Amoxicillin / Clavulanic Acid and Cefotaxime Resistance in *Salmonella* Minnesota and *Salmonella* Heidelberg from Broiler Chickens

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Abstract

This study investigated the resistance of various *Salmonella* strains to beta-lactam antibiotics. *Salmonella* Minnesota (36 strains) and *Salmonella* Heidelberg (24 strains) were isolated from broiler chickens and carcasses by the Disk Diffusion Test and resistance genes *bla*CTX-M-8, *bla*ACC-1 and *bla*CMY-2 were detected by PCR. Of the 60 strains tested, 80% were resistant to at least one antibiotic. Specifically, 66.7% were resistant to amoxicillin/clavulanic acid and 75% were resistant to cefotaxime. Among the amoxicillin/clavulanic acid resistant strains, the *bla*CMY-2 gene was detected in 40%, *bla*ACC-1 in 37.5% and *bla*CTX-M-8 in 7.5%. Among the cefotaxime resistant strains, we detected the genes *bla*CTX-M-8 in 13.3%, *bla*ACC-1 in 33.3%, and *bla*CMY-2 in 31.1%. The presence of cefotaxime- and amoxicillin/clavulanic acid-resistant *Salmonella* in poultry, and the prevalence of extended spectrum betalactamases and AmpC-betalactamases in these strains are of huge concern to public health and economy.

Introduction

Europe leads the list of rigorous buyers of imported poultry meat. Through the Rapid Alert System for Food and Feed (RASFF) protocol published by the European Commission on Food Safety and Health, there was a significant increase in notifications regarding poultry products from Brazil during 2013 and 2014 due to the presence of *Salmonella*. In particular, *Salmonella* Heidelberg was the most prevalent serotype in Brazilian chicken meat in 2013 (RASFF, 2014, 2015). Recent studies in Brazil have also shown an increased rate of infection in chickens by *Salmonella* Heidelberg and Minnesota (Cardoso *et al*., 2015; Voss-Rech *et al*., 2015), which was credited to improved measures

controlling for Enteritidis and Typhimurium serotypes, thereby increasing the frequency of other serotypes.

A study conducted in Brazil from 2003-2012 found changes over time in the dynamics of *Salmonella* spp. serotypes isolated from animals (including birds), food, and humans. Specifically, there was a decrease in detection of Enteritidis and Typhimurium serotypes, but an increase (starting in 2008) in Minnesota, Mbandaka, Senftenberg, Agona, Schwarzengrund, Infantis, and Panama serotypes (ANVISA, 2012). Similarly, the Agriculture, Livestock and Food Supply Ministry reported an increase in *Salmonella*

Minnesota percentage in broiler chickens from 0.96% in 2004-2008 to 9.38% in 2009-2010 (Freitas, 2011). Among the *Salmonella* spp. serotypes that can infect humans, Heidelberg seems to be the most invasive and capable of causing diseases with greater severity than other paratyphoid serotypes. Heidelberg is also among the most commonly isolated serotypes in both birds (and humans in Canada and USA), and is one of the five main serotypes associated with human salmonellosis (CDC, 2008; 2014; FDA, 2010; PHAC, 2014).

Consumption of poultry products – mainly chicken meat – has often been associated with salmonellosis (WHO, 2016). *Salmonella* spp. can enter the food chain from animal production and technological processing through cross-contamination and product commercialization. The use of antimicrobials in the majority of human salmonellosis cases is not recommended, but in systemic infections, such as those caused by *Salmonella* Heidelberg, drugs such as third generation cephalosporins are commonly used (Hoffmann *et al.*, 2014). A study conducted in the Netherlands from 1999-2013 years, revealed that among 200 *Salmonella* Heidelberg strains isolated from human infections, animal production, and poultry meat, 23.5% were resistant to extended-spectrum cephalosporins (Liakopoulos *et al.*, 2016). Resistance has alarming implications on food-borne transmission and public health, and has been reported in several countries (Hoffmann *et al.*, 2014; Liakopoulos *et al.*, 2016).

