



## Isolation and Antimicrobial Resistance of *Escherichia coli* and *Salmonella enterica* subsp. *enterica* (O:6,8) in Broiler Chickens\*

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### ABSTRACT

**Background:** The third largest poultry flock in Northeast Brazil is located in Ceará State. Some pathogens are commonly disseminated in broiler chicken flocks, such as the bacteria from the Enterobacteriaceae family. Among these, some strains of *Escherichia coli* are frequently associated with different pathological manifestations in domestic animals, while bacteria from the genus *Salmonella* are considered the most frequent enteric pathogens reported causing foodborne infections in humans. Therefore, this study aimed to evaluate the prevalence and antimicrobial resistance of *Salmonella* sp. and *Escherichia coli* strains isolated from broiler chickens in the Metropolitan Region of Fortaleza city, Brazil.

**Materials, Methods & Results:** Samples were collected from July-2014 to March-2015 in ten broiler chicken farms located in the Metropolitan Region of Fortaleza city, Brazil, with birds in pre-slaughter age. From each farm, 100 individual cloacal swabs were randomly collected from broilers independent of clinical status. Distinct methodologies were used in order to provide optimal isolation conditions for both the bacterial species. For *Escherichia coli*, the methodology consisted in enrichment with BHI broth, plating in EMB agar and biochemical identification, after which some isolates were maintained in nutrient agar for antimicrobial resistance evaluation. For the isolation of *Salmonella* sp., a standard method was used with pre-enrichment, selective enrichment, selective plating and biochemical identification steps. Antimicrobial susceptibility test (AST) was performed with disk diffusion technique and the following antibiotics were tested: ampicillin, ceftiofur, ciprofloxacin, trimethoprim-sulfamethoxazole, polymyxin B, gentamycin, cloranfenicol, tetracycline, azithromycin and fosfomicin. According to the methodology used, 95.9% of samples were positive for *Escherichia coli* and the most frequent resistance was to trimethoprim-sulfamethoxazole. *Salmonella* sp. was isolated from 0.2% of the samples, which were identified as the serotype *Salmonella enterica* subsp. *enterica* O:6,8. Both isolates presented the same antimicrobial resistance profile, which were resistant to six, out of ten tested antibiotics (trimethoprim-sulfamethoxazole, tetracycline, ciprofloxacin, azithromycin, chloramphenicol and ceftiofur).

**Discussion:** The low prevalence of *Salmonella* observed in this study have also been reported by other studies performed in poultry farms in Ceará State, which suggests a good status for this pathogen in the local industry, however further efforts in order to eradicate this pathogen must be applied. The salmonella serotype detected in this study is rarely reported in the literature, especially from the poultry industry. In Brazil, the use of tetracyclines, quinolones and penicillins as feed additives or growth promoters is prohibited; however, a high resistance to drugs from these groups was detected. In addition, multidrug resistant *E. coli* isolates presented more elevated rates than other studies reported in the literature with antibiotics commonly used in the poultry industry and this may indicate an excessive use of these drugs in the production routine. These results should serve as a warning for surveillance programs to evaluate the incidence of these microorganisms as well as their antimicrobial resistance rates, which may be an important tool for control and prevention in meat poultry production.

**Keywords:** antibiotic, multidrug resistance, poultry industry.

## INTRODUCTION

Brazil is the third largest producer of poultry meat in the world, close behind of United States and China. However, it is the leading exporter and 69.8% of the total volume of broiler chicken production is destined for domestic market, while 30.2% is exported [35]. The third largest poultry flock in Brazilian North-east region is located in Ceará State, with 28.3 million birds [16].

Some pathogens are easily disseminated in poultry flocks due to the high density of birds in rearing, such as the members of Enterobacteriaceae family, which are distributed worldwide and may be found in soil, water, fruits, vegetables, grains, flowers, trees and animals [15]. *Escherichia coli* is a member of the intestinal microbiota of some birds, however some strains are frequently associated with several pathological manifestations in domestic animals related to environmental conditions and management practice [10,11,17]. Bacteria from the genus *Salmonella* are the most common enteric pathogens associated with foodborne infections originated from poultry products consumed by humans, but also responsible for severe economic losses [14].

