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Genetic Analysis of *Salmonella* spp. with Resistance to Extended-Spectrum Cephalosporins and Fluoroquinolones Isolated in North and Latin America (SENTRY Antimicrobial Surveillance Program)

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AMENDED ABSTRACT

Background: Emerging antimicrobial resistant (R) *Salmonella* spp. (SAL) requires increased efforts to appropriately test susceptibility (S). The SENTRY Program monitored SAL and detected nalidixic acid (NA)-R strains with elevated fluoroquinolones (FQ) MICs and strains with extended-spectrum ß-lactamase (ESBL) phenotypes. Genetic analysis was used to characterize the R mechanisms.
Methods: Stool and bloodstream isolates (328) from North and Latin America were tested by NCCLS broth microdilution methods. 9 sites forwarded 23 (7.0%) NA-R strains. FQ comparators included ciprofloxacin (CIP), levofloxacin (LEV), gatifloxacin (GAT) and garenoxacin (GRN). 19 isolates were studied to determine mutations in the QRDR. 9 sites contributed 11 (3.4%) strains which met NCCLS ESBL criteria (≥ 2 μg/ml) for aztreonam (ATM) or ceftazidime (CAZ) or ceftriaxone (CRO). ESBL confirmation was evaluated by Etest ESBL strips and the enzymes were characterized by PCR and gene sequencing.
Results: Among the NA-R SAL isolates, FQ MICs were elevated (8- to 32-fold) compared to wild type strains. CIP (MIC_{90}, 0.25 μg/ml) was more potent than LEV, GAT and GRN (0.25 - 0.5 μg/ml). Single *gyrA* mutations were responsible for elevated FQ MICs and included D87Y (5), S83F (7), D87N (5) and S83Y (2). The ESBL-phenotype isolates had the following MIC patterns: CAZ (≥ 16 μg/ml), cefoxitin (≥ 32), ATM (4 - >16) and CRO (8 - 32). All strains were S to cefepime, carbapenems and FQs at current breakpoints. Tetracycline co-R was common (73%). No strains were inhibited by clavulanic acid consistent with all isolates producing the identified CMY-2.
Conclusions: Contemporary isolates of SAL must be monitored for antimicrobial R. FQ may be compromised among isolates with QRDR mutations detected using NA as a screening agent. SAL with ESBL phenotypes were likely to harbor CMY-2 (not a true ESBL) and remain S to cefepime and carbapenems which can still be used for serious invasive SAL infections.

INTRODUCTION

Pathogens that cause gastroenteritis became a focused objective of the SENTRY Antimicrobial Surveillance Program in 2001. Antimicrobial resistant enteric bacilli that produce diarrheal symptoms and are capable of serious invasive infection have been reported worldwide. Emergence and dissemination of extended-spectrum ß-lactamase enzymes (ESBL) and the development of fluoroquinolone resistance have recently become important clinical concerns. The production of plasmid-mediated CMY-2 Amp C ß-lactamase in *Salmonella* spp. and *E. coli* is particularly problematic since resistance can be transfered to other species and clonal dissemination can cause large outbreaks.

Commonly prescribed empiric treatments such as ceftriaxone could be compromised for at-risk patients including children who are more prone to sequela. The widespread use of fluoroquinolones has led to increased rates of resistance among bacterial species that cause human disease. Although the development of high-level fluoroquinolone-resistance in *Salmonella* spp. remains rare, strains with elevated MICs have been associated with point mutations in the quinolone resistance determining region (QRDR). It has been shown that single *gyrA* mutations mediate resistance to nalidixic acid and increase the MIC to broader spectrum fluoroquinolones. It has also been demonstrated that fluoroquinolone treatment for strains with elevated fluoroquinolone MICs, although susceptible using current NCCLS breakpoint criteria, have led to clinical failure. This has recently led to discussion regarding whether the current breakpoint criteria used for *Salmonella* spp. and fluoroquinolones are appropriate.

The SENTRY Program documented *Salmonella* spp. isolates in North America and Latin America with resistance to extended-spectrum cephalosporins and fluoroquinolones during routine surveillance in 2001. These strains were analyzed genetically to determine the resistance mechanisms responsible.