The use of Beta-lactams in animal feed as a growth performance additive is banned in Brazil (Brasil, 2009). Extended-spectrum beta-lactam resistance is usually due to intracellular production of extended spectrum betalactamases (ESBL). The most frequently encountered ESBLs belong to the TEM, SHV and CTX-M groups, in which their encoding genes are found in plasmids (which normally harbor other genes that confer resistance to aminoglycosides, chloramphenicol, sulfonamides, trimethoprim, and tetracycline) (Cánton *et al.*, 2012). CTX-M enzymes are able to hydrolyze third generation cephalosporins (Bonnet, 2008). AmpC-betalactamases are enzymes encoded by genes with a chromosomal origin called *ampC* (Hanson, 2003) and have also been detected in *Salmonella* spp. that lack this chromosomal gene. Enzyme production in this bacterium is mediated by plasmid genes (Pérez-Pérez and Hanson, 2002).

The objective of this work is to characterize antimicrobial resistance of *Salmonella* Minnesota and Heidelberg strains isolated from live chickens and carcasses against amoxicillin/clavulanic acid and cefotaxime. We will do so using the Disk Diffusion Test, followed by the detection of resistance genes using Polymerase Chain Reaction (PCR).

Materials and Methods
Samples
60 *Salmonella enterica* strains (36 Minnesota and 24 Heidelberg serotypes) were studied in isolates from nine live chickens and 51 slaughtered chickens from slaughterhouses with Federal Inspection Service located in the South and West-Center regions of Brazil. Isolation occurred from 2012-2013 according to U.S. FDA Bacteriological Analytical Manual (Hammack *et al.*, 2014). Strains were serotyped at the Enterobacteria Laboratory (Department of Bacteriology) in Oswaldo Cruz Foundation in Rio de Janeiro State (IOC, FIOCRUZ, RJ, Brazil).

Disk Diffusion Test
The susceptibility of samples to antimicrobial agents Amoxicillin/Clavulanic Acid (20/10 µg) and Cefotaxime (30 µg) was evaluated by the Disk Diffusion Test according to previously established methods (Bauer *et al.*, 1966), following resistance parameters provided by CLSI (2013).

Polymerase Chain Reaction (PCR)
PCR was performed, following DNA thermal extraction, for genotypic resistance characterization. Specific primer pairs were used to detect ESBL (which confer resistance to third generation Cephalosporins) and AmpC-betalactamases (which confer resistance to Beta-Lactams, except Carbapenem and are resistant to Clavulanic Acid) (Table 1).

Each reaction contained 29.25 µL of PCR water, 5.0 µL of DMSO, 5.0 µL of 10X Buffer, 2.0 µL 50 mM $MgCl_2$, 2.5 µL of each primer (10 pmol/µL; Invitrogen), 1.0 µL of 10 mM DNTP, 0.25 µL 5 U/µL Taq Polymerase (Ludwig Biotec), and 2.5 µL of DNA (final volume of 50 µL). PCR reactions included denaturation at 94°C for 10 minutes, 35 cycles at 94°C for 30 seconds, 52°C for 30 seconds, and 72°C for 60 seconds, with a final extension at 72°C for 10 minutes (Nadjar *et al.*, 2000; Decré *et al.*, 2002; Jouini *et al.*, 2007). Positive controls used were *Salmonella* Heidelberg (IOC 200/14), and

Klebsiella pneumoniae (CCBH 3589) provided by Enterobacteria Laboratory (Department of

Bacteriology) in Oswaldo Cruz Institute Foundation in Rio de Janeiro State, Brazil.

