




Isolation, characterization, and resistance profile of *Salmonella* spp. from chicken cuts

[*Isolamento, caracterização e perfil de resistência de Salmonella spp., provenientes de cortes de frango*]

A.C.P. Silva¹ , T.P. Morais² , K.B.P.B. Casari³ 

¹Graduate, Universidade Estadual do Oeste do Paraná, Centro de Ciências da Saúde, Francisco Beltrão, PR, Brasil

²Undergraduate, Universidade Estadual do Oeste do Paraná, Centro de Ciências da Saúde, Francisco Beltrão, PR, Brasil

³Universidade Estadual do Oeste do Paraná, Centro de Ciências da Saúde, Francisco Beltrão, PR, Brasil

ABSTRACT

The present study aimed at isolating and characterizing *Salmonella* spp. from chicken cuts marketed in Francisco Beltrão, PR, and verify the resistance profile of the isolates against antimicrobials used in human therapy. Samples of chicken cuts (n=40) were purchased from supermarkets and submitted to microbiological analysis for the detection of *Salmonella* spp. The suspected colonies underwent biochemical testing for the identification of enterobacteria. Four colonies were selected from each sample positive for *Salmonella* spp., totaling 28 isolates that were tested for antimicrobial sensitivity. Colonies that showed resistance to ceftriaxone were subjected to extended-spectrum beta-lactamases (ESBL). Among the analyzed chicken samples, seven (17.5%) showed biochemical behavior characteristic of *Salmonella* spp. Among the 28 isolates, seventeen different resistance profiles were found, of which 46.42% (n=13) had a multi-resistance profile, and 21.4% (n=6) of the isolates had a phenotype for ESBL production. The strains of *Salmonella* spp. isolated from chicken cuts found in this study showed a high level of resistance to antimicrobials of different classes and of last generations, these data serve as a warning, as they put the human treatment of salmonellosis at risk.

Keywords: antimicrobials, microbiota, microorganisms, poultry

A pesquisa objetivou isolar e caracterizar Salmonella spp., a partir de cortes de frangos comercializados na cidade de Francisco Beltrão – PR, bem como verificar o perfil de resistência dos isolados em relação aos antimicrobianos utilizados na terapêutica humana. Amostras de cortes de frango (n=40) foram adquiridas em supermercados e submetidas à análise microbiológica para detecção de Salmonella spp. As colônias suspeitas foram submetidas a provas bioquímicas para identificação de enterobactérias. Quatro colônias foram selecionadas de cada amostra positiva para Salmonella spp., totalizando 28 isolados, que foram testadas quanto à sensibilidade a antimicrobianos. As colônias que apresentaram resistência à ceftriaxona foram submetidas à pesquisa de betalactamases de espectro estendido (ESBL). Das amostras de frango analisadas, sete (17,5%) apresentaram comportamento bioquímico característico de Salmonella spp. Entre os 28 isolados, foram encontrados 17 perfis diferentes de resistência, tendo 46,42% (n=13) apresentado perfil de multirresistência e 21,4% (n=6) apresentado fenótipo para produção de ESBL. As cepas de Salmonella spp. isoladas de cortes de frango, encontradas neste estudo, apresentaram alto índice de resistência a antimicrobianos de diferentes classes e de últimas gerações. Esses dados servem de alerta, uma vez que coloca em risco o tratamento da salmonelose humana.

Palavras-chave: antimicrobianos, microbiota, microrganismos, avicultura

INTRODUCTION

Chicken meat is widely consumed by Brazilians since it is considered a healthy, nutritious food, with high protein content. However, this food constitutes a vehicle for pathogenic

microorganisms and can cause infections in the consumer population (Welker *et al.*, 2010).

Maintenance of the health of chickens is essential to guarantee the quality of the food that reaches the consumer's table. However, the integrated production system and the innumerable processes

that the food undergoes until it reaches the consumer contribute to the birds' poor health, thus increasing the risk of contamination (Welker *et al.*, 2010; Zagonel *et al.*, 2017).

