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# Distribution, diversity, virulence genotypes and antibiotic resistance for *Salmonella* isolated from a Brazilian pork production chain

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ABSTRACT

Pigs infected with *Salmonella* are an important source of contamination at slaughterhouses. We characterized the distribution, virulence genotypes and antimicrobial-resistance phenotypes for *Salmonella* isolates that were collected from different stages of a pork production chain. Each of ten pig lots were sampled for feed (n = 10), water (n = 10), barn floor (n = 10), lairage floor (n = 10), mesenteric lymph nodes (n = 100), tonsils (n = 100), processing environment (n = 120), pork cuts (n = 40) and carcasses after bleeding (n = 100), after singeing (n = 100), after evisceration (n = 100), and after final rinsing (n = 100). *Salmonella* was isolated according to ISO 6579, and after confirmation the isolates were subjected to serogrouping, macro-restriction digests and pulsed-field gel electrophoresis (PFGE), detection of virulence-related genes and antimicrobial-resistance phenotyping. *Salmonella* was recovered from barn floors from 3 pig farms (3/10), lairage floors (7/10), carcasses after bleeding (2/100) and final washing (1/100), palatine tonsils (45/100), mesenteric lymph nodes (43/100), utensils (3/120) and cuts (4/40). The most prevalent serogroup was O: 4 (82%) followed by O:3 (7.7%); O:9 (5.1%); O:8 (2.6%) and O:7 (2.6%). Recovered strains (n = 109) were classified into 24 different pulsotypes (*XbaI* restriction digest), which were arranged into five different clusters. Fourteen different virulence genotypes were observed based on 15 loci, and all isolates were positive for *invA*, *sitC*, *pagC* and *tolC*. There was a high prevalence of antimicrobial resistance against streptomycin (90.5%), tetracycline (88.1%), ampicillin (81.0%), chloramphenicol (71.4%), and ciprofloxacin (50.0%). No strain was resistant to ertapenem, meropenem or kanamycin. A majority (80.9%) of isolates were considered multidrug resistant (resistant to ≥3 antibiotic classes). This study provides valuable insight about the epidemiology of *Salmonella* in swine production, and despite the low presence of this pathogen in carcasses and meat cuts, the majority of isolates was multidrug resistant.

## 1. Introduction

Nontyphoidal *Salmonella enterica* is a common cause of foodborne disease outbreaks in Brazil and other countries (Brasil, 2016; EFSA and ECDC, 2016). Pork products are commonly linked to these outbreaks (EFSA and ECDC, 2016). Once pigs become asymptomatic carriers of *Salmonella*, there is an increased probabililty that food products will be contaminated during slaughter and processing (Argüello et al., 2013) with widespread contamination potentially occurring earlier during production, transport, and lairage (Argüello et al., 2013; Simons et al., 2016). The prevalence of *Salmonella* on carcasses reportedly varies between 1.5% and 24% depending on the country and the methods used during surveillance (Bohaychuk et al., 2011; Cabral et al., 2017; Kich et al., 2011; Marier et al., 2014; Pesciaroli et al., 2017).

The widespread use of antibiotics in different steps of swine production can favor resistant strains of bacteria including *Salmonella* (ECDC et al., 2017; Lopes et al., 2015). It is further likely that use of different antibiotics will select for emergence of multidrug-resistant strains, and the spread of the resistance is facilitated by mobile elements (Bennett, 2008). The emergence of quinolone resistance is of particular concern because ciprofloxacin is an important antibiotic for treating sepsis, particularly for pediatric cases (Kuang et al., 2018). In Brazil previous studies reported 24% of *Salmonella* prevalence in pork carcasses and the high rate of antimicrobial resistance was described in

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strains isolated from pork production (Kich et al., 2011; Lopes et al., 2015). The official guidelines for swine slaughtering in Brazil has recently changed, being based in a risk analysis approach and focusing on microbial hazards, such as *Salmonella* (Brasil, 2018). Because of the importance of *Salmonella* in swine production, this study aimed to characterize the distribution, diversity, virulence genotypes and antibiotic resistance of this pathogen at different stages of pork production.

## 2. Material and methods

### *2.1. Sampling*

Ten sampling locations were selected at different steps of pork production. Finishing farms were selected based on the existence of an intensive breeding system with a similar number of pigs in all the farms (average 1000), and the same company provided the piglets and feed. All selected pig farms (n = 10) sent stock to the same slaughterhouse (n = 1), which was inspected by the Brazilian Ministry of Agriculture, following the Brazilian standards of production, quality and safety. At pig farms, the following samples were collected: 200 g of feed from the top of feeder pigs (n = 10), 200 mL of water from the bite drinkers (n = 10), barn floor [n = 10, sampled by footprint as described by Botteldoorn et al., 2003]. After transport, pigs were sampled at the slaughterhouse including the lairage floor (n = 10, as sampled in barn floors), swine carcasses (10 carcasses per lot, n = 100), processing environment (n = 120), and pork cuts (n = 40).

