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High prevalence, low counts and uncommon serotypes of *Listeria monocytogenes* in *linguiça*, a Brazilian fresh pork sausage

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ABSTRACT

Linguiça is a highly popular and appreciated pork product in Brazil, frequently consumed undercooked. Aiming at collection of data for a future risk assessment, this study evaluated the prevalence and counts of *Listeria monocytogenes* in *linguiça* samples collected at retail level in Sao Paulo, SP, Brazil. ISO methods were used for detection and enumeration of the pathogen (11290-1 and 11290-2, respectively). Isolates were submitted to Simplex-PCR for *hlyA* gene and those with biochemical features of *L. monocytogenes* and *hlyA* positive were serotyped using a Multiplex PCR. Ninety percent of the samples were positive for *Listeria* spp., and *L. monocytogenes* was detected in 42% of the samples, with counts below 10^2 CFU/g in all samples. A prevalence of uncommon serotypes 4a and 4c was observed.

1. Introduction

Listeria monocytogenes is an important human foodborne pathogen, and causes listeriosis, a severe invasive illness in humans, which may result in death. Listeriosis has high hospitalization and fatality rates, with high costs per case. The most susceptible hosts are pregnant women, newborn, the elderly and those with a compromised immune system. Several serotypes of *L. monocytogenes* have been identified, but most human infections are caused by strains belonging to serotypes 1/2a, 1/2b and 4b (Graves, Swaminathan, & Hunter, 2007; Rocourt & Bille, 1997; Thévenot, Dernburg, & Vernozy-Rozand, 2006).

Infection has been associated with a variety of foods, including meat, cheese, milk, vegetables and fish (Swaminathan, Cabanes, Zhang, & Cossart, 2007). Foods can be contaminated from a variety of sources because *L. monocytogenes* is widely distributed in the environment. Occurrence of *L. monocytogenes* has been reported in dairies (Brito et al., 2008; Catão & Ceballos, 2001; Kells & Gilmour, 2004; Moshtaghi & Mohamadpour, 2007; Rudolf & Scherer, 2001; Silva, Destro, Hofer, & Tibana, 2001), fruits and vegetables (Aguado, Vitas, & García-Jalón, 2004; Beuchat, 1996; Fröder et al., 2007; Pao, Ettinger, Khalid, Mebrahtu, & Mullins, 2008; Silva et al., 2007), seafood (Cruz et al., 2008; Destro, Leitão, & Farber, 1996; Parihar, Barbuddhe, Danielsson-Tham, & Tham, 2008; Souza, Destro, & Martinis, 2008) and meats (Aragon-Alegro et al., 2005; Assis, Destro, Franco, & Landgraf, 2000; Ceylan, Demi'Rkaya, & Adigüzel, 2008; Jalali & Abedi, 2008; Rodrigues, Landgraf, & Destro, 2002; Sakate, Aragon, Raghiante, Landgraf, & Destro, 2003).

L. monocytogenes can be found in raw pork meat, the processing environment and finished products (López et al., 2007, 2008; Thévenot et al., 2006; Wesley et al., 2008). Pork meat and processed pork products have been implicated in listeriosis outbreaks in several countries (Goulet et al., 1998; Jacquet et al., 1995; Loncarevic et al., 1997).

Linguiça is a popular pork meat product in Brazil. It is a fresh (not cured or fermented) sausage prepared with minced pork, fat, curing salts and spices, filled in natural gut casings. The pH is around 6.0 and Aw is high (>0.98), and according to Brazilian regulations, the product should be sold under refrigeration. This product requires heat treatment before consumption, being consumed mostly fried in oil or baked in ovens or barbecue grills. However, the consumption of partially or undercooked products is common due to consumers' habits and preference. Despite being injured by curing salts (i.e. sodium nitrites) and spices in *linguiça*, *L. monocytogenes* is able to revert the injuries (Ngutter & Donnelly, 2003). Due to favorable Aw and pH conditions and tolerance to high salt concentration (up to 12%) and low temperature (0 °C), growth of the pathogen in *linguiça* during storage is likely to occur (Gandhi & Chikindas, 2007; Martinis & Franco, 1998; Swaminathan et al., 2007; Thévenot et al., 2006; Zuliani et al., 2007), compromising safety.

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In Brazil, some studies were conducted on the occurrence of *Listeria* spp. and *L. monocytogenes* in pork meat products (Aragon-Alegro et al., 2005; Silva et al., 2004). However, they only investigated the prevalence of the pathogen in the products, while enumeration was not carried out. In addition, little is known about the most prevalent serotypes in pork meat products.

This study aimed to determine the prevalence and counts of *L. monocytogenes* in *linguiça*, and to evaluate the prevalent serovars, as part of an approach to the building of quantitative risk assessment models to estimate the risks associated to this pathogen in this type of food.

2. Materials and methods

2.1. Samples

One hundred samples of refrigerated raw *linguiça* (fresh pork sausage), belonging to different lots and brands, were purchased in different retail outlets in Sao Paulo, Brazil, between January and March, 2008. This is the summer season in Brazil, when *linguiça* consumption is enhanced due to open-air barbecues. The samples were transported to the laboratory under refrigeration in polystyrene boxes and promptly analyzed for *L. monocytogenes*.

2.2. L. monocytogenes detection and enumeration

Detection and enumeration of *L. monocytogenes* were carried out according to ISO 11290-1 and 11290-2 methods, respectively (Anonymous, 1996, 1998). All culture media and selective supplements were from Oxoid Ltd. (Oxoid, Hampshire, UK) unless otherwise mentioned.

