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Original Article

Molecular and Phenotypic Detection of the Resistance Profile to β-Lactams and Colistin of Salmonella spp. Isolated from Broilers' Litter

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ABSTRACT

This study aimed to analyze 19 isolates of *Salmonella* spp., from broiler litter swabs in the State of São Paulo, by typing and analyzing the detection of resistance genes associated with ESBL's (Extended Spectrum Beta Lactamase), AMPC (C-type cephalosporinases) and carbapenemases by molecular and phenotypic techniques. A PCR microarray platform (Check and Trace by Check-Points) was used to identify the isolated serotype. The isolates were also evaluated for identification of carbapenemase genes, MCR 1-2 (colistin resistance), AmpC (C-type cephalosporins), and ESBLs (β-lactamases resistance). To identify phenotypic antibiotic resistance, the minimal inhibitory concentration (MIC) was evaluated with the antibiotics meropenem, amoxicillin, and ceftriaxone. The most prevalent serotypes identified were *S.* Infantis and *S.* Saintpaul, with a prevalence of 15.07% (3/19). Other strains identified were *S. Cerro, S.* Sandiego*, S.* Kentucky*, S.* Alachua*, S.* Javiana*, S.* Livingstone*, S.* Typhimurium*, S.* Heidelberg*,* non-enteric *Salmonella,* and a *Salmonella* not typifiable by the typing kit. All samples were negative for identifying carba resistance genes, MCR, ESBL, and AmpC. In the phenotypic profile, meropenem was the least resistant, while amoxicillin and ceftriaxone showed a high resistance pattern. The results show that phenotypic resistance is not associated with the presence of resistance genes studied here. In addition, the resistant bacteria found in MIC have resistance mechanisms not associated with the genes studied here. Additional measures must be implemented to prevent the indiscriminate use of antimicrobial agents therapeutically or as growth promoters.

INTRODUCTION

Salmonella infections are still a worldwide concern to public health, considered as one of the most clinically important agents to cause human disease. *Salmonella* strains' genetic makeup allows them to adapt to several environments, making it more difficult to eliminate the bacteria. For years, antimicrobial agents have been used on a large scale in poultry production for therapeutic, prophylactic, and growth promoters, which exert selective pressure on the bacteria, contributing to its resistance (Chantziaras *et al.*, 2014). In particular, the emergence of new multidrug-resistant strains (mainly those resistant to β-lactam antimicrobials from 3rd and 4th generation and to carbapenemases) brings a new challenge in terms of treatment efficiency in infections caused by gram-negative bacteria (Eng *et al.*, 2015). The prevention and control of foodborne diseases have been cited as a challenge, and antimicrobial resistance among foodborne pathogens as a growing problem (Van Seventer & Hamer 2017).

Selective pressure and the presence of resistance genes are the two main factors involved in the development of antibiotic resistance bacteria (Witte, 2000). Genes encoding antimicrobial resistance can be located on the chromosome or on plasmids. Chromosomal DNA is relatively more stable, while plasmid DNA is easily transported from one strain to another by bacterial conjugation, allowing for joint gene transfer, including those of antimicrobial resistance (Witte, 2000).

Among several antimicrobials, resistance to β-lactams and colistin stand out due to their importance for human health. Gene's expression of extended-spectrum β-lactamases (ESBLs), β-lactamases, C-type Cephalosporinase (AmpC), or carbapenemases (carba) are mechanisms that deserve to be emphasized concerning resistance to β-lactams, as are the genes' expression of MCR 1-2 for colistin resistance. Thus, research to detect the presence of resistance genes encoding β-lactamases and colistin in bacteria has been carried out worldwide.

In this context, this work aimed to analyze 19 isolates of *Salmonella* spp., collected from broiler flocks' litter in the State of São Paulo, through the isolates' typification, molecular profile analysis of resistance to β- lactams (ESBLs, AmpC, carba) and colistin (MCR 1-2) as well as identifying the phenotypic resistance profile by determining the minimal inhibitory concentration (MIC) to meropenem, amoxicillin, and ceftriaxone.

