Quinolones resistance in *Salmonella* spp. isolated from broilers and chickens' carcasses under federal inspection[1]

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ABSTRACT.- Carneiro A.L.O.C., Silva R.L., Rodrigues I.B.B.E., Machado S.C., Cunha N.C., Nascimento E.R., Pereira V.L.A. & Abreu D.C.L. 2020. **Quinolones resistance in *Salmonella* spp. isolated from broilers and chickens' carcasses under federal inspection**. *Pesquisa Veterinária Brasileira 40(7):519-524*. Núcleo de Diagnóstico Avícola, Faculdade de Veterinária, Universidade Federal Fluminense, Rua Vital Brasil Filho 64, Niterói, RJ 24230-340, Brazil. E-mail: analuoccarneiro@gmail.com

We analyzed 77 *Salmonella* spp. strains, from which 20 were isolated from broilers (cloacal swabs) and 57 from chickens from slaughterhouses under federal inspection. The following serotypes were identified: *Salmonella* Saint Paul (29), *Salmonella* Heidelberg (27), *Salmonella* Anatum (9), *Salmonella* Cerro (5), *Salmonella* Senftenberg (5), *Salmonella enterica* (O: 4,5) (1) and *Salmonella enterica* (O: 9.12) (1). Fifteen strains (19.5%) were resistant to enrofloxacin, six (7.8%) to ciprofloxacin, and 26 (33.8%) to nalidixic acid in the Disk Diffusion Test. The fifteen enrofloxacin resistant strains were selected for the PCR to detect the genes *gyrA, gyrB, parC,* and *parE,* and genetic sequencing to identify mutations in these genes. Five strains (33.3%) had point mutations in the *gyrA* gene, and one (6.7%) presented a point mutation in the *parC* gene. None of the 15 strains had mutations in the *gyrB* and *parE* genes, and none had more than one mutation in the *gyrA* gene or the other genes. The presence of point mutations in the strains studied corroborates with the phenotypic resistance observed to nalidixic acid. However, it did not explain the resistance to fluoroquinolones found in the 15 strains. Other mechanisms may be related to the fluoroquinolones resistance, highlighting the need for additional mutation screening.

INDEX TERMS: Quinolones, *Salmonella* spp., broilers, chicken carcass, fluoroquinolones, antimicrobial resistance.

RESUMO.- [**Resistência à quinolonas em *Salmonella* spp. isoladas de frangos vivos e carcaças sob inspeção federal.**] Foram analisadas neste estudo 77 estirpes de *Salmonella* spp., 20 isoladas de frangos vivos (suabes de cloaca) e 57 isoladas de carcaças, provenientes de abatedouros frigoríficos sob Inspeção Federal. Foram identificados os seguintes sorotipos: *Salmonella* Saint Paul (29), *Salmonella* Heidelberg (27), *Salmonella* Anatum (9), *Salmonella* Cerro (5), *Salmonella* Senftenberg (5),

Salmonella enterica (O: 4,5) (1) e *Salmonella* enterica (O: 9,12) (1). Do total de estirpes estudadas, 15 (19,5%) se mostraram resistentes à enrofloxacina, seis (7,8%) à ciprofloxacina e 26 (33,8%) ao ácido nalidíxico no Teste de Difusão em Disco. Foram selecionadas as 15 estirpes resistentes à enrofloxacina para a realização da PCR para detecção dos genes *gyrA, gyrB, parC e parEe* para sequenciamento genético do produto da PCR para identificação de mutações nesses genes. Cinco estirpes (33,3%) apresentaram mutações pontuais no gene *gyrA* e uma (6,7%) apresentou mutação pontual no gene *parC*. Nenhuma das 15 estirpes apresentou mutações nos genes *gyrB* e *parE* e nenhuma apresentou mais de uma mutação no gene *gyrA* ou nos outros genes. A existência apenas de mutações pontuais em alguns genes das estirpes analisadas está de acordo com a resistência fenotípica observada ao ácido nalidíxico, mas não explica a resistência às fluoroquinolonas encontrada nas

15 estirpes. Outros mecanismos de resistência podem estar relacionados à resistência encontrada às fluoroquinolonas e estudos adicionais são necessários para investigar sua presença.

INTRODUCTION

Bacteria of the genus *Salmonella* are essential pathogens for causing diseases in animals and man, and are considered of great significance for both public and animal health.

