Resíduo de fluoroquinolona em carne de frango e efeito da mistura de fezes na resistência de Salmonella Heidelberg à enrofloxacina

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Residue of fluoroquinolone in poultry meat and effect of feces mixture on Salmonella Heidelberg resistance to enrofloxacin

Residuos de fluoroquinolonas en carne de pollo y efecto de la mezcla de heces sobre la resistencia de Salmonella Heidelberg al enrofloxacino

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RESUMO

O objetivo deste trabalho foi validar uma metodologia para detecção de enrofloxacina em tecidos de frango e pesquisar a influência da mistura de fezes de frango na resistência de *Salmonella* spp. à enrofloxacina. Enrofloxacina (10 mg.kg^{-1}) foi administrado oralmente e os tecidos (músculo, fígado e rim) coletados até o 10º dia da última dose e analisados por LCMS. Um total de 124 amostras de fezes foram coletadas da cama de aviários foram submetidas ao isolamento de *Salmonella* spp. e paralelamente, a mistura fecal foi adicionada de solução contendo enrofloxacina (10 µg.mL^{-1}) e *Salmonella* spp. isolada e identificada. O limite de quantificação e detecção de LCMS foram 20 e 10 µg.kg^{-1}, respectivamente e o tempo de depleção nos tecidos foi de 3 dias. Quando exposta à enrofloxacina na presença de mistura fecal, *Salmonella* Heidelberg mostrou menor susceptibilidade (39,8%) à enrofloxacina do que quanto exposta isoladamente (100%). Considerando todos os parâmetros de validação analisados, LCMS foi sensível e aplicável à análise de rotina de resíduos de fluoroquinolonas. Diferenças de susceptibilidade à enrofloxacina na presença de material fecal indicam a necessidade de considerar as condições ambientais na resistência de patógenos, principalmente à classes de antimicrobianos de uso na medicina veterinária e humana.

Palavras chaves: antibiótico. Resistência. LCMSMS

ABSTRACT

The objective of this work was to validate a methodology for the detection of enrofloxacin and in chicken tissues and to investigate the influence of the mixture of chicken feces on the resistance of Salmonella spp. to enrofloxacin. Enrofloxacin (10 mg.kg^{-1}) was administered orally, tissues (muscle, liver and kidney) were collected until the 10th day of the last dose and analyzed by LCMS. A total of 124 feces samples were collected from poultry litter and Salmonella spp were isolated and in parallel, feces were added of a solution containing enrofloxacin (10 µg.mL^{-1}) followed by Salmonella spp. isolation and identification. The limit of quantification and detection of the LCMS was 20 and 10 µg.kg^{-1}, respectively, and the withdrawal period was 3 days. When exposed to enrofloxacin in the presence of a fecal mixture, Salmonella Heidelberg showed less susceptibility (39.8%) to enrofloxacin than when exposed alone (100%). Considering all the validation parameters analyzed, this LCMS was sensitive and applicable to the routine analysis of fluoroquinolone residues. Differences in susceptibility to enrofloxacin in the presence of fecal material indicate the need to consider environmental conditions in the resistance of pathogens, mainly to the classes of antimicrobials used in veterinary and human medicine.

