Brazilian Journal of Poultry Science
Revista Brasileira de Ciência Avícola

e-ISSN: 1806-9061 2024 / v.26 / n.2 / 001-006

http://dx.doi.org/10.1590/1806-9061-2023-1888

Original Article

Resistance Profile of Salmonella spp. to Third Generation Cephalosporins and Quinolones in Chicken Carcasses from Rio de Janeiro, Brazil

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■Keywords

Antimicrobial susceptibility, cephalosporins, chicken carcasses, Salmonella spp., quinolones.

Submitted: 30/November/2023
Approved: 09/April/2024

ABSTRACT

Salmonella spp. is one of the major bacterial causes of foodborne gastroenteritis in humans. The aim of this study was to investigate antimicrobial susceptibility to cephalosporins and quinolones, and to identify the genetic mechanisms related to this resistance in strains of *Salmonella* spp. Seventy chicken carcass samples were collected from slaughterhouses in the state of Rio de Janeiro, Brazil. The phenotypic profile was detected by the disk-diffusion method and the search for genes encoding betalactamases, and resistance to quinolones was evaluated by PCR. The search for mutations in *gyr*A and *par*C was carried out by sequencing these genes. Eleven strains of *Salmonella* spp. of different serotypes were isolated. All the strains were resistant to at least one of the antimicrobials tested, and 63.64% (7/11) showed resistance to three or more antimicrobials. In the phenotypic test for ESBL production, 36.36% (4/11) of the strains were considered positive. PCR detected the resistance genes bla_{CMY-2}, *qnr*B, bla_{CTX-M}, and bla_{TEM}. Among the isolates, 45.45% (5/11) simultaneously presented the bla_{CTX-M}, bla_{TEM}, *qnr*B genes and a mutation (Thr-57→Ser) in *par*C. Point mutations in the *par*C gene were detected in all the analyzed samples. Genes such as bla_{SHV}, *qnr*A, *qnr*C, *qnr*D, *qnr*S, *aac*(6')-Ib, *qep*A, and *oqx*AB were not detected. The study identified *Salmonella* spp. resistant to cephalosporins and quinolones, with resistance genes and mutations in *par*C, highlighting concerns about the adoption of biosecurity measures, responsible use of antimicrobials, and surveillance of resistant strains in the poultry chain.

INTRODUCTION

Salmonella spp. colonize the gastrointestinal tract of birds and mammals and is recognized as a major cause of gastroenteritis in humans. It is often transmitted through food, with undercooked chicken meat being one of the main sources of infection (EFSA & ECDC, 2022). In Brazil, serovars such as Heidelberg, Typhimurium, Infantis, Schwarzengrund, Enteritidis, and Agona are commonly detected in poultry and their derived products (Monte *et al.*, 2019).

Antimicrobials belonging to the beta-lactam and quinolone classes are the drugs of choice for treating salmonellosis in humans when antibiotic therapy is required (WHO, 2023). Beta-lactams, which include penicillins and cephalosporins, act by inhibiting the synthesis of the bacterial cell wall (Darini & Andrade, 2017). Quinolones, including ciprofloxacin, enrofloxacin, and nalidixic acid, inhibit bacterial DNA replication by blocking the activity of DNA gyrase type II and topoisomerase type IV enzymes (Correia *et al.*, 2017).

Monte *et al.* (2019) describes increased *Salmonella* spp. resistance to these antimicrobials. Resistance is a global public health concern, as

it can make the treatment of bacterial diseases more challenging or even impossible (EFSA & ECDC, 2022). One of the mechanisms of antimicrobial resistance involves the production of enzymes known as beta-lactamases. Extended-spectrum cephalosporins can be hydrolyzed by beta-lactamases belonging to molecular class C (such as AmpC, like bla_{CMY-2}) or by molecular class A extended-spectrum beta-lactamases (ESBLs) like bla_{CTX-M}, which are further subdivided into groups 1, 2, 8, 9, and 25, along with variants of bla_{SHV} and bla_{TEM}. ESBL-producing bacteria are resistant to penicillins, 1st to 4th generation cephalosporins, and monobactams like aztreonam (Darini & Andrade, 2017).

