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Identification of *Salmonella* isolated from pork sausage and evaluation of thermal and antimicrobial resistance of isolates

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Pork products are important reservoir of *Salmonella*. In Brazil, the consumption of pork sausage is high and the occurrence of *Salmonella* in product is usual. *Salmonella* occurrence was studied in twenty samples of fresh pork sausage. Pulsed-Field Gel Electrophoresis (PFGE) was performed to characterize *Salmonella* isolates. Their antimicrobial and heat resistance were also evaluated. *Salmonella* was detected in five samples of industrial pork sausage and six isolates were obtained and identified by phenotypic and genotypic methods: *Salmonella enterica* subsp. *houtenae* (two isolates) and serovars of *S. enterica* subsp. *enterica:* Bareilly, Typhimurium, Paratyphi C and Paratyphi B. *Salmonella houtenae* and the serovars Paratyphi B and Typhimurium were resistant to three or more of antimicrobial agents tested. The *Salmonella* cocktail presented high heat resistance in pork sausage with *D*-values at 58, 62 and 65°C of 10.99, 5.29 and 2.16 min, respectively, and a *z*-value of 10.1°C. The evaluation of thermal and antimicrobial resistance of *Salmonella* can be useful for researchers and food industry involved in the management of pork product quality and resulting in improvements in microbiological safety. According to our results, the binomial effect of time and temperature can be utilized to pork industry in designing and estimating thermal processes specific for sausage. To consumers, a longer heating time ensures the microbiological quality of sausage and reduces the risk of salmonellosis.

Key words: Pulsed-field gel electrophoresis, pork product, food safety, salmonellosis.

INTRODUCTION

Swine is an important reservoir of *Salmonella* and considered asymptomatic carriers of microorganism (Berends et al., 1996; Vieira-Pinto et al., 2006). Contamination of pork products during slaughter represents an important vehicle for *Salmonella* spp. dissemination to humans (Oliveira et al., 2012). During further processing of meat such as cutting and mincing, *Salmonella* from contaminated pork cuts may then spread into meat preparations.

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Abbreviations: PFGE, Pulsed-Field Gel Electrophoresis; **TSI,** agar triple sugar iron; **LIA,** lysine iron agar; **TSB,** tryptone soy broth; **TSBP,** tryptone soy broth with 0.3% yeast extract; **MPN,** most probable number; CFU, colony-forming units.

At retail and at consumer level, cross contamination, improper storage and insufficient cooking can additionally increase the risk to consumers (Gonzales-Barron et al., 2012). In Brazil, the consumption of pork sausage is high and the occurrence of *Salmonella* in product is usual (Marques et al., 2006; Borowsky et al., 2007; Mürmann et al., 2009), which increases the population's exposure to the pathogen. Thus, the periodic outbreaks of salmonellosis caused by pork clearly demonstrate the need for improved tracking and tracing of *Salmonella* spp. in the pork production chain (Pielaat, 2011). For genotype characterization, the Pulsed-Field Gel Electrophoresis (PFGE) technique has been shown to be highly effective for epidemiological studies of some serovars of *Salmonella* (Willford et al., 2007). Also, other factors need be evaluated such as the multidrug and heat resistance. The antimicrobial resistance in *Salmonella* has lead to failure of treatment of salmonellosis and other diseases caused by bacterial pathogens (Travers and Barza, 2002) and there are few data in the literature on the inactivation of the pathogen in pork sausage by heat treatment.

The study of the *Salmonella* profiles circulating in production chain may contribute to better preventive measures to control meat products and a better protection of public health. In this sense, the objective of this study was to detect and identify *Salmonella* serovars in industrial pork sausage and evaluate antimicrobial resistance profiles and heat resistance of these isolates when challenged in a pork sausage model system.

MATERIALS AND METHODS

Sample collection

Twenty samples of fresh pork sausage industrial registered in the Ministry of Agriculture Livestock and Supply of Brazil were collected into sealed and refrigerated packages in commercial establishments from four cities from Minas Gerais state. The samples were directly transported in refrigerated isothermal boxes to the laboratory and immediately analyzed.

Isolation and phenotypic identification of *Salmonella* spp.

