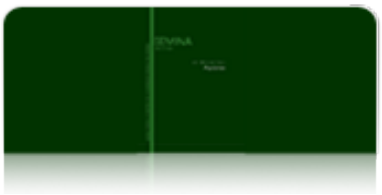

Semina: Ciências Agrárias
ISSN: 1676-546X
ISSN: 1679-0359
Universidade Estadual de Londrina

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Semina: Ciências Agrárias, vol. 39, no. 4, 2018, July-August, pp. 1533-1545
Universidade Estadual de Londrina

DOI: https://doi.org/10.5433/1679-0359.2018v39n4p1533

Available in: https://www.redalyc.org/articulo.oa?id=445759824013

DOI: 10.5433/1679-0359.2018v39n4p1533

Detection of *Salmonella* spp. and diarrheagenic *Escherichia coli* in fresh pork sausages

Pesquisa de *Salmonella* spp. e *Escherichia coli* diarreiogênica em linguiças suínas frescas

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Abstract

The presence of pathogenic microorganisms in meat products may result in foodborne diseases and economic losses to their producers. Small industries in the region of Londrina, Paraná, produce sausages that are commercialized in free fairs, small markets, bars, and restaurants in the city. Although these industries are inspected by the Municipal Inspection Service of Londrina, there are no data about the pathogenic microorganisms present in these products. The objective of this study was to investigate the presence of *Salmonella* spp. in sausages produced and sold in the region of Londrina, Paraná, and identify *eae*, *bfp, stx*1, *stx*2, *hlyA*, *ipaH, elt, est, aggR, aap*, and *AA probe* genes in *Escherichia coli* strains isolated from these samples. Forty-six samples of three types of sausages (fresh pork, Tuscan, and Calabresa) produced by four different producers (brands A, B, C, and D) were analyzed. *Salmonella* spp. was isolated from 13 (28.3%) and *E. coli* from 33 (71.3%) of the analyzed samples. Seven (53.8%) of 13 samples contaminated with *Salmonella* spp. were from brand A. *Salmonella* spp. contamination was the highest in the Tuscan sausage samples (8/17, 41.7%) when compared with the fresh pork sausage samples of all brands analyzed. *E. coli* was isolated from 12 of 13 samples contaminated with *Salmonella* spp. One sample of Calabresa sausage was contaminated with atypical enteropathogenic *E. coli* serotype O108:H9 that has the *eae* and *hlyA* genes. The results suggest contamination of the processing plant and/or raw meat used in the manufacture of sausages. A better inspection of the industries is required to ensure that Good Manufacturing Practices are followed by which the contamination of products by pathogenic bacteria can be prevented.

Key words: Pork meat. Enteropathogenic bacteria. Virulence genes.

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Received: June 23, 2017 - Approved: May 28, 2018

Resumo

A presença de microrganismos patogênicos em produtos cárneos pode levar a doenças de origem alimentar e perdas econômicas aos seus produtores. Pequenas indústrias da região de Londrina, Paraná, produzem embutidos que são comercializados em feiras livres, pequenos mercados, bares e restaurantes da cidade. Embora essas indústrias sejam fiscalizadas pelo Serviço de Inspeção Municipal de Londrina, não existem dados sobre microrganismos patogênicos presentes nesses produtos. Os objetivos deste estudo foram pesquisar a presença de *Salmonella* spp. em amostras de linguiças produzidas e comercializadas na região de Londrina, Paraná, e identificar os genes de virulência *eae*, *bfp, stx*1, *stx*2, *hlyA*, *ipaH, elt, est, aggR, aap* e *AA probe* das cepas de *E. coli* isoladas dessas amostras. Quarenta e seis amostras de três tipos de linguiça (suína fresca, toscana e calabresa) produzidas por quatro diferentes produtores (marcas A, B, C e D) foram analisadas. *Salmonella* spp. foi isolada em 13 (28.3%) amostras e *E. coli* em 33 (71.3%) amostras. Das 13 amostras contaminadas com *Salmonella* spp., sete (53.8%) foram da marca A. A contaminação por *Salmonella* spp. foi maior nas amostras de linguiça toscana (8/17 – 41.7%) quando comparada com a contaminação encontrada nas amostras de linguiça suína (4/22 – 18.2%), independente da marca analisada. *E. coli* foi isolada de 12 das 13 amostras contaminadas com *Salmonella* spp. Uma amostra de linguiça calabresa estava contaminada com *E. coli* enteropatogênica clássica atípica do sorotipo O108:H9, que apresentava genes *eae* e *hlyA*. Os resultados obtidos sugerem contaminação da planta de processamento e ou das matérias primas utilizadas na fabricação das linguiças. Uma fiscalização mais adequada das indústrias se torna necessária para que as Boas Práticas de Fabricação sejam atendidas e, consequentemente, prevenida a contaminação do produto por bactérias patogênicas.

Palavras-chave: Carne suína. Bactérias enteropatogênicas. Genes de virulência.

Introduction

The prevention of meat product contamination is a challenge faced by the meat industry. The presence of pathogenic microorganisms in these food products may result in foodborne diseases and economic losses to their producers, such as the need to dispose of the contaminated batch and lawsuits by consumers who become ill due to the *ingestion of* the contaminated product.

Many studies have reported the isolation of diarrheagenic *E. coli* and *Salmonella* spp. in pork meat and its derivates (MÜRMANN et al., 2007; LIMA et al., 2011; CHARIMBA et al., 2012; RANTSIOU et al., 2012; CABRAL et al., 2014). According to the Brazilian Health Ministry, 6,848 outbreaks of foodborne diseases were reported between 2007 and 2016 that resulted in 121,283 cases of illness, 17,517 hospitalizations (14.5%), and 111 deaths (0.09%). The main etiologic agents responsible for the outbreaks were *Salmonella* spp. (7.3%), *Escherichia coli* (7.3%), and *Staphylococcus aureus* (5.7%). The contaminated food was not identified in majority of the reported outbreaks (66.9%). Mixed foods were the main cause of the outbreaks where the contaminated food had been identified (2,266, 33.1%). Raw pork meat and derivatives and pork offal were responsible for 103 (4.5%) of the reported outbreaks that had known etiology (BRASIL, 2017).

