Updated Correlations Between Susceptibility Testing Results for Nalidixic Acid and Fluoroquinolones When Testing *Salmonella* spp.

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AMENDED ABSTRACT

Background: The CLSI and EUCAST both recommend the use of nalidixic acid susceptibility testing result to screen for fluoroquinolone resistance among *Salmonella* spp. strains. We evaluated the correlation between susceptiblity results for nalidixic acid and fluoroquinolone compounds among clinical *Salmonella* spp. strains.

Methods: 134 *Salmonella* spp. strains (110 [88.7%] nalidixic acid-resistant) were collected from Europe (47), Asia-Pacific (36), USA (29) and Latin America (22). 56 (50.9%) nalidixic acid-resistant and 16 nalidixic acid-susceptible strains were *S. typhi/paratyphi/typhimurium*. Isolates were susceptibility tested by the CLSI broth microdilution and disk diffusion methods against nalidixic acid, ciprofloxacin, levofloxacin, moxifloxacin and gatifloxacin. Correlation between MIC and disk results for each drug as well as between nalidixic acid and fluorquinolone MIC results were evaluated.

Results: Among nalidixic acid-resistant strains, 31.8% had nalidixic acid MIC of >1024 µg/ml (MIC_{50}, 512 µg/ml). Ciprofloxacin, levofloxacin, moxifloxacin and gatifloxacin were active against nalidixic acid-resistant strains with $MIC_{50/90}$ of 0.25/1, 0.5/1, 0.5/2 and 0.25/1 µg/ml, respectively. 95.5 and 85.5% of nalidixic acid-resistant strains were susceptible to ciprofloxacin according to the current CLSI and EUCAST breakpoints, respectively. Nalidixic acid-susceptible strains exhibited FQ MIC values 4- to 16-fold lower compared to nalidixic acid-resistant strains, with $MIC_{50/90}$ of 0.015-0.12/0.03-0.12 µg/ml. Categorical agreement between broth microdilution and disk diffusion results ranged from 93.7 to 95.5% with only minor errors. There was a good correlation between MIC results for nalidixic acid and the fluoroquinolones tested.

Conclusions: Susceptibility results for nalidixic acid correlated well with those of ciprofloxacin, levofloxacin, moxifloxacin and gatifloxacin. Although resistance to nalidixic acid predicted decreased susceptibility to fluoroquinolones, the vast majority of nalidixic acid -resistant strains remained susceptible to the fluoroquinolone agents according to current CLSI and EUCAST breakpoint criteria per PK/PD analyses. Further evaluation of nalidixic acid screening to predict fluoroquinolone resistance as well as fluoroquinolone breakpoints for *Salmonella* spp. appears necessary.

Nalidixic acid MIC (µg/ml) \ Ciprofloxacin MIC (µg/ml)	≤0.002	0.004	0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16
>1024								2	20	9		1ᶜ		3
1024							1	4	3	1				
512							11	11	5					
256						1	16	16	3	1	1			
128														
64														
32									1ᵃ					
16														
8					1	1	2							
4					14									
2			1		3									
1	1				1									
0.5														
0.25														
0.12														
≤0.06														

a. Reproducible result

INTRODUCTION

Resistance to antimicrobial agents among species of Enterobacteriaceae, including *Salmonella* spp., is increasing and becoming a serious therapeutic concern. For example, resistance to β-lactams continues to become more complex due to the numerous inactivating extended-spectrum- and metallo-β-lactamases that have become endemic in some geographic regions. Cross-resistance to other antimicrobial classes is commonly associated with strains that produce these β-lactamase enzymes, including the wide spectrum fluoroquinolones.

Salmonella spp. that are resistant to nalidixic acid and with reduced susceptibility to fluoroquinolones have been documented for many years. This is mainly attributed to mutations in the quinolone resistance-determining region (QRDR). However, new determinants of quinolone resistance have been detected and include transmissible mechanisms such as *qnr* genes and *aac*(6')-*Ib*-cr which are associated with target protection and enzymatic modification, respectively. These fluoroquinolone resistance mechanisms have been detected in several species of Enterobacteriaceae, including *Salmonella* spp.

First-step QRDR mutations and strains with *qnr* genes and *aac*(6')-*Ib*-cr often have phenotypically lower levels of "resistance" to the fluoroquinolones. MIC values are typically increased above those of the wildtype population, but remain susceptible according to the currently established susceptibility breakpoint criteria. It has been documented that strains harbouring these resistance mechanisms may be associated with clinical treatment failures.