Detection of the *Salmonella* spp. was conducted as previously described by Pignato et al. (1995). For isolation and identification, 25 g of each sample were aseptically transferred to 225 ml of pre-enrichment broth base "Salmosyst" (Merck), homogenized in stomacher for 4 min and incubated at 37°C/6 h. For the selective Salmosyst enrichment, 10 ml of pre-enrichment broth base was supplemented with one selective supplement tablet (Merck) and incubated for 18 h at 37°C. From each tube, a loopful of culture was streaked on Rambach agar (Merck), and the plates were incubated at 37°C/24 h. Typical colonies on Rambach agar were selected and transferred to tubes containing agar triple sugar iron (TSI) (Himedia) and lysine iron agar (LIA) (Himedia), and incubated at 37°C/24 h. Characteristic strains of *Salmonella* spp. were tested for differential staining of gram, catalase and oxidase. API20E kit (BioMérieux) was used to complement these biochemical tests and the final identification was performed using the API LAB plus software (BioMérieux).

Bacterial DNA extraction and PCR analyses

DNA sequences from six isolated and phenotypically identified strains of *Salmonella* spp. were analyzed. The DNA of strains was extracted using a QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. The PCR analyses, reaction was carried out in a final volume of 50 µl containing 25 µl of TopTaq master mix (Qiagen), 1 µl of each primer (27f /1512r), 2 µl of DNA and 21 µl of free water RNase. The amplification was carried out as follows: template DNA was denatured for 5 min at 95°C followed for 30 cycles, then denatured at 92°C for 60 s, and annealed at 55°C for 60 s, and primer extension was carried out at 72°C for 60 s. The tubes were then incubated for 10 min at 72°C for the final extension. After amplification the samples were stored at 4°C. The unpurified PCR products were sequenced by Macrogen Inc. (Seoul, South Korea), and sequences were then compared to those available in the GenBank database using the BLAST algorithm (National Centre for Biotechnology Information, Maryland, USA).

Pulsed field gel electrophoresis (PFGE)

The methods previously described by Pang et al. (2005) were used to perform a restriction digestion with *Not*I (Invitrogen). The conditions used were 6 V/cm for 25 h at 12°C with pulsed time ranging from 2 to 10 s. The gels were stained with Sybr Green (Invitrogen) and photographed. The similarity between *Salmonella* strains was determined based on the presence or absence of amplicons detected by PFGE. The gels were analyzed determining the diversity of amplicons. The hierarchical clustering was performed using the program Systat 8.0, based on similarity matrices that were generated via the agreement method (simple matching) using the Ward algorithm and Euclidean distance.

Antimicrobial susceptibility testing

The antimicrobials used in this test were the following: amikacin (30 µg/disc), tetracycline (30 µg/disc), cephalothin (30 µg/disc), cefotaxime (30 µg/disc), ceftazidime (30 µg/disc), aztreonam (30 µg/disc), cefoxitin (30 µg/disc), ceftriaxone (30 µg/disc), chloramphenicol (30 µg/disc), sulphazotrin (25 µg/disc), gentamycin (10 µg/disc) and ampicillin (10 µg/disc). Isolates of *Salmonella* were grown on Case agar (Merck) for 18 h at 37°C. The bacterial population was inoculated in 4 ml of sterile distilled water to achieve the n° 0.5 McFarland turbidity standard (Probac, Brazil). A swab was used to spread the inoculum across the surface of Muller Hinton Agar (Merck), and antimicrobials disks (DME Polisensidisc® 4 × 6 - Specialized Diagnostic Microbiology, São Paulo, Brazil) were applied to the plate. Isolates resistance was assessed by measuring the inhibition of bacterial growth after incubation for 18 h at 37°C. The breakpoints to antimicrobial agents considered for *Salmonella* were those recommended by the CLSI (2011, Table 2) for *Enterobacteriaceae*. *Escherichia coli* ATCC 25922 was used as positive control.

Heat resistance tests in pork sausage

The thermo-tolerance of *Salmonella* strains was tested in fresh pork sausages. In this test, the inoculum contained a cocktail of the 6 isolated serovars of *Salmonella* in stationary phase. They were re-