Among the microorganisms that can cause diseases in humans, the one of greatest clinical importance is the bacterium *Salmonella* spp. Despite all technological development and the adoption of hygiene measures by breeders and poultry industries, infections by *Salmonella* spp. are the most common foodborne diseases worldwide, with most serotypes of the genus being pathogenic to humans. Research has revealed the presence of this bacterium in at least one-third of the broiler samples analyzed (Yamaguchi *et al.*, 2013; Silva and Menão, 2016; Zagonel *et al.*, 2017; Montezani *et al.*, 2018).

For years, antimicrobial agents have been used on a large scale in the production of chickens for therapeutic, prophylactic purposes and as growth promoters. The long-term use of antimicrobial agents in farm animals exerts selective pressure on bacteria, contributing to the development of resistance. Since these animals are intended for human consumption, it is possible that these microorganisms, as well as their resistance genes, may be incorporated into the human microbiota through direct contact with the bird or the ingestion of food or water contaminated with the bacteria (Chantziaras *et al.*, 2014; Lai *et al.*, 2014).

In this context, the present study aimed at isolating and characterizing *Salmonella* spp. from chicken cuts marketed in Francisco Beltrão – PR and verify the resistance profile of the isolates against antimicrobials used in human therapy.

MATERIAL AND METHODS

From April to August 2019, samples of chicken meat (n = 40) were purchased in supermarkets in Francisco Beltrão – PR. The samples consisted of chilled chicken cuts (n=24), including thighs and drumsticks (n=15), wing drumette (n=7), and breast fillet (n=2); and frozen chicken cuts (n=16), including thighs and drumsticks (n=13), and breast fillet (n=3). The samples were obtained under conventional packaging and commercial conditions (Table 1).

The investigation of *Salmonella* spp. was carried out according to Brasil (2003) and followed the methodologies recommended by Silva *et al.* (2010). Suspicious colonies were screened by biochemical testing using a kit for the identification of enterobacteria (NEWPROV), which includes biochemical tests such as deamination of L-tryptophan, production of H₂S, glucose fermentation, gas production, decarboxylation of lysine and ornithine, indole production, motility, use of citrate as a carbon source, and rhamnose fermentation.

Four colonies were selected from each sample that exhibited colonies with biochemical behavior of *Salmonella* spp., totaling 28 isolates. The samples were subjected to rapid slide agglutination test using polyvalent flagellar serum and were sent to the AQUACEN laboratory of the Veterinary School of the Federal University of Minas Gerais (UFMG) for confirmation of the *Salmonella* genus using the MALDI –TOF methodology (Benagli *et al.*, 2011).

The sensitivity test to antimicrobials was conducted using the agar diffusion method, according to recommendations of the Clinical and Laboratory Standards Institute (Performance..., 2005). The tested antimicrobial agents included ampicillin (10µg), amoxicillin+clavulanic acid (20/10µg), cephalothin (30µg), cephalexin (30µg), nalidixic acid (30µg), ciprofloxacin (5µg), azithromycin (15µg), gentamicin (10µg), sulphazotrin (25µg), meropenem (10µg), nitrofurantoin (300µg), imipenem (10µg), ceftriaxone (30µg), and cefepime (30µg). Reference strains of the American Type Culture Collection were used, namely: *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 (provided by the Microbiology Laboratory of the Regional Hospital of Southwest Paraná), and *Salmonella* Typhimurium ATCC 14028 (supplied by the Oswaldo Cruz Institute). The results were read and interpreted according to CLSI (Performance..., 2005) standards.

Multidrug resistance was defined as resistance to three or more classes of antimicrobials and isolates with resistance to third generation cephalosporins (ceftriaxone) were subjected to phenotypic evaluation for the production of ESBL, performed using the disc approximation technique, according to CLSI standards (Performance..., 2007). For this study, a

descriptive statistical analysis of the antimicrobial resistance and sensitivity profile was performed, calculating the absolute and relative frequency.

RESULTS AND DISCUSSION

Among the total samples analyzed (n = 40), seven lots exhibited colonies with biochemical behavior

suggestive of the genus *Salmonella* spp. (17.5%), five of which were from chilled samples and two from frozen samples, including cuts of thigh and drumstick and wing drumette, as described in Table 1. All isolates submitted to serology and referred for identification by MALDI-TOF were confirmed for the *Salmonella* genus.