Table 1. Pair of primers for the study of AmpC-betalactamases and Extended Spectrum Betalactamase (ESBL) in strains of *Salmonella* Minnesota and *Salmonella* Heidelberg

Resistance genes	Primers
*bla*CMY-2-F	5'-ATG ATG AAA AAA TCG TTA TGC-3'
*bla*CMY-2-R	5'-TTG CAG CTT TTC AAG AAT GCG-3'
*bla*ACC-1-F	5'-CAC CGA AGC CGT TAG TTG AT-3'
*bla*ACC-1-R	5'-AAG TGG GTT CGC TGA GTA AA-3'
*bla*CTX-Mgp-8-F	5'-TGA TGA GAC ATC GCG TTA AG-3'
*bla*CTX-Mgp-8-R	5'-TAA CCG TCG GTG ACG ATT TT-3'

*bla*CMY-2 – CMY-2 betalactamase (AmpC-betalactamase group); *bla*ACC-1 – ACC-1 betalactamase (AmpC-betalactamase group); *bla*CTX-Mgp-8 – CTX-M-8 betalactamase (ESBL group); F – Forward; R – Reverse.

Statistical analysis
Data collected were subjected to non-parametric test (Test G: Williams) using Bioestat 5.3 program (Ayres *et al.*, 2007).

Results
We observed resistance to at least one of the two antimicrobials tested in 80% (48/60) of the *Salmonella* strains, with 66.7% (40/60) resistant to Amoxicillin/Clavulanic Acid, and 75% (45/60) resistant to Cefotaxime (Table 2). 23 out of the 40 strains that yielded resistance to Amoxicillin/Clavulanic Acid were Heidelberg

serotype (all isolated from carcasses) while the remaining 17 strains were Minnesota serotype. Of these 17 Minnesota serotypes, four were isolated from live chickens while 13 were from carcasses. 22 out of the 45 *Salmonella* strains resistant to Cefotaxime were Heidelberg serotype (isolated from carcasses) and 23 were Minnesota serotype (six were isolated from live chickens and 17 were from carcasses). Resistance to both antimicrobials was observed in 61.7% (37/60) of the *Salmonella* strains and sensitivity to both antimicrobials was observed in 20% (12/60) of them.

Table 2. Phenotypic profile of *Salmonella* Minnesota and Heidelberg by the Disk Diffusion Test of Amoxicillin/Clavulanic Acid and Cefotaxime

Serotypes*	Sources	AMC R*	CTX R*	AMC E CTX R*	Total of Resistant Samples	Total of Samples
Salmonella Minnesot	Live chickens**	1,6% (1/60)	5% (3/60)	5% (3/60)	7 (11,7%)	9
	Carcasses**	1,6% (1/60)	8,3% (5/60)	20% (12/60)	18 (30%)	27
Salmonella Heidelberg	Carcasses	1,6% (1/60)	0% (0/60)	36,6% (22/60)	23 (38,3%)	24
Total		3	8	37	48 (80%)	60

* Test G: Williams, *P* < 0.05; ** Test G: Williams, *P* > 0.05; AMC: Amoxicillin/Clavulanic Acid; CTX: Cefotaxime; R – Resistant.

With respect to *Salmonella* Minnesota, the resistance profiles were similar between *Salmonella* from live chicken versus carcass. Regarding the resistance of *Salmonella* Minnesota and *Salmonella* Heidelberg to the antimicrobials (Amoxicillin/Clavulanic Acid, and Cefotaxime), there was a significant difference (*P* < 0.05) between the observed proportions, with the highest proportion of resistance found for Amoxicillin/Clavulanic Acid and Cefotaxime together, and the lowest for

Amoxicillin/Clavulanic Acid alone (Table 2).

After PCR was performed, of the 40 amoxicillin/clavulanic acid resistant *Salmonella* strains, *bla*CTX-M-8 gene was detected in 7.5% (3/40), *bla*CMY-2 gene in 40% (16/40) and *bla*ACC-1 gene in 37.5% (15/40) (Table 3). Three *bla*CTX-M-8 positive strains were Minnesota serotype, one isolated from live chicken and two from carcasses. Among *bla*CMY-2 positive strains, two were Heidelberg serotype from carcasses and 14 were Minnesota serotype, with

four from live chicken and ten from carcasses. The *bla*ACC-1 gene was found in 15 strains of *Salmonella* Minnesota, three of which were

isolated from live chickens and 12 isolated from carcasses.