L. monocytogenes detection consisted of a resuscitation step in which 25 g of sample were transferred to sterile plastic bags containing 225 mL of half-Fraser (HF) broth without supplements, homogenized using a Stomacher 400-laboratory blender (Seward Medical, London, UK), and incubated at 20 °C for 1 h. After this resuscitation step, selective supplement SR-166 was added to the HF broth, following incubation at 30 °C for further 23 h. Then, 0.1 mL of the HF broth was transferred to tubes containing 10 mL of Fraser Broth (FB plus supplement SR 156) and incubated at 35 °C for 48 h. The culture was streaked onto duplicate plates of *Listeria* selective agar (Oxford) and Palcam agar, supplemented with SR 140E and SR 150E, respectively. The plates were incubated for 48 h at 35 °C and observed for the presence of typical *Listeria* colonies. Presumptive *Listeria* colonies were selected and submitted to biochemical and PCR tests for identification and serotyping. Results were expressed as absence or presence of *L. monocytogenes* per 25 g.

For enumeration of *L. monocytogenes*, the same HF broth used for *L. monocytogenes* detection was used. After the resuscitation period and before the addition of the supplement SR-166, aliquots of HF broth were taken and submitted to serial decimal dilutions in 0.1% peptone water and 0.1 mL of each dilution was plated on Palcam agar in duplicate. After incubation at 35 °C for 48 h, presumptive *Listeria* colonies were enumerated and submitted to biochemical and genotypic tests for identification and serotyping. The results were expressed as the number of *L. monocytogenes* per gram (CFU/g).

For identification of *Listeria* spp., typical colonies in Oxford and Palcam agar plates were submitted to Gram staining, catalase production, motility test at 25 °C, haemolysis on blood sheep agar and fermentation of rhamnose and xylose (Anonymous, 1996, 1998; Pagotto, Daley, Farber, & Warburton, 2001). Suspected colonies were also subjected to a PCR for the listerolysin (*hlyA*) gene, as described by Blais, Phillippe, Pagotto, and Corneau (2008).

2.3. Monocytogenes serotyping

Strains identified as *L. monocytogenes* by biochemical tests and PCR simultaneously were serotyped using the Multiplex PCR described by Doumith, Buchrieser, Glaser, Jacquet, and Martin (2004).

2.4. Determination of ISO methods detection limits

Four different *L. monocytogenes* strains isolated from *linguiça* in the present study were selected and individually inoculated in 10 mL of tryptic soy broth supplemented with 6% yeast extract (TSBYE) and incubated at 35 °C for 18–24 h. Then, 0.1 mL of these cultures were transferred to 100 mL bottles containing TSBYE and incubated at 35 °C for 18–24 h under agitation (120 rpm) (New Brunswick, Edison, USA). After repeating the cultivation procedure, cultures were centrifuged (Hettich Zentrifugen, Tuttingen, Germany) at 1600*g* for 30 min at 4 °C. Pellets were washed three times with 0.1% sterile peptone water, under the same conditions. A pool of *L. monocytogenes* strains was prepared by mixing 1 mL of each washed suspension. The pool was used for inoculation of *linguiça* to achieve the following levels of contamination: 10^3 CFU/g, 10^2 CFU/g, 10^1 CFU/g, 10^0 CFU/g, 10^1 CFU/25 g and 10^0 CFU/25 g. The *linguiça* samples were not submitted to any decontamination treatment in order to simulate the same microbiological conditions found in the non-inoculated samples. Negative control samples consisted of non-inoculated *linguiça*, previously tested for absence of *Listeria* spp. All experiments were carried out in triplicate. After inoculation, *linguiça* samples were analyzed for *Listeria* spp. according to ISO 11290-1 (detection method) and ISO 11290-2 (enumeration method). All culture media were from Oxoid Ltd. (Basingstoke, UK).

3. Results and discussion

The positivity for *Listeria* spp. in *linguiça* samples was 90%. According to the ISO method, which is based on biochemical identification of suspected colonies in Oxford and Palcam agar plates, the positivity for *L. monocytogenes* was 42% (Table 1). However, when the PCR for detection of gene *hlyA* was applied, the positivity was reduced to 29% of the samples. Other studies have also reported discrepant results for genotypic and phenotypic characterization of *L. monocytogenes* (Allerberger, Dierich, Petranyi, Lalic, & Bubert, 1997; O'Grady, Sedano-Balbás, Maher, Smith, & Barry, 2008; Strom, 1998).

Several authors have used PCR for identification of *L. monocytogenes*, which has been proven to be a rapid and highly specific method (O'Grady, Sedano-Balbás, Maher, Smith, & Barry, 2008; O'Grady et al., 2009). Besides, this method is easily interpretable and considered more powerful than biochemical tests, since it targets different marker genes, providing identification and species confirmation in the same step (Doumith et al., 2004). In fact, phenotypic identification methods have the disadvantage of being influenced by environmental conditions of the cells, while in molecular-based methods the nucleotide sequence of the DNA does not change (Boer & Beumer, 1999). Considering that *linguiça* may contain up to 150 ppm of nitrite (Oliveira, Araújo, & Borgo,

Table 1
Prevalence of *L. monocytogenes* in *linguiça* samples, according to the isolates identification method.

Isolates identification method	Positivity for *L. monocytogenes* (%)
Biochemical tests	42
Multiplex PCR 16S rRNA	46
Biochemical tests and Multiplex PCR	29