Table 1. Number of samples analyzed and positive for *Salmonella* spp. isolated from chicken cuts marketed in Francisco Beltrão – PR, according to cut and marketing conditions

Cuts	Marketing condition	Total samples analyzed	Total positive samples
Wing drumette	Chilled	N=7	N=1
Thigh and drumstick	Frozen	N=13	N=2
Thigh and drumstick	Chilled	N=15	N=4
Breast fillet	Frozen	N=3	N=0
Breast fillet	Chilled	N=2	N=0

N= Number of samples

The frequency of 17.5% positivity for *Salmonella* spp. in the lots analyzed in the present survey was similar to other studies found in the literature. Cardoso *et al.* (2015) verified a 14.6% presence of *Salmonella* spp. in chicken samples in São Paulo state between 2000 and 2010. However, the frequency of batches destined for commercialization contaminated by *Salmonella* spp. is quite variable. In a study by Zagonel *et al.* (2017), the presence of this bacterium was found in 100% of chilled chicken meat samples sold in the Alto do Vale do Peixe region in Santa Catarina. Meanwhile, Trainotti *et al.* (2013), when analyzing 50 samples of chicken meat, found no serotype of *Salmonella* spp., with all samples meeting the standard of absence of bacteria in 25 g of the analyzed product.

It is worth mentioning that, in the Brazilian trade, chicken meat can be found frozen or chilled. These forms of storage are not able to destroy bacterial cells of *Salmonella* spp., however, a reduction in viable cells is expected in freezing. (D'aoust; Maurer, 2007). In the present study, of the seven samples positive for *Salmonella* spp, five were marketed in the chilled form and only two in the frozen form. Other studies have also shown a greater number of positive samples for *Salmonella* spp. in chilled chicken samples. In the study by Almeida *et al.* (2000), of the 15 frozen chicken cuts analyzed, the presence of bacteria of the genus *Salmonella* was detected in seven, and

in 15 of chilled chicken, 13 positive samples were observed for this pathogen.

It is known that the application of good manufacturing practices and the Hazard Analysis and Critical Control Points (HACCP) assist in controlling the presence of these microorganisms in the production line. According to Yamatogi *et al.* (2016), the moment of slaughter constitutes the main critical point of contamination of chicken meat. However, some stages that precede slaughter, such as capture and transport, are also crucial considering the contamination and proliferation of pathogenic microorganisms, since they generate a stressful environment for the birds, resulting in the disturbance of intestinal functions and increasing the spread of bacteria in the feces, leading to contamination of the carcass, and spread of the pathogen in the production line.

Regarding the sensitivity profile, no isolate was sensitive to all the tested antimicrobials. The antimicrobial susceptibility test results (Table 1 and Fig. 1) revealed that all isolates analyzed showed sensitivity to meropenem, whereas 100% were resistant to nalidixic acid, representative of the class of first-generation quinolones. When subjected to ciprofloxacin, a third-generation quinolone, no resistance was observed. The strains showed only intermediate resistance and sensitivity.