Both bacterial species present adaptive capacity that favors persistence in bird flocks and outbreaks recurrence, which enhances the importance of studying the behavior of these microorganisms for public and animal health sake. In this context, bacterial resistance to antibiotics represent a serious problem of clinical and public health concern, since scientific evidence indicate that the use of these drugs in animals destined to human consumption is possibly the main cause of emergence and distribution of resistant strains [1,33]. Therefore, this study aimed to evaluate the prevalence of *Salmonella* sp. and *Escherichia coli* in broiler chickens from the Metropolitan Region of Fortaleza and the antimicrobial resistance of the isolated strains.

## MATERIALS AND METHODS

### *Samples*

Sampling was performed from July-2014 to March-2015 in ten broiler chicken farms located in the Metropolitan Region of Fortaleza city, Brazil, with birds in pre-slaughter age (35 to 38 days). From each farm, 100 individual cloacal swabs were randomly collected, independent of clinical status of the birds.

Samples were transported in microtubes containing 300 µL of Brain Heart Infusion (BHI)<sup>1</sup> broth refrigerated in isothermal box containing recyclable ice and sent to the Laboratory of Ornithological Studies (LABEO) located in the State University of Ceará (UECE) in Fortaleza, Brazil.

### *Bacteriological procedure*

Distinct methodologies were used in order to provide optimal conditions of isolation for both bacterial species. For the isolation of *Escherichia coli*, a previously described methodology [18] was used with modifications. Briefly, after arriving at LABEO, 200 µL of the BHI suspension used for sample transportation was incubated, after which a loopful of the suspension was streaked in plate containing EMB Levine agar<sup>1</sup>. Colonies with morphological characteristics (dark colored colonies with a brilliant sheen) of *E. coli* were selected and submitted to identification with the following biochemical tests: Triple-Sugar-Iron agar (TSI)<sup>2</sup>, Lysine-Iron-Agar (LIA)<sup>2</sup>, Sulfide-Indole-Motility agar (SIM)<sup>2</sup>, Citrate<sup>2</sup>, Malonate<sup>2</sup>, Voges-Proskauer<sup>2</sup> and Methyl Red<sup>2</sup>. Part of the isolates from each farm after biochemical confirmation were maintained in nutrient agar for evaluation of phenotypic antimicrobial resistance.

For the isolation of *Salmonella* sp., recommendations from the Brazilian Ministry of Agriculture, Livestock and Supply (MAPA) [6] were followed with modifications. Briefly, the following procedure was performed: immediately after arriving in LABEO, aliquots of 100 µL were transferred from BHI with samples to microtubes containing 1mL of buffered peptone water<sup>2</sup>. After incubation, 10 µL and 100 µL were respectively transferred to microtubes containing 1mL of the broths Rappaport-Vassiliadis and Selenite-Cystine added 40 µg/mL of Novobiocin<sup>3</sup> and then incubated. Afterwards, samples were streaked in at least two solid selective media: Brilliant Green agar<sup>2</sup> added Novobiocin<sup>3</sup> (40 µg/mL) and Hektoen Enteric agar<sup>2</sup> or *Salmonella-Shigella*<sup>1</sup> agar. After incubation, two to three colonies from each sample with morphological characteristics compatible with *Salmonella* sp. were submitted to biochemical identification with the following tests TSI<sup>2</sup>, LIA<sup>2</sup> and urea broth<sup>4</sup>. In all bacteriological incubation steps, standard temperature was 37°C in bacteriological incubator and the duration was 24 h. Isolates with biochemical profile compatible with *Salmonella* spp. were tested with polyvalent

antiserum O<sup>5</sup> in a rapid slide agglutination test and when positive were sent to the reference Laboratory of Enterobacteria (LABENT) located in FIOCRUZ/RJ for serotype identification.