Keywords: *Antibiotics. Resistance. LCMSMS*

RESUMEN

El objetivo de este trabajo fue validar una metodología para la detección de enrofloxacino en tejidos de pollo e investigar la influencia de la mezcla de heces de pollo en la resistencia de Salmonella spp. a la enrofloxacina. Se administró enrofloxacina (10 mg.kg^{-1}) por vía oral y se recogieron los tejidos (músculo, hígado y riñón) hasta el décimo día de la última dosis y se analizaron por LCMS. Se recogieron un total de 124 muestras de heces de la cama de las aves y se sometieron a aislamiento de Salmonella spp. y en paralelo, a la mezcla fecal se le añadió una solución que contenía enrofloxacina (10 µg.mL^{-1}) y Salmonella spp. aislado e identificado. El límite de cuantificación y detección de LCMS fue de 20 y 10 µg.kg^{-1}, respectivamente, y el tiempo de agotamiento del tejido fue de 3 días. Cuando se expuso a enrofloxacino en presencia de una mezcla fecal, Salmonella Heidelberg mostró menos susceptibilidad (39,8%) a enrofloxacino que cuando se expuso sola (100%). Teniendo en cuenta todos los parámetros de validación analizados, LCMS fue sensible y aplicable al análisis de rutina de los residuos de fluoroquinolonas. Las diferencias en la susceptibilidad al enrofloxacino en presencia de materia fecal indican la necesidad de considerar las condiciones ambientales en la resistencia de los patógenos, principalmente a las clases de antimicrobianos utilizados en medicina veterinaria y humana.

***Descriptores**: Antibiotico,Resistencia. LCMSMS.*

INTRODUCTION

Fluoroquinolone is a class of antimicrobial that stand out due to the good bioavailability after oral administration, relatively low toxicity and favourable pharmacokinetics. A broad-spectrum antimicrobial activity of these fluoroquinolones includes Gram negative, Gram positive, anaerobic bacteria, *Mycoplasma* spp. and others (ALDRED et al., 2014).

Unreasonable use of antibiotics in the veterinary, particularly in the livestock production has led antibiotic resistance, the great public health concern. Enrofloxacin- ENR (6-Fluoro-1-(4-fluorophenyl)-7-(4-methylpiperazin-1-yl)- 4-oxo-1,4-dihydro-quinoline-3-carboxylic acid) and Ciprofloxacin - CIP (1-Ethyl-7-methyl-4-oxo-[1,8]naphthyridine-3-carboxylic acid) are quinolone derivatives. ENR was specially developed for veterinary use and CIP is widely used in both human and veterinary medicine (RUSU et al., 2015).

Salmonellosis is a concern to public health and the poultry industry. In intensive poultry farming fluoroquinolone is used to treat chronic respiratory diseases, colibacillosis and cholera (SINDAN, 2020). The development of fluoroquinolone resistance has led to alert the international authorities. Quinolones derivates are classified as extremely important antibiotics, because they are used as a therapy in human medicine and they are used to treat diseases caused by organisms that can be transmitted from non-human sources or disease caused by organisms that may acquire resistance from non-human sources (WHO, 2015).

The constant sub-therapeutic antibiotic exposition is the mainly responsible for resistant bacteria selection. Antibiotics of veterinary use may result in residue in meat and animal excretion may pollute soil and water, and humans are also exposed by the residue accumulated in vegetables and drinking water (MUHAMMAD et al, 2020).

Antibiotics have been reported to form residues at varying concentrations in animal, including edible tissues as muscle, liver and kidney. Many risk factors are responsible for the antibiotic residues. Fluoroquinolones were added to water, differences in diet and water, eventually there is differences in the doses consumed between the individuals, with one receiving a higher dose than others. The animal age and disease status result in different adsorption, distribution, metabolism and the excretion. There are constant detectable levels of residues due to improper withdrawal (clearence and depletion) period. The interval time necessary between the last administration

of antibiotic and the time need for the animal to metabolize and reduce drug residue in a safe concentration for public consumption must be respected (MANYI-LOH et al, 2018). Analytical methods have been developed for the analysis of fluoroquinolone antibiotics in different matrices. Various regulatory agencies around the word have established the maximum residue limit (MRL) of antibiotics in poultry. The Brazilian Health Regulatory Agency (Anvisa) has set LMR of ENR + CIP to 100 $\mu g.kg^{-1}$ for muscle and fat/skin and 300 $\mu g.kg^{-1}$ por kidney and liver (BRASIL, 2019). Detection of these antibiotics at low concentrations in complex matrix requires the use of a highly sensitive and selective technique, and its validation is also necessary.