Table 3. Presence of resistance genes using PCR in *Salmonella* Minnesota and Heidelberg strains resistant to Amoxicillin/Clavulanic Acid and/or Cefotaxime (assessed using the Disk Diffusion Test)

Samples	Antimicrobials Resistance genes	Amoxicillin/Clavulanic Acid			Cefotaxime			Amoxicillin/Clavulanic Acid + Cefotaxime*		
		*bla*CMY-2	*bla*ACC-1	*bla*CTX-M-8	*bla*CMY-2	*bla*ACC-1	*bla*CTX-M8	*bla*CMY-2*	*bla*ACC-1*	*bla*CTX-M-8*
Salmonella Minnesota	Live chickens*	100% (1/1)	100% (1/1)	0% (0/1)	0% (0/3)	33,3% (1/3)	66,7% (2/3)	100% (3/3)	66,7% (2/3)	33,3% (1/3)
	Carcasses*	100% (1/1)	100% (1/1)	0% (0/1)	0% (0/5)	20% (1/5)	20% (1/5)	75% (9/12)	91,6% (11/12)	16,6% (2/12)
Salmonella Heidelberg	Carcasses	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/0)	0% (0/0)	0% (0/0)	9,1% (2/22)	0% (0/22)	0% (0/22)
Total		2	2	0	0	2	3	14	13	3

*Test G: Williams, *P* < 0.05; *bla*CMY-2 – CMY-2 betalactamase (AmpC-betalactamase group)*;* *bla*ACC-1 – ACC-1 betalactamase (AmpC-betalactamase group)*;* *bla*CTX-M-8 – CTX-M-8 betalactamase (ESBL group).

Among the 45 Cefotaxime resistant strains, the *bla*CTX-M-8 gene was detected in 13.3% (6/45), *bla*CMY-2 in 31.1% (14/45) and *bla*ACC-1 in 33.3% (15/45). These *bla*CTX-M-8 genes were detected in six Minnesota strains, three of which were isolated from live chickens and three from carcasses. Among *bla*CMY-2 positive strains, two were Heidelberg serotype isolated from carcasses and 12 were Minnesota serotype (three of which were isolated from live chicken and nine from carcasses). The *bla*ACC-1 gene was also found in 15 Minnesota strains resistant to Cefotaxime, three of which were isolated from live chicken and 12 from carcasses.

Considering the antimicrobial with greater resistance rate, a comparison was made between the frequency of detection of the resistance gene according to *Salmonella* Minnesota origin (live chicken vs carcass). We found that the highest frequency was for CMY-2 in live chicken, followed by ACC-1 in carcass, and finally, CTX-M-8 in carcass (Table 3).

Discussion
From all the samples we studied, 80% demonstrated resistance to at least one of the antimicrobials tested. 66.7% were resistant to Amoxicillin/Clavulanic Acid and 75% were

resistant to Cefotaxime. Other studies have found similar resistance frequencies in *Salmonella* spp. strains, such as Cortez *et al.* (2006) who found 72.4% resistance to Cefotaxime and 55.2% to Amoxicillin/Clavulanic Acid from poultry slaughterhouses isolates in São Paulo state in 2003 and 2004. In contrast, Minharro *et al.* (2015) observed 100% Amoxicillin/Clavulanic Acid resistance after analyzing *Salmonella* strains isolated from carcasses, livers, and hearts of slaughtered chickens in Tocantins state in 2011 and 2012. These studies, together with our results, reflect the high rate of resistance to beta-lactams in strains of *Samonella* spp. in Brazil today.

From the 40 Amoxicillin/Clavulanic Acid resistant *Salmonella* strains identified by the Disk Diffusion Test, the *bla*CMY-2 gene was detected in 40% (16/40). The presence of this gene in Amoxicillin/Clavulanic Acid resistant *Salmonella* Heidelberg isolated from chickens or derived products has been reported in Canada and United States, relating it to outbreaks induced by *Salmonella* Heidelberg in humans (Andrysiak *et al.*, 2008; Folster *et al.*, 2011). It was demonstrated, as in our study, that 31.1% of cefotaxime resistant strains and 40% of Amoxicillin/Clavulanic Acid resistant strains