*Phenotypic antimicrobial resistance*

Antimicrobial susceptibility test (AST) was performed in the isolates with the disk diffusion method [4]. The following antibiotic disks<sup>1</sup> with respective concentrations were used: ampicillin (10 µg), ceftiofur (30 µg), ciprofloxacin (5 µg), trimethoprim-sulfamethoxazole (25 µg), polymyxin B (300 U.I.), gentamycin (10 µg), chloramphenicol (30 µg), tetracycline (30 µg), azithromycin (15 µg) e fosfomicin (200 µg). Inhibition zone diameters were measured and values were interpreted according to manufacturer’s specifications and to standards established by the Clinical and Laboratory Standards Institute (CLSI) [8].

**RESULTS**

According to the methodology used in this study, 95.9% were positive for *Escherichia coli* from a total of 1000 samples analyzed. Antimicrobial susceptibility testing revealed that all *E. coli* strains studied were resistant to trimethoprim-sulfamethoxazole and the following resistance rates were registered:

tetracycline (95.4%), ciprofloxacin (91.4%), ampicillin (87.3%), chloramphenicol (51.1%), azithromycin (48.8%) ceftiofur (42.5%), fosfomicin (33.3%), gentamycin (27.6%) and polymyxin B (1.1%) [Figure 1].

Results showed that 98.3% (171/174) of the tested *E. coli* strains were resistant to three or more antibiotics from different drug groups, while most strains (24.7%) were resistant to five, out of ten tested antimicrobials, and no isolate was susceptible to all of the antibiotics. The maximum number of antibiotics to which a single strain presented resistance was nine, out of ten tested, which occurred in eight isolates (Table 1).

*Salmonella* was isolated from 0.2% (2/1000) of individual cloacal swab samples from a single farm. According to the reference laboratory, the two isolates were identified as *Salmonella enterica* subsp. *enterica* O:6,8 and neither presented flagellar structure. At the time of collection, we observed that some birds were apparently healthy, while others presented respiratory, intestinal and/or locomotor clinical signs and birds from which the *Salmonella* strains were isolated presented some of these symptoms. AST results showed the same antimicrobial resistance profile for both isolates, which were resistant to six, out of ten tested antibiotics: trimethoprim-sulfamethoxazole, tetracycline, ciprofloxacin, azithromycin, chloramphenicol and ceftiofur (Table 2).

**Table 1.** Multi-drug resistance of *Escherichia coli* isolated from broiler chickens.

Number of antibiotics	Resistant <i>E. coli</i> isolates (%)
None	0 (0%)
1	0 (0%)
2	3 (1.7%)
3	17 (9.8%)
4	28 (16.0%)
5	43 (24.7%)
6	39 (22.4%)
7	18 (10.4%)
8	18 (10.4%)
9	8 (4.6%)
10	0 (0%)

**Table 2.** Antimicrobial resistance profiles of *Salmonella enterica* subsp. *enterica* (O:6,8) isolates from cloacal swab samples of broiler chickens.

Isolate	Antibiotics									
	CIP	GEN	AZI	AMP	TET	SUT	CLO	CTF	POL	FOS
<i>Salmonella enterica</i> subsp. <i>enterica</i> (O:6,8)	R	S	R	S	R	R	R	R	S	S
<i>Salmonella enterica</i> subsp. <i>enterica</i> (O:6,8)	R	S	R	S	R	R	R	R	S	S

CIP= ciprofloxacin (5 µg); GEN= gentamycin (10 µg); AZI= azithromycin (15 µg); AMP= ampicillin (10 µg); TET= tetracycline (30 µg); SUT= trimethoprim-sulfamethoxazole (25 µg); CLO= chloramphenicol (30 µg); CTF= ceftiofur (30 µg); POL= polymyxin B (300 UI); FOS= fosfomicin (200 µg); S: susceptible; R: resistant.

