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OCURRENCE OF *STAPHYLOCOCCUS AUREUS* AND MULTIPLEX PCR DETECTION OF CLASSIC ENTEROTOXIN GENES IN CHEESE AND MEAT PRODUCTS

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ABSTRACT

Multiplex PCR was used to investigate the presence of enterotoxins genes (*sea, seb, sec, sed* and *see*) and *femA* gene (specific for *Staphylococcus aureus*) in coagulase-positive staphylococci (CPS) isolated from cheese and meat products. From 102 CPS isolates, 91 were positive for *fem*A, 10 for *sea*, 12 for *sed* and four for *see*.

Key words: *Staphylococcus aureus*, enterotoxins, multiplex PCR

Staphylococcus aureus is the predominant specie involved in staphylococcal food-poisoning outbreaks, although other coagulase-positive *Staphylococci*, such as *S. intermedius* and *S. hyicus*, may also be enterotoxigenic (5,34,35). *S. aureus* may produce a large variety of enterotoxins (A, B, C, D, E, G, H, I, J, K, L, M, N, O, P, Q, R and U), but 95% of poisoning outbreaks are caused by classical enterotoxins: A, B, C, D and E (24). Enterotoxin A is the most frequently produced (8,21). Staphylococcal enterotoxins are thermostable and also resistant to gastrointestinal proteases such as pepsin, explaining its ability in remaining active after ingestion (7,8,36).

The amount of staphylococcal enterotoxins required for establishment of typical symptoms of food poisoning is very low, ranging from 20 ng to 1 µg (32). Which corresponds to approximately 10^5 staphylococci colony-forming units per gram of food (19). Several studies evaluated the capability of staphylococcal strains isolated from foods to produce the classical enterotoxins A, B, C, D and E (2,5,8,13,15,18,20,26,31,33). In Brazil, several studies reported counts of coagulase-positive *Staphylococci* above the maximum levels allowed by the Brazilian legislation (11) in sausages (colonial sausage), milk and milk products (1,3-6,10,12-14,23,25).

The aim of the present work was to evaluate the presence of by coagulase-positive *Staphylococci* (CPS) in meat and milk-derived products commercialized in Santa Catarina, SC, Brazil and to detect the presence of genes for classical staphylococcal enterotoxins A, B, C, D and E (*sea, seb, sec, sed* and *see*) and for gene *fem*A, specific for *S. aureus* species, using multiplex PCR.

A total of 72 food samples including mozzarella (15 samples), American cheese (15 samples), colonial cheese (15 samples), colonial sausage (18 samples) and salaminho (09 samples) were collected from markets in Alto Uruguai Catarinese region (AMAUC), in Santa Catarina state, Brazil, from 2005 to 2007.

The mozzarella (A, B, C) and American cheese brands analyzed in this work are under Federal Inspection Service (SIF), while colonial cheese brands are under different inspection services: State Inspection (SIE) (G brand), Municipal Inspection (SIM) (H brand) and SIF (I brand). Five (A, B, C, D, E) out of six brands of colonial sausages are inspected by SIE, and G brand is inspected by SIM (Concórdia - SC). Salaminho brands (A, B, C) are inspected by SIF.

For *S. aureus* enumeration (17), serial dilutions of food homogenates were plated on Baird Parker agar (Oxoid) with 5% egg yolk tellurite emulsion (Oxoid) and incubated at 35ºC for

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48 h. After this period, typical colonies (circular, smooth, convex, gray to jet-black, frequently with light-colored (off-white) margin, surrounded by opaque zone and frequently with an outer clear zone) were counted and five colonies were transferred to MacConkey agar (Oxoid) and blood agar. Colonies that grew in blood agar but not in MacConkey were tested for Gram coloration, coagulase, catalase, oxidase, urease and maltose.

Total DNA was extracted from 5 mL of a coagulase-positive staphylococcal culture grown at 35ºC (± 2ºC) for 16-24 h in Brain Heart Infusion (Merck) broth. DNA was isolated using the WizardÒ Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA) and lisozyme 10 $mg.mL^{-1}$ (Sigma Aldrich). Enterotoxigenic *S. aureus* strains ATCC 13565 (*sea*), ATCC 14458 (*seb*), ATCC 19095 (*sec*), ATCC 23235 (*sed*) e ATCC 27664 (*see*) were used as positive controls and *Staphylococcus xylosus* ATCC 29971 as negative control.