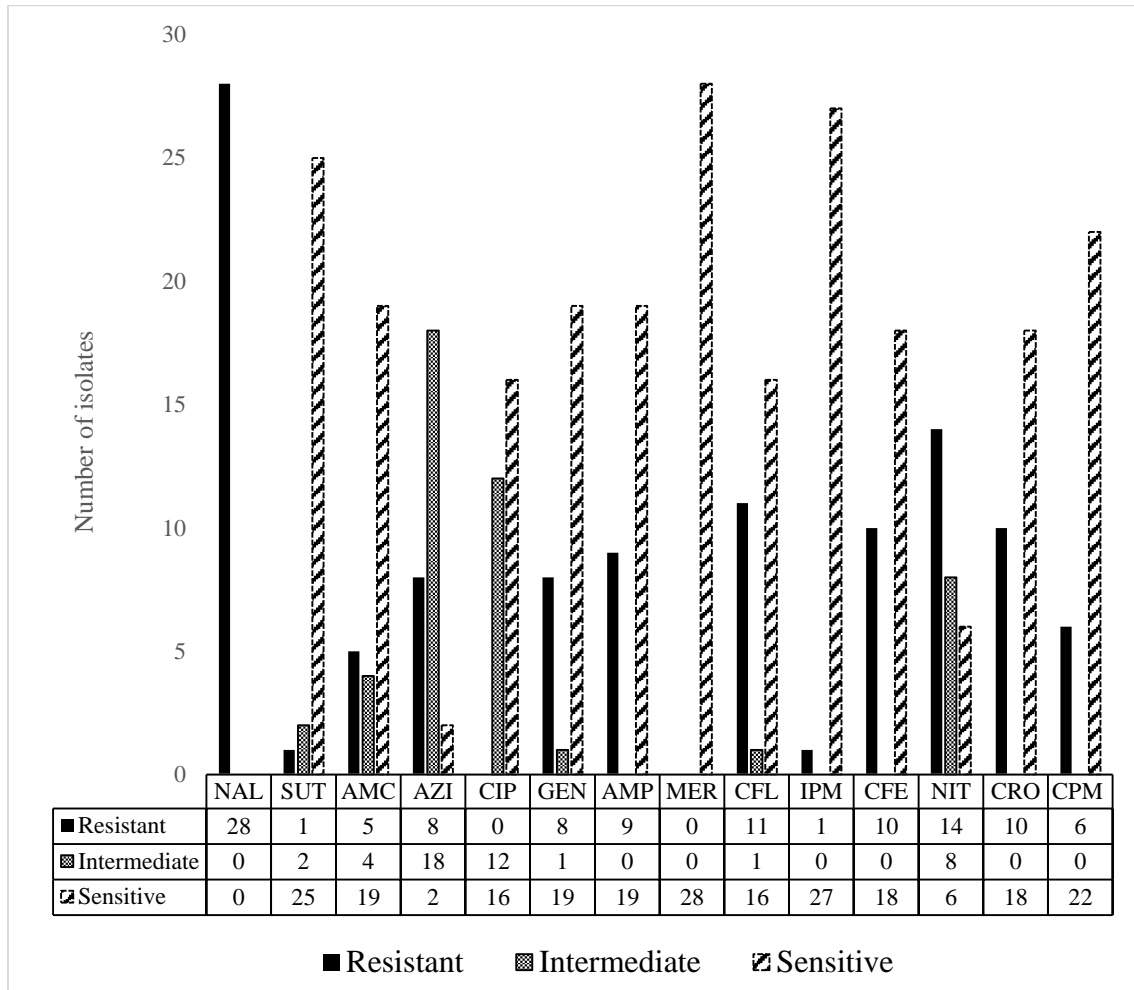
Table 1. Antimicrobial resistance profile of the isolates selected for the *Salmonella* spp. antibiogram test from broiler cuts marketed in Francisco Beltrão, PR in 2019