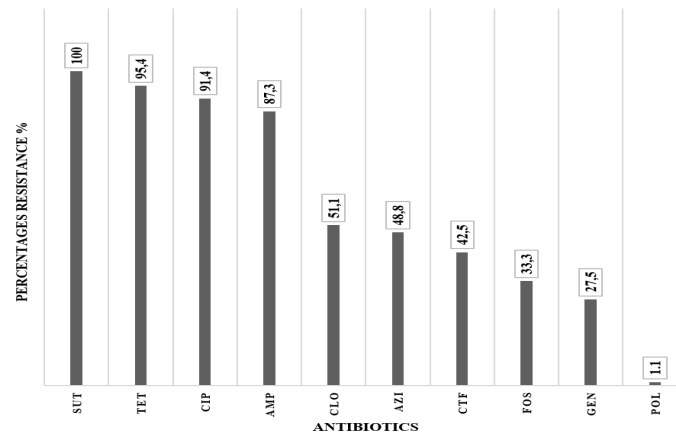


Figure 1. Antimicrobial resistance of *E. coli* isolated from broiler chickens to 10 antibiotics tested individually using disk diffusion method.

## DISCUSSION

A low prevalence of *Salmonella* was detected in this study, which have been reported by other authors in surveys performed in the local poultry industry of Ceará State, where this pathogen was not isolated from the feces of ten broiler chicken flocks [24]. However, a rough strain of *Salmonella enterica* subsp. *enterica* and *Salmonella* Newport were isolated from 32 samples from feces of layer hens, while no strain was isolated from 40 meconium samples of layer chicks, also showing low prevalence rates [31]. Recently, a study revealed that 0.58% out of 510 broiler day-old chicks sampled from hatcheries located in Ceará State were positive for *Salmonella* Enteritidis, which indicates that birds may arrive in farms already infected [2]. The low prevalence of *Salmonella* sp. reported in this study as well as in other studies suggest a good status of this pathogen in the local poultry industry, however further efforts to eradicate this pathogen should be reinforced. There are scarce reports about the serotype of salmonella detected in this study. A single study [26] reported one strain (0.1%) from 207 strains of salmonella isolated from drag swab samples collected from litter of broiler chicken farms in Goiás and São Paulo State, which may indicate that this serotype is rare, possibly emergent, or even it is hard to isolate with conventional methods.

An elevated resistance rate to trimethoprim-sulfamethoxazole (100%) was found in this study; however, a lower result (28%) has been reported in 69 *E. coli* strains isolated from feces of healthy broiler chicken [28]. A study performed with 91 cloacal swab samples from healthy broiler chickens revealed a con-

siderably high resistance rate to this antibiotic (68.1%) [34], which was similar to another report with 86 *E. coli* strains isolated from air sacs of birds with respiratory signs and identified a 66.7% of isolates resistant to this antibiotic association [7]. However, a study performed in Pernambuco State with 16 *E. coli* from cecal content of healthy broiler chickens revealed a low resistance rate to trimethoprim-sulfamethoxazole (43.7%) when compared to the present study, while 13 birds that presented clinical signs of respiratory disease revealed a high resistance rate (84.6%) [3]. In addition, recent studies verified elevated resistance in 25 *E. coli* isolates from cloacal swab samples of healthy broiler chickens to the same antibiotic (84%) [2], unlike what was observed with 60 feces samples of intensive reared broiler chickens, which revealed 45% of resistance to trimethoprim-sulfamethoxazole [19].

