Antimicrobial Resistance and Virulence Profiles of *Salmonella* Isolated from Butcher Shops in Minas Gerais, Brazil

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ABSTRACT

*Salmonella* can contaminate finished products of butcher shops, mainly through cross-contamination of utensils exposed to raw materials. To identify the main sources of contamination with this foodborne pathogen in four butcher shop environments, surface samples were obtained from employees’ hands, cutting boards, knives, floor of the refrigeration room, meat grinders, and meat tenderizers (32 samples per area) and analyzed for *Salmonella* using the International Organization for Standardization method 6579, with modifications. Suspect isolates were identified by PCR (targeting *ompC*), and confirmed *Salmonella* isolates were subjected to pulsed-field gel electrophoresis (after treatment with restriction enzyme *XbaI*), analyzed for the presence of virulence genes (*invA, sefA, and sprC*), and screened for resistance to 12 antimicrobials. *Salmonella* isolates was identified only on cutting boards (five samples) from three butcher shops. Fifteen isolates were confirmed as *Salmonella* belonging to four pulse types (similarity of 71.1 to 100%). The *invA* gene was detected in 13 isolates, and the *sefA* was found in 8 isolates; no isolate carried *sprC*. All tested isolates were resistant to clindamycin and sensitive to amikacin and cefotaxime, and all isolates were resistant to at least 3 of the 12 antimicrobials tested. The results indicate the importance of cutting boards as a source of *Salmonella* contamination in butcher shops. The presence of multidrug-resistant *Salmonella* strains possessing virulence genes highlights the health risks for consumers.

*Salmonella* is considered an important foodborne pathogen worldwide. It is the causative agent of salmonellosis and has been implicated in numerous cases and outbreaks associated with the consumption of contaminated foods, including meat products (24, 26). This persistent occurrence of *Salmonella* in foods highlights its relevance as a public health concern and demands proper control by food industries and retail establishments (5).

In the meat processing arena, *Salmonella* can contaminate finished products mainly by improper handling and cross-contamination by raw materials (17). This scenario is particularly relevant in butcher shop environments, where raw cuts of meat of different types (e.g., beef, poultry, and pork) are handled by different employees using various types of equipment and utensils (e.g., knives, tenderizers, grinders, slicers) on various food contact surfaces (31). Once present in one area of the shop, *Salmonella* can easily contaminate the end products and become a potential hazard for consumers (13).

Despite the known virulence potential of *Salmonella*, several studies have revealed another relevant concern related to this foodborne pathogen: its resistance to antimicrobials (23, 35). The overuse of antimicrobials for feeding and treatment of meat-producing animals is considered the main cause of the development of such resistance by *Salmonella* (10), resulting in difficulties in effectively treating humans in cases and outbreaks of salmonellosis caused by resistant strains (23).

Because of the importance of *Salmonella* as a microbiological hazard associated with meat products, the present study was conducted to identify the main points of contamination with this foodborne pathogen in butcher shop environments and to identify the pathogenic characteristics and antimicrobial resistance patterns of the isolates obtained.

MATERIALS AND METHODS

Butcher shop selection and sampling. Four butcher shops located in Viçosa, Minas Gerais state, Brazil, were included in the present study based on the agreement of the owners and inspection by official fiscalization services. All selected butcher shops processed raw meat from different animals and produced a small variety of meat products (mainly fresh sausages) for sale directly to consumers. Each butcher shop was visited eight times at 2-week intervals during usual hours of activity, and 32 surface samples were obtained from each of the following: employees’ hands, plastic cutting boards, knives, floor of the refrigeration room, meat grinders, and meat tenderizers. Samples were obtained during the usual processing and handling of meat products in each butcher shop without specific cleaning procedures. Selected places were sampled by surface swabbing of two 50-cm² areas (defined with a sterile template of 5 by 10 cm) with two sterile sponges (3M

