CIBELI VIANA

Salmonella IN A BRAZILIAN PORK PRODUCTION CHAIN: OCCURRENCE, DIVERSITY, VIRULENCE GENOTYPES AND ANTIBIOTIC RESISTANCE PROFILE

Thesis presented to the Universidade Federal de Viçosa, as part of the requirements of the Veterinary Medicine Graduate Program, to obtain the title of *Doctor Scientiae*.

Advisor: Luís Augusto Nero
Co-advisor: Luciano dos Santos Bersot

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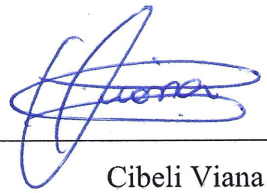

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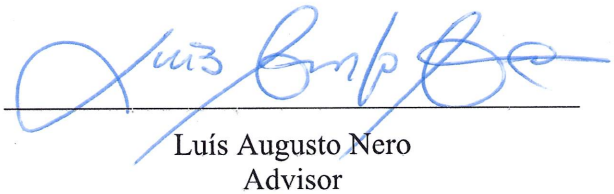

Luís Augusto Nero
Advisor

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“Nothing ever goes away until it has taught us what we need to know”.

Pema Chödrön

ABSTRACT

VIANA, Cibeli, D.Sc., Universidade Federal de Viçosa, February, 2020. ***Salmonella*** **in a Brazilian pork production chain: occurrence, diversity, virulence genotypes and antibiotic resistance profile.** Advisor: Luís Augusto Nero. Co-advisor: Luciano dos Santos Bersot.

Salmonella spp. is considered one of the main foodborne pathogens associated to food poisoning outbreaks worldwide. *Salmonella* strains have been detected in several countries. The aim of this study was to track *Salmonella* spp. contamination in pork production chain during slauthering and processing. Also, the aim was to provide a deep characterization of the isolates by molecular analysis. The collections were carried out in ten different farms, which 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 sent stock to the same slaughterhouse. At pig farms, the following samples were collected: feed from the top of feeder pigs (n=10), water from the bite drinkers (n = 10), barn floor (n = 100). 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), blood samples (n = 100), processing environment (n = 120), and pork cuts (n = 40). Also, portions of diaphragm (n = 100), palatine tonsils (n = 100) and mesenteric lymph nodes (n=100) were sampled from each pig carcass after evisceration. Blood and meat juice were subjected to ELISA to detect antibodies against *Salmonella*, and other samples were subjected to *Salmonella* detection by ISO 6579. 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. Also, 41 isolates were selected to whole-genome sequencing. *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%). Based on ELISA, *Salmonella* positive samples were: 86 and 46 blood serum (20% and 40% cut-offs) and 68 and 46 meat juice (20% and 40% cutoffs). Optical density readings from blood serum and meat juice presented a high and significant correlation ($r = 0.93$, $p < 0.001$). 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*. Whole-genome sequencing and in silico serotyping demonstrated that the S. enterica serovar Typhimurium was the most common serotype (n = 17), but eight additional servoars were identified. Eight multilocus sequence types were identified with ST19 being most common (n = 21). Several plasmids replicons were detected, with Col (RNAI) the most abundant (n = 30), followed by IncR (n = 22), IncI1 (n = 10) and IncA/C2 (n = 10). High rates os resistance were observed, mainly to streptomycin, tetracycline, ampicillin, chloramphenicol and ciprofloxacin. Only two isolates were resistant to third-generation cephalosporins and no isolates were resistant to two tested carbapenems. Twenty-six unique antimicrobial-resistance genes were identified with *bla*$_{\text{TEM-1A}}$ and *bla*$_{\text{TEM-1B}}$ likely responsible for most beta-lactam resistance and *floR* responsible for most chloramphenicol resistance. At the time of collection, the sampled farms were adding ciprofloxacin to feed, and this may have contributed to the high prevalence of resistance to this antibiotic. A majority of isolates were considered multidrug resistant (resistant to 3 or more antibiotic classes). This study provides valuable insight about the epidemiology of *Salmonella* in swine production. Despite the low presence of this pathogen in carcasses and meat cuts, the presence of the pathogen in lymph nodes and tonsils emphasizes the importance of slaughterhouse hygiene measures. Considering the antimicrobial resistance global scenario, the high occurrence of multidrug resistant isolates from pork production is a serious data. The antibiotic use must be controlled, and it is recommended that antibiotics considered to be of critical importance to human health should be avoided in animal use.

Keywords: Ciprofloxacin. ELISA. MLST. Swine. WGS.

RESUMO

VIANA, Cibeli, D.Sc., Universidade Federal de Viçosa, fevereiro de 2020. ***Salmonella*** **em uma cadeia de produção de carne suína brasileira: ocorrência, diversidade, genótipos de virulência e perfil de resistência antimicrobiana.** Orientador: Luís Augusto Nero. Coorientador: Luciano dos Santos Bersot.

Salmonella spp. é mundialmente considerada um dos principais patógenos associados com surtos de origem alimentar. Estirpes de *Salmonella* multirresistentes aos antibióticos tem sido detectadas em diversos países do mundo. O objetivo desse trabalho foi rastrear a contaminação de *Salmonella* spp. na cadeia de produção de suínos durante o abate e processamento além de realizar a caracterização molecular dos isolados. As coletas foram realizadas em dez diferentes granjas de suínos de sistema intensivo com média de 1000 suínos/granja. Todos as granjas abatiam os animais em um mesmo matadouro-frigorífico. Nas granjas as amostras coletadas foram: ração (n=10), água (n = 10) e swab do piso (n = 10). Posteriormente, no local do abate, as seguintes amostras foram coletadas: piso da pocilga de espera (n = 10), carcaça de suínos (n = 100), swabs de produto final (n = 40), amostras de sangue (n = 100), amostras do diafragma (n = 100), tonsilas palatinas (n = 100), linfonodos mesentéricos (n = 100) e amostras do ambiente de processamento (n = 120). Sangue e suco da carne (proveniente do pilar do diafragma) foram submetidos ao teste de ELISA e as demais amostras a detecção microbiológica de *Salmonella* de acordo com a ISO 6579. Nos isolados confirmados foram realizadas as analíses de soroagrupamento, PFGE e pesquisa de genes de virulência e resistência antimicrobiana. Além disso, foram selecionados 41 isolados para sequenciamento completo do genoma. *Salmonella* spp. foi detectada em pisos de granjas (03/10), pocilgas de espera (07/10), carcaça após a sangria (2/100) carcaça após a lavagem final (1/100), tonsilas (45/100), linfonodos mesentericos (43/100), utensílios (3/120) e cortes (4/40). Os isolados encontrados nas amostras pertenciam aos sorogrupos: O:4 (82%), O:3 (7,7%), O:9 (5,1%), O:8 (2,6%) and O:7 (2,6%). Baseado no teste de ELISA, 86 e 46 amostras do plasma sanguíneo foram positivas (ponto de corte 20% e 40%), enquanto 68 e 46 amostras de suco da carne foram positivas (ponto de corte 20% e 40%). Foi verificado uma alta correlação ($r = 0.93$, $p < 0.05$), entre as leituras da densidade ótica do soro e do suco de carne. Baseado na análise de PFGE, 109 cepas foram alocadas em 24 diferentes padrões de pusotipos, arranjados em cinco diferentes clusters. Catorze diferentes perfis de virulência foram observados de acordo com a combinação dos genes detectados e todos os isolados foram positivos para o gene *invA*, *sitC*, *pagC* e *tolC*. Com

base na análise do genoma, a sorotipificação *in silico* identificou nove diferentes sorovares, sendo que *Salmonella enterica* sorovar Typhimurium foi o mais comum (n = 17). A análise do MLST revelou oito ST diferentes, em que O ST-19 foi o mais comum (n = 21). Detectou-se múltiplos replicons de plasmídeos, sendo que os mais abundantes foram Col (RNAI) (n = 30), IncR (n = 22), IncI1 (n = 10) e IncA/C2 (n = 10). Altas taxas de resistência antimicrobiana foram observadas no estudo, principalmente frente aos seguintes antimibrocianos: estreptomicina, tetraciclina, ampicilina, cloranfenicol e ciprofloxacina. Apenas dois isolados foram resistentes a cefalosoprinas de terceira geração e nenhum isolado foi resistente aos carbapenens testados. Vinte e seis genes de resistência foram identificados, sendo *bla*$_{\text{TEM-1A}}$ e *bla*$_{\text{TEM-1B}}$ os maiores responsáveis pela resistência aos beta-lactâmicos e *floR* o gene responsável pela maioria das resistências ao cloranfenicol. Durante o período em que as coletas foram realizadas, a adição de ciprofloxacina de maneira preventiva era feita via ração, fato que pode ter contribuído para a alta prevalência de resistência a este antibiótico. A maioria dos isolados avaliados foram considerados multirresistentes (resistentes a 3 ou mais classes de antibióticos). Este estudo elucidou pontos importantes da epidemiologia de *Salmonella* na cadeia de produção de carne suína. Apesar da baixa ocorrência do patógeno em carcaça e cortes finais, a presença do mesmo em linfonodos e tonsilas deixa claro a importância da adoção de medidas de higiene pelo abatedouro. Considerando o cenário mundial de resistência a antibióticos, a alta ocorrência de isolados multirresistentes provenientes da produção de suínos é um dado de extrema importância, visto que o uso de antibióticos deve ser controlado e é recomendado que os antibióticos considerados de importância crítica à saúde humana sejam evitados em uso animal.

Palavras-chave: Ciprofloxacina. ELISA. MLST. Suínos. WGS.

FIGURE LIST

CHAPTER 1

CHAPTER 2

CHAPTER 3

TABLE LIST

CHAPTER 1

CHAPTER 2

CHAPTER 3

SUMMARY

INTRODUCTION

Salmonella spp. is considered one of the main foodborne pathogens associated to food poisoning outbreaks worldwide (BRASIL, 2018a; EFSA; ECDC, 2018). This pathogen is responsible for more than one million of illnesses and 450 deaths per year in the United States, where these diseases are systematically reported (CDC, 2019); in Brazil, even with the deficient reports of the foodborne diseases by the Ministry of Health, *Salmonella* spp. is described as the main bacterial agente enrolled in the cases and outbreaks of foodborne diseases (BRASIL, 2018a). Usually, most of the salmonellosis cases are self-limiting, although some cases may require hospitalization and antibiotics are typically used only to treat people with severe illness (CDC, 2019). Salmonellosis have been associated with different serotypes, specially *S.* Enteritidis, *S.* Typhimurium and its monophasic variant (*Salmonella* 1,4,[5],12:i-) (EFSA; ECDC, 2018). In general, *S.* Enteritidis is associated with poultry and its products, while *S.* Typhimurium is associated with a wide range of hosts, including pigs (CAMPOS; MOURÃO; PEIXE; ANTUNES, 2019). Also, a strong association between monophasic variants of *S.*Typhimurium and pig chain has been observed (EFSA; ECDC, 2018).

Pigs are asymptomatic carriers of *Salmonella* spp. and they can act as shedders under stress, spreading the pathogen in their feces (SIMONS; HILL; SWART; KELLY *et al.*, 2016). Also, they can harbor this pathogen in their lymph nodes, tonsils and oral cavity (GUERRA FILHO; YAMATOGI; POSSEBON; FERNANDES *et al.*, 2016; KICH; COLDEBELLA; MORES; NOGUEIRA *et al.*, 2011; PESCIAROLI; CUCCO; DE LUCA; MASSACCI *et al.*, 2017). The prevalence of *Salmonella* spp. in swine carcasses is variable, ranging from 1.5% to 24%, (BOHAYCHUK; GENSLER; BARRIOS, 2011; CABRAL; PANZENHAGEN; DELGADO; SILVA *et al.*, 2017; KICH; COLDEBELLA; MORES; NOGUEIRA *et al.*, 2011; PALA; TEDDE; SALZA; UDA *et al.*, 2019; PESCIAROLI; CUCCO; DE LUCA; MASSACCI *et al.*, 2017). Thus, pork products are considered as important sources of *Salmonella* spp. being often associated with human salmonellosis reported in different countries (EFSA; ECDC, 2018; WHO, 2019).

Official programs for controlling *Salmonella* spp. in swine have been developed in several countries over the years. The monitoring programmes were established to decrease contamination of the pathogen in slaughtering process and to control its presence in swine carcasses immediately after slaughtering (CAMPOS; MOURÃO; PEIXE; ANTUNES, 2019; MARTÍNEZ-AVILÉS; GARRIDO-ESTEPA; ÁLVAREZ; DE LA TORRE, 2019). Brazilian Ministry of Agriculture had regulamented in 2018 an official program to control and to

monitore *Salmonella* in pig slaughtering (BRASIL, 2018c). This current program includes microbiological criteria for self-control and official control of pig and bovine carcasses, based on a scenario observed in Brazil, that considers *Salmonella* as the main bacterial hazard associated to pork (BRASIL, 2018c). Then, a rigorous control of the whole production chain is conducted by the pork companies to achieve the required hygiene and biosecurity standards. In addition, the intensive food production systems request high productivity and efficiency, leading to the development of strategies to avoid the dissemination and to mitigate the persistance of pathogens.

The antibiotics are widely used in the production chain of animal origin foods. Van Boeckel et al. (2017), based on a literature review, reported that 130.000 tons of antibiotcs were consumed by the livestock production system worldwide in 2013, and it is assumed that 200.000 tons will be used in 2030. Poultry and pork chains consume around 75% of the antibiotcs used in animal production, and these antibiotcs are used for different purposes (VAN BOECKEL; GLENNON; CHEN; GILBERT *et al.*, 2017). In swine production system, the antibiotics may be used for therapeutic treatment, prophylaxis and growth promotion and they can lead to increase in selection and spread of antibiotic-resistant bacteria (CHANTZIARAS; BOYEN; CALLENS; DEWULF, 2014; MAGOURAS; CARMO; STÄRK; SCHÜPBACH-REGULA, 2017; POSTMA; BACKHANS; COLLINEAU; LOESKEN *et al.*, 2016). Since 2006, the European Union banned antibiotics as a growth promoter or prophylaxys through animal feed. However, the therapeutic use is still allowed if approved by European Medicines Agency (ECDC; EFSA; EMA, 2017). According OIE (2018), 155 countries submitted completed reports about the use of antibiotic in animals and 45 of them stated the use of antibiotics as growth promoters, including Brazil.