ESBLs are encoded by genes that can be plasmid-mediated or chromosomally expressed. The production of ESBLs encoded by plasmid-borne genes is concerning, as these genetic elements carry resistance genes to other antimicrobials, such as quinolones, and can facilitate the transmission of resistance genes to other bacteria (Woodford *et al.*, 2009).

Resistance mechanisms against quinolones in bacteria are associated with the presence of plasmid-mediated genes, overexpression of efflux pumps, and mutations in genes located in chromosomes. Chromosomal genes are linked to high levels of quinolone resistance and, when plasmid mediated (PMQR), they confer an intermediate but still clinically relevant level of resistance (Correia *et al.*, 2017). Three mechanisms of PMQR action have been identified: *qnr* genes (*qnr*A, *qnr*B, *qnr*C, *qnr*S, and *qnr*VC) that protect target enzymes; a mutation in the *aac*(6')-Ib gene, which confers the ability to acetylate certain quinolones; and the *oqx*AB and *qep*A genes, which encode mobile efflux pumps (Correia *et al.*, 2017). Chromosomal mutations in the quinolone resistance-determining regions (QRDR) of the *gyr*A and *par*C genes are frequently associated with quinolone resistance in *Salmonella* spp., and have been the main mechanism of antimicrobial resistance in this class for many years (Correia *et al.*, 2017).

The detection of resistance genes in *Salmonella* spp. from poultry products is critical for public health. The presence of these genes can contribute to the dissemination of resistant microorganisms in the environment, affecting other animals and humans. This poses challenges in the treatment of hospital infections in humans, exacerbating the difficulties associated with antimicrobial resistance (EFSA & ECDC, 2022).

In this context, this study aimed to investigate the antimicrobial susceptibility to cephalosporins and quinolones in *Salmonella* spp. strains, as well as the characterization of resistance genes from chicken carcasses collected in slaughterhouses in the state of Rio de Janeiro.

MATERIALS AND METHODS

Isolation of *Salmonella* spp. Strains

This research was submitted to the Ethics Committee on the Use of Animals of Universidade Federal Fluminense, under number 697. Seventy samples of chicken carcasses from slaughterhouses under the supervision of the State Inspection Service (SIE) located in the state of Rio de Janeiro, Brazil, were collected in the years 2016 and 2022. The carcasses were randomly collected after the drip process and stored in sterile bags. All samples were refrigerated and transported in isotermic containers to the Avian Health Laboratory of the Faculdade de Veterinária at Universidade Federal Fluminense.

Isolation was performed according to ISO 6579-1:2017. 400 ml of 1% Peptone Saline Solution (PSS) was added to the bags containing the carcasses. The bags were shaken for 60 seconds to allow the solution to cover the entire surface of the carcasses. Subsequently, 10 ml of the washing solution was transferred to a sterile container and incubated at 37 ± 1°C for 18 ± 2 hours. Rappaport Vassiliadis and Tetrationate broths were used for the selective enrichment and selective plating steps, followed by MacConkey and Brilliant Green agars. For biochemical identification, Triple Sugar Iron, L-Lysine decarboxylation, Urea agar, and Voges-Proskauer media were employed. Strains were serotyped at the National Reference Laboratory for Enterobacteriaceae at the Oswaldo Cruz Foundation in Rio de Janeiro, Brazil (IOC, FIOCRUZ, RJ, Brazil).

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility of the isolates was determined using the disk diffusion method in Muller Hinton agar, following the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2022). The tested antimicrobial disks included ceftazidime (CAZ-30µg), ceftriaxone (CRO-30µg), ceftiofur (CTF-30µg), cefotaxime (CTX-30µg), amoxicillin-clavulanic acid (AMC-30µg), nalidixic acid (NAL-30µg), enrofloxacin (ENO-5µg), and ciprofloxacin (CIP-5µg).

Double Disk Test (DDT) was performed on *Salmonella* spp. strains to phenotypically assess ESBL production. On Muller Hinton agar, disks containing cephalosporins cefotaxime, ceftazidime, and ceftriaxone were placed alongside a beta-lactamase inhibitor disk (amoxicillin-

clavulanic acid), as recommended by EUCAST (2017). When inhibition zones around any of the cephalosporin disks were enlarged or there was a distortion of the halo towards the amoxicillin-clavulanic acid disk, the test was considered positive.

