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Prevalence of mechanisms decreasing quinolone-susceptibility among *Salmonella* spp. clinical isolates

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Summary. Fluoroquinolone treatment failure has been reported in patients with nalidixic acid-resistant *Salmonella* infections. Both chromosomal- and plasmid-mediated quinolone-resistance mechanisms have been described. The objective of this study was to identify the prevalence of these mechanisms in a collection of 41 *Salmonella* spp. clinical isolates causing acute gastroenteritis, obtained in the Hospital Clinic, Barcelona. The minimum inhibitory concentrations (MICs) of nalidixic acid and ciprofloxacin were determined by Etest. Mutations in the quinolone-resistance determining regions (QRDRs) of the *gyrA, gyrB*, and *parC* genes and the presence of the *qnr, aac(6′)-Ib-cr*, and *qepA* genes were detected by PCR and DNA sequencing. All isolates showed constitutive expression of an efflux pump. None of the isolates were ciprofloxacin-resistant, whereas 41.5% showed nalidixic acid resistance associated with a mutation in *gyrA* and overexpression of an efflux pump. Although *qnrS1*, *qnrB6*, and *qepA* were found in four isolates, the expression of these genes was not associated with decreased quinolone susceptibility. Mutations in the *gyrA* gene and overexpression of an efflux pump were critical for nalidixic acid resistance and decreased susceptibility to ciprofloxacin in these isolates. However, plasmid-mediated quinolone resistance did not seem to play a major role. To our knowledge, this is the first description of *qepA* in *Salmonella*. [**Int Microbiol** 2010; 13(1):15-20]

Keywords: *Salmonella* · quinolones · efflux pumps · gene *gyrA* · plasmid-encoded genes · antibiotic resistance

Introduction

Non-typhoidal *Salmonella* isolates typically cause a self-limiting gastroenteritis, leading to bacteremia in 1–4% of cases. Bacteremia can result in complications, such as osteomyelitis, visceral abscesses, and endocarditis. These complications occur more frequently in the elderly and immunosuppressed [14]. Furthermore, *Salmonella* infection is often more serious in the developing world, where the levels of antibiotic resistance are higher [2,21].

Antimicrobial therapy is rarely necessary for *Salmonella* infection, but in cases of systemic salmonellosis, fluoroquinolones are used for treatment in adults, and third-generation cephalosporins for treatment in children [20]. Since its introduction, nalidixic acid resistance has steadily increased in *Salmonella*. However, despite wide use of fluoroquinolones such as ciprofloxacin, the levels of resistance to these antimicrobials remain low [15]. Resistance to quinolones is mainly due to: (i) mutations in the quinolone-resistance determining regions (QRDRs) of the target genes (*gyrA* and *gyrB*, which encode DNA gyrase, and *parC* and *parE*, which encode topoisomerase IV), and (ii) low accumulation of the antimicrobial within the cell, mostly associated

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with increased efflux due to overexpression of the AcrAB-TolC efflux pump [9]. Plasmid-mediated quinolone resistance is also emerging: *qnr, qepA* and *aac(6′)-Ib-cr* are plasmid-encoded genes that confer quinolone resistance. Qnr comprises a group of pentapeptide repeat proteins that protect bacteria against quinolones in a dose-dependent manner [22]. QepA is a plasmid-encoded efflux pump that significantly affects susceptibility to norfloxacin [23], and Aac(6′)-Ib-cr is a modified aminoglycoside *N*-acetyltransferase [18] that acetylates some fluoroquinolones, including ciprofloxacin.

The aim of this study was to understand the role of the different mechanisms of quinolone resistance that generate decreased susceptibility to quinolones among a collection of *Salmonella* spp. clinical isolates.

Materials and methods

Bacteria. Bacterial clinical isolates were obtained from *Salmonella-Shigella* agar plates (BD) inoculated with stool samples of patients with acute gastroenteritis during the period July 2007–October 2008 in the Department of Clinical Microbiology, Hospital Clinic, Barcelona, Spain. There were 41 isolates of *Salmonella enterica*: 19 *S.* Typhimurium; 19 *S.* Enteritidis; 1 *S.* Hadar; 1 *S.* Muenchen and 1 *S.* Choleraesuis. Specific antisera were used for typing the serovar.

Susceptibility testing. Antimicrobial susceptibility testing was performed using Etests (AB Biodisk, Solna, Sweden) on Mueller Hinton plates (Oxoid) following the manufacturer's recommendations. When the susceptibility of the isolates was too high to be measured by Etest, the MICs of those isolates were determined by the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI, formerly National Committee for Clinical Laboratory Standards, NCCLS) [7] and as described in [8].