This study was conducted to determine the Clinical and Laboratory Standards Institute (CLSI) and EUCAST recommendations for using the nalidixic acid disk diffusion and/or MIC testing result to screen for fluoroquinolone resistance and the correlation between susceptibility results for this quinolone agent and four fluoroquinolone compounds tested against clinical isolates of *Salmonella* spp. from a worldwide collection.

MATERIALS AND METHODS

Bacterial isolates. A total of 134 *Salmonella* spp. were selected and included 110 nalidixic acid-resistant (MIC, ≥32 µg/ml) strains of the following species; *S. paratyphi* (21), *S. typhi* (30), *S. typhimurium* (5), other *Salmonella* spp. (54). Also included were 24 nalidixic acid-susceptible strains (MIC, ≤16 µg/ml); *S. paratyphi* (5), *S. typhi* (5), *S. typhimurium* (6) and other *Salmonella* spp. (8). Isolates were collected during 2005-2009 from diverse geographic regions, including Europe (47), Asia-Pacific (36; 22 from India), United States (29) and Latin America (22). Three *E. coli* isolates were included as controls with nalidixic acid MIC values of >1024, 128 and 0.25 µg/ml.

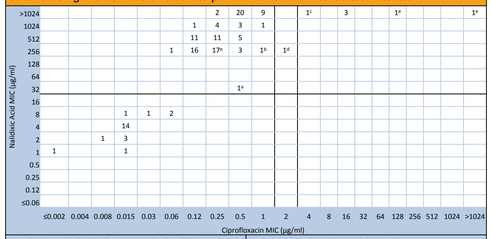
Antimicrobial susceptibility testing. Isolates were tested for antimicrobial susceptibility using two CLSI methods. The broth microdilution method (CLSI; M07-A8, 2009) was performed using reference frozen-form panels with cation-adjusted Mueller-Hinton broth produced by JMI Laboratories (North Liberty, Iowa, USA). Isolates were also tested using the disk diffusion method (M02-A10, 2009). The following antimicrobials (disk content/dilution range) were tested; nalidixic acid (30-µg/0.06 – 1024 µg/ml), ciprofloxacin (5-µg/0.002 – 1024 µg/ml), levofloxacin (5-µg/0.002 – 1024 µg/ml), moxifloxacin (5-µg/0.002 – 1024 µg/ml) and gatifloxacin (5-µg/0.002 – 1024 µg/ml). Interpretations were determined using current CLSI (M100-S20-U, 2010) and EUCAST (Version 1.1, April 2010) breakpoint criteria. No interpretive breakpoints have been established for gatifloxacin by EUCAST or moxifloxacin by the CLSI.

Molecular characterization. Screening of plasmid-mediated quinolone resistance determinates was performed by PCR and sequencing. The following genes were screenend: *qnrA* (*qnrA1* -*qnrA6*), *qnrB* (*qnrB1* -*qnrB19*), *qnrS* (*qnrS1* -*qnrS3*) and *aac(6')-Ib*. Primers targeting 16S rRNA were utilized in every reaction mixture as extraction and internal amplification control. Positive and negative controls were used in every PCR batch.

RESULTS

- Ciprofloxacin ($MIC_{50/90}$, 0.25/1 µg/ml), levofloxacin ($MIC_{50/90}$, 0.5/1 µg/ml), moxifloxacin ($MIC_{50/90}$, 0.5/2 µg/ml) and gatifloxacin ($MIC_{50/90}$, 0.25/1 µg/ml) were active against nalidixic acid-resistant strains (data not shown). Among nalidixic acid-resistant strains, 95.5% were susceptible to ciprofloxacin according to the current CLSI MIC breakpoint criteria (Figure 1).
- Figure 2 is a scattergram of the MIC and disk diffusion results for nalidixic acid which shows that the currently applied nalidixic acid breakpoint criteria produced only a 3.7% error rate (all minor) using the CLSI guidelines and no errors using EUCAST guidelines (Tables 1 and 2).
- The scattergram analysis of ciprofloxacin as shown in Figure 3 demonstrates that higher error rates were observed for this fluoroquinolone using the CLSI (5.2%) and more conservative EUCAST (14.2%; unacceptable, all minor) breakpoint criteria compared to nalidixic acid (Tables 1 and 2).
- No categorical errors for levofloxacin and only minor errors (3.7%) were observed for gatifloxacin using the CLSI recommended breakpoints (Table 1), whereas error rates were common (unacceptable) for levofloxacin (21.6%) and moxifloxacin (28.3%) using EUCAST disk diffusion breakpoint criteria (Table 2) which included major and very major errors.
- Among the strain collection, including 110 nalidixic acid-resistant *Salmonella* spp., all were negative for *qnrA*, one isolate was positive for *qnrB* and three isolates were positive for *qnrS* (Figure 1); **only 3.0% of *Salmonella* spp**.
- All four *Salmonella* spp. strains with documented *qnr* resistance determinants were resistant to nalidixic acid but only one strain (25.0%) was resistant to ciprofloxacin according to current CLSI breakpoints (see Figure 1).