The faecal material is composed of a complex mixture of excretion metabolism of poultry, microbial community (AHN et al., 2013). And the influence of this mixture interaction with bacteria and antibiotic resistance is complex and needs studies.

Considering the emergence health and global environmental pollution problem of antibiotic resistance. The present study aimed to evaluate by LC-MS/MS the withdraws period of commercial ENR and CIP administered in broiler chickens and asses the influence of these broiler fecal mixture on the sensitivity of *Salmonella* Heildeberg to ENR.

Materials And Methods

Reagents and analytical standards

HPLC grade methanol, acetonitrile, formic acid, anhydrous sodium acetate and sodium sulfate were purchased from Sigma-Aldrich (St. Louis, USA), glacial acetic acid from Merck (Darmstadt, Germany) and dispersive phase containing C-18 and PSA from Agilent Technologies (Santa Clara, USA). Water was purified through a Milli-Q system (Millipore, Bedford, USA). Analytical standards of enrofloxacin (ENR), ciprofloxacin (CIP) and enrofloxacin d5 (ENR-d5, internal standard) were purchased from Sigma-Aldrich (St. Louis, USA). Millex filters with 0.22 µm porosity from Millex (Darmstadt, Germany). Individual stock standard solutions were prepared for each compound at a concentration of 100 $\mu g.mL^{-1}$, dissolving each standard in methanol and stored at -20 ºC. The standard working solutions were diluted from the stock solution in a water-methanol solution (80:20 v/v) to a final concentration of 0.8 $\mu g.mL^{-1}$ for each compound. The working solutions were stored at 2-8 °C. The commercial enrofloxacin 10% (m/v) for veterinary use were purchased from Hipra (Girona, Spain).

Animal and treatments

The experimental protocol was approved by the Ethics committee for animal experimentation protocol number 004/2020 (UNIR). The study was performed with 120 broiler chickens (*Gallus gallus domesticus*, Cobb®) in an experimental farm of an agroindustry of state of Santa Catarina,. The maximum density recommendation was maintained, not exceeding 39 $kg.m^{-2}$ (ABPA, 2016). After allocated, one day old broiler chickens were kept for acclimatization at a temperature of 25±5 ºC with 50-60% relative humidity and with free access to water. The feed provided was in accordance with the nutrient requirements required for broilers and free of any antimicrobial drugs. On day 32 of the experiment animals were randomly divide into control and experimental groups. For the experimental group, ENR (10 $mg.kg^{-1}$) was provided for 5 consecutive days, orally, through drinking water. Eight broiler chickens from each treatment were euthanized by intravenous injection of the thiopental anesthetic (150 $mg.kg^{-1}$), on days 1 to 10 after treatment. For the control treatment,

tissue collections took place on days 1, 3, 5, 7 and 10. The two kidneys, liver (clean without gallbladder) and muscle (pectoralis major) were collected separately and stored at -20 °C until LC-MS/MS analysis.

Sample preparation

Blank samples of poultry muscle, liver and kidney tissue were weighed (2 g ± 0.05 g) and added in tubes containing 200 µL of the internal standard (ENR-d5) to obtain a concentration of 80 $\mu L.kg^{-1}$ and the working solutions of ENR and CIP (50 µL to 300 µL, depending on the desired concentration level). The samples were left in the dark for 30 min at room temperature. A volume of 10 mL of a solution of water:acetronitrile (80:20 v/v), acidified with 5% glacial acetic acid, was added to the samples. The tubes were shaken manually for 1 min and then 4 g of Na_2SO_4 and 1g of sodium acetate were added. The tubes were centrifuged at 4000 rpm for 10 min. The supernatant was transferred to the 2 mL tubes contained in the dispersive kit, composed of C-18 and PSA. The tubes were vortexed for 30 s and centrifuged at 14000 rpm for 20 min at 4 ºC. The extracts were collected and filtered through 0.22 µm nylon filters and then analyzed by LC-MS/MS.