Sample (CFU)	Antimicrobials													
	Nal	Sut	Amc	Azi	Cip	Gen	Amp	Mer	Cfl	Ipm	Cfe	Nit	Cro	Cpm
1(1)	R	S	S	R	I	S	S	S	S	S	S	R	S	S
1(2)	R	S	S	R	I	S	S	S	S	S	S	R	S	S
1(3)	R	S	S	R	I	S	S	S	S	S	S	R	S	S
1(4)	R	S	S	I	S	S	S	S	S	S	S	I	S	S
2(1)	R	S	R	I	S	S	R	S	R	S	R	S	R	S
2(2)	R	S	R	I	S	S	R	S	R	S	R	S	R	S
2(3)	R	S	R	I	I	R	R	S	R	S	R	S	R	S
2(4)	R	S	R	I	S	S	R	S	R	S	R	S	R	S
3(1)	R	S	S	I	S	S	S	S	S	S	S	I	S	S
3(2)	R	S	S	S	S	S	S	S	S	S	S	I	S	S
3(3)	R	S	S	I	S	S	S	S	S	S	S	I	S	S
3(4)	R	S	S	I	S	S	S	S	S	S	S	I	S	S
4(1)	R	S	S	I	S	S	S	S	S	S	S	I	S	S
4(2)	R	S	S	I	S	S	S	S	S	S	S	R	S	S
4(3)	R	S	S	I	S	S	S	S	S	S	S	R	S	S
4(4)	R	S	S	I	S	S	S	S	S	S	S	S	S	S
5(1)	R	S	S	I	I	S	S	S	R	S	S	R	S	S
5(2)	R	S	S	R	I	S	S	S	S	S	S	R	S	S
5(3)	R	S	S	I	S	S	S	S	S	S	S	R	S	S
5(4)	R	R	S	R	I	S	S	S	I	S	R	R	S	S
6(1)	R	S	I	R	S	R	R	S	R	S	R	I	R	R
6(2)	R	S	R	R	I	R	R	S	R	S	R	R	R	R
6(3)	R	I	I	I	I	R	R	S	R	R	R	R	R	R
6(4)	R	S	I	I	I	I	R	S	R	S	R	R	R	R
7(1)	R	S	S	I	I	R	S	S	S	S	S	R	S	S
7(2)	R	S	S	I	S	R	S	S	R	S	R	I	R	R
7(3)	R	S	S	S	S	R	S	S	S	S	S	S	S	S
7(4)	R	S	S	I	S	R	R	S	R	S	R	R	R	R

CFU- Colony Forming Unit.

NAL- Nalidixic Acid, SUT- Sulfazotrim, AMC- Amoxicillin + Clavulanic Acid, AZI- azithromycin, CIP- Ciprofloxacin, GEN- Gentamicin, AMP- Ampicilin, MER- Meropenem, CFL- Cephalotin, IPM – Imipenem, CFE- Cephalexin, NIT- Nitrofurantoin, CRO- Ceftriaxone, CPM- Cefepime.

R- Resistant, S- Sensitive, I- Intermediate.



NAL- Nalidixic Acid, SUT- Sulfazotrim, AMC- Amoxicillin + Clavulanic Acid, AZI- azithromycin, CIP- Ciprofloxacin, GEN- Gentamicin, AMP- Ampicillin, MER- Meropenem, CFL- Cephalotin, IPM – Imipenem, CFE- Cephalexin, NIT- Nitrofurantoin, CRO- Ceftriaxone, CPM- Cefepime.

Figure 1. Number of isolates with intermediate resistance, resistance and sensitivity to antimicrobials tested.

In relation to the other antimicrobial tested, the strains of *Salmonella* spp. showed resistance to nitrofurantoin (50%), cephalothin (39.3%), cephalexin (39.3%), ceftriaxone (35.7%), ampicillin (32.1%), gentamicin (28.6%), azithromycin (28.6%), cefepime (21.4%), amoxicillin with clavulanate (17.9%), sulphazotrin (3.6%), and imipenem (3.6%).

Considering the total isolated, 42.9% (n=12) were resistant to at least one of the beta-lactam agents tested, in which the first-generation cephalosporins, cephalexin and cephalothin, and ampicillin showed the highest percentage of resistance within this class of antimicrobials

(resistance percentages of 39.3%, 39.3%, and 32.1, respectively).

The high resistance index found herein was similar to other studies that also reported a rate of 100% resistance to at least one of the antimicrobials tested in strains of *Salmonella* spp (Pandini *et al.*, 2015).

Ten isolates (35.7%) were resistant to third generation cephalosporins (ceftriaxone) and underwent phenotypic analysis regarding ESBL production. Among the ten isolates tested, six revealed this resistance mechanism phenotype, evidenced by halo distortion and the formation of a “phantom zone” in the approach disc test.

In addition to resistance against third generation cephalosporins, 21.4% of the tested microorganisms were resistant to cefepime, a fourth-generation cephalosporin, and one isolate (6-5) showed resistance to imipenem, a representative of the carbapenem class.