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Representative strains of each of the PCR using the protocol recommended by PulseNet and 5\% with some modifications. Reactions of 25 bp for invA and 9 bp for ompC with some modifications. Aliquots (40 ml) of each sample homogenate (corresponding to 40 cm² of surface area) were transferred to tryptic soya broth (TSB) and incubated at 37°C for 24 h. The obtained cultures were subjected to Rappaport-Vassiliadis and Muller-Kauffmann tetrahionate-novobiocin broths and incubated at 41.5°C for 24 h and 37°C for 24 h, respectively (all culture media were from Oxoid unless otherwise noted). The cultures from both broths were streaked onto xylose lysine deoxycholate agar and incubated at 37°C for 24 h. Suspected Salmonella colonies were transferred to lysine iron agar (LIA) and triple sugar iron agar (TSI) slants and incubated at 37°C for 24 h. Colonies that presented typical LIA and TSI reactions were confirmed as Salmonella according to ISO 6579 (12) with some modifications. Aliquots (40 ml) of each sample homogenate (corresponding to 40 cm²) were centrifuged at 4°C for 15 min at 1,000 × g. The supernatant was discarded, and the pellet was resuspended with 10 ml of 1% (wt/vol) buffered peptone water and incubated at 37°C for 24 h. The obtained cultures were transferred to Rappaport-Vassiliadis and Muller-Kauffmann tetrahionate-novobiocin broths and incubated at 41.5°C for 24 h and 37°C for 24 h, respectively (all culture media were from Oxoid unless otherwise noted). The cultures from both broths were streaked onto xylose lysine deoxycholate agar and mannitol lysine crystal violet brilliant green agar and incubated at 37°C for 24 h. Suspected Salmonella colonies were transferred to lysine iron agar (LIA) and triple sugar iron agar (TSI) slants and incubated at 37°C for 24 h.

Colonies that presented typical LIA and TSI reactions were streaked onto tryptic soya agar and incubated at 37°C for 24 h; isolated colonies were transferred to tryptic soya broth (TSB) and incubated at 37°C for 24 h. The obtained cultures were subjected to DNA extraction and purification using the Wizard Genomic DNA Purification kit (Promega Corp., Madison, WI). The extracted DNA was used in a PCR assay to detect a sequence of the ompC gene typical of Salmonella according to the protocol described by Alvarez et al. (1) with some modifications. Reactions of 25 μl were composed of 2.0 μl of DNA, 12.5 μl of GoTaq Green Master Mix (Promega), 8.5 μl of nuclease-free water (Promega), and 1 μl of each primer (both at 10 pmol/μl: 5’-ATCGCTGACTTATG-3’ and 3’-CGGTTTGGCTTATAGTGCTG3’). The PCR conditions were 95°C for 2 min; 30 cycles at 93°C for 1 min, 42°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 5 min. The PCR products were subjected to electrophoresis on 2.0% (wt/vol) agarose gels, stained in a GelRed (Biotium, Inc., Hayward, CA) bath, and visualized with a transilluminator. PCR products of 204 bp were considered positive for Salmonella.

Microbiology, St. Paul, MN) previously moistened with 10 ml of buffered peptone (0.1%, wt/vol) saline (0.85%, wt/vol) solution (BPS; Oxoid Ltd., Basingstoke, UK). Samples were kept under refrigeration for up to 60 min until analysis. For hand samples, the palm and fingers were swabbed because of their direct contact with meat products. For microbiological analyses, each set of two sponges per sample were transferred to a sterile bag, and 80 ml of BPS was added (total volume of 100 ml per sample) and homogenized for 60 s (Stomacher 400 circulator, Seward Ltd., Worthington, UK). For the analysis, 1 ml of the homogenate corresponded to 1 cm² of each sample.

Salmonella detection. All samples were subjected to microbiological analysis to detect Salmonella according to ISO 6579 (12) with some modifications. Aliquots (40 ml) of each sample homogenate (corresponding to 40 cm²) were centrifuged at 4°C for 15 min at 1,000 × g. The supernatant was discarded, and the pellet was resuspended with 10 ml of 1% (wt/vol) buffered peptone water and incubated at 37°C for 24 h. The obtained cultures were transferred to Rappaport-Vassiliadis and Muller-Kauffmann tetrahionate-novobiocin broths and incubated at 41.5°C for 24 h and 37°C for 24 h, respectively (all culture media were from Oxoid unless otherwise noted). The cultures from both broths were streaked onto xylose lysine deoxycholate agar and mannitol lysine crystal violet brilliant green agar and incubated at 37°C for 24 h. Suspected Salmonella colonies were transferred to lysine iron agar (LIA) and triple sugar iron agar (TSI) slants and incubated at 37°C for 24 h.