Pig carcasses were sampled by swabbing rectangular sterile sponges (3 × 8 cm) in four delimited areas of 100 $cm^2$ according ISO 17604, (ISO, 2015) in the following slaughtering steps: a) after bleeding; b) after singeing; c) after evisceration; d) after final rinse. These steps are described as the most significant contamination steps in the slaughter line (Argüello et al., 2013). Also, portions of palatine tonsils (n = 100) and mesenteric lymph nodes (n = 100) were sampled from each pig carcass after evisceration. Environmental samples were collected by swabbing sterile sponges from the surfaces, which were directly in contact with the meat cuts, included knives (n = 40), steel gloves (n = 40), cutting boards (n = 20) and conveyor belts (20), being sampled by sterile sponges in four limited areas of 100 $cm^2$ to complete a sample. Pork cuts included ribs (n = 10), loin (n = 10), shoulder (n = 10) and legs (n = 10), also sampled by swabbing of four limited areas of 100 $cm^2$. Sponges used for sampling were previously moistened with 10 mL of buffered peptone water (BPW, 0.1%, w/v, Oxoid Ltd., Basingstoke, England). Palatine tonsils and mesenteric lymph nodes portions were transferred to sterile plastic bags. All samples were kept at 4 °C until microbiological analysis.

### *2.2. Salmonella detection*

Sponges used for surface sampling were transferred to sterile bags with 160-mL buffered peptone saline (BPS, with peptone at 0.01%, w/v and NaCl at 0.85%, w/v). The diluted samples were homogenized for 1 min at 230 rpm (Stomacher Seward 400®, Seward), and aliquots (40 mL) were centrifuged at 2000 ×*g* for 15 min. Supernatants were discarded and pellets were re-suspended 10-mL Buffered Peptone Water (1%, w/v) (Oxoid) and incubated at 37 °C for 18 h. Portions of palatine tonsils (10 g) and mesenteric lymph nodes (10 g) were transferred to sterile bags with 90-mL BPW (1%, w/v); aliquots of 25-mL or -g of water and feed were transferred to sterile bags with 225-mL BPW (1%, w/v) and incubated at 37 °C for 18 h.

Following ISO 6579 (ISO, 2002), aliquots of cultured BPW were transferred to Rappaport Vassiliadis Soya broth (Oxoid) and Muller-Kauffman Tetrathionate Novobiocin (Oxoid) broth and incubated at 42 °C and 37 °C, respectively, for 24 h. Afterwards, cultures were streaked onto Xylose Lysine Deoxycholate Agar (Oxoid) and Mannitol Lysine Crystal Violet Brilliant Agar (Oxoid), and were incubated at 37 °C for 24 h. Up to three colonies that appeared typical of *Salmonella* were selected and re-streaked onto trypticase soya agar (Oxoid) and subjected to biochemical tests [triple sugar iron, lysine iron agar, urease and malonate, according to Andrews et al., 2007] for identification.

Isolates that presented biochemical profile typical for *Salmonella* were subjected to PCR targeting the *invA* and *ompC* gene sequences. DNA was obtained as described by Dias et al. (2016), and the PCR reactions followed Swamy et al. (1996) for *invA* and Alvarez et al. (2004) for *ompC* with *Salmonella* Abony NCTC 6017 used as a positive control. PCR products were subjected to electrophoresis on 1.5% (w/v) agarose gels, stained with GelRed (Biotium Inc., Hayward, CA, USA) and visualized with a UV transilluminator. Primer sequences, PCR conditions and interpretation are described in the Supplementary Table 1. Based on PFGE profiles and molecular assays, 42 isolates were subjected to serogrouping test using *Salmonella* antisera according to manufacturer instructions (Denka Seiken Co., Ltd., Japan).

### *2.3. Isolates characterization*

#### *2.3.1. Macro-restriction digest and pulsed-field gel electrophoresis*

At least one confirmed *Salmonella* isolate per sample was selected for fingerprinting by macro-restriction digest and pulsed-field gel electrophoresis (PFGE), following PulseNet protocol (Centers for Disease Control and Prevention, Atlanta, GA, USA) as described by Ribot et al. (2006) with some modifications. Genomic DNA was digested with *Xba*I (50 U, Promega Corp., Madison, WI, USA) for 2 h at 37 °C. A CHEFDR III (Bio-Rad) was used to run the gels with the following parameters: initial switch time of 2.2 s, final switch time of 63.8 s, running time of 19 h, 6 V/cm and angle of 120°. *S. enterica* serovar Braenderup (ATCC BAA664) was subjected to same protocol and used as a reference pattern for every gel. After electrophoresis, gels were stained with GelRed (Biotium) and bands patterns were detected by using a UV transilluminator. Results were recorded with a digital camera and were then analyzed using Bionumerics 6.6 (Applied Maths, Ghent, Belgium) using 1.5% for optimization and 5% of tolerance. Resulting Dice coefficients were used to generate a dendrogram (unweighted pair group method with arithmetic mean, UPGMA).

