Isolation and molecular characterization of quinolone resistant *Salmonella* spp. from poultry farms

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Abstract

The antimicrobial susceptibility of 94 *Salmonella* strains isolated from different poultry farms in Chile (broiler and laggin hens) were analyzed by the dilution plates method. Thirty-nine of them were resistant to flumequine, nalidixic acid and oxolinic acid with MIC values higher than 64 μg/ml. These quinolone resistant strains were analyzed in order to determine the presence of mutations in the QRDR region of *gyrA* gene by AS-PCR-RFLP analysis. 51.3% of the strains showed mutations at codon Ser 83 and 41.0% showed mutations at codon Asp 87. No mutations were observed on codon Gly 81. These mutations were confirmed by sequenciation of one representative strain from different RFLP pattern. Likewise, no double mutations were observed. Over 90% of the quinolone resistant strains presented mutations at the QRDR region of the *gyrA* gene. Three phenotypically resistant strains did not show any mutations on the QRDR region of *gyrA* gene. However, other molecular resistant mechanism could be involve. This is the first study that demonstrate the emergency of quinolone and fluoroquinolone resistance in Chilean *Salmonella* strains isolated from poultry thus indicating the requirement of monitoring programmes in veterinary medicine.

Keywords: *Salmonella*; *Resistance*; *Quinolones*; *gyrA*; *Poultry*

1. Introduction

Salmonella spp. is one of the most important pathogens responsible for gastrointestinal infections in humans, poultry and its derivatives being one of the

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best known sources of contamination. An increase of strains showing resistance and multiple resistance against different antibiotics have been found from isolates from pigs, poultry, and cattle in recent year (Esaki et al., 2004).

Salmonella typhimurium DT104, resistant to streptomycin, cloramphenicol, amoxicillin, sulfonamides, and tetracyclines, is one of the strains that most frequently displays multiple resistance characteristics (Kristiansen et al., 2003), additional resistance to quinolones, such as nalidixic and ciprofloxacin acid, has been described in recent years, thus increasing the importance of public health area (Piddock, 2002). Quinolones are widely used for treatment and prevention in animal production systems, particularly in the avicultural area. The Federal and Drug Administration (FDA), in docket N° 00N-1751, has prohibited the use of enrofloxacin in poultry, due to the increased resistance displayed by *Campylobacter* spp. (FDA, 2000).

The antibiotic effect of quinolones depend on its ability to bind to the DNA gyrase complex inducing a conformational change in the enzyme (Hawkey, 2003). In *Salmonella* spp., as well as in *E. coli* and in the majority of gram-negative microorganisms, resistance to quinolones is determined fundamentally by two mechanisms: one of them corresponds to the alteration of the DNA gyrase binding site due to mutation between aminoacids 67 and 106 of the QRDR region (Quinolone Resistance Determining Region) of *gyrA* gene. The other mechanism corresponds to structural changes of the porins sof the outer membrane of gram-negative microorganisms, which produce an alteration of the permeability of the bacteria to the antimicrobial agent (Reyna et al., 1995).

The most frequent mutation in *gyrA* gene which confer resistance to nalidixic acid, are those located at the Ser 83 or Asp 87 aminoacids. The mutation in Ser 83 can change the aminoacid to Phe, Tyr, or Ala, while the mutation in Asp 87 can change the aminoacid for Gly, Asn, or Tyr. Double mutations for both aminoacids confer resistance to ciprofloxacin, as observed in isolated of *Salmonella* spp. (Reyna et al., 1995). A less frequent mutation associated to resistance is found on aminoacid Gly 81, which is substituted for Asp or Cys (Giraud et al., 1999).

In Chile there is not available information about quinolones resistance, included ciprofloxacin, in

Salmonella strains isolated from poultry. The purpose of this study was to determinate whether quinolone-resistant *Salmonella* spp. from poultry present mutations in QRDR region of the *gyrA* gene.

2. Materials and methods

2.1. Isolation of Salmonella

The analysis was done in 94 strains isolated from 30 poultry farms from the central zone of Chile (Broiler and laggin hens). Feces samples were collected between the months of March and June (2004). The samples were collected at the cloacal level, and enriched in Rappaport Vassiliadis broth (Difco®) at 37 °C for 24, 48, and 72 h, streaked every 24 h in XLD agar (Murray and Barton, 1993). Suspicious colonies were identified with conventional biochemical tests for *Salmonella* spp., and with fast agglutination with polyvalent A-I and Vi sera (Difco®) (Murray and Barton, 1993). The strains that did not agglutinate were analyzed by a quick diagnostic BBL-Crystal kit (B&D Co.). One colony per sample was selected. Serotyping was performed by the Public Health Institute of Chile.