Van Boeckel et al. (2015) estimated the antibiotics consumption around the world in 2014, and revealed average consumptions of 45 mg of antibiotic per kg of beef cattle, 148 mg per kg of chicken and 172 mg per kg of produced pigs. In Brazil, a study of 25 pig production systems revealed an average 358.0 mg of antibiotics per kg of produced pig, considering a variation of 5.4 to 586.0 mg (DUTRA, 2019). The same study demonstrated that the animals are exposed to 2 to 11 (average of 7) different antibiotics over their lifetime. As an additional estimative, the animals are exposed to at least one antibiotic class during 66.3% of their lifetime. From these results, it is clear the excessive use of antibiotics in Brazil when compared to the world scenario (DUTRA, 2019).

In order to control the excessive use of antibiotics, the Brazilian Ministry of Agriculture established in 2017 a national program that aims the identification and surveillance of antibiotic

resistance in agriculture. This program was designed with a 5-year implementation (2018-2022), considering an approach based on “One health”, establishing an integration among human, animal and environmental health. The program aims to promote strategic actions such as: epidemiological studies, strengthening the implementation of infection prevention and control measures, promoting rational use of antimicrobials and their resistance (BRASIL, 2017).

On a global level, antibiotics are widely available with no restriction or control, and as a result it may be used improperly (OIE, 2018). One of the direct consequence of excessive or mislead use of antibiotics is the potentially emergence of resistant strains and the horizontal spread of resistance genes in the environment and the food production chain (AARESTRUP, 2015; CHANTZIARAS; BOYEN; CALLENS; DEWULF, 2014; POSTMA; BACKHANS; COLLINEAU; LOESKEN *et al.*, 2016).Antibiotic resistance is recognized as a global health concern, and controlling its spread and transmission is considered as one of the biggest worldwide health challenges (FERRI; RANUCCI; ROMAGNOLI; GIACCONE, 2017). The antibiotic resistance may be classified as innate or acquired. Innate resistance is a consequence of the selected and functional characteristics of the microorganism and the acquired resistance may occur through the horizontal gene transfer or mutations in chromosomal genes (BLAIR; WEBBER; BAYLAY; OGBOLU *et al.*, 2015).

Antibiotic resistance and pathogenicity are often associated with horizontal gene transfer and the acquisition of new resistant genes harbored on mobile genetic elements, such as plasmids, integrons, transposons, insertion sequences, and phage-related elements (SOUCY; HUANG; GOGARTEN, 2015). Horizontal gene transfer has been identified as one of the major responsable to spread antibiotic resistance genes in the enviroment, it may be associated with transmission between different species of microorganisms (FOUNOU; FOUNOU; ESSACK, 2016). This mechanism is able to create a resistant bacteria population and the selective pressure, due to the continuous use of antibiotics, increases substiantially the number of resistant bacteria. Also, antibiotic resistant bacteria may act as a reservoir of resistance genes for other bacteria, even without the related antibiotics the gene might be conserved within bacteria population (HOLMES; MOORE; SUNDSFJORD; STEINBAKK *et al.*, 2016). Typically, bacteria have become resistant to antibiotic through a several mechanisms, that can be characterized in three categories. (1) Efflux pump or changes in permeability or transport of the antimicrobial, (2) Inactivation of the antibiotic by hydrolysis or modification and (3) modification or replacement of the antibiotic target (SOUCY; HUANG; GOGARTEN, 2015).

A number of studies has indicated that *Salmonella* harbors a large number of resistance

genes and consequently present a high rate of resistance against several antibiotics (ALMEIDA; SERIBELLI; MEDEIROS; RODRIGUES *et al.*, 2018; FRYE; JACKSON, 2013; MCDERMOTT; TYSON; KABERA; CHEN *et al.*, 2016; MICHAEL; BUTAYE; CLOECKAERT; SCHWARZ, 2006; PORNSUKAROM; VAN VLIET; THAKUR, 2018). Thus, *Salmonella* is considered an important agent to contribute with the antibiotic resistance global problem (MONTE; LINCOPAN; BERMAN; CERDEIRA *et al.*, 2019). The deep understanding of the pathogen's epidemiology associated with the knowledge about the resistant determinants genes in the bacterial population is a significant tool, which can be used to promote new strategies and approaches to surveillance of mutli-drug resistant *Salmonella* in the food chain.

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OBJECTIVES

The aim of this study was to track *Salmonella* spp. in pork production chain during the slaughtering and processing through conventional and alternative methodologies. Also, the aim was to provide a deep characterization of the isolates by molecular analysis. Considering the main goal, specific objectives were:

To assess the adequacy of using an ELISA test as an alternative to screen and support tool to predict the presence of *Salmonella* in the key sites of swine carcasses;

To characterize *Salmonella* spp. strains isolated from different steps of a pork production chain in Brazil according the the distribution, diversity, virulence genotypes and phenotipyc antibiotic resistance.

To perform a deep analysis in the specific isolates to verify the genetic diversity and the presence of antimicrobial resistance genes, as well as, the occurrence of mutations.

CHAPTER 1. Comparison of meat juice serology and bacteriology for surveillance of *Salmonella* in the Brazilian pork production chain

Short title: Serology for *Salmonella* detection in pork

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Abstract

This study assessed an ELISA based assay to detect *Salmonella* in swine as a potential tool to predict the presence of *Salmonella* in swine carcasses. The following samples were collected from ten swine batches: blood (n = 100); environment (barn floor, n = 10, and lairage floor, n = 10); meat juice (n = 100, obtained after defrosting of diaphragm); tonsils (n = 100); mesenteric lymph nodes (n = 100); and carcasses after bleeding (n = 100), after singeing (n = 100), after evisceration (n = 100), and after final rinsing (n = 100). Blood and meat juice were subjected to ELISA to detect antibodies against *Salmonella*, and other samples were subjected to *Salmonella* detection by ISO 6579. *Salmonella* was detected in 3 samples from barn floors, 7 lairage floors, 45 tonsils, 43 mesenteric lymph nodes and in 3 carcasses. Based on ELISA, *Salmonella* positive samples were: 86 and 46 blood serum (20% and 40% cut-offs) and 68 and 46 meat juice (20% and 40% cut-offs). Optical density (OD) readings from blood serum and meat juice presented a high and significant correlation ($r = 0.93$, $P < 0.001$), and a substantial agreement for *Salmonella* detection ($K = 0.69$, ELISA 40% cut-off). The agreement between ELISA and microbiological analysis for *Salmonella* detection in pig carcasses were absent or poor, with the exception of results obtained by ELISA 40% cut-off from blood serum and meat juice with mesenteric lymph nodes ($K = 0.49$ and 0.50, respectively) and tonsils ($K = 0.29$ and 0.30, respectively). Based on the obtained results, Meat juice can be considered an alternative to blood serum as a matrix for ELISA for preliminary detection of *Salmonella*, allowing the identification of potential sources of contamination during slaughtering.

Keywords: *Salmonella*; pig; ELISA; surveillance; conventional isolation

1. Introduction

Pigs are usually asymptomatic carriers of *Salmonella*, excreting the pathogen intermittently or when stressed. In such a condition, pigs can spread *Salmonella* widely in the pork production chain (ARGÜELLO; ALVAREZ-ORDONEZ; CARVAJAL; RUBIO *et al.*, 2013; MANNION; EGAN; LYNCH; FANNING *et al.*, 2008; SILVA; DIAS; FERRONATTO; GUERRA *et al.*, 2012; SIMONS; HILL; SWART; KELLY *et al.*, 2016). Several studies have demonstrated the prevalence of *Salmonella* in pig farms and its spread to slaughterhouses, and highlight the relevance of infected animals as carriers of this pathogen to processing facilities (ARGÜELLO; ALVAREZ-ORDONEZ; CARVAJAL; RUBIO *et al.*, 2013; BUNCIC; SOFOS, 2012; RODRIGUEZ; PANGLOLI; RICHARDS; MOUNT *et al.*, 2006; SILVA; DIAS; FERRONATTO; GUERRA *et al.*, 2012). This evidence has led to rigorous control of *Salmonella* from the first steps of production to processing of end products, conducted by the pork companies and guided by official inspection services.

Different official programs for *Salmonella* spp. control in swine have been discussed and developed in several countries; most of these programs were based on the Danish model, which considers the serology of animals as the main monitoring tool (MAINAR-JAIME; CASANOVA-HIGES; ANDRES-BARRANCO; VICO, 2018; WEGENER; HALD; LO FO WONG; MADSEN *et al.*, 2003). However, some countries failed in adopting serology-based programs due to different reasons, like distinct *Salmonella* levels in pig farms, leading to distinct approaches for data interpretation and guidance, and the complexity of *Salmonella* transmission among animals and producing systems, jeopardizing proper prediction of positive results (BLAHA, 2017; BRITISH PIG EXECUTIVE, 2012; BROSSÉ, 2015; GRADASSI; CAMINITI; GALLETTI; SANTI *et al.*, 2015; MAINAR-JAIME; CASANOVA-HIGES; ANDRES-BARRANCO; VICO, 2018; WEGENER; HALD; LO FO WONG; MADSEN *et al.*, 2003; ZDOLEC; DOBRANIC; FILIPOVIC, 2015). Because of these limitations, some countries have adopted the microbiological detection method for *Salmonella* isolation from the key sites of pig carcasses, such as tonsils and mesenteric lymph nodes (MLN), and achieved reliable results, as occurs in Sweden (WEGENER; HALD; LO FO WONG; MADSEN *et al.*, 2003).

Kich *et al*. (2007) developed an enzyme-linked immunosorbent assay (ELISA) capable of detecting antibodies against most of the prevalent *Salmonella* serotype found in the Brazilian pork production chain; despite this, up to this date, no official program has been established in the country to monitor this pathogen in swine production (KICH; SCHWARZ; SILVA;

COLDEBELLA *et al.*, 2007). This study aimed to assess the adequacy of using an ELISA based assay as an alternative to screen and support tool to predict the presence of *Salmonella* in the key sites of swine carcasses.

2. Materials and Methods

2.1. Sampling

A pork production chain located in Paraná State, Brazil, and subjected to official inspection by the Brazilian Ministry of Agriculture, was selected for the present study with the agreement of the owners. Two days before transport to the slaughterhouse, the swine batches were visited and samples from barn floors were obtained by footprint, as described by (BOTTELDOORN; HEYNDRICKX; RIJPENS; GRIJSPEERDT *et al.*, 2003). Each swine batch is from a different farm. After arrival at the slaughterhouse, the lairage floor was sampled by footprint (BOTTELDOORN; HEYNDRICKX; RIJPENS; GRIJSPEERDT *et al.*, 2003) and 10 animals per batch were randomly selected. During the slaughtering steps, the following samples of the selected animals were collected: blood (after bleeding), carcass surface after bleeding (400 cm²), carcass after singeing (400 cm²), carcass after evisceration (400 cm²), carcass after final washing (400 cm²), and from the diaphragm, palatine tonsils and mesenteric lymph nodes. The samples were collected from the same carcasses throughout slaughter steps. Surface samples of carcasses were obtained by swabbing with sterile sponges, which were moistened previously with 10 mL of buffered peptone water (BPW; 0.1%, w/v, Oxoid Ltd., Basingstoke, England), on four 100 cm² areas, as described by ISO 17604 (ISO, 2015b). All samples were stored at 4° C until analysis.

2.2. *Salmonella* detection by microbiological method

All samples, except for blood and diaphragm, were subjected to *Salmonella* detection according ISO 6579 (ISO, 2002), with some modifications. Samples obtained by overshoes and swabbing were transferred to sterile bags, added with BPW (0.1%, w/v, Oxoid) to create a final volume of 200 mL and homogenized for 1 min at 230 rpm (Stomacher 400, Seward, Worthing, England). Portions of 12.5 g of tonsils and mesenteric lymph nodes were transferred to sterile bags, added with 112.5 mL of BPW (1%, w/v, Oxoid) and homogenized as described above. Aliquots of 40 mL of the homogenates obtained were centrifuged at 2,000 × *g* for 15 min, the supernatant was discarded, and the pellet obtained was suspended with 10 mL of BPW (1%,

w/v, Oxoid), and then incubated at 37° C for 24 h. The cultures obtained were transferred to Rappaport Vassiliadis Soya broth (Oxoid) and Muller-Kauffman Tetrathionate Novobiocin broth (Oxoid), and incubated at 42° C and 37° C, respectively, for 24 h. The cultures were then streaked onto agar plates containing Xylose Lysine Deoxycholate Agar (Oxoid) and Mannitol Lysine Crystal Violet Brilliant Agar (Oxoid), and incubated at 37° C for 24 h; colonies that presented the typical morphology of *Salmonella* were selected and subjected to biochemical tests for identification. Cultures identified as *Salmonella* were subjected to PCR targeting *invA*, as described by (SWAMY; BARNHART; LEE; DREESEN, 1996) and *ompC*, as described by (ALVAREZ; SOTA; VIVANCO; PERALES *et al.*, 2004) to confirm the identification with *Salmonella* Abony NCTC 6017 used as a positive control. Thirty-nine isolates were selected based on their sample origin and subjected to agglutination assays using *Salmonella* antisera (Denka Seiken Co., Tokyo, Japan) in order to identify their serogroups.

2.3. *Salmonella* detection by serology

Blood and diaphragm samples were processed and subjected to an ELISA assay to detect *Salmonella* infection detection by serology, as described by (KICH; SCHWARZ; SILVA; COLDEBELLA *et al.*, 2007). Just after sampling, blood samples were centrifuged at 2,000 × *g* for 15 min, and the serum obtained was stored in sterile flasks at -80° C; also, fragments of the collected diaphragms were frozen at -80° C for 24 h, thawed at 4° C for 24 h, the meat juice obtained was collected in sterile flasks and stored at -80° C. Just prior to the ELISA assay, serum and meat juice samples were thawed at 4° C for 8 h, diluted at 1:400 and 1:30, respectively, in Phosphate Buffer Sodium (PBS) supplemented with Tween 20 (0.05%, v/v) and bovine serum albumin (1%, v/v) (PBS-TA), pH 7.4, and transferred in triplicates to 96 well plates previously prepared with the antigen (1:2,000). After incubation at 37° C for 30 min, the plates were washed with PBS-TA, and 100 µL of anti-pig IgG conjugated to horseradish peroxidase, diluted at 1:25,000 in PBS-TA, were added per well. The plates were incubated at 37° C for 1 h, washed with tap water, and added to 100 µL per well of a substrate (3.5 µL H_2O_2, 230 µL 10 N NaOH, and 10 µL 3,39,5,59 tetramethyl-benzidine) for color reaction. The plates were incubated at 25° C for 15 min, when the reaction was stopped by adding 50 µL of 2 M sulphuric acid in each well. The optical density at 450 nm was assessed using a plate reader (Titertek Multiscan, McLean, VA). The matrices from a known positive and negative animals were used as a positive and negative control, respectively. The results for both matrices were expressed as OD% obtained by “sample-to-positive (S/P)” ratio multiplied by 100, using the

following equation:

$$S/P = \frac{(Median\ OD\ of\ sample - Median\ OD\ of\ negative\ control)}{(Median\ OD\ of\ positive\ control - Median\ OD\ of\ negative\ control)} \times 100$$

In this study two different cut-off values were evaluated: samples were considered positive with OD% > 20% or OD% > 40% (KICH; SCHWARZ; SILVA; COLDEBELLA *et al.*, 2007).

