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Surveillance of quinolone resistance in *Salmonella enterica* from Argentina

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INTRODUCTION

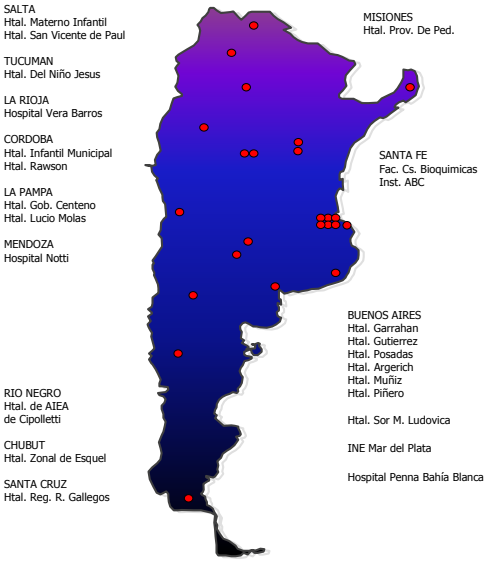


Figure 1. WHONET - Argentina Working Group

Fluoroquinolones are agents with high activity against a wide range of gram-positive and gram-negative bacteria. This fact, plus the advantage of an oral dosing, make this family of antibiotics useful for the treatment of gastrointestinal infections including *Salmonella* species infections. The wide use of antimicrobial agents, including fluorquinolones, in food animal production raises the incidence of antibiotic resistance in zoonotic food-borne *Salmonella enterica* infections in most industrialized countries (N. Engl. J. Med. 341:1420-25, 1999). During the last years, several treatment failures with ciprofloxacin (CIP) and other fluoroquinolones have been reported with strains that already harbored a single mutation in the *gyr*A gene (J. Antimicrob. Chemother. 37:351-356, 1996).

In gram-negative bacteria, point mutations in the gyrA gene coding for the A subunit of gyrase are primarily responsible for the development of resistance to quinolones. Other mechanisms of resistance include impermeability and efflux pumps. Resistance mutations of *gyr*A have been clustered in a region of the gene product between amino acids 67 and 106 termed the quinolone resistance-determining region (QRDR). Amino acid changes at Ser-83 (to Phe, Tyr or Ala) or at Asp-87 (to Gly, Asn or Tyr) are the most frequently observed in nalidixic acid-resistant strains with decreased-susceptibility to CIP (Microb. Drug Resist. 2:299-302, 1996). Double mutations at both residues 83 and 87 have been identified in fluoroquinolone-resistant clinical isolates of *E. coli* and *Salmonella* spp. (Antimicrob. Agents Chemother. 37:696-701, 1993). It has been suggested that resistance to nalidixic acid (NAL) may be an indicator of decreased suceptibility to CIP (Clin. Microbiol. Infect. 3:541-543, 1997).

THE AIMS OF THIS STUDY WERE:

- **to asses a retrospective quinolone resistance surveillance in clinical isolates of *Salmonella enterica* subsp. *enterica* collected in Argentina from 1999 to 2001.**
- **to determine the mutations in the QRDR that confers quinolone resistance in these isolates.**
- **to determine the prevalence of these mutations.**

MATERIALS AND METHODS

1) Bacterial isolates.

A total of 753 clinical *Salmonella enterica* isolates representative of different regions of the country were studied. These isolates were collected during 1999 and 2001 by laboratories that participate in the WHONET-Argentina Network for antimicrobial resistance surveillance (Figures 1 and 2)

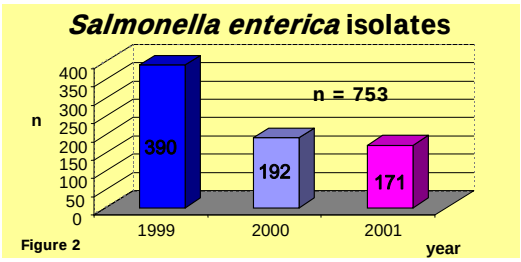


Figure 2

2) Biochemical identification and serotyping.