Eight diarrheagenic *E. coli* (DEC) groups are known: shiga toxin-producing *E. coli* (STEC), which includes the subgroup enterohemorrhagic *E. coli* (EHEC); typical and atypical enteropathogenic *E. coli* (EPECt and EPECa); enterotoxigenic *E. coli* (ETEC); enteroinvasive *E. coli* (EIEC); enteroaggregative *E. coli* (EAEC); diffusely adherent *E. coli* (DAEC); and adherent invasive *E. coli* (AIEC). Enteroaggregative hemorrhagic *E. coli* (EAHEC) O104:H4 is an emerging *E. coli* pathotype associated with an outbreak that occurred in Germany in May 2011. The genes used for genetic characterization of these groups are *stx*1

and *stx*2 (STEC); *stx*1, *stx*2, and *eae* (EHEC); *eae* (EPECa); *eae* and *bfp* (EPECt); enterotoxin genes LT and ST and colonization factors (ETEC); *ipaH* and *ial* (EIEC); and *aggR, aap*, and *AA probe* (EAEC) (CROXEN et al., 2013). Genes for the genetic characterization of AIEC have not yet been characterized and there is no agreement among authors for the genetic characterization of DAEC (NATARO; KAPER, 1998; CROXEN et al., 2013). EAHEC O104:H4 is an enteroaggregative *E. coli* with the *stx*2a encoding gene (BEUTIN; MARTIN, 2012).

The incidence of gastroenteritis caused by EAEC and EPEC, specially EPECa, has increased in Brazil (REGUA-MANGIA et al., 2004; FRANZOLIN et al., 2005; MORENO et al., 2010; LOZER et al., 2013; DIAS et al., 2016). In the last few years, EPECa was isolated as the cause of gastroenteritis in Brazil in more cases than EPECt, and as a shiga toxin-producing *E. coli* (STEC), it is an emerging food pathogen (SCHMIDT, 2010).

EPECa serotypes have already been reported in the feces of pig, and pork meat may be a vehicle of transmission of this bacterium to humans. The infection of pig herds by *Salmonella* tends to be a persistent problem in food production systems. Submandibular lymph nodes and tonsils of pigs contaminated by *Salmonella* spp. may remain in the carcasses after slaughter, and combined with head region muscles, be used in processed meat manufacturing.

Brazil is the fourth largest producer and exporter of pork meat. In 2016, Brazil produced about 3.7 million tons and 19.6% of this was exported. In Brazil, the per capita consumption of pork meat in 2016 was 14.4 kg. Paraná is the second largest producer of pork meat and produced 13.2% of the total of the pork meat exported by Brazil in 2016. In this year, 13.2% of swine slaughtered in Brazil was in Paraná (ABPA, 2017).

In Londrina, Paraná, Brazil and the *surrounding areas* there are some meat processing industries that commercialize their products in free fairs, small markets, bars, and restaurants of the city. The Municipal Inspection Service of Londrina is responsible for the inspection of these industries. However, there are no data about the pathogenic microorganisms present in these products.

The aims of this study were to evaluate the presence of *Salmonella* spp. in sausage samples produced and commercialized in Londrina, and identify *eae*, *bfp, stx*1, *stx*2, *hlyA*, *ipaH, elt, aggR, aap*, and *AA probe* genes in *E. coli* strains isolated from the sausage samples.

Material and Methods

Sampling

Forty-six samples of three types of fresh sausages (22 fresh pork, 17 Tuscan, and 7 Calabresa) produced by four different producers of Londrina, Paraná, Brazil and the *surrounding* areas were purchased weekly from March to July 2015 in four supermarkets of Londrina. The brands were nominated as A, B, C, and D. The number of samples from brands C and D were smaller because these brands were not always available for purchase.

Salmonella spp. detection

Salmonella spp. detection was performed according to Silva et al. (2017). Twenty-five grams of each sample was homogenized in 225 mL of buffered peptone water in a Stomacher blender (Stomacher 400, Seward, Worthing, England). After incubation at 35°C for 24 h, 1.0 mL and 0.1 mL aliquots of the non-selective enrichment were inoculated, respectively, in 10 mL of Selenite Cystine Broth (SC) (Himedia Laboratories Pvt. Ltd., Mumbai, India) and in 10 ml of Rappaport-Vassiliadis (RV) broth (Himedia Laboratories). The SC broth was incubated at 35°C and RV broth at 42°C for 24 h. Hektoen agar (Himedia Laboratories) and Xylose Lysine Deoxycholate (XLD) (Himedia Laboratories) used as selective

and differential media, were incubated at 35°C for 24 h. Biochemical screening using motility, production of indole, decarboxylation of lysine, and hydrolysis of urea was carried out with the characteristic colonies of *Salmonella* spp. The confirmation of *Salmonella* genus was performed using the agglutination technique with polyvalent antiserum anti-*Salmonella* spp. according to the manufacturer's recommendation (Probac do Brasil, São Paulo, Brasil).

Isolation of E. coli

E. coli isolation was carried out using traditional culture media according to Silva et al. (2017). Twenty-five grams of each sample was homogenized in 225 mL of buffered peptone water in a Stomacher blender (Stomacher 400). Aliquots of 1 mL were inoculated in tubes containing 10 mL of sodium lauryl sulfate (SLS) (Himedia Laboratories) and incubated at 35°C for 24 h. After incubation, SLS tubes were inoculated in eosin methylene blue (EMB) (Himedia Laboratories) and xylose lysine deoxycholate agar (XLD) (Himedia Laboratories) and incubated at 35°C for 24 h. The colonies that were lactose positive and non-H_2S producing were submitted to the following biochemical screening: production of indole, lactose fermentation with gas production, and citrate as the sole source of carbon. The *E. coli* strains were stored in nutrient agar (Himedia Laboratories).

DNA extraction of E. coli strains for use in PCR

E. coli strains stored in nutrient agar were resuspended in sterile saline solution and cultivated on MacConkey agar (Himedia Laboratories Pvt. Ltd., Mumbai, India). After incubation at 35°C for 24 h, colonies were resuspended in 200 µL of sterile water, boiled in a water bath for 10 min, and centrifuged at 10,000 ×*g* for 5 min. The supernatant was used as DNA target in the amplification reactions.

