High occurrence of β-lactamase-producing *Salmonella* Heidelberg from poultry origin

Andrei I. S. Souza\(^1,2\), Mauro M. S. Saraiva\(^3,4,5,6\)*, Monique R. T. Casas\(^3\), Gustavo M. Oliveira\(^1\), Marita V. Cardozo\(^4\), Valdinete P. Benevides\(^1,2\), Fernanda O. Barbosa\(^1,5\), Oliveira C. Freitas Neto\(^6\), Adriana M. Almeida\(^1\), Angelo Berchieri Junior\(^1\)

\(^1\) Department of Veterinary Pathology, Laboratory of Avian Pathology, School of Agricultural and Veterinary Sciences, São Paulo State University (Unesp), Jaboticabal, São Paulo, Brazil, \(^2\) Agricultural and Livestock Microbiology Postgraduation Program, School of Agricultural and Veterinary Sciences, São Paulo State University (Unesp), Jaboticabal, São Paulo, Brazil, \(^3\) Nucleus of Enteric Diseases and Infections by Special Pathogens of the Center for Bacteriology of the Adolfo Lutz Institute, São Paulo, São Paulo, Brazil, \(^4\) Department of Veterinary Pathology, Laboratory of Microbiology, School of Agricultural and Veterinary Sciences, São Paulo State University (Unesp), Jaboticabal, São Paulo, Brazil, \(^5\) Veterinary Medicine Postgraduation Program, School of Agricultural and Veterinary Sciences, São Paulo State University (Unesp), Jaboticabal, São Paulo, Brazil, \(^6\) Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil

* saraiva_ufba@hotmail.com, mauro.saraiva@unesp.br

Abstract

*Salmonella* Heidelberg is commonly reported in foodborne outbreaks around the world, and chickens and poultry products are known as important source of these pathogen. Multidrug-resistant S. Heidelberg strains are disseminated into poultry production chain, which can lead to severe clinical infections in humans and of difficult to treat. This study aimed at evaluating the β-lactam susceptibility and genotypic relatedness of *Salmonella* Heidelberg at Brazilian poultry production chain. Sixty-two S. Heidelberg strains from poultry production chain (poultry, poultry meat and poultry farm) were used. All strains were evaluated to antimicrobial susceptibility by diffusion disk test, as well as β-lactam resistance genes. Genotypic relatedness was assessed by Pulsed-Field Gel Electrophoresis, using *XbaI* restriction enzyme. Forty-one strains were characterized as multidrug-resistant according to phenotype characterization. The resistance susceptibility revealed 31 distinct profiles, with higher prevalence of streptomycin (61/62), nalidixic acid (50/62), tetracycline (43/62) and β-lactam drugs (37/62). *bla\(_{CMY-2}\)* was the more frequent β-lactamase gene found (38/62); other resistance genes found were *bla\(_{CTX-M}\)* (2/62), *bla\(_{SHV}\)* (3/62) and *bla\(_{TEM}\)* (38/62). No carbapenemase genes was found. The Pulsed-Field Gel Electrophoresis showed 58 different profiles. Strains with a larger number of antimicrobial resistance were grouped into ten major clusters apart from others. The spread of resistance by *ampC* continues to rise, thereby turning concern to public health, since the β-lactam antimicrobials are used as a therapeutic treatment in humans.

Introduction

The non-typhoid *Salmonella* (NTS) serovar Heidelberg (SH) is frequently found affecting humans and animals [1–5]. This pathogen has been commonly isolated in food-borne...
outbreaks from humans through consumption of poultry and pork-derived products, as well as dairy products [6]. SH is a pathogen of nonspecific host characterized by has a variety of infection sources and easy bacterial dissemination, due to their antigenic composition [7].

In the last years, SH has been reported causing outbreaks at 13 USA states, which 33 hospitalizations [2], and confirmed as the most frequent serovar involved in human diseases (21.6%) linked to poultry meat consumption (49.9%) [5]. Moreover, the high prevalence of multidrug-resistant (MDR) SH has been identified, including third generation cephalosporins [8–10], critical importance drugs to public health.

The antimicrobial use in animal production is a common practice, but it has a different procedure from different parts of the world. In the United States of America and European Union, the use of antimicrobials is limited to veterinarian prescription, and they should not be used to animal performance purpose. On the other hand, in Brazil some antibiotic groups are allowed to be used in animal production system to treat or to prevent infections or even as growth promoters [11,12]. Veterinary prescription is also required but sometimes failures in the official surveillance of antimicrobial use can lead to misuse. It is known that off-label use of some antimicrobials make a selective pressure which have been associated with quickly increased of bacterial resistance in Enterobacteriaceae species, as E. coli, from farms animals [13,14].

Currently, the class of β-lactam antimicrobials have been widely used to treat serious infections in humans and animals, including third and fourth generations of cephalosporins [15,16]. However, the bacterial resistance to cephalosporins has been found in Salmonella serovars, including Heidelberg from humans [4], poultry [9,17], and poultry meat [5], all of them presenting a diverse MDR pattern. Moreover, strains of MDR SH have been reported in the USA in outbreaks caused by chicken meat [10].

In Enterobacteriaceae species, the enzymatic inhibition is the main β-lactam resistance mechanism found. Both, Extended Spectrum β-Lactamase (ESBL) and Restrict Spectrum β-Lactamase (AmpC) are most common enzymes synthetized by Salmonella spp. [18–21], as well as most frequently found in Enterobacteriaceae isolated from poultry meat [22].

