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Antibiotics susceptibility of quinolones against *Salmonella* spp. strains isolated and molecularly sequenced for gyrA gene

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ABSTRACT

Drug-resistant *Salmonella* is frequently detected in most parts of the world, and its rate of resistance has increased significantly in recent years. However, this study aimed to evaluate the minimum inhibitory concentration (MIC, determined with the Kirby-Bauer method) of quinolones in 86 *Salmonella* spp. strains isolated from pigs. Both the inside and outside of the QRDR region of strains were sequenced. The DNA sequence of the QRDR region of *Salmonella* spp. revealed the mutations S83F, D87N and S83Y. The region outside the QRDR showed a mutation in L582G. Forty-five isolates of *Salmonella* ssp. were categorized as quinolone-resistant; out of these, 16 corresponded to *Salmonella enterica* and isolates showed intermediate resistance (6.25%) to nalidixic acid. Three isolats (18.6%) were resistant to ampicillin; two (12.5%) were resistant to carbenicillin. Moreover, three (18.7%) isolates were resistant to gentamicin, nitrofurantoin and pefloxacin, and 8 (50%) were resistant to trimethoprim/sulfamethoxazole. Six percent of *Salmonella* spp. strains showed less resistance to antimicrobial agents compared to *S. Thyphimurium* (18%). The resistance to individual quinolones varied by serotypes. For *S. anatum* and *S. Reading*, it was 12.25%, and for *S. choreaeaesuis*, *S. typhimurium* monofasica, 6.25%. In contrast, *S. agona*, *S. bredeney* and *S. london* were sensitive to these antibiotics. In conclusion, quinolones have become the drugs of choice for the treatment of severe *Salmonella* infections. The study of mutations outside the QRDR region opens up new insights about the resistance of *Salmonella* to fluoroquinolones.

1. Introduction

Quinolone-resistant *Salmonella* spp. isolates are increasingly frequent in clinical and environmental settings; this has been attributed mainly to target gene alterations, efflux, bacterial cell membrane alterations and transferable quinolone resistance [1]. Mutations in the quinolone resistance-determining regions (QRDRs) of these genes alter the conformation of the active sites of the enzymes, preventing the binding and subsequent inhibitory action of quinolones [2]. Quinolones, originally represented by nalidixic acid and more recently by fluoroquinolones, initiate bactericidal activity by trapping the covalent protein-DNA complexes formed by bacterial DNA gyrase or topoisomerase IV [3]. The DNA gyrase and topoisomerase IV are two important enzymes that play an essential role in bacterial DNA replication, and are targets for the inhibitory activity of quinolones. Both gyrase and topoisomerase IV are tetrameric enzymes, each encoded by two pairs of genes (gyrA and gyrB for DNA gyrase and parC and parE for topoisomerase IV) [4]. The most common mutation in gyrA leads to a change at codon 83 from serine to phenylalanine or tyrosine; a second mutation at codon 87 of the gyrA gene changes aspartate to asparagine. Target alteration is the most common resistance mechanism found in *Salmonella* strains isolated worldwide [5].

The uptake of quinolones by bacteria can be reduced by altering the cell membranes and increasing the expression of efflux pumps. The quinolones mechanisms were categorized into four groups: (i) mutations in the local repressor gene; (ii) mutations in the global regulatory gene; (iii) mutations in the promoter region of the transporter gene; (iv) insertion elements upstream of the transporter gene [6].

Salmonella strains may gain resistance to quinolones through a decreased expression of porins and/or an increased expression of

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nonspecific, energy-dependent efflux systems such as the AcrAB–TolC efflux pumps. However, these mechanisms produce only a low level of resistance toward quinolones and are only clinically important when they are concurrent with alterations in target enzymes such as DNA gyrase [7,8].

Among the strains that are resistant to quinolones and/or fluoroquinolones, the most prevalent mechanism is amino acid substitutions in the quinolone resistance-determining region (QRDR) of the gyrA and parC genes, but a mechanism that has been showing a growing importance is plasmid-mediated quinolone resistance associated with the qnrA, qnrB, qnrC, qnrD and qnrS genes; however, the frequency of these mechanisms is different [9,10]. Codon 57 of parC has been found outside the QRDR of this gene, which makes it possible that the mechanism of the resistance conferred by this mutation is different to that of the other QRDR mutations [11].

The aim of this work is to study changes in the susceptibility of *Salmonella* sp. isolated from pigs to nalidixic acid and fluoroquinolone, and to analyse and detect mutations of the gyrA gene in quinolone-resistant isolates. Sampling and experimental characterization of mutations outside and inside the QRDR of gyrA sequences of *Salmonella* sp. isolates were performed, and the minimum inhibitory concentration values of known quinolones vs. isolated mutants of *Salmonella* sp. were measured.

2. Materials and methods

2.1. Isolation of Salamonella spp. and susceptibility to quinolones

Isolates of *Salamonella* were obtained from pigs (an average of 1600 animals), and a total of 320 bile and lymph samples were taken for the chemical identification and antibiotic resistance tests of the isolates.

All bacterial cultures were incubated at 37 °C in atmospheric conditions. Suspect colonies were identified using the API 20E system (bioMérieux). The cultures were enriched in buffered peptone broth for 18–24 h, and then in brilliant green and XLD agar to identify *Salmonella* species. Serotyping was performed using the agglutination test at the National Institute of Epidemiological Diagnosis and Reference in Mexico.