Positive and negative controls used in the PCR assays

The diarrheagenic *E. coli* strains used as positive controls in PCR assays were EHEC ATCC® 43889™ (*stx*2+, *eae*+, and *hlyA*+), EPEC 2348/69 (*eae*+ and *bfp*+), EHEC H30 (*stx*1+), EIEC O152 (*ipaH+),* ETEC H10407 (*elt*+), ETEC B41 (*est*+), and EAEC O42 (*aggR*+). *E. coli* K12, Hb101, and DH5α were used as negative controls.

PCR conditions for the detection of the virulence genes of diarrheagenic E. coli

The virulence genes of diarrheagenic E. *coli* were detected using the following virulence markers: *eae* (a gene that encodes intimin present in EPEC and EHEC), *bfp* (a gene that encodes *bfp* present in typical EPEC), *aggR* (a transcriptional activator for AAFs in EAEC), *elt* and *est* (genes that encoded LT and ST toxins in ETEC), *ipaH* (a gene present in the invasion plasmid that encodes the H antigen in EIEC), *stx*1 and *stx*2 (genes that encode the shiga toxin in STEC), and *hlyA* (a gene that encodes enterohemolysin in EHEC). The primer sequences and sizes of the amplified DNA are described in Table 1. The conditions used in the PCR assays are presented in Table 2.

Table 1. Primers used for detection of the virulence genes of diarrheagenic *E. coli*.

Genes	Primers (5′-3′)	PCR product (bp)
hlyA[1]	(F) GCATCATCAAGCGTACGTTCC (R) AATGAGCCAAGCTGGTTAAGCT	534
eae[1]	(F) GACCCGGCACAAGCATAAGC (R) CCACCTGCAGCAACAAGAGG	384
*stx*1[1]	(F) ATAAATCGCCATTCGTTGACTAC (R) AGAACGCCCACTGAGATCATC	180
*stx*2[1]	(F) GGCACTGTCTGAAACTGCTCC (R) TCGCCAGTTATCTGACATTCTG	255
elt[2]	(F) GGCGACAGATTATACCGTGC (R) CGGTCTCTATATTCCCTGTT	190
ipaH[2]	(F) TTCCTTGACCGCCTTTCCGATACCGTC (R) GCCGGTCAGCCACCCTCTGAGAGTAC	600
bfp[2]	(F) AATGGTGCTTGCGCTTGCTGC (R) GCCGCTTTATCCAACCTGGTA	326
aap[3]	(F) CTTGGGTATCAGCCTGAATG (R) AACCCATTCGGTTAGAGCAC	310
aggR[3]	(F) CTAATTGTACAATCGATGTA (R) AGAGTCCATCTCTTTGATAAG	457
AA probe[3]	(F) CTGGCGAAAGACTGTATCAT (R) CAATGTATAGAAATCCGCTGTT	629

[1] Paton & Paton, 1998; [2] Aranda et al., 2004; [3] Cerna, Nataro & Estrada-Garcia, 2003.

Table 2. The amplification conditions used in the PCR assay for the detection of *eae*, *bfp, stx*1, *stx*2, *hlyA*, *ipaH, elt, aggR, aap*, and *AA probe* genes of *E. coli* isolated from sausage samples.

Genes	PCR reaction mixture*	Amplification conditions	References
eae, *stx*1, *stx*2, and *hly*A	2.5 µL of DNA template in a total reaction volume of 25.0 µL containing 1.5 U of *Taq* DNA polymerase, 0.4 mM of dNTPs, 2.5 mM of $MgCl_2$, 20.0 pmol/L of each primer, 2.5 µL of PCR buffer 10x**	Initial denaturation at 95°C for 5 min; 15 cycles of 95°C for 1 min, 2 min at 65°C, 1.5 min at 72°C; 20 cycles of 94°C for 1 min, 2 min at 60°C, 2.5 min at 72°C; and a final extension at 72°C for 7 min.	Paton & Paton (1988), with some modification
ipaH, *est*, and *elt*	2.5 µL of DNA template in a total reaction volume of 25.0 µL containing 1.0 U of *Taq* DNA polymerase, 0.4 mM of dNTPs, 2.5 mM of $MgCl_2$, 20.0 pmol/L of each primer, 2.5 µL of PCR buffer 10x**	Initial denaturation at 95°C for 5 min, followed by 40 cycles of 95°C for 1 min, 2 min at 54°C, 1.0 min at 72°C; and a final extension at 72°C for 7 min.	Aranda (2004), with some modification
aap, aggR, and *AA probe*	2.5 µL of DNA template in a total reaction volume of 20.0 µL containing 1.0 U of *Taq* DNA polymerase, 0.4 mM of dNTPs, 2.5 mM of $MgCl_2$, 10.0 pmol/L of *aap* primer, 15.0 pmol/L of *aggR* primer, and 20.0 pmol/L *AA probe* primer, 2.5 µL of PCR buffer 10x**	Initial cycle at 50°C for 2 min, denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 40 s, 40 s at 59°C, 40 s at 72°C; and a final extension at 72°C for 7 min.	Cerna, Nataro & Estrada-Garcia (2003), with some modification

continue

continuation

bfp	2.5 µL of DNA template in a total reaction volume of 25.0 µL containing 1.0 U of *Taq* DNA polymerase, 0.4 mM of dNTPs, 2.5 mM of $MgCl_2$, 20.0 pmol/L of *bpf* primer, 2.5 µL of PCR buffer 10x**	Initial cycle at 50°C for 2 min, denaturation at 95°C for 3 min, followed by 40 cycles of 95°C for 40 s, 40 s at 59°C, 40 s at 72°C; and a final extension at 72°C for 7 min.	Aranda (2004), with some modification

* Invitrogen Life Technologies, Alameda, CA, EUA
** 20 mM Tris-HCl, 50 mM KCl, pH 8.4.