In this scenario, wherein ESBL/AmpC-producing bacteria is not only limited to hospitals and healthcare system but reaches food animals and food chain production [22,23], the spread of resistant Salmonella Heidelberg is a relevant public health issue. Furthermore, in view of the recent concern in the field with the frequent appearance of the this serovar resistant to different antimicrobials, this work aimed to evaluate the β-lactam susceptibility and genotypic relatedness of Salmonella Heidelberg, to provide information on the Brazilian scenario.

Materials and methods

Salmonella Heidelberg isolates

Sixty-two SH isolates were used: 20 from the Avian Pathology Laboratory (FCAV, Unesp Jaboticabal, São Paulo, BR) database and 42 from Adolfo Lutz Institute (IAL, São Paulo, BR) database (S1 Table). All SH isolates were obtained from poultry-relatedness samples, and categorized into three types: Poultry (sampled from cloacal swabs and cecal contents); Poultry farm (sampled from drag swabs, poultry feeders and drinkers); Poultry meat (sampled from product ready to consumption, in nature or processed). All strains were submitted to serovar confirmation by molecular assay using specific primers [24] (S1 File).

Antimicrobial susceptibility testing

All 62 SH strains were submitted to the antimicrobial susceptibility using the disk diffusion test [25] and breakpoints used according to the recommendations of the Clinical and
Laboratory Standards Institute [26]. The antimicrobials used are shown in S2 Table. Strains which presented resistance to three or more antimicrobial drug class used were considered MDR.

**β-lactam resistance genes**

**DNA extraction.** All 62 SH strains were subjected to DNA extraction using the PureLink™ Genomic DNA Kit (K182001, Invitrogen—Thermo Fisher Scientific, USA) following manufacturer’s recommendations. The extracted DNA quantity was evaluated by spectrophotometer, DeNovix DS-11 + (DeNovix Inc., Delaware, USA), in nanogram per microliter (ng/μL).

**Resistance genes.** The presence of β-lactam resistance genes was evaluated by polymerase chain reaction (PCR) using specific primers [27] (S3 Table). The master mix concentrations and amplification conditions used in this study are described in S4 and S5 Tables.

The fragments were visualized from 5 μL of the amplicons along with 1 μL of Loading Dye buffer (Invitrogen, Thermo Fisher Scientific, USA) in the 1.5% agarose gel (Sigma Aldrich, Missouri, USA) stained with Sybr Safe DNA Gel Stain (Invitrogen, Thermo Fisher Scientific, USA). It was submitted to electrophoretic run under the 4 V/cm conditions of the well (Bio-Rad Laboratories, USA) for 45 minutes. Then, the gel was subjected to UV light in a Gel Doc EZ Gel Documentation System (Bio-Rad Laboratories, USA).

**Pulsed-Field Gel Electrophoresis (PFGE)**

PFGE was performed using XbaI (Sigma Aldrich, Missouri, USA) protocol [28]. Then, dendrogram was constructed using the Bionumerics version 7.1 (Applied Maths, Sint-Martens-Latem, Belgium) applying the Unweighted Pair Group Method with Arithmetic Mean method using the Dice coefficient with 1% tolerance and 0.5% of optimization.

**Results and discussion**

**Antimicrobial susceptibility testing**

According to the phenotypic susceptibility test, the prevalence of resistance was observed for streptomycin (S; 98.3%), nalidixic acid (NAL; 80.6%), tetracycline (T; 69.3%), cefotaxime (CTX; 59.7%), ampicillin (AMP; 58.1%), amoxicillin (AMX; 58.1%), cefoxitin (FOX; 56.4%), amoxicillin–clavulanate (AMC; 56.4%) and ceftiofur (CEF; 54.8%). In contrast, the lowest prevalence of resistance was observed for chloramphenicol (C; 1.6%) and imipenem (IMP; 4.8%). No resistance was observed against norfloxacin (NOR), amikacin (AK), gentamicin (GM) and trimethoprim-sulfamethoxazole (SXT) (Table 1).

Based on antimicrobial susceptibility test, 41/62 (66.2%) of SH strains were resistant to three or more drug class and identified as MDR; nineteen (30.6%) of them resistant to five antimicrobial classes of the seven used in this work. These results revealed 31 different resistant profile from all SH, with the most frequent pattern identified were NalFoxCtxAmcTNiTtAxMamp (12.1%) and NalFoxCtxAmcTNiTtAxMamp (11.3%) (S1 Table).

In this study, *Salmonella* Heidelberg showed resistance to some antimicrobials, as C, Nit and T, that are prohibited in Brazil in animal production since early 2000s [29,30]. However, the tetracycline is a commonly antimicrobial used in animals of production. Previous studies have reported resistance to T after it was used as a growth promoter or performance enhancers in food animals [11,31]. The selective pressure by antimicrobial presence on environment favors the exponential resistance spread on gut, oral cavity and feces [32].

It is noteworthy that the prevalence of resistance among the 62 SH in this study was highest to NAL, S and β-lactams class drugs, including cephalosporin group ones. These results lead to
a public health concern, since extended-spectrum cephalosporin (ESC) is indicated as first-line antibiotics for the gastrointestinal infections treatment caused by *Salmonella* spp. in humans [33].

In the present work, we found high occurrence of strains resistant to all β-lactam subclass used, including those combined to β-lactamase inhibitor (AMC). Penicillin associated to adjuvants has been used as alternative to β-lactam resistant bacteria in human infections caused both Gram-positive and Gram-negative species [15]. Furthermore, the drugs inhibitors, as clavulanic acid, acts under penicillinases and cephalosporinases, being used to distinguish ESBL-producing from AmpC-producing isolates [32,34]. In our results, all FOX resistant samples also presented resistance to AMC, suggesting that they are AmpC-producing SH and then confirmed by PCR.