2.4. Data analysis

The results obtained for pig batches (barns and lairage floors) were considered with the corresponding pig carcasses sampled during slaughtering. Additionally, a pig carcass was considered positive for *Salmonella* spp. by the microbiological detection method when the pathogen was isolated in at least one slaughtering step. ELISA results were compared considering the different cut-offs adopted in the readings; and the results obtained by ELISA (20% and 40% cut-off, from blood and meat juice) and microbiological detection (barn and lairage floor) were considered as indicative of the presence of *Salmonella* spp. in tonsils, mesenteric lymph nodes and carcasses. The agreement of results for *Salmonella* spp. in different samples for a same animal, but obtained by different methodologies, were calculated and compared by the Cohen's Kappa index using the software OpenEpi (DEAN; SULLIVAN; SOE, 2006), and by the McNemar test ($P < 0.05$), using the software XLStat (Addinsoft Inc., New York, NY, USA). ODs from blood serum and meat juice samples were compared by linear regression ($P < 0.05$), using the software XLStat (Addinsoft).

3. Results

Table 1 shows the results for *Salmonella* spp. in the samples obtained. The pathogen was detected mainly from the lairage floors (7/10), followed by tonsils (45/100), and mesenteric lymph nodes (43/100). Selected *Salmonella* confirmed isolates were characterized as belonging to serogroups O:4 (32/39), O:3 (3/39), O:9 (2/39), O:8 (1/39) and O:7 (1/39). Based on the serological results from blood serum and meat juice, higher frequencies of *Salmonella* spp. seropositive animals were observed when considering the ELISA 40% cut-off (Table 1). Individual OD readings for each tested sample are presented in the Supplementary Table 1.

Table 1. Frequencies of positives results to *Salmonella* sp. based on different approaches, samples and protocols in Paraná state, Brazil, 2018.

Method	Protocol specification	Sample	Tested	*Salmonella* spp.
Isolation	ISO 6579	Barn floor	10	3
		Lairage floor	10	7
		Pig carcass after bleeding	100	2
		Pig carcass after singeing	100	0
		Pig carcass after evisceration	100	0
		Pig carcass after end washing	100	1
		*Pig carcass**	*100*	*3*
		Tonsil	100	45
		Mesenteric lymph node	100	43
Serology	Cut off 20% (20CO)	Blood serum	100	86
		Meat juice	100	68
	Cut off 40% (40CO)	Blood serum	100	46
		Meat juice	100	37

* Considering at least one *Salmonella* spp. positive result in the different slaughtering steps of a same pig carcass

The correlation between serum and meat juice was very high (r = 0.93, P < 0.001; Figure 1), and Table 2 shows the agreement of *Salmonella* spp. positive results in these samples obtained by ELISA, when considering 20% and 40% cut-offs. Despite the absence of agreement by McNemar (P < 0.05), OD readings presented a high and significant correlation index (Figure 1) and the Cohen's Kappa indices indicated a substantial agreement of the data obtained for samples when considering ELISA 40% cut-off (Table 2). A moderate agreement was observed by Cohen's Kappa test when the data obtained from blood serum with 40% cut-off and meat juice with 20% cut-off were compared (Table 2).

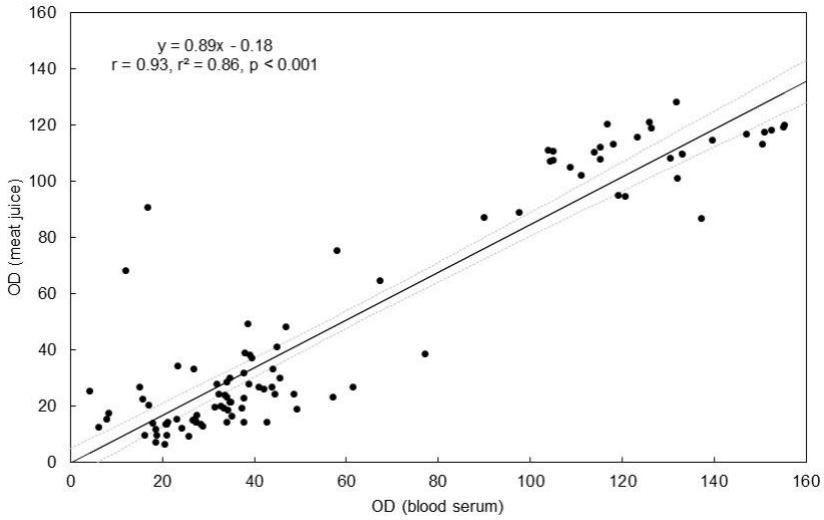

Figure 1. Dispersion of optical density readings (λ = 450 nm, values multiplied by 100) of serum and meat juice from 100 finishing pigs slaughtered in Paraná state, Brazil, 2018. r =

correlation index, r^2 = coefficient of determination of the adopted model, p = level of significance.

Figure 2 shows the analysis of the equivalent results obtained among the collected samples, when considering serum (ELISA), and meat juice (ELISA), as references for predicting the contamination of *Salmonella* spp. in tonsils, mesenteric lymph nodes and carcasses. The results obtained by ELISA 20% cut-off did not present significant agreement with results obtained by microbiological detection, while the results obtained by ELISA 40% cut-off presented significant agreement with results of microbiological detection from tonsils and mesenteric lymph nodes, using McNemar (Table 3). When considering the Cohen's Kappa, the results from blood serum and meat juice obtained by ELISA 40% cut off presented moderate agreement with the conventional isolation of mesenteric lymph nodes (Figure 2).

Table 4 shows the analysis of the coincident results of *Salmonella* spp. obtained only by microbiological detection method, when considering as a reference the presence of positive results in the floors of barns and lairage. Based on the data obtained in this analysis, the presence of *Salmonella* spp. in barn and lairage floors did not present significant association or agreement with the detection of the pathogen in the key contamination points of pig carcasses during slaughtering, based on McNemar and Cohen's Kappa (Table 4).

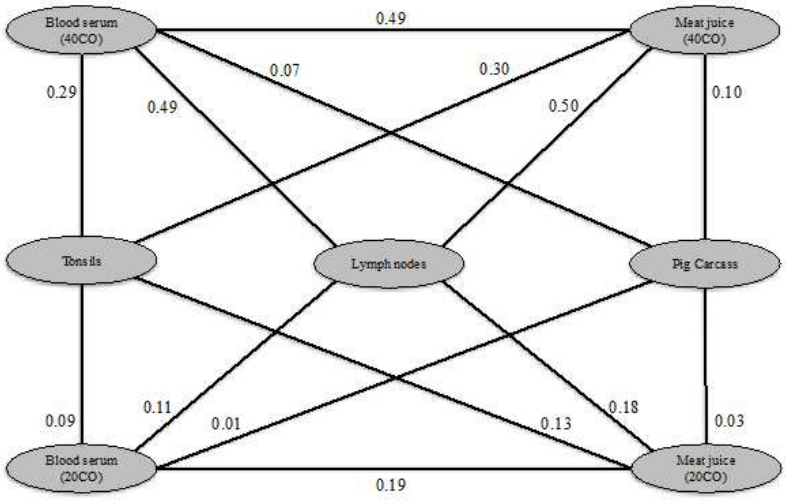

Figure 2. Comparison of *Salmonella* spp. detection by ELISA (20% and 40% cut-offs, 20CO and 40CO, respectively, in 100 blood serum samples and 100 meat juice samples) and conventional isolation (100 tonsils, 100 mesenteric lymph nodes and 100 pig carcasses) in

Paraná state, Brazil, 2018. The numbers between sample types are the Kappa reference value (K). Cohen's Kappa interpretation: K < 0.00 - poor agreement; 0.00 < K < 0.20 - slight agreement; 0.21 < K < 0.40 - fair agreement; 0.41 < K < 0.60 - moderate agreement; 0.61 < K < 0.80 - substantial agreement; 0.81 < K < 1.00 - almost perfect agreement.

Table 2. Comparison of *Salmonella* spp. detection in 100 blood serums (BS) and 100 meat juices (MJ) obtained by ELISA, considering 20% and 40% cut-offs (20CO and 40CO, respectively) in Paraná state, Brazil, 2018.

Comparison	Coincident		Divergent		McNemar[1]		Cohen's Kappa[2]		
	positive	negative	pos × neg	neg × pos	Q	P	K	CI 95%	P
Blood serum (20CO) × Meat juice (20CO)	62	8	24	6	9.63	0.002	0.19	0.02 - 0.36	0.031*
Blood serum (40CO) × Meat juice (40CO)	34	51	12	3	4.27	0.040	0.69	0.50 - 0.89	< 0.0001
Blood serum (20CO) × Meat juice (40CO)	35	12	51	2	43.47	< 0.0001	0.11	0.00 - 0.22	0.029
Blood serum (40CO) × Meat juice (20CO)	44	30	2	24	16.96	< 0.0001	0.49	0.32 - 0.67	< 0.0001

Q: McNemar reference value, K: Kappa reference value, P: level of significance, CI 95%: confidence interval at 95%. [1] McNemar interpretation: p values higher than 0.05 indicate equivalency of results. [2] Cohen's Kappa interpretation: K < 0.00 - poor agreement; 0.00 < K < 0.20 - slight agreement; 0.21 < K < 0.40 - fair agreement; 0.41 < K < 0.60 - moderate agreement; 0.61 < K < 0.80 - substantial agreement; 0.81 < K < 1.00 - almost perfect agreement. *Chi-square test with Yates correction, due to one of the expected frequencies lower than 5.

Table 3. Comparison of *Salmonella* spp. detection in 100 blood serums, 100 meat juices obtained by ELISA considering 20% and 40% cut-offs (20CO and 40CO, respectively) and conventional isolation (100 mesenteric lymph nodes, 100 tonsils and 100 pig carcasses) in Paraná state, Brazil, 2018.

Comparison	Coincident		Divergent		McNemar[1]	
	positive	negative	pos × neg	neg × pos	Q	P
Blood serum (20CO) × Lymph nodes	40	11	46	3	36.00	0.000
Blood serum (20CO) × Tonsils	41	10	45	4	32.65	0.000
Blood serum (20CO) × Carcasses	3	14	83	0	81.01	0.000
Meat juice (20CO) × Lymph nodes	34	23	34	9	13.40	0.000
Meat juice (20CO) × Tonsils	34	21	34	11	10.76	0.001
Meat juice (20CO) × Carcasses	3	32	65	0	63.02	< 0.0001
Blood serum (40CO) × Lymph nodes	32	43	14	11	0.16	0.689
Blood serum (40CO) × Tonsils	28	37	18	17	0.00	1.000
Blood serum (40CO) × Carcasses	3	54	43	0	41.02	< 0.0001
Meat juice (40CO) × Lymph nodes	28	48	9	15	1.04	0.307
Meat juice (40CO) × Tonsils	24	42	13	21	1.44	0.230
Meat juice (40CO) × Carcasses	3	63	34	0	32.03	< 0.0001

Q: McNemar reference value, P: level of significance, CI 95%: confidence interval at 95%. [1]McNemar interpretation: p values higher than 0.05 indicate equivalency of results.

Table 4. Comparison of *Salmonella* spp. detection by conventional isolation in 10 barn floors[1], 10 lairage floors[1], 100 tonsils, 100 mesenteric lymph nodes and 100 pig carcasses in Paraná state, Brazil, 2018.

Comparison	Coincident		Divergent		McNemar[2]		Cohen's Kappa[3]		
	positive	negative	pos × neg	neg × pos	Q	P	K	CI 95%	P
Barn floors × Lymph nodes	18	46	11	25	4.69	0.030	0.24	0.05 - 0.42	0.007
Barn floors × Tonsils	16	42	13	29	5.36	0.021	0.12	-0.06 - 0.31	0.096
Barn floors × Pig carcasses	3	71	26	0	24.04	< 0.0001	0.14	0.04 - 0.24	0.018*
Lairage floors × Lymph nodes	36	23	33	7	15.63	< 0.0001	0.23	0.06 - 0.40	0.004
Lairage floors × Tonsils	36	22	33	9	12.60	0.000	0.19	0.02 - 0.36	0.016
Lairage floors × Pig carcasses	3	31	66	0	64.02	< 0.0001	0.03	-0.02 - 0.07	0.293*

[1] results for barn and lairage floors were obtained per animal lot (10) and extrapolated to corresponding pigs (100). Q: McNemar reference value, K: Kappa reference value, p: level of significance, CI 95%: confidence interval at 95%. [2] McNemar interpretation: p values higher than 0.05 indicate equivalency of results. [3] K: Kappa reference value, P: level of significance, CI 95%: confidence interval at 95%. Cohen's Kappa interpretation: K < 0.00 - poor agreement; 0.00 < K < 0.20 - slight agreement; 0.21 < K < 0.40 - fair agreement; 0.41 < K < 0.60 - moderate agreement; 0.61 < K < 0.80 - substantial agreement; 0.81 < K < 1.00 - almost perfect agreement. *Chi-square test with Yates correction, due to one of the expected frequencies lower than 5.