Detection of specific genes for *S. aureus* (*fem*A) and for enterotoxins A, B, C, D, and E was carried out according to Mehrotra, Gehua, Johnson (27), with some modifications, yelding the expected amplicons: 102 bp for *sea*, 132 bp for *fem*A, 164 bp for *seb*, 209 bp for *see*, 278 bp for *sed*, 451 bp for *sec*.

For multiplex PCR detection of *sec* and *fem*A genes, amplification reactions were performed in final volume of 25 mL containing PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.4 mM of each primer, 1.25 U Taq DNA polymerase and 100-300 ng of template DNA. Reactions were carried out in Minicycler™ (MJ Research, Inc. Watertown, MA) with the following program: initial denaturation at 94ºC for 5 min followed by 35 cycles of 94ºC for 2 min, 57ºC for 2 min and 72ºC for 60 s with a final extension at 72ºC for 7 min.

For multiplex PCR detection of *sea, seb, sed, see* and *fem*A genes, amplification reactions were performed in final volume of 50 mL containing PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl), 3 mM $MgCl_2$, 0.2 mM of each dNTP, 400 nM of each primer, 1.25 U Taq DNA polymerase and 100-300 ng of template DNA. Amplification profile was standardized in 94ºC for 5 min followed by 35 cycles of 94ºC for 2 min, 52ºC for 2 min and 72ºC for 3 min with a final extension at 72ºC for 7 min.

PCR products were separated by electrophoresis at 80 V for 70 min in 2% agarose gel and stained with ethidium bromide. Gels were visualized in a UV transilluminator and images were digitalized with a digital camera (CANON Powershot A70).

Presence of coagulase-positive staphylococci (CPS) was detected in 33 out of 72 analyzed samples. In American and colonial cheeses, from 30 samples analyzed, 19 presented contamination by CPS. However, presence of CPS was not detected in the 15 mozzarella samples. 28.8% of samples contaminated with CPS presented counts with levels above 10^3 $CFU.g^{-1}$ which is the upper limit established by RDC nº 12 of the Brazilian National Sanitary Control Agency (11). Counts in samples inspected by municipal, state and federal inspection services differed significantly, being those in SIF inspected samples significantly lower (mean of $1.4x10^3$ $CFU.g^{-1}$) than the samples inspected by the other two services ($1.5x10^5$ and $1.7x10^5$ $CFU.g^{-1}$, for SIE and SIM, respectively). In 6 samples of colonial cheese, CPS counts were above 10^5 $CFU.g^{-1}$. According to FDA (19), in foods with such high counts of CPS the presence of staphylococcal enterotoxin is likely. In 9 out of 18 samples of colonial sausages, concentration of CPS was above the limit established by the Brazilian legislation (11), and in 6 samples, the counts were above than 10^5 $CFU.g^{-1}$. All salaminho samples were negative for CPS.

From the 200 *Staphylococcus* spp. isolates, 116 originated from American and colonial cheeses and 84 from colonial sausage. 102 (51%) strains were characterized as coagulase-positive staphylococci (CPS), being 67 from colonial sausage and 35 from American and colonial cheeses. The remaining 98 isolates (49%) were coagulase-negative staphylococci (Table 1).

CPS isolates were analyzed by multiplex PCR in order to detect *fem*A, *sea, seb, sec, sed* and *see* genes (Fig. 1). 91 out of 102 isolates were positive for *fem*A gene. All *fem*A gene negative CPS isolates (11 isolates) have been isolated from colonial sausage. The *sed, sea and see* genes were detected in 12, 10 and 4 isolates, respectively (Table 1). None of the isolates was positive for *seb* or *sec* genes.

The *fem*A gene was detected in all 35 CPS isolates from cheeses, confirming the biochemical characterization. From cheese samples, *sed* was detected in ten isolates and *see* was detected in one isolate (Tab.1). In *sed* gene positive isolates, amplification of fragments with 600 and 700 bp was also observed (Fig.1A). The presence of these two extra fragments was observed only when the *sed* fragment was also present. Other authors have also reported the presence of unexpected fragments in some dairy products evaluated by multiplex PCR for the presence of enterotoxin genes (16).

Among the 56 CPS isolates from colonial sausage that were positive for *fem*A, seven were also positive for *sea* and one for *sed* (Fig. 1B, Table 1). Three isolates amplified the enterotoxin genes *sea* and *see* simultaneously.