MATERIAL AND METHODS

Nineteen *Salmonella* strains were analyzed. The strains were collected from the litter of a 30-day-old broiler flock in the State of São Paulo and isolated in the Biological Institute, located in Descalvado-SP. The strains were randomly chosen within *Salmonella* spp. library isolated from broiler houses in the state of São Paulo between January and July 2020. The strains were not identified as serotypes and molecular serotyping was performed using a PCR microarray platform (Check and Trace, Check-Points). Each position on the microarray represents a specific DNA marker associated with a unique *Salmonella* target sequence. Targets only become visible if DNA markers match exactly the equivalent DNA sequences of the *Salmonella* isolate. The software provided with the test converts these scores to acquainted serotypes.

Isolates were resuspended in BHI broth and incubated in XLD agar for PCR, using the commercial kit Check MDR CT103XL (Check Points B.V., Netherlands), for molecular typing and identification of carba, MCR 1-2, AmpC, and ESBLs genes. DNA was extracted using the commercial kit's reagents Check MDR CT103XL (Check-Points B.V., Netherlands). DNA extraction was performed by using the DNeasy Blood&Tissue Kit (Qiagen) adapted to the supplier's protocol. Initially, the material was a pure colony isolated on TSA agar (Tryptic Soy Agar). In a microtube of 1.5ml, 180µl of ATL buffer and 20µl of proteinase K solution were added. These colonies were resuspended in this solution, homogenized by vortexing, and incubated at 56ºC in a heating block for 1 hour. The following steps were carried out according to the supplier's recommendation. The final eluted volume was diluted to a concentration of 1:5 and used in the working solution. The commercial kit Check MDR CT103XL (Check Points B.V., Netherlands) was used according to the manufacturer's guidelines and consisted of 3 steps: DNA recognition, amplification, and detection. Specific molecular recognition of DNA target sequences and subsequent amplifications were performed using universal primers. The multiplex binding detection reaction generated DNA molecule collections that were further amplified using a single pair of amplimers using PCR. PCR products were then sorted by hybridization to a low-density DNA microarray. Positive hybridization was detected using a biotin marker incorporated into one of the PCR primers. The samples were then inserted into the ATR03 single-channel tube reader after the detection reaction was complete, and the images were acquired and interpreted with software provided by the manufacturer (Check Points, Wageningen, Netherlands), following the protocol described by Cuzon *et al.*, 2012.

For phenotypic antibiotic resistance identification, broth microdilution methodology was used to determine the MIC for three antimicrobial agents (Meropenem, Amoxicillin, and Ceftriaxone). Results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2015).

Salmonella isolates were inoculated onto nutrient agar plates and incubated at 37ºC for 24 hours. Isolated colonies were collected and suspended in sterile saline (0.9%) and diluted to a final concentration of 1 x 10^5 CFU per well (0.5 McFarland Standard) in Mueller Hinton broth. Afterward, different concentrations of meropenem, amoxicillin, and ceftriaxone were added to the wells. Positive and negative controls were added for analysis. The analyzes were performed in triplicate. The plate was incubated at 37°C for 24-48 hours, and the minimum inhibitory concentration was determined for each sample.

RESULTS AND DISCUSSION

Among the 19 samples of *Salmonella* spp., 12 serotypes were identified (Table 1). The most prevalent serotypes were *Salmonella* Infantis (15.7%) and *Salmonella* Saintpaul (15.7%), followed by *Salmonella* Cerro (10.5%), *Salmonella* Sandiego (10.5%), *Salmonella* Kentucky (10.5%), *Salmonella* Alachua (5.2%), *Salmonella* Javiana (5.2%), *Salmonella* Livingstone (5.2%), *Salmonella* Typhimurium (5.2%), *Salmonella* Heidelberg (5.2%), Non-enteric *Salmonella* (5.2%) and *Salmonella* genovar 3076 (5.2%), which consists of a strain whose subspecies is not included in the typing kit database (Table 1).

Salmonellosis is one of the most common human foodborne infections worldwide (Webster, 2009). From January to June 2020, an outbreak of 473 people with this disease was reported in the United States (*S*. Braenderup, *S*. Muenchen, *S*. Thompson, *S*. Typhimurium). Also, between late 2020 and early 2021, other outbreaks affecting around 200 people were reported, involving *S*. Newport, *S*. Thompson, *S*. Enteritidis, *S*. Potsdam, and *S*. Miami strains (Popa & Popa, 2021).