4. Discussion

Based on the results obtained by microbiological detection, the presence of *Salmonella* spp. in the barns and lairage floors indicates excretion by the animals in the producing environment, and is confirmed by the presence of positive results in tonsils and lymph nodes (Table 1). ELISA results confirmed these results in the sampled animals (Table 1). The stressing conditions that the animals are subjected during transport and slaughtering are highly associated with *Salmonella* excretion by the animals, supporting the observed results (ARGÜELLO; ALVAREZ-ORDONEZ; CARVAJAL; RUBIO *et al.*, 2013; MANNION; EGAN; LYNCH; FANNING *et al.*, 2008; SIMONS; HILL; SWART; KELLY *et al.*, 2016). In addition, fasting periods higher than 4 h are considered critical to *Salmonella* spp. excretion by carrier animals (EICHER; ROSTAGNO; LAY, 2017; MARTÍN-PELÁEZ; PERALTA; CREUS; DALMAU *et al.*, 2009). Mesenteric lymph nodes are considered the key sites to monitor *Salmonella* spp. in carriers animals (EFSA, 2006) and have led to the adoption of proper control measures during slaughtering; similar frequencies of *Salmonella* spp. in mesenteric lymph nodes were previously recorded in similar studies conducted in Brazil (CABRAL; PANZENHAGEN; DELGADO; SILVA *et al.*, 2017; KICH; COLDEBELLA; MORES; NOGUEIRA *et al.*, 2011). Tonsils are also usually screened for the presence of *Salmonella* spp. as an indicator of carrier animals and the potential entrance of the pathogen into the slaughtering process (SILVA; FARIA; PAULA; MARTINS *et al.*, 2009; VAN DAMME; MATTHEUS; BERTRAND; DE ZUTTER, 2018; ZDOLEC; DOBRANIC; FILIPOVIC, 2015). Despite being detected in the animals, *Salmonella* spp. were isolated on only three carcasses, which indicates good manufacturing practices in the slaughterhouse selected for the present study (Table 1). These data emphasize the importance of slaughterhouse hygiene measures, where further procedures might be adopted to reduce the risk of contamination, based on serological data.

Also, despite being routinely studied for *Salmonella* spp. detection and monitoring in swine, tonsils and mesenteric lymph nodes are considered important points of contamination during slaughtering. When these tissues are opened or removed, *Salmonella* spp. spreading can occur to the pig carcass and to the environment (BIASINO; DE ZUTTER; MATTHEUS; BERTRAND *et al.*, 2018; VAN DAMME; MATTHEUS; BERTRAND; DE ZUTTER, 2018). Contaminated feces can also increase the chances of contamination by *Salmonella* spp. in the slaughterhouse (CASANOVA-HIGES; ANDRES-BARRANCO; MAINAR-JAIME, 2017; CORBELLINI; JUNIOR; COSTA; DUARTE *et al.*, 2016; MAINAR-JAIME; CASANOVA-HIGES; ANDRES-BARRANCO; VICO, 2018; SILVA; DIAS; FERRONATTO; GUERRA *et*

al., 2012). The lower is the presence and the load of *Salmonella* spp. in the animal intestines, the lower the chances for carcass contamination during slaughtering, highlighting the relevance of proper monitoring of the animals (BAPTISTA; DAHL; NIELSEN, 2010; PESCIAROLI; CUCCO; DE LUCA; MASSACCI *et al.*, 2017).

The most prevalent serogroup identified amongst the characterized isolates was the O:4, which includes *S.* Typhimurium, usually identified in the Brazilian pork production chain (BIASINO; DE ZUTTER; MATTHEUS; BERTRAND *et al.*, 2018; CABRAL; PANZENHAGEN; DELGADO; SILVA *et al.*, 2017; KICH; COLDEBELLA; MORES; NOGUEIRA *et al.*, 2011; PARADA; CARRANZA; ALVAREZ; PICHEL *et al.*, 2017). These results are in agreement with the data obtained by ELISA, once the protocol used considers the detection of IgG against the lipopolysaccharide antigens O1, O4, O5 and O12, from *S.* Typhimurium. Due to antigenic similarity, the adopted ELISA assay is also able to detect other *Salmonella* serotypes, like Agona, Derby, Bredeney and Panama, which are highly prevalent in swine from Southern Brazil (KICH; COLDEBELLA; MORES; NOGUEIRA *et al.*, 2011; KICH; COSTA; TRIQUES; NIOGUEIRA *et al.*, 2016; KICH; SCHWARZ; SILVA; COLDEBELLA *et al.*, 2007; SCHWARZ; CALVEYRA; SELLA; BESSA *et al.*, 2009; SILVA; DIAS; FERRONATTO; GUERRA *et al.*, 2012).

The frequency of animals that presented positive results for *Salmonella* spp. by serological tests were higher when compared to the frequency of positive results in the microbiological detection procedure, when considering tonsils and mesenteric lymph nodes (Table 1), as already reported by Kich *et al.* (2011) and *Silva et al.* (2012). Antibodies against *Salmonella* spp. are produced in an infected pig after 7 to 15 days of contact with the pathogen, but not all animals become carriers, as they are able to eliminate it completely; thus, many animals will present serological results for *Salmonella* spp. without becoming infected (GRADASSI; CAMINITI; GALLETTI; SANTI *et al.*, 2015; KICH; COSTA; TRIQUES; NIOGUEIRA *et al.*, 2016; KICH; SCHWARZ; SILVA; COLDEBELLA *et al.*, 2007; VICO; ENGEL; BUIST; MAINAR-JAIME, 2010). However, the isolation of *Salmonella* spp. in tonsils and mesenteric lymph nodes from animals that were negative by serological assays is also possible; previous studies have demonstrated that *Salmonella* spp. can reach the intestinal lymphoid tissues within 2 h after the infection, and it can be isolated from intestine of pigs; but, several days are necessary for the production of immunoglobulins (BOUGHTON; EGAN; KELLY; MARKEY *et al.*, 2007; HURD; GAILEY; MCKEAN; ROSTAGNO, 2001; ROSTAGNO; EICHER; LAY, 2011).

A substantial agreement by Cohen's Kappa test was observed among *Salmonella* results

obtained by ELISA 40% cut off (Table 2), as well as a high and significant correlation index (Figure 1). Thus, some agreement among OD readings can be observed despite the differences in the composition of both matrices, which are usually considered the major cause of differences in their performance in ELISA-based assays (MAINAR-JAIME; ATASHPARVAR; CHIRINO-TREJO; BLASCO, 2008; VICO; MAINAR-JAIME, 2011). Despite these differences, meat juice would be an important alternative to blood serum, because fragments of this diaphragm muscle are easily collected during slaughter, which allows for the establishment of a pattern of historical data of *Salmonella* spp. contamination in the pork production chain: the high and significant correlation of OD readings from blood serum and meat juice supports this interpretation (Figure 1). Immunoglobulins in meat juice are present in lower levels when compared to blood serum (STEINBACH; METHNER; MEYER, 2003; SZABO; SCHERER; ROESLER; APPEL *et al.*, 2008); this might be a relevant factor in determining the low sensitivity of this matrix for ELISA based assays (GRADASSI; CAMINITI; GALLETTI; SANTI *et al.*, 2015; VICO; MAINAR-JAIME, 2011).

No serological data from blood serum and meat juice, with a 20% cut-off, were in agreement with data from a *Salmonella* spp. isolation from tonsils, mesenteric lymph nodes and carcasses based on McNemar ($P < 0.05$; Table 3) and Cohen's Kappa test (Figure 2). However, despite presenting some data in disagreement, with a 40% cut off in serological tests, there was allowed a significant agreement with microbiological detection in tonsils and mesenteric lymph nodes by the McNemar test ($P > 0.05$; Table 3). Moderate agreement indices by the Cohen's Kappa test were observed for *Salmonella* results by ELISA 40% cut-off from blood serum and meat juice when compared to microbiological detection from mesenteric lymph nodes ($K = 0.49$ and 0.50 respectively) (Figure 2).

Better agreement results can be obtained if the cut-off is changed, but it is a procedure that must be adopted with care. The cut-off value at which the combination of sensitivity and specificity is maximal is not always the best choice for an assay, and may lead to relevant economic and public health implications due to inadequate data interpretation. Ideally, the cut-off value for an assay must be adjusted based on the history of the prevalence of the target organism in the studied region, and the development of a surveillance program (KICH; SCHWARZ; SILVA; COLDEBELLA *et al.*, 2007; LO FO WONG; DAHL; WINGSTRAND; VAN DER WOLF *et al.*, 2004). Because many studies demonstrate that the *Salmonella* spp. prevalence in swine production, based on ELISA assays with a 40% cut-off, most commercial kits for *Salmonella* detection are standardized with this value (ARGÜELLO; MANZANILLA; LYNCH; WALIA *et al.*, 2018; CASANOVA-HIGES; ANDRES-BARRANCO; MAINAR-

JAIME, 2017; GRADASSI; CAMINITI; GALLETTI; SANTI *et al.*, 2015; MAINAR-JAIME; ATASHPARVAR; CHIRINO-TREJO; BLASCO, 2008). Lowering the cut-off value may result in an increase of false positive results: thus, lowering of cut-off will not necessarily improve agreement among serological and microbiological results. The relevance of adopting different cut-off values for *Salmonella* serological tests has already been reported in the literature (GRADASSI; CAMINITI; GALLETTI; SANTI *et al.*, 2015; KICH; COSTA; TRIQUES; NIOGUEIRA *et al.*, 2016; METHNER; RAMMLER; FEHLHABER; ROSLER, 2011).

Additionally, independent of the adopted cut-off, the ELISA assay considered in this study was designed based on the antigenic formula of *S.* Typhimurium (1,4,5,12:i:1,2), the most prevalent in southern Brazil and presents an antigenic formula similar to other serotypes commonly isolated in this region (KICH; COLDEBELLA; MORES; NOGUEIRA *et al.*, 2011; KICH; COSTA; TRIQUES; NIOGUEIRA *et al.*, 2016; KICH; SCHWARZ; SILVA; COLDEBELLA *et al.*, 2007; SCHWARZ; CALVEYRA; SELLA; BESSA *et al.*, 2009; SILVA; DIAS; FERRONATTO; GUERRA *et al.*, 2012). However, other serogroups that do not present similar antigenic formula to *S.* Typhimurium (group O:4) were isolated, such as O:3, O:9, O:8 and O:7, which may have contributed to some disagreement in the results (Figure 2). Considering the differences in the antigenic formulas of some of the identified *Salmonella* serogroups, the adopted ELISA assay may have failed to identify truly infected animals, as was already observed in previous studies (GRIMONT; WEILL, 2007; VICO; ENGEL; BUIST; MAINAR-JAIME, 2010).

Finally, based on data from Table 4, the presence of *Salmonella* spp. in barns and lairage does not necessarily indicate the presence of the same pathogen in tonsils and mesenteric lymph nodes, nor a contamination of pig carcasses. Thus, these data indicate that *Salmonella* carriers are not necessarily excreting it (DE BUSSER; MAES; HOUF; DEWULF *et al.*, 2011). According to the McNemar test and Cohen's Kappa, no significant agreement was observed when the barn floor was used as a reference for a contamination. A similar result was observed when the detection of *Salmonella* spp. in lairage was considered as a reference for subsequent contamination in slaughterhouse, which presented even more positive results.

5. Conclusions

According to the results, the best agreement between the tests for predicting the occurrence of *Salmonella* in swine (MLN and tonsils) was found to be the ELISA test with a

cut-off of 40% (independent of the matrix: blood serum or meat juice). In spite of the divergences and restrictions of each method, if used as an additional tool, the serological test is important for distinguishing herds with a greater probability of contamination from herds with a lesser probability of contamination at the time of slaughter. Considering such results, slaughterhouses can adopt preventive procedures to avoid *Salmonella* spp. spread in their facilities, leading them to take the proper decisions for the next batches coming from the same farm and also collect the data for epidemiological studies of the animals. The microbiological detection protocol is essential for monitoring the contamination of carcasses in the slaughterhouse and should be used in association with serology.

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Supplementary material

Supplementary Table 1. Individual optical density readings (λ = 450 nm, values multiplied by 100) obtained of blood serum and meat juice obtained from 100 swine slaughtered in Paraná state, Brazil.

Animal	Optical density	
	Blood serum	Meat juice
1	108.6	105.0
2	132.9	109.9
3	125.8	121.2
4	48.4	24.4
5	40.8	26.9
6	113.9	110.6
7	155.3	120.1
8	115.1	112.1
9	131.6	128.3
10	41.9	26.1
11	26.7	33.3
12	24.1	12.4
13	25.6	9.6
14	31.3	19.9
15	32.1	24.6
16	38.4	49.5
17	26.5	15.2
18	23.3	34.4
19	18.7	9.8
20	22.9	15.5
21	28.6	13.1
22	20.8	9.7
23	43.6	27.1
24	15.5	22.5
25	32.6	20.2
26	35.0	16.6
27	27.2	14.5
28	33.9	14.6
29	42.6	14.4
30	7.8	15.4
31	16.1	9.8
32	16.7	90.7
33	34.5	30.1
34	61.4	26.8
35	34.8	21.4
36	37.6	14.5
37	37.6	23.1
38	39.3	37.3
39	17.7	14.0
40	20.3	6.6
41	37.7	32.0
42	137.1	86.8
43	15.0	26.9
44	111.0	102.4
45	155.0	119.5
46	131.9	101.2
47	33.2	19.5
48	150.8	117.8
49	49.1	19.0

50	152.3	118.4
51	21.0	14.5
52	33.5	24.2
53	34.0	28.8
54	44.3	24.4
55	56.9	23.4
56	27.0	15.5
57	8.1	17.6
58	20.7	13.7
59	37.1	19.4
60	28.3	13.6
61	89.9	87.4
62	97.4	89.1
63	146.9	116.9
64	126.2	119.0
65	130.3	108.3
66	116.7	120.4
67	104.3	107.1
68	104.8	110.7
69	150.5	113.2
70	139.6	114.7
71	18.4	7.3
72	120.6	94.6
73	44.0	33.5
74	77.1	38.7
75	34.1	18.6
76	27.2	14.9
77	20.9	13.6
78	34.6	21.6
79	46.7	48.2
80	45.5	30.3
81	18.4	11.9
82	44.8	41.3
83	37.8	38.9
84	123.2	115.9
85	17.0	20.5
86	31.7	27.9
87	39.0	38.2
88	12.0	68.2
89	6.0	12.6
90	38.7	27.9
91	4.1	25.6
92	57.9	75.4
93	104.9	107.5
94	67.2	64.6
95	117.9	113.2
96	115.1	107.9
97	33.9	23.2
98	27.3	17.0
99	119.0	95.0
100	103.9	111.2

CHAPTER 2. Distribution, diversity, virulence genotypes and antibiotic resistance for *Salmonella* isolated from a Brazilian pork production chain

Short title: Pork *Salmonella* in Brazil

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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.