Over to fifty percent of studied SH shown cephalosporin-resistance. These results are in accordance with previous works which reported cephalosporin-resistance in *Salmonella* from food-producing animals and poultry meat, since early 2000s [9,35,36]. Third-generation cephalosporin, as ceftiofur, have been frequently used in day-old chicks together with Marek’s vaccine [16], and it is related to short-term antimicrobial resistance in Enterobacteriaceae [13,14]. Despite that CEF is not a common cephalosporin used in human medicine, previous studies have shown a strict relationship between it and CTX resistance in Enterobacteriaceae [13,37]. We found complete agreement in that CEF and CTX resistance. This result suggests that ceftiofur could be used to access cefotaxime resistance by study model in poultry origin bacteria.

### Table 1. Antimicrobial resistance frequency in phenotypic test of 62 *Salmonella* Heidelberg strains collected from poultry, poultry meat and poultry farms.

| Antimicrobials            | Poultry | Poultry meat | Poultry farms | Total (%) |
|---------------------------|---------|--------------|---------------|-----------|
| Ciprofloxacin             | 0/10    | 0/20         | 1/32          | 1.6% (1/62) |
| Nalidixic acid            | 9/10    | 20/20        | 21/32         | 80.6% (50/62) |
| Enrofloxacin              | 2/10    | 4/20         | 12/32         | 29.1% (18/62) |
| Norfloxacin               | 0/10    | 0/20         | 0/32          | 0         |
| Amikacin                  | 0/10    | 0/20         | 0/32          | 0         |
| Kanamycin                 | 1/10    | 0/20         | 7/32          | 12.9% (8/62) |
| Streptomycin              | 10/10   | 19/20        | 32/32         | 98.3% (61/62) |
| Gentamicin                | 0/10    | 0/20         | 0/32          | 0         |
| Ampicillin                | 8/10    | 17/20        | 11/32         | 58.1% (36/62) |
| Amoxicillin               | 8/10    | 17/20        | 11/32         | 58.1% (36/62) |
| Imipenem                  | 0/10    | 0/20         | 3/32          | 4.8% (3/62) |
| Ceftriaxime               | 8/10    | 15/20        | 11/32         | 54.8% (34/62) |
| Cefotaxime                | 8/10    | 17/20        | 12/32         | 59.7% (37/62) |
| Cefoxitin                 | 6/10    | 17/20        | 12/32         | 56.4% (35/62) |
| Amoxicil-clavulanate      | 8/10    | 17/20        | 10/32         | 56.4% (35/62) |
| Nitrofurantoin            | 10/10   | 9/20         | 8/32          | 43.5% (27/62) |
| Chloramphenicol           | -       | -            | 1/32          | 1.6% (1/62) |
| Tetracycline              | 9/10    | 20/20        | 14/32         | 69.3% (43/62) |
| Sulfamethoxazole-trimethoprim | 0/10 | 0/20       | 0/32          | 0         |

Quinolones and fluoroquinolones [Ciprofloxacin, Nalidixic acid, Enrofloxacin, Norfloxacin]; Aminoglycosides [Amikacin, Kanamycin, Streptomycin, Gentamicin]; β-lactam [Penicillins (Ampicillin, Amoxicillin), Carbapenems (Imipenem), Cephalosporins (Ceftiofur, Cefotaxime, Cefoxitin)]; β-lactam / β-lactamase inhibitor combinations [Amoxicil-clavulanate]; Nitrofurans [Nitrofurantoin]; Phenicols [Chloramphenicol]; Tetracyclines [Tetracycline]; Sulfonamide / Folate pathway inhibitors [Sulfamethoxazole-trimethoprim]

https://doi.org/10.1371/journal.pone.0230676.t001
β-lactam resistance genes

The frequency of ESBL, ampC and Carbapenemase genes in Salmonella Heidelberg from poultry origins are shown in Table 2. Extended spectrum β-lactamase genes were identified in seven of 62 SH strains studied: bl\(\text{a}_{\text{CTX-M}}\), 3.22% (2/62); bl\(\text{a}_{\text{SHV}}\), 4.83% (3/62); bl\(\text{a}_{\text{TEM-1}}\), 3.22% (2/62). ESBL-producing Enterobacteriaceae harboring these genes are frequently found from animal and human origin [5,13,38,39]. None of other studied ESBL genes—bl\(\text{a}_{\text{PSE}}\) and bl\(\text{a}_{\text{OXA-2}}\)—were found in this study. The low prevalence of these determinants has been reported from animal hosts bacteria, whereas from humans, these isolates are often found [19,40].

Differently from ESBL determinants, the bl\(\text{a}_{\text{CMY-2}}\) gene was found in 62.3% (38/62) of SH strains (Table 2). The CMY-2 is related to resistance with second generation cephalosporins and penicillins through the production of β-lactamase AmpC [41] and it has been reported as major determinant of CEF resistance in Enterobacteriaceae from poultry source [42–44]. No others plasmid-mediated ampC genes investigated in our study were found in SH strains, including bl\(\text{a}_{\text{FOX}}\) and bl\(\text{a}_{\text{CMY-1}}\).

Usually, AmpC β-lactamase can occur as both a plasmid-mediated gene and hyperproduction of chromosomal ampC. The last mechanisms has instead of have been reported as the major one from animal origin bacteria [45,46], and it be related to mutations in enzyme regulatory [45]. Although chromosomal AmpC overproduction is the common resistance mechanism in AmpC-producing bacteria, in our results all CEF resistant SH shown plasmid-mediated cephamycinase. The increase of plasmid-mediated bl\(\text{a}_{\text{CMY-2}}\) is associated with the CEF use in animal treatment infections, leading a new selective pressure to bacteria [5,16,35,47,48].