Colonies that presented typical LIA and TSI reactions were streaked onto tryptic soya agar and incubated at 37°C for 24 h; isolated colonies were transferred to tryptic soya broth (TSB) and incubated at 37°C for 24 h. The obtained cultures were subjected to DNA extraction and purification using the Wizard Genomic DNA Purification kit (Promega Corp., Madison, WI). The extracted DNA was used in a PCR assay to detect a sequence of the ompC gene typical of Salmonella according to the protocol described by Alvarez et al. (1) with some modifications. Reactions of 25 μl were composed of 2.0 μl of DNA, 12.5 μl of GoTaq Green Master Mix (Promega), 8.5 μl of nuclease-free water (Promega), and 1 μl of each primer (both at 10 pmol/μl: 5’-ATCGCTGACTTATG-3’ and 3’-CGGTTTGGCTTATAGTGCTG3’). The PCR conditions were 95°C for 2 min; 30 cycles at 93°C for 1 min, 42°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 5 min. The PCR products were subjected to electrophoresis on 2.0% (wt/vol) agarose gels, stained in a GelRed (Biotium, Inc., Hayward, CA) bath, and visualized with a transilluminator. PCR products of 204 bp were considered positive for Salmonella.

Based on the results, samples were classified as positive or negative for Salmonella for each 40 cm² of surface area swabbed.

XbaI macrorestriction and PFGE. Isolates identified as Salmonella by PCR were also subjected to PCR protocols to detect virulence genes. The primers utilized in these protocols were those described by Swamy et al. (27) for invA (5’-TGTATCCGCTATTTT-GACCA-3’ and 5’-CTGACTGCTACCTTGATGAG-3’), and spoV (5’-GGAAATACCATCTAAATGA-3’ and 5’-CCCAGGGCATATCTCAGG-3’). For each pair of primers, PCR products of 25 μl were composed of 12.5 μl of GoTaq Green Master Mix, 2.0 μl of the DNA, 8.5 μl of nuclease-free water, and 1 μl of each primer (at 10 pmol/μl). For invA and spoV, the PCR conditions were 93°C for 5 min; 30 cycles at 93°C for 1 min, 42°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 5 min. For spoV, the PCR conditions were 94°C for 5 min; 40 cycles at 94°C for 1 min, 61°C for 1 min, and 72°C for 1.5 min; and a final extension at 72°C for 5 min. The PCR products were subjected to electrophoresis and were stained and visualized as described above. PCR products of 521 bp (invA), 669 bp (spoV), and 310 bp (spoV) were considered positive for each respective screened gene.

Virulence genes. All isolates identified as Salmonella by PCR were also subjected to PCR protocols to detect virulence genes. The primers utilized in these protocols were those described by Swamy et al. (27) for invA (5’-TGTATCCGCTATTTT-GACCA-3’ and 5’-CTGACTGCTACCTTGATGAG-3’), and spoV (5’-GGAAATACCATCTAAATGA-3’ and 5’-CCCAGGGCATATCTCAGG-3’). For each pair of primers, PCR products of 25 μl were composed of 12.5 μl of GoTaq Green Master Mix, 2.0 μl of the DNA, 8.5 μl of nuclease-free water, and 1 μl of each primer (at 10 pmol/μl). For invA and spoV, the PCR conditions were 93°C for 5 min; 30 cycles at 93°C for 1 min, 42°C for 1 min, and 72°C for 2 min; and a final extension at 72°C for 5 min. For spoV, the PCR conditions were 94°C for 5 min; 40 cycles at 94°C for 1 min, 61°C for 1 min, and 72°C for 1.5 min; and a final extension at 72°C for 5 min. The PCR products were subjected to electrophoresis and were stained and visualized as described above. PCR products of 521 bp (invA), 669 bp (spoV), and 310 bp (spoV) were considered positive for each respective screened gene.

Antimicrobial resistance. Representative strains of each pulse type identified after XbaI macrorestriction and PFGE analysis and the analysis for virulence genes were cultured in TSB and incubated at 37°C until they reached a turbidity close to 0.5 on the MacFarland scale. Each culture was swabbed on the