The isolated samples were frozen at −70 °C in a brain/heart broth containing 15% glycerol.

2.2. Antimicrobial and susceptibility determination

All isolates were analyzed by the plate dilution method, following the recommended norms of the National Commitee for Clinical Laboratory Standards (NCCLS). The antimicrobials used were: nalidixic acid (Sigma®, 98%), oxolinic acid (Sigma®, 98%), flumequine (Sigma®, 94.6%), ciprofloxacin (USP Standard, 100%) and enrofloxacin (Lab Chile®, 100%). *E. coli* ATCC 25922 (NCCLS, 1999, 2001) was used as control strain. For oxolinic acid, the breakpoint for nalidixic acid was used as reference.

2.3. Analysis of QRDR region of gyrA gene

The strains that showed antimicrobial resistance were analyzed by AS-PCR-RFLP to assess mutations at QRDR region of *gyrA* gene.

Table 1
Fragment size (bp) patterns of the QRDR region of *gyr*A gene in *Salmonella typhimurium* used as control

Strain name	Pattern	Fragment size (bp)		Mutated QRDR region *gyrA* gene
		Before *Hinf* I	After *Hinf* I	
Sensitive	I	195-80	137-43-22-15	None
BN 99 (resistant)	II	195-80	152-43-37	Codon 83 (TCC /TTC)
BNJ 18 (resistant)	III	195-80	137-58-22	Codon 87 (GAC/AAC)
BN18/42 (resistant)	IV	195	137-43-15	Codon 81 (GGC/TGC)
BN 82/61 (resistant)	V	195-80	195-80	Codon 83 (TCC/TTC) Codon 87 (GAC/GGC)
BN 18/61 (resistant)	VI	195	137-58	Codon 81 (GGC/TGC) Codon 87 (GAC/GGC)

Source: Giraud et al., 1999.

2.3.1. Amplification and digestion of QRDR region of gyrA gene

Each strain was cultivated in Luria–Bertani broth (Difco®) at 37 °C for 18 h and bacteria lysated were obtained as described by Ling et al. (2003).

For the amplification, as reference, studies described by Weigel et al., 1998 and Giraud et al., 1999, were used. Mix reactions contained 1 µl of each primer (25 pmol), 1 µl (200 µM) of deoxynucleotide triphosphates (Promega®), 3 µl (1.5 mM) of magnesium chloride, 5 µl of buffer Taq (10 mM) and 0.2 µl (0.5 U) of polymerase Taq (Promega®) and 3 µl of lysated.

The mix was placed on an eppendorf® thermocycler, programmed for 35 cycles as follows: 1 min at 94 °C, 1 min at 57 °C, and 1 min at 72 °C, with a final extension of 10 min at 72 °C. Ten microliters of each amplification was run in a 1% gel with Tris–borate–EDTA buffer (Winkler®) at 80 volts for 45 min. DNA ladder (100 bp) (Promega®) was used as molecular weight marker. The gel was stained with ethidium bromide and the DNA bands were visualized in an UV transilluminator.

Each amplified sequences was digested with *Hinf* I endonuclease restriction enzyme (New England®). 16.5 µl of each amplification product was mixed with 5 U of *Hinf* I, 0.1 µl of BSA (New England®), and 2 µl of B buffer (New England®) and incubated at 37 °C for 2 h. Ten microliters of the digested fragments and 10 µl of the non-digested fragments were run in 3% agarose gel in Tris–borate–EDTA buffer (Winkler®), at 80 volts for 40 min. The gel was stained with ethidium bromide and the DNA bands were visualized with a UV transilluminator (Giraud et al., 1999). DNA ladder (25 pb) (Promega®) was

used as a molecular weight marker. The Table 1 shows the restrictions patterns using the control strains donated by Dr. Elizabeth Chaslus Dancla, INRA Institute, France.

2.3.2. Sequencing of the QRDR region of gyrA gene

In order to confirm the mutations that were found, the QRDR region of *gyrA* gene of a sensitive strain and a resistant strain representative of each pattern obtained was sequenced. Free primer and nucleotides were removed with a DNA purification kit (Qiagen S.A®) and sent frozen at −20 °C to the Molecular Genetic Laboratory at Faculty of Science of the University of Chile for analysis. The results of the sequencing were analyzed using *Salmonella typhimurium* NTCT 74, Gene Bank access code X78977.