Some serotypes, known as paratypical, may not cause clinical disease in birds. However, most of them can multiply and remain in their digestive tract for some time, spreading in the farm environment. By contaminating poultry origin products to human consumption, they may be responsible for human outbreaks of food infection (Berchieri Jr. & Freitas Neto 2009, Andreatti Filho 2009, Gast 2013). The paratypical serotypes most commonly described in poultry environments, birds and their products, are the serotypes Enteritidis, Albany, Heidelberg, Mbandaka and Newport (Brasil 2008, Dutil et al. 2010, FDA 2012, Robinson 2013, Cardoso et al. 2015, Pandini et al. 2015, PHAC 2017).

Salmonella infection leads to different clinical signs in humans, with gastrointestinal infection being the most common and generally self-limiting manifestation (Rezende et al. 2016, Draper et al. 2017).

Another important fact related to *Salmonella* spp. is the constant description of resistance to various antimicrobials, such as those of the quinolone class (Duarte et al. 2009, Mion et al. 2014, Casas et al. 2016, Neves et al. 2016, Rodrigues et al. 2017). Resistance in *Salmonella* spp. quinolones have been recorded in countries such as China, Hong Kong, Italy, the United Kingdom and Brazil (Eaves et al. 2004, Duarte et al. 2009, Lai et al. 2014, Lin et al. 2015,García-Fernández et al. 2015, Casas et al. 2016). Some countries showed a significant increase in resistance, such as China, which observed an increase from 4.4% to 41.5% of ciprofloxacin resistance, from 2009 to 2012 (Lai et al. 2014). Several studies have linked the therapeutic and prophylactic use of quinolones in animals with the emergence and spread of resistance from these pathogens (Yan et al. 2011, Cheng et al. 2012, Finley et al. 2013).

Quinolones, antimicrobials with a full application for the treatment of diseases in both humans and animals, were developed to identify nalidixic acid in the 1960s. In the 1980s, fluoroquinolones were developed and considered second-generation quinolones, with a spectrum of action on Gram-negative and Gram-positive bacteria. Enrofloxacin, a fluoroquinolone developed exclusively for use in animals, has broad antibacterial activity and is commonly used in Brazil's poultry production. Ciprofloxacin, in addition to its use in poultry production, is also used to treat human salmonellosis (Ito et al. 2005, Górniak 2011).

Salmonella spp. may develop chromosome and plasmid resistance to quinolones. Chromosome-mediated resistance can cause overexpression of efflux pumps or changes in porins present in the outer bacterial membrane, reducing the accumulation of antimicrobials in the bacteria. Another mechanism mediated by chromosomes is the conformational alteration of enzymes responsible for the replication of bacterial DNA (García-Fernández et al. 2015). This type of mutation is the most frequent in *Salmonella* (Sinwat et al. 2018). It occurs by specific mutations that result in amino acid substitutions in the coded enzymes DNA Gyrase and Topoisomerase IV in the subunits encoded by the *gyrA*, *gyrB*, *parC*, or *parE* genes. These enzymes are involved in the bacterial DNA replication process, which is essential for bacterial survival. Mutations in *gyrA*, *gyrB*, *parC*, or *parE* in the regions that are part of the quinolone binding site are called Quinolone Resistance Determining Region (QRDR). The mutations alter these enzymes' structure, preventing quinolones from becoming connected to this site, reducing bacterial susceptibility to quinolones (Gouvêa et al. 2015, Thong et al. 2015, Sinwat et al. 2018). In *Salmonella* spp. mutations in *gyrA* are the most frequent, which can be explained due to the positioning of the enzyme DNA Gyrase in the bacterial DNA replication fork, which makes the inhibitory action of quinolone more effective than the enzyme Topoisomerase IV. Consequently, this may have created a selective pressure that caused the DNA Gyrase genes, especially the *gyrA* gene, to mutate more frequently to avoid quinolones' inhibitory action (Thong et al. 2015). Mutations in the *parC* gene for Topoisomerases IV occur less frequently, and mutations in the *gyrB* and *parE* genes have been considered rare in *Salmonella* (Kim et al. 2011, Yang et al. 2012, Thong et al. 2015, Lin et al. 2015).

Although antimicrobials are not indicated in most cases of salmonellosis in humans, its use becomes necessary in severe cases of systemic infections (Boxstael et al. 2012, WHO 2018). However, the antimicrobials' lack of susceptibility increases the risk of treatment failures (Park et al. 2019).