<table>
<thead>
<tr>
<th>Sample point</th>
<th>n</th>
<th>Shop 1</th>
<th>Shop 2</th>
<th>Shop 3</th>
<th>Shop 4</th>
<th>Total</th>
</tr>
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<tbody>
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<td>0</td>
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</tr>
<tr>
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<td>1</td>
<td>0</td>
<td>3</td>
<td>5</td>
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</tr>
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* A 100 cm² area was sampled per point. For employees’ hands, the front (palm) and fingers of the hands were sampled for a total area of 100 cm².
surface of brain heart infusion agar plates, and Etest strips (bioMérieux, Marcy l’Etoile, France) with antimicrobials were placed onto the agar surfaces. The antimicrobials tested were amikacin, cefotaxime, cefoxitin, cephalexin, clindamycin, imipenem, kanamycin, minocycline, sulfamethoxazole, tetracycline, tobramycin, and trimethoprim-sulfamethoxazole. Plates were incubated at 37°C for 24 h, and the MIC of each antimicrobial for each strain was defined based on the presence of an inhibition halo around the strip. Based on these data, the manufacturer’s instructions, and Clinical and Laboratory Standards Institute standards (6), the strains were classified as resistant (total or intermediate) or sensitive to each antimicrobial.

RESULTS AND DISCUSSION

The positive results for samples from each butcher shop that were analyzed for Salmonella are presented in Table 1. Salmonella was found only on the surfaces of cutting boards. These food contact surfaces are known to harbor pathogens such as Salmonella. These bacteria can adhere and form biofilms on a variety of materials, such as stainless steel and plastic (11, 30). This finding highlights the role of cutting boards in the cross-contamination by Salmonella in butcher shop environments, indicating a possible source of contamination of handled meat cuts and products and representing a hazard for consumers (15, 21). Based on the adherence of Salmonella and its presence on cutting boards, the need for effective cleaning procedures to reduce the chances of cross-contamination of finished products is evident (20).

Berends et al. (3) highlighted the relevance of continuous cleaning procedures for Salmonella control in butcher shop environments and identified cutting boards as a relevant source of contamination by this foodborne pathogen, as found in the present study. In previous studies, various sources of Salmonella have been identified in butcher shop environments, including cutting boards, knives, tables, and finished products such as pork cuttings and poultry (7, 9, 29).

From the Salmonella-positive samples, 15 isolates were obtained and confirmed by PCR as possessing the *ompC* gene, which is involved in the production of structural proteins from the outer bacterial membrane and was detected using primers designed specifically for this pathogen (1, 16). These isolates were designated S01 through S15, and their genetic profiles and PFGE results are presented in Figure 1. The isolates were grouped into four pulse types, each sharing 71.1 to 100% sequence similarity. Isolates from butcher shops 1 and 4 had identical genetic profiles, indicating a common source of Salmonella contamination. In contrast, isolates from butcher shop 4 collected at different times had different genetic profiles, indicating continuous contamination by different Salmonella strains in this environment. Previous studies have revealed continuous contamination by Salmonella in different food processing environments and the occurrence of identical strains in different locations (2, 33, 34) as observed in the present study.

Despite having identical PFGE genetic profiles, the isolates differed in their virulence genes (Fig. 1). The PFGE
The antimicrobial resistance results and MICs are listed in parentheses: R, resistant; I, intermediate resistance; S, sensitive. AB, absence of halo formation, which indicates resistance.

The identification of strains that were resistant to cephalothin (Table 2) is a concern, because this antimicrobial agent is usually used to treat salmonellosis (10). Another concern is the resistance of S11 to imipenem, which was considered by Hur et al. (10) as one of the few options for treatment of Salmonella infections. Resistance of Salmonella strains to multiple antimicrobials is usually associated with the genetics of these microorganisms and can be exacerbated by the use of subclinical doses of antimicrobials in animal feed and for disease treatment (4). Thus, the occurrence of multidrug-resistant Salmonella strains in foods of animal origin can easily be associated with the increasing number of salmonellosis cases and outbreaks that are not responsive to standard clinical treatments (13, 23, 35).

Salmonella is usually identified as resistant to multiple antimicrobials because of its ability to produce beta lactamases, enzymes that inhibit the activity of beta-lactam antibiotics (10, 13). The resistance patterns found in this study among Salmonella strains isolated from the butcher shop environment means that consumers may be exposed to these pathogens, mainly through cross-contamination at the retail level and in the home, representing a potential health risk.

In conclusion, cutting boards were an important point of contamination by Salmonella in these butcher shop environments, mainly because of the possibility of cross-contamination of finished products. Despite the observed low prevalence of this foodborne pathogen, the identification of isolates that possess virulence genes and are multidrug resistant highlights the risk of Salmonella infection for consumers.

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