Keywords: *Salmonella*; pork; distribution; antibiotic resistance; ciprofloxacin

1. Introduction

Nontyphoidal *Salmonella enterica* is a common cause of foodborne disease outbreaks in Brazil and other countries (BRASIL, 2016; EFSA; ECDC, 2016). Pork products are commonly linked to these outbreaks (EFSA; 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; ALVAREZ-ORDONEZ; CARVAJAL; RUBIO *et al.*, 2013) with widespread contamination potentially occurring earlier during production, transport, and lairage (ARGÜELLO; ALVAREZ-ORDONEZ; CARVAJAL; RUBIO *et al.*, 2013; SIMONS; HILL; SWART; KELLY *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; GENSLER; BARRIOS, 2011; CABRAL; PANZENHAGEN; DELGADO; SILVA *et al.*, 2017; KICH; COLDEBELLA; MORES; NOGUEIRA *et al.*, 2011; MARIER; SNOW; FLOYD; MCLAREN *et al.*, 2014; PESCIAROLI; CUCCO; DE LUCA; MASSACCI *et al.*, 2017).

The widespread use of antibiotics in different steps of swine production can favor resistant strains of bacteria including *Salmonella* (ECDC; EFSA; EMA, 2017; LOPES; PISSETTI; PELLEGRINI; SILVA *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; ZHANG; XU; SHI *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 strains isolated from pork production (KICH; COLDEBELLA; MORES; NOGUEIRA *et al.*, 2011; LOPES; PISSETTI; PELLEGRINI; SILVA *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, 2018b). 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 1,000), 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 x 8 cm) in four delimited areas of 100 cm² according ISO 17604, (ISO, 2015a) 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; ALVAREZ-ORDONEZ; CARVAJAL; RUBIO *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 cm² 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 cm². 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 2,000 × *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 18h. 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 18h.

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 *XbaI* (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 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, Figure 1). Each pulsotype included 1 to 41 isolates that shared identical band patterns (100% of similarity) (Figure 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; BARNHART; LEE; DREESEN, 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 *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 (Figure 1) and belonging to serogroups O:4 and O:3.

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[1](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)*
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)*
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)

[1] as indicated in Figure 1; * ND: not digest with XbaI.

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

virulence profile	virulence genes	n	pulsotypes[1]
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

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

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

class[1]	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%)

[1] 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[1]	n	Pulsotypes[2]
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[3]
5	AMP-CHL-STR-TET-CIP	7	A3, A4, C3, B3, E1[3]
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

[1] 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; [2] as indicated in Figure 1; [3] both resistance patterns presented one isolate each without PFGE profile.

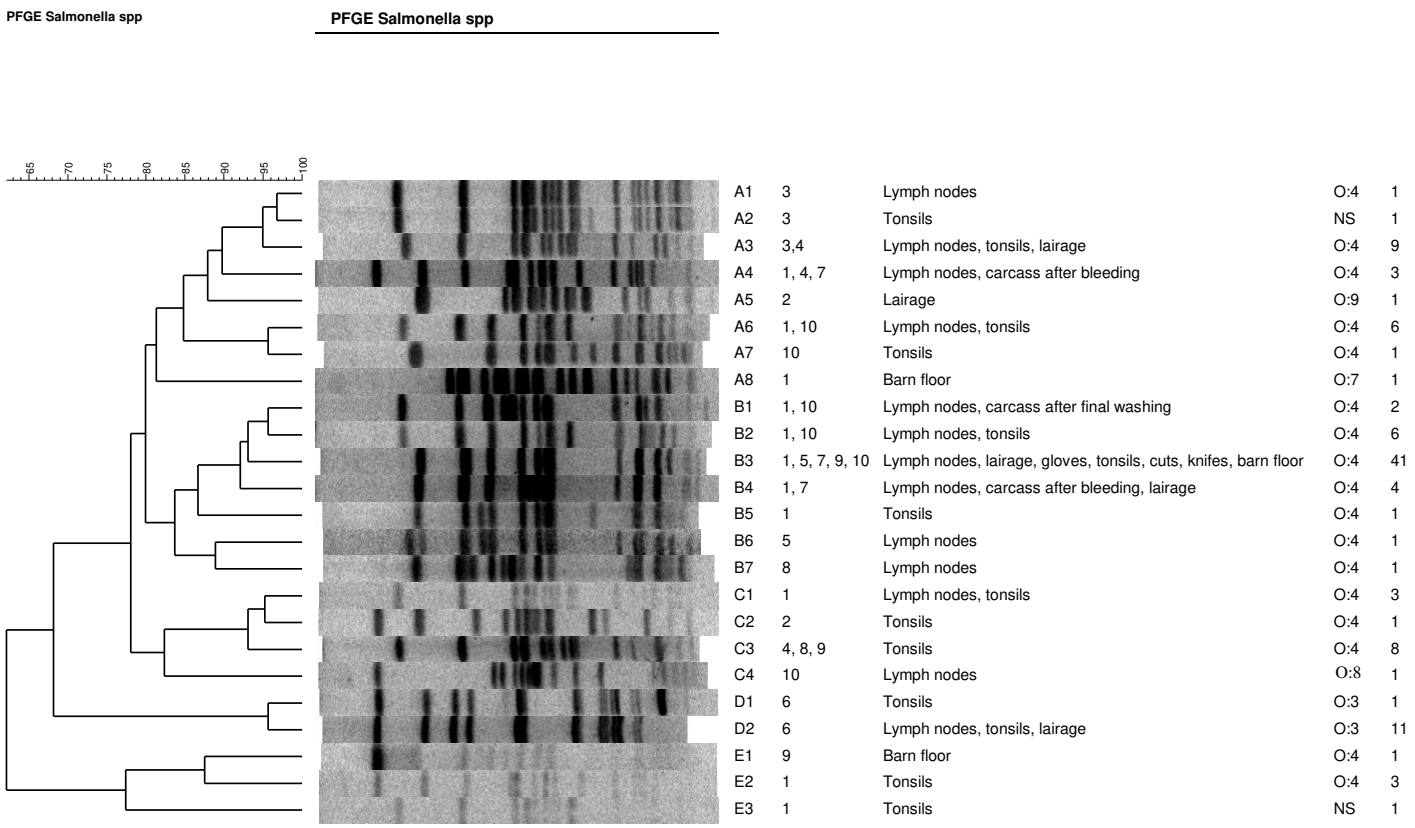

Figure 1: Schematic representation (pattern, day, sources, serogroups and n° of isolates) of 24 unique band patterns from *Salmonella* strains that were isolated from farms and slaughterhouses in Brazil. Macro-restriction was completed with XbaI. Identity was estimated using the Dice coefficient (5%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

4. Discussion

Based on the obtained results, the frequency of *Salmonella enterica* isolation increased from farms to lairage (Table 1). As pig are asymptomatic carriers of *Salmonella* spp., they can act as shedders under stress and transmit the pathogen in their feces (SIMONS; HILL; SWART; KELLY *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).

Salmonella enterica was isolated from 43% of mesenteric lymph node samples (Table 1), as previously observed in similar studies (CABRAL; PANZENHAGEN; DELGADO; SILVA *et al.*, 2017; KICH; COLDEBELLA; MORES; NOGUEIRA *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; CUCCO; DE LUCA; MASSACCI *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; PANZENHAGEN; DELGADO; SILVA *et al.*, 2017; VAN DAMME; MATTHEUS; BERTRAND; DE ZUTTER, 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; DE ZUTTER; MATTHEUS; BERTRAND *et al.*, 2018; VAN DAMME; MATTHEUS; BERTRAND; DE ZUTTER, 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; SEEGERS; MAGRAS, 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 among *Salmonella* positive herds (ARGÜELLO; ALVAREZ-ORDONEZ; CARVAJAL; RUBIO *et al.*, 2013; DUGGAN; MANNION; PRENDERGAST; LEONARD *et al.*, 2010). *Salmonella* spp. frequencies in pig carcasses are variable, being directly associated to the adoption of adequate hygienic procedures (BONARDI; BASSI; BRINDANI; D'INCAU *et al.*, 2013; CABRAL; PANZENHAGEN; DELGADO; SILVA *et al.*, 2017; KICH; COLDEBELLA; MORES; NOGUEIRA *et al.*, 2011; MARIER; SNOW; FLOYD; MCLAREN

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; PANZENHAGEN; DELGADO; SILVA *et al.*, 2017; KICH; COLDEBELLA; MORES; NOGUEIRA *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; ALVAREZ-ORDONEZ; CARVAJAL; RUBIO *et al.*, 2013; GOMES-NEVES; ANTUNES; TAVARES; THEMUDO *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, Figure 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; DE ZUTTER; MATTHEUS; BERTRAND *et al.*, 2018; BONARDI, 2017; CABRAL; PANZENHAGEN; DELGADO; SILVA *et al.*, 2017; KICH; COLDEBELLA; MORES; NOGUEIRA *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; 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; PIRAS; TORPDAHL; MAZZA *et al.*, 2017; LI; PAN; KANG; GENG *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 (Figure 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 (Figure 1). Isolates from pulsotype A3 were isolated during two consecutive sampling efforts and in mesenteric lymph nodes, palatine tonsils and in the lairage (Figure 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 (Figure 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; ANTUNES; TAVARES; THEMUDO *et al.*, 2012; HERNÁNDEZ; GÓMEZ-LAGUNA; LUQUE; HERRERA-LEÓN *et al.*, 2013; KICH; COLDEBELLA; MORES; NOGUEIRA *et al.*, 2011; PATCHANEE; TANSIRICHAROENKUL; BUAWIRATLERT; WIRATSUDAKUL *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 (Figure 1). As pigs are *Salmonella* reservoirs (ARGÜELLO; ALVAREZ-ORDONEZ; CARVAJAL; RUBIO *et al.*, 2013), the presence of different strains in lymphatic tissues would be expected and a plausible explanation for these results (Figure 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; BASSI; BRINDANI; D'INCAU *et al.*, 2013; GOMES-NEVES; ANTUNES; TAVARES; THEMUDO *et al.*, 2012; HERNÁNDEZ; GÓMEZ-LAGUNA; LUQUE; HERRERA-LEÓN *et al.*, 2013; KICH; COLDEBELLA; MORES; NOGUEIRA *et al.*, 2011; PATCHANEE; TANSIRICHAROENKUL; BUAWIRATLERT; WIRATSUDAKUL *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; WILSON; CHESSA; ANDREWS-POLYMENIS *et al.*, 2005; SKYBERG; LOGUE; NOLAN, 2006). *spaN* and *sipB* have some relationship with entry into non-phagocytic cells and killing of macrophages (CHEN; KANIGA; GALÁN, 1996). Other specific virulence genes are related with the survival and replication within macrophages, such as *spiA*, *sifA*, *pagC* and *msgA* (BOHAYCHUK; GENSLER; BARRIOS, 2011; ZHANG; FAN; GE;

YAN *et al.*, 2013). *spvB* and *spvC* contribute in adhesion and systemic infection against the host cells (IBARRA; STEELE-MORTIMER, 2009). *sitC* and *iroN* are responsible to encode products for iron uptake (HAGHJOO; GALÁN, 2004; SKYBERG; LOGUE; NOLAN, 2006). *cdtB* encodes the cytolethal-distending toxin (HAGHJOO; 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 (Figure 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; AKTAS; TUGRUL, 2018; ZOU; KEELARA; THAKUR, 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; EFSA; EMA, 2017). The emergence of MDR *Salmonella* has been considered one of the main concerns related to global health (LOPES; PISSETTI; PELLEGRINI; SILVA *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; HENDRIKSEN; LE HELLO; WEILL *et al.*, 2016).

Swine-related samples and pork products usually present MDR *Salmonella* (BARILLI; BACCI; STELLAVILLA; MERIALDI *et al.*, 2018b; CALAYAG; PACLIBARE; SANTOS; BAUTISTA *et al.*, 2017; COLELLO; RUIZ; PADÍN; ROGÉ *et al.*, 2018; FOIS; PIRAS; TORPDAHL; MAZZA *et al.*, 2017; LOPES; PISSETTI; PELLEGRINI; SILVA *et al.*, 2015; SINWAT; ANGKITTITRAKUL; COULSON; PILAPIL *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; PIRAS; TORPDAHL; MAZZA *et al.*, 2017; LOPES; PISSETTI; PELLEGRINI; SILVA *et al.*, 2015; PATCHANEE; TANSIRICHAROENKUL; BUAWIRATLERT; WIRATSUDAKUL *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;

PISSETTI; PELLEGRINI; SILVA *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; BACCI; STELLAVILLA; MERIALDI *et al.*, 2018b; CAMERON-VEAS; FRAILE; NAPP; GARRIDO *et al.*, 2018; FOIS; PIRAS; TORPDAHL; MAZZA *et al.*, 2017; LOPES; PISSETTI; PELLEGRINI; SILVA *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; BACCI; STELLAVILLA; MERIALDI *et al.*, 2018b; CAMERON-VEAS; FRAILE; NAPP; GARRIDO *et al.*, 2018). The absence of resistance to carbapenems in *Salmonella* isolates (Table 3) is in agreement with similar studies (CALAYAG; PACLIBARE; SANTOS; BAUTISTA *et al.*, 2017; FARDSANEI; SOLTAN DALLAL; DOURAGHI; MEMARIANI *et al.*, 2018); *Salmonella* is usually susceptible to carbapenems, being considered as the last choice for treatment of salmonellosis caused by resistant strains (FERNÁNDEZ; GUERRA; RODICIO, 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; FRAILE; NAPP; GARRIDO *et al.*, 2018; GUERRA FILHO; YAMATOGI; POSSEBON; FERNANDES *et al.*, 2016; JIU; ZHU; KHAN; SUN *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 pig, 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; DENG; FANG; YANG *et al.*, 2017; KUANG; ZHANG; XU; SHI *et al.*, 2018). The veterinary analogue for ciprofloxacin is enrofloxacin, and enrofloxacin use will select for ciprofloxacin resistance (MCDERMOTT; BODEIS; ENGLISH; WHITE *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; DAVIES; THRELFALL, 2005; KUANG; ZHANG; XU; SHI *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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Supplementary Table 1. Primers used to detection of virulence genes according to the protocol described by Skyberg et al., 2006.