In the present study 36 strains displayed resistant to AMC, all of them presenting bl\(\text{a}_{\text{CMY-2}}\) gene. The β-lactamase inhibitors use associated with β-lactams has confirmed the resistance caused by AmpC production, since the clavulanate acid (inhibitor) acts under the ESBL-producing strains [15] however, not under the AmpC-producing strains.

No carbapenemase coding genes were found, despite three different SH presented resistance against IMP (Table 2). The bl\(\text{a}_{\text{NDM}}\) and bl\(\text{a}_{\text{OXA-48}}\) gene-determinants, tested in this study, have been found in Salmonella enterica from different regions of the world [49–52]. Though no gene was found, we assume that IMP resistance in SH strains studied here, could harbor other carbapenemase group like those belonging the class A, usually found in others Enterobacteriaceae (e.g. Klebsiella pneumoniae and Escherichia coli) [53].

Genotypic relatedness by PFGE

According to PFGE analysis carried from restriction using XbaI, studied SH strains were clustered in ten major groups as shown in Fig 1. Fifty-eight PFGE patterns were found among 62 Salmonella Heidelberg indicating a large diversity of these serovar from poultry sources. Studies in the USA have shown large diversity between Salmonella enterica serovar Heidelberg

Table 2. Resistance genes frequency of 62 Salmonella Heidelberg strains collected from poultry, poultry meat and poultry farms.

| Gene          | Poultry | Poultry meat | Poultry farms | Total (%) |
|---------------|---------|--------------|---------------|-----------|
| ESBL bl\(\text{a}_{\text{CTX-M}}\) | 0/10    | 1/20         | 1/32          | 2 (3.22)  |
| ESBL bl\(\text{a}_{\text{SHV}}\)  | 0/10    | 1/20         | 2/32          | 3 (4.83)  |
| ESBL bl\(\text{a}_{\text{TEM-1}}\) | 1/10    | 0/20         | 1/32          | 2 (3.22)  |
| AmpC bl\(\text{a}_{\text{CMY-2}}\) | 8/10    | 17/20        | 13/32         | 38 (62.3) |
| Carbapenemase bl\(\text{a}_{\text{NDM}}\) | 0/10    | 0/20         | 0/32          | 0         |
| Carbapenemase bl\(\text{a}_{\text{OXA-48}}\) | 0/10    | 0/20         | 0/32          | 0         |

https://doi.org/10.1371/journal.pone.0230676.t002
### PFGE Dendrogram of *Salmonella* Heidelberg Isolates