Detection of target gene mutations and presence of plasmid-encoded determinants. PCR was carried out to screen for mutations in the QRDRs of the *gyrA*, *gyrB*, and *parC* genes, and for the presence of *qnr*, *qepA*, and *aac(6′)-Ib-cr*. Bands of the correct size were excised and purified using the Wizard SV Gel and PCR Clean-Up System kit (Promega, Madison, WI, USA), then quantified using a GeneQuest spectrophotometer (Cecil CE2302) and sent to Macrogen (Seoul, Korea) for sequencing. The results were analyzed using BLAST (PubMed) or by direct comparison with the gene sequences for the appropriate serotype. The primers used for PCR amplification and sequencing are listed in Table 1.

Statistical analysis. SPSS 15.0 for Windows was used for statistical analysis. Normality was assessed using histograms. Chi-square tests were done for comparisons of proportions. The Wilcoxon matched pairs test was applied for analysis of paired subjects when differences were not normal.

Results

Antimicrobial susceptibility testing revealed that all isolates were susceptible to ciprofloxacin (MICs: 0.012–0.75 μg/ml), whereas 41.5% of *Salmonella* isolates were nalidixic acid-resistant (MICs: 64–512 μg/ml). Of the 19 isolates of *S.* Enteritidis, 16 were resistant to nalidixic acid, whereas all 19 *S.* Typhimurium isolates were susceptible. Among the remaining three species, only *S.* Hadar was resistant to nalidixic acid. The results are shown in Table 2.

Table 1. List of primers used in this study

Gene	Primer (5′ to 3′)	Temperature (°C)	Reference
gyrA	AAATCTGCCCGTGTCGTTGGT	58	[8]
	GCCATACCTACTGCGATACC		
gyrB	GAATACCTGCTGGAAAACCCAT	57	[8]
	CGGATGTGCGAGCCGTCGACGTCCGC		
parC	AAGCCGGTACAGCGCCGCATC	57	[8]
	GTGGTGCCGTTCAGCAGG		
qnrA	ATTTCTCACGCCAGGATTTG	55	[19]
	GATCGGCAAAGGTTAGGTCA		
qnrB	GATCGTGAAAGCCAGAAAGG	55	[19]
	ACGATGCCTGGTAGTTGTCC		
qnrS	ACGACATTCGTCAACTGCAA	55	[19]
	TAAATTGGCACCCTGTAGGC		
aac(6′)-Ib-cr	CCCGCTTTCTCGTAGCA	55	This work
	TTAGGCATCACTGCGTCTTC		
qepA	CGTGTTGCTGGAGTTCTTC	59	[3]
	CTGCAGGTACTGCGTCATG		

Table 2. MIC determinations in the presence and absence of PAβN, and detection of target gene mutations and plasmid-encoded genes

Isolates	MIC (μg/ml)				QRDR mutation	Plasmid-encoded genes
	NAL[a]		CIP[a]		GyrA	*qnr* or *qepA*
Salmonella Enteritidis						
16435	128	(8)[b]	0.125	(0.094)	D87Y[c]	–
24097	256	(8)	0.19	(0.19)	D87Y	–
21380	128	(8)	0.19	(0.19)	D87Y	–
19505	128	(8)	0.19	(0.19)	D87Y	–
22679	128	(8)	0.19	(0.19)	D87Y	–
27341	512	(128)	0.19	(0.19)	D87Y	–
20055	512	(8)	0.19	(0.19)	D87Y	–
12345	512	(8)	0.19	(0.19)	D87Y	–
12333	128	(8)	0.19	(0.125)	D87Y	–
22601	8	(3)	0.016	(0.016)	–	*qnrB6*
29860	256	(8)	0.38	(0.19)	S83F	–
33910	64	(16)	0.19	(0.125)	D87Y	–
42565	64	(8)	0.19	(0.094)	D87Y	–
37453	64	(8)	0.25	(0.19)	D87Y	–
37141	32	(8)	0.19	(0.19)	D87Y	–
35397	8	(2)	0.023	(0.016)	–	–
44819	256	(8)	0.25	(0.125)	D87Y	–
43735	64	(16)	0.19	(0.125)	D87Y	–
53908	8	(1.5)	0.012	(0.012)	–	–
Salmonella Typhimurium						–
14630	6	(2)	0.016	(0.012)	–	–
13920	6	(4)	0.016	(0.012)	–	–
26986	6	(1.5)	0.023	(0.016)	–	*qnrS1*
27562	8	(1.5)	0.016	(0.016)	–	–
249	8	(1.5)	0.016	(0.012)	–	*qepA*
566	12	(4)	0.023	(0.016)	–	–
21389	6	(1)	0.016	(0.016)	–	–
13197	8	(2)	0.016	(0.016)	–	–
12397	8	(3)	0.016	(0.012)	–	–
7660	6	(4)	0.012	(0.016)	–	–
40456	6	(1)	0.016	(0.016)	–	–
26563	12	(6)	0.016	(0.016)	–	–
27224	8	(1)	0.016	(0.008)	–	–
30010	12	(8)	0.023	(0.016)	–	–