Analytical Validation

LC-MS/MS analyses were performed on a 1260 Infinity liquid chromatography system coupled to an Agilent 6460 triple quadrupole (Agilent Technologies, Santa Clara, USA). The Agilent SB C18 chromatographic column (100 mm × 3.5 mm, 2.1 µm) was used (Agilent Technologies, Santa Clara, USA). The mobile phases used in the experiment were 0.1% v/v formic acid in H_2O (A) and 0.1% v/v formic acid in methanol (B). The flow was kept constant at 0.6 ml/min. The injection volume was 3 µL. The AJS ESI ionization source was operated in a positive mode, considering precursors ions 360.2 (ENR), 332.1 (CIP) and (ENR-d5). The conditions of the mass spectrometer were capillary voltage, 4000 V; temperature, 325 °C; gas flow (N2), 8 L/min. MRM (Multiple Reaction Monitoring) transitions and mass spectrometer conditions were shown in Table 1.

Table 1 - Multiple reaction monitoring (MRM) transitions and mass spectrometer conditions for each analyte

Analyte	Precursor ion	Production		DP (V)	CE (V)
Enro	360.2	342.2[a]	316.2[b]	120	15
Cipro	332.1	314.1[a]	231.1[b]	120	12
Enro-d5	365.2	347.1[a]	245.0[b]	120	30

[a] quantification ion; [b] confirmation ion; DP: Declustering potential; CE: Collision energy

The analytical method was fully validated taking into consideration the European Decision Commission 2002/657 (EC, 2002), the Guidelines on analytical methods validation of Brazilian National Institute of Metrology, Quality and Technology (INMETRO, 2011) and the guidelines for the design and implementation of the national food safety guarantee program associated with the use of veterinary medicines in food-producing animals (CODEX, 2009). Broiler tissues (muscle, liver and kidney) were used as blank samples of matrix. The following parameters were evaluated: linearity, selectivity, precision, accuracy, limit of quantification (LOQ), limit of detection (LOD), decision limits (CCα) and detection capability (CCβ). Analytical curves were determined for target tissue samples from untreated broiler chickens and were spiked with known concentrations of ENR and CIP. The calibration curve

of ENR and CIP used was in the range of 20 - 120 $\mu g.kg^{-1}$ for all tissues.

Commercial Enrofloxacin susceptibility

A total of 124 broiler faeces samples, not previously treated with fluoroquinolones, were collected from three different commercial aviaries in the North Region of Paraná State, Brazil. The chickens had the same characteristics as the flock age, feed and treatment. The samples were collected under aseptic conditions and processed for enrofloxacin sensitivity teste and *Salmonella* spp. isolation. Faeces samples were pre-enriched with BHI broth. Aliquots of 1 mL of enriched samples were added in 9 mL tubes containing commercial enrofloxacin at 10 $\mu g.mL^{-1}$ in Buffered Peptone Water (1%). The solutions were incubated at 36 ± 1 ºC for 18 to 24 hours. Subsequently, *Salmonella* spp. were isolated to verify whether there was growth of the genera after contact with antimicrobial.

In parallel, aliquots (1 mL) of the BHI enriched feaces sample were inoculated in tetrathionate broth for *Salmonella* spp. isolation. *Salmonella* spp. isolation and identification were performed according to the official methodology of Brazilian Ministry of Agriculture, Livestock and Food Supply (BRASIL, 1995). The typical *Salmonella* spp. colonies were characterized antigenically by rapid agglutination test and the serovars were identified by Microarray Check & Trace *Salmonella*®. After isolation, *Salmonella* spp. strains were standardized at 1.5×10^8 $CFU.mL^{-1}$ (0.5 on the McFarland scale) in NaCl 0.85% solution, through a turbidimeter (Highmed HMTDP-1000). Afterwards, 1 mL of the solution was inoculated in 9 mL of Enrofloxacin (10 $\mu g.mL^{-1}$) diluted in Buffered Peptone Water (1%). Tubes were incubated at 36 ± 1 ºC for 18 to 24 hours. *Salmonella* spp. absence was confirmed inoculating samples without turbidity on Nutrient Agar. All the experiment was performed in triplicate.