Brazilian data on *Salmonella* human outbreaks are often limited, but a survey of confirmed cases of deaths caused by *Salmonella* recorded in the Notifiable Diseases Information System (NDIS) was carried out

Table 1 – *Salmonella* spp. serotypes. of 19 isolates from broiler litter in the State of São Paulo from Jan to Jun 2020.

Identified serotype	Nº of samples		Nº of resistant (antibiotic) by MIC
	N	%	
Salmonella Infantis	3	15.70% (3/19)	1 (amoxicillin)
Salmonella Saintpaul	3	15.70% (3/19)	1 (amoxicillin)
Salmonella Cerro	2	10.50% (2/19)	1 (ceftriaxone)
Salmonella Sandiego	2	10.50% (2/19)	0
Salmonella Kentucky	2	10.50% (2/19)	0
Salmonella Alachua	1	5.20% (1/19)	0
Salmonella Javiana	1	5.20% (1/19)	0
Salmonella Livingstone	1	5.20% (1/19)	0
Salmonella Typhimurium	1	5.20% (1/19)	1 (ceftriaxone)
Salmonella Heidelberg	1	5.20% (1/19)	1 (amoxicillin and ceftriaxone)
Non-enteric *Salmonella*	1	5.20% (1/19)	0
Salmonella genovar 3076	1	5.20% (1/19)	1 (amoxicillin and ceftriaxone)

from January 1st, 2013, and December 31st, 2017. Death rates were used in accordance with Brazilian regions and age groups between 2013 and 2017 based on the records made by NDIS and the Brazilian Institute of Geography and Statistics (BIGS), with the results: 2013 - 07 deaths, 2014 – 18 deaths, 2015 – 11 deaths, 2016 – 10 deaths, 2017 – 17 deaths (Furquim *et al.*, 2021).

From a global perspective, *Salmonella* Enteritidis has been the most isolated serotype in affected humans, followed by *S.* Typhimurium. On a global scale, besides these two serotypes, there are several others often isolated, such as *Salmonella* Newport (mainly isolated in North and Latin America and Europe), *S.* Infantis (distributed worldwide), *S.* Virchow (mainly recorded in Asia, Europe, and Oceania), *S.* Hadar (frequently in Europe) and *S.* Agona (frequently in North and Latin America and Europe) (Hendriksen *et al.*, 2011).

Hydrolysis of β-lactam antibiotics by β-lactamases is the most common resistance mechanism for this class of antimicrobial agents in clinically important gram-negative bacteria (Bush & Jacoby, 2010). From 19 *Salmonella* samples tested, all were negative for the presence of carba, ESBL, and AmpC resistance genes. In the identification analysis of antibiotic resistance phenotype, no samples showed 100% of resistance or sensitivity to the antimicrobials used (Table 1). The fact that some samples had high resistance to the tested antimicrobials but did not show resistance genes in the molecular analysis can be explained by the different ways in which bacteria develop resistance mechanisms. The detection of β-lactamases has some limitations, such as the presence of other resistance mechanisms in the same microorganism (permeability alterations, for example) and the simultaneous production of other β-lactamases, also occurring hyperproduction of a β-lactamase, phenotypically confusing the microorganism classification. Therefore, these factors can interact, making the phenotypic test reading different (Gralha, 2011).

Some usual resistance mechanisms include the reduction of the intracellular antibiotic concentration by cellular permeability reduction or by antibiotic efflux, which is a mechanism of particular interest

since some of the efflux pumps can expel several classes of antibiotics from the bacterial cell, which may contribute to the emergence of multidrug-resistant (MDR) phenotypes (Paulsen *et al.*, 1996).

Carbapenems resistance in Gram-negative microorganisms may occur due to multiple mechanisms, such as lipopolysaccharides alteration, efflux pumps overexpression, porins loss, mutations in polysaccharide capsule and β-lactamases enzymes (especially carbapenemases) production (Pitout *et al.*, 2015). As the tested genes are associated with this third condition - production of enzymes (β-lactamases) that degrade carbapenems, the resistance found in strains that did not have carba, ESBL, and AmpC genes, may have occurred due to these two previous conditions (either decreased permeability of the outer membrane to antimicrobials by loss or reduced expression of outer membrane proteins, or efflux pumps overexpression, which reduce antimicrobial concentration within the cells). Resistance to meropenem may have occurred due to the presence of another gene associated with the production of the β-lactamase enzyme (which was not tested), such as the Metallo-beta-lactamase carbapenemase (Bertoncheli & Horner, 2008).