had the *bla*CMY-2 gene, implying the involvement of AmpC betalactamases which promotes resistance to third-generation cephalosporins and Beta-lactam + betalactamases inhibitor (Clavulanic Acid) combinations. It also shows the gene emergence, which may be a consequence of the use of these drugs in poultry farms in different parts of the world. Recently, an increase of extended-spectrum cephalosporins resistance in *Salmonella* Heidelberg in the Netherlands was attributed to frequent occurrence of strains carrying the IncI1/ST12 plasmid encoding *bla*CMY-2 gene in production animals and poultry products imported from Brazil (Liakopoulos *et al.*, 2016). Another gene from the AmpC betalactamase family, *bla*ACC-1, was detected in our study in 35.4% (17/48) of Amoxicillin/Clavulanic Acid and/or Cefotaxime resistant samples. The presence of this gene in samples resistant to Cephalosporins and Penicillins resistant samples has also been reported by other authors in Tunisia and India (Ktari *et al.*, 2009; Gokul *et al.*, 2010).

The CTX-M gene has already been detected in different *Salmonella* spp. serotypes in many countries. In Brazil, Silva *et al.* (2013) analyzed 93 *Salmonella* Schwarzengrund and Agona strains isolated from different stages of the poultry production cycle in 2008 and 2009, and observed the presence of the CTX-M-2 gene in 14% of the samples. Fernandes *et al.* (2009) studied 153 *Salmonella* Typhimurium strains isolated from humans and animals between 2003 and 2004 and detected the presence of the *bla*CTX-M gene in 6.5% of the samples. In general, ESBL confer resistance to third generation Cephalosporins and are inhibited by betalactamase inhibitors, whereas AmpC-betalactamases are not inhibited (Alvarez *et al.*, 2004). This was observed in this study, where almost all isolates with the presence of *bla*ACC-1 and *bla*CMY-2 were resistant to Amoxicillin with Clavulanic Acid, a betalactamase inhibitor. With the increased prevalence of ESBL-producing bacteria, rapid and accurate identification has proved to be increasingly important clinically. Despite the variety of available methods, ESBL identification by conventional phenotypic methods is difficult in routine laboratory tests due to the large number of betalactamases variants in association

to these ESBL with AmpC and in association with metallo-betalactamases or to outer membrane permeability modification (Grimm *et al.*, 2004; Drieux *et al.*, 2008). Thus, genotypic determination has the potential to identify the resistance-causing gene and translate this into clinically useful information that may assist in improving diagnostic practice and salmonellosis treatment.

The detection of *bla*CTX-M-8, *bla*CMY-2, and *bla*ACC-1 resistance genes in *Salmonella* Minnesota and *Salmonella* Heidelberg highlights the diversity of resistance genes against antimicrobials. The possibility of plasmids transmission of these genes to other serotypes or other bacterial species generates a need for more comprehensive studies, considering that the Heidelberg serotype plays an important role in zoonosis.

In this study, samples were obtained from slaughterhouses and poultry farms of south and west-center regions of Brazil, and the *Salmonella* Minnesota serotype corresponded to 60% of the studied strains. Voss-Rech *et al.* (2015) detected this serotype presence in 40.24% of the strains when analyzing 82 *Salmonella* spp. strains isolated from poultry farms within the same regions and showed the highest occurrence of the Minnesota serotype in Mato Grosso do Sul state. Our findings reinforce the prevalence of this serotype in these regions, characterized as centers of poultry export.

Conclusion

The presence of Amoxicillin/Clavulanic Acid and/or Cefotaxime resistant *Salmonella* Heidelberg and *Salmonella* Minnesota in both live chicken and carcass indicates the serotypes' potential as sources of antibacterial resistance transmission, with possible implications both for collective health and the Brazilian poultry export trade. Measures that establish the appropriate use of antibiotics in animal production in an effort to prevent the development of drug-resistant *Salmonella* strains are of vital importance. These efforts can reduce the impact on public health and consolidate the Brazilian market in the international scene by adding value to the product and making it more competitive.

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