The use of tetracyclines, quinolones and penicillins as feed additives or growth promoters in broiler chickens is prohibited in Brazil [6], however this study found high resistance rates to drugs from these groups (tetracycline, ciprofloxacin and ampicillin). Similar findings were recently found in *E. coli* samples from feces of broiler chickens reared in intensive system in the Metropolitan Region of Curitiba with the following rates, respectively 83%, 23% and 100% [19]. A previous study [28] revealed lower resistance rates to tetracycline (48%) and ampicillin (42%) when compared to this study, while another study showed higher values in *E. coli* samples, which 67% were resistant to tetracycline and 84.6% were resistant to ampicillin [34]. In Canada, the antimicrobial resistance profiles of 600 *E. coli* isolates from broiler chickens was identified and high antimicrobial resistance

rate to tetracycline (69.2%) was found, however no resistance to ciprofloxacin was identified [21]. These findings reveal that bacterial resistance to antibiotics should be constantly monitored due to the variation observed during time and in different geographic locations.

Multidrug resistance rates found in *E. coli* samples from this study were higher than other reports, which identified in 65.7% (46/70) of analyzed strains [28] and 81.6% (49/60) in isolates from intensive reared broiler chickens. However, a multidrug resistance rate of 94.2% (33/35) in isolates that were resistant to three or more antibiotics from different groups was reported [3], which is a value closer to the result found in this study. Therefore, studies found in the literature along with this research show worrying rates of resistance to antibiotics commonly used in poultry industry, which may indicate an excessive use of these drugs in the production routine.

As for the resistant *Salmonella*, the low number of isolates in this study may not reflect the real situation in the studied population and recent studies with a higher number of isolates show different results. However, a low resistance rate to tetracycline (30.8%) have been reported in 39 *Salmonella* isolates from broiler farms sampled with drag swabs and none was resistant to ciprofloxacin [25]. Studies with AST in 18 strains of *Salmonella* isolated from drag swabs of poultry in São Paulo State showed 100% susceptibility of serotypes to chloramphenicol and tetracycline, with a low resistance rate to trimethoprim-sulfamethoxazole (11%) [13]. In addition, resistance profile of 53 *Salmonella* isolates from poultry origin to different antibiotics in Goiás State revealed elevated sulfonamide resistance rates (73.6%), while trimethoprim-sulfamethoxazole and tetracycline were low, both with 13.2%, followed by ampicillin and enrofloxacin, both with 5.7% [23]. These authors found higher values of resistance to the antibiotics most commonly used in poultry industry, which was also identified in this study.

Ceftiofur is a third generation cephalosporin, which is a group of drugs used to treat humans with salmonellosis, which is worrying that both *Salmonella* isolates in this study were resistant. However, other studies reported low resistance rates to this antibiotic, such as 15.4% (2/13) in *Salmonella* isolates from cloacal swab samples of broiler chickens [5]. In addition, 250 *Salmonella* isolates from frozen chicken carcasses in fifteen Brazilian cities, including Fortaleza, revealed

that 28% were resistant to ceftiofur [21]. Antimicrobial resistance of salmonella isolates to ceftiofur is constantly reported and public health concern has increased the interested in this type of study [9].

Although MAPA prohibits the use of chloramphenicol since 1998 in Brazil, resistance to this drug in both bacterial species was registered. This finding was reported in a study [28], in which *E. coli* isolated from the intestinal microbiota of chickens in conventional rearing system at different ages presented high resistance rate to chloramphenicol (52%) and the authors related this to the presence of resistance genes present in some plasmids. In another study, the antimicrobial resistance profiles of 19 different *Salmonella* sp. serotypes from broiler chickens in Paraná State was identified and revealed that 51% of serotypes were resistant to at least one of the tested drugs, among which chloramphenicol and gentamycin presented the lowest rates (2.6%) [25].

## CONCLUSION

This study reports the occurrence of multidrug resistant *E. coli* isolates, as well as the existence of multiresistant *Salmonella enterica* subsp. *enterica* (O:6,8) in the intestinal microbiota of broiler chickens in farms located in Fortaleza, Brazil. These results should serve as a warning for surveillance programs to monitor the incidence and antimicrobial resistance of these microorganisms, which may be an important tool for control and prevention in meat poultry production.

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