#### *2.3.2. Virulence-related genes*

Isolates selected for PFGE were also subjected DNA extraction, as described above, and to a panel of PCR reactions to detect the presence of several virulence-related genes: *sitC*, *pagC*, *tolC*, *sifA*, *msgA*, *orgA*, *spiA*, *sipB*, *prgH*, *iroN*, *spaN*, *cdtB*, *spvB*, *spvC* and *sopB*. Primers, conditions of the reactions and interpretation of the results are described in the Supplementary Table 1. The obtained PCR products were subjected to electrophoresis in a 1.5% agarose gel stained with GelRed™ (Biotium) and products were observed with UV illumination. *Salmonella* Typhimurium ATCC 13311 was used as positive control to *cdtB* gene and *Salmonella* Abony NCTC 6017 was used as positive control to other genes (except *spvB*).

#### *2.3.3. Antibiotic resistance*

Based on PFGE profiles, 42 *Salmonella* isolates were selected and subjected an antibiotic susceptibility assay based on minimal inhibitory concentrations (MIC), as described by the Clinical & Laboratory Standards Institute (CLSI, 2017). Mueller Hinton brroth media (Oxoid) was used for these assays, with the following antibiotics: ampicillin, cefoxitin, chloramphenicol, streptomycin, ceftazidime, gentamicin, tetracycline, ciprofloxacin, ertapenem, meropenem, kanamycin and trimethoprim/sulfamethoxazole. All antibiotics were purchased from Sigma-Aldrich. *Escherichia coli* ATCC 25922 was used as a pan-susceptible quality control.

## 3. Results

Approximately 82% of recovered *Salmonella* isolates came from palatine tonsils and mesenteric lymph nodes (Table 1). Few isolates

**Table 1**
Frequencies positive results for *Salmonella enterica* (positive samples/total of tested samples), number of isolates and identified pulsotypes obtained from 10 lots of a pork production chain located in Brazil.

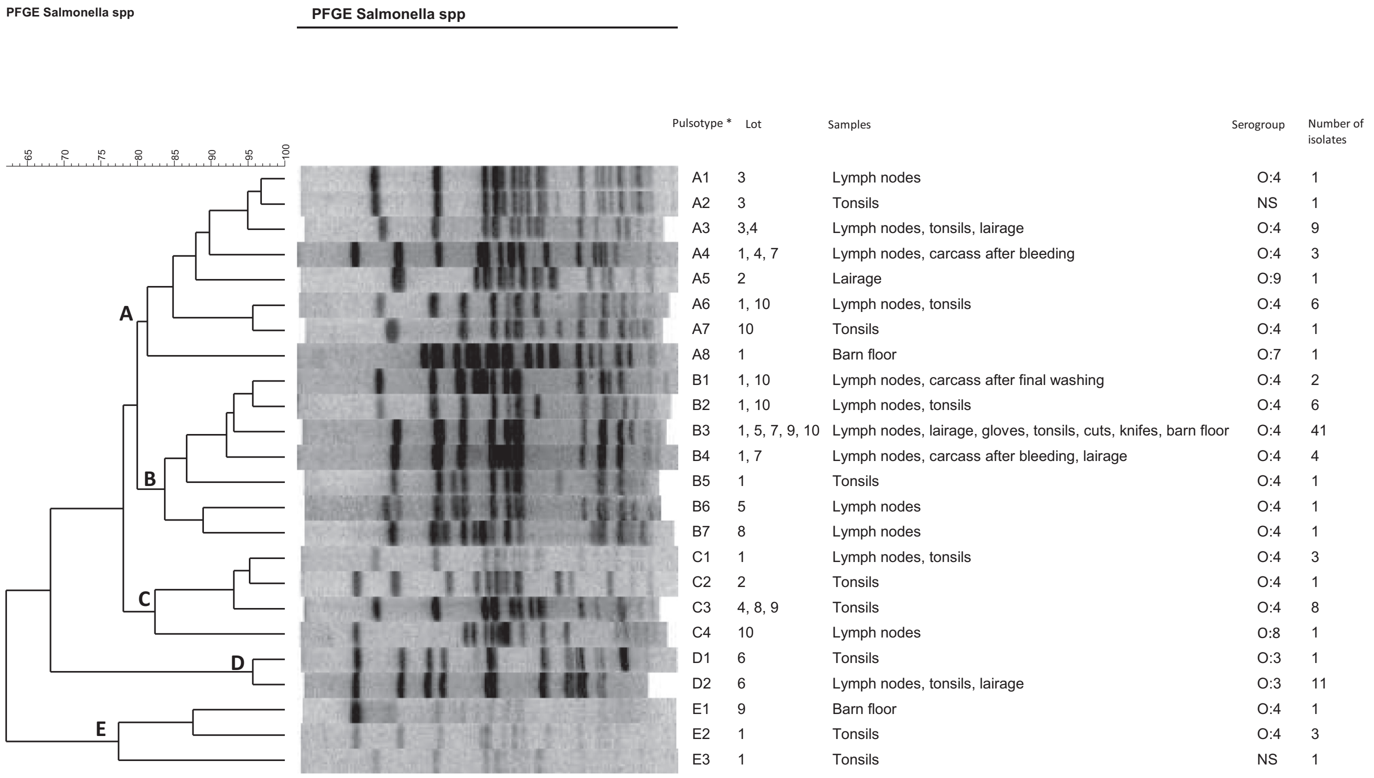
| Sample | *Salmonella enterica*/n | Isolates | Pulsotype[a] (s) |
|---|---|---|---|
| Farm | | | |
| Barn floor | 3/10 | 3 | A8 (1), B3 (1), E1 (1) |
| Water | 0/10 | – | – |
| Feed | 0/10 | – | – |
| Slaughterhouse | | | |
| Lairage floor | 7/10 | 7 | A3 (1), A5 (1), B3 (2), B4 (1) D2 (1) ND (1)[b] |
| Carcass after bleeding | 2/100 | 2 | A4 (1), B4 (1) |
| Carcass after buckling | 0/100 | – | – |
| Carcass after evisceration | 0/100 | – | – |
| Carcass after washing | 1/100 | 1 | B1 (1) |
| Palatine tonsil | 45/100 | 49 | A3 (3), A2 (1), A6 (2), A7 (1), C1, (1) C2 (1), C3 (8), B3 (18), B2 (2), B5 (1), D1 (1) D2 (4) E2 (3), E3(1), ND (2)[b] |
| Mesenteric lymph node | 43/100 | 43 | A3 (5), A4 (2), A1 (1), A6 (4), C1 (2), B6 (1) B7 (1), B3 (13), B1 (1), B2 (4), B4 (2), D2 (6), C4 (1) |
| Processing | | | |
| Knife | 1/40 | 1 | B3 (1) |
| Steel glove | 2/40 | 2 | B3 (2) |
| Cutting board | 0/40 | – | – |
| Conveyor belt | 0/40 | – | – |
| Pork cut | 4/40 | 4 | B3 (4) |