**Fig 1.** PFGE dendrogram of *Salmonella* Heidelberg isolates collected from poultry, poultry meat and poultry farms.

| Key  | Resistance Profile          | Resistance Genes          | Isolation Source |
|------|----------------------------|---------------------------|------------------|
| SH13 | NalKska                    | blacMY-2 (CIT)            | Poultry farm     |
| SH15 | Nal                         |                          | Poultry farm     |
| SH14 | NalKska                    | blacMY-2 (CIT)            | Poultry farm     |
| SH16 | Nal                         |                          | Poultry farm     |
| SH17 | S                           |                          | Poultry farm     |
| SH18 | Nal                         |                          | Poultry farm     |
| SH44 | Nal Fox Cef Ctx Amc T Nal Amx Amp S | blacMY-2 (CIT) | Poultry farm     |
| SH43 | Nal Fox Cef Ctx Amc T Nal Amx Amp S | blacMY-2 (CIT) | Poultry farm     |
| SH05 | Nal Enr Fox Cef Ctx Amc T Amx Amp S Ka | blacMY-2 (CIT) | Poultry farm     |
| SH04 | Nal Enr Fox Cef Ctx Amc T Amx Amp S | blacMY-2 (CIT) | Poultry farm     |
| SH03 | Nal                         |                          | Poultry farm     |
| SH02 | Nal                         |                          | Poultry farm     |
| SH01 | EnvS                        |                          | Poultry farm     |
| SH45 | Amp Amp S                   |                          | Poultry farm     |
| SH40 | Nal T Amx Amp S             |                          | Poultry meat     |
| SH39 | Nal Enr Fox Cef Ctx Amc T Nal Amx Amp S | blacMY-2 (CIT) | Poultry farm     |
| SH23 | Nal T Nal Amx Amp S         |                          | Poultry farm     |
| SH24 | Nal T Nal S                 |                          | Poultry farm     |
| SH41 | Nal Cip Emb Fox Cef Ctx Amc T S | blacMY-2 (CIT) | Poultry farm     |
| SH42 | Nal Enr Fox Cef Ctx Amc T Amx Amp S | blacMY-2 (CIT) | Poultry farm     |
| SH37 | Nal Enr Amx Amp S           | blacMY-2 (CIT)            | Poultry farm     |
| SH38 | Nal Fox Cef Ctx Amc T Nal S | blacMY-2 (CIT)            | Poultry farm     |
| SH34 | Nal Enr Fox Cef Ctx Amc T Nal Amx Amp S | blacMY-2 (CIT) | Poultry farm     |
| SH21 | Nal T Nal S                 |                          | Poultry farm     |
| SH22 | Nal Fox Cef Ctx Amc T Nal S | blacMY-2 (CIT)            | Poultry farm     |
| SH31 | Nal Cef Ctx Amc T Nal Amx Amp S | blacMY-2 (CIT) | Poultry farm     |
| SH33 | Nal Enr Fox Cef Ctx Amc T Nal Amx Amp S | blacMY-2 (CIT) | Poultry farm     |
| SH32 | Nal Cef Ctx Amc T Nal Amx Amp S | blacMY-2 (CIT) | Poultry farm     |
| SH12 | S                           |                          | Poultry farm     |
| SH27 | Nal Fox Cef Ctx Amc T Amx Amp S | blacMY-2 (CIT) | Poultry meat     |
| SH29 | Nal Fox Cef Ctx Amc T Amx Amp S | blacMY-2 (CIT) | Poultry meat     |
| SH28 | Nal Fox Cef Ctx Amc T Amx Amp S | blacMY-2 (CIT) | Poultry meat     |
| SH26 | Nal Enr Fox Cef Ctx Amc T | blacMY-2 (CIT)            | Poultry meat     |
| SH25 | Nal T Nal S                 |                          | Poultry farm     |
| SH30 | Nal Fox Cef Ctx Amc T Amx Amp S | blacMY-2 (CIT) | Poultry farm     |
| SH11 | S                           |                          | Poultry farm     |
| SH09 | Cef Imp Amx Amp             |                          | Poultry farm     |
| SH10 | Fox Cef T S                 | blacMY-2 (CIT)            | Poultry farm     |
| SH20 | S                           |                          | Poultry farm     |
| SH19 | S                           |                          | Poultry farm     |
| SH46 | Nal Fox Cef Ctx Amc T Nal S Ka | blacMY-2 (CIT) | Poultry farm     |
| SH07 | Nal Enr Fox Cef Ctx Amc T Nal Amx Amp S | blacMY-2 (CIT) | Poultry farm     |
| SH08 | Nal Enr Fox Cef Ctx Imp Amc T Nal Amx Amp S | blacMY-2 (CIT) | Poultry farm     |
| SH59 | Nal Fox Cef Ctx Amc T Nal Amx Amp S | blacMY-2 (CIT) | Poultry meat     |
| SH80 | Nal Fox Cef Ctx Amc T Nal Amx Amp S | blacMY-2 (CIT) | Poultry meat     |
| SH47 | Nal T Nal Amx Amp S         |                          | Poultry farm     |
| SH48 | Nal Fox Cef Ctx Amc T S     |                          | Poultry farm     |
| SH61 | Nal Enr Fox Cef Ctx Amc T Amx Amp S | blacMY-2 (CIT) | Poultry meat     |
| SH62 | Nal Enr Fox Cef Ctx Amc T Amx Amp S | blacMY-2 (CIT) | Poultry meat     |
| SH35 | Amp Amp S                   |                          | Poultry farm     |
| SH36 | Nal Fox Cef Ctx Amc T S     |                          | Poultry farm     |
| SH51 | Nal Fox Cef Ctx Amc T Nal Amx Amp S | blacMY-2 (CIT) | Poultry meat     |
| SH52 | Nal Fox Cef Ctx Amc T Amx Amp S | blacMY-2 (CIT) | Poultry meat     |
| SH53 | Nal Fox Cef Ctx Amc T Nal Amx Amp S | blacMY-2 (CIT) | Poultry meat     |
| SH50 | Nal Fox Cef Ctx Amc T Amx Amp S | blacMY-2 (CIT) | Poultry meat     |

[https://doi.org/10.1371/journal.pone.0230676.g001](https://doi.org/10.1371/journal.pone.0230676.g001)
from food-producing animals [54]. Despite ours results shown high heterogeneity between isolates from different poultry sources (poultry, poultry meat and poultry farm), study with Heidelberg serovar from turkey-associated sources reveled extensive similarity between those isolates [55].

Comparing PFGE with antimicrobial resistance results (phenotype and genotype ones), no relatedness between PFGE and resistance profiles. We assumed that these findings were due genetic diversity among SH isolates from poultry sources. Similarly, Lynne et al found large genetic diversity work with this serovar from cattle and swine sources [54].

Conclusions

Our results showed high resistance characterized by both phenotypes and genotypes being related to AmpC. Strains resistant to a larger number of antimicrobials were also grouped into ten major clusters by PFGE. The present study reinforces that Salmonella Heidelberg from poultry origin is a serious hazard to public health that can act as foodborne pathogen. Therefore, measures to reduce antimicrobial resistance and to control Salmonella Heidelberg should be seriously addressed by the Brazilian poultry sector.

Supporting information

S1 Table. Information of the Salmonella Heidelberg strains used, including the antimicrobial susceptibility profile.
(DOC)

S2 Table. Class, concentrations and abbreviations of each antimicrobial drug used to disk diffusion test in this study.
(DOCX)

S3 Table. Primers sequences to evaluate the presence or absence of the β-lactamase resistance gene.
(DOC)

S4 Table. PCR master mix of β-lactam resistance genes.
(DOC)

S5 Table. Amplification conditions of β-lactam resistance genes.
(DOC)

S1 File. Master mix and PCR conditions to SH identification.
(DOC)

Acknowledgments

We are also grateful to the Avian Pathology Laboratory team (Unesp), for their technical support, even as to Adolfo Lutz Institute for allowing us to work with the SH strains, to Microbiology Laboratory (Unesp) in analysis of PFGE and the last, but not least, to the Biochemistry of Microorganisms and Plants Laboratory (Unesp) for allowing the use of Bionumerics software.

Author Contributions

Conceptualization: Andrei I. S. Souza, Mauro M. S. Saraiva, Oliveiro C. Freitas Neto, Angelo Berchieri Junior.

Data curation: Andrei I. S. Souza, Mauro M. S. Saraiva.
Formal analysis: Andrei I. S. Souza, Mauro M. S. Saraiva, Marita V. Cardozo.

Investigation: Andrei I. S. Souza, Mauro M. S. Saraiva.