(*continued*)

Table 2. *Continued*

Isolates	MIC (μg/ml)				QRDR mutation	Plasmid-encoded genes
	NAL[a]		CIP[a]		GyrA	*qnr* or *qepA*
43968	6	(1)	0.016	(0.008)	–	–
39645	8	(0.75)	0.016	(0.008)	–	–
44615	6	(2)	0.016	(0.012)	–	–
49758	6	(1.5)	0.016	(0.016)	–	–
32962	4	(0.5)	0.016	(0.012)	–	–
Salmonella Choleraesuis						
24271	6	(0.5)	0.016	(0.012)	–	*qnrS1*
Salmonella Hadar						
46324	512	(8)	0.75	(0.38)	D87N	–
Salmonella Muenchen						
49958	12	(1)	0.016	(0.012)	–	–

[a] NAL: nalidixic acid; CIP: ciprofloxacin.
[b] Numbers in parenthesis represent the MICs determined in the presence of PAβN (20 μg/ml).
[c] D: aspartic acid; Y: tyrosine; S: serine; F: phenylalanine; N: asparagine; –: no mutation found.

All nalidixic acid resistant isolates had a mutation in the QRDR of the *gyrA* gene. All substitutions observed were in codons Ser83 and Asp87: 15 out of the 16 *S.* Enteritidis resistant isolates carried the substitution D87Y, whereas the remaining isolate carried the substitution S83F. The amino acid change D87N was detected in the *S.* Hadar isolate (Table 2).

The association between nalidixic acid resistance and mutations in the *gyrA* gene was statistically significant (Pearson χ^2 of 48, on one degree of freedom, $P < 0.001$). Only one *S.* Typhimurium isolate (40456) showed a mutation in the *parC* gene, T57S (data not shown), which was associated neither with decreased susceptibility to ciprofloxacin nor with an increased MIC of nalidixic acid. Among these isolates, no mutations were detected in the *gyrB* gene (data not shown).

According to the results presented in Table 2, which shows antimicrobial susceptibility data and mutations in the QRDR of target genes, the *Salmonella* isolates were divided into two groups. The first group comprised the 24 nalidixic acid susceptible isolates in which no mutation in the target genes was detected. These isolates had a phenotype highly susceptible to ciprofloxacin (MICs ≤0.023 μg/ml). When the MICs were measured in the presence of the efflux pump inhibitor PAβN, to evaluate the contribution of efflux pump activity, a 1.5- to 12-fold decrease in the nalidixic acid values was observed. However, a slight decrease (1.3- to 2-fold) was detected in 15 of the 24 isolates when we measured the MIC of ciprofloxacin; the remaining isolates did not show any change in their MICs.

The second group comprised those 17 isolates with a mutation in the *gyrA* gene. They had a nalidixic acid resistance phenotype, and in all but one isolate, resistance decreased below the clinical breakpoints of susceptibility when the MICs were measured in the presence of PAβN. A 4- to 64-fold decrease in the nalidixic acid MIC was detected in these resistant isolates. This was a considerably greater reduction than that observed in the nalidixic acid susceptible isolates, suggesting an increase in efflux pump activity. Furthermore, the isolates belonging to this group were less susceptible to ciprofloxacin (MICs of ciprofloxacin: 0.19–0.75 μg/ml). When the MICs of ciprofloxacin were measured in the presence of PAβN, ten of these nalidixic acid resistant isolates showed a 1.3- to 2-fold decrease, whereas no change was detected in any of the other seven isolates. In all but two isolates (MIC of 0.094 μg/ml), susceptibilities to ciprofloxacin remained decreased in the presence of PAβN.

