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Polymerase Chain Reaction Detection of Enterotoxins Genes in Coagulase-Negative Staphylococci Isolated from Brazilian Minas Cheese

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Abstract

For a long time, *Staphylococcus aureus* has been always thought to be the only pathogenic species among *Staphylococcus*, while coagulase-negative staphylococci (CNS) were classified as contaminant agents. However, molecular techniques have shown that these microorganisms also possess enterotoxin-encoding genes. The aim of this study was to analyze the frequency of genes for staphylococcal enterotoxins SEA, SEB, SEC, and SED in CNS strains isolated from Minas soft cheese and to assess the *in vitro* production of toxins. CNS were found in 65 (72.2%) samples of cheese: 23 were *Staphylococcus saprophyticus*, 16 *Staphylococcus warneri*, 10 *Staphylococcus epidermidis*, 9 *Staphylococcus xylosus*, 3 *Staphylococcus haemolyticus*, 2 *Staphylococcus schleiferi* subsp. *schleiferi*, and 1 each *Staphylococcus capitis* subsp. *urealyticus* and *Staphylococcus caprae*. Seventeen (26.2%) CNS strains had genes for enterotoxins, and *sea* was more frequently found (18.5%), followed by *sec* in three and *seb* in two strains, whereas the *sed* gene was not found. *S. saprophyticus* showed enterotoxin genes in 6 of 23 isolates, but only *sea* was observed. On the other hand, five strains of *S. warneri* showed the *sea*, *seb*, or *sec* gene. In spite of the presence of these enterotoxin genes, these strains did not produce enterotoxins *in vitro*. It is essential to understand the real role of CNS in food, and based on the presence of enterotoxin genes, CNS should not be ignored in epidemiological investigations of foodborne outbreaks.

Introduction

Enterotoxigenic *Staphylococcus aureus* is one of the major pathogens causing food poisoning worldwide (Dinges *et al.*, 2000). It has been considered the only pathogenic species among *Staphylococcus*, while coagulase-negative staphylococci (CNS) have been classified just as contaminant agents (Kloos and Bannerman, 1994). Recently, several authors have suggested that this group of microorganisms should be studied regarding the presence of enterotoxin genes (Rodriguez *et al.*, 1996; Veras *et al.*, 2008; Rall *et al.*, 2010).

In Brazil, Minas cheese is the most popular cheese consumed all over the country. It is a soft and fresh white cheese with high pH, 55% moisture content, and low percentage of salt (1.4%–1.6%). It is produced on both industrial and domestic scales by adding lactic cultures or by direct acidification of milk (Carvalho *et al.*, 2007). In a minor-scale production, small producers use raw milk in nonhygienic conditions. Besides the nonpasteurized milk, *Staphylococcus* genus from the food handler may contaminate Minas cheese (Carmo *et al.*, 2004). The present study aimed to analyze the frequency of the *sea*, *seb*, *sec*, and *sed* genes in CNS strains isolated from samples of Minas soft cheese and to evaluate their ability to produce enterotoxins *in vitro*.

Materials and Methods

Microbiological analysis

We analyzed 90 samples of Minas cheese from 16 different brands collected from supermarkets and dairy product stores in Botucatu, São Paulo State, Brazil. Twenty-five grams of each sample was homogenized in 225 mL of buffered sterilized water using a Stomacher Lab Blender 400 (Seward) for 30 s, and several decimal dilutions were performed using the same diluent.

CNS identification

Serial dilutions were plated on Baird–Parker agar with 5% egg yolk tellurite emulsion and incubated at 35°C for 48 h.

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Five characteristic colonies were tested for catalase and coagulase. After this screening test, antibiogram test for bacitracin (0.04 U) and furazolidone (100 μg) was applied to discriminate CNS from *Kocuria* (Bannerman and Peacock, 2007). Finally, the strains were submitted to API Staph (Biomérieux).

Polymerase chain reaction test for genes encoding staphylococcal enterotoxins

DNA was extracted using a commercial kit (Mini Spin Kit; GE Healthcare) following the supplier's instructions. The primers used to detect the SE genes and the polymerase chain reaction were performed according to Johnson *et al.* (1991).

Samples of *sea*, *seb*, and *sec* amplicons were sequenced and the partial sequences were confirmed as corresponding to GenBank accessions numbers M18970, M11118, and X05815, respectively.

In vitro *enterotoxin production*

Enterotoxin production was analyzed according to Donnelly *et al.* (1967). The supernatants were tested for SEA, SEB, SEC, and SED using the reversed passive latex agglutination assay method (RPLA) (Oxoid–SET-RPLA) according to the manufacturer's instructions. American Type Culture Collection (ATCC) strains were used as positive controls.