Antimicrobial susceptibility

The susceptibility to antimicrobials of 123 isolated *Salmonella* strains was determined by the disk diffusion test according to Clinical and Laboratory Standards Institute (CLSI, 2006). The disks of amoxicillin (10 μg), amoxicillin with clavulanic acid (10 μg), kanamycin (5 μg), gentamicin (10 μg), neomycin (10 μg), ceftiofur (10 μg), colistin (10 μg), enrofloxacin (5 μg), norfloxacin (5 μg), ciprofloxacin (10 μg), fosfomycin (50 μg), lincomycin + spectiomycin (10 μg) and tetracycline (10 μg) were used. Antibiotics were chosen based on drugs that are used from the point of view of human and veterinary view and WHO guidance and survey carried out in poultry establishments, indicating the most used antimicrobials in the treatment of broilers (CLSI, 2006; Paraná, 2005).

Statistical analysis

Data were submitted for analysis of variance and means were compared using Tukey test t a 5% significance level ($P<0.05$) (JASP Team; 2020)

RESULTS AND DISCUSSIONS

The regression equations and the determination coefficient for muscle, liver and kidney showed adequate linearity in the range of 20 $\mu g.kg^{-1}$ to 120 $\mu g.kg^{-1}$. The determination coefficient above values 0.99 fit the requirement criteria established by Regulatory Agencies (INMETRO, 2011; BRASIL, 2003). The limit of quantification of the method was determined by the first level of the calibration curve (20 $\mu g.kg^{-1}$) of each analyte and tissue studied (BRASIL, 2011). The detection limit of the method was estimated

at 10 μg.kg^{-1}, as being half of the LOQ. In which the signal / noise ratios were higher than 3 for all the analytes and tissues studied. The method had proven selectivity since no interfering transcripts were observed in the retention times for the studied analytes (Table 2).

The percentage of recovery obtained for muscle, liver and kidney was 94,0%, 101,2% and 97,7%, respectively, for ENR and 103,4%, 97,8% and 100.8% for CIP and RSD values less than 12%. Besides that, the extraction procedure using QuEChERS (quick, easy, cheap, effective, rugged and safe) and acetonitrile as the extraction phase, resulted in excellent recovery and linearity values. Acetonitrile and SPE cartridges are widely used in the extraction of quinolones in several tissues, showing excellent recovery and linearity (LUCATELLO et al., 2015). Which demonstrates that the method fit the established criteria and can be used in this validation.

The limit of quantification of the method was determined by the first level of the calibration curve (20 μg.kg^{-1}) of each analyte and tissue studied (BRASIL, 2011). The detection limit of the method was estimated at 10 μg.kg^{-1}, as being half of the LOQ . In which the signal / noise ratios were higher than 3 for all the analytes and tissues studied. The method had proven selectivity since no interfering transcripts were observed in the retention times for the studied analytes (Table 2).

Table 2. Linearity and sensitivity of Liquid Chromatograph analysis.

Tissue	Analyte	Slope	y-intercept	r^2	%RSD	LOD	LOQ
Muscle	ENR	156.91	1154.5	0.9986	5,66 - 7,19	10	20
	CIP	204.82	411.76	0.9943	7,27 – 9,01	10	20
Liver	ENR	390.4	2763.8	0.9931	4,90 – 6,84	10	20
	CIP	462.91	7285.5	0.9906	10,21 – 11,26	10	20
Kidney	ENR	822.1	1345.3	0.9972	6,30 – 12,86	10	20
	CIP	247.09	2473.8	0.9913	8,13 – 11,49	10	20

Concentration in μg.kg^{-1}

Then the detection limit of the method was estimated at 10 μg.kg^{-1}, as being half of the LOQ. In which the signal / noise ratios were higher than 3 for all the analytes and tissues studied. The results of the evaluated parameters are in accordance with current regulations (BRASIL, 2011; CODEX, 2009; EC, 2002).