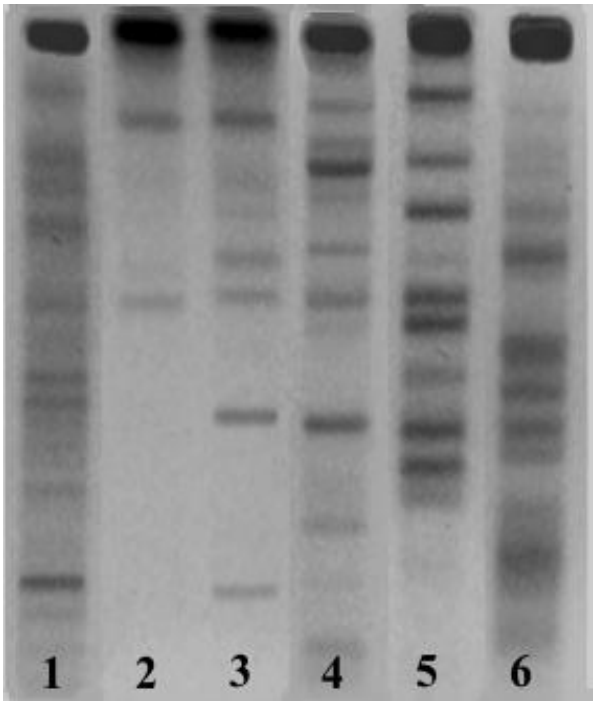

Figure 1. Pulsed field gel electrophoresis (PFGE) patterns for *Not*I-digested genomic DNA of *Salmonella* strains from sausage.
1. *Salmonella* Paratyphi B; 2. *Salmonella houtenae;* 3. *Salmonella houtenae;* 4. *Salmonella* Typhimurium; 5. *Salmonella* Bareilly*;* 6. *Salmonella* Paratyphi C.

suspended in 2 ml of Tryptone Soy Broth (TSB) (Merck) with a concentration around 10^{10} CFU/ml. The cocktail was added in 80 g of pork sausage. The sausages were prepared under aseptic conditions using the following formula: 74% lean pork ham, 20% fat pork, 2.0% NaCl, 0.5% Antioxidant Ibracor L600® (IBRAC Additives & Spices, São Paulo, Brazil), 1.0% Cure LF® (IBRAC Additives & Spices, São Paulo, Brazil), 1.0% garlic paste, 0.5% chili pepper and 1% cold water. The inoculated mixture was filled into a natural casing of 26 mm diameter. The sausages were kept in a sterile and closed plastic packet. Heat resistance trials were performed by completely submerging of these packets in a circulating water bath kept in the temperatures: 58, 62 and 65°C and in the times: 0, 5, 10 and 15, in triplicate for each time/temperature. After each time, the packets were removed and immediately cooled in water and ice. They were then stored at refrigeration temperature and survival (cell vialbility of *Salmonella* strains) counts were performed within 2 h.

Enumeration of bacteria surviving heat-treatment

Survivors counts were performed by the most probable number (MPN) dilution technique (De Man, 1983), using a series of three tubes per dilution in media TSB with the addition of 0.3% yeast extract (Himedia) (TSBP). Bacterial growth was evaluated by turbidity of the broth TSBP after 48 h of incubation at 37°C. The presumptive *Salmonella* survival was confirmed by their recovering on Rambach Agar (Merck) at 37°C for 24 h. Thus, counts were performed.

Statistical analysis

The number of *Salmonella* isolates which were survivors as a function of time was evaluated by regression analysis using SISVAR® (Lavras, Brazil) software, version 4.5. Survival curves of *Salmonella* isolates were obtained by plotting $\log_{10}$ of the surviving cells number/g versus heating time as suggested by Quintavalla et al. (2001). The line for survival plots was determined by linear regression analysis and the *D*-values (decimal reduction time) were calculated from the resulting regression equations. The *z*-values were evaluated by the linear regression of $\log_{10}$ *D*-values versus heating temperatures. The counts were subjected to analysis of variance (ANOVA) and the means were compared by Scott-Knott test, with $P < 0.01$.

RESULTS AND DISCUSSION

A total of 20 samples of industrial sausages were investigated, *Salmonella* was detected in five samples. Six isolates were obtained and identified by phenotypic testes. These isolates were also identified by comparative analysis of 16S rRNA gene sequences and identified with a similarity of 99 to 100% to *Salmonella enterica* subsp. *houtenae* (AB273733.1) and serovars of *S. enterica* subsp. *enterica*: Bareilly (U92196.1), Typhimurium (AP011957.1), Paratyphi C (EU118097.1) and Paratyphi B (DQ344539.1). *S. houtenae* was detected in two samples of industrial sausage. Two subspecies of *Salmonella*: *houtenae* and *enterica* serovar Typhimurium were detected in the same sample. In this study, all *Salmonella* isolates from pork sausage identified are described in scientific literature as human pathogenic. The PFGE characterization identified 6 different profiles for 5 different serovars, displaying 3 to 10 bands (Figure 1). The lines 2 and 3 (Figure 1) show 2 chromosomal patterns for a same serovar identified as *S. houtenae* by 16 S sequencing, showing genetic differences even into the serovar level. In this survey, it was possible observed that *Salmonella* Typhimurium and *Salmonella* Bareilly were more distant from others *Salmonella* species by chromosomal pattern analysis obtained from PFGE method. Cluster analysis (Figure 2) grouped *S. houtenae* strains with *Salmonella* Paratyphi B and *Salmonella* Paratyphi C. The *S. houtenae* strains, *Salmonella* Paratyphi B and *Salmonella* Paratyphi C are more genotypic related to each other that *Salmonella* Typhimurium and *Salmonella* Bareilly strains.