Methodology: Andrei I. S. Souza, Mauro M. S. Saraiva, Gustavo M. Oliveira, Marita V. Cardozo, Valdine P. Benevides, Fernanda O. Barbosa, Adriana M. Almeida.

Project administration: Andrei I. S. Souza, Mauro M. S. Saraiva, Angelo Berchieri Junior.

Resources: Monique R. T. Casas, Angelo Berchieri Junior.

Supervision: Mauro M. S. Saraiva.

Writing – original draft: Andrei I. S. Souza, Mauro M. S. Saraiva, Fernanda O. Barbosa, Oliveira C. Freitas Neto, Angelo Berchieri Junior.

Writing – review & editing: Mauro M. S. Saraiva, Oliveira C. Freitas Neto, Angelo Berchieri Junior.

References

1. Glen LM, Lindsey RL, Folster JP, Pecic G, Boerlin P, Gilmour MW, et al. Antimicrobial Resistance genes in Multidrug-Resistant *Salmonella enterica* Isolated from Animals, Retail meats, and Humans in the United States and Canada. Microb Drug Resist. 2013.

2. (CDC) Centers for Disease Control and Prevention. Multistate Outbreak of *Salmonella Heidelberg* Infections Linked to Chicken (Final Update). 2013a. https://www.cdc.gov/salmonella/heidelberg-02-13/index.html.

3. Folster JP, Pecic G, Singh B, Duval B, Rickert R, Ayers S, et al. Characterization of Extended-Spectrum Cephalexin-Resistant *Salmonella enterica* Serovar Heidelberg Isolated from Food Animals, Retail Meat, and Humans in the United States. Foodborne Pathog Dis. 2012. https://doi.org/10.1089/fpd.2012.1130 PMID: 22755514

4. Patchanee P, Zewde BM, Tadesse DA, Hoet A, Gebreyes WA. Characterization of multidrug-resistant *Salmonella enterica* serovar Heidelberg isolated from humans and animals. Foodborne Pathog Dis. 2008. https://doi.org/10.1089/fpd.2008.0149 PMID: 18991546

5. Zhao S, White DG, Friedman SL, Glenn A, Blickenstaff K, Ayers SL, et al. Antimicrobial resistance in *Salmonella enterica* serovar Heidelberg isolates from retail meats, including poultry, from 2002 to 2006. Appl Environ Microbiol. 2008. https://doi.org/10.1128/AEM.01249-08 PMID: 18757574

6. (EFSA) European Food Safety Authority. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. EFSA Journal. 2017. https://doi.org/10.2903/j.efsa.2017.5077

7. Butaye P, Michael GB, Schwarz S, Barrett TJ, Brisabois A, White DG. The clonal spread of multidrug-resistant non-typhi *Salmonella* serotypes. Microbes Infect. 2006. https://doi.org/10.1016/j.micinf.2005.12.020 PMID: 16714135

8. Medeiros MA, Oliveira DC, Rodrigues DP, Freitas DR. Prevalence and antimicrobial resistance of *Salmonella* in chicken carcasses at retail in 15 Brazilian cities. Rev Panam Salud Publica. 2011; 30(6): 555–560. https://doi.org/10.1590/s1020-498920110001200010 PMID: 22358402

9. Campos J, Mourão J, Silveira L, Saraiva M, Correia CB, Maçãs AP, et al. Imported poultry meat as a source of extended-spectrum cephalexin-resistant CMY-2-producing *Salmonella* Heidelberg and *Salmonella* Minnesota in the European Union, 2014–2015. Int J Antimicrob Agents. 2018. https://doi.org/10.1016/j.ijantimicag.2017.09.006 PMID: 28919197

10. (CDC) Centers for Disease Control and Prevention. Multistate Outbreak of Multidrug-Resistant *Salmonella Heidelberg* Infections Linked to Foster Farms Brand Chicken (Final Update). 2013a. https://www.cdc.gov/salmonella/heidelberg-10-13/advice-consumers.html.

11. Antunes P, Mourão J, Campos J, Peixe L. Salmonellosis: the role of poultry meat. Clin Microbiol Infect. 2016. https://doi.org/10.1016/j.cmi.2015.12.004 PMID: 26708671

12. Van Boeckel TP, Brower C, Gilbert M, Grenfell BT, Levin SA, Robinson TP, et al. Global trends in antimicrobial use in food animals. Proc Natl Acad Sci U S A. 2015. https://doi.org/10.1073/pnas.1503141112 PMID: 25792457