There were statistically significant differences at the 5% level in the MICs of ciprofloxacin depending on the presence of the efflux pump inhibitor ($P = 0.001$). In the nalidixic acid resistant isolates with mutations in the *gyrA* gene, there were also statistically significant differences at the 5% level in the MICs of nalidixic acid that depended on the presence of PAβN ($P > 0.001$). The *qnr* gene was found in three isolates: a gene with 100% identity with *qnrS* was detected in one *S.* Choleraesuis and one *S.* Typhimurium, and a gene with 100% identity with *qnrB6* was found in one *S.* Enteritidis (Table 2). However, the quinolone susceptibility levels of these isolates

were similar to those of isolates without the *qnr* genes. A gene with 100% identity to *qepA* was found in one *S.* Typhimurium isolate, although it was not associated with any phenotype of decreased susceptibility to quinolones, not even to norfloxacin (MIC of 0.125 μg/ml without PAβN and 0.094 μg/ml with PAβN). The *aac(6′)-Ib-cr* gene was not found in this work.

Discussion

Nalidixic acid resistance has increased among *Salmonella* clinical isolates over the last few years, whereas ciprofloxacin resistance remains low [15]. Single mutations in the *gyrA* gene have been found to be sufficient for high-level nalidixic acid resistance in *Salmonella* [11]. Increased efflux of the antimicrobial has also been reported to be a common mechanism and generally represents the first step in the acquisition of fluoroquinolone resistance [12]. AcrAB/TolC is the main efflux pump involved in determining intrinsic levels of resistance in Enterobacteriaceae, according to basal levels of expression, and confers quinolone resistance when overexpressed [16].

The most important mechanisms producing nalidixic acid resistance are point mutations in the *gyrA* gene and increased efflux contribution. In this study, all of the isolates resistant to nalidixic acid had a single mutation in *gyrA*. Furthermore, the presence of PAβN, an efflux pump inhibitor, rendered all but one of the nalidixic acid resistant isolates susceptible, and decreased the MIC of the susceptible isolates to a lesser extent. However, the MICs of ciprofloxacin decreased only slightly in the presence of PAβN. These results reinforce the idea of a constitutive expression of an efflux system, mainly affecting nalidixic acid extrusion. This efflux system might be overexpressed in those isolates with a mutation in *gyrA*, suggesting a synergistic effect between efflux pumps and QRDR mutations. However, efflux pump activity could be less important in decreasing ciprofloxacin susceptibility levels, at least at the low levels of resistance observed in these isolates. It seems likely that overexpression of the AcrAB-TolC efflux pump is responsible for this effect, due to the high prevalence of the efflux pump among clinical isolates and in vitro mutants of *Salmonella* [5,6,8], although a concomitant overexpression of another efflux pump cannot be ruled out. Furthermore, this study concurs with previous research that the T57S substitution detected in ParC is not associated with a quinolone resistance phenotype since it has been found in both resistant and susceptible isolates [1].

Concerning the plasmid-encoded determinants, a decreased susceptibility to the quinolones nalidixic acid, ciprofloxacin, or norfloxacin has been reported and ascribed to the presence of the *qnr*, *aac(6′)-Ib-cr*, and *qepA* genes [18,22, 23], increasing the MICs 16- to 32-fold, 2- to 4-fold, and 32- to 64-fold, respectively [17]. The highest prevalence of these genes has been found among Enterobacteriaceae, especially in *Escherichia coli*, *Enterobacter* spp., *Klebsiella pneumoniae*, and *Salmonella* spp. The *qnr* genes have been detected worldwide, with *qnrB* being the most prevalent variant. However, despite their worldwide spread, the prevalence of the *qnr* genes is still low in *Salmonella* spp. (0.2–3%, reaching 9.8% among isolates showing decreased susceptibility to fluoroquinolones). The *aac(6′)-Ib-cr* gene may be even more widespread than *qnr*, whereas the prevalence of *qepA* is apparently low, perhaps reflecting the fact that few studies of *qepA* prevalence have been performed [17]. Three out of the 41 isolates (7.3%) tested in this study were positive for the presence of *qnr* genes (*qnrB6* and *qnrS1*); only one isolate (2.4%) showed the presence of *qepA*. However, *aac(6′)-Ib-cr* was not detected. To our knowledge, the *qepA* gene has not yet been reported in *Salmonella* spp. [4,10,13], suggesting that ours is the first description of the *qepA* gene and the *qnrB6* variant in *Salmonella*. Note that the clinical isolates harboring these plasmid-encoded genes did not show any significant change in their MICs when compared with isolates susceptible to nalidixic acid and negative for the presence of *qnr* or *qepA*.

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