The PFGE is an approach to measure the genetic diversity of the entire genome, which may have arisen as a result of mutation. Such mutation may remove or create recognition sites through insertion, deletion, translocation and inversion or by mobile genetic elements. The assumption is that as time passes and organisms spread, divergence may occur (Bakeri et al., 2003; Abassi et al., 2010). Laconcha et al. (2000) studied genotypic characterization by PFGE of *Salmonella* strains and concluded that this technique represents a suitable tool for the

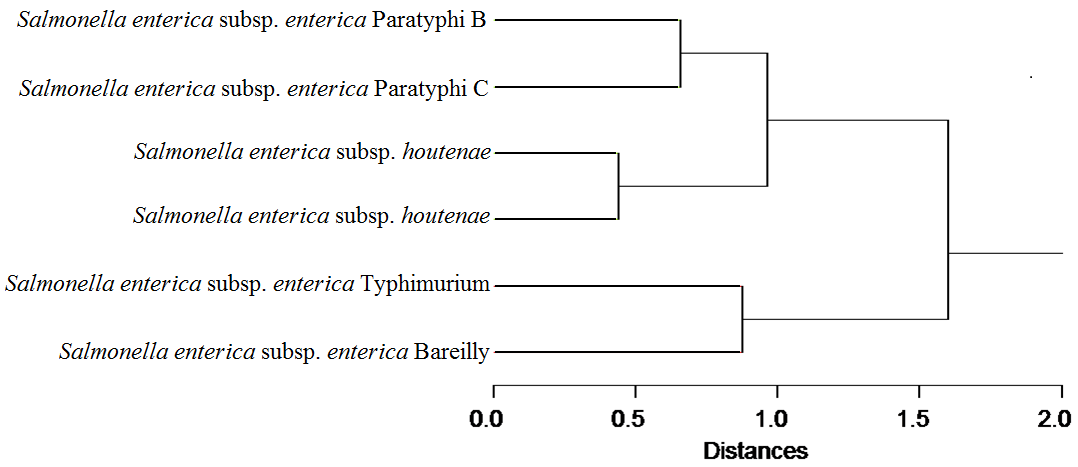

Figure 2. Cluster analysis of chromosomal pattern of *Salmonella* strains isolated from commercial Brazilian sausage.

Table 1. Antimicrobial susceptibility[a] of *Salmonella* isolates from pork sausages.

Antimicrobials	*Salmonella* isolates					
	Paratyphi B	*Houtenae*	*Houtenae*	Typhimurium	Bareilly	Paratyphi C
Cefotaxime	R	R	I	R	I	R
Ampicillin	R	R	R	R	R	I
Amikacin	R	R	R	R	R	R
Ceftazidime	I	R	S	R	I	I
Cephalothin	R	R	R	R	I	R
Sulfazothrim	S	R	S	S	S	S
Cefoxitin	R	R	S	R	S	R
Gentamicin	R	S	R	R	R	R
Tetracycline	I	S	R	S	S	S
Ceftriaxone	R	I	S	S	S	I
Chloramphenicol	R	S	I	R	S	S
Aztreonam	I	I	S	R	I	I

[a] R: Resistant; I: intermediary; S: susceptible.

epidemiological typing of *Salmonella* strains.

In the antimicrobial susceptibility test, all six *Salmonella* isolates in this study were resistant to amikacin (Table 1). Sulfazothrim was the antimicrobial in which all the serovars of *S. enterica* subsp. *enterica* were sensitive. The two isolates of *S. houtenae* showed different susceptibility to the following antimicrobial agents: ceftazidime, sulfazothrim, cefoxitin, gentamicin and tetracycline. According to Schwarz et al. (2010), the resistance of three or more classes of antimicrobial agents can be referred to as multi-resistance. In our studies, *S. houtenae* and the serovars Paratyphi B and Typhimurium isolated from pork sausage were multidrug-resistant (MDR). The serovar Typhimurium was resistant to five classes of antimicrobial agents. The large profile of antimicrobial resistance for these *Salmonella* isolates from pork sausage might be a great risk for public health. Kim et al. (2011) reported that *Salmonella* spp. isolated from pigs at slaughterhouses in Korea demonstrated an appreciable broad-spectrum, (multi)-antimicrobial resistance. Thus, a continuous antibiotic surveillance program may be worthwhile.