This study aimed to verify resistance to quinolones, enrofloxacin, ciprofloxacin, and nalidixic acid by mutating the genes *gyrA*, *gyrB*, *parC* and *parE* in strains of *Salmonella* spp., isolated from live chickens and carcasses from slaughterhouses with Federal Inspection Service.

MATERIALS AND METHODS

Obtaining strains of *Salmonella* spp. In this study, we used 77 strains of *Salmonella* spp., 20 of which were isolated from live chickens (cloaca swabs), and 57 from carcasses from slaughterhouses under federal inspection. The samples were sent to the "Laboratório de SanidadeAvícola", "Faculdade de Veterinária", of the "Universidade Federal Fluminense" (UFF), included in nutrient Agar (VWR Chemicals).

Strains serotyping. The strains were reactivated in Brain Heart Infusion (BHI) broth, included in nutrient Agar and sent to the "Laboratório de Enterobactérias" of the "Instituto Oswaldo Cruz"/"FundaçãoInstituto Oswaldo Cruz" (Fiocruz) for serotyping.

Antimicrobial susceptibility test. The 77 strains studied were subjected to the Disc-Diffusion test (CLSI 2018) to verify resistance to nalidixic acid, ciprofloxacin, and enrofloxacin.

Polymerase chain reaction (PCR) of the *gyrA*, *gyrB*, *parC*, and *parE* genes. PCR was performed at the "Laboratório de Epidemiologia Molecular" of UFF. For the extraction of bacterial DNA, strains of *Salmonella* spp. resistant to enrofloxacin by the Disc-Diffusion test were reactivated in BHI broth and subjected to the thermal method (AndreattiFilho et al. 2011). To detect chromosomal mutations in the DNA Gyrase and Topoisomerase IV enzymes of the strains of *Salmonella* spp., The *gyrA*, *gyrB*, *parC*, and *parE* genes were amplified by PCR, in a thermocycler (Thermo Electron Corporation), according to Kim et al. (2011). Primers were used to amplify the quinolone resistance region (Quinolone Resistance Determining Region - QRDR)

in the *gyrA*, *gyrB*, *parC*, and *parE* genes, producing fragments of 610, 660, 950 and 897 base pairs (bp), respectively (Table 1).

Purification, preparation, and genetic sequencing of samples. The samples were purified using the GFX™ PCR DNA and Gel Band Purification Kit according to the manufacturer's instructions and subsequently sent to Subunit RPT01A - DNA Sequencing, RJ from Fiocruz and sequenced in the DNA sequencer ABI 3730 (AppliedBiosystems/USA).

Detection of mutation in QRDR. The files containing the nucleotide sequences of the quinolone resistance region of the *gyrA*, *gyrB*, *parC*, and *parE* genes of the *Salmonella* spp. Samples were read in the BioEdit program (BioEdit Sequence Alignment Editor) to check the sequences' quality and generate new files in the "Fasta" format. All sequences obtained were compared with those of GenBank using the Blast algorithm[4].For the detection of chromosomal mutations in the QRDR region of the *gyrA*, *gyrB*, *parC*, and *parE* genes, the files in "Fasta" format were read by the ResFinder program available on the Center for Genomic Epidemiology website[5], according to Zankari et al. (2012).

RESULTS

The most frequent serotypes of *Salmonella enterica* were Saint Paul (29/77, 37.7%), Heidelberg (27/77, 35.1%) and Anatum (9/77, 11.7%). Cerro and Senftenberg had a frequency of 6.5% (5/77), and serotypes O: 4.5 and O: 9.12 had a frequency of 1.3% (1/77).

After undergoing the Disc-Diffusion test, for the evaluation of resistance to the antimicrobials enrofloxacin, ciprofloxacin and nalidixic acid, 19.5% (15/77) of the strains showed resistance to enrofloxacin, 7.8% (6/77) ciprofloxacin and 33.8% (26/77) to nalidixic acid (Table 2).

The Heidelberg serotypes, O: 4.5 and O: 9.12, were resistant to the three antimicrobials tested. Of these, the Heidelberg serotype showed the highest frequency of resistance to enrofloxacin (11/15, 73.3%), Ciprofloxacin (4/6, 66.7%) and nalidixic acid (20/26, 76.9%). The Senftenberg serotype did not show resistance to any of the three antimicrobials tested. The complete result of the resistance profile is shown in Table 2.