All amplification reactions were performed in Veriti® thermocycler (Applied Biosystems®, Foster City, CA, USA). Aliquots of 10 µL of each reaction were homogenized with 1 µL 20x Red Gel (Biotium®, Hayward, CA, USA). The electrophoresis was carried out on 2% agarose in 1x Tris-Borate-EDTA buffer [Tris (Invitrogen®) 90 mM; boric acid (Nuclear, Diadema, São Paulo) 90 mM, EDTA (Nuclear) 2 mM] at 70 V for 100 min. A DNA fragment of 1 Kb (Invitrogen®) was used as molecular size marker. Gels were viewed under ultraviolet light (UV) (Loccus Biotecnologia Molecular, Cotia, São Paulo, Brasil) and photo-documented (L-Pix Image Versão 1.21, Loccus Biotecnologia Molecular). Samples showing bands corresponding to the *hly*A gene in the multiplex PCR were confirmed by uniplex PCR using only the *hly*A primers.

Serotyping of diarrheagenic E. coli

After detection of the virulence genes of diarrheagenic *E. coli* by PCR, one of the *E. coli* strains that harbored the *eae* and *hly*A genes was serotyped at the Health Public Department, Medicine Faculty, National University of Mexico, Mexico City.

Results and Discussion

Salmonella spp. were isolated in 13 (28.3%) and *E. coli* in 33 (71.3%) of the 46 pork sausage samples analyzed in this study. The contamination by *Salmonella* spp. was higher in Tuscan sausage samples (8/17, 41.7%) than in fresh pork sausage samples (4/22, 18.2%). *E. coli* were isolated in 12 of 13 samples positive for *Salmonella* spp. (Table 3). Seven (53.8%) of 13 samples contaminated with *Salmonella* spp. were from brand A. Additionally, five from the eight samples of Tuscan sausages positive for *Salmonella* spp. (62.5%) were also from this brand. All samples of fresh pork and Tuscan sausage from brand A were contaminated by *E. coli*. A high percentage of contamination by *E. coli* also was found in fresh pork and Tuscan sausages from brand B (12/15, 80.0%).

Table 3. Results of the detection of *Salmonella* spp. and *E. coli* in samples of Calabresa, fresh pork, and Tuscan sausages analyzed.

Producer (No. samples analyzed)	Type of sausage	No. of samples	Samples contaminated with *E. coli*	Samples contaminated with *Salmonella*
A (n = 18)	Calabresa	2	0	0
	Fresh pork	9	9	2
	Tuscan	7	7	5
B (n = 18)	Calabresa	3	0	0
	Fresh pork	10	10	2
	Tuscan	5	2	1
C (n = 5)	Calabresa	0	0	0
	Fresh pork	2	1	0
	Tuscan	3	1	1
D (n = 5)	Calabresa	2	1 [a]	1
	Fresh pork	1	1	0
	Tuscan	2	1	1

[a] Sample contaminated with EPECa O108:H9 that harbored the genes *eae* and *hlyA*.

The number of samples from brands C (n=5) and D (n=5) were smaller than those from brands A (n=18) and B (n=18). However, *E. coli* and *Salmonella* were also isolated in samples from these two brands. Two out of seven samples of Calabresa sausage from brand D were contaminated with *Salmonella* spp. One of the samples was also contaminated with *E. coli* serotype O108:H9 that harbored the genes *eae* and *hlyA*.

Other studies conducted in the South of Brazil have reported percentages of isolation of *Salmonella* spp. in sausages similar to that found in this work (13/46, 28.3%). Mürmann et al. (2009) and Souza et al. (2014) detected *Salmonella* spp. in 24.4% (84/336) and 30.0% (6/20) of the fresh sausage samples analyzed in Porto Alegre, RS and in Cascavel, PR, respectively. In Niteroi, Rio de Janeiro, the percentage of fresh sausages contaminated with *Salmonella* spp. was higher than that found in the present study and ranged from 37.0% (10/27) to 53.0% (58/91) (LIMA et al., 2011; CABRAL, et al., 2014). However, in other studies conducted in Brazil, the contamination of pork meat and its derivatives was much lower, with percentages ranging from 5.0 to 17.9% (CHAVES et al., 2000; LOBO et al., 2001; SPRICIGO et al., 2008a; BEZERRA et al., 2012).

The prevalence of *Salmonella* spp. in the brand B samples may be related to the contamination of the pork meat or other ingredients used in the production of some batches. Two fresh pork and one Tuscan sausage sample was collected at an interval of four months. *Salmonella* spp. was not isolated from samples collected in the intermediate period. However, it should be noted that *E. coli* was isolated in 12 (80.0%) of the 15-fresh pork and Tuscan sausage samples of this brand, which indicated inadequate hygienic-sanitary conditions

of processing, or the contamination of raw pork meat.

Lima et al. (2011) in Niterói, Rio de Janeiro analyzed the surface of sausages using swabs and subsequently the meat from these same samples. The meat of 33.0% of the samples was contaminated with *Salmonella* spp. while contamination was only present on the surface in 11.0% of the samples. These results indicated that the contamination probably occurred due to the use of pork raw meat contaminated with *Salmonella*. In another study conducted by Cabral et al. (2014), there was no statistical difference in the *Salmonella* contamination of sausages analyzed in their original packaging compared to those repackaged by the market itself or sold in bulk to the consumer. These authors have suggested that food handling and storage in retail outlets appear to play a minor role in the contamination of sausages and that the main cause of contamination of the product is the contaminated raw pork meat used in the manufacture of sausages.

Infection of swine herds by *Salmonella* spp. tends to be a persistent problem in production systems. Castagna et al. (2004b) reported a high percentage of pigs carrying *Salmonella* spp. in submandibular lymph nodes and tonsils, which may remain in the carcass after slaughter. These parts can be used along with the head muscles in the manufacture of sausages and mechanically separated meat. These ingredients, when contaminated, may be responsible for the presence of *Salmonella* in those products. The removal of the tonsils from carcass after slaughter does not eliminate the risk of contamination, but it can be reduced in about 30.0%. The removal of the submandibular lymph nodes during processing is difficult because they are firmly attached to the musculature of the head (CASTAGNA et al., 2004a).