To determine the heat resistance, a cocktail of *Salmonella* isolated and identified in this study was inoculated into fresh prepared pork sausage. The thermal inactivation curves were linear in all temperatures evaluated (58, 62 and 65°C). The determination of R^2 coefficient of the regression curves was always higher

Table 2. Enumeration using log_{10} (CFU/g) at time points (minutes) and heat-resistance (expressed in *D*-values) of a cocktail of six *Salmonella* isolates in fresh pork sausage.

Temperature (°C)	Time (min)					Equation	*D*-values (min)
	0	5	10	15	Average		
58	10.173[a]	9.903[a]	9.586[a]	8.756[a]	9.605[a]	y = -0.091x + 10.29 R^2 = 0.92	10.99
62	10.196[a]	9.873[b]	8.836[b]	7.386[b]	9.073[b]	y = -0.189x + 10.49 R^2 = 0.93	5.29
65	10.190[a]	9.860[b]	6.396[c]	3.623[c]	7.517[c]	y = -0.463x + 10.99 R^2 = 0.92	2.16
Average	10.186	9.878	8.273	6.588	8.731		

For each column, mean values with different letters are significant ($P < 0.001$) according to the Scott–Knott test.
Standard error medium (SEM) = 0.007.

than 0.90 (Table 2). The regression curves of temperatures 58, 62 and 65°C presented a reduction of (CFU/g) 0.091, 0.189 and 0.463 log/min of microorganism, respectively. Thus, the decimal reduction times (*D*-values) of the *Salmonella* cocktails in the sausage decreased substantially with an increase in temperature (Table 2). The *D*-values for 58, 62 and 65°C were 10.99, 5.29 and 2.16 min, respectively. The *z*-value was 10.1°C. There is limited information about thermal inactivation of *Salmonella* in pork sausage (Mattick et al., 2002). In pork meat containing curing additives, Quintavalla et al. (2001) reported that for six different strains of *Salmonella* inoculated in product, the D_{58}-value ranged from 2.79 to 4.8 min and the *z*-value ranged from 4.1 to 4.8°C. Juneja et al. (2001) inoculated a cocktail of eight serovars of *Salmonella* in pork meat (8.5% fat) and determined that D_{58}, $D_{62.5}$ and D_{65} values were 6.68, 1.62 and 0.87 min, respectively, and the *z*-value was 7.1°C. The D-value and *z*-value determined in this work were higher than the values calculated for pork meat in previous studies. This difference may be due to the fat content of the sausage. The protective effects of fat may be due to the lowest heat conductivity or reduced water activity in the fat portion (Senhaji, 1977). In general, high fat content results in high thermal resistance (Juneja et al., 2001). Typically, fresh pork sausage contains between 10 to 40% of fat (according to manufacturing industries in Brazil).

In the enumeration of cell viability of *Salmonella*, there was an interaction ($p<0.01$) between heated sausages at different temperatures and their heating time (Table 2). For each temperature, the reduction (log_{10} CFU/g) of *Salmonella* was significant at each time point. At 5 min, there was no difference between the temperatures of 62 and 65°C for inactivation of the microorganism. At this time, the reduction of the microorganism was still low, only 0.32 and 0.33 log units from the initial time for 62 and 65°C, respectively. From 10 min, the temperature of 65°C was more effective in reducing of *Salmonella*, with population decline to 3.794 log units in relation to the initial time. In the pork meat chain, the specific time and temperature binomial is also valid for other processes and stages. In animal slaughter, there is a wide variation in the scalding process; Brazilian legislation on the technical standards for slaughtering pigs cites between 62 to 72°C and 2 to 5 min (Brazil, 1995). These standards may not be effective for pathogen inactivation.

Conclusion

Brazilian industrial pork sausage may be vehicles for transmitting *S. enterica*. *S. houtenae* and the serovars Paratyphi B and Typhimurium. The isolates obtained were genotypically different and resistant to three or more classes of antimicrobial agents. According to the results of this study, the time and temperature binomial of the inactivation of microorganisms in sausages is higher than the binomial typically employed for meat, probably due to the fat content in the product. Thus, heating times of 10.99, 5.29 and 2.16 min with internal temperature of 58, 62 and 65°C, respectively, in fresh sausage, ensure a higher level of microbiological quality and offer less risk of salmonellosis to consumers of product. The resulting kinetic parameter can be useful to pork industry in designing and estimating thermal processes specific for sausage.

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