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Molecular study of quinolone resistance mechanisms and clonal relationship of *Salmonella enterica* clinical isolates

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ABSTRACT

In the last few years, the number of *Salmonella enterica* strains resistant to nalidixic acid has steadily increased. In a previous study, the quinolone susceptibility phenotype and genotype of 38 *S. enterica* clinical isolates (19 *S. enterica* serovar Typhimurium and 19 *S. enterica* serovar Enteritidis) were determined. Forty-two percent of the isolates showed nalidixic acid resistance associated with a mutation in *gyrA* together with putative overexpression of efflux pump(s). In this study, mutations in the quinolone resistance-determining region (QRDR) of *parE* and the regulators of AcrAB (*acrR*, *marRAB*, *soxRS* and *ramR*) were analysed. Intracellular accumulation of ciprofloxacin and nalidixic acid was determined. Gene expression of the efflux pump components *acrB*, *tolC*, *acrF* and *emrB* was also assessed. In addition, an epidemiological study of the isolates by multilocus sequence typing (MLST) and pulsed-field gel electrophoresis (PFGE) was performed. No mutations were detected in *parE*, whereas two amino acid substitutions were found in two susceptible strains in MarR (I84L) and AcrR (N214T) in one strain each, although both were suggested to be polymorphisms. No changes in the gene expression of *acrB*, *tolC*, *acrF* and *emrB* were detected between nalidixic-acid-resistant and -susceptible strains. Intracellular accumulation was not useful to reveal differences. Epidemiological analysis showed an important clonal relatedness among the *S.* Enteritidis isolates, whereas major divergence was seen for *S.* Typhimurium. Altogether, these results suggest the presence of previously undiscovered drug efflux pump(s) and confirm the high clonality of *S.* Enteritidis and the genetic divergence of *S.* Typhimurium.

1. Introduction

Salmonellosis is one of the major causes of food-borne gastroenteritis in humans worldwide. Currently, among the high diversity of *Salmonella enterica* serovars, *S. enterica* serovar Typhimurium and *S. enterica* serovar Enteritidis are the two major aetiological agents. Although antimicrobial resistance in *S.* Enteritidis is reported to be lower than in *S.* Typhimurium [1], in the last few years the prevalence of multidrug-resistant *S.* Enteritidis isolates has increased. Of special concern is the increase in resistance to nalidixic acid and the decrease in susceptibility to fluoroquinolones such as ciprofloxacin, which is one of the first-choice agents to treat salmonellosis.

Quinolone resistance in Enterobacteriaceae is mainly due to point mutations in the quinolone resistance-determining regions (QRDRs) of the target genes that encode both the A and B subunits of the two type II topoisomerases, namely DNA gyrase (*gyrA* and *gyrB*) and DNA topoisomerase IV (*parC* and *parE*). Amino acid substitutions are mostly located in two particular positions in GyrA (S83 and D87) and in ParC (S80 and E84). However, mutations in *gyrB* and *parE* have also been described [2].

Resistance to quinolones is also due to changes in membrane permeability causing a reduction in concentration of these drugs in the cytoplasm. This phenotype can be achieved by a decrease in the expression of porins or by overexpressing efflux systems. The major efflux system in *Salmonella* is AcrAB-TolC, a tripartite multidrug efflux system belonging to the resistance nodulation-division (RND) family [3]. Other efflux systems such as EmrAB, which belongs to the major facilitator superfamily (MFS), and AcrEF, an RND member, have previously been reported to show increased expression levels in fluoroquinolone-resistant mutants [4].

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Table 1
List of primers used in this study.

Gene	Primers (5′–3′)	Annealing temperature (°C)	Reference
For sequencing			
Quinolone resistance-determining region (QRDR)			
parE	SparE.1 CCTGCGGCCCGGCGTTGCCGGGG	62	[8]
	SparE.2 CGCCCGCCTTCTCTTCTTCCGTCAGCGCG		
Regulatory genes			
soxRS	Ssox.1 GGCACTTTGCGAAGGCGTTACCA	54	[8]
	Ssox.2 GGGATAGAGCGAAAGACAA		
marRAB	Smar.1 AGCGGCGGACTTGTCATAGC	58	[8]
	Smar.2 ACGGTGGTTAGCGGATTGGC		
acrR–acrA	Sacr.1 CAGTGGTTCCGTTTTTAGTG	58	[8]
	Sacr.2 ACAGAATAGCGACACAGAAA		
ramR	SramR.1 CGTGTCGATAACCTGAGCGG	62	[15]
	SramR.2 AAGGCAGTTCCAGCGCAAAG		
For real-time PCR			
acrB	AcrB_RT_F TTTTGCAGGGCGCGGTCAGAATAC	60	[5]
	AcrB_RT_R TGCGGTGCCCAGCTCAACGAT		
tolC	TolC_RT_F GTGACCGCCCGCAACAAC	60	This study
	TolC_RT_R ATTCAGCGTCGGCAGGTGAC		
16S	16S_RT_F GCGGCAGGCCTAACACAT	60	[7]
	16S_RT_R GCAAGAGGCCCGAACGTC		
acrF	SacrF.RT.1 TACCCAGGACGACATCTCTGA	60	This study
	SacrF.RT.2 CACACCATTCAGACGGCTGAT		
emrB	SemrB_RT.F CCGTCGTCCTGATGACGTTA	60	This study
	SemrB_RT.R CCGTTCGGTATGCGTTTCAC		

