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Genotypic and antimicrobial characterization of pathogenic bacteria at different stages of cattle slaughtering in southern Brazil

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ABSTRACT

Meat can be contaminated in different stages of the slaughtering process and the identification of these stages is the starting point to implement adequate control measures. The objectives of this study were to assess the presence of pathogenic microorganisms in cattle carcasses, to identify the most important contamination points of the slaughtering process, and to evaluate the possible risk factors related to them in a cattle slaughterhouse. To this aim, 108 cattle carcasses were sampled at three stages of the slaughtering process: Point 1 (hides after bleeding); Point 2 (carcasses after hide removal); and Point 3 (carcasses immediately after division). *Escherichia coli* O157:H7, *Listeria monocytogenes* and *Salmonella* Livingstone were isolated from the carcasses. Phenotypic and genotypic characterization indicated that there was cross-contamination among animals, since bacteria with identical genotypic and phenotypic profiles were isolated from different animals at the same sampling day. Furthermore, this is the first report about the isolation of *E. coli* O157:H7 in a bovine slaughterhouse from southern Brazil.

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1. Introduction

Pathogens can contaminate meat at different stages of the slaughtering process (Gill, 2007; Nouichi & Hamdi, 2009), and appropriate control measures must be in place in order to remove or prevent microbial contamination. Among pathogenic bacteria that can contaminate cattle meat, *Salmonella* spp., *Listeria monocytogenes*, and *Escherichia coli* O157:H7 are frequently pointed out as responsible for foodborne illnesses (Nouichi & Hamdi, 2009; Antic et al., 2010). According to Scallan et al. (2012) and the Ministry of Health/Brazil (MS - Ministry of Health, 2014), animal products are recognized as the major cause of foodborne diseases worldwide and the effective prevention of outbreaks depends on, among others factors, the microbiological quality of raw material used.

Another issue related to foodborne diseases is the pathogenic bacteria featuring antimicrobial resistance, which can be a serious threat to public health, as it makes the treatment of infectious diseases difficult (Arslan & Eyi, 2010). The increased use of antimicrobials in animal production and human medicine is a significant factor in the emergence of pathogens resistant to antibiotics (Silva, Hovarth, G., & Tondo, 2014; Nespolo, Saba, Rossatelli, Fairbrother, & Júnior, 2014). Based on these facts, information about sources of resistant pathogens that can contaminate meat is of great importance and is one of the main reasons of the present study, which aimed to assess the contamination of cattle hides and carcasses at different stages of the slaughtering process in southern Brazil. Moreover, we characterized the pathogenic organisms according to their antimicrobial profiles and genotypic characteristics.

2. Materials and methods

2.1. Industry characterization

The industry in which the present study was carried out was chosen due to its facilities and infrastructure management operating under the Brazilian Federal Inspection Service, with a slaughtering capacity of 500

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to 700 animals/day. Besides, it was located in the central region of the Rio Grande do Sul State (RS), receiving cattle from several districts.

2.2. Sampling

From September 2010 to February 2012 (6 sampling days), samples from 108 animals of different Rio Grande do Sul regions and farms were collected. Three points of the cattle slaughtering process were assessed: Point 1 (P1): animal hides, just after bleeding; Point 2 (P2): carcasses, just after hide removal and before evisceration; and Point 3 (P3): after carcasses are cut into two large pieces for handling — totaling 324 samples. Carcasses were sampled using the sponge method, applied on the pectoral region according to the European Community guidelines (Commission Regulation — EC, 2007). Each point was sampled using four sponges, each of them scrubbed on a 100 cm^2 region (400 cm^2/point; 1200 cm^2/carcass) (Andrews & Hammack, 1998). After collection, the four sponges from each point were placed into a sterile plastic bag and stored at 4 °C until analysis. Table 1 shows the sample features and animals in this study.

2.3. Microbiological analyses

For each plastic bag containing the sponges, 200 mL of sterile 0.85% saline solution was added. The bags were then placed into a Stomacher (Seward, USA) at low speed for removal of the adhered material over the sponges.

2.3.1. Total count of aerobic mesophilic microorganisms

Aerobic Plate Count Petri films (3M Health Care, USA) were used to estimate the total count of mesophilic microorganisms. Five dilutions were plated in duplicate and incubated at 37 °C for 48 h. Red colonies were included in the count regardless of size or color intensity. The results were expressed as the log number of colony forming units (CFU) per unit area (cm^2). This method was performed in accordance with International Organization for Standardization (ISO) 4833:2003, 2003.

2.3.2. Counting of generic E. coli

Coliforms and *E. coli* Petri films (3M Health Care, USA) were used to estimate the count of generic *E. coli*. Five dilutions of the initial material were plated in duplicate and incubated at 35 °C for 48 h. Blue colonies with gas production were counted as *E. coli*. The results were expressed as log of CFU/cm^2. This method was performed in accordance with International Organization for Standardization (ISO) 21528-2:2004, 2004.