It is almost impossible to avoid cross-contamination of carcasses during the slaughter of swine. The use of only Good Manufacturing Practices in the slaughter process does not seem to be effective in reducing the meat contamination by *Salmonella* spp. More effective measures are related to the production of *Salmonella* spp. free swine and decontamination of the carcasses (BERENDS, 1998).

The percentage of contamination, 12.6%, found in pork meat samples produced in Portugal is much higher than the percentage reported in the European Union (0.7%). This difference in contamination can be explained by the fact that Portugal does not have a national program for the control of *Salmonella* in primary production, although it has a monitoring system for the contamination of pork carcasses after slaughter. Control strategies should be implemented on farms to reduce carcass contamination because cross-contamination during slaughter is the most common form of contamination of pork meat and its derivatives (XAVIER et al., 2014).

The highest frequency of *Salmonella* isolation in the present study was in the Tuscan sausage samples from brand A (71.4%). Samples were collected weekly over a period of three months, suggesting possible contamination of the processing plant and not contamination of a single batch of the product. Two of the nine fresh pork sausage samples from the same brand were also positive for *Salmonella*.

Alban et al. (2002) reported contamination of <50 to 400 CFU of *Salmonella* spp./cm^2 on the surface of the swine carcass, with a reduction of 2.0 to 3.0 log during the *sausage manufacture*. Mataragas et al. (2015) found that *Salmonella enterica* contamination can be reduced by 1.0 log or more during the sausage making *process*, in which changes in pH and water activity occur. Inhibition of multiplication occurs at pH values below 5.3 and therefore, the time and temperature at which raw meat remains at a pH above 5.3 are critical (LUCKE, 2000; ICMSF, 2005).

Although the reduction of contamination during sausage production may occur in Brazil, Mürmann et al. (2007) reported a count ranging from 0.03

NMP g^{-1} to 460 NMP g^{-1} and Spricigo et al. (2008b) found counts ranging from <3 NMP g^{-1} to 1100 NMP g-1 in the 54 positive samples analyzed. The Brazilian legislation defines that *Salmonella* spp. must be absent in 25 g of the analyzed sample (BRASIL, 2001).

None of the *E. coli* isolates analyzed in this study presented virulence genes used in the genetic characterization of enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIE), or shiga toxin-producing *E. coli* (STEC). A DEC strain was detected in a Calabresa sausage sample from brand D. This isolate harbored the genes *eae* and *hlyA* and was identified as EPECa O108: H9. The O108 serogroup was already associated with an outbreak of human gastroenteritis in the city of Mairiporã, SP, Brazil (VIEIRA et al., 2016) and the serotype O108: H9 has already been isolated from swine feces (FROHLICHER el al., 2008; MALIK et al., 2006). Although there is no evidence of transmission from animals to humans, it is believed that some isolates of EPECa could be zoonotic pathogens (GOMES et al., 2016).

The prevalence of infections caused by EPEC varies considerably according to age, socioeconomic status, and geographic region of the populations studied. EPECt isolates have always been strongly associated with childhood diarrhea; however, recent studies have shown a higher frequency of EPECa as a cause of diarrhea in Brazil and other South American countries (GOMES et al., 2004; ARAUJO et al., 2007; OCHOA et al., 2009; HANNAOUI et al., 2010; LOZER et al., 2013; RUGÉLES et al., 2010; SCHMIDT, 2010; ASSIS et al., 2014; GOMES et al., 2016).

Isolates of EPEC are classified as typical when they have the adhesion factor plasmid (EAF) and the *bfp* gene, in addition to the *eae* gene. The serogroups of EPECa differ from those of EPECt, indicating that few EPECa isolates are EPEC isolates that have lost the EAF plasmid (SCHMIDT, 2010). Unlike EPECt, many EPECa isolates are found in several sick or healthy animal species. Recently, Borges et al. (2017) isolated EPEC (typical and atypical) and STEC in wild birds and feral pigeons in the city of São Paulo.

E. coli O108: H9 strain isolated in this study from Calabresa sausage did not present the *stx* gene. Enterohemorrhagic *E. coli* (EHEC) is a subtype of STEC that encodes, besides the *stx* gene, *hly*A and *eae* genes. The *eae* gene is responsible for the attaching and effacing (AE) lesion (CROXEN et al., 2013). According to Bielaszewska et al. (2007), EHEC isolates may lose *stx* genes during infection, isolation, or subculture. We do not believe that the serotype O108: H9 isolated in this study had lost the *stx* gene because it is not one of the classic serotypes of shiga toxin-producing *E. coli* (O157:H7, O26, O45, O103, O111, O121, and O145) (CROXEN et al., 2013).

Some brands of Calabresa sausages are subject to a cooking process during their production, allowing their consumption without previous cooking. Tuscan and pork sausages, however, are raw and prepared in barbecue or oven grills, baked in conventional ovens, or deep fried. Therefore, the risk for human health that this product may present is more related to the cross contamination to other foods, especially those ingested raw, or the ingestion of the sausage after inadequate thermal process (ESCARTIN et al., 2000; GORMAN et al., 2002).

The standardization and control of all stages of the sausage process are crucial to obtain a product free of contamination. Excessive handling, inappropriate sanitation of instruments and equipment, the absence or deficiency of Good Manufacturing Practices and Hazard Analysis Critical Control Points (HACCP) system affect the sanitary quality of this type of food. In addition, the lack of proper preparation instructions can lead to cross-contamination and consumption of contaminated products.

Conclusion

Salmonella spp. and *E. coli* were isolated in 28.3% and 71.3% of the analyzed samples, respectively. A sample of Calabresa sausage was contaminated with atypical enteropathogenic *E. coli* from serotype O108: H9, which harbored the *eae* and *hlyA* genes. The results suggest possible contamination of the processing plant and/or raw meat used in the manufacture of sausages. A better inspection of the industries is required to ensure that good manufacturing practices are followed and thus the contamination of the product by pathogenic bacteria be prevented.

Acknowledgment

The authors express their gratitude to the CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for the Master's scholarship of Paola Bianca Barbosa Cavalin and to the CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for the financial support. The serotyping of *E. coli* was performed with the financial support of the *Dirección General de Asuntos del Personal Académico (DGAPA), Programa de Apoyo a Proyectos de Investigación e Innovación Tecnológica* (PAPIIT), project IN216417, UNAM, Mexico.