Expression of AcrAB is controlled by AcrR, the local repressor of AcrAB, and the global regulators MarA (*marRAB*), SoxS (*soxRS*) and RamA. Mutations in *marR*, *soxR* and *ramR* lead to an increase in the expression of the transcriptional activators MarA, SoxS and RamA, respectively, which in turn interact with the *acrAB* promoter, thereby increasing the amount of AcrAB produced and effectively enhancing efflux. Otherwise, mutations in the local repressor *acrR* impair its repressive function and hence AcrAB can be overproduced [5].

Quinolone resistance in *Salmonella* can also be plasmid-mediated through the transmission of several determinants: Qnr, a pentapeptide repeat protein family that protects the target topoisomerases from quinolone inhibition; the Aac(6′)-Ib-cr protein, which acetylases fluoroquinolones; and two plasmid-encoded efflux pumps, QepA (MFS-type) and OqxAB (RND family) [2].

In this study, mutations in the QRDR of *parE* and in the regulators of AcrAB (*acrR*, *marRAB*, *soxRS* and *ramR*) were analysed, as was the expression of some efflux pumps components (acrB, tolC, acrF and emrB), quinolone accumulation and clonal relatedness of in quinolone-resistant and -susceptible *Salmonella* enterica clinical isolates.

2. Materials and methods

2.1. Bacterial strains

Forty-one *S. enterica* clinical strains were isolated during the period 2007–2008 in the Department of Clinical Microbiology of the Hospital Clínic, Barcelona (Spain). Both chromosomal (mutations in the QRDRs of the *gyrA*, *gyrB* and *parC* genes) and plasmid-mediated quinolone resistance mechanisms [presence of *qnr*, *aac(6′)-Ib-cr* and *qepA* genes] were initially characterised in a previous study [6]. Among these, 19 *S.* Typhimurium and 19 *S.* Enteritidis were recovered for further study.

2.2. Screening for mutations in the quinolone resistance-determining region of parE and the multidrug resistance regulatory genes (acrR, marRAB, soxRS and ramR) and promoter regions

Amplification of the QRDR of *parE* and the regulatory genes *acrR*, *marRAB*, *soxRS* and *ramR* including their promoter regions was carried out by PCR. PCR products were purified with a Gel Extraction Kit (Omega Bio-tek, Norcross, GA) and were sent for sequencing at Macrogen Inc. (Amsterdam, The Netherlands). Screening for mutations was done using BioEdit® software (Ibis Biosciences, Carlsbad, CA) through alignment with the corresponding template sequences, obtained from the genome of *S.* Enteritidis (RefSeq NC_003197.1) and *S.* Typhimurium (RefSeq NC_011294.1). The primers used for PCR amplification and sequencing are listed in Table 1.

2.3. RNA extraction and DNase treatment

Five *S.* Enteritidis isolates, comprising three nalidixic-acid-resistant isolates (44819, 20055 and 12345) and two nalidixic-acid-susceptible isolates (22601 and 35397) were selected for RNA extraction, as well as one *S.* Typhimurium strain that overexpresses AcrB (50–64) [8] to ensure the validity of the results.

RNA extraction was carried out as previously described [8]. Briefly, mid-logarithmic phase cultures [optical density at 600 nm (OD_{600}) = 0.4–0.6] were treated with RNAprotect™ Bacteria Reagent (QIAGEN, Hilden, Germany) and were harvested by centrifugation. Pellets were re-suspended in a mixture of TE [Tris–ethylene diamine tetra-acetic acid (EDTA)] and lysozyme and RNA extraction was achieved using an RNeasy® Mini Kit (QIAGEN). All of the procedures were performed according to the manufacturer's recommendations.

To remove contamination by genomic DNA (gDNA), all samples were treated with a DNA-free DNase Kit (Ambion, Austin, TX) and PCR was conducted to confirm the loss of gDNA. Quantification of the decontaminated RNA was accomplished with an ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Five independent RNA extractions of each isolate were performed.

2.4. cDNA synthesis and real-time PCR

Retro-transcription of 500 ng of each RNA sample was performed according to the manufacturer's instructions (Takara, Dalian, China). Then, the cDNA template was diluted 1/5 to a final amount of ca. 40 ng/μL.

The genes selected for gene expression analysis were a*crB*, *tolC*, *acrF* and *emrB*, and the 16S gene was used as an endogenous control.