MATERIALS AND METHODS

Participating SENTRY Program sites in North America and Latin America were requested to forward 25 consecutive isolates of species considered producers of diarrheal disease. The site of isolation was to be blood or stool cultures. This yielded 328 *Salmonella* spp. that were tested for susceptibility to numerous antimicrobial classes including third- and fourth-generation cephalosporins and fluoroquinolones. Isolates were tested using reference NCCLS broth microdilution methods in validated panels (TREK Diagnostics). Among the isolates tested, 23 strains were resistant to nalidixic acid (≥ 16 μg/ml). Breakpoint criteria for nalidixic acid of ≤ 8 μg/ml for susceptible and ≥ 16 μg/ml for resistant were used to classify fluoroquinolone-resistance with potential clinical failure. Nineteen strains were genetically analyzed to determine mutations in the QRDR by PCR and sequencing.

Eleven additional strains had MIC results (≥ 2 μg/ml) to ceftazidime, ceftriaxone and aztreonam, consistent with NCCLS initial screening criteria for ESBL production. Isolates were further tested using ceftazidime and/or ceftriaxone Etest strips (AB BIODISK, Solna, Sweden) with and without clavulanic acid as a phenotypic confirmatory test. All strains were genetically analyzed to determine the enzyme(s) responsible for the resistance.

The quinolone resistance determining regions (QRDRs) of genes *parC*, *parE*, *gyrA* and *gyrB* were amplified by PCR using primers and cycling conditions previously described. Sequencing was performed in both strands by the dideoxy-chain termination method with a Perkin Elmer Biosystems 377 DNA sequencer and sequence analysis performed using the Lasergene software package (DNASTAR, Madison, WI).

All genes encoding ß-lactamases also were aligned and generic primers designed for PCR analysis. In most cases PCR annealing was carried out at 48ºC but gradient PCR was also used over a range of 38-60ºC. Sequencing of enzymes was carried out using DuPont Automated systems and analyzed using DNAstar.

RESULTS

- Twenty-seven *Salmonella* spp. isolates with nalidixic acid-resistant MIC results had ciprofloxacin MIC values of ≤ 0.03 - 0.5 μg/ml (Table 1). Ciprofloxacin was slightly more potent against nalidixic acid-susceptible (MIC_{90}, ≤ 0.03 μg/ml) and -resistant (MIC_{90}, 0.25 μg/ml) *Salmonella* spp. compared to the other fluoroquinolones tested.
- Figure 1 shows the distribution of ciprofloxacin MIC results for *Salmonella* spp. isolates with nalidixic acid MIC values of ≤ 8 μg/ml and ≥ 16 μg/ml. Isolates that were susceptible to nalidixic acid at ≤ 8 mg/ml had ciprofloxacin MICs of ≤ 0.03 μg/ml (> 99.0%) and strains with nalidixic acid values of ≥ 16 μg/ml had a uni-model distribution of ciprofloxacin MICs between 0.03 - 0.5 μg/ml.
- All *Salmonella* spp. strains with a nalidixic acid MIC of ≥ 32 μg/ml that were tested for QRDR mutations had a *gyrA* mutation (Figure 1). Mutations were Ser83Phe (S83F, seven), Asp87Asn (D87N, five), Asp87Tyr (D87Y, five) and Ser83Tyr (S83Y, two). No mutations were found in *gyrB*, *parC* or *parE*.
- The *Salmonella* spp. isolated in North America from nine medical centers had ESBL phenotypes that were negative using the ESBL Etest strip (Table 2). All strains were characterized for ß-lactamase production and all were positive for a CMY-2 ß-lactamase. No *Salmonella* spp. isolates in Latin America met the NCCLS criteria for ESBL production.
- Table 2 shows that the *Salmonella* spp. isolates with ESBL phenotypes were susceptible to cefepime (0.25 - 1 μg/ml), imipenem (0.12 - 0.5 μg/ml), ciprofloxacin (≤ 0.03 μg/ml) and gentamicin (≤ 1 μg/ml). Most strains were resistant to tetracycline (> 8 μg/ml) and susceptible to trimethoprim/sulfamethoxazole (≤ 0.5 μg/ml).