References

ALBAN, L.; OLSEN, A. M.; NIELSEN, B.; SORENSEN, R.; JESSEN, B. Qualitative and quantitative risk assessment for human salmonellosis due to multi-resistant *Salmonella* Typhimurium DT 104 from consumption of Danish dry-cured pork sausages. *Preventive Veterinary Medicine*, Amsterdam, v. 52, n. 3-4, p. 251-265, 2002.

ASSIS, F. E. A.; WOLF, S.; SUREK, M.; DE TONI, F.; SOUZA, E. M.; PEDROSA, F. O.; FARAH, S. M. S. S.; PICHETH, G.; FADEL-PICHETH, C. M. T. Impact of *Aeromonas* and diarrheagenic *Escherichia coli* screening in patients with diarrhea in Paraná, Southern Brazil. *The Journal of Infection in Developing Countries*, Sassari, v. 8, n. 12, p. 1609-1614, 2014.

ARAUJO, J. M.; TABARELLI, G. F.; ARANDA, K. R. S.; FABBRICOTTI, U. F.; MENDES, C. M. F.; SCALETSKY, I. C. A. Typical enteroaggregative and atypical enteropathogenic types of *Escherichia coli a*re the most prevalent diarrhea-associated pathotypes among Brazilian children. *Journal of Clinical Microbiology*, Washington, v. 45, n. 10, p. 3396-3399, 2007.

ARANDA, K. R.; FAGUNDES-NETO, U.; SCALETSKY, I. C. Evaluation of multiplex PCRs for diagnosis of infection with diarrheagenic *Escherichia coli* and *Shigella* spp. *Journal of Clinical Microbiology*, *Washington,* v. 42, n. 1, p. 5849-5853, 2004.

ASSOCIAÇÃO BRASILEIRA DE PROTEÍNA ANIMAL – ABPA. Relatório Anual ABPA 2016**.** Disponível em: <http://abpa-br.com.br/setores/avicultura/publicacoes/relatorios-anuais>. Acesso em: 27 out. 2017.

BERENDS, B. R.; VAN KNAPEN, F.; MOSSEL, D. A. A.; BURT, S. A.; SNIJDERS, J. M. A. Impact on human health of *Salmonella* spp. on pork in The Netherlands and the anticipated effects of some currently proposed control strategies. *International Journal of Food Microbiology*, Amsterdam, v. 44, n. 3, p. 219-229, 1998.

BEUTIN, L.; MARTIN, A. Outbreak of Shiga Toxin–Producing *Escherichia coli* (STEC) O104:H4 infection in Germany causes a paradigm shift with regard to human pathogenicity of STEC strains. *Journal of Food Protection*, Des Moines, v. 75, n. 2, p. 408-418, 2012.

BEZERRA, M. V. P.; ABRANTES, M. R.; SILVESTRE, M. K. S.; SOUSA, E. S.; ROCHA, M. O. C.; FAUSTINO, J. G.; SILVA, J. B. A. Avaliação microbiológica e físico-química de linguiça toscana no Município de Mossoró, RN. *Arquivos do Instituto Biológico*, São Paulo, v. 79, n. 2, p. 297-300, 2012.

BIELASZEWSKA, M; PRAGER, R.; KÖCK, R.; MELLMANN, A.; ZHANG, W.; TSCHÄPE, H.; TARR, P. I.; KARCH, H. Shiga toxin gene loss and transfer in vitro and in vivo during enterohemorrhagic *Escherichia coli* O26 infection in humans. *Applied and Environmental Microbiology*, Washington, v. 73, n. 10, p. 3144-3150, 2007.

BORGES, C. A.; CARDOZO, M. V.; BERALDO, L. G.; OLIVEIRA, E. S.; MALUTA, R. P.; BARBOZA, K. B.; WERTHER, K.; ÁVILA, F. A. Wild birds and urban pigeons as reservoirs for diahrreagenic *Escherichia coli* with zoonotic potential. *Journal of Microbiology*, Seoul, v. 55, n. 5. p. 344-348. 2017.

BRASIL. Ministério da Saúde. Agência Nacional de Vigilância Sanitária. Resolução RDC nº 12, de 02 de janeiro de 2001. Aprova o regulamento técnico sobre

padrões microbiológicos para alimentos. *Diário Oficial [da] República Federativa do Brasil*, Brasília, 10 jan. 2001. Seção 1, p. 45-53.

_____. Ministério da Saúde. Secretaria de Vigilância em Saúde. *Surtos de doenças transmitidas por alimentos no Brasil***.** [S.l.: s.n.], dez. 2016. Disponível em: <http://portalarquivos.saude.gov.br/ images/pdf/2016/ dezembro/09/Apresentacao-Surtos-DTA-2016.pdf>. Acesso em: 10 jan. 2017.

CABRAL, C. C.; CONTE-JUNIOR, C. A.; SILVA, J. T.; PASCHOALIN, V. M. F. *Salmonella* spp. contamination in fresh pork and chicken sausages marketed in Niterói and Rio de Janeiro, Brazil. *Journal fur Verbraucherschutz und Lebensmittelsicherheit*, Heidelberg, v. 9, n. 3, p. 243-249, 2014.

CASTAGNA, S. M. F.; SCHWARZ, P.; CANAL, C. W.; CARDOSO, M. Presença de *Salmonella* sp. no trato intestinal e em tonsilas/linfonodos submandibulares de suínos ao abate. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, Belo Horizonte, v. 56, n. 3, p. 300-306, 2004b.

CASTAGNA, S. M. F.; SCHWARZ, P.; CANAL, C. W.; CARDOSO, M. R. I. Prevalência de suínos portadores de *Salmonella* sp. ao abate e contaminação de embutidos tipo frescal. *Acta Scientiae Veterinariae*, Porto Alegre, v. 32, n. 2, p. 141-147, 2004a.

CERNA, J. F.;NATARO, J. P.; ESTRADA-GARCIA, T. Multiplex PCR for detection of three plasmid-borne genes of enteroaggregative *Escherichia coli* strains. *Journal of Clinical Microbiology*, *Washington*, v. 41, n. 5, p. 2138-2140, 2003.