Of the 15 strains resistant to enrofloxacin, five (33.3%) had mutations in the *gyrA* gene. These mutations were isolated. The five mutations found occurred at codon 83, leading to a change from the amino acid Serine to Phenylalanine (TCC → TTC). One of the 15 strains (6.7%) showed a mutation in the *parC* gene. The mutation found occurred at codon 84, leading to a change from the amino acid Glutamic Acid to Lysine (GAA → AAG). None of the 15 strains had mutations in the *gyrB* and *parE* genes or more than one mutation in the *gyrA* gene or the other genes (Table 3).

DISCUSSION

The *Salmonella* serotypes identified in this study may cause mild gastrointestinal diseases (Rezende et al. 2016, Draper et al. 2017) to more severe extraintestinalconditions, as verified by the Heidelberg serotype, responsible for cases of septicemia, myocarditis, and death (Dutil et al. 2010). This fact makes production birds and their derived products essential

vehicles for the dissemination of this agent. The Heidelberg serotype was one of the most isolated in this study and in the work of Pandini et al. (2015). They detected *Salmonella* Heidelberg in 12.82% of isolates from swine trawlers from broiler farms in the state of Paraná. These same authors detected serotypes different from those isolated in this study, such as the Mbandaka and Newport serotypes, with frequencies of 10.25% and *S.* Schwarzengrund, *S.* Enteritidis, *S.* Livingstone, and *S.* Orion, with 7.70% frequency each. Cardoso et al. (2015) also detected serotypes different from those isolated in this study in chicken carcasses from slaughterhouses in the state of São Paulo/SP and the most frequent serotype was Enteritidis (49.4%) followed by *S.* Albany serotypes (15.7%), *S.*Infantiles (11.2%), *S.* Agona (5.6%), *S.* Tennessee (4.5%), *Salmonella* spp. (3.4%), *S.* Kentucky (2.3%), *S.* Montevideo (1.1%), and *S.* Newport (1.1%). The Heidelberg and O: 4.5 serotypes, also isolated in the present study, were the least frequent, with an isolation frequency of 3.4% and 2.3%, respectively. The serotype most frequently isolated in this study was Saint Paul. In 2014, this serotype was the third most reported in Australia, accounting for 11% of all salmonellosis reports in this country (Draper et al. 2017).

Salmonella resistance to quinolones has been detected worldwide (Eaves et al. 2004, Lai et al. 2014, Lin et al. 2015, García-Fernández et al. 2015). In a study carried out in Recife, Brazil, Duarte et al. (2009) analyzed the susceptibility to antimicrobials of 19 strains of *Salmonella* spp., isolated from chicken carcasses. They found a percentage of 5.2% resistance for the three quinolones tested (enrofloxacin, ciprofloxacin, and norfloxacin). Another study, carried out in São Paulo, detected 16 strains resistant (17.5%) to ciprofloxacin in 91 strains of *Salmonella* spp. (Casas et al. 2016). These percentages were like those found in this study. The presence of *Salmonella* strains resistant to quinolones in foods, especially those used for treatment in humans, such as nalidixic acid and ciprofloxacin, is worrying because when its use becomes necessary for the treatment of serious infections it can increase the risk of failure (Park et al. 2019).

The existence of point mutations in some genes of the strains analyzed corroborates with the phenotypic resistance observed to nalidixic acid. However, it does not explain the resistance to fluoroquinolones found in the 15 strains. In this study, we only found point mutations in *gyrA*, and *parC* and, therefore, resistance to fluoroquinolones in strains of *Salmonella* detected by the phenotypic test may be due to other resistance mechanisms than the mutation in the

Table 1. Primers, nucleotide sequences and size of amplicons used for PCR amplification of the*gyrA*, *gyrB*, *parC* and *parE* genes in strains of *Salmonella* spp.

