μg/mL) [71]. Azithromycin has been shown to yield higher cure rates and lower mean duration of fever than OFO for the treatment of *Salmonella* Typhi with DCS [72, 73] but is not approved for the treatment of salmonellosis in the United States.

TECHNICAL HURDLES TO IMPLEMENTING THE NEW MIC BREAK POINTS IN THE UNITED STATES

Implementation of the 2012 CIP break points for *Salmonella* by a clinical laboratory is complicated in the United States by the requirement of commercial test system manufacturers to adhere to antimicrobial break points set by the US Food and Drug Administration, which for CIP, are currently the same for all *Enterobacteriaceae* (Table 2). No commercial MIC panels produced in the United States currently contain CIP concentrations low enough to allow use of the 2012 CLSI *Salmonella* break points (Table 2). To use the 2012 breakpoints, laboratories may consider determining CIP MIC by Etest, which appears to correlate well with MICs obtained by agar dilution for *Salmonella* Typhi with high-level FQ resistance [74] and will reliably detect DCS [19, 75]. Because Etest is a Food and Drug Administration–approved commercial test, laboratories will need to perform a verification study before applying the new break points. The extent of this verification study is at the discretion of the laboratory director, but reliability of Etest to detect CIP-susceptible, -intermediate, and -resistant results with use of the new break points should be confirmed. As an alternative to using Etest, laboratories could perform both NAL and CIP DD in parallel for isolates with CIP MICs ≤1 μg/mL, thereby testing for the more common NAL^R DCS phenotype and for PMQR by evaluation of CIP DD zone diameters. At the very least, laboratories should interpret CIP MICs using the old break points, while providing a comment that *Salmonella* isolates with CIP MICs ≤1 μg/mL

but >0.06 μg/mL are associated with delayed responses or clinical failure after FQ therapy.

TWO SETS OF BREAK POINTS FOR CIPROFLOXACIN AND *SALMONELLA*: CHALLENGES FOR REPORTING

Another source of confusion for many laboratories is the question of when to apply the new CIP break points. The CLSI intended that the break points be used for all typhoidal *Salmonella* (ie, Typhi and Paratyphi serovars), including those recovered from intestinal sources, although serovar Paratyphi is not explicitly addressed in M100-S22. Because some clinical laboratories rely on their local public health laboratory to subtype *Salmonella* and identify Typhi and Paratyphi serovars, incorrect CIP break points could be applied to *Salmonella* Typhi and Paratyphi recovered from stool samples that are awaiting public health laboratory identification. We encourage clinical laboratories to routinely rule out these organisms to ensure that CIP susceptibility results are interpreted correctly and, moreover, to provide a timely detection of these serious pathogens. Identification of *Salmonella* Typhi and Paratyphi can be accomplished by evaluating reactions on a triple sugar iron agar (TSI) slant, an automated system, or API 20E (bioMérieux), with subsequent confirmation by a public health laboratory.

CLSI states that the 2012 break points are to be applied to nontyphoidal *Salmonella* serovars only when isolated from extra-intestinal sources. However, it is the authors' opinion that laboratories should consider applying the new break points to all *Salmonella* isolates when susceptibility testing is performed, regardless of the specimen from which the isolate was recovered. There are no clinical data to suggest that intestinal DCS *Salmonella* will respond better to CIP therapy than would extra-intestinal isolates; indeed, it is recognized that treatment of uncomplicated salmonellosis prolongs fecal shedding [5, 6]. Thus, a request for susceptibility testing of *Salmonella* isolates may indicate a complicated infection and/or immunocompromised host, in which CIP therapy may fail for isolates with DCS. In recognition of this fact, the CLSI voted in June 2012 to apply the revised break points to all *Salmonella* species, if susceptibility testing is warranted. Laboratories should be clear that routine susceptibility testing of fecal isolates of nontyphoidal *Salmonella* is discouraged, because therapy is rarely indicated and a susceptibility report may prompt some clinicians to treat.

The CLSI has not yet revised the *Salmonella* break points for other FQs, with the exception of OFO and levofloxacin, for which MIC break points alone have been proposed but are not yet published. The DCS/NAL^R phenotype confers decreased susceptibility of *Salmonella* Typhi and Paratyphi A to OFO [76], which is a racemic mixture of active and inactive levofloxacin enantiomers. The DCS phenotype, irrespective of NAL phenotype, confers reduced susceptibility to OFO, norfloxacin, and levofloxacin in nontyphoidal *Salmonella* [19]. OFO treatment outcomes are as poor as CIP outcomes for patients infected with DCS/NAL^R *Salmonella* Typhi [73, 77]. Similarly, *Salmonella* isolates with nonsusceptible levofloxacin or OFO MICs using the proposed 2013 break points (eg, ≥0.25 μg/mL, as indicated by PK/PD [78], clinical [77], and microbiological data [20]) are suggestive of a DCS phenotype and should prompt caution in using levofloxacin or OFO for treatment. Alternative agents, such as ceftriaxone, if the isolate is ceftriaxone susceptible, or azithromycin may be treatment options in these cases. Gatifloxacin remains active and clinically effective against FQ-resistant *Salmonella* Typhi with *gyrA* mutations [29, 79], but this FQ is unavailable for systemic use in the United States and many other countries because of adverse effects on glucose metabolism. The CLSI will be evaluating additional FQ break points in the near future.

CONCLUSIONS

FQ resistance among *Salmonella* is a pressing worldwide concern, but recognition of resistance has become complicated over the past decade by a growing number of FQ resistance mechanisms. Although the CLSI has addressed this issue in a limited fashion by introducing a new CIP break point for *Salmonella* isolates (Table 2), it is critical that clinicians and laboratory workers be aware of limitations associated with this strategy. In the United States, *Salmonella* susceptibility to CIP is optimally tested by obtaining an MIC and interpreting according to the 2012 CLSI break points (Table 2), regardless of isolate serovar or source. Use of the 2012 interpretive criteria for all *Salmonella* isolates will reduce confusion for clinical laboratories and more reliably predict the appropriateness of CIP for the treatment of *Salmonella* infections that warrant therapy. Until the manufacturers of commercial AST systems are able to incorporate the lower CIP dilutions required to detect DCS, laboratories may perform a CLIA verification study using Etest to obtain a CIP MIC for implementation of the new break points. However, because of the complexity of such a verification study, testing may be performed using DD for CIP and NAL to detect both DCS and high-level FQ resistance. In resource-limited countries, performing a NAL DD alone to predict DCS may be a viable option; however, surveillance in these countries for increasing prevalence of strains with DCS/NAL^S phenotypes is warranted.

Note

Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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