CHARIMBA, G.; HUGO, C.; HUGO, A. The incidence of diarrhoeagenic *Escherichia coli* in minced beef and boerewors. *Food Research International*, Essex, v. 47, n. 2, p. 353-358, 2012.

CHAVES, G. M. C.; GONÇALVES, P. M. R.; FRANCO, R. M.; CARVALHO, J. C. A. P. Avaliação bacteriológica de lingüiça frescal suína comercializada no Município do Rio de Janeiro, RJ. *Higiene Alimentar*, São Paulo, v. 14, n. 13, p. 48-52, 2000.

CROXEN, M.; LAW, R.; SCHOLZ, R.; KEENEY, K.; WLODARSKA, M.; FINLAY, B. Recent advances in understanding enteric pathogenic *Escherichia coli*. *Clinical Microbiology Reviews*, Washington, v. 26, n. 4, p. 823-880, 2013.

DIAS, R. C.; DOS SANTOS, B. C.; DOS SANTOS, L. F.; VIEIRA, M. A.; YAMATOGI, R. S.; MONDELLI, A. L.; SADATSUNE, T.; SFORCIN, J.M.; GOMES, T. A.; HERNANDES, R. T. Diarrheagenic *Escherichia coli* pathotypes investigation revealed atypical enteropathogenic *E.coli* as putative emerging diarrheal agents in children living in Botucatu, São Paulo State, Brazil. *APMIS: Acta Pathologica, Microbiologia, et Immunologica Scandinavica*, Copenhagen, v. 124, n. 4, p. 299-308, 2016.

ESCARTIN, E. F.; LOZANO, J. S.; GARCIA, O. R. Quantitative survival of native *Salmonella* serovars during storage of frozen raw pork. *International Journal of Food Microbiology*, Amsterdan, v. 54, n. 1-2, p. 19-25. 2000.

FRANZOLIN, M. R.; ALVES, R. C.; KELLER, R.; GOMES, T. A.; BEUTIN, L.; BARRETO, M. L.; MILROY, C.; STRINA, A.; RIBEIRO, H.; TRABULSI, L. R. Prevalence of diarrheagenic *Escherichia coli* in children with diarrhea in Salvador, Bahia, Brazil. *Memórias do Instituto Oswaldo Cruz*, Rio de Janeiro, v. 100, n. 4, p. 359-363, 2005.

FROHLICHER, E.; KRAUSE, G.; ZWEIFEL, C.; BEUTIN, L.; STEPHAN, R. Characterization of attaching and effacing *Escherichia coli* (AEEC) isolated from pigs and sheep. *BMC Microbiology*, London, v. 8, n. 144, p.144, 2008.

GOMES, T. A. T.; ELIAS, W. P.; SCALETSKY, I. C. A.; GUTH, B. E. C.; RODRIGUES, J. F.; PIAZZA, R. M. F.; FERREIRA, L. C. S.; MARTINEZ, B. M. Diahrreagenic *Escherichia coli*. *Brazilian Journal of Microbiology*, São Paulo, v. 47, p. 3-30, 2016. Supplement 1.

GOMES, T.; IRINO, K.; GIRÃO, D. M.; GIRÃO, V. B. C.; GUTH, B. E. C.; VAZ, T. M. I.; MOREIRA, F. C.; CHINARELLI, S. H.; VIEIRA, M. A. M. Emerging enteropathogenic *Escherichia coli* strains? *Emerging Infectious Diseases*, Atlanta, v. 10, p. 1851-1855, 2004.

GORMAN, R.; BLOMFIELD, S.; ADLEY, C. A study of cross-contamination of food-borne pathogens in the domestic kitchen in the Republic of Ireland. *International Journal of Food Microbiology*. Amsterdan, v. 76, n. 1-2, p. 143-150. 2002.

HANNAOUI, E.; VILLALOBOS, L.; MARTÍNEZ, R.; MALDONADO, A.; BASTARDO, J.; HAGEL, I. Diarrheagenic *Escherichia coli* associated with acute diarrhea in children of Cumaná, Venezuela. *Investigacion Clinica*, Maracaibo, v. 51, n. 4, p. 489-500, 2010.

INTERNATIONAL COMMISSION ON MICROBIOLOGICAL SPECIFICATIONS FOR FOODS – ICMSF. Microorganisms in Foods 6. Microbial Ecology of Food Commodities. 2. ed. New York: Kluwer Academic, 2005. (Chapter 1).

LIMA, B. R. C. C.; CANTO, A. C. V. C. S.; NASCIMENTO, R. S.; FRANCO, R. M.; NASCIMENTO, E. R. Prevalência de *Salmonella* spp.

na superfície e no interior de linguiça frescal suína comercializada no município de Niterói, Rio de Janeiro, Brasil. *Revista Brasileira de Medicina Veterinária*, São Paulo, v. 33, n. 3, p. 133-136, 2011.

LOBO, M. V.; UGALDE, M. G.; FRIES, L. L. M.; KUBOTA, E. H. Avaliação microbiológica de salames coloniais comercializados no Município de Santa Maria - RS. *Higiene Alimentar*, São Paulo, v. 15, n. 88, p. 57-61, 2001.

LOZER, D.; SOUZA, T. B.; MONFARDINI, M. V.; VICENTINI, F.; KITAGAWA, S. S.; SCALETSKY, I. C.; SPANO, L. C. Genotypic and phenotypic analysis of diarrheagenic *Escherichia coli* strains isolated from brazilian children living in low socioeconomic level communities. *BMC Infectious Diseases*, London, v. 13, n. 1, p. 1471-2334, 2013.

LÜCKE, F. Utilization of microbes to process and preserve meat. *Meat Science*, Amsterdam, v. 56, n. 2, p. 105-115, 2000.

MALIK, A.; TÓTH, I.; BEUTIN, L.; SCHMIDT, H.; TAMINIAU, B.; DOW, M. A.; MORABITO, S.; OSWALD, E.; MAINIL, J.; NAGY, B. Serotypes and intimin types of intestinal and faecal strains of eae + *Escherichia coli* from weaned pigs. *Veterinary Microbiology*, Amsterdan, v. 114, n. 1, p.82-93, 2006.

