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Prevalence, genetic characterization and antimicrobial resistance of *Salmonella* isolated from fresh pork sausages in Porto Alegre, Brazil

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ABSTRACT

A total of 336 samples of fresh pork sausage randomly obtained from supermarkets and butcher shops in Porto Alegre, Brazil, were examined for the presence of *Salmonella* serovars. *Salmonella enterica* was detected in 82 (24.4%) of the samples, with a most probable number count ranging from 0.03 MPN g^{-1} to 460 MPN g^{-1}. Strains belonging to the most isolated *S. enterica* serovars (Brandenburg, Panama, Derby and Typhimurium) were further characterized by *Xba*I-macrorestriction, resulting in a total of 17 profiles. Resistance to tetracycline was the most prevalent among the *Salmonella* isolates. *S. panama* and *S. typhimurium* presented the greatest number of resistance phenotypes.

1. Introduction

Salmonella is the leading cause of food-borne illness in southern Brazil, with foods of animal origin being reported as the major vehicle of this pathogen (Costalunga & Tondo, 2002; Geimba, Tondo, Oliveira, Canal, & Brandelli, 2004). Although contaminated eggs and raw or undercooked poultry are involved in most human salmonellosis cases worldwide (Rabsch, Tschäpe, & Bäumler, 2001), it was estimated that pork causes 15–20% of all human cases of *Salmonella* infection (Berends, Urlings, Snijders, & Van Knapen, 1996).

The southern region of Brazil is the most important area for swine production in the country. Its pork output accounted for two thirds of the country's total production, which consisted of 2.8 million tons in 2006. Approximately 70% of the pork in Brazil is consumed in the form of processed meat products (sausages, bacon and ham). Among these, fresh pork sausages are widely consumed in southern Brazil, and are usually served roasted in restaurants and households.

Pork contamination by *Salmonella* can occur at multiple stages along the production chain. Moreover, the level of *Salmonella* infection of pigs on farms can be increased during transport, lairage and slaughtering-plant operations (Swanenburg, Urlings, & Snijders, 2001; Vieira-Pinto, Tenreiro, & Martins, 2006).

Studies conducted in southern Brazil showed a high carriage rate of *Salmonella* in pigs at slaughter (Bessa, Costa, & Cardoso, 2004), which was associated with the increased isolation of *Salmonella* from batches of minced meat included in the production of fresh sausage (Castagna, Schwarz, Canal, & Cardoso, 2004). Thus, a survey to estimate the frequency and level of *Salmonella* contamination of fresh pork sausage at retail outlets in Porto Alegre, southern Brazil, was conducted.

2. Material and methods

2.1. Sample collection

Fresh pork sausage samples ($n = 336$) prepared in industrial food processing plants, including prepackaged ($n = 155$) and store-packaged sausage samples ($n = 181$), were randomly purchased from 36 butcher's shops and supermarkets in Porto Alegre, Brazil. Stores were located in five central boroughs of the city and were identified using phone books. Thirty three retail stores were visited twice and in three stores only one sampling was conducted. Sampling visits were made every Monday for eight months (April to November 2005). On each sampling day, three stores were randomly chosen in one of the five boroughs. Five prepackaged or store-packaged raw pork sausages were randomly selected in each store and transported on ice to the laboratory. All sampled pork sausages were stored under refrigeration at the stores.

2.2. Salmonella isolation

Approximately 8 g of minced meat were removed aseptically from the casing of three sausages belonging to the same purchased sample and put in a stomacher bag, until a 25 g-aliquot was obtained. The remaining sausage portions were stored in sterile flasks