The decision limit (CCα) and detection capability (CCβ) of ENRO and CIP for muscle, liver and kidney were determined by analyzing blank samples fortified. The CCα and CCβ of ENR were 62.33 and 74.45 μg.kg^{-1} for muscle, 61.22 and 72.24 μg.kg^{-1} for liver and 61.96 and 73.98 μg.kg^{-1}for kidney. And for CIP the respectively values of CCα and CCβ were: 64.78 and 81.09 μg.kg^{-1} for muscle, 66.83 and 82.39 μg.kg^{-1} for liver and 65.33 and 80.57 μg.kg^{-1} for kidney. The decision limit (CCα) and the detection capacity (CCβ) consider the variability of the method and the statistical risk when making decisions. Therefore, the values found were considered satisfactory for the analysis (Table 3). As shown in Table 3, ENR and CIP were extracted satisfactorily at

the three levels of fortification and for all studied tissues.

Recovery ranged from 92.47 to 100.91% for ENRO and 87.33 to 98.33% for CIP in muscle and liver, respectively. All these recovery values were according to the established limits of 70-120% (CODEX, 2009; BRASIL, 2011). The CV value in relation to repeatability and ranged from 4.9 to 12.86 % and CV reproducibility ranged from 4.52 to 12.23%. All these CV values were below the maximum acceptable value (CV <20%) for the tissues studied (BRASIL, 2011; CODEX, 2009).

Analysis of poultry tissue revealed ENR residues in around the world samples. The concentration in commercial samples ranged from ng.kg^{-1} to µg.kg^{-1}. In Brazil, National Plan for the Control of Residues contaminants (PNCRC) reports of 2017 and 2019, analyzing 607 and 3310 poultry meat samples in respectively period, showed that just one sample presented ENR residue (563.1 µg.kg^{-1}) above LMR (BRASIL, 2020). Although ENR has not been frequently detected, high concentrations of residue in samples indicate the need for constant monitoring.

After administration enrofloxacin is biotransformed in CIP (MORALES-GUTIÉRREZ et al., 2015). For fluoroquinolones residue in poultry meat, Brazilian authorities had established LMR for the sum of ENR+CIP (BRASIL, 2019). To ensure safe chicken meat to consumers, the withdrawal time must be respected. Antimicrobials (ENR, CIP and ENR+CIP) were not detected after 3, 4 and 7 days after the last dose exposition in chicken kidney, muscle and liver tissues, respectively (Table 3).

Table 3. The validation parameters recovery, coefficient of variation for repeatability and reproducibility for ENR and CIP obtained for muscle, liver and kidney samples.

Tissue	**Level**	**Recovery (%)**		**CV Repeatability (%)**		**CV Reproducibility (%)**	
	(µg.kg^{-1})	**ENR**	**CIP**	**ENR**	**CIP**	**ENR**	**CIP**
Muscle	25	95.75	89.36	7.19	8.90	7.96	8.60
	50	97.30	94.94	7.52	9.01	7.39	9.95
	75	92.47	87.33	5.66	7.27	6.50	7.01
Liver	25	100.91	98.33	4.9	10.21	4.52	10.06
	50	98.77	97.53	6.84	10.26	6.72	9.49
	75	93.21	95.72	8.02	7.45	9.23	8.21
Kidney	25	99.65	92.67	12.86	11.49	12.23	11.06
	50	98.06	96.81	7.29	9.35	7.33	9.29
	75	98.9	94.98	6.3	8.13	6.5	8.13

Studies on fluoroquinolone residues in poultry products to determine withdrawal period reports 5 – 10 days to bring the levels of residues below de LMR (GOUVEA et al., 2015). Fourten enrofloxacin products are registered at MAPA (SINDAN, 2020), the withdrawal period of these products ranges from 2 to 10 days. The tissues depletion experiment showed that, after 3 days the mean values of ENR+CIP were 48.73,

147.4 μg.kg^{-1} in muscle, and liver, respectively and not detected in kidney (Table 4). All these values are acceptable and did not exceed the LMR (100 μg.kg^{-1} for muscle and 300 μg.kg^{-1} for liver) established by Brazilian Health Minister (BRASIL, 2019).