MATARAGAS, M.; BELLIO, A.; ROVETTO, F.; ASTEGIANO, S.; DECASTELLI, L.; COCOLIN, L. Risk-based control of food-borne pathogens *Listeria monocytogenes* and *Salmonella enterica* in the Italian fermented sausages Cacciatore and Felino. *Meat Science*, Amsterdam, v. 103, n. 5, p. 39-45, 2015.

MORENO, A. C.; FILHO, A. F.; GOMES, A.; RAMOS, S. T.; MONTEMOR, L. P.; TAVARES, V. C.; FILHO, S.; IRINO, K.; MARTINEZ, M. B. Etiology of childhood diarrhea in the northeast of Brazil: significant emergent diarrheal pathogens. *Diagnostic Microbiology and Infectious Dise*ase, New York, v. 66, n. 1, p. 50-57, 2010.

MÜRMANN, L; SANTOS, M. C. M.; CARDOSO, M. Curvas de crescimento e destruição termica de serovares de *Salmonella* spp. isolados de linguiça frescal de carne suína. *Acta Scientiae Veterinariae*, Porto Alegre, v. 37, n. 4, p. 329-335, 2007.

MÜRMANN, M. C. S. Prevalence, genetic characterization and antimicrobial resistance of *Salmonella* isolated from fresh pork sausages in Porto Alegre, Brazil. *Food Control*, Vurrey, v. 20, n. 3, p. 191-195, 2009.

NATARO, J. P.; KAPER, J. B. Diarrheogenic *Escherichia coli*. *Clinical Microbiology Review*, Washington, v. 11, n. 1, p. 142-201, 1998.

OCHOA, T. J.; RUIZ, J.; MOLINA, M.; DEL VALLE, L. J.; VARGAS, M.; GIL, A. I.; ECKER, L.; BARLETTA, F.; HALL, E.; CLEARY, T.G.; LANATA, C.F. High frequency of antimicrobial drug resistante of diarrheagenic *Escherichia coli* in infants in Peru. *The American Journal of Tropical Medicine and Hygiene*, Cleveland, v. 81, n. 2, p. 296-301, 2009.

PATON, A. W.; PATON, J. C. Detection and Characterization of Shiga Toxigenic *Escherichia coli* by Using Multiplex PCR Assays for *stx*1, *stx*2, *eaeA*, Enterohemorrhagic *E. coli hlyA*, *rfbO111*, *andrfbO157*. *Journal of Clinical Microbiology*, Washington, v. 36, n. 2, p. 598-602, 1998.

RANTSIOU, K.; ALESSANDRIA, V.; COCOLIN, L. Prevalence of Shiga toxin-producing *Escherichia coli* in food products of animal origin as determined by molecular methods. *International Journal of Food Microbiology*, Amsterdam, v. 154, n. 1-2, p. 37-43, 2012.

REGUA-MANGIA, A. H.; GOMES, T. A.; VIEIRA, M. A.; ANDRADE, J. R.; IRINO, K.; TEIXEIRA, L. M. Frequency and characteristics of diarrhoeagenic *Escherichia coli* strains isolated from children with and without diarrhea in Rio de Janeiro. *Journal of Infection*, London, v. 48, n. 2, p. 161-167, 2004.

RÚGELES, L. C.; BAI, J.; MARTÍNEZ, A. J.; VANEGAS, M. C.; GÓMEZ-DUARTE, O. G. Molecular characterization of diarrheagenic *Escherichia coli* strains from stools samples and food products in Colombia. *The Journal of Infection in Developing Countries*, Sassari, v. 8, n. 12, p. 1609-1614, 2010.

SCHMIDT, M. A. LEEways: tales of EPEC, ATEC and EHEC. *Cellular Microbiology*, Oxford, v. 12, n. 11, p. 1544-1552, 2010.

SILVA, N.; JUNQUEIRA, V. C. A.; SILVEIRA, N. F. A.; TANIWAKI, M. H.; GOMES, R. A. R.; OKAZAKI, M. M. *Manual de métodos de análise microbiológica de alimentos e água*. 5. ed. São Paulo: Editora Edgard Blucher Ltda., 2017.

SOUZA, M.; PINTO, F. G. S.; BONA, E. A. M.; MOURA, A. C. Qualidade higiênico-sanitária e prevalência de sorovares de *Salmonella* em linguiças frescais produzidas artesanalmente e inspecionadas, comercializadas no oeste do Paraná, Brasil. *Arquivos do Instituto Biológico*, São Paulo, v. 81, n. 2, p. 107-112, 2014.

SPRICIGO, D. A.; MATSUMOTO, S. R.; ESPÍNDOLA, M. L.; FERRAZ, S. M. Prevalência, quantificação e resistência antimicrobiana de sorovares de *Salmonella* isolados de lingüiça frescal suína. *Ciência e Tecnologia de Alimentos*, Campinas, v. 28, n. 4, p. 779-785, 2008b.

SPRICIGO, D. A.; MATSUMOTO, S. R.; ESPÍNDOLA, M. L.; VAZ, E. K.; FERRAZ, S. M. Prevalência e perfil de resistência a antimicrobianos de sorovares de *Salmonella* isolados de lingüiças suínas tipo frescal em Lages, SC. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, Belo Horizonte, v. 60, n. 2, p. 517-520, 2008a.

VIEIRA, M. A.; SANTOS, L. F.; DIAS, R. C. B.; CAMARGO, C. H.; PINHEIRO, S. R. S.; GOMES, T. A. T.; HERNANDES, R. Atypical enteropathogenic *Escherichia coli* as aetiologic agents of sporadic and outbreak-associated diarrhoea in Brazil. *Journal of Medical Microbiology*, Edinburgh, v. 65, n. 9, p. 998-1006, 2016.

XAVIER, C.; GONZALES-BARRON, U.; PAULA, V.; ESTEVINHO, L.; CADAVEZ, V. Meta-analysis of the incidence of foodborne pathogens in Portuguese meats and their products. *Food Research International,* Essex, v. 55, n. 1, p. 311-323, 2014.