Results

CNS were found in 65 of 90 samples (72.2%): 23 (35.4%) were *Staphylococcus saprophyticus*, 16 (24.6%) *Staphylococcus warneri*, 10 (15.4%) *Staphylococcus epidermidis*, 9 (13.8%) *Staphylococcus xylosus*, 3 (4.6%) *Staphylococcus haemolyticus*, 2 (3.1%) *Staphylococcus schleiferi* subsp. *schleiferi*, and each one of (1.5%) *Staphylococcus capitis* subsp. *urealyticus* and *Staphylococcus caprae*. Simultaneous presence of two species in the same sample was also observed: one of them showing *S. saprophyticus* and *S. warneri*, and the other *S. warneri* and *S. epidermidis*.

Table 1 shows the results of molecular tests for the *sea*, *seb*, *sec*, and *sed* genes detection, and *sea* was the most frequent gene (18.5%). *S. warneri* was the only species presenting strains with the *sea*, *seb*, and *sec* genes.

Table 1. Genotypic Profile of the *sea*, *seb*, *sec*, and *sed* Genes in Coagulase-Negative Staphylococci Isolated from Minas Cheese

		Classic enterotoxins genes			
Species	n	sea	seb	sec	sed
Staphylococcus saprophyticus	23	6	—	—	—
Staphylococcus warneri	16	2	1	2	—
Staphylococcus xylosus	9	2	—	1	—
Staphylococcus haemolyticus	3	1	1	—	—
Staphylococcus schleiferi subsp. *schleiferi*	2	—	—	—	—
Staphylococcus capitis subsp. *urealyticus*	1	1	—	—	—
Staphylococcus epidermidis	10	—	—	—	—
Staphylococcus caprae	1	—	—	—	—
Total	65	12	2	3	—

Despite the presence of the enterotoxin genes, these strains did not produce enterotoxins *in vitro*.

Discussion

S. aureus is one of the most prevalent pathogens in food, causing several outbreaks (Veras *et al.*, 2008), but the potential of CNS to cause foodborne disease is not clearly elucidated.

The enterotoxin genes in CNS have been described using polymerase chain reaction techniques. Herein, 17 (26.2%) CNS strains presented genes for classical enterotoxin production. A similar percentage was observed by Rall *et al.* (2010), with 19.4% of CNS isolated from food handlers. A lower prevalence was observed by Vernozy-Rozand *et al.* (1996), with 5.3% of the microorganisms isolated from goat milk and cheese presenting enterotoxin genes, and by Rosec *et al.* (1997), who found that all of 264 CNS strains were negative for these genes. On the other hand, *sec* and *sed* were present in 57.1% of CNS isolated by Rodriguez *et al.* (1996). Veras *et al.* (2008) found five out of eight (62.5%) of CNS encoding such genes.

The *sea* gene was found more frequently (18.5%), while *sed* was not observed. The SE genes s*eb* and *sec* were found in similar percentages (3.1% and 4.6%, respectively). Rall *et al.* (2010) observed that *sea* also occurred more frequently among classical enterotoxin genes (24.1% of CNS).

Despite the presence of classical enterotoxin genes in 26.2% of CNS strains, none of them produced enterotoxins *in vitro* as evaluated by RPLA method. This result is in agreement with Harvey and Gilmour (1988, 1990), who showed that none of 353 and 384 strains of CNS isolated from goats milk and milk powders produced enterotoxins, respectively. One may speculate that it might be due to the low amounts of SEs production, which is not detectable by the method. Enterotoxin was produced using sac culture methods, which are considered to be the most efficient techniques (Robbins *et al.*, 1974), and according to the manufacturer's instructions, the sensitivity of the Kit has been reported to be 0.5 ng/mL. Besides, all ATCC controls produced enterotoxins. Thus, probably these isolates were not expressing the enterotoxin genes. According to Robbins *et al.* (1974), CNS poorly produce enterotoxins even in optimal conditions. Besides, defects in toxin expression may occur due to point mutations that convert the toxin genes to silent genes (Okoji *et al.*, 1993).

However, several authors have shown that enterotoxin production may be highly variable. Crass and Bergdoll (1986) observed that 10% of CNS isolated from food and animals were enterotoxin producers. A percentage of 5.3% was reported by Vernozy-Rozand *et al.* (1996), whereas Zell *et al.* (2008) reported that 45.7% of CNS isolated from food produced enterotoxins. Veras *et al.* (2008) observed that 62.5% of these microorganisms isolated from outbreaks were enterotoxin producers as well. Taken together, our data and those from literature point out that CNS should not be ignored in epidemiological studies of foodborne outbreaks, because the presence of genes for enterotoxins indicates the potential ability of those species to synthesize the proteins. Therefore, further investigations on the role of such genes are still required.

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Disclosure Statement

No competing financial interests exist.

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