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for up to seven days at 4 °C. To each stomacher bag, 250 mL of buffered peptone water (BPW, Merck, Darmstadt, Germany) was added as pre-enrichment media, and the mixture was homogenized. After incubation at 37 °C for 18 h, aliquots of 0.1 mL or 1.0 mL were transferred to Rappaport-Vassiliadis broth (RV, Merck) or Tetrathionate broth (Difco Laboratories, Detroit, USA), respectively. After incubation at 42 °C for 24 h, broth cultures were streaked onto both XLT4 (Difco) agar and Brilliant Green Lactose Saccharose (BPLS, Merck) agar. Plates were incubated at 37 °C for 24 h. Up to 5 colonies were selected from each plate and were streaked on triple sugar iron agar (TSI, Merck), lysine decarboxylase iron agar (LIA, Merck) and inoculated into urea broth (Merck). Colonies presumptively identified as *Salmonella* were submitted to slide agglutination tests using poly-O-antiserum (Probac, São Paulo, Brasil) and were serotyped at the Brazilian *Salmonella* Reference Institute (Instituto Fundação Oswaldo Cruz, Rio de Janeiro, Brazil).

2.3. Salmonella quantification

Fresh pork sausage samples from which *Salmonella* was isolated were submitted to estimation of *Salmonella* using a most probable number (MPN) technique (Sinell, Pietzsch, Klingbeil, & Benner, 1990). From each positive sample, which was kept refrigerated, three portions of each 10 g, 1 g and 0.1 g were taken aseptically and added individually to tubes with BPW. After incubation of the pre-enrichment tubes at 37 °C for 18 h, aliquots of 0.1 mL were transferred to 9.9 mL of RV (Merck). Following incubation of the tubes of selective enrichment media at 42 °C for 24 h, the samples were streaked onto XLT4 (Difco) agar. After 24 h incubation at 37 °C, suspected colonies from each plate were confirmed as *Salmonella* by biochemical tests and agglutination using poly-O-antiserum (Probac). The number of tubes in each dilution, from which colonies were confirmed as *Salmonella*, was used to estimate *Salmonella* quantification using the MPN table (BAM, 1998).

2.4. Macrorestriction analysis

Genomic DNA of *Salmonella* isolates, belonging to representative serovars, was extracted as previously described (Liebisch & Schwarz, 1996; Schwarz & Liebisch, 1994). Slices of DNA-containing agarose plugs were digested with 20 units of *Xba*I (Promega, Madison, WI, USA) at 37 °C for 18 h. The respective fragments were separated by pulsed field gel electrophoresis (PFGE) in 1% PFGE-certified Agarose gel (BioRad, Hercules, CA, USA) in a CHEF DR II system (BioRad, California, USA) at 5.6 V/cm with 0.5× TBE as the running buffer. In order to avoid the DNA degradation of *Salmonella panama* isolates, 50 μm of Thiourea (Acros Organics, Geel, Belgium) was added to the running buffer. The pulse times were increased from 10 to 30 s during the first 11 h and subsequently from 30 to 50 s during the next 13 h. The gel was stained with ethidium bromide (2 μg/ml, Sigma, St. Louis, MO, USA) and photographed under UV-illumination. The *Xba*I fragments of *S. typhimurium* LT2 served as size standards (Liu, Hessel, & Sanderson, 1993). Patterns produced by PFGE were compared using the GelCompar II software package (Applied Maths, Kortrijk, Belgium). Dendrograms were constructed by the unweighted pair-group method with arithmetic averages (UPGMA) using the Dice coefficient.

2.5. Antimicrobial susceptibility testing

Antimicrobial resistance was determined by agar disk diffusion tests using disks with the following antimicrobials (Cefar Diagnóstica, São Paulo, Brazil): amikacin (Am, 30 μg), ampicillin (Ap, 10 μg), cefaclor (Ce, 30 μg), ciprofloxacin (Ci, 5 μg), chloramphenicol (Cm, 30 μg), gentamicin (Ge, 10 μg), nalidixic acid (Na, 30 μg), tetracycline (Te, 30 μg), tobramycin (To, 10 μg), streptomycin (Sm, 10 μg), sulfamethoxazole-trimethoprim (St, 25 μg), and sulfonamide (Su, 300 μg). The testing was conducted and evaluated according to the document M100-S15 of the Clinical and Laboratory Standards Institute (CLSI/NCCLS, 2005). *Escherichia coli* ATCC 25922 was used for quality control testing.

