PCR MOLECULAR TYPING TO TRACE *SALMONELLA* SPP. IN ALHEIRA PRODUCTION LINES

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Introduction

Alheira is a traditional Portuguese cooked meat product with specific characteristics, with respect to its raw material and its manufacturing technology. Previous studies showed that this product might contain some pathogens that could affect the safety of the product. In Esteves (2005), 96 samples of alheira from different production lines were analysed and *Salmonella* spp. was detected in 12.5% of them.

The study of the occurrence of a microorganism throughout a production line can be used to determine the main source and the spread of food product contamination (Samelis and Metaxopoulus, 1999). Several authors established that molecular typing methodologies of typing could be used to identify the major sources and routes of contamination of specific microorganisms in processing plants (Aguado *et al.*, 2001; Peccio *et al.*, 2003). The aim of the present study was to determine the source(s) and the type (s) of *Salmonella* contamination in alheira, by examining the occurrence of the microorganism throughout the production line of the product, and by PCR typing *Salmonella* isolates obtained from different locations at the production lines.

Materials and Methods

Experimental design: The production of 16 batch of Alheira was examined along the process. *Salmonella* spp. was isolated from various locations along the production line: samples of raw materials and seasoning (poultry P, pork R; bread B; casing T; casing desalting water WI; seasoning E); swabs of different contact surfaces (table I; reservoir Q; equipment S; handler's hands M); product in several stages of manufacturing (cooked meat Z; batter before the stuffing L); water from final washing (WII) and finished product (H), with a total of 14 different sampling points. For each sampling point, four independent samples were considered. 85 strains were isolated for further molecular typing.

Primer selection: A random Amplified Polymorphic DNA (RAPD) method using the primer M13R2 (5' GGA AAC AGC TAT GAT CAT GA- 3') and a Repetitive-Element PCR (rep-PCR) method using the primers ERIC1R (5'-ATG TAA GCT CCT GGG GAT TCA G-3'), ERIC 2 (5' AAG TAA GTA ACT GGG GTG AGT G- 3'), BOX1R (5' CTA CGG CAA GGC GAC GCT GAC G- 3') and BOXA2R (5' ACG TGG TTT GAA GAG ATT TTG G- 3') were evaluated for typing purposes. A preliminary study was carried out, testing all 5 primers in a sub-collection of 15 isolates of *Salmonella* spp, to choose the primer(s) which yielded the best results, in order to fulfil the objectives of the current work. The remaining *Salmonella* spp. collection was typed by the chosen PCR primers.

Salmonella spp. detection: The presence of *Salmonella* was investigated in 25 g, 25ml and 100 square cm of meat samples, water and contact surfaces respectively. Detection was automatically performed by an Enzyme Linked Fluorescent assay (MiniVIDAS-BioMérieux 99088), using the test sets and sample preparation as recommended by the manufacturer. Preenrichment was performed in a non selective medium (Buffered Peptone Water) followed by enrichment procedures in two different selective media Selenite Cystine and Rapapport-Vassiliadis (Oxoid), incubated at 35-37ºC and 41-42ºC for 6-8h, respectively. Presumptive *Salmonella* positive samples were streaked on plates of selective media, SM ID and Hektoen Enteric agar (BioMérieux). The confirmation/ identification of *Salmonella* was done by API 20E system (BioMérieux).Antigenic analysis by the agglutination test using polyvalent antisera (Bacto *Salmonella* O Poly A-I and Vi antisera, Difco) was also performed.

Molecular typing: DNA was extracted from pure cultures using Dneasy tissue kit (Quiagen), according to the manufacturer indications. DNA was quantified and the purity evaluated by spectophotometry using GeneQuant pro RNA/DNA Calculator (Amersham Pharmacia Biotech., 80-2109-99). PCR mixtures (25 µl) consisted of: 2.5 µl of Buffer (10x) (200mM Tris-Hcl, pH 8.4, 500m MKCl) (Invitrogen); 1.5 mM of $MgCl_2$ for the primer M13R2 and 3.5 mM for the others (Invitrogen); 200 µM dNTP (Roche Molecular Biochemical); 20 picomoles each primer (Roche); 1 U of Taq DNA polymerase (Invitrogen); 100 ng of genomic DNA. PCR reactions were performed in a Thermal Cycler (Perkin-Elmer Corp. 2700) as follows: initial denaturation at 95ºC for 5 min; 40 cycles with denaturation at 95ºC for 0.30 s, primer annealing for 1 min (temperatures for each primer: M13R2 38ºC; ERIC1R 65ºC; ERIC2- 68ºC; BOXA2R 65ºC; BOX1R- 68ºC), and primer extension at 72ºC for 1.30 min; followed by a final extension at 72ºC for 5 min and cooling to 4ºC. PCR products were resolved by agarose gel (1.5%) electrophoresis (80 mA, 90 minutes), in 0.5x Tris-acetate- EDTA buffer stained with 0.1 µg/ml of ethidium bromide (Sigma). The profiles obtained were observed under UV light, and the image was captured by a video camera.

Data analysis: Band profiles were analysed using Bio Profile software. Reproducibility was tested on 15% of the isolates. The similarity was estimated using Dice coefficient /UPGMA algorithm (NtsysPC 2.1). A cluster was defined with at least a group of three isolates and a limit a 95% similarity.

Results and Discussion

Salmonella spp. was detected in 4% of the samples along the production lines. Among the different incoming materials, casing and raw meat samples presented positive *Salmonella* samples. As occurrence data was not enough to identify sources to trace the pathogen in the processing plants, molecular typing methodologies such as RAPD and rep-PCR were considered for this purpose. Composite RAPD with M13R2 and ERIC 2 profiles were used to interpret the molecular typing results. Nine different clusters were obtained. In order to trace the *Salmonella* spp. contamination throughout the production line, three distinct areas were considered: area 1, reception; area 2, processing; area 3, finished product. Table 1 shows different profiles presented in each area, as well as the different origins of the typed isolates.

Table 1: Distribution of clusters in different areas of the alheira production lines.

Areas in processing plant	Isolates origin Sampling point	Clusters*
1 Reception	Casing, casing desalting water	*A, B, D, E, I*
	Pork, poultry	*A,B,C, D, E, F, G, H*
2 Processing	Table surface	*B, D*
	Hands	*B, D*
	Washing water after stuffing	*B*
3 Finished product	Alheira	*A, B, D, I*

* Clusters (A, B, C, D, E, F, G, H, I).

Clusters ***C, E, F, G*** e ***H*** included isolates exclusively from reception area - from raw meat and from casing. Clusters ***A, B, D*** and ***I*** consist of by isolates from two or more of the defined areas in production lines. These clusters included isolates from final product and from casing related samples. The occurrence of isolates from these clusters in alheira could be explained by the use of contaminated casings, as a direct vehicle for *Salmonella* spp.. The presence of isolates from cluster ***B*** and ***D***, in the processing area, respectively in table surfaces and in hands of workers suggests the possibility of cross contamination between intermediate manufacturing stages throughout contaminated surfaces. Considering the production lines and the production batches, it was possible to identify the sources and to trace the spread of the isolates from these clusters.

Conclusion

The presence of *Salmonella* spp. in alheira could be attributed to the use of contaminated casings, as a direct vehicle or by cross contamination through contaminated table surfaces and hands of workers. The knowledge of *Salmonella* spp. contamination sources and spreading along the production line will allow us to define new strategies towards the control of *Salmonella* spp. in the alheira production lines.

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