The identification of strains was performed by biochemical (1986, 4th ed., p. 181-340, Elsevier Science Publishing Co., N. Y.) and serological tests according to the standard international scheme for serotyping *Salmonella* (2001, 8th ed., WHO Collaborating Centre for Reference and Research on *Salmonella*, Inst. Pasteur, Paris). For serotyping, somatic and flagellar antisera were used which were prepared by Antigens and Antisera Division of National Production Institute – ANLIS "Dr. Carlos G. Malbrán".

3) Antimicrobial and susceptibility testing.

The NAL- and CIP-resistance screening was performed by disk diffusion. The antimicrobial susceptibility of each isolate with resistance or reduced susceptibility to nalidixic acid was confirmed by the agar dilution method according to NCCLS guidelines. The breakpoints employed for disk diffusion method (susceptible -S-, intermediate -I- and resistant -R- categories, respectively) were ≥19 mm, 14-18 mm and ≤13 mm to NAL and ≥21 mm, 16-20 mm and ≤15 to CIP. For the agar dilution method, the breakpoints were ≤16 µg/ml (S) and ≥32 µg/ml (R) for NAL, and ≤1 µg/ml (S), 2 µg/ml (I) and ≥4 µg/ml (R) to CIP. *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922 were used as internal quality controls for this method.

4) PCR amplification.

The NAL intermediate and resistant strains were studied. The analogous region to the QRDR of the *gyr*A gene were amplified by PCR. These primers (5´-ACGTATTGGGCAAYGACTGGA-3´ and 5´-CAACGAAATCGACCGTCTCTT-3') amplified a fragment of 281 bp of that region.

5) DNA sequencing and sequence analysis .

DNA sequencing of amplicons was performed on both strands by the method of Sanger *et al.*, using the BigDye terminators methodology. The sequences were obtained in an ABI Prism 377 DNA Sequencer. Multiple nucleotide sequence alignments were performed with the CLUSTAL facilities of the PCGENE software package (Intelligenetics, Inc.).

RESULTS AND DISCUSSION

Hospital distribution of NAL intermediate and resistant strains

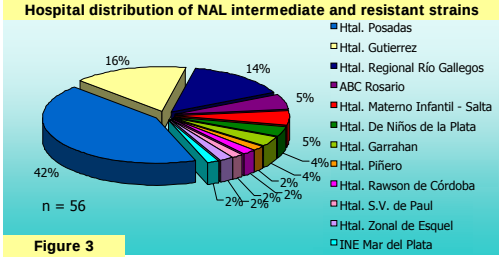


Figure 3

Serotypes of NAL intermediate and resistant *Salmonella*

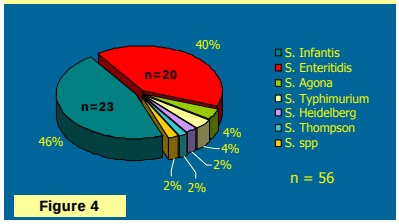


Figure 4

Nalidixic acid susceptibility profile

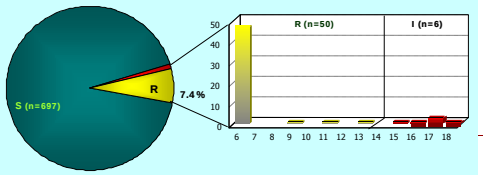


Figure 5

Resistant *Salmonella enterica* strains belonged to 12 different institutions (Figure 3). These strains corresponded to 7 different serotypes (Figure 4), being *Salmonella Infantis* (n=23) and *Salmonella Enteritidis* (n=20) the most prevalent serovars. About 7.4 % (n=56, including 50 resistant and 6 intermediate strains) shown resistance to NAL (Figures 5 and 6).