3. Results

Salmonella enterica was detected in 82 (24.4%) of the fresh pork sausages collected at retail level in Porto Alegre, Brazil. Positive samples were identified in fresh pork sausages belonging to 12 of 22 brands included in this study. No statistical difference ($P = 0.64$) was detected in the frequency of *S. enterica* isolation between pre-packaged (36/155) and store-packed (45/181) samples. Except for one strain identified as belonging to the subspecies *houtenae*, the remaining isolates belonged to the subspecies *enterica*. Isolates were assigned to 12 serovars of *S. enterica* subsp. *enterica* (Table 1), most of them belonging to serogroups B (58%) and D1 (22.2%).

S. enterica could not be re-isolated from 20 samples during the quantification assays. In the remaining 62 positive samples the level of *S. enterica* varied from 0.03 MPN g^{-1} to 460 MPN g^{-1} (Table 1), with a median value of 0.23 MPN g^{-1}. The single fresh pork sausage sample with a previous isolation of *S. enterica* subsp. *houtenae* demonstrated a low level of contamination (0.03 MPN g^{-1}).

Strains belonging to the four most commonly isolated *S. enterica* serovars (Brandenburg, Panama, Derby and Typhimurium) were further characterized by *Xba*I-macrorestriction, resulting in a total of 17 profiles with 10–24 fragments each (Fig. 1). *S. derby* and *S. brandenburg* presented the highest diversity. The similarities among the different profiles in these serovars varied in the range 38.7–96.8%. Contrarily, *S. panama* was the most homogeneous with only two different profiles and an overall similarity of 83.3%. Similar *Xba*I-macrorestriction profiles were distributed among fresh pork sausages samples of different brands (Fig. 1). Nevertheless, members of some profiles (Br3, De3 and Pa1) were obtained from sausage samples of the same brand collected during different sampling events (data not shown).

One isolate from each positive sausage sample was submitted to antimicrobial resistance testing. Of a total of 82 strains analyzed, sixty-four (82%) displayed resistance to at least one antimicrobial agent. The isolates showed an overall resistance to 11 of the 14

Table 1
Distribution and quantification of *Salmonella enterica* subsp. *enterica* serovars isolated from fresh pork sausages samples collected in Porto Alegre, Brazil

Salmonella enterica serovars (Serogroup)	Total isolates	Negative samples	MPN g^{-1} [a]				
			0.03–0.1	0.1–1.0	1.0–10	10–100	460
Brandenburg (B)	14	6	3	5			
Panama (D1)	12	2	4	5		1	
Derby (B)	11	1	3	6	1		
Typhimurium (B)	8	1	4	3			
Agona (B)	6	1	2	2		1	
Infantis (C1)	5	–	2	1			2
S. enterica O:4,5 (B)	5	2	1	1		1	
S. enterica O:9,12 (D1)	4	–	2	2			
Schwarzengrund (B)	3	1	2				
Senftenberg (E4)	3	2				1	
Ohio (C1)	2	–		1	1		
Orion (E1)	2	–		2			
Cerro (K)	2	1			1		
S. enterica rough	2	1		1			
Münster (E1)	1	1					
S. enterica O:6,7 (C1)	1	–		1			
Total	81	20	22	31	2	4	2

[a] Most probable number.