In recent work conducted in Egypt, cloacal swabs were collected from commercial broilers to detect *Salmonella*. Isolation rates were 3.4% in clinically healthy birds and 11.1% in birds with diarrhea symptoms. All *Salmonella* isolates belonged to serotypes of public health concern - Typhimurium, Kentucky, and Infantis. For the antibiotic susceptibility test, the disk diffusion test was performed, and from 20 samples, 19 showed resistance to more than one antibiotic, of which 19.04% were ESBL negative, which contained the CMY II gene and was resistant to a cephalosporin (Sabry *et al.*, 2020).

In another study performed in South Korea, resistance mechanisms and molecular characteristics of *Salmonella Virchow* isolates were investigated in feces samples and cattle, pigs, and poultry carcasses collected from 2010 to 2017. Most of the resistant samples (96,4%) were from poultry. All strains which were resistant to extended-spectrum cephalosporins produced CTX-M-15-type ESBL and CMY II-type AmpC β-lactamase, highlighting the urgent importance of biosecurity practices in the poultry industry (Na *et al.*, 2020).

For meropenem, one sample (5.2%) had sensitivity, while others (94.7%) had intermediate resistance. In a recent study in which *Salmonella* spp. from chicken carcasses were analyzed regarding the resistance profile, 100% of the isolates were sensitive to meropenem (Tuon *et al.*, 2014). Similar data were found in a study evaluating *Salmonella* spp. isolated from broiler houses in Paraná, which found 100% of sensitivity to meropenem in *Salmonella* serotypes tested, including serovar Heidelberg (Pandini *et al.*, 2015). In human medicine, meropenem is one of the drugs of choice for initiating empirical treatment in patients with severe infection and with an unknown etiologic agent (Tuon *et al.*, 2014), and the World Health Organization (WHO) recommends the complete restriction of all classes of important antimicrobials in human medicine for use as growth promoters of food-producing animals (BRASIL, 2020). The peculiarities of this antimicrobial and its low availability in poultry may justify the greater sensitivity of the tested strains in this work.

Regarding amoxicillin, 13 samples (68.4%) showed intermediate resistance, and 04 (21.05%) were highly resistant. In a study with isolates of *Salmonella* spp. from a broiler slaughtering plant in the State of São Paulo to evaluate the resistance profile to antimicrobial agents, from a total of 29 samples, 16 (55.2%) were resistant to amoxicillin. Intermediate results were observed, which should be considered resistant since using these antimicrobial drugs as sensitive would only select resistant strains. Nine samples (31.03%) showed intermediate behavior to amoxicillin (Cortez *et al.*, 2006). Galdino *et al.*, 2013 analyzed the resistance profile to different antimicrobials in 18 samples of *Salmonella* spp. from broiler litter flocks in 2009, and the data indicated that the greatest resistance seen was to amoxicillin, with 27.7%. The high resistance profile to amoxicillin can be explained by the wide use of this antimicrobial in poultry. A study concerning the use of drugs in laying poultry in Brazil described the most used antimicrobials. For therapeutic purposes, among several drugs, amoxicillin is mentioned (Brasil, 2006).

For ceftriaxone, 15 samples (78.9%) showed intermediate resistance and 04 samples (21.05%) had high resistance in this work. In a similar study conducted in Cuba, from 28 isolates of *Salmonella* spp., an important number of 06 (21.4%) showed resistance to ceftriaxone, which is the antibiotic of the first choice for the treatment of non-invasive salmonellosis in adults and especially in children (Sonali *et al.*, 2012). Interestingly, ceftriaxone is not used in poultry production, but probably another antibiotic of the same class may be inducing cephalosporin resistance in broiler isolates, such as ceftiofur, a third-generation cephalosporin used in the poultry industry.