In this study, 17 different resistance profiles (P1 to P17) were identified, as shown in Table 2. The most frequent profiles were P3, represented by five of the 28 isolates (17.8%), characterized by resistance to nalidixic acid, intermediate resistance to azithromycin and nitrofurantoin, and

sensitivity to the other tested antimicrobials, and P6, with four isolates (14.2%) exhibiting resistance to nalidixic acid, azithromycin, and nitrofurantoin, intermediate resistance to ciprofloxacin, and sensitivity to the other tested antimicrobials.

Of the total of isolated, 42.9% presented a multidrug resistance profile, showing resistance to three or more different classes of antimicrobials, 21.4% of which exhibited an ESBL production phenotype, evidenced by the approach disc test (Table 2).

Table 2. Resistance profiles and intermediate resistance of 28 isolates of *Salmonella* spp. from chicken cuts marketed in Francisco Beltrão, PR

Resistance profiles	Antimicrobial resistance	Classes ¹	ESBL ²	Isolates ³ (%)
P1	NAL (NIT)	2	-	1 (3.5)
P2	NAL, GEN	2	-	1 (3.5)
P3	NAL (AZI), (NIT)	3	-	5 (17.8)
P4	NAL (AZI), (CFL)	3	-	1 (3.5)
P5	NAL, NIT (AZI)	3	-	3 (10.7)
P6	NAL, AZI, NIT (CIP)	3	-	4 (14.2)
P7	NAL, GEN, CRO, CPM (AZI), (NIT)	5	+	1 (3.5)
P8	NAL, AMC, AMP, CFL, CFE, CRO (AZI)	3	-	3 (10.7)
P9	NAL, CFL, NIT (AZI), (CIP)	4	-	1 (3.5)
P10	NAL, GEN, NIT (AZI), (CIP)	4	-	1 (3.5)
P11	NAL, AMC, GEN, AMP, CFL, CFE, CRO (AZI), (CIP)	4	-	1 (3.5)
P12	NAL, AMP, CFL, CFE, NIT, CRO, CPM (AMC), (AZI), (CIP), (GEN)	5	+	1 (3.5)
P13	NAL, AZI, GEN, AMP, CFL, CFE, CRO, CPM (AMC), (NIT).	5	+	1 (3.5)
P14	NAL, GEN, AMP, CFL, CFE, NIT, CRO, CPM (AMC), (AZI)	5	+	1 (3.5)
P15	NAL, SUT, AZI, CFE, NIT (CIP), (CFL)	5	-	1 (3.5)
P16	NAL, AMC, AZI, GEN, AMP, CFL, CFE, NIT, CRO, CPM (CIP)	5	+	1 (3.5)
P17	NAL, GEN, AMP, IPM, CFL, CFE, NIT, CRO, CPM (SUT), (AMC), (AZI), (CIP)	6	+	1 (3.5)

* Profiles in parentheses: strains with intermediate resistance to antimicrobials.

1 Number of classes of antimicrobials to which the isolates were resistant.

2 ESBL phenotypic research using the disk approximation method.

3 Number of isolates that presented a determined resistance profile.

+ Positive result, - Negative result.

NAL- Nalidixic Acid, SUT- Sulfazotrim, AMC- Amoxicillin + Clavulanic Acid, AZI- azithromycin, CIP- Ciprofloxacin, GEN- Gentamicin, AMP- Ampicilin, MER- Meropenem, CFL- Cephalotin, IPM – Imipenem, CFE- Cephalexin, NIT- Nitrofurantoin, CRO- Ceftriaxone, CPM- Cefepime.

Isolation, characterization...

Resistance to beta-lactams was found in 42.9% of the isolates, with resistance to at least one representative of this class. Among the beta-lactams, cephalosporins showed a higher percentage of resistance, including third- and fourth-generation representatives.

For many years, conventional treatment for *Salmonella* spp. was performed using a combination of sulfamethoxazole with trimethoprim and a representative of the penicillin group, such as amoxicillin or ampicillin. However, the high resistance that *Salmonella* strains have shown to penicillin rendered third- and fourth generation cephalosporins more suitable for the treatment of these infections (Sánchez-Vargas *et al.*, 2011). Therefore, the data found in this study causes concern, since the isolates were also resistant to these drugs.