[a] As indicated in Fig. 1.
[b] ND: not digest with *Xba*I.

were recovered from other sampling locations, although 10% of pork cuts were positive for *Salmonella*.

Macro-restriction digest differeniated 109 strains into 24 distinct pulsotypes (3 isolates did not digest with *Xba*I), which were arranged into five different clusters (A–E, Fig. 1). Each pulsotype included 1 to 41 isolates that shared identical band patterns (100% of similarity) (Fig. 1). Clusters A and B encompassed 72.5% of the isolates (23 and 56 isolates, respectively) with 83.8% and 81.8% similarity within clusters, respectively and the pulsotype B3 is the biggest one, including 41 isolates. Serogrouping was possible for 39 isolates (3 isolates did not show agglutination), being characterized as belonging to O:4 (32/39), O:3 (3/39), O:9 (2/39), O:8 (1/39) and O:7 (1/39).

Virulence related genes were detected in all 112 tested isolates, consistent with what we should expect for pathogenic strains of *S. enterica*; despite *invA* being considered for genus identification, this gene is related to enterocytes invasion and a relevant virulence factor (Swamy et al., 1996), being detected in all *Salmonella* isolates. Besides *invA*, the isolates presented a minimum of ten virulence-related genes simultaneously, being possible the characterization of 14 virulence profiles (VP, Table 2). All tested isolates presented amplification for



| Pulsotype * | Lot | Samples | Serogroup | Number of isolates |
|---|---|---|---|---|
| A1 | 3 | Lymph nodes | O:4 | 1 |
| A2 | 3 | Tonsils | NS | 1 |
| A3 | 3,4 | Lymph nodes, tonsils, lairage | O:4 | 9 |
| A4 | 1, 4, 7 | Lymph nodes, carcass after bleeding | O:4 | 3 |
| A5 | 2 | Lairage | O:9 | 1 |
| A6 | 1, 10 | Lymph nodes, tonsils | O:4 | 6 |
| A7 | 10 | Tonsils | O:4 | 1 |
| A8 | 1 | Barn floor | O:7 | 1 |
| B1 | 1, 10 | Lymph nodes, carcass after final washing | O:4 | 2 |
| B2 | 1, 10 | Lymph nodes, tonsils | O:4 | 6 |
| B3 | 1, 5, 7, 9, 10 | Lymph nodes, lairage, gloves, tonsils, cuts, knifes, barn floor | O:4 | 41 |
| B4 | 1, 7 | Lymph nodes, carcass after bleeding, lairage | O:4 | 4 |
| B5 | 1 | Tonsils | O:4 | 1 |
| B6 | 5 | Lymph nodes | O:4 | 1 |
| B7 | 8 | Lymph nodes | O:4 | 1 |
| C1 | 1 | Lymph nodes, tonsils | O:4 | 3 |
| C2 | 2 | Tonsils | O:4 | 1 |
| C3 | 4, 8, 9 | Tonsils | O:4 | 8 |
| C4 | 10 | Lymph nodes | O:8 | 1 |
| D1 | 6 | Tonsils | O:3 | 1 |
| D2 | 6 | Lymph nodes, tonsils, lairage | O:3 | 11 |
| E1 | 9 | Barn floor | O:4 | 1 |
| E2 | 1 | Tonsils | O:4 | 3 |
| E3 | 1 | Tonsils | NS | 1 |

**Fig. 1.** Schematic representation (pattern, day, sources, serogroups and n° of isolates) of 24 unique band patterns from *Salmonella* strains that were isolated from farms and slaughterhouses in Brazil. Macro-restriction was completed with *Xba*I. Identity was estimated using the Dice coefficient (5% tolerance). *NS: Non serogrouped.

