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 blaCTX-M-8, blaACC-1 and blaCMY-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 blaCMY-2 gene was detected in 40%, blaACC-1 in 37.5% and blaCTX-M-8 in 7.5%. Among the cefotaxime resistant strains, we detected the genes blaCTX-M-8 in 13.3%, blaACC-1 in 33.3%, and blaCMY-2 in 31.1%. The presence of cefotaxime- and amoxicillin/clavulanic acid-resistant Salmonella in poultry, and the prevalence of extended spectrum beta-lactamases and AmpC-beta-lactamases in these strains are of huge concern to public health and economy.

Keywords
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Salmonella
Resistance
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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...
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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 beta-lactamases (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-beta-lactamases 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-beta-lactamases (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 MgCl₂, 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

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

*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

<table>
<thead>
<tr>
<th>Serotypes*</th>
<th>Sources</th>
<th>AMC R*</th>
<th>CTX R*</th>
<th>AMC E CTX R*</th>
<th>Total of Resistant Samples</th>
<th>Total of Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella Minnesota</em></td>
<td>Live chickens**</td>
<td>1.6% (1/60)</td>
<td>5% (3/60)</td>
<td>5% (3/60)</td>
<td>7 (11.7%)</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Carcasses**</td>
<td>1.6% (1/60)</td>
<td>8.3% (5/60)</td>
<td>20% (12/60)</td>
<td>18 (30%)</td>
<td>27</td>
</tr>
<tr>
<td><em>Salmonella Heidelberg</em></td>
<td>Carcasses</td>
<td>1.6% (1/60)</td>
<td>0% (0/60)</td>
<td>36.6% (22/60)</td>
<td>23 (38.3%)</td>
<td>24</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>3</td>
<td>8</td>
<td>37</td>
<td>48 (80%)</td>
<td>60</td>
</tr>
</tbody>
</table>

* 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
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)

<table>
<thead>
<tr>
<th>Antimicrobials Resistance genes</th>
<th>Amoxicillin/Clavulanic Acid</th>
<th>Cefotaxime</th>
<th>Amoxicillin/Clavulanic Acid + Cefotaxime*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>blaCMY-2 blaACC-1</td>
<td>blaCTX-M-8</td>
<td>blaCMY-2</td>
</tr>
<tr>
<td><strong>Salmonella Minnesota</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Live chickens*</td>
<td>100% (1/1)</td>
<td>100% (1/1)</td>
<td>0% (0/1)</td>
</tr>
<tr>
<td>Carcasses*</td>
<td>100% (1/1)</td>
<td>100% (1/1)</td>
<td>0% (0/1)</td>
</tr>
<tr>
<td><strong>Salmonella Heidelberg</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carcasses</td>
<td>0% (0/1)</td>
<td>0% (0/1)</td>
<td>0% (0/1)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

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

Among the 45 Cefotaxime resistant strains, the *blaCTX-M-8* gene was detected in 13.3% (6/45), *blaCMY-2* in 31.1% (14/45) and *blaACC-1* in 33.3% (15/45). These *blaCTX-M-8* genes were detected in six Minnesota strains, three of which were isolated from live chickens and three from carcasses. Among *blaCMY-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 *blaACC-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 isolated from live chickens and 12 isolated from carcasses.
had the blaCMY-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 blaCMY-2 gene in production animals and poultry products imported from Brazil (Liakopoulos et al., 2016). Another gene from the AmpC betalactamase family, blaACC-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 blaCTX-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 blaACC-1 and blaCMY-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 blaCTX-M-8, blaCMY-2, and blaACC-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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