In the present study, multiresistant isolates were found, that is, resistant to three or more different classes of antimicrobials. Multidrug resistance is a worldwide public health problem. In studies carried out in Spain and Iran, a 100% frequency of multidrug-resistant *Salmonella* spp. strains was found present in chicken cuts (Alvarez-Fernández *et al.*, 2012; Fallah *et al.*, 2013). In Turkey, 92.85% of the *Salmonella* spp. exhibited multiresistance profiles (Siriken *et al.*, 2015). In Brazil, these data are also high. A study conducted by ANVISA, which monitors the prevalence and the sensitivity profile to antimicrobials against *Salmonella* spp. and *Enterococcus* spp. isolated from frozen chicken carcasses, revealed that 76.8% of the 250 samples of *Salmonella* spp. analyzed were classified as multiresistant (Brasil, 2012). Later, Lopes *et al.* (2016) reported that 60% of *Salmonella* Typhimurium isolates showed multidrug resistance.

Resistance to several different classes of antimicrobials is a global alert issue, limiting treatment options for these infections, as well as presenting a high potential for dissemination since bacteria have the ability to transfer resistance genes to other pathogenic microorganisms (Iglesias *et al.*, 2017; Fardsanei *et al.*, 2018).

When analyzing the different resistance and multiresistance profiles found in this study, it was observed that resistance to beta-lactams was associated with resistance to drugs in the group of quinolones (NAL) and/or nitrofurans (NIT),

aminoglycosides (GEN), and macrolides (AZI). These multidrug-resistance phenotypes in *Salmonella* spp. have been associated with the presence of plasmids that carry resistance genes to several antimicrobials, such as strains that produce ESBL enzymes, responsible for conferring resistance to beta-lactams, which generally exhibit co-resistance to quinolones and aminoglycosides (Fernandes *et al.*, 2009; Uma *et al.*, 2010). Upon performing phenotypic analysis to detect ESBL, six isolates, corresponding to 21.4% of the total samples, presented the phenotype of this resistance mechanism.

Only a few drugs belonging to the carbapenem class retain their activity against ESBL-producing enterobacteria (Pitout, 2010). The occurrence of a resistance profile that includes imipenem (P17), found in this study, is probably due to carbapenem usage in Brazil as a therapeutic alternative to fight these infections. These data highlight the need for the rational use of carbapenems in order to prevent the emergence of new multiresistant microorganisms.

A study carried out in Brazil, between 2007 and 2011, with 12,582 strains of *Salmonella* spp., indicates the exponential growth of isolates with multidrug-resistant profiles, including third generation cephalosporins (Costa *et al.*, 2013). The increasingly high frequency of these multidrug-resistant strains of *Salmonella* spp. may be related to inappropriate antimicrobial usage in the poultry sector and the spread of antimicrobial resistance genes from one bacterium to another. In order to avoid the spread of resistance, caution and responsibility in antimicrobial use in veterinary and human medicine are necessary, in addition to continuous epidemiological monitoring (Lai *et al.*, 2014).

Knowing the profile of sensitivity and resistance to antimicrobials of *Salmonella* spp. isolated from food intended for human consumption is extremely imperative since it is an important epidemiological marker and provides data that guide therapy in veterinary and human medicine, both of which use these same antimicrobial agents to treat infections (Brasil, 2012).

It is worth mentioning that optimization by the poultry industries in the production chain and guidance to food handlers regarding hygienic-sanitary aspects, combined with the application of

antimicrobial control as growth promoters and prophylactic agents are vital points to avoid the selection and dissemination of resistance to antimicrobials (Lai *et al.*, 2014).

CONCLUSIONS

The strains of *Salmonella* spp. isolated from chicken cuts, found in this study, showed a high level of resistance to antimicrobials of different classes and of last generations. The highest percentage of resistance was obtained for the group of beta-lactams, including the production of ESBL. The presence of *Salmonella* spp. with a multi-resistance profile was also observed, especially co-resistance among beta-lactams, fluoroquinolones, and aminoglycosides. These data serve as a warning due to the ability to transfer resistance from one bacterium to another, generating high-potential dissemination, which places the human treatment of salmonellosis at risk.

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