Figure 1. Molecular characterization and distribution of ciprofloxacin versus nalidixic acid MIC results obtained when testing *Salmonella* spp. and three *E. coli* control strains using the current CLSI breakpoint criteria for Enterobacteriaceae.



a. Reproducible MIC results. *qnrB*-positive strain (Chile, Santiago).
b. *qnrS*-positive *Salmonella virchow* (Sweden, Linkoping).
c. *qnrS*-positive *Salmonella hadar* (Sweden, Linkoping).
d. *qnrS*-positive *Salmonella virchow* (Sweden, Varjo).
e. Includes an *E. coli* control strain.

Figure 2. Distribution of nalidixic acid MIC and disk diffusion zone diameter results obtained when testing nalidixic acid-susceptible and -resistant *Salmonella* spp. and three *E. coli* control strains using the current CLSI and EUCAST breakpoint criteria for Enterobacteriaceae (solid vertical lines indicate the CLSI breakpoint criteria and the single vertical dashed line indicates the EUCAST breakpoint for the disk diffusion method).

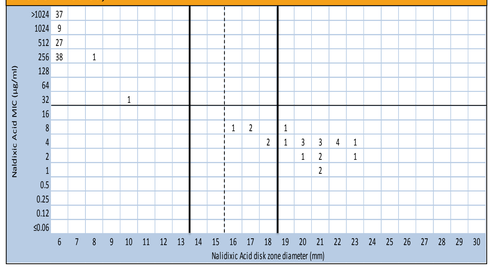


Figure 3. Distribution of ciprofloxacin MIC and disk diffusion results obtained when tested against nalidixic acid-susceptible and -resistant *Salmonella* spp. and three *E. coli* control strains using the current CLSI and EUCAST breakpoint criteria for Enterobacteriaceae (solid vertical lines indicate the CLSI breakpoint criteria and the single vertical dashed line indicates the EUCAST breakpoint for the disk diffusion method).

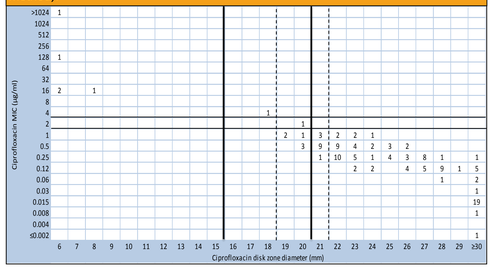


Table 1. Categorical agreement between disk diffusion and broth microdilution test results for nalidixic acid and three fluoroquinolones tested against 134 *Salmonella* spp. isolates using the current CLSI breakpoint criteria.

Antimicrobial agent	Error rate (%) Minor	Major	Very major
Nalidixic acid[a]	3.7	0.0	0.0
Ciprofloxacin	5.2	0.0	0.0
Levofloxacin	0.0	0.0	0.0
Gatifloxacin	3.7	0.0	0.0

a. No intermediate MIC breakpoint has been established by the CLSI (M100-S20, 2010).

Table 2. Categorical agreement between disk diffusion and broth microdilution test results for nalidixic acid and three fluoroquinolones tested against 134 *Salmonella* spp. isolates using the current EUCAST breakpoint criteria.

Antimicrobial agent	Error rate (%) Minor	Major	Very major
Nalidixic acid[a]	0.0	0.0	0.0
Ciprofloxacin	14.2	0.0	0.0
Levofloxacin	20.1	1.5	0.0
Gatifloxacin	26.1	0.0	2.2

a. No intermediate MIC or disk diffusion zone diameter breakpoints have been established by EUCAST.

CONCLUSIONS

- Susceptibility results for nalidixic acid correlated well with those of ciprofloxacin, levofloxacin, moxifloxacin and gatifloxacin.
- Although resistance to nalidixic acid predicted decreased susceptibility to fluoroquinolones, the vast majority of nalidixic acid-resistant strains remained susceptible to the fluoroquinolone agents according to current CLSI (95.5%) and EUCAST (85.5%) breakpoint criteria.
- Further evaluation of nalidixic acid screening to predict fluoroquinolone resistance as well as fluoroquinolone breakpoints for *Salmonella* spp. appears necessary.

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