3. Results

From the 94 *Salmonella* spp. strains isolated, 39 strains were resistant to flumequine, nalidixic acid, and oxolinic acid simultaneously, while only two strains were resistant to nalidixic acid and oxolinic acid. All the strains were sensitive to ciprofloxacin and enrofloxacin. The most frequent seroptype were *S. enteritidis* and *S. heidelberg*.

The 39 resistant strains were used to amplify the QRDR following the protocol as described by Giraud et al., 1999. All of them amplified two bands of 195 and 80 bp (Fig. 1).

From the 39 resistant strains, 3 RFLP patterns with enzyme *Hinf*. Three strains phenotypically resistant
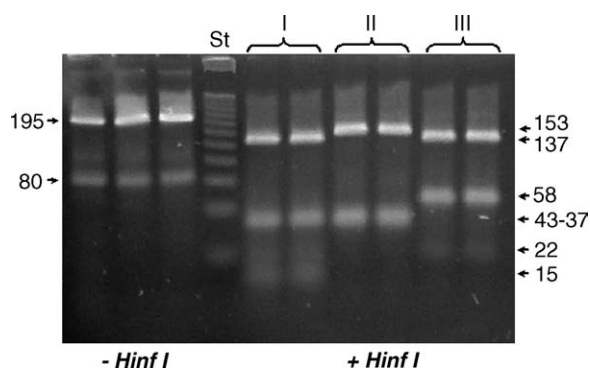
Fig. 1. AS-PCR-RFLP patterns of quinolone resistant strains of *Salmonella* islolated from poultry. Before restriction (–*Hinf* I), amplification were similar for all strains, three samples are showed at left side of the agarose gel. After restriction (+*Hinf* I), three different pattern were obtained, two representative strain of each pattern are showed at right side of the gel. St is 25 bp ladder molecular size standard. Arrows indicate molecular size (bp) of DNA fragments.

to quinolones presented pattern I (137-43-22-15 bp). This pattern corresponded to the sensitive control strain (Table 1). Sequencing confirmed that the strains did not show any mutations of the QRDR region of *gyrA* gene (Table 2).

Pattern II (152-43-37 bp) was obtained in 20 of the resistant strains. This pattern corresponds to the one belonging to strain BN99 (Table 1). Sequencing of a

representative strain confirmed a mutation on codon 83 of the QRDR region of *gyrA* gene (Table 2).

Pattern III (137-58-22 bp), obtained in 16 of the resistant strains, corresponded to strain BNJ18 (Table 1). Sequencing of a representative strain confirmed mutation in codon 87 of the QRDR region of *gyrA* gene (Table 2).

Mutations in codon 83 and 87 of the QRDR region of *gyrA* gene were observed in the serotypes *S. enteritidis* and *S. Heidelberg*, the most frequent serotypes identified.

No patterns were found that suggested mutation in codon 81 or restriction patterns for double mutations of the QRDR region of *gyrA* gene.

4. Discussion

The relationship between *gyrA* mutations and quinolone and fluoroquinolone resistance has been demonstrated in various gram-negative bacteria (Weigel, 1998). In this study, 39 strains were phenotypically resistant to flumequine, nalidixic acid, and oxolinic acid, 36 of them presented simple mutations in codon Ser 83 or Asp 87 of *gyrA* gene and no mutations were observed in three strains. All analyzed strains (94), were phenotypically sensitive to enrofloxacin and ciprofloxacin. Our results are consistant with those of

Table 2
Phenotypic and genotypic characterization of quinolone resistant *Salmonella* strains isolated from poultry