Target gene	Function	Primer sequences	Annealing temperature (°C)[1]	Size (bp)	Reference
invA	Invasion (identification)	TTGTTACGGCTATTTTGACCA CTGACTGCTACCTTGCTGATG	42	521	Swamy et al. (1996)
ompC	Identification	ATCGCTGACTTATGCAATCG CGGGTTGCGTTATAGGTCTG	57	204	Alvarez et al. (2004)
cdtB	Toxin biosynthesis	ACAACTGTCGCATCTCGCCCCGTCATT CAATTTGCGTGGGTTCTGTAGGTGCGAGT	66.5	268	Skyberg et al. (2006)
sifA	Filamentous structure formation	TTTGCCGAACGCGCCCCCACACG GTTGCCTTTTCTTGCGCTTTCCACCCATCT	66.5	449	Skyberg et al. (2006)
iroN	Iron acquisition	ACTGGCACGGCTCGCTGTCGCTCTAT CGCTTTACCGCCGTTCTGCCACTGC	66.5	1,205	Skyberg et al. (2006)
spiA	Survival within macrophage	CCAGGGGTCGTTAGTGTATTGCGTGAGATG CGCGTAACAAAGAACCCGTAGTGATGGATT	66.5	550	Skyberg et al. (2006)
sitC	Iron acquisition	CAGTATATGCTCAACGCGATGTGGGTCTCC CGGGGCGAAAATAAAGGCTGTGATGAAC	66.5	768	Skyberg et al. (2006)
pagC	Survival within macrophage	CGCCTTTTCCGTGGGGTATGC GAAGCCGTTTATTTTTGTAGAGGAGATGTT	66.5	454	Skyberg et al. (2006)
msgA	Survival within macrophage	GCCAGGCGCACGCGAAATCATCC GCGACCAGCCACATATCAGCCTCTTCAAAC	66.5	189	Skyberg et al. (2006)
orgA	Host recognition/invasion	TTTTTGGCAATGCATCAGGGAACA GGCGAAAGCGGGGACGGTATT	66.5	255	Skyberg et al. (2006)
sipB	Entry into nonphagocytic cells, killing of macrophages	GGACGCCGCCCGGGAAAAACTCTC ACACTCCCGTCGCCGCCTTCACAA	66.5	875	Skyberg et al. (2006)
spvB	Growth within host	CTATCAGCCCCGCACGGAGAGCAGTTTTTA GGAGGAGGCGGTGGCGGTGGCATCATA	66.5	717	Skyberg et al. (2006)
tolC	Host recognition/invasion	TACCCAGGCGCAAAAAGAGGCTATC CCGCGTTATCCAGGTTGTTGC	66.5	161	Skyberg et al. (2006)
prgH	Host recognition/invasion	GCCCGAGCAGCCTGAGAAGTTAGAAA	66.5	756	Skyberg et al. (2006)

		TGAAATGAGCGCCCCTTGAGCCAGTC			
sopB	Host recognition/invasion	CGGACCGGCCAGCAACAAAACAAGAAGAAG TAGTGATGCCCGTTATGCGTGAGTGTATT	66.5	220	Skyberg et al. (2006)
spaN	Entry into nonphagocytic cells, killing of macrophages	AAAAGCCGTGGAATCCGTTAGTGAAGT CAGCGCTGGGGATTACCGTTTTG	66.5	504	Skyberg et al. (2006)
spvC	Growth within host	CGGAAATACCATCTACAAATA CCCAAACCCATACTTACTCTG	42	669	Skyberg et al. (2006)

[1] PCR conditions for: *invA*: 93 °C for 5 min; then 30 cycles at 93 °C for 1 min, 42 °C for 1 min, and 72 °C for 2 min, followed by 72 °C for 5 min as final extension; *ompC*: 95 °C for 2 min; then 30 cycles of 95 °C for 1 min, 57 °C for 1 min, 72 °C for 2 min and a final extension at 72 °C for 5 min; *sitC*, *pagC*, *tolC*, *sifA*, *msgA*, *orgA*, *spiA*, *sipB*, *prgH*, *iroN*, *spaN*, *cdtB*, *spvB*, *spvC and sopB* : 95 °C for 5 min, 25 cycles of 94 °C for 30 sec, 66,5 °C for 30 sec, 72°C for 2 min and a final extension at 72 °C for 10 min; *spvC*: 93 °C for 5 min, then 30 cycles at 93 °C for 1 min, 42 °C for 1 min, and 72 °C for 2 min, followed by 72 °C for 5 min as final extension

CHAPTER 3. Phenotypic and genotypic characterization of non-typhoidal *Salmonella* isolated from a Brazilian pork production chain

Short title: *S. enterica* from Brazil

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Abstract

Pork products are important sources of foodborne non-typhoidal *Salmonella* in Brazil where antibiotics are commonly used throughout the pork production process and this has the potential to selectively favor antibiotic-resistant strains. We characterized the genotypic and phenotypic diversity of 41 *S. enterica* isolates that were isolated in Brazil. Isolates were collected from ten swine farms and one slaughterhouse. Whole-genome sequencing and *in silico* serotyping demonstrated that the *S. enterica* serovar Typhimurium was the most common serotype (n = 17), but eight additional servoars were identified. Eight multilocus sequence types were identified with ST19 being most common (n = 21). Several plasmids replicons were detected, with Col (RNAI) the most abundant (n = 30), followed by IncR (n = 22), IncI1 (n = 10) and IncA/C2 (n = 10). Minimum inhibitory concentration assays showed that the principle resistance phenotypes were for streptomycin (90.2%), tetracycline (87.8%), ampicillin (80.5%), chloramphenicol (70.7%) and ciprofloxacin (51.2%). Only two isolates were resistant to third-generation cephalosporins and no isolates were resistant to two tested carbapenems. Twenty-six unique antimicrobial-resistance genes were identified with *bla*$_{\text{TEM-1A}}$ and *bla*$_{\text{TEM-1B}}$ likely responsible for most beta-lactam resistance and *floR* responsible for most chloramphenicol resistance. Six strains were positive for *mcr-1*. At the time of collection, the sampled farms were adding ciprofloxacin to feed and this may have contributed to the high prevalence of resistance to this antibiotic.

Keywords: Antibiotic resistance; Ciprofloxacin, *Salmonella* Typhimurium; Sequence type; Swine; WGS;

1. Introduction

Non-typhoidal *Salmonella* is a major foodborne pathogen (BRASIL, 2018a; EFSA; ECDC, 2018), responsible for more than one million illnesses and 450 deaths per year in the United States alone (CDC, 2019). *S. enterica* can be separated into over 2,500 recognized serovars, of which serovars Typhimurium and Enteritidis are the most common causes of human salmonellosis (WHO, 2019). Pork products are considered important sources of *Salmonella* (EFSA; ECDC, 2018; WHO, 2019), with the prevalence of *Salmonella* in swine carcasses ranging from 1.5% to 24% depending on the husbandry processing conditions and practices (BERSOT; QUINTANA CAVICCHIOLI; VIANA; KONRAD BURIN *et al.*, 2019; BOHAYCHUK; GENSLER; BARRIOS, 2011; CABRAL; PANZENHAGEN; DELGADO; SILVA *et al.*, 2017; KICH; COLDEBELLA; MORES; NOGUEIRA *et al.*, 2011; PALA; TEDDE; SALZA; UDA *et al.*, 2019; PESCIAROLI; CUCCO; DE LUCA; MASSACCI *et al.*, 2017).

Antibiotic resistance is recognized as a global health concern, and controlling its spread and transmission is considered a significant challenge worldwide (FERRI; RANUCCI; ROMAGNOLI; GIACCONE, 2017). The use of antimicrobials in animals for therapeutic treatment, prophylaxis and growth promotion can increase the selection and spread of antibiotic-resistant bacteria in food production chains (CHANTZIARAS; BOYEN; CALLENS; DEWULF, 2014; MAGOURAS; CARMO; STÄRK; SCHÜPBACH-REGULA, 2017; POSTMA; BACKHANS; COLLINEAU; LOESKEN *et al.*, 2016). It is estimated that the average global input of antibiotics ranges from 45 mg of antimicrobials for every kg of beef product, 148 g for every kg of poultry and 172 mg for every kg of pork (VAN BOECKEL; BROWER; GILBERT; GRENFELL *et al.*, 2015). In Brazil, Dutra (2019) reported that at least seven antibiotics were used in pork production with an average 358 mg of antimicrobials per kg of pork product. With over twice the global average of antibiotic inputs, we surmise that antimicrobial-resistant pathogenic bacteria like *Salmonella* will be common in the Brazilian pork production chain (BARILLI; BACCI; STELLAVILLA; MERIALDI *et al.*, 2018a; CALAYAG; PACLIBARE; SANTOS; BAUTISTA *et al.*, 2017; FOIS; PIRAS; TORPDAHL; MAZZA *et al.*, 2017). The aim of this study was to characterize *S. enterica* strains isolated from a pork production chain in Brazil, including both phenotypic and molecular data.

2. Material and Methods

2.1. Bacterial strains and whole-genome sequencing

A culture collection composed by 41 isolates identified as *S. enterica* was obtained from a pork production chain for the present study. Ten finishing farms were selected based on the existence of an intensive breeding system with a similar number of pigs in all the farms (average approximately 1,000 head), and the same parent company provided the piglets and feed. All of the farms sent animals to the same slaughterhouse, which was inspected by the Brazilian Ministry of Agriculture, following the Brazilian standards of production, quality and safety. Methodological details regarding sample collection, bacterial isolation, and isolate characterization are available in Viana et al. 2019. Bacterial isolates were obtained from lairage (n=4/41 samples) and barn floors (n=2/41) at pig the farms. Slaughterhouse isolates were collected from mesenteric lymph nodes (n=18/41), tonsils (n=14/41), swine carcasses (n=2/41) and knives (n=1/41) (only one isolate was retained from any single sample). All isolates were identified as *Salmonella* based on the PCR amplification of *invA* and *ompC* genetic markers and based on serogrouping (Denka Seiken Co., Ltd., Japan). Subsequent characterization included macro-restriction digests (XbaI) and pulse-field gel electrophoresis (PFGE), PCR screening for select virulence related genes, and characterization of antibiotic susceptibility (Viana et al., 2019; Supplementary Table).

All selected isolates were subjected to genomic DNA extraction by using the DNeasy UltraClean microbial kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. The concentration of DNA was estimated by spectrophotometric measurement (NanoDrop 2000, Thermo Scientific, Wilmington, DE) and confirmed by using a Qubit dsDNA BR assay kit (Invitrogen, Carlsbad, California, USA) with a Qubit fluorometer (Invitrogen). DNA samples were sequenced at MicrobesNG (University of Birmingham, Birmingham, UK). The Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer protocol with the following modifications: 2 ng of DNA was used instead of the recommended 1 ug, and PCR elongation time was increased to 1 min from 30 seconds. DNA quantification and library preparation were carried out on a Microlab STAR automated liquid handling system (Hamilton, Reno, Nevada, USA). Libraries were sequenced using an Illumina HiSeq with a 250-bp paired-end protocol. Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15. *De novo*

assembly was performed on samples using SPAdes version 3.7, and contigs were annotated using Prokka 1.11. Among the 41 sequenced isolates, there was a median of 90 contigs (range: 46 to 190) with 50X coverage (range: 30 to 387) per genome (Supplementary Material – S1).

2.2. *In silico* analysis

For serovar prediction, the 41 *Salmonella* genomes were analyzed using Seqsero (www.denglab.info/SeqSero) and e SISTR-type (https://lfz.corefacility.ca/sistr-app/) software packages. SeqSero uses curated databases of *Salmonella* serotype determinants (*rfb* gene cluster, *fliC* and *fljB* alleles) (ZHANG; YIN; JONES; ZHANG *et al.*, 2015) to identify serovar. The SISTR module considers O (somatic) antigen, H (flagellar: H1 and H2) antigen, and/or serogroup-specific probes what were orginally designed for a *Salmonella* Genoserotyping Array (SGSA) (YOSHIDA; KRUCZKIEWICZ; LAING; LINGOHR *et al.*, 2016).

The multi-locus sequence type (MLST) for each sequenced isolate was determined by using MLST 2.0 (https://cge. cbs.dtu.dk/services/MLST/). For MLST, seven housekeeping genes were evaluated: *thrA* (aspartokinase+homoserine dehydrogenase), *purE* (phosphoribosylaminoimidazole carboxylase), *sucA* (alpha ketoglutarate dehydrogenase), *hisD* (histidinol dehydrogenase), *aroC* (chorismate synthase), *hemD* (uroporphyrinogen III cosynthase), and *dnaN* (DNA polymerase III beta subunit) (LARSEN; COSENTINO; RASMUSSEN; FRIIS *et al.*, 2012). Plasmid sequences were identified using PlasmidFinder 2.0 (https://cge.cbs. dtu.dk/services/PlasmidFinder/) with a minimum of 80% identity threshold (CARATTOLI; ZANKARI; GARCÍA-FERNÁNDEZ; VOLDBY LARSEN *et al.*, 2014).

2.3. Antimicrobial-resistance testing

Antimicrobial-resistance profiles of the *Salmonella* isolates (Viana et al., 2019, Supplementary Table), were confirmed by using a minimal inhibitory concentration (MIC) assay as described by the Clinical & Laboratory Standards Institute (CLSI, 2017). Mueller Hinton broth (Oxoid) was used 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 (St Louis, MO, USA). *Escherichia coli* ATCC 25922 was used as a negative control strain. Isolates resistant to three or more antimicrobial classes were

characterized as multidrug resistant (ECDC; EFSA; EMA, 2017).

Identification of antimicrobial resistance genes was performed by using the ResFinder 3.1 webserver [https://cge.cbs.dtu.dk/services/ResFinder/ (ZANKARI; HASMAN; COSENTINO; VESTERGAARD *et al.*, 2012)]. To ensure a comprehensive analysis using all known resistance determinants, a secondary inquiry was performed using Resistance Gene Identifier (RGI), a resistome prediction tool that uses BLAST queries against curated antimicrobial genes and SNPs available in the Comprehensive Antibiotic Resistance Database (JIA; RAPHENYA; ALCOCK; WAGLECHNER *et al.*, 2017). The databases include antimicrobial genes for β-lactams, fluoroquinolones, aminoglycosides, tetracyclines, phenicols, trimethoprim, sulphonamides, lincosamide, colistin and macrolides. Resistance determinants were identified if they presented a minimum of 98% of identity and a minimum of 60% sequence length identity. Point mutations in the quinolone resistance determining region (QRDR) of the *gyrA*, *gyrB*, *parC*, and *parE* were identified by using ResFinder 3.1 with settings of threshold of 90%, and minimum length of 60%.