However, the detection of fluoroquinolone metabolism products has been reported in the literature. Morales-Gutiérrez et al. (2015) identified 31 metabolites of ENR. In our study, these metabolites and their effects on bacterial resistance were not researched.

Another concern is about the residues excreted after antimicrobial ingestion. ENR was detected in poultry litter and soil representing a source of pollution to the environmental (LEAL et al., 2012). To evaluate the effect of feacal mixture on *Salmonella* spp. resistance, poultry feaces samples were analyzed. *Salmonella* spp. were isolated in 123 of 124 fecal samples. All the strains were identified as *Salmonella* Heidelberg (Table 4).

Table 4. ENR and CPR concentrations for muscle, liver and kidney collection days.

Tissue	Time after last Dose (days)	Concentration ± SD (μgkg^{-1})		
		ENR Mean	CIP	ENR+CIP
Muscle	1	727.33 ± 84.83^{a}	98.51 ± 14.09^{a}	825.84 ± 98.92^{a}
	2	192.45 ± 45.41^{b}	19.25 ± 4.46^{b}	211.7 ± 49.87^{b}
	3	24.63 ± 6.53^{c}	24.1 ± 4.14^{c}	48.73 ± 10.67^{c}
	4-10	nd	nd	Nd
Liver	1	1330.31 ± 71.77^{a}	986.63 ± 66.49^{a}	1158.48 ± 138.26^{a}
	2	200.1 ± 7.35^{b}	171.27 ± 8.65^{b}	371.37 ± 16.00^{b}
	3	77.46 ± 3.44^{c}	69.94 ± 6.51^{c}	147.4 ± 9.95^{c}
	4	52.8 ± 8.55^{cd}	43.91 ± 8.90^{cd}	96.71 ± 17.45^{cd}
	5	35.02 ± 9.51^{de}	30.51 ± 4.95^{de}	65.53 ± 14.46^{de}
	6	12.97 ± 2.03^{e}	18.03 ± 2.89^{e}	31.00 ± 4.92^{e}
	7-10	Nd	nd	nd
Kidney	1	374.71 ± 9.07^{a}	91.01 ± 1.75^{a}	465.72 ± 10.82^{a}
	2	64.37 ± 2.68^{b}	19.74 ± 2.73^{b}	84.11 ± 5.41^{b}
	3-10	nd	nd	nd

nd: not detected. Limit of detection: 10 μg.kg^{-1}

The National Program of Monitoring Prevalence and Bacterial Resistance in Chicken (PREBAF), susceptibility profile to antimicrobials from strains of *Salmonella* spp. isolated on chicken carcasses demonstrated the high incidence of *S.* Heidelberg in the country and the resistance of this serotype to four different classes of antimicrobials, including quinolones (BRASIL, 2012). This serovar is associated with resistance to several antimicrobials used in chicken production, tetracyclines, beta-lactams

and nalidixic acid (PANDINI et al., 2015). Demonstrating the importance of studying this pathogen and evaluating its effective control.

In our study, when isolated all the strains were susceptible to commercial enrofloxacin (10). But in presence of fecal samples, after enrofloxacin exposition, *S.* Heildeberg was isolated from 60.2% of samples (Table 4). Feces samples were collected from commercial aviary, from where poultry were feed treated with Narasin, Nicarbazin, Tylosin, Monensin, Enramycin Although poultry were not previoulsy treated with fluoroquinolone, microorganism presents in feaces were able to develop resistance mechanisms. Ahn et al. (2013) reported that sterilized fecal extract and ENR (0.06 μg.mL^{-1}) decreased the susceptibilities of *Salmonella enterica*, altering fatty acids composition and cell permeability. In our study the ENR was not analyzed in poultry feaces, but its presence in poultry litter is not discarded and also resistance gene.