Several authors have also reported that enterotoxin genes *sea* and *sed* are the most common in staphylococci isolated from foodstuffs (5,7,8,30,33). In Brazil, Carmo *et al*. investigated the presence of staphylococci in minas fresh cheese and raw milk and observed that the isolates were able to produce enterotoxins A, B and C (13). Cheese produced in Serra da Canastra, MG, also contained *S. aureus* isolates able to produce enterotoxins B and C (9).

Detection of the enterotoxin genes in the CPS isolates coming from a food is not an indication that the toxins are effectively present in this food. Morandi *et al.* (28) evaluated 107 CPS isolates from dairy products, and observed that enterotoxin genes were detected in 67% isolates, but only 52% of the isolates were capable to produce enterotoxins. These

Table 1. Prevalence of *femA, sea, seb, sec, sed* and *see* genes in coagulase-positive *Staphylococci* strains isolated from foods.

Type of Food	Number of Isolates	CPS	CPS *femA* positive	*CPS sea positive*	*CPS se bpositive*	*CPS sec positive*	*CPS sed positive*	*CPS see positive*
Colonial and American Cheeses	116	35	35	0	0	0	11	1
Colonial Sausage	84	67	56	10	0	0	1	3*
Total	200	102	91	10	0	0	12	4

* These strains were also positive for the presence of *sea* gene.
CPS = Coagulase-positive *Staphylococci.*

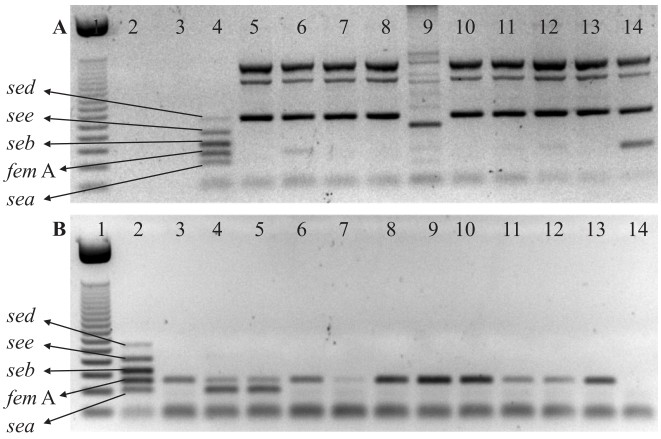

Figure 1. Multiplex PCR of CPS strains isolated from A) cheese, B) colonial sausage. **A)** lane 1: Ladder 50bp (Promega); lane 2: water; lane 3: negative control (*Staphylococcus xylosus* ATCC 29971)*;* lane 4: positive control (mix of four ATCC strains – 13565 *sea*, 14458 *seb*, 23235 *sed* and 27664 *see*); lanes 5 – 14: *S. aureus* strains isolated from cheese. **B)** Lane 1: Ladder 50 bp (Promega); lane 2: positive control (mix of four ATCC strains – 13565 *sea*, 14458 *seb*, 23235 *sed* and 27664 *see*); lanes 3 – 13: *S. aureus* isolated from colonial sausage; lane 14: negative control (*Staphylococcus xylosus* ATCC 29971).

authors reported that the results obtained by multiplex PRC and immunoassay tests were in agreement when tested for the presence of enteroxins A, C and D (16).

Results of the present work confirm that multiplex PCR is a rapid and sensitive method for screening of enterotoxigenic coagulase-positive *Staphylococci*, being highly specific. Results also indicate that better hygiene practices and better inspection are required in the production of the foods included in the study.

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RESUMO

Ocorrência de *Staphylococcus aureus* e detecção por PCR multiplex de genes de enterotoxinas clássicas em queijo e derivados cárneos

PCR multiplex foi empregado para investigar a presença de genes de enterotoxinas estafilocócicas (*sea, seb, sec, sed* e *see*) e do gene *fem*A, específico para *S.aureus*, em cepas de estafilococos coagulase positiva (ECP) isoladas de queijos e derivados cárneos. De 102 cepas, 91 foram positivas para *fem*A, 10 para *sea*, 12 para *sed* e 4 para *see*.

Palavras-chave: *Staphylococcus aureus*; enterotoxinas, PCR multiplex

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