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Distribution of *Salmonella* clonal groups in four Brazilian feed mills

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ABSTRACT

Feed is one of the major vehicles for *Salmonella* transmission to pigs, and its spread during the milling process may play an important role in feed contamination. Therefore, a cross-sectional study was carried out in four feed mills in order to evaluate the frequency of *Salmonella* isolation at various stages of feed production and to track the spread of genotypically related isolates grouped by pulsed-field gel electrophoresis (PFGE). From a total of 1269 analyzed samples, 63 (4.96%) showed the presence of *Salmonella*. Evaluated feed mills (A, B, C and D) presented 3.5% ($n = 11/317$), 1.7% ($n = 5/289$), 7.5% ($n = 23/308$) and 7.0% ($n = 25/355$) positive samples, respectively. Twenty-three serovars were identified, with the most frequently detected being Montevideo ($n = 14$, 22.2%), Anatum ($n = 8$, 12.7%) and Senftenberg ($n = 8$, 12.7%). The isolation of *Salmonella* was significantly higher ($p = 0.002$) in samples with the presence of total coliforms (36/489; 7.36%) than in the coliform-negative samples (27/780; 3.46%). Conveyors (OR = 4.43, 95% CI: 2.43–8.09) were the most likely sites of *Salmonella* isolation, followed by dust settled on the feed mill's floor (OR = 2.88, 95% CI: 1.41–5.33). Isolates indistinguishable on PFGE or belonging to pulsotypes with a high similarity (>95%) were identified in serovars Agona, Infantis, Montevideo, Orion, Senftenberg and Worthington. In particular, clonal groups of serovars Montevideo and Senftenberg were found to be disseminated among different sample types (ingredients, dust collected from the premise's floor and complete feed) or to be endemic in the feed mills. The dissemination of *Salmonella* clonal groups demonstrates the importance of control measures to avoid dust and debris accumulation on equipment surfaces.

1. Introduction

Salmonella infection in humans is a foodborne disease, frequently associated with the consumption of contaminated eggs, poultry meat and pork (Voetsch et al., 2004). Feed is considered to be one of the major vehicles of *Salmonella* transmission for poultry and swine, and thus should be targeted in control programs (EFSA, 2010). Although few studies have been able to demonstrate the real proportion of foodborne diseases in humans attributed to contaminated feed (Crump, Griffin, & Angulo, 2002), the isolation of *Salmonella* serovars associated with human infections in ingredients and complete feed (Li et al., 2012; Wierup & Häggblom, 2010) stresses the importance of *Salmonella* monitoring and control during feed production.

All feed ingredients are potential vehicles of *Salmonella* (Davies & Wales, 2013; Wierup & Häggblom, 2010). Moreover, several factors, such as dust, the presence of animal vectors and poor hygiene conditions, may contribute to feed contamination and recontamination during processing (EFSA, 2008; Torres, Piquer, Algarra, de Frutos, & Sobrino, 2011). However, *Salmonella* counts in feed may be low and the contamination highly clustered (Jones & Ricke, 1994). Therefore, feed sampling conducted in storage bins has not been considered suitable for *Salmonella* contamination monitoring programs (Davies & Wray, 1997; Malmqvist, Jacobsson, Hägbblom, Cerenius, Sjöland, & Gunnarsson, 1995), which must include sampling of ingredients, complete feed, and dust or spilling collected throughout the production process (Davies & Hilton, 2000). Additionally, the quantification of indicator

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microorganisms, such as coliforms, can be adopted to assess decontamination process efficacy and to evaluate the sanitary conditions of feed-mill production lines (Jones & Richardson, 2004; Veldman, Vahl, Borggreve, & Fuller, 1995).

Although common *Salmonella* serovars have been frequently identified in ingredients as well as in several points of the feed processing, studies of animal feed contamination have rarely used genotypic methods to identify transient or endemic clonal groups in feed mills. Genotypic approaches, such as pulsed-field gel electrophoresis (PFGE), have been successfully adopted for determining the spread of *Salmonella* clonal groups on farms and at slaughter (De Busser et al., 2011; Kich et al., 2011; Molla et al., 2010; Silva et al., 2012), and therefore, they may also contribute to tracking *Salmonella* in feed mills.

In Brazil, the Ministry of Agriculture and Livestock Production is in charge of feed-mill inspection, which is conducted according to the Normative Instruction 4 (IN4; Brasil, 2007). This document sets the hygiene standards and good manufacturing practices required for all feed mills, and it includes a checklist for assessing the compliance level of each feed mill with the regulations. In spite of that, there is still a lack of data concerning the contamination and distribution of *Salmonella* in Brazilian feed mills. Thus, the objectives of this study were to assess: (1) the frequency of *Salmonella* isolation at different production stages in Brazilian feed mills; (2) the association between the isolation of *Salmonella* and coliforms; and (3) the cross-contamination and persistence of *Salmonella* clonal groups throughout the feed processing.