DNA Extraction

Bacterial isolates DNA was extracted using the Wizard® Genomic DNA Purification kit (Promega, Brazil) following the manufacturer's protocol. The quality and concentration of DNA were measured using a NanoDrop Spectrophotometer (Biodrop®).

Detection of *bla*, PMQR and QRDR Genes

The resistance genes examined in this study are presented in Table 1. The reactions were conducted in a thermal cycler (Bio-Rad T100). Subsequently, the amplicons obtained from the PCR were subjected to electrophoresis in a 1.5% agarose gel at 94 V for 40 minutes. The visual inspection of the gel was performed using the L-Pix Photodocumenter for gel electrophoresis (Loccus, Brazil).

For sequencing, PCR was performed with primers for amplification of *gyrA* and *parC* genes. Amplicon purification was conducted using the QIAquick PCR purification kit (Qiagen), following the manufacturer's instructions, with subsequent sequencing in the DNA sequencer ABI 3730 (Applied Biosystems) on the Fiocruz sequencing platform, Oswaldo Cruz Institute (IOC).

Detection of QRDR Mutation

The files containing the nucleotide sequences of the quinolone resistance region in the *gyr*A and *par*C

Table 1 – Genes, sequence, size, and primer references that were used in this study.

Genes	Sequence (5'- 3')	Size (bp)	References
bla_{SHV}	F: ATGCGTTATATTCGCCTGTG R: TGCTTTGTTATTCGGGCCAA	747	Monstein *et al.*(2007)
bla_{TEM}	F: TCGCCGCATACACTATTCTCAGAATGA R: ACGCTCACCGGCTCCAGATTTAT	445	
bla_{CTX-M}	F: ATGTGCAGYACCAGTAARGTKATGGC R:TGGGTRAARTARGTSACCAGAAYCAGCGG	593	
bla_{CMY-2}	F: ATGATGAAAAAATCGTTATGC R: TTGCAGCTTTTCAAGAATGCGC	1143	Koeck *et al.* (1997)
$bla_{CTX-M-1}$	F: AAAAATCACTGCGCC AGTTC R: AGCTTATTCATCGCCACG TT	415	Woodford *et al.* (2006)
$bla_{CTX-M-2}$	F: AGCTTATTCATCGCCACGTT R: CGACGCTACCCCTGCTATT	552	
$bla_{CTX-M-8}$	F: TCGCGTTAAGCGGATGATGC R: AACCCACGATGTGGGTAGC	666	
$bla_{CTX-M-9}$	F: CAA AGAGAGTGCAACGGATG R: ATTGGA AAGCGTTCATCACC	205	
$bla_{CTX-M-25}$	F: GCACGATGACATTCGGG R: ACCCACGATGTGGGTAGC	327	
*qnr*A	F: AGAGGATTTCTCACGCCAGG R: TGCCAGGCACAGATCTTGAC	580	Kraychete *et al.* (2016)
*qnr*B	F: GGMATHGAAATTCGCCACTGA R: TTTGCYGYYCGCCAGTCGAAA	264	
qnrC	F: GCGAATTTCCAAGGGGCAAA R: ACCCGTAATGTAAGCAGAGCAA	135	
*qnr*D	F: AGGTGTAGCATGTATGGAAAAGC R: ACATTGGGGCATTAGGCGTT	691	
qnrS	F: GCAAGTTCATTGAACAGGGT R: TCTAAACCGTCGAGTTCGGCG	428	
aac(6')Ib	F: TTGCGATGCTCTATGAGTGGCTA R: CTCGAATGCCTGGC GTGTTT	482	Park *et al.* (2006)
*qep*A	F: GCAGGTCCAGCAGCGGGTAG R: CTTCCTGCCCGAGTATCGTG	199	Yamane *et al.* (2008)
*oqx*AB	F: GACAGCGTCGCACAGAATG R: GGAGACGAGGTTGGTATGGA	339	Chen *et al.* (2012)
*gyr*A	F: CGAGAGAAATTACACCGGTCA R: AGCCCTTCA ATGCTGATGTC	610	Kim *et al.* (2011)
*par*C	F: ATGAGCGATATGGCAGAGC R: GCGAACAGATGGTTCATCAC	950	

bp: base pair

genes of *Salmonella* spp. were read in the BioEdit program (BioEdit Sequence Alignment Editor) to assess sequence quality and generate new files in "Fasta" format. All obtained sequences were compared with those in the GenBank using the BlastN algorithm**.**

Statistical Analysis

Fisher's exact test was employed to determine if there was a difference between the frequencies of phenotypic resistance and resistance genes. Values with $p<0.05$ were considered significant, and the analysis was conducted using the BioEstat 5.3 software.

