DETERMINATION OF THE MAIN CONTAMINATION SOURCES OF ALHEIRA WITH STAPHYLOCOCCUS AUREUS USING A PCR BASE TYPING METHODOLOGY

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Introduction

Alheira is a traditional meat product whit specific characteristics, both considering its raw materials and its manufacturing technology. It was verified in previous works the occurrence of a high contamination rate by S. aureus (Esteves et al, 2000).

The study of the occurrence of a microorganism throughout a production line, determines with some accuracy the main sources and ways used in the contamination of that food product (Samelis and Metaxopoulos,1999).

Works by several authors (Aguado et al, 2001; Vogel et al, 2001; Peccio et al, 2003), have shown that the molecular typing methodologies may bring an important contribution in determining the food contamination source by specific microorganisms. The molecular typing methodology is applied with the purpose of identifying different strains (Struelens, 1998), making it possible to establish with greater accuracy the contamination sources and ways used by bacteria to the contamination of the final product.

Objectives

The aim of the present work was to identify the source(s) and the way(s) used by S. aureus in the Alheira's contamination, using different methodologies. The study of the occurrence of S. aureus throughout a food product's production line, and PCR based typing methodology applied to isolates obtained from different locations at the production line.

Methodology

Experimental design

It was followed the production of 16 lots of Alheira. S. aureus was isolated from various locations along the production line: samples of raw materials and condiments (poultry -P, pork -R; bread - B; gut – T; gut desanting water - WI; spices - E); swab 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), in a total of 14 different sampling points. For each situation, four samples were considered. It was obtained 170 isolates for posterior molecular typing.

Randomly Amplified Polymorphic DNA (RAPD), and Repetitive Element PCR Typing (rep-PCR), were the PCR based methodologies used. A preliminary study was carried out, testing 5 primers in a sub-collection of 15 isolates of S. aureus, with the purpose of choosing the primer which offers the best results, considering the objectives of the current work.

S. aureus search

The search was made in 10 g, 10 ml or in 100 square cm of samples or the contact surfaces respectively. Samples were homogenised in peptone (0.3%) and NaCl (0.85%). After pre-enrichment in Chapman broth (Difco) presumptive S. aureus were isolated in Baird Parker Agar (Difco) supplemented with egg yolk tellurite and sulfametazine. Typical colonies were confirmed by Gram staining and the tube coagulase test was performed using coagulase plasma rabbit (BBL TM).

Molecular typing

DNA extraction from pure culture was made using Dneasy tissue kit (Quiagen), basically according to manufacturer indications. The lysis step was adapted to improve the final DNA yield. Lysosyme (5µg/µl), lysostaphine (0,03µg/µl), and mutanolysine (0,1 U/µl) (Sigma) were added to the lysis buffer. Samples were incubated (30 min, 37ºC) to ensure an efficient lysis. Quantification and DNA purity evaluation were done by spectophotometry using GeneQuant pro RNA/DNA Calculator (Amersham Pharmacia Biotech., 80-2109-99).

PCR mixture (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 (Tib Molbiol Syntheselabor); 1 U of Taq DNA polymerase (Invitrogen); 1 µg of genomic DNA.

PCR reaction was 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 10% of the isolates. The similarity of the DNA patterns was estimated by Dice coefficient and clustering was achieved by unweighted pair group method using arithmetic average linkage (UPGMA) using NtsysPC 2.1software. It was considered as a cluster formation limit a 95% similarity. A cluster was defined with at least a group of three isolates.

Results & Discussion

The number of samples with S. aureus is presented in table 1. We can point out the diversity of sampling point, including raw materials, contact surfaces and product in the various fabrication stages where S. aureus was isolated. This indicates the possibility of the existence of a continuous product contamination, throughout its manufacture, as well as the possibility of the occurrence of cross contaminations. The increase of the occurrence of S. aureus from the early stages of fabrication (cooked meat - Z) to the final product (Alheira - H) could be reflex of the contamination throughout the manufacturing operations, or the occurrence of environmental conditions favourable to the growth of the microorganisms eventually present.