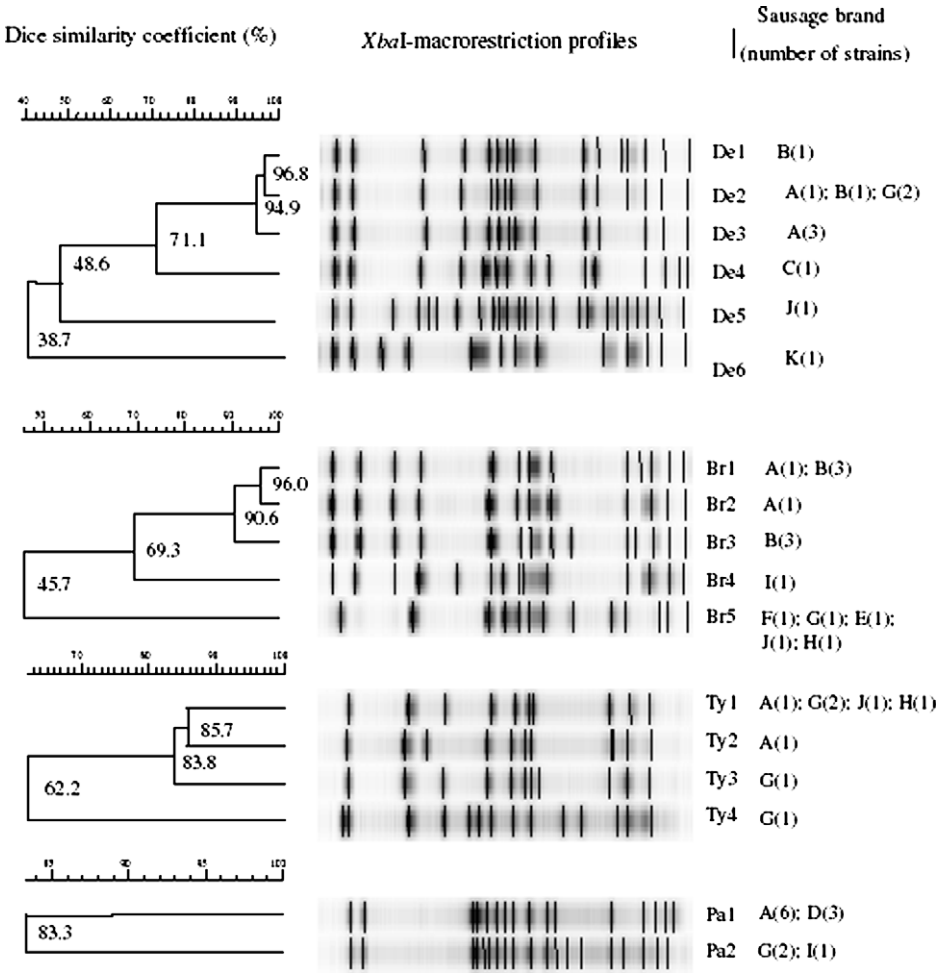

Fig. 1. XbaI-macrorestriction profiles identified in the most representative *Salmonella serovars* (*De*: *S. derby*; *Br*: *S. brandenburg*; *Ty*: *S. typhimurium*; *Pa*: *S. panama* isolated from fresh pork sausage samples in Porto Alegre, Brazil. The dendrogram presents the similarity determined by Dice coefficient and UPGMA clustering.

drugs tested. The highest frequency of resistance was observed to tetracycline (70.7%) followed by sulfonamide (54.9%). None of the isolates were resistant to amikacin, cefaclor or ciprofloxacin. The frequency of resistance against antimicrobials varied among serovars (Table 2).

Among the 64 isolates which showed resistance, 55 (85.9%) were found to be resistant to more than one antimicrobial. Thirty different multidrug-resistance (MDR) patterns were found, and most of them were represented by only one strain. Among the most prevalent serovars, the MDR pattern StSuTe was the most prevalent.

4. Discussion

The present study demonstrated that *S. enterica* was isolated from 24.4% of fresh pork sausages obtained from supermarkets and butcher's shops in Porto Alegre, Brazil. Fresh pork sausages were used as test items because they are widely available in grocery stores and commonly consumed in this region. Frequencies of *Salmonella* isolation from pork sausages vary widely among different countries, and rates between 1.7% in Ireland (Boughton et al., 2004) to 88.3% in Mexico (Escartin, Castillo, Hinojosa-Puga, & Saldaña-Lozano, 1999) have been reported.