RESULTS

Antimicrobial Susceptibility Test Profile

Eleven strains of *Salmonella* spp. were obtained, which were serotyped as *Salmonella enterica* subsp. *Enterica* (rough) (n=2), Mbandaka (n=2), Typhimurium (n=2), Heidelberg (n=1), Schwarzengrund (n=2), Agona (n=1), and Senftenberg (n=1).

The strains exhibited three different phenotypic profiles of antimicrobial resistance (Tab. 2). Among the isolates, 63.64% (7/11) showed resistance to three or more antimicrobials, and all strains were resistant to at least one of the tested antimicrobials (Table 2).

All strains were resistant to at least one antimicrobial from the quinolone class. Within this class, 100% of the isolates were resistant to nalidixic acid and enrofloxacin, and 63.64% (7/11) were resistant to ciprofloxacin. Among the tested samples, 27.27% (3/11) showed resistance to all tested cephalosporins (Figure 1). In the studied strains, the difference in susceptibility was statistically significant only for antimicrobials from the quinolone classes ($p<0.05$).

Regarding the phenotypic ESBL production test, 36.36% (4/11) of the strains were considered producers of this enzyme (Table 2).

Detection of *bla*, *PMQR*, and QRDR Genes

The association of genes encoding resistance to cephalosporins (bla_{TEM}, bla_{CTX-M}) and quinolones (*qnr*B and *par*C mutation) was detected in 45.45% (5/11) of the studied strains, which belonged to the Mbandaka (ID 4), Typhimurium (ID 6 and 7), Schwarzengrund (ID 8), and Agona (ID 9) serovars. Strains positive for *bla* belonged to group 2, and the bla_{CMY-2} gene was detected in only one sample, identified as S. *enterica* subsp. *enterica* (ID 1).

The highest gene frequency detected in this study was *qnr*B, with 63.64% of positive strains. The *qnr*B gene, on its own, was associated with *par*C mutation in 18.18% (2/11) of the strains (Table 1). Other plasmid-borne genes investigated in this study (bla_{SHV}, *qnr*A, *qnr*C, *qnr*D, *qnr*S, *aac*(6')-Ib, *qep*A, and *oqx*AB) were not detected.

The values associated with the frequencies of the resistance genes bla_{TEM} and bla_{CTX-M}, as well as *qnr*B, were considered statistically significant. This suggests a statistically relevant difference in their occurrence or expression compared to other genes ($p<0.05$).

Detection of QRDR Mutation

Sequencing analysis resulted in the identification of point mutations in the *par*C gene in all analyzed samples (Table 2). The mutations found occurred at codon 57, leading to amino acid substitutions from threonine to serine. Mutations in *gyr*A were not detected.

Table 2 – Phenotypic and genotypic characteristics of *Salmonella* spp. serovars subspecies enterica isolated from chicken carcasses in the state of Rio de Janeiro.

ID	Serovars	Resistance Profile	ESBL phenotype	Resistance Genes
1	ser. Enterica	CRO,CTF,CAZ,CTX, AMC,ENO,NAL,CIP	+	bla_{CMY-2}
2	ser. Enterica	ENO,NAL	-	*qnrB*
3	Mbandaka	CRO,CTF,CAZ,CTX, AMC,ENO,NAL,CIP	+	-
4	Mbandaka	ENO,NAL,CIP	-	bla_{CTX-M}, bla_{TEM}, *qnrB*
5	Heidelberg	CRO,CTF,CAZ,CTX, AMC,ENO,NAL,CIP	+	-
6	Typhimurium	ENO,NAL,CIP	-	bla_{CTX-M}, bla_{TEM}, *qnrB*
7	Typhimurium	ENO,NAL,CIP	-	bla_{CTX-M}, bla_{TEM},*qnrB*
8	Schwarzengrund	ENO,NAL,CIP	+	bla_{CTX-M},bla_{TEM},*qnrB*
9	Agona	ENO,NAL	-	bla_{CTX-M},bla_{TEM},*qnrB*
10	Senftenberg	ENO,NAL	-	*qnrB*
11	Senftenberg	ENO,NAL	-	-

Legend. ID: Identification, CAZ: ceftazidime, CRO: ceftriaxone, CTF: ceftiofur, CTX: cefotaxime, AMC: amoxicillin-clavulanic acid, NAL: nalidixic acid, ENO: enrofloxacin, and ciprofloxacin (CIP), -: Negative, +: positive.