Serotype	n	MIC (µg/ml)			Pattern (before *Hinf* I after *Hinf* I)			Mutated QRDR region *gyrA* gene
		Nal[*]	Oa[*]	Flu[*]				
S. enteritidis	1	1280	1280	0.125	III	195-80	137-58-22	Codon 87 (GAC/AAC)
S. heidelberg	8	1280	1280	640	II	195-80	152-43-37	Codon 83 (TCC/TTC)
S. enteritidis	7	1280	1280	640	II	195-80	152-43-37	Codon 83 (TCC/TTC)
S. heidelberg	5	1280	1280	640	III	195-80	137-58-22	Codon 87 (GAC/AAC)
S. senftenberg	3	1280	1280	640	III	195-80	137-58-22	Codon 87 (GAC/AAC)
S. give	1	1280	1280	640	II	195-80	152-43-37	Codon 83 (TCC/TTC)
S. enteritidis	5	1280	1280	640	III	195-80	137-58-22	Codon 87 (GAC/AAC)
S. enteritidis	1	1280	1280	640	I	195-80	137-43-22-15	N[**]
S. agona	2	1280	1280	640	II	195-80	152-43-37	Codon 83 (TCC/TTC)
S. enteritidis	1	1280	1280	0.125	II	195-80	152-43-37	Codon 83 (TCC/TTC)
S. heidelberg	1	640	640	320	I	195-80	137-43-22-15	N[**]
S. senftenberg	1	1280	1280	640	II	195-80	152-43-37	Codon 83 (TCC/TTC)
S. give	1	1280	1280	640	III	195-80	137-58-22	Codon 87 (GAC/AAC)
S. enteritidis	1	640	640	320	I	195-80	137-43-22-15	N[**]

Isolated from poultry.
[*] Nal: nalidixic acid; Oa: oxolinic acid; Flu: flumequine.
[**] N: none mutated QRDR region *gyrA* gene.

Giraud et al. (1999), who point out that, in strains of *Salmonella* spp., resistance to ciprofloxacin and enrofloxacin are manifested phenotypically only when there are double mutation in *gyrA* gene, and that simple mutations are related to nalidixic acid and flumequine resistance.

In resistant strains, percentage of mutation in codons Ser 83 or Asp 87 were similar, of 51.3 and 41%, respectively. The percentage of strains with mutations in codon Ser 83 were similar to the one reported by Liebana et al. (2002) in strains isolated from different animals, and slightly inferior to the one reported by Giraud et al. (1999), in strains isolated from poultry. This mutation in codon Ser 83 has also been reported by Ling et al. (2003) in *Salmonella* strains isolated from clinical cases in human patients, but with percentages closer to 20%.

The percentage of strains with mutation in Asp 87 is consistent with studies done by Griggs et al. (1996) and Liebana et al. (2002), who obtained high percentages of mutations in codon 87 in isolates of *Salmonella* from different animals. However, in a study done by Giraud et al. (1999), the authors observed a low percentage of *Salmonella* strains isolated from poultry with mutations in this codon. This variability in percentages of strains that show simple mutations in codon 87 has also been reported in isolated of *Salmonella* of human origin (Griggs et al., 1996; Ling et al., 2003).

On the other hand, even though different authors have associated simple mutations of codons Ser 83 or Asp 87 to flumequine and nalidixic acid only, we also have observed that these strains were resistant to oxolinic acid, which was to be expected due to the structural similarity between the two molecules.

It is important to emphasize that the three *Salmonella* spp. strains that were phenotypically resistant to flumequine, nalidixic acid, and oxolinic acid presented the same restriction pattern as the sensitive strain. It is likely that these strains developed other molecular resistance mechanisms, such as the overexpression of efflux pumps, mutation on *gyrB*, *parC*, and *parE* genes (Ettiene et al., 2003), or the alteration of the outer bacterial membrane's permeability mediated by porins that frequently affect Omp F or *marA*, *soxS*, *robA* regulators gene (Hawkey, 2003). Future studies will focus to study the different resistance mechanisms in detail.

The change in codons Ser 83 or Asp 87 has been related to different levels of quinolone resistance (elevated MIC values). In the phenotypical studies of the isolates analyzed in this study, all resistant strains presented MIC values higher than 64 μg/ml for flumequine, nalidixic acid, and oxolinic acid. Giraud et al. (1999) observed MIC levels higher than 128 μg/ml only for nalidixic acid and only 8 μg/ml for flumequine in poultry-isolated *Salmonella* strains (chicken, turkey, and ducks). The elevated MIC levels against flumequine we found here could be explained by the presence of other resistance mechanisms which could be acting simultaneously with the mutation in *gyrA* gene.

Given the ocurrence of quinolone and fluoroquinolone resistance in *Salmonella* strains worldwide, including Chile, it seems highly recommended the development of vigilance programs in veterinary medicine at the government level in Chile, in order to know phenotypical and genotypical aspects of antimicrobial resistant strains.

Acknowledgement

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