Table 2
Antimicrobial resistance frequency among *Salmonella* isolates from fresh pork sausages by serovars

Antimicrobial	Number of strains (%) serovar of *Salmonella* isolate				
	Brandenburg (n = 14)	Panama (n = 12)	Derby (n = 11)	Typhimurium (n = 8)	All other serovars (n = 36)
Ampicillin	0	10 (83.0)	1 (9.1)	5 (62.5)	9 (25.0)
Chloramphenicol	1 (7.1)	9 (75.0)	2 (18.2)	3 (37.5)	10 (27.8)
Gentamicin	0	1 (8.0)	0	1 (12.5)	0
Nalidixic acid	5 (35.7)	6 (50.0)	0	4 (50.0)	5 (13.9)
Tetracycline	13 (92.9)	9 (75.0)	11 (100)	7 (87.5)	18 (50.0)
Tobramycin	0	0	0	1 (12.5)	1 (2.8)
Streptomycin	1 (7.1)	9 (75.0)	5 (45.5)	1 (12.5)	7 (19.4)
Sulfamethoxazole-trimethoprim	12 (85.7)	4 (33.0)	2 (18.2)	1 (12.5)	5 (13.9)
Sulfonamide	12 (85.7)	10 (83.0)	9 (81.8)	3 (37.5)	11 (30.6)

Among the 82 *Salmonella* isolates from pork sausages in this study, only one strain did not belong to *S. enterica* subsp. *enterica*. The relevance of this finding is uncertain, since in Brazil less than 1% of 4581 *Salmonella* strains isolated from non-human sources have been identified as *S. enterica* subsp. *houtenae* (Tavechio et al., 2002), and human infections associated with this subspecies are considered rare. Nevertheless, a case of bacteremia in a 33-year-old HIV-infected patient caused by *S. enterica* subsp. *houtenae* was diagnosed in Rio de Janeiro (Lourenço, Reis, Valls, Asensi, & Hofer, 2004). In this case, close contact with animal reservoirs or the contamination through ingested food were indicated as possible sources of infection.

S. enterica subsp. *enterica* strains belonging to serogroups B and D1 accounted for 77.8% of all isolates. These serogroups were also the most prevalent in porcine samples collected at slaughterhouses in southern Brazil (Bessa et al., 2004; Castagna et al., 2004), and seem to be the most relevant to the swine industry in this region. The most isolated serovars in our study are frequently isolated from pork (Botteldoorn, Herman, Rijpens, & Heyndrickx, 2004; Escartin et al., 1999) and were reported as the cause of several outbreaks in humans (Herikstad, Motarjemi, & Tauxe, 2002). *S. typhimurium* specifically constitutes a major pathogen responsible for salmonellosis in humans (Mead et al., 1999; Rabsch et al., 2001). Contrary to this, only *S. typhimurium* is among the top 10 serovars isolated from human patients and from non-human sources in Brazil (Taunay et al., 1996; Tavechio, Fernandes, Neves, Dias, & Irino, 1996). In this country, *S.* Enteritidis has been the most prevalent serovar from every kind of source (Tavechio et al., 2002), and was also identified in 97% of foods involved in outbreaks occurring in the same region of our study (Geimba et al., 2004). In both studies poultry meat, eggs and homemade mayonnaise accounted for the majority of foods implicated in food borne outbreaks, while pork represented less than 1% of the samples.

In accordance to other reports (Escartin, Saldaña-Lozano, & Garcia, 2000; Sinell et al., 1990), *Salmonella* could not be re-isolated from 20 sausage samples in our study using the quantitative assay, although corresponding portions of the same samples had been positive in the qualitative assay. The heterogeneous distribution and the overall low number of salmonellae in the sample may be pointed as the cause of failure in the re-isolation of salmonellae from meat (Sinell et al., 1990). Similar to other studies of *Salmonella* quantification in pork, the great majority of samples in our study demonstrated a level of contamination below 100 MPN g^{-1} (Escartin, Saldaña-Lozano, Rodríguez, Martínez-Gonzáles, & Torres, 1995; Giovannini et al., 2004; Sinell et al., 1990). In spite of the generally low level of contamination found, and the habit of eating pork sausages well-done in southern Brazil, every *Salmonella*-positive sample may represent a potential health hazard. Furthermore, contaminated sausages may also represent a potential source of cross contamination in the kitchen environment and for ready-to-eat foods, since they are handled and prepared in the raw state.