2. Material and methods

2.1. Study design

A cross-sectional study was conducted in four feed mills located in the south and southeast regions of Brazil. The selection criteria to include the feed mills in the study were a minimal monthly feed production volume of 4000 tons, and their agreement to participate. The production flowchart of each plant was examined to define a minimum of 50 sampling points, including different production areas. In each feed mill, six repetitions of the complete sampling plan were conducted on different days.

2.2. Feed-mill characterization

Feed mills comprised the following areas: ingredient reception (bulk or bags), storing area (external and internal bins), warehouses and tanks, grinder, dosage scale, mixer, pellet mill, cooler, final product bagging and expedition.

Feed mills B, C and D produced both mash and pelleted feeds, whereas only mash feed was processed in mill A. The production of feed mills A, B, C and D was, respectively, 16,000, 4000, 5000 and 38,000 ton/month. All feed mills, except D, had a sector for cleaning grains before storage and use. The reception of bulk ingredients was performed in bins, while bagged ingredients remained stored in warehouses. In feed mills B and C, all ingredients were submitted to physical and chemical analyses before milling, while monitoring of bacterial contamination in ingredients was not performed in any of the feed mills. The distribution of the ingredients to external and internal bins was performed by different types of conveyor belts and carriers. The expedition of complete feed (mashed or pelleted) was performed in bulk in trucks or bagged. Regarding the compliance with the Normative Instruction 4 (Brasil, 2007), feed mills A and B were classified as “in total compliance”, whereas C and D were classified as “in implementation process” at the time of the study.

2.3. Sample collection

Up to 200 g of each sample (raw ingredients, aggregated debris and dust from the inner surface of equipment [bins, conveyors, mills, scales, pelleting and coolers], dust settled on the floor and debris from premises and trucks) were collected. Each sample was a pool of five to ten individually collected aliquots in order to increase the representativeness of the sample (Richardson, 2008). Samples were individually collected in sterile plastic bags. During the sampling procedures, the operator wore clean disposable gloves that were changed before each sample collection.

2.4. Salmonella detection

Aliquots (25 g) of each sample were pre-enriched in 225 mL buffered peptone water (BPW, Oxoid, UK) incubated at 37 °C overnight. *Salmonella* isolation was performed based on FSIS (2008), with selective enrichment in Rappaport-Vassiliadis (RV, Merck, Germany) and Tetrathionate (Difco Laboratories, USA) broths at 42 °C for 24 h. Afterwards, aliquots of each broth were plated onto both xylose-lactose-tergitol 4 (XLT-4, Oxoid) and brilliant-green phenol-red lactose sucrose (BPLS, Merck, Germany) agar plates before being incubated at 37 °C for 24–48 h. *Salmonella* presumptive colonies were selected from each positive sample for biochemical testing (Triple Sugar Iron [TSI], Oxoid); Lysine Iron Agar [LIA] (Oxoid); and O-nitrophenyl-β-D-galactopyranoside [ONPG] (Oxoid), followed by agglutination testing with somatic polyvalent serum (Probac, Brazil). Isolates that showed a positive reaction in agglutination tests were shipped to the Fundação Instituto Oswaldo Cruz (FIOCRUZ) for serotyping according to the Kauffmann–White scheme.

2.5. Enumeration of coliforms

From the initial sample suspension (10^{-1}) in buffered peptone water, further decimal dilutions until 10^{-6} were performed. Aliquots (1 mL) of each dilution were transferred to sterile Petri dishes in duplicate. About 15 mL of Neutral Red Violet Bile agar (VRBA, Oxoid) were poured into each Petri dish. Afterwards, a 5-mL VRBA overlay was added to each plate. Upon incubation at 37 °C for 48 h, typical colonies (pink surrounded by a purple precipitate) were counted (Kornacki & Johnson, 2001). The number of colony-forming units per gram of sample ($CFU.g^{-1}$) was obtained by multiplying the average number of colonies by the reciprocal of the dilution that presented between 30 and 300 colonies on the plate.

2.6. Genotyping by pulsed-field gel electrophoresis

Salmonella isolates belonging to common serovars and isolated from the same feed-mill plant were submitted to PFGE. The bacterial suspension was embedded in agarose, lysed, washed, and digested with XbaI and BlnI restriction enzymes (New England Biolabs, Beverly, MA) overnight (12–16 h) at 37 °C, essentially as described in Ribot et al. (2006). Electrophoresis was performed in 1% agarose gel using 0.5X Tris-borate-EDTA buffer on a Chef II (BioRad Laboratories, Hercules, CA) at 6 V/cm for 19 h at 14 °C with an initial switch time of 2 min and 16.0 s, and a final switch time of 63.8 s. Gels were stained for 30 min at room temperature with ethidium bromide (Invitrogen, Carlsbad, CA), destained and photographed. *Salmonella* Braenderup (ATCC BAA-664) was included as a reference. Pattern images were acquired using a Kodak Gel Logic 2200 system and analyzed using the Bionumerics software program, Version 2.0 (Applied Maths BVBA, Saint-Martens-Latem-Belgium). Similarities between isolate fingerprints were determined on the basis of the Dice correlation coefficient. A band