RESULTS

Table 1. Fluoroquinolone potencies among *Salmonella* spp. isolates that were susceptible or resistant to nalidixic acid from North America and Latin America.[a]

		MIC (μg/ml)		
Nalidixic acid (no.)	Fluoroquinolone	50%	90%	Range
Susceptible (301)	Ciprofloxacin	≤0.03	≤0.03	≤0.03-0.06
	Levofloxacin	≤0.03	0.06	≤0.03-0.25
	Gatifloxacin	≤0.03	0.06	≤0.03-0.25
	Garenoxacin	0.06	0.12	≤0.03-0.5
Resistant (27)	Ciprofloxacin	0.12	0.25	≤0.03-0.5
	Levofloxacin	0.25	0.5	≤0.03-1
	Gatifloxacin	0.25	0.5	≤0.03-0.5
	Garenoxacin	0.25	1	0.06-4

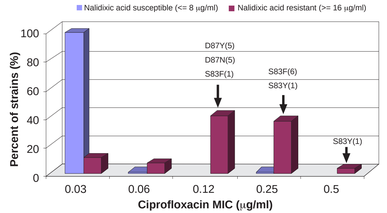
a. Breakpoint criteria for nalidixic acid of ≤ 8 μg/ml (susceptible) and ≥ 16 μg/ml (resistant) were used to classify fluoroquinolone resistance with potential clinical failure [NCCLS, M2-A8, 2003].

Table 2. Susceptibility characterization for *Salmonella* spp. with ESBL-phenotypes (NCCLS screen-positive) isolated in North America from SENTRY Program (2001) participant hospitals.

	Antimicrobial agents MIC (μg/ml):									Clavulanic	ß-
Country/ strain	Cefta-zidime	Cef-triaxone	Aztreo-nam	Cefe-pime	Imi-penem	Cipro-floxacin	Genta-micin	Tetra-cycline	Trim/ Sulfa	acid inhibition[a]	lactamase enzyme
USA											
272G	>16	32	>16	0.5	0.25	≤0.03	≤1	>8	≤0.5	Negative	CMY-2
330G	>16	32	16	0.5	0.12	≤0.03	≤1	>8	≤0.5	Negative	CMY-2
701G	>16	16	16	0.5	0.25	≤0.03	≤1	>8	≤0.5	Negative	CMY-2
821G	>16	16	16	1	0.25	≤0.03	≤1	>8	≤0.5	Negative	CMY-2
1231G	16	16	8	0.25	0.25	≤0.03	≤1	>8	≤0.5	Negative	CMY-2
1374G	16	8	4	0.25	0.25	≤0.03	≤1	>8	≤0.5	Negative	CMY-2
Canada											
554G	>16	32	16	1	0.5	≤0.03	≤1	≤4	>2	Negative	CMY-2
684G	>16	16	>16	1	0.25	≤0.03	≤1	>8	≤0.5	Negative	CMY-2
1275G	16	8	8	0.5	0.5	≤0.03	≤1	≤4	≤0.5	Negative	CMY-2
1366G	>16	32	16	1	0.25	≤0.03	≤1	>8	≤0.5	Negative	CMY-2
1409G	16	8	16	0.5	0.25	≤0.03	≤1	≤4	≤0.5	Negative	CMY-2

a. Results of NCCLS confirmatory test

Figure 1: Distribution of ciprofloxacin MIC results for *Salmonella* spp. isolates susceptible and resistant to nalidixic acid (NCCLS, M2-A8) and the *gyrA* mutations responsible for elevated ciprofloxacin MIC values.



CONCLUSIONS

- Utilizing a nalidixic acid MIC of ≥ 16 μg/ml and/or a ciprofloxacin MIC of 0.12 μg/ml or greater, would adequately detect *Salmonella* spp. isolates with single step *gyrA* mutations that may not fully respond to fluoroquinolone therapy.
- A discord between NCCLS susceptibility breakpoints for the disk diffusion test (MIC correlate, ≤ 8 μg/ml) and the MIC reference method (MIC, ≤ 16 μg/ml) could confuse clinical laboratories that may be trying to identify mutant strains of *Salmonella* spp.
- North American isolates of *Salmonella* spp. with ESBL phenotypes were detected which were not inhibited by clavulanic acid and were characterized as CMY-2 (Amp C) ß-lactamase producers. True ESBL producing *Salmonella* spp. remain very rare, in contrast to the commonly detected CMY-series of enzymes. The isolates remained susceptible to cefepime, carbapenems, aminoglycosides, fluoroquinolones and trimethoprim/sulfamethoxazole.

SELECTED REFERENCES

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