Primer	Sequence (5' to 3')	pb
gyrA - F	CGA GAG AAA TTA CAC CGG TCA	610
gyrA - R	AGC CCT TCA ATG CTG ATG TC	
gyrB - F	CTG CTT TAC CAA CAA CAT CC	660
gyrB - R	TTG TCC GGG TTG TAC TCG TC	
parC - F	ATG AGC GAT ATG GCA GAG C	950
parC - R	GCG AAC AGA TGG TTC ATC AC	
parE - F	GCG GAA GAT ATC TGG GAT CG	897
parE - R	CAG CAG CAT ATC CAT CAT CG	

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4   Available at <available at https://blast.ncbi.nlm.nih.gov/Blast.cgi>

5   Available at <http://www.genomicepidemiology.org/>

Table 2. Frequency of isolation and resistance assessment against the antimicrobials enrofloxacin, ciprofloxacin and nalidixic acid in strains of *Salmonella* spp. (n=77) isolated from live chickens and carcasses in slaughterhouses with Federal Inspection Service

Serotype	Frequency (%)		Resistance					
			Enrofloxacin (%)		Ciprofloxacin (%)		Nalidixic acid (%)	
Saint Paul	29	37.7	1	6.7	0	0.0	2	7.7
Heidelberg	27	35.1	11	73.3	4	66.7	20	76.9
Anatum	9	11.7	0	0.0	0	0.0	1	3.8
Cerro	5	6.5	1	6.7	0	0.0	1	3.8
O: 4.5	1	1.3	1	6.7	1	16.7	1	3.8
O: 9.12	1	1.3	1	6.7	1	16.7	1	3.8
Senftenberg	5	6.5	0	0.0	0	0.0	0	0.0
Total	77	100.0	15	100.0	6	100.0	26	100.0
	100%		19.5%		7.8%		33.8%	

Table 3. Mutations in the *gyrA*, *gyrB*, *parC* and *parE* genes found in the 15 strains of *Salmonella* spp. resistant to enrofloxacin, ciprofloxacin and nalidixic acid

Serotype	Phenotypic susceptibility			Mutation in (position)	Nucleotide change	Amino Acid change
	ENO	CIP	NAL. AC.			
Saint Paul	R	I	R	-	-	-
Heidelberg	R	I	R	*gyrA* (S83F)	TCC to TTC	Serine to Phenylalanine
Heidelberg	R	I	R	-	-	-
Heidelberg	R	R	I	*gyrA* (S83F)	TCC to TTC	Serine to Phenylalanine
Heidelberg	R	I	R	-	-	-
Heidelberg	R	I	R	-	-	-
Heidelberg	R	I	R	*gyrA* (S83F)	TCC to TTC	Serine to Phenylalanine
Heidelberg	R	I	R	-	-	-
Heidelberg	R	R	R	*parC* (E84K)	GAA to AAG	Glutamic acid to Lysine
Heidelberg	R	R	R	*gyrA* (S83F)	TCC to TTC	Serine to Phenylalanine
Heidelberg	R	R	R	-	-	-
Heidelberg	R	I	R	-	-	-
Cerro	R	I	R	-	-	-
O: 9.12	R	R	R	-	-	-
O: 4.5	R	R	R	*gyrA* (S83F)	TCC to TTC	Serine to Phenylalanine

ENO = Enrofloxacin, CIP = ciprofloxacin, NAL. AC. = nalidixic acid, R = resistant, I = intermediate.

studied genes. The quinolones' targets are the enzymes DNA Gyrase and Topoisomerase IV, which subunits are encoded by the genes *gyrA* and *gyrB* (DNA Gyrase) and *parC* and *parE* (Topoisomerase IV). One of the main resistance mechanisms developed by *Salmonella* is the mutation in these genes, preventing the binding of the antimicrobial molecule to enzymes and thereby ensuring the survival of these bacteria (Thong et al. 2015, Sinwat et al., 2018). Point mutations in the *gyrA*, *gyrB*, *parC*, or *parE* genes lead to resistance to first-generation quinolones. Resistance to fluoroquinolones in Enterobacteriaceae generally results from two or more mutations in the quinolone resistance genes determining region of DNA Gyrase and Topoisomerase IV (Thong et al. 2015 & Campioni et al. 2017).

CONCLUSIONS

Saint Paul and Heidelberg's serotypes were the most frequently identified in this study. The Heidelberg serotype was the one with the highest percentage of resistance to enrofloxacin, ciprofloxacin, and nalidixic acid.

The presence of point mutations in the *gyrA* and *parC* genes was predominant in strains of *Salmonella* spp. isolated from live chickens and carcasses but did not explain the phenotype of these strains' resistance to the antimicrobials enrofloxacin and ciprofloxacin.

The presence of strains resistant to these antimicrobials in birds and their products is worrying, and the results obtained indicated the presence of other resistance mechanisms that should be investigated.

Conflict of interest statement.- There are no conflicts of interest.

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