**Table 2**
Virulence profile of *Salmonella enterica* isolated from pig farms and swine slaughterhouse environments.

| Virulence profile | Virulence genes | n | Pulsotypes[a] |
|---|---|---|---|
| VP1 | *invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC* | 77 | C2, C3, B6, B7, B3, A6, B2, A4, B4, D1, D2, C3, D2, A7 |
| VP2 | *invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-cdtB-iroN-tolC* | 17 | A3, A4, A1, A2, A5, C3 E2 |
| VP3 | *invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-cdtB-iroN-tolC* | 3 | B3, E2 |
| VP4 | *invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-spvC-spvB-iroN-tolC* | 3 | C1, B1 |
| VP5 | *invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-cdtB-iroN-tolC* | 3 | C1, B3, E3 |
| VP6 | *invA-sifA-spiA-sitC-pagC-orgA-sipB-prgH-sopB-spaN-cdtB-iroN-tolC* | 1 | A3 |
| VP7 | *invA-sifA-spiA-sitC-pagC-msgA-sipB-prgH-sopB-spaN-cdtB-iroN-tolC* | 1 | A4 |
| VP8 | *invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-cdtB-tolC* | 1 | C4 |
| VP9 | *invA-sifA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC* | 1 | B3 |
| VP10 | *invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-spvC-spvB-tolC* | 1 | B1 |
| VP11 | *invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-iroN-tolC* | 1 | B4 |
| VP12 | *invA-sifA-spiA-sitC-pagC-msgA-orgA-prgH-sopB-spaN-tolC* | 1 | B5 |
| VP13 | *invA-spiA-sitC-pagC-msgA-orgA-sipB-spaN-iroN-tolC* | 1 | A8 |
| VP14 | *invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-tolC* | 1 | D2 |

[a] As indicated in Fig. 1, except for 1 isolate from VP1 and 2 isolates from VP2, that did not present PFGE profile.

*invA*, *sitC*, *pagC* and *tolC*, and 99.1% for *sifA*, *spiA*, *msgA*, *orgA* and *sipB*, 96.4% for *prgH* and *iroN*, 95.5% for *sopB*, 92.9% for *spaN*, 23.2% for *cdtB*, and only 3.6% for *spvB* and *spvC*.

For antibiotic resistance, no strains were resistant to ertapenem, meropenem or kanamycin, while there was a high frequency of resistance to streptomycin, tetracycline, ampicillin and chloramphenicol (Table 3). Resistance against ciprofloxacin was observed in 50% of strains. Approximately 81% of isolates were resistant to three or more classes of antibiotics (Table 4). One isolate was resistant to eight antibiotic classes while only one isolate was pansusceptible. The simultaneous resistance to ampicillin, chloramphenicol, streptomycin and tetracycline was the most recorded pattern, presented by 11 isolates from pulsotype clusters A, B and D (Fig. 1) and belonging to serogroups O:4 and O:3.

## 4. Discussion

Based on the obtained results, the frequency of *Salmonella enterica* isolation increased from farms to lairage (Table 1). As pigs are asymptomatic carriers of *Salmonella* spp., they can act as shedders under stress and transmit the pathogen in their feces (Simons et al., 2016). Several stressing conditions can increase *Salmonella* spp. excretion by animals in the pre-slaughtering, especially the high density and long duration during the transport, prolonged fasting period and long time on lairages (Bonardi, 2017).

**Table 3**
Frequencies of *Salmonella enterica* isolates (n = 42) obtained from the pork production chain with resistance to different antibiotics.

| Class[a] | Antibiotic | Resistance (%) |
|---|---|---|
| β-Lactam | Ampicillin | 34 (81.0%) |
| Cephem | Ceftazidime | 2 (4.8%) |
| | Cefoxitin | 2 (4.8%) |
| Phenicols | Chloramphenicol | 30 (71.4%) |
| Quinolones | Ciprofloxacin | 21 (50.0%) |
| Carbapenems | Ertapenem | 0 (0.0%) |
| | Meropenem | 0 (0.0%) |
| Aminoglycosides | Gentamicin | 7 (16.7%) |
| | Kanamycin | 0 (0.0%) |
| | Streptomycin | 38 (90.5%) |
| Tetracyclines | Tetracycline | 37 (88.1%) |
| Sulfonamides | Sulfamethoxazole + trimethoprim | 8 (19.0%) |

[a] Concentrations evaluated described according to CLSI (2017). Susceptibility breakpoints were used as follows, ampicillin: ≤8; ceftazidime: ≤4; cefoxitin: ≤8; chloramphenicol: ≤8; ciprofloxacin: ≤0.06; ertapenem: ≤0.5; meropenem: ≤1; gentamicin: ≤4; kanamycin: ≤16; tetracycline: ≤4; sulfamethoxazole + trimethoprim: ≤2/38; to streptomycin was used the same breakpoint for netilmicin (another aminoglycoside) ≤8.