2.3.3. Isolation and identification of Salmonella spp.

During the pre-enrichment step, 25 mL of the suspension was mixed with 225 mL of buffered peptone saline water (BPW; Oxoid, England), and incubated for 18 h at 37 °C. Subsequently, 1 mL and 0.1 mL were transferred to 10 mL of Muller–Kauffmann tetrathionate–novobiocin (MKTTn, Oxoid, England) and Rappaport–Vassilliadis with soy broths (RVS, Oxoid, England) and incubated at 37 °C and 41.5 °C, respectively, for 24 h. Then, a loopful of each RVS and MKTTn broth was inoculated in xylose lysine deoxycholate (XLD, Oxoid, England) and mannitol lysine crystal violet brilliant green agar (MLCB, Oxoid, England) and incubated at 37 °C for 24 h. Candidate colonies were purified on nutrient agar and confirmed biochemically using API 20E kits (BioMérieux, USA) and serologically using poly "O" and "H" antisera (Probac, Brazil). This method was performed in accordance with International Organization for Standardization (ISO) 6579, 2001.

Table 1
Description of cattle samples collected in southern Brazil.

Variable	Legend	Score	Number of samples collected (%)
Sampling point	P1[a]	0	108 (33.33)
	P2[b]	1	108 (33.33)
	P3[c]	2	108 (33.33)
Animal age	Calf	0	57 (52.78)
	Adult	1	51 (47.22)
Region in RS	South	0	29 (26.85)
	Central	1	13 (12.04)
	West	2	48 (44.44)
	Midwest	3	18 (16.66)
Season of sampling	Spring	0	20 (18.51)
	Summer	1	32 (29.62)
	Autumn	2	25 (23.14)
	Winter	3	31 (28.70)

State of Rio Grande do Sul, southern Brazil.
[a] P1: hide after bleeding.
[b] P2: carcasses after hide removal but before evisceration.
[c] P3: carcasses immediately after division into two parts.

2.3.4. Isolation and identification of Listeria spp.

For *Listeria* spp. isolation, 40 mL of the homogenized suspension (after removal of adhered material over the sponges, as described above) was centrifuged at 2000 rpm for 15 min. The pellet was resuspended in half Fraser broth (Oxoid, England) and incubated at 30 °C for 24 h. After this, 0.1 mL was transferred to 10 mL Fraser broth (Oxoid, England) and incubated at 37 °C for 48 h. After the incubation, a loopful was inoculated on selective Agar Chromogenic and Agar Oxford (Oxoid, England) and incubated at 37 °C for 48 h. For biochemical characterization, three to five candidate colonies of *Listeria* spp. were transferred to trypticase soy agar plates (TSA, Oxoid, England) supplemented with 0.6% yeast extract (TSA–YE, Difco, USA). The plates were incubated at 37 °C for 48 h. The colonies were submitted to analysis at oblique light incidence transmitted at 45° (Henry method) and further subjected to biochemical identification using the following tests: catalase, carbohydrate fermentation, β-hemolysis, Voges–Proskauer (VP), methyl red (MR), gram staining, motility and CAMP test (McFaddin, 2000). After biochemical testing, the candidate isolates of *Listeria* spp. were tested by agglutination of "O" and "H" as recommended by Donker-Voet (1959) and Seeliger and Höhne (1979). This method was carried out in accordance with International Organization for Standardization (ISO) 11290-1 and 11290-2, 2002.

2.3.5. Isolation and identification of E. coli O157:H7

After processing on a Stomacher, 25 mL of the homogenized suspension was mixed to 225 mL of modified tryptone soy broth (Oxoid, England) containing 0.45 mg of novobiocin (Laborclin, Brazil) and incubated at 41.5 °C for 24 h. Target microorganisms were concentrated by immunomagnetic separation (IMS) using magnetic particles (Dynabeads, Invitrogen, USA) coated with anti-O157 antibodies. The pellet was inoculated onto cefixime tellurite sorbitol MacConkey agar and sorbitol MacConkey agar (both Oxoid, England). Sorbitol-negative colonies were characterized by the following biochemical tests: indol, beta-glucuronidase analysis and agglutination with *E. coli* O157 antiserum (Probac, Brazil). This method was applied in accordance with International Organization for Standardization (ISO) 16654-2001, 2001.

2.4. Molecular typing

2.4.1. DNA extraction

Salmonella spp. DNA was extracted using a Wizard® Genomic DNA kit (Promega, USA), following the manufacturer's instructions. Genomic DNA of *Listeria* spp. and *E. coli* O157:H7 were extracted by heat, as previously described (Doumith, Buchrieser, Glaser, Jacquet, & Martin, 2004; Border, Howard, Plastow, & Siggens, 1990; Paula, Geimba, Amaral, & Tondo, 2010).

2.4.2. Multiplex polymerase chain reaction (PCR)

Multiplex PCR was used to analyze the virulence of *Salmonella* spp., *Listeria* spp. and *E. coli* O157:H7 isolates. The sequences and references of the oligonucleotides used in this study are shown in Table 2. The presence of *invA*, *sefA* and *spvC* genes was evaluated in *Salmonella* isolates. *Listeria* spp. serotype identification was carried out by the presence of *lmo1118*, *lmo0737*, *ORF 2110*, *ORF 2819*, *prs* and *hlyA* genes. The

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