Ceftiofur was once commonly administered to day-old chicks, along with Marek's vaccine in commercial hatcheries to prevent disease in broilers (Webster, 2009). The use of ceftiofur in poultry production has also been responsible for increased resistant isolates of E. coli and *Salmonella* Heidelberg in Canada (Dutil *et al.*, 2010). According to Frye & Cray (2007), the genetic element responsible for most of the resistance to ceftiofur in *Salmonella* spp. isolated from animals in the USA appears to be the BlaCMY gene, as they were able to isolate this gene from the plasmids of resistant *Salmonella* spp., thus inferring that the resistance increase is related to the gene passage through the plasmid among the different *Salmonella* serotypes. This same finding was reported in another study, where the nineteen isolates resistant to Ceftiofur carried the BlaCMY gene (Alcaine *et al.*, 2005). However, in a study conducted by Frye & Cray (2007), 17% of resistant strains did not have the BlaCMY gene or any of the other resistant β-lactamases genes detected by PCR, raising a concern that other undetected mechanisms are associated with ceftiofur resistance.

The emergence of bacterial resistance to cephalosporin and fluoroquinolone classes is a major concern as both are widely used in human infection treatments, and resistance to these drugs may result in serious complications to treatments (Hur *et al.*, 2012). The National Antimicrobial Resistance Monitoring System (NARMS) has presented data (from 1996 to 2007) that are more comprehensive, reporting the emergence of non-typhoid *Salmonella* isolates that are resistant to nalidixic acid and ceftriaxone. This phenomenon has increased concern among public health authorities regarding clinical management and infection prevention (Crump *et al.*, 2011).

The antimicrobial resistance effect on bacteria of animal origin has been extensively studied. The focus has been to withdraw drugs for treatments in humans that are being used as growth promoters or as prophylactic drugs in the production of animals' feeding. Bacteria of animal origin can reach the human population in several ways: water sources contamination, contamination at slaughtering, farm effluents, and others. This becomes particularly important with enteric bacteria. Individuals who are most exposed, such as meat industry workers, animal handlers, and veterinarians, tend to have a higher degree of antimicrobial resistance than the general population. However, it is almost impossible to quantify the transfer of this resistance since the same active ingredient may have also been used in humans (Boerlin & White, 2013).

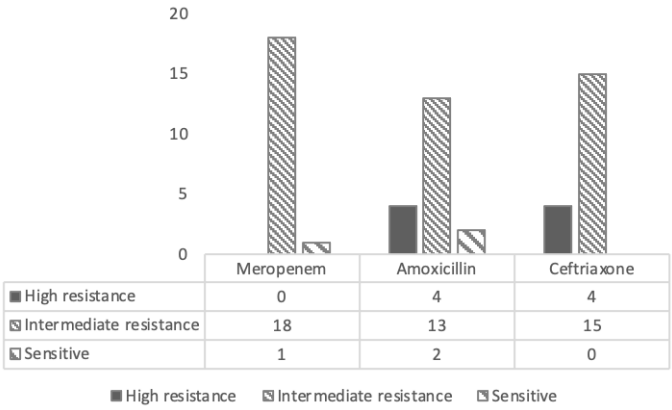

Figure 1 – Number of isolates with resistance, intermediate resistance, and sensitivity to the tested antimicrobial agents.

From these results, the high resistance and intermediate results to amoxicillin and ceftriaxone, and meropenem are a close attention concern for the indiscriminate use of antibiotics in the treatment of infections and in addition to animal feeds, which can contribute to resistant strains selection. The importance of biosecurity prophylactic measures to contain the spread of diseases or prevent the flock's affection is also highlighted.

CONCLUSION

According to the data from this study, the most relevant serotypes were *S*. Infantis, *S*. Heidelberg, and *S*. Typhimurium. Despite strains of *Salmonella* spp. did not show carba, MCR, ESBL, and AmpC resistance genes, some isolates showed a high resistance profile and an intermediate profile by MIC, bringing great concern since there was high resistance to the tested drugs and no expression of resistance genes. This event may have occurred due to resistance processes by spontaneous mutation and selection related to genes not studied in this work. This phenotypic resistance, however, may contribute to the emergence of multidrug-resistant phenotypes.

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