2.4. Data analysis

Each antibiotic susceptibility test result (resistant or susceptible) was compared with the presence or absence of a corresponding resistance gene as identified by *in silico* analysis. If the isolate presented at least one of the resistance genes related to the given antimicrobial, this was classified as having correspondence between phenotype and genotype. The overall "coherence" between resistant phenotypes and genotypes was then calculated using the software OpenEpi (DEAN; SULLIVAN; SOE, 2013). The phenotypic results were considered the "Gold Standard" for this test. Diagnostic sensitivity represents the proportion of true positives while diagnostic specificity represents the proportion of true negatives.

3. Results

Based on WGS, the 41 selected *S. enterica* isolates represented nine different serovars with serovar Typhimurium being the most common (n = 17; Table 1). These results were consistent with serogroup agglutination tests reported by Viana et al. (2019). MLST analysis identified eight different sequence types with ST19 being the most common (n = 21) (Table 1). Multiple plasmids replicons were detected in the 41 *S. enterica* isolates with Col (RNAI) being

the most abundant (Figure 1). The number of plasmids replicons ranged from 1 to 8 per each strain, and only one isolate did not present any plasmid.

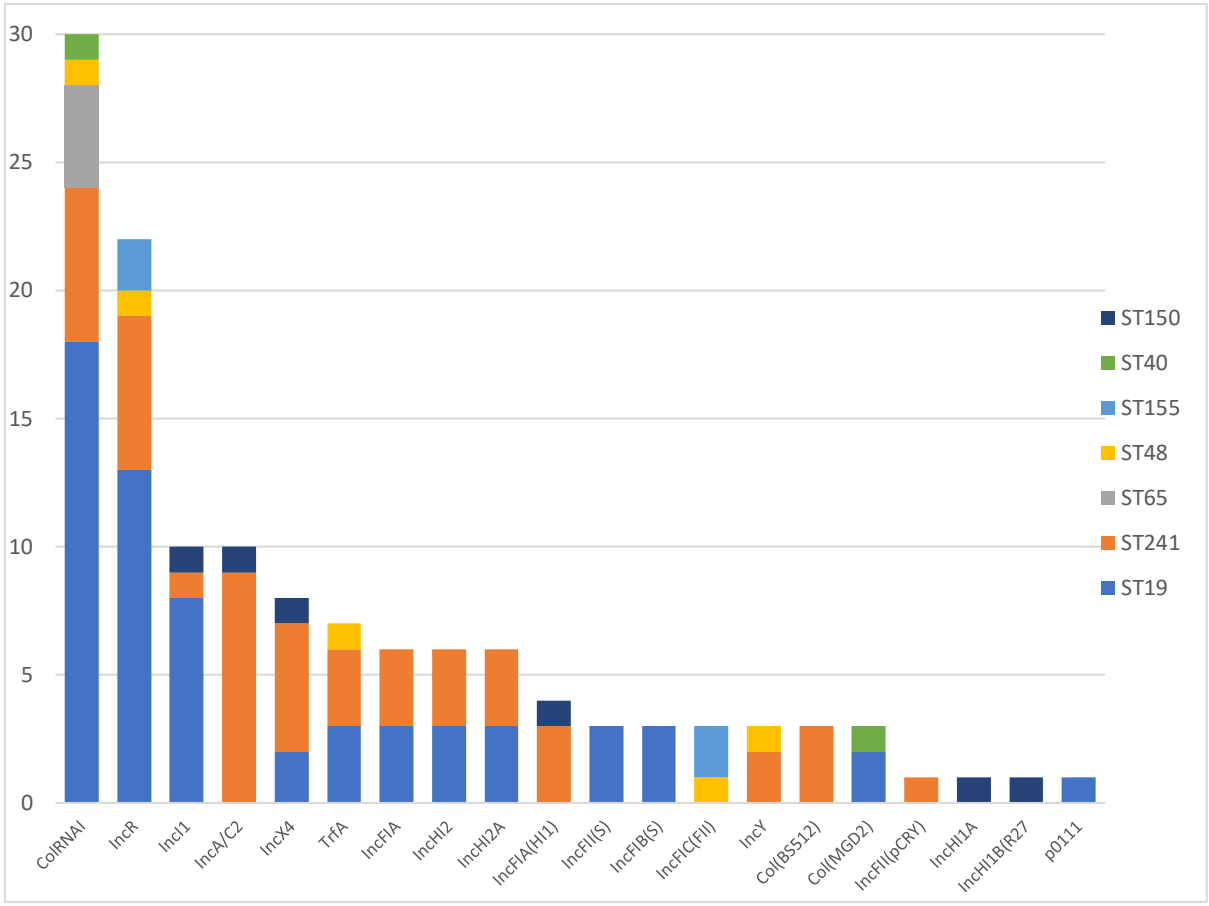

Figure 1. Numbers of sequenced plasmids replicons detected by PlasmidFinder in the different STs profile of *Salmonella*.

Table 1. Multilocus sequence types for *Salmonella enterica* isolates (n = 41) obtained from the pork production chain.

Sequence Type	Alelle profile[a]	Serovar	No. (n) of isolates
ST-19	10, 7, 12, 9, 5, 9, 2	Typhimurium	17
		Monophasic variant of Typhimurium I 4,[5],12:i:-	4
ST-241	43, 47, 49, 16, 41, 15, 3	Bredeney	9
ST-65	11, 10, 13, 32, 10, 13, 4	Brandenburg	4
ST-48	22, 11, 25, 21, 10, 23, 23	Panama	2
ST-155	10, 60, 58, 66, 6, 65, 16	London	2
ST-413	15, 70, 93, 78, 113, 6, 68	Mbandaka	1
ST-40	19, 20, 3, 20, 5, 22, 22	Derby	1
ST-150	61, 12, 10, 65, 54, 63, 57	Bovismorbificans	1

[a] Allele number for *aroC, dnaN, hemD, hisD, purE, sucA,* and *thrA,* respectively (one for each ST).

MIC assays showed that the *S. enterica* isolates presented resistance mainly to streptomycin (90%), tetracycline (87.8%), ampicillin (80.5) and chloramphenicol (70.7%) (Table 2). A high frequency of isolates (73.2%) were considered multidrug resistant. One isolate was resistant to eight antimicrobials classes, while only one isolate (2.4%) was pansusceptible. The simultaneous resistance to ampicillin, chloramphenicol, streptomycin and tetracycline was the most frequent resistance profile, identified in 10 isolates. No isolates were resistant to ertapenem, meropenem or kanamycin.

A total of 26 different resistance genes were identified in the *S. enterica* isolates (Table 3). Except for three isolates, all presented at least one antimicrobial-resistance gene, 33 positives for at least five antibiotic-resistance genes, and five isolates were positive for more than 10 genes. For most of the tested antibiotics, the phenotypic and genotypic results were in agreement with 93.2% overall diagnostic sensitivity and 92.0% overall diagnostic specificity (Table 4). The greatest discordance was evident for kanamycin and streptomycin resistance for which several isolates were genotypically positive but phenotypically negative.

Regarding mutations in the quinolone resistance-determining regions, 16 isolates exhibited corresponding mutations in the *parC* gene (Thr (57) to Ser). Within *gyrA*, 15 isolates presented replacement in Asp (87) to Asn, and six isolates presented mutations in amino acid 83 with three having a change from Ser to Phe and the other three changing from Ser to Tyr. The mutation in *gyrA* 83 was consistent with resistance to ciprofloxacin, but other mutations in this region were not clearly correlated with resistance. One isolate presented two different mutations, Thr (57) to Ser in *parC* gene and Glu (466) to Asp in *gyrB*, but this isolate was sensitive to ciprofloxacin. All ciprofloxacin-resistant strains had either the *gyrA* (83, Ser->Phe; n = 3) mutation or harbored a corresponding quinolone resistance gene (Table 3).

Table 2. Frequencies of *Salmonella enterica* isolates (n = 41) obtained from the pork production chain with resistance to different antibiotics.

class[1]	antibiotic	resistance (%)
β-lactam	ampicillin	33 (80.5%)
cephem	ceftazidime	2 (4.9%)
	cefoxitin	2 (4.9%)
phenicols	chloramphenicol	29 (70.7%)
quinolones	ciprofloxacin	21 (51.2%)
carbapenems	ertapenem	0 (0.0%)
	meropenem	0 (0.0%)
aminoglycosides	gentamicin	7 (17.1%)
	kanamycin	0 (0.0%)
	streptomycin	37 (90.2%)
tetracyclines	tetracycline	36 (87.8%)
sulfonamides	sulfamethoxazole + trimethoprim	8 (19.5%)

[1] 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 3. Frequencies of resistance genes observed in *Salmonella enterica* isolates (n = 41) obtained from the pork production chain with resistance to different antibiotics.

class	resistance gene	all isolates (n=41)	Typhimurium (n=16)	Typhimurium I 4,[5],12:i:- (n=5)	Bredeney (n=9)	Brandenburg (n=4)	London (n=2)	Panama (n=2)	Derby (n=1)	Bovismorbificans (n=1)	Mbandaka (n=1)
β-lactam											
	bla$_{\text{TEM-1A}}$	15	13	0	0	0	1	0	0	1	0
	bla$_{\text{TEM 1B}}$	17	1	4	9	0	1	2	0	0	0
	bla$_{\text{CMY-2}}$	1	0	1	0	0	0	0	0	0	0
	bla$_{\text{CTXM-8}}$	1	0	0	0	0	0	0	0	1	0
phenicols											
	floR	28	12	3	9	0	2	1	0	1	0
	cmlA1	1	0	1	0	0	0	0	0	0	0
quinolones											
	qnrE1	6	0	3	3	0	0	0	0	0	0
	qnrS1	9	0	0	6	0	2	1	0	0	0
	qnrB19	2	0	0	0	1	0	0	1	0	0
	oqxA	2	2	0	0	0	0	0	0	0	0
	oqxB	2	2	0	0	0	0	0	0	0	
aminoglycosides											
	strA (aph 3)	27	14	3	9	0	0	0	0	1	0
	strB (aph 6)	27	14	3	9	0	0	0	0	1	0
	aac(3')-Ila	5	0	2	3	0	0	0	0	0	0
	aac(6')-Iaa	26	16	5	0	0	2	0	1	1	1
	aadA1	8	0	4	3	0	0	1	0	0	0
	aadA2	13	0	1	9	0	2	0	1	0	0
	aac(3)-IId	1	0	1	0	0	0	0	0	0	0
tetracyclines											
	tet(A)	16	0	1	9	0	2	1	1	0	0
	tet(B)	20	17	2	0	0	0	1	0	1	0
sulfonamides											
	sul1	7	0	3	3	0	0	0	1	0	0
	sul2	24	13	0	9	0	0	1	0	1	0
	sul3	1	0	1	0	0	0	0	0	0	0
trimethoprim											
	dfrA1	6	0	2	3	0	0	1	0	0	0
	dfrA8	3	0	0	0	0	2	0	0	1	0
	dfrA12	1	0	1	0	0	0	0	0	0	0
colistin											
	mcr-1	8	0	2	5	0	0	0	0	1	0

Table 4. Phenotypical (PHE) and genotypical (GEN) results for antimicrobial resistance of *Salmonella enterica* isolates (n = 41) obtained from a pork production chain in Brazil, 2018.

Antibiotic class	coherent results		incoherent results		Sensitivity%	Specificity%
	both resistant	both susceptible	PHE resistant & GEN susceptible	GEN resistant & PHE susceptible		
β-lactam						
Ampicillin	31	7	2	1	93.9	87.5
cephem						
Cefoxitin	1	39	1	0	50	100
Ceftazidime	2	39	0	0	100	100
phenicols						
Chloramphenicol	28	11	1	1	96.5	91.7
quinolones						
Ciprofloxacin	18	19	3	1	85.7	95
aminoglycoside						
Streptomycin	31	2	6	2	83.8	50
Gentamicin	6	35	0	0	100	100
Kanamycin	0	15	0	26	NC	NC
Carbapenems						
Ertapenem	0	0	0	0	NC	NC
Meropenem	0	0	0	0	NC	NC
tetracyclines						
Tetracycline	36	0	0	5	100	0
sulfonamides						
Sulfamethoxazole	8	10	0	23	NC	NC
Total	153	151	13	11	93.2	92

NC: not calculated. Coherent results: if the specific gene was present and the isolate was resistant to the correlated antibiotic or if the specific gene was absent and the isolate was susceptible to the correlated antibiotic. Incoherent results: if the specific gene was present, but the isolate was susceptible to the correlated antibiotic or if the specific gene was absent, but the isolate was resistant to the correlated antibiotic.

4. Discussion

Within Brazilian pork production systems, antibiotics are used to treat and prevent disease, and to promote growth (explicitly or implicitly). During our farm visits we documented specific instances whereby producers were using amoxicillin, doxycycline, kanamycin, neomycin, florfenicol, fosfomycin, tulathromycin, colistin and tiamulin. Furthermore, rather than using the veterinary analog for ciprofloxacin, called enrofloxacin, these farms were adding ciprofloxacin directly to feed (this practice had ended by the time this study was completed). If concentrations are sufficient, routine use of antibiotics such ciprofloxacin, amoxicillin, doxycycline, and florfenicol will likely favor antibiotic-resistant strains of *S. enterica* relative to susceptible strains. Serovars such as Typhimurium are well known to be resistant to five or more antibiotics [e.g., phage type DT104, (LEEKITCHAROENPHON; HENDRIKSEN; LE HELLO; WEILL *et al.*, 2016)] and are likely to be favored in the presence of long-term antibiotic exposure on pig farms.