The presence of genes associated with the type IV secretion system (T4SS) indicates that this system is functional in strains of *S.* Heidelberg and highlights a probable ability to acquire foreign DNA by horizontal transfer between bacteria (DEBLAIS et al., 2018). Antimicrobial supplementation on the fecal microbiota of broilers have shown that there are significant changes in the microbial association of gastrointestinal tract (COSTA et al., 2017). The gastrointestinal tract has a wide variety of microorganisms and the use of antimicrobials is likely to promote the spread of resistance genes in the microbiota, explaining the decrease in sensitivity of *Salmonella* associated with microbiota.

Disk diffusion test showed that all *Salmonella* strains (123 isolated) showed that isolated *S.* Heidelberg were more susceptible (>99%) to norfloxacin (5 μg) and CIP (10 μg) than ENR (30.1%), 69.1% of strains showed intermediate resistance to ENR. One mechanism of quinolone resistance is the mutation in the target site gyrase and/or topoisomerase IV. Another mechanism is plasmid-mediated resistance, with the efflux of quinolones increasing along with a decreasing in the interaction of the drug with gyrase (topoisomerase IV). Resistance maybe also developed by chromosome-mediated quinolone resistance. In this case there is a decrease in the influx of the drug into the cell (NAEEM et al., 2016).

Table 5 .Sensitivity of *Salmonella* Heidelberg exposed to Commercial enrofloxacin (10 μg.mL^{-1}) in feces and after isolated.

***Salmonella* Heibelberg**	**Resistant (%)**	**Susceptible (%)**
In feces sample	60.2	39.8
After isolation	0	100

n=123

All the 123 isolated strains were resistant to amoxicillin, amoxicillin with clavulanic acid and tetracycline (10 μg) and susceptible to gentamicin, colistin (10 μg), fosfomycin (50 μg), lincomycin + spectiomycin (10 μg). And strains were partially susceptible to kanamycin (93.5%) and neomycin (59.4%), and resistant to ceftiofur (99.2%).

In Brazil, according to the National Program of Monitoring Prevalence and Bacterial Resistance in Chicken (PREBAF), *S.* Heidelberg isolated from chicken carcass were resistant to four different classes of antimicrobials, including quinolones (BRASIL, 2012). Demonstrating the importance of studying this pathogen and evaluating its effective control. The resistance was also reported for *S.* Enteritidis and *S.* Typhimurium to this class of antimicrobials in Brazil (PRIBUL et al., 2017). A meta-analysis study to

evaluating the profile and temporal evolution of the antimicrobial resistance of nontyphoidal Salmonella from humans and poultry over the past 20 years in Brazil, showed highest levels of resistance to sulfonamides, nalidixic acid and tetracycline in poultry isolates (VOSS-RECH ET AL., 2017). The resistance of nalidixic acid in Enterobacteriaceae is generally correlated to reduced susceptibility to ciprofloxacin and enrofloxacin (LAI et al., 2014). Assessing the prevalence of resistance over time can improve the ability to treat diseases in humans and animals. The incidence of resistance to different serovars poses a threat to public health since these classes of antimicrobials are commonly used in the treatment of salmonellosis in humans and animals.

Conclusions

Bacterial resistance to veterinary and human antimicrobials is a public health concern. Many studies report methods with increasingly lower limits of detection and quantification. Considering all validation parameters analyzed the method presented in our study was sensitive and applicable for routine analysis of fluoroquinolones residues in poultry industries in developing countries. Withdrawal period of 3 days was enough to decrease ENR and CIP under the MRL. But antimicrobial resistance is also an emergent problem in the complex environmental, feces mixture from chicken litter decreased *S* Heidelberg susceptibility for ENR. Integrated actions by the productive, sanitary, and environmental sector are necessary to control this problem.

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