Susceptibility profiles distribution for NAL and CIP (diffusion method)

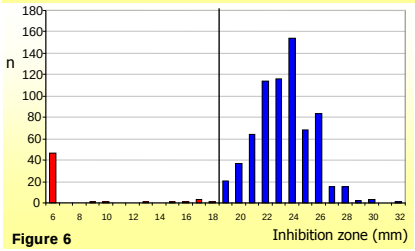


Figure 6

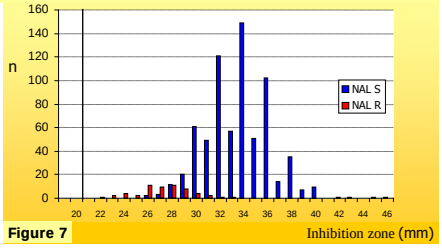


Figure 7

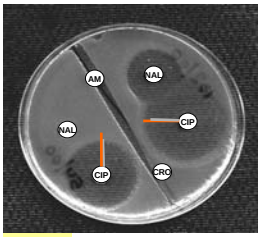


Figure 8

MIC results			
NAL inhibition zone	n	MIC to NAL (µg/ml)	MIC to CIP (µg/ml)
06 mm(R)	47	128-> 1024	0.125-1
≥07-13 mm (R)	3	32	0.06
14-18 mm (I)	6	4-8	0.03-0.06
≥ 19 mm (S)	697	≤4	≤ 0.015

Table 1

All the strains were susceptible to CIP, but a reduction in the inhibition zone to this drug was detected in the NAL-R strains (Figures 7 and 8). The strains without inhibition zones to NAL had MIC to CIP three-fold higher than the NAL susceptible strains (Table 1).

NAL-resistant strains

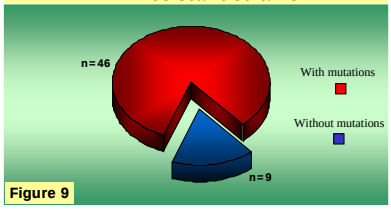
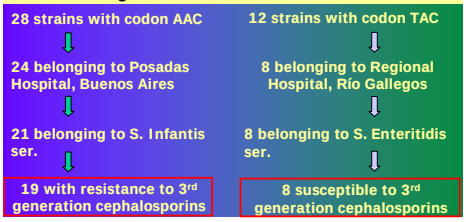


Figure 9

Table 2. Sequences of QRDR fragment of *gyrA* gene in NAL-resistant isolates

Codon number	Original codon	Original amino acid	Mutations detected	Changed amino acid	N° of strains (n=46)
83	TCC	Ser	TTC	Phe	4
			TTT		2
87	GAC	Asp	AAC	Asn	28
			TAC	Tyr	12

Fig. 10. Mutations in codon 87



Forty-six (83%) strains shown mutations in some codon belonging to QRDR amplified fragment. No mutations were detected in 9 (17%) strains that expressed a resistance to NAL ≥07 (09 to 18 mm) by diffusion method (Fig. 9, Table 2). One strain was not analyzed. Four different mutations were found in QRDR, that codified 3 amino acids: Phe, Asn and Tyr. The more frequent mutation (51%) was the substitution in codon 87 (GAC → AAC, Table 2); in most cases the mutations in this codon were detected in only 2 hospitals (Figure 10).

CONCLUDING REMARKS

- **Neither CIP resistant nor intermediate strains were found, but all the isolates intermediate or resistant to NAL showed decreased susceptibility to CIP.**
- **The diffusion test, using the NAL disc, was the best phenotypic methodology to detect fluorquinolones decreased susceptibility.**
- **No mutations in *gyr*A gene were found in the strains with intermediate susceptibility to NAL (inhibition zone ≥14 and ≤ 18) .**
- **No mutations in *gyr*A gene were found in the NAL-resistant strains with inhibition zone between ≥ 07 and ≤ 13 mm.**
- **Mutations in *gyr*A gene were detected only in the NAL-resistant strains without inhibition zone.**
- **The more frequent mutation detected was GAC → AAC in codon 87 inside the QRDR region of *gyrA* gene.**
- **No mutations in both 83 and 87 codons were found.**