**Table 4**
Antibiotic resistance profiles of *Salmonella enterica* isolates obtained from different steps of a pork production chain located in Brazil. Multidrug resistance is indicated by the dotted line.

| Simultaneous resistance | Resistance pattern[a] | n | Pulsotypes[b] |
|---|---|---|---|
| 9 | AMP-CHL-STR-GEN-SXT-TET-CIP-CFX-CAZ | 1 | B5 |
| 7 | AMP-CHL-STR-GEN-SXT-TET-CIP | 2 | A3, A8 |
| 6 | AMP-CHL-STR-GEN-TET-CIP | 3 | A1, A2, B7 |
| 6 | AMP-CHL-STR-SXT-TET-CIP | 3 | B1, D1, D2 |
| 6 | AMP-CHL-STR-SXT-TET-CAZ | 1 | C4 |
| 6 | AMP-CHL-STR-TET-CIP-CFX | 1 | no PFGE profile[c] |
| 5 | AMP-CHL-STR-TET-CIP | 7 | A3, A4, C3, B3, E1[c] |
| 5 | AMP-STR-GEN-TET-CIP | 1 | C1 |
| 4 | AMP-CHL-STR-TET | 11 | B6, B3, A6, B4, D2 |
| 4 | AMP-STR-TET-CIP | 1 | B1 |
| 4 | CHL-STR-TET-CIP | 1 | E3 |
| 3 | AMP-SXT-TET | 1 | A5 |
| 3 | AMP-STR-TET | 1 | B2 |
| 2 | AMP-TET | 1 | B3 |
| 2 | STR-CIP | 1 | A9 |
| 2 | STR-TET | 1 | C2 |
| 1 | STR | 3 | A4, E2, A7 |
| 1 | TET | 1 | A6 |
| 0 | No resistance | 1 | E2 |

[a] AMP: ampicillin, CHL: chloramphenicol, STR: streptomycin, GEN: gentamicin, CAZ: ceftazidime, CFX: cefoxitin, CIP: ciprofloxacin, ETP: ertapenem, MER: meropenem, KAN: kanamycin, TET: tetracycline, SXT: sulfamethoxazole + trimethoprim.

[b] As indicated in Fig. 1.

[c] Both resistance patterns presented one isolate each without PFGE profile.

*Salmonella enterica* was isolated from 43% of mesenteric lymph node samples (Table 1), as previously observed in similar studies (Cabral et al., 2017; Kich et al., 2011). As feces are considered the main sources of initial contamination by *Salmonella* spp. in a slaughterhouse, the lower is the frequency of this pathogen in the pig intestine, the lower is the likelihood of carcass contamination (Pesciaroli et al., 2017). In the palatine tonsils, 45% of samples were positive for *Salmonella enterica* (Table 1), at higher frequencies when compared to similar studies (Cabral et al., 2017; Van Damme et al., 2018). The presence of *Salmonella* spp. in lymphatic tissues represents a risk for contamination, once such sites are cut or removed from carcasses during slaughtering and processing, leading to a potential cross contamination along the facilities (Biasino et al., 2018; Van Damme et al., 2018). *Salmonella enterica* isolates from these samples were identified as belonging to a diversity of pulsotypes (Table 1), demonstrating the harbouring potential of the lymphatic tissue for the pathogen (Fosse et al., 2009).

Despite the presence of *Salmonella enterica* in the lymphatic tissue and potential spreading during slaughtering, only three pig carcasses presented positive results: two before bleeding and one after end washing. Low frequencies of *Salmonella* spp. in pig carcasses can be explained by proper handling during slaughtering, leading to a reduced cross contamination risk even amongst *Salmonella* positive herds (Argüello et al., 2013; Duggan et al., 2010). *Salmonella* spp. frequencies in pig carcasses are variable, being directly associated to the adoption of adequate hygienic procedures (Bonardi et al., 2013; Cabral et al., 2017; Kich et al., 2011; Marier et al., 2014). Because of that, *Salmonella* spp. frequencies in pig carcasses are variable, being directly dependent of characteristics of the studied facility and good manufacturing practices adopted and conducted by the slaughterhouse employed (Cabral et al., 2017; Kich et al., 2011). Two of three *Salmonella* isolates obtained from pig carcasses were serotyped as O:4, as the majority of serogroup identified amongst the isolates obtained from palatine tonsils and mesenteric lymph nodes, indicating these potential sources of contamination.