The potential sources of the high *Salmonella* prevalence found in pork sausages were investigated by analyzing *Xba*I-macrorestriction profiles of strains belonging to the most isolated *Salmonella* serovars. Although strains of some profiles (Br3, De3 and Pa1) were obtained from sausage samples of the same brand, in most cases similar profiles were distributed among fresh pork sausages samples of different brands. Furthermore, most *Salmonella* strains isolated from sausage samples of the same brand presented different profiles or belonged to different serovars. These results reflect the complexity of the *Salmonella* transmission chain, which provides many opportunities for infection/reinfection and cross contamination on pre-harvest and post-harvest stages. Moreover, as demonstrated in previous studies (Castagna et al., 2004; Duffy et al., 2001), pork products, such as sausages, which are exposed to more extensive handling than regular meat have a higher risk of *Salmonella* contamination at food processing plants. Furthermore, in butchers' shops many opportunities for cross contamination of store-packed products are also present. During cutting and handling, the microbial load is redistributed throughout the product and can increase due to exposure to other contamination sources, such as knives and work tables (Berends et al., 1996; Reij & den Aantrekker and ILSI Europe Risk Analysis in Microbiology Task Force, 2004). In this sense, cross contamination at retail level could explain store-packed sausage samples of different brands purchased in the same store which presented *Salmonella* strains with a common *Xba*I-macrorestriction profile in two sampling events of our study.

Multidrug-resistant *Salmonella* spread by food animals represents an additional public health risk (Cruchaga et al., 2001). In this study, we demonstrated the widespread occurrence of antimicrobial resistance to tetracycline and sulfonamide in *Salmonella* strains isolated from fresh pork sausages. This finding is in accordance with previous studies of *Salmonella* strains isolated from slaughtered pigs in Brazil (Michael, Cardoso, & Schwarz, 2006; Oliveira et al., 2002; Bessa et al., 2007). Moreover, a relatively high frequency of chloramphenicol resistance in isolates tested in this study and elsewhere (Bessa et al., 2007) was also detected. Tetracycline and sulfonamide have been widely used in pig production in Brazil, and this fact can explain the high rates of resistance which were found. On the other hand, chloramphenicol was banned from animal production in Brazil more than a decade ago. The occurrence of resistance against this antimicrobial could possibly be due to the emergence and spread of multi-drug resistant *Salmonella* isolates that harbour physically linked resistance to this drug, as previously reported (Briggs & Fratamico, 1999). Contrary to the increasing incidence of ciprofloxacin-resistant *S. typhimurium* reported in other countries (Angulo, Johnson, Tauxe, & Cohen, 2000; Threlfall, Ward, & Rowe, 1997), we did not find resistance to ciprofloxacin. However, a marked resistance to nalidixic acid was detected in the most prevalent serovars (except for *S. derby*) isolated from fresh pork sausages. This is a matter of concern, since nalidixic acid resistance has been associated with a decrease in susceptibility to fluoroquinolones, which is used to treat salmonellosis in humans (Gorman & Adley, 2004).

In our study, 30 different MDR patterns were found, and two serovars commonly involved in food borne outbreaks, *S. panama* and *S. typhimurium*, presented the greatest number of resistance phenotypes, and may reflect a reservoir of resistance in animals which can be transmitted to humans. Contrarily, the most common resistant phenotype presented by *S. typhimurium* DT104 strains (resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline) (Beaudin et al., 2002) was not detected in strains of our study. As antibiotic usage varies among countries, different patterns of resistance phenotypes and genotypes can be expected. Thus, the monitoring of antimicrobial resistance patterns presented by pathogenic strains isolated from different sources and regions is an important issue.

Our data confirms that raw pork sausages may be vehicles for transmitting *S. enterica* in southern Brazil. To diminish contamination rates by *Salmonella* in fresh pork sausages, it is critical that risk reduction strategies are used throughout the food chain. These strategies should include on-farm practices which reduce pathogen carriage, increased hygiene at slaughter and meat processing, continued implementation of HACCP systems, and increased consumer education efforts.

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