Considering the spread occurrence of Staphylococcus in the processing environment, the simple search of the bacteria does not seem to be elucidative about the main contamination sources and ways used by S. aureus to contaminate the Alheira. Viewing the possibility of the simultaneous existence, along the production line, of more than one strain of S. aureus, the distinction between isolates in the final product and in several point along the production line, through molecular methodology, will allow to determine the used source and way of contamination (Khambaty et al.,1994). Between the five tested primers, M13R2 was chosen because it originated very clear and only moderately complex band profiles, with a good discriminative power.

Figure 1 represents the dendrogram resulting from the analysis of profiles obtained from the amplification of 170 isolates of S. aureus, using the M13R2 primer. The use of this primer lead to the formation of 42 different band profiles that resulted in the formation of 10 different clusters. 26% of the isolates were not included in any cluster. Clusters B and F, which include 46% of all the typed isolates, were the predominant band profiles.

Considering the elements of each clusters and the sampling points they come from, it was verified that the same pattern was observed in isolates from several locations of the production line, except for clusters E and J which include exclusively isolates from poultry (P) and pork (R), indicating that raw material presents characteristic patterns not found elsewhere in the production line, result in agreement with Lam and collaborators (1995).

Table 2 shows the several clusters, distributed by the 3 distinct areas throughout the Alheira's production line, as well as the different origins of the typed isolates.

Isolates from clusters H and I belong to raw materials reception area (samples of pork-R and poultry- P) and processing area (handler's hands -M and different stages of the Alheira's manufacturing, cooked meat - Z and batter before stuffing - L).

Fresh meat could never be a primary contamination vehicle, considering it is cooked before its addiction in to the batter. The presence of isolates from these clusters in different production stages might be explained by the occurrence of cross contamination, probably throughout handlers, once the came RAPD pattern was also observed in their hands.

Clusters B, D and F present isolates from each one of the three areas. In the raw materials reception area, there are isolates originated from raw material (P and R), as well as in gut (T and WI). In the processing area they are found in the handler's hands (M), in some equipment's surfaces (Q) and in some of the analysed manufacturing stages (Z e L). The isolates from these clusters in the final product (H) might result both from cross contamination with raw material, where the handler seems to have an important role as a vehicle, or directly through contaminated gut.

Analysing the distribution of clusters A and C, we can verify that they are constituted only by isolates from processing and final product area. The fact that the occurrence of these patterns is not related to the raw materials means that its origin may be established specifically within the working environment. From the analysis of cluster C, we can say it is totally constituted by isolates from the handler's hands (M) and final product - Alheira (H), belonging to the same production lot (not presented data), which implies as primary origin of S. aureus the handler's hands.

Conclusions

The presence of S. aureus in Alheira might result both from cross contamination with raw material and gut. It was also shown that the handler have an important role has vehicle for cross contamination, and, it was also observed that they might be the primary source of these microorganisms.

Considering the objectives of this work, we may also conclude that the use of the RAPD molecular typing methodology, using the M13R2 primer, lead to a vast and extremely valid amount of information, concerning the sources and ways of contamination used by S. aureus in the contamination of Alheira, thus making it easier to establish strategies directed towards the control of this microorganism in this product.

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Tables and Figures

Table 1. Percentage (number) of samples with S. aureus

Sampling location (n=64)	Presence of S. aureus % (n)	
Water (WI)	6	(4)
Gut (T)	13	(8)
Bread (B)	5	(3)
Pork Meat (R)	50	(32)
Poultry Meat (P)	44	(28)
Spices (E)	2	(1)
Equip. Surf. (S)	3	(2)
Table Surf. (I)	-	-
Tank Surf. (Q)	6	(4)
Hand Surf. (M)	16	(10)
Cooked Meat (Z)	25	(16)
Dough (L)	22	(14)
Water (WII)	2	(1)
Alheira (H)	34	(22)
Total (n=896)		

Table 2. Clusters distributed by the 3 distinct areas throughout the Alheira's production line, as well as the different origins of the typed isolates.

Areas in production line	Isolates origin - Sampling location	Clusters
1 Raw materials and condiments reception area	WI; T;	B; D; F
	B; R; P; E;	B; D; E; F; G; H; I; J;
2 Processing area	S; Q,	A; F;
	M;	B; C; D; F; H, I;
	Z;	A; B; D; F; I;
	L;	A; B; D; F; G; H;
3 Finished product area	H;	B; C; D; F;

Figure 1. Dendrogram resulting from the analysis of profiles obtained from the amplification of 170 isolates of S. aureus, using the M13R2 primer.