The same pattern was observed in the samples obtained from the processing environment of slaughterhouse: only steel glove and knife samples were positive for *Salmonella enterica*, and all isolates were identified as belonging to pulsotype B3 (Table 1). Utensils are often contaminated with *Salmonella enterica* and can contribute significantly with the cross-contamination between carcasses and meat (Argüello et al., 2013; Gomes-Neves et al., 2012). As result, some pork cut samples (n = 4) were positive for *Salmonella enterica*, and all isolates were identified also as belonging to pulsotype B3 and serogroup O:4 (Table 1, Fig. 1). As observed in studies focusing on swine slaughtering, *Salmonella* spp. frequencies in processing and pork cuts are variable and directly dependent on cross contamination in the facilities. Duggan et al. (2010) described that 1.1% of pork cuts were positive for *Salmonella* spp. in Ireland, Valero et al. (2014) detected the pathogen in 8.3% of pork cuts obtained from the retail sale in Spain and Colello et al. (2018) showed 8% of positive samples in pork meat and minced meat from retail markets in Argentina.

The serogroup O:4 is the most associated serogroup with swine production and pork products, the main serotypes allocated in this serogroup are Typhimurium and Derby (Biasino et al., 2018; Bonardi, 2017; Cabral et al., 2017; Kich et al., 2011). Also, the serotype Typhimurium is the second most associated to human salmonellosis outbreaks in European Union, and pork was the main food related to these outbreaks in 2014 (EFSA and ECDC, 2016). Despite being isolated in lower frequencies than serogroup O:4, *Salmonella* from serogroup O:3 is also associated to pork products, as described previously (Fois et al., 2017; Li et al., 2014), the main serotypes in this group are Anatum and London.

Based on genetic profiles obtained by PFGE, *Salmonella* isolates presented high similarity indexes (Fig. 1). The same pulsotype was detected in lymph nodes and carcasses from different lots; this situation was observed for *Salmonella* isolates from pulsotype A4, B1 and B4 (Fig. 1). Isolates from pulsotype A3 were isolated during two consecutive sampling efforts and in mesenteric lymph nodes, palatine tonsils and in the lairage (Fig. 1). B3 was the most frequent characterized pulsotype, being described in several sampling days and different samples, including mesenteric lymph nodes and tonsils, as well as environmental and pork cuts samples (Fig. 1), demonstrating the spread of them in the pork production chain. Also, PFGE analysis has been proven to be useful and accurate for tracking contamination sources, allowing the identification of *Salmonella* persistence, cross contamination and distribution in swine production and pork processing (Gomes-Neves et al., 2012; Hernández et al., 2013; Kich et al., 2011; Patchanee et al., 2016).

The *Salmonella* genetic profiles obtained by PFGE also allowed the identification of a diversity of strains present in the pork production chain: different strains of *Salmonella*, belonging to different PFGE clusters, were isolated from similar samples in same sampling days (Fig. 1). As pigs are *Salmonella* reservoirs (Argüello et al., 2013), the presence of different strains in lymphatic tissues would be expected and a plausible explanation for these results (Fig. 1). Several isolates obtained from the first pig lot presented eleven different genetic profiles (A4, A6, A8, B1, B2, B3, B4, B5, C1, E2, E3), demonstrating their genetic diversity and indicating the relevance of the production environment as sources of *Salmonella* strains, as previously described (Bonardi et al., 2013; Gomes-Neves et al., 2012; Hernández et al., 2013; Kich et al., 2011; Patchanee et al., 2016).

Results for virulence-related genes demonstrated the pathogenic potential of *Salmonella* isolates (Table 2). As the isolates presented simultaneously a minimum of ten virulence-related genes, this is a strong evidence of the pathogenic potential of the *Salmonella* strains present in the studied pork chain. Several genes are important in *Salmonella* virulence; *invA*, *spaN*, *sipB*, *tolC*, *prgH*, *sopB* and *orgA* are associated with the ability to invade the intestinal epithelial cells (Raffatellu et al., 2005; Skyberg et al., 2006). *spaN* and *sipB* have some relationship with entry into non-phagocytic cells and killing of macrophages (Chen et al., 1996). Other specific virulence genes are related with the survival and replication within macrophages, such as *spiA*, *sifA*, *pagC* and *msgA* (Bohaychuk et al., 2011; Zhang et al., 2013). *spvB* and *spvC* contribute in adhesion and systemic infection against the host cells (Ibarra and Steele-Mortimer, 2009). *sitC* and *iroN* are responsible to encode products for iron uptake (Haghjoo and Galán, 2004; Skyberg et al., 2006). *cdtB* encodes the cytolethal-distending toxin (Haghjoo and Galán, 2004). Four *Salmonella* isolates harboured both *spv* genes included in this study, all from serogroup O:4 and from VP4 and VP10 (Table 2). Based on their genetic profiles, two of these isolates presented the pulsotype B1 and the other two the pulsotype C1, indicating their close genetic relationship (Fig. 1). Despite the observations, no trend was observed related to *Salmonella* VP, serotype and pulsotype.