DISCUSSION

We identified different serovars of *Salmonella* with a high incidence of resistance to cephalosporins and quinolones in broiler carcasses. *Salmonella* Mbandaka, Heidelberg, Typhimurium, and Schwarzengrund are commonly detected in poultry and poultry products from Brazil (Monte *et al.*, 2019). The presence of these serovars in food represents a risk to human health, since they have been described to cause both enteric and systemic disease.

Moreover, antimicrobial resistance emerges as a significant worldwide concern in the 21st century, resulting in numerous human fatalities and economic setbacks across different sectors (Pokharel *et al.*, 2020). Antibiotic-resistant bacteria, including *Salmonella* spp., are present in food animals and can be transmitted to humans through the consumption of contaminated food, direct interaction with animals, or exposure to contaminated environmental sources like water (EFSA & ECDC, 2022).

The detection of CMY-2, CTXM-2, and TEM genes in *Salmonella* spp. is important because genes can be easily transferred through food. That is because ESBL enzymes are often carried on plasmids, DNA fragments that can be transferred between different bacterial strains and/or different species. This facilitates the rapid dissemination of antimicrobial resistance (Woodford *et al.*, 2009).

Human salmonellosis does not typically necessitate antibiotic treatment; however, in cases involving immunosuppressed patients, persistent diarrhea, or invasive infections, antibiotic intervention becomes essential. Commonly prescribed first-line oral antibiotics for treating salmonellosis include cephalosporins and quinolones. The limitation of therapeutic options can be triggered by the presence of strains encoding the production of Extended-Spectrum Beta-Lactamases (ESBL) (EFSA & ECDC, 2022).

The Double Disk Test stands out as an effective technique for the preliminary identification of bacterial strains producing ESBL (EUCAST, 2017). In this study, its efficacy was evident, as positive results were correlated with both the detection of resistance genes in PCR and the method of antimicrobial resistance through the disk diffusion method. By integrating different techniques to detect and characterize ESBL strains, the approach was refined, ensuring a more reliable identification of strains resistant to beta-lactams.

Regarding quinolone resistance, all *Salmonella* spp. strains analyzed exhibited resistance to at least one quinolone tested. Resistance to quinolones is usually related to mutations in the *gyr*A gene, but this mutation was not detected in this study. DNA sequencing detected the presence of mutations at position Thr-57→Ser of the *par*C gene in all studied strains, which could explain the resistance to quinolones. Eaves *et al.* (2004) previously described this mutation to confer higher levels of resistance to nalidixic acid and lower levels to ciprofloxacin. The use of antimicrobials in animal production may select these mutated strains, leading to their spread in the poultry chain (Vidovic & Vidovic, 2020)

The *qnr*B gene, recognized for conferring moderate levels of resistance to quinolones (Dias *et al.*, 2022), was detected in 63.64% of the strains. Our results corroborate previous studies from Brazil (Pribul *et al.*, 2017; Saidenberg *et al.*, 2023), and demonstrate that *qnrB* is the most common *qnr* allele in Brazilian *Salmonella* strains.

These findings emphasize the complexity of the problem and the necessity for comprehensive approaches in monitoring and understanding antimicrobial resistance in *Salmonella* spp., offering important information for more effective therapeutic strategies and preventive control measures.

CONCLUSION

The study analyzed chicken carcass isolates from slaughterhouses in the state of Rio de Janeiro and detected *Salmonella* spp. resistant to cephalosporins and quinolones, plasmid-borne resistance genes bla_{CTX-M}, bla_{CMY2}, and *qnr*B, as well as mutations in *par*C encoding resistance to these antimicrobials. The results raise concerns regarding food safety, calling for improvements in biosafety measures in slaughterhouses, responsible antimicrobial use, and surveillance of resistant strains in animal and human products to prevent the spread of resistant bacteria.

ACKNOWLEDGMENTS

This work was supported by the Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (E-26/010.002133/2019), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, code 001).

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