The antimicrobial susceptibility test discs (BBL Sensi-Disc; Becton Dickinson) used here had the following antibiotic dosages: 30 μg of nalidixic acid, 5 μg of ofloxacin, 10 μg of norfloxacin and 5 μg of ciprofloxacin (Becton Dickinson). The antibiotic resistance of the isolates was determined using the Kirby-Bauer disc diffusion test on Mueller-Hinton agar, following the method described by Refs. [12,13]. The bacterial inoculum was standardized to a concentration of 0.5 on the McFarland scale, and the agar plates were inoculated and incubated at 37 °C for 24 h.

The growth inhibition zones were measured and compared using interpretation tables, which was used to categorize the bacteria as resistant, intermediate or sensitive (Table 1). The inhibition zones were calculated in agreement with the recommendations of the National Committee for Clinical Laboratory Standards [14].

Table 1
Antimicrobial susceptibility pattern of *Salmonella* spp. isolates according to the Kirby-Bauer disc diffusion assay.

Quinolone	Resistance	Intermediate	Sensitive
Nalidixic acid	≤13	14–18	≥19
Norfloxacin	≤12	13–16	≥17
Ciprofloxacin	≤15	16–20	≥21
Ofloxacin	≤12		≥16

2.2. Polymerase chain reaction (PCR) amplification and sequencing of the quinolone resistance-determining region of the gyrA gene

2.2.1. DNA extraction

Forty-five isolates of *Salmonella* ssp. were categorized as quinolone-resistant; out of these, 16 corresponded to *Salmonella enterica* spp. according to serological analysis. DNA was extracted from pure cultures of *S. agona*, *S. anatum*, *S. bredeney*, *S. choleraesuis*, *S. enteritidis* and *S. reading*, which were inoculated into 9 ml brain heart infusion and grown overnight at 37 °C for 18 h to a cell density of approximately 108 CFU/ml. The cells were washed twice by centrifuging 1 ml of cell suspension for 10 min at 16.000 g; the supernatant was removed and the pellet was suspended in 1 ml of sterile physiological salt solution (0.85% NaCl).

The method involves chemical lysis by chaotropic salt and enzymatic digestion with proteinase K and thermal lysis. DNA is bound to a silica-gel membrane in a spin-column, while PCR inhibitors are, supposedly, not retained. The bound DNA is then washed, eluted in buffer and stored at −20 °C. The extraction and purification of DNA were performed using a DNA extraction mini kit (Qiagen, Courtaboeuf, France).

2.2.2. Primers and probes used in the PCR assay

The gyrA gene was amplified with the program 3 plus using four pairs of primers (Table 2) that were designed for sequencing the gyrA gene (2637 bp) based on the nucleotide sequence of the LT2 chromosome of *Salmonella typhimurium* (STM 2272; NCBI). A PCR was performed with a final volume of 50 μl for each oligonucleotide, containing a concentration of 0.2 mM, 5 microliters of PCR buffer, 200 mM of deoxynucleotides (dNTPs), 2.5 μl of MgCl2, 0.5 U of Taq polymerase (Promega, Madison, WI, USA), and about 100 ng of chromosomal DNA.

The reactions were carried out in a Thermocycler (applied Biosystems 3730 DNA analyzer xl) under the following conditions: 94 °C for 2 min, 35 cycles at 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1.30 min and 72 °C for 10 min.

One microliter of PCR product was run on 1.5% agarose gel with TAE buffer (1X) at 120 V for 20 min. A DNA ladder (Promega 1 kb DNA Ladder, USA, Madison, WI 53711-5399 and GeneRuler Express DNA Ladder) was used as molecular weight marker. The gel was stained with ethidium bromide and the DNA bands were visualized on a UV transilluminator.

2.2.3. GyrA sequencing

The DNA sequencing of the gyrA gene was performed on an ABI PRISM® 310 (Macrogen, Rockville, USA). Direct double sequencing was performed using the two DNA strands (forward and reverse). The sequencing results were analyzed and compared to a published DNA sequence of gyrA of *Salmonella enterica* subsp. *enterica typhimurium* str. LT2 (STM 2272) with the GenBank access code AE006468 (ID1253794).

2.2.4. Software analysis

The sequencing reactions were performed using the BigDye

Table 2
Primers for amplification of the gyrA gene.

	Sequence	Scale	Purity	No. of Bases
prim1_28_F	GGTTAGATGAGCGACCTTGC	25 N	DSL	20
prim1_794_R	GTTTTGGCGTCAGCTTCAAC	25 N	DSL	20
prim2_37_F	CGGTCGTGGCAAAGTGTA	25 N	DSL	18
prim2_728_R	TTTCTGCAAACGCAGATCC	25 N	DSL	19
prim3_37_F	ATGGCTGGAGCCAGAATTT	25 N	DSL	19
prim3_819_R	GCATGACTTCGTCAGAACCA	25 N	DSL	20
prim4_40_F	ATTGGCGTTGACCTGACTTC	25 N	DSL	20
prim4_649_R	CCCTCGCACAGCAATAACAT	25 N	DSL	20