13. Saraiva MMS, Moreira Filho ALB, Freitas Neto OC, Silva NMV, Givisiez PEN, Gebreyes WA, et al. Off-label use of cefotin in one-day chicks triggers a short-term increase of ESBL-producing *E. coli* in the gut. PLoS One. 2018. https://doi.org/10.1371/journal.pone.0203156 PMID: 30204766
14. Gibbons JF, Boland F, Egan J, Fanning S, Markey BK, Leonard FC. Antimicrobial Resistance of Faecal Escherichia coli isolates from Pig Farms with Different Durations of In-feed Antimicrobial Use. Zoonoses Public Health. 2016. https://doi.org/10.1111/zph.12225 PMID: 2635644
15. Bush K, Bradford PA. β-Lactams and β-Lactamase Inhibitors: An Overview. Cold Spring Harb Perspect Med. 2016. https://doi.org/10.1101/cshperspect.a025247 PMID: 27329032
16. Landoni MF, Albarellol G. The use of antimicrobial agents in broiler chickens. Vet J. 2015. https://doi.org/10.1016/j.tvjl.2015.04.016 PMID: 25981931
17. Baron S, Jouy E, Larvor E, Eono F, Bougeard S, Kempf I. Impact of third-generation-cephalosporin administration in hatcheries on faecal Escherichia coli antimicrobial resistance in broilers and layers. Antimicrob Agents Chemother. 2014. https://doi.org/10.1128/AAC.03106-14 PMID: 24982086
18. Arlet G, Barrett TJ, Butaye P, Cloeckaert A, Mulvey MR, White DG. Salmonella resistant to extended-spectrum cephalosporins: prevalence and epidemiology. Microbes Infect. 2006. https://doi.org/10.1016/j.micinf.2005.12.029 PMID: 16714134
19. Miriagou V, Tassios PT, Legakis NJ, Tzouvelekis LS. Expanded-spectrum cephalosporin resistance in non-typhoid Salmonella. Int J Antimicrob Agents. 2004. https://doi.org/10.1016/j.ijantimicag.2004.03.006 PMID: 15194124
20. Jeon HY, Seo KW, Kim YB, Kim DK, Kim SW, Lee YJ. Characteristics of third-generation-cephalosporin-resistant Salmonella from retail chicken meat produced by integrated broiler operations. Poult Sci. 2018. https://doi.org/10.3382/ps/pey514 PMID: 30535173
21. Fitch FM, Carmo-Rodrigues MS, Oliveira VG, Gaspari MV, Dos Santos A, Freitas JB, et al. β-Lactam Resistance Genes: Characterization, Epidemiology, and First Detection of blaCTX-M-1 and blaCTX-M-14 in Salmonella spp. Isolated from Poultry in Brazil-Brazil Ministry of Agriculture’s Pathogen Reduction Program. Microb Drug Resist. 2015. https://doi.org/10.1089/mdr.2015.0143 PMID: 26380894
22. Daehre K, Projahn M, Semmler T, Roesler U, Friese A. Extended-Spectrum β-Lactamase-/AmpC Beta-Lactamase-Producing Enterobacteriaceae in Broiler Farms: Transmission Dynamics at Farm Level. Microbial Drug Resistance. 2017. https://doi.org/10.1016/j.mdr.2017.0150 PMID: 28981392
23. (ECDC) European Centre for Disease Preventions and Control. Surveillance of antimicrobial resistance in Europe—Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) 2017. ECDC. 2018.
24. Park SH, Ricke SC. Development of multiplex PCR assay for simultaneous detection of Salmonella genus, Salmonella subspecies I, Salm. Enteritidis, Salm. Heidelberg and Salm. Typhimurium. J Appl Microbiol. 2015. https://doi.org/10.1111/jam.12676 PMID: 25358641
25. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. Am J Clin Pathol. 1966; 45(4): 493–496. PMID: 5325707
26. CLSI. Performance standards for antimicrobial susceptibility testing: 27 ed. CLSI supplement M100. Wayne, PA: Clinical and Laboratory Standards Institute, 2017.
27. Carlson SA, Bolton LF, Briggs CE, Hurd HS, Sharma VK, Fedorka-Cray PJ, et al. Detection of multiresistant Salmonella typhimurium DT104 using multiplex and fluorogenic PCR. Mol Cell Probes. 1999. https://doi.org/10.1006/mcpr.1999.0240 PMID: 10369747
28. Ribot EM, Fair MA, Gautam R, Cameron DN, Hunter SB, Swaminathan B, et al. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of Escherichia coli O157:H7, Salmonella, and Shigella for PulseNet. Foodborne Pathog Dis. 2006. https://doi.org/10.1089/fpd.2006.3.59 PMID: 16602980
29. (Brazil) Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa n° 09, 27 de Junho de 2003. 2003. http://www.agricultura.gov.br/assinato/insumos-agropecuarios/insumos-pecuarios/alimentacao-animais/arquivos-alimentacao-animais/legislacao/instrucao-normativa-no-9-de-27-de-junho-de-2003.pdf/view
30. (Brazil) Ministério da Agricultura, Pecuária e Abastecimento. Instrução Normativa n° 26, 09 de Julho de 2009 (Portaria n° 193/1998). 2009. http://www.agricultura.gov.br/assinato/insumos-agropecuarios/insumos-pecuarios/alimentacao-animais/arquivos-alimentacao-animais/legislacao/instrucao-normativa-no-26-de-9-de-julho-de-2009.pdf/view.
31. Chopra I, Roberts M. Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. J Vet Diagn Invest. 2001. https://doi.org/10.1128/MMBR.65.2.232-280.2001
32. Van Hoek AH, Mevius D, Guerra B, Mulany P, Roberts AP, Aarts HJ. Acquired antibiotic resistance genes: an overview. Front Microbiol. 2011. https://doi.org/10.3389/fmicb.2011.00203 PMID: 22046172
33. Abou-Shaabab M, Ali AA, Rao PGM, Majid A. Drug utilization review of cephalosporins in a secondary care hospital in United Arab Emirates. International Journal of Clinical Pharmacy. 2016. https://doi.org/10.1007/s11996-016-0392-4 PMID: 27817172

34. Wright GD. Antibiotic Adjuvants: Rescuing Antibiotics from Resistance. Trends Microbiol. 2016. https://doi.org/10.1016/j.tim.2016.07.008 PMID: 27522372

35. Duttil L, Irwin R, Finley R, Ng LK, Avery B, Boerlin P, et al. Ceftiofur resistance in Salmonella enterica serovar Heidelberg from chicken meat and humans, Canada. Emerg Infect Dis. 2010. https://doi.org/10.3201/eid1601.090729 PMID: 20031042