S. Typhimurium was the most prevalent serovar identified in our panel of isolates, followed by Bredeney and monophasic variants of *S.* Typhimurium. Pork-associated serovar Typhimurium have also been commonly detected in Brazil and European Union (BERSOT; QUINTANA CAVICCHIOLI; VIANA; KONRAD BURIN *et al.*, 2019; BIASINO; DE ZUTTER; MATTHEUS; BERTRAND *et al.*, 2018; BONARDI, 2017; CABRAL; PANZENHAGEN; DELGADO; SILVA *et al.*, 2017). Serovar Typhimurium is the second most commonly associated cause of salmonellosis outbreaks in European Union, and pork was the main food source for these outbreaks in 2014 (EFSA; ECDC, 2018). A similar strong association has been reported between pork-isolates of *Salmonella* and monophasic variants of serovar Typhimurium (EFSA; ECDC, 2018). All the isolates (n = 10) with the most frequent resistance profile (ampicillin – chloramphenicol – streptomycin – tetracycline) were identified as serovar Typhimurium. The most common antibiotic-resistance profiles of serovar Typhimurium in the U.S. include the same four resistance phenotypes plus resistance to sulfa antibiotics [“penta-resistance,” commonly phage type DT104 (LEEKITCHAROENPHON; HENDRIKSEN; LE HELLO; WEILL *et al.*, 2016)]. For the current study we combined sulfa and trimethoprim antibiotics in the same test, which probably underestimated the prevalence of sulfa-only resistance for these isolates. Other studies reported high frequencies *Salmonella* strains with resistance to streptomycin, tetracycline and ampicillin (FOIS; PIRAS; TORPDAHL; MAZZA *et al.*, 2017; PATCHANEE; TANSIRICHAROENKUL;

BUAWIRATLERT; WIRATSUDAKUL *et al.*, 2016; PORNSUKAROM; VAN VLIET; THAKUR, 2018).

Multilocus sequence type 19 (ST19) was closely associated with serovar Typhimurium, consistent with previous reports (ACHTMAN; WAIN; WEILL; NAIR *et al.*, 2012; ASHTON; NAIR; PETERS; BALE *et al.*, 2016). Ashton et al. (2016) evaluated 6,887 isolates of *S. enterica* in a reference laboratory, the concordance between the serotype and the sequence type from MLST was 96%. Twenty different plasmids replicons were identified of which ColRNAI was most common, which corresponds to an earlier report about common presence of ColRNAI in *Salmonella* serovars isolated from people, food animals and agriculture environmental sources in United States (PORNSUKAROM; VAN VLIET; THAKUR, 2018). High frequencies of antibiotic resistance were identified, mainly against streptomycin, tetracycline, ampicillin, chloramphenicol (Table 2) and 33 strains were resistant to three or more antibiotic classes, being characterized as multidrug resistant (ECDC; EFSA; EMA, 2017). All the pig farms involved in this study have been using florfenicol on a regular basis for prophylaxis (growing and finishing steps), which may explain the high rate of resistance to a chloramphenicol, another phenicol.

Resistance to ciprofloxacin for our study was relatively high (51.2%), but comparable to other reports from Spain, Brazil and China (CAMERON-VEAS; FRAILE; NAPP; GARRIDO *et al.*, 2018; GUERRA FILHO; YAMATOGI; POSSEBON; FERNANDES *et al.*, 2016; JIU; ZHU; KHAN; SUN *et al.*, 2017). Interestingly, Pornsukarom et al. (2018) did not detect resistance to ciprofloxacin for *Salmonella* recovered from different sources in United States, where in 2013 the prevalence of ciprofloxacin-resistant *Salmonella* was approximately 0.5% for swine-source isolates (CDC, 2016). In the U.S. the only approved floroquinolone for food animals is enrofloxacin and this is limited to therapeutic applications (FDA, 2019). Limited fluoroquinolone exposure in the U.S., in contrast to our study farms, may explain the relatively low prevalence of ciprofloxacin-resistance in U.S. swine isolates of *S. enterica*.

When we compared presence of antibiotic-resistance genes from *in silico* analysis with their corresponding phenotypes, diagnostic sensitivity and specificity were 93.2% and 92%, respectively (Table 4). These results are similar to other reports (MCDERMOTT; TYSON; KABERA; CHEN *et al.*, 2016; NEUERT; NAIR; DAY; DOUMITH *et al.*, 2018; PORNSUKAROM; VAN VLIET; THAKUR, 2018; WILSON; FOX; FEGAN; KURTBÖKE, 2019). The genes *tet*(A) and *tet*(B) were found in 39% and 48.8% of the isolates, respectively, with a 100% correspondence between genotype and phenotypes. Tetracycline is one of the most

frequently used antibiotics in pig production in several countries (CHANTZIARAS; BOYEN; CALLENS; DEWULF, 2014), and these genes encode a membrane-associated efflux protein and both of them have been reported for *Salmonella* isolates (MICHAEL; BUTAYE; CLOECKAERT; SCHWARZ, 2006). Interestingly, none of the *in silico* analysis identified a *tet*(G) sequence, which is expected for *S. enterica* Typhimurium phage type DT104 (LEEKITCHAROENPHON; HENDRIKSEN; LE HELLO; WEILL *et al.*, 2016). It is possible that this otherwise widespread phage type in the U.S. (CARROLL; WIEDMANN; DEN BAKKER; SILER *et al.*, 2017; LEEKITCHAROENPHON; HENDRIKSEN; LE HELLO; WEILL *et al.*, 2016) is not prevalent in the swine population that we sampled.

The most common resistance mechanism for chloramphenicol are efflux pumps that are encoded by *floR* and *cmlA*. (FRYE; JACKSON, 2013; MICHAEL; BUTAYE; CLOECKAERT; SCHWARZ, 2006). *floR* is commonly found with serovar Typhimurium (MEUNIER; BOYD; MULVEY; BAUCHERON *et al.*, 2002). For the current study, the second highest *in silico* diagnostic sensitivity was for resistance to chloramphenicol (96.5%) and *floR*, which was observed in 28 isolates. The efflux pump encoded by *cmlA1* was observed only for one isolate.

For ampicillin resistance we primarily observed *bla*$_{\text{TEM-1A}}$ (36.6%) and *bla*$_{\text{TEM-1B}}$ (41.5%). *bla*$_{\text{TEM-1}}$ is often plasmid mediated and it is able to hydrolyze penicillin and 1st-generation cephalosporins (UR RAHMAN; ALI; ALI; KHAN *et al.*, 2018). One isolate presented *bla*$_{\text{CMY-2}}$ and another had *bla*$_{\text{CTXM-8}}$, both of which convey extended-spectrum beta-lactamase resistance phenotypes. The isolate that harbored *bla*$_{\text{CMY-2}}$ also presented phenotypic resistance to cefoxitin and ceftazidime, while the isolate with *bla*$_{\text{CTXM-8}}$ was resistant to ceftazidime (MICHAEL; BUTAYE; CLOECKAERT; SCHWARZ, 2006).

In our study, the most prevalent genes associated with streptomycin resistance were *strA* and *strB*, which co-occurred. These genes confer resistance only to streptomycin and they are commonly linked to each other (MICHAEL; BUTAYE; CLOECKAERT; SCHWARZ, 2006). We observed 26 isolates that were sensitive to kanamycin despite harboring *aac(6')-Iaa*, which is a chromosomal-encoded aminoglycoside acetyltransferase that should confer resistance to tobramycin, kanamycin, and amikacin (SALIPANTE; HALL, 2003).

Another interest finding was the occurrence of *mcr-1* gene in eight isolates. Colistin resistance due to plasmid-mediated *mcr-1* was first reported in 2015 (LIU; WANG; WALSH; YI *et al.*, 2016). The only isolates containing the IncX4-type plasmid in our panel were also positive for *mcr-1*. This result is consistent with earlier reports suggesting that IncX4 plasmids

might be responsible for the spread of the *mcr-1* gene in Latin America (MENDES OLIVEIRA; PAIVA; LIMA, 2019; RAU; DE LIMA-MORALES; WINK; RIBEIRO *et al.*, 2019).

5. Conclusion

The obtained results highlight that both, phenotypic and genotypic tests, are important to characterize the resistance profiles in *Salmonella*. Despite some disagreement and limitations, the WGS has become a useful method to predict the antimicrobial resistance and it allows a more complete analysis of the genes and mechanisms, which may contribute to take promptly decisions regarding emerging resistance genes. Furthermore, the presence of a high number of *Salmonella* MDR and the significant number of resistant genes and plasmids, emphasize the wide genomic diversity of *Salmonella* observed in our study and how challenging is interpret the transference of resistance elements between the microorganisms.

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Supplementary Table 1. Samples isolated and identified by Viana et al. (2019) obtained from pig farms and swine slaughterhouse environments in a production chain located in Brazil.

Sample	Source	Serogroup	PFGE Profile[1]	Virulence profile[2]	Resistance pattern[3]
01	Floor of barn	O:7	A8	*invA-spiA-sitC-pagC-msgA-orgA-sipB-spaN-iroN-tolC*	STR
02	Carcass after final washing	O:4	B1	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-spvC-spvB-iroN-tolC*	AMP-STR-TET-CIP
03	Carcass after bleeding	O:4	A4	*invA-sifA-spiA-sitC-pagC-msgA-sipB-prgH-sopB-spaN-cdtB-iroN-tolC*	STR
04	Knives	O:4	B3	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	AMP-CHL-STR-TET
05	Lairage	O:4	B4	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-iroN-tolC*	AMP-CHL-STR-TET
06	Tonsils	O:4	E2	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-cdtB-iroN-tolC*	STR
07	Tonsils	NS*	E3	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-cdtB-iroN-tolC*	CHL-STR-TET-CIP
08	Tonsils	O:4	B3	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-cdtB-iroN-tolC*	AMP-CHL-STR-TET
09	Tonsils	O:4	E2	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-cdtB-iroN-tolC*	No resistance
10	Lymph nodes	O:4	A6	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	AMP-CHL-STR-TET
11	Lymph nodes	O:4	C1	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-spvC-spvB-iroN-tolC*	AMP-STR-GEN-TET-CIP
12	Lymph nodes	O:4	B2	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	AMP-STR-TET
13	Lymph nodes	O:4	A6	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	TET
14	Tonsils	O:4	C2	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	STR-TET
15	Lairage	O:9	A5	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-cdtB-iroN-tolC*	AMP-SXT-TET
16	Lymph nodes	O:4	A3	*invA-sifA-spiA-sitC-pagC-orgA-sipB-prgH-sopB-spaN-cdtB-iroN-tolC*	AMP-CHL-STR-GEN-SXT-TET-CIP
17	Lymph nodes	O:4	A1	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-cdtB-iroN-tolC*	AMP-CHL-STR-GEN-TET-CIP
18	Tonsils	NS*	A2	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-cdtB-iroN-tolC*	AMP-CHL-STR-GEN-TET-CIP
19	Lymph nodes	O:4	A4	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-cdtB-iroN-tolC*	AMP-CHL-STR-TET-CIP
20	Lymph nodes	O:4	A3	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-cdtB-iroN-tolC*	AMP-CHL-STR-TET-CIP
21	Tonsils	O:4	C3	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-cdtB-iroN-tolC*	AMP-CHL-STR-TET-CIP
22	Tonsils	O:4	B3	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	AMP-CHL-STR-TET
23	Lymph nodes	O:4	B6	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	AMP-CHL-STR-TET
24	Lymph nodes	O:4	B3	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	AMP-CHL-STR-TET
25	Lymph nodes	O:3	D2	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	AMP-CHL-STR-SXT-TET-CIP
26	Tonsils	O:3	D1	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	AMP-CHL-STR-SXT-TET-CIP
28	Lymph nodes	O:4	B4	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	AMP-CHL-STR-TET
29	Tonsils	O:4	B3	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	AMP-TET
30	Lymph nodes	O:4	A4	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	STR-CIP
31	Lymph nodes	O:4	B3	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	AMP-CHL-STR-TET
32	Lymph nodes	O:4	B3	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	AMP-CHL-STR-TET
33	Lymph nodes	O:4	B7	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	AMP-CHL-STR-GEN-TET-CIP
34	Floor of barn	O:4	E1	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	AMP-CHL-STR-TET-CIP

35	Lairage	O:4	B3	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	AMP-CHL-STR-TET-CIP
36	Tonsils	O:4	B5	*invA-sifA-spiA-sitC-pagC-msgA-orgA-prgH-sopB-spaN-tolC*	AMP-CHL-STR-GEN-SXT-TET-CIP-CFX-CAZ
37	Tonsils	O:4	C3	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	AMP-CHL-STR-TET-CIP
38	Lymph nodes	O:4	B1	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-spvC-spvB-tolC*	AMP-CHL-STR-SXT-TET-CIP
39	Lymph nodes	O:8	C4	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-cdtB-tolC*	AMP-CHL-STR-SXT-TET-CAZ
40	Tonsils	O:4	A7	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-iroN-tolC*	AMP-CHL-STR-GEN-SXT-TET-CIP
41	Tonsils	O:4	**	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-cdtB-iroN-tolC*	AMP-CHL-STR-TET-CIP-CFX
42	Lairage	O:9	**	*invA-sifA-spiA-sitC-pagC-msgA-orgA-sipB-prgH-sopB-spaN-cdtB-iroN-tolC*	AMP-CHL-STR-TET-CIP

*NS: Non serogrouped; **No PFGE profile.

All isolates were submitted and positive to *ompC* gene for identification.

[1]PFGE profile from *Salmonella* strains that were isolated from farms and slaughterhouses in Brazil. Macro-restriction was completed with XbaI. Identity was estimated using the Dice coefficient (5% tolerance).

[2]PCR conditions for: *invA*: 93 °C for 5 min; then 30 cycles at 93 °C for 1 min, 42 °C for 1 min, and 72 °C for 2 min, followed by 72 °C for 5 min as final extension; *ompC*: 95 °C for 2 min; then 30 cycles of 95 °C for 1 min, 57 °C for 1 min, 72 °C for 2 min and a final extension at 72 °C for 5 min; *sitC*, *pagC*, *tolC*, *sifA*, *msgA*, *orgA*, *spiA*, *sipB*, *prgH*, *iroN*, *spaN*, *cdtB*, *spvB*, *spvC and sopB* : 95 °C for 5 min, 25 cycles of 94 °C for 30 sec, 66,5 °C for 30 sec, 72°C for 2 min and a final extension at 72 °C for 10 min; *spvC*: 93 °C for 5 min, then 30 cycles at 93 °C for 1 min, 42 °C for 1 min, and 72 °C for 2 min, followed by 72 °C for 5 min as final extension.

[3]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; ² as indicated in Figure 1; ³ both resistance patterns presented one isolate each without PFGE profile.