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).
When investigated, most isolates with DCS were found to harbor single mutations in the \textit{gyrA} gene, which encodes a subunit of DNA gyrase and is located in the quinolone resistance–determining region (QRDR) \cite{25}. Mutations in \textit{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 \cite{26–28}. Genotyping has identified at least 15 independent \textit{gyrA} mutations that have occurred within a decade among \textit{Salmonella} Typhi from Asia and Africa, suggestive of rapid evolution of DCS, which is maintained through selective pressure \cite{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 \textit{Salmonella} Typhi infection \cite{23, 31–42}, even with adequate CIP doses \cite{43, 44} and documented therapeutic drug concentrations \cite{45}. Similarly, reports have documented poor FQ treatment outcomes for systemic infections caused by DCS/NAL\(^R\) nontyphoidal serovars of \textit{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\(^R\) \textit{Salmonella} harbor the DCS phenotype, the CLSI recommended in 2004 that laboratories screen extra-intestinal \textit{Salmonella} with CIP MICs ≤1µg/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 \textit{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 \textit{Salmonella} FQ resistance mechanisms. There are now numerous reports of NAL-susceptible isolates with DCS (DCS/NAL\(^R\)) \cite{46–51}. This phenotype appears to be mediated by

![Figure 1.](cid:2012:55 (15 October))\) Distribution of 2010 US \textit{Salmonella} ciprofloxacin (CIP) minimum inhibitory concentrations (MICs), measured by broth microdilution. Shaded area indicates the decreased CIP susceptibility MIC zone. Data adapted from \cite{20}.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Phenotype</th>
<th>Ciprofloxacin MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild Type (no resistance)</td>
<td>Usually susceptible</td>
<td>0.008–0.06</td>
</tr>
<tr>
<td>Chromosomal \textit{gyrA} (single(^n)mutation)</td>
<td>Usually resistant</td>
<td>0.12–2.0</td>
</tr>
<tr>
<td>Chromosomal \textit{gyrB} (single(^n)mutation)</td>
<td>Usually susceptible</td>
<td>0.12–0.5</td>
</tr>
<tr>
<td>Chromosomal \textit{gyrA}, \textit{gyrB} (multiple mutations)</td>
<td>Resistant</td>
<td>≥4.0</td>
</tr>
<tr>
<td>PMQR (such as \textit{qnr} or \textit{aac} ((6′)-lb-cr))</td>
<td>Often susceptible</td>
<td>0.12–2.0</td>
</tr>
</tbody>
</table>

Abbreviations: MIC, minimum inhibitory concentration; PMQR, plasmid-mediated quinolone resistance.
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-NAL5 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 µg/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 µg/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 µg/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 is difficult to interpret, which may relate to local materials, strains, or other factors. Because the DCS/NAL5 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 2. Interpretive Criteria for Ciprofloxacin and Salmonella

<table>
<thead>
<tr>
<th>Criterium</th>
<th>CIP MIC (µg/mL) Interpretive Criteria</th>
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<tbody>
<tr>
<td>CLSI (M100 S21; all Salmonella)</td>
<td>≤1.0 2.0 ≥4.0</td>
</tr>
<tr>
<td>CLSI (M100 S22; extra-intestinal &amp; S. Typhi)</td>
<td>≤0.06 0.12–1.0 ≥2.0</td>
</tr>
<tr>
<td>CLSI (M100 S22; intestinal Salmonella)</td>
<td>≤1.0 2.0 ≥4.0</td>
</tr>
<tr>
<td>CLSI (proposed M100 S23, all Salmonella)</td>
<td>≤0.06 0.12–1.0 ≥2.0</td>
</tr>
<tr>
<td>EUCASTa</td>
<td>≤0.5 1.0 ≥2.0</td>
</tr>
<tr>
<td>FDA</td>
<td>≤1.0 2.0 ≥4.0</td>
</tr>
</tbody>
</table>

* 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.
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<sup>8</sup> 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<sup>8</sup> 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. Gatifloxin 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<sup>5</sup> 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.
References