Besides the presence of virulence-related genes, pathogenicity of *Salmonella* isolates is also highly associated to their resistance profiles to antibiotics, a characteristic that can jeopardize the success of clinical treatment of salmonellosis (Unlu et al., 2018; Zou et al., 2012). Amongst the tested isolates (n = 42), high frequencies of resistance were identified for ampicillin (81%), chloramphenicol (71.4%), streptomycin (90.5%) and tetracycline (88.1%) (Table 3), and 34 (81%) isolates presented resistance to three or more antibiotic classes, being characterized as multi-drug resistant (MDR, Table 4) (ECDC et al., 2017). The emergence of MDR *Salmonella* has been considered one of the main concerns related to global health (Lopes et al., 2015). The most prevalent resistance pattern observed was AMP-CHL-STR-TET, in 11 strains, which belonged to a five different pulsetypes. This pattern is one of the typical characteristics of *Salmonella* Typhimurium DT104, a relevant phage type that is historicaly recognized by its emergence and high capacity of spread (Leekitcharoenphon et al., 2016).

Swine-related samples and pork products usually present MDR *Salmonella* (Barilli et al., 2018; Calayag et al., 2017; Colello et al., 2018; Fois et al., 2017; Lopes et al., 2015; Sinwat et al., 2016). As observed in the present study (Table 3), other studies had demonstrated high frequencies *Salmonella* strains with resistance to streptomycin, tetracycline and ampicillin (Fois et al., 2017; Lopes et al., 2015; Patchanee et al., 2016). These substances are usually employed in different steps of swine production, resulting in a massive selective pressure in the bacterial population of animals, including *Salmonella* (Lopes et al., 2015). However, Almeida et al. (2018) demonstrated lower frequencies of resistance against these antibiotics (48.9% to streptomycin, 30% to tetracycline and 35.6% to ampicillin) in *Salmonella* strains recovered in Brazil.

Considering the other tested antibiotics (cephems, carbapenems, aminoglycosides - gentamicin and kanamycin, and sulphonamides), only a few *Salmonella* isolates, or even none, presented resistance (Tables 3 and 4). Although low, the observed frequencies of resistance were higher when compared to data from other studies (Barilli et al., 2018; Cameron-Veas et al., 2018; Fois et al., 2017; Lopes et al., 2015). Despite the low frequencies of resistance to cephems, the presence of resistant strains is still a concern for public health: resistance to cephems is an indicative that these antibiotics are being currently used during swine production, and that they can became non-effective for clinical treatment of children and immune compromised patients (Barilli et al., 2018; Cameron-Veas et al., 2018). The absence of resistance to carbapenems in *Salmonella* isolates (Table 3) is in agreement with similar studies (Calayag et al., 2017; Fardsanei et al., 2018); *Salmonella* is usually susceptible to carbapenems, being considered as the last choice for treatment of salmonellosis caused by resistant strains (Fernández et al., 2018). As observed for virulence-related genes, none trend was observed amongst *Salmonella* serotypes, pulsotypes and antibiotic resistance profiles.

Resistance to ciprofloxacin was considered particularly high (50%). This rate was similar when compared to reports from other countries (Cameron-Veas et al., 2018; Guerra Filho et al., 2016; Jiu et al., 2017), but it is unusually high relative to the United States where ciprofloxacin resistant *Salmonella* was approximately 0.5% in 2013 (CDC, 2016). Also, Sinwat et al. (2016) showed 0.5% of ciprofloxacin resistant strains isolated from pigs, pork and humans in Thailand and Laos. Ciprofloxacin resistance is mainly attributed to mutations in the quinolone resistance-determining regions, and the large-scale use of fluoroquinolone antimicrobials will selectively favor such mutations (Cao et al., 2017; Kuang et al., 2018). The veterinary analogue for ciprofloxacin is enrofloxacin, and enrofloxacin use will select for ciprofloxacin resistance (McDermott et al., 2002). Interestingly, all pig lots included in the present study were using ciprofloxacin rather than enrofloxacin, and the antibiotic was being used for prophylaxis (growing and finishing steps). The recorded prevalence of ciprofloxacin resistance is alarming in part because fluoroquinolones are an important therapeutic for invasive gastrointestinal infections caused by MDR strains (Hopkins et al., 2005; Kuang et al., 2018).

## 5. Conclusion

Results obtained in this study indicate that, due the high occurrence of *Salmonella* in tonsils and lymph nodes, pigs are potential sources of contamination for carcasses and slaughterhouse environment and a strict control should be implemented in order to avoid this contamination. Also, the high rate of virulence genes found in *Salmonella* isolates and the concomitant presence of a high number of *Salmonella* MDR are a significant public health concern. Monitoring the antimicrobial susceptibility is necessary to identify emerging resistant strains in the pork production whereas the isolates can be transferred to humans by the food chain. Our results provide valuable insight about the epidemiology of *Salmonella* in swine production and may help to develop control programs implement measures at both the primary and slaughterhouse levels.

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## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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