36. Liakopoulos A, Geurts Y, Dierikx CM, Brouwer MS, Kant A, Wit B, et al. Extended-Spectrum Cephalosporin-Resistant Salmonella enterica serovar Heidelberg Strains, the Netherlands. Emerg Infect Dis. 2016. https://doi.org/10.3201/eid2207.151377 PMID: 27314180

37. Hiki M, Shimizu Y, Kawanishi M, Ozawa M, Abo H, Kojima A, et al. Evaluation of the relationship between the minimum inhibitory concentration of ceftiofur and third-generation cephalosporins in Escherichia coli isolates from food-producing animals. J Vet Diagn Invest. 2017. https://doi.org/10.1177/1040638717713794 PMID: 28613139

38. Fernandez AS, Paterson DL, Ghiardi-Rodrigues AC, Adams-Haduch JM, Tavechio AT, Doi Y. CTX-M-2-Producing Salmonella Typhimurium Isolated from Pediatric Patients and Poultry in Brazil. Microbial Drug Resistance. 2009.

39. Djeffal S, Bakour S, Murname B, Elground R, Agabou A, Chabou S, et al. Prevalence and clonal relationship of ESBL-producing Salmonella strains from humans and poultry in northeastern Algeria. BMC Vet Res. 2017. https://doi.org/10.1186/s12917-017-1050-3 PMID: 28506272

40. Giuriatti J, Stefani LM, Brisola MC, Crecencio RB, Bittner DS, Faria GA. Salmonella Heidelberg: Genetic profile of its antimicrobial resistance related to extended spectrum β-lactamases (ESBLs). Microb Pathog. 2017. https://doi.org/10.1016/j.micpath.2017.05.040 PMID: 28578094

41. Clothier KA, Byrne BA. Phenotypic and Genotypic Characterization of Animal-Source Salmonella Heidelberg Isolates. J Vet Med. 2016. https://doi.org/10.1155/2016/6380890 PMID: 26881274

42. Tiba-Casas MR, Camargo CH, Soares FB, Doi Y, Fernandes SP. Emergence of CMY-2-Producing Salmonella Heidelberg Associated with IncI1 Plasmids Isolated from Poultry in Brazil. Microb Resist. 2018. https://doi.org/10.1089/mdr.2018.0044 PMID: 30256175

43. Martin LC, Weir EK, Poppe C, Reid-Smith RJ, Boerlin P. Characterization of blaCMY-2 Plasmids in Salmonella and Escherichia coli Isolates from food Animals in Canada. Appl Environ Microbiol. 2011. https://doi.org/10.1128/AEM.06498-11 PMID: 22156427

44. Heider LC, Hoet AE, Wittum TE, Khaita ML, Love BC, Huston CL, et al. Genetic and Phenotypic Characterization of the blaCMY Gene from Escherichia coli and Salmonella enterica Isolated from Food-Producing Animals, Humans, the Environment, and Retail Meat. Foodborne Pathog and Dis. 2009.

45. Bush K, Jacoby GA. Updated functional classification of beta-lactamases. Antimicrob Agents Chemother. 2010. https://doi.org/10.1128/AAC.01009-09 PMID: 19995920

46. Jacoby GA. AmpC β-Lactamases. Clin Microbiol Rev. 2009. https://doi.org/10.1128/CMR.00036-08 PMID: 19136439

47. Aarestrup FM, Hasman H, Olsen I, Sørensen G. International spread of blaCMY-2-mediated cephalosporin resistance in a multiresistant Salmonella enterica serovar Heidelberg isolate stemming from the importation of a boar by Denmark from Canada. Antimicrob Agents Chemother. 2004. https://doi.org/10.1128/aaac4.8.1916-1917.2004

48. Carattoli A. Animal reservoirs for extended spectrum beta-lactamase producers. Clin Microbiol Infect. 2008. https://doi.org/10.1111/j.1469-0691.2007.01851.x PMID: 18154535

49. Fischer J, Schmoger S, Jahn S, Helmuth R, Guerra B. NDM-1 carbapenemase-producing Salmonella enterica subsp. enterica serovar Corvallis isolated from a wild bird in Germany. 2013. Journal of Antimicrobial Chemotherapy. https://doi.org/10.1093/jac/dkt260 PMID: 23818284

50. Day MR, Meunier D, Doumith M, de Pinna E, Woodford N, Hopkins KL. Carbapenemase-producing Salmonella enterica isolates in the UK. 2015. Journal of Antimicrobial Chemotherapy. https://doi.org/10.1093/jac/dkt075 PMID: 25795771

51. Ktari S, Le Héloïs S, Ksibi B, Courdavault L, Mnif B, Maalej S, et al. Carbapenemase-producing Salmonella enterica serotype Kentucky ST198, North Africa. 2015. Journal of Antimicrobial Chemotherapy. https://doi.org/10.1093/jac/dkv276 PMID: 26377865

52. Mairi A, Pantel A, Sotto A, Lavigne JP, Touati A. OXA-48-like carbapenemases producing Enterobacteriaceae in different niches. 2018. European Journal of Clinical Microbiology & Infectious Diseases. https://doi.org/10.1007/s10096-017-3112-7 PMID: 28990132

53. Codjoe F, Donkor E. Carbapenem resistance: a review. 2018. Medical Sciences.
54. Lynne AM, Kaldhone P, David D, White DG, Foley SL. Characterization of antimicrobial resistance in Salmonella enterica serotype Heidelberg isolated from food animals. 2009. Foodborne Pathogens and Disease.

55. Kaldhone P, Nayak R, Lynne AM, David DE, McDermott PF, Logue CM, et al. Characterization of Salmonella enterica serovar Heidelberg from turkey-associated sources. 2008. Applied and Environmental Microbiology.