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Short communication

PCR detection of staphylococcal enterotoxin genes in *Staphylococcus aureus* strains isolated from raw and pasteurized milk

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Abstract

Milk is considered a nutritious food because it contains several important nutrients including proteins and vitamins. Conversely, it can be a vehicle for several pathogenic bacteria such as *Staphylococcus aureus*. This study aimed to analyze the frequency of genes encoding the staphylococcal enterotoxins (SEs) SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI and SEJ in *S. aureus* strains isolated from raw or pasteurized bovine milk. *S. aureus* was found in 38 (70.4%) out of 54 raw milk samples at concentrations of up to 8.9×10^5 CFU/ml. This microorganism was present in eight samples of pasteurized milk before the expiration date and in 11 samples analyzed on the expiration date. Of the 57 strains studied, 68.4% were positive for one or more genes encoding the enterotoxins, and 12 different genotypes were identified. The gene coding for enterotoxin A, *sea*, was the most frequent (16 strains, 41%), followed by *sec* (8 strains, 20.5%), *sed* (5 strains, 12.8%), *seb* (3 strains, 7.7%) and *see* (2 strains, 5.1%). Among the genes encoding the other enterotoxins, *seg* was the most frequently observed (11 strains, 28.2%), followed by *sei* (10 strains) and *seh* and *sej* (3 strains each). With the recent identification of new SEs, the perceived frequency of enterotoxigenic strains has increased, suggesting that the pathogenic potential of staphylococci may be higher than previously thought; however, further studies are required to assess the expression of these new SEs by *S. aureus*, and their impact in foodborne disease. The quality of Brazilian milk is still low, and efforts from the government and the entire productive chain are required to attain consumer safety.

Keywords: *S. aureus*; Staphylococcal enterotoxins; Raw milk

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1. Introduction

Foods of animal origin, especially milk and dairy products, are associated with foodborne disease (Asao et al., 2003; Jorgensen et al., 2005). The fact that *Staphylococcus aureus* is the main etiological agent of bovine mastitis in Brazil makes milk a great vehicle for its dispersion (Stamford et al., 2006). This information is crucial because 35–42% of the milk marketed in Brazil is raw (IBGE, 2004).

Milk is a good substrate for *S. aureus* growth and enterotoxin production. In addition, enterotoxins retain their biological activity even after pasteurization (Asao et al., 2003). According to Anderson et al. (1996), staphylococcal enterotoxins (SEs) are very resistant to heat; staphylococcal enterotoxin A (SEA), for example, retains some biological activity after 28 min at 121 °C. In Brazil, the majority of small dairy farms still employ poor technology and show deficiencies in sanitation and animal sanitary control, which results in a low-quality product. Regardless of the efforts of Brazilian Ministry of Agriculture, it is still common to find non-refrigerated milk containers by the roads waiting for collection by the cooperative's truck. In spite of this problem, most of the milk produced in Brazil is processed (IBGE, 2004). However, in small or even in larger cities, commercialization of raw milk, now called ''informal marketing'', is still usual, even though it is prohibited by law since 1952. According to data from IBGE (2004), approximately 35–42% of the milk produced in Brazil seems to be marketed raw. This practice increases the risk of staphylococcal food poisoning due to inadequate storage conditions before pasteurization. Classic antigenic SEs have been identified as SEA, SEB, SEC1, SEC2, SEC3, SED and SEE (Bergdoll and Robbins, 1973). Ren et al. (1994) have sequenced the gene that codes for toxin H. In 1998, Munson et al. identified and characterized *seg* and *sei*, while Zhang et al. (1998) found the gene *sej* in the same plasmid that encoded *sed*. Recently, several other toxins, termed enterotoxins SEK, SEL, SEM, SEN, SEO, SEP, SEQ, SER and SEU, have been described, and their genes have been sequenced (Jarraud et al., 2001; Kuroda et al., 2001; Orwin et al., 2001; Orwin et al., 2003; Letertre et al., 2003; Omoe et al., 2003).

Considering the facts above, the present study aimed to analyze the frequency of genes encoding the staphylococcal enterotoxins SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI and SEJ in *S. aureus* strains isolated from raw and pasteurized bovine milk.

2. Materials and methods

2.1. Milk samples

The five farms are located in Assis city area, in west of São Paulo State, Brazil. A total of 54 bulk tank milk samples from these farms were collected before pasteurization. Two more samples of the same lot were also collected; one was analyzed immediately after the thermal process, and the other was tested on the expiration date. A total of 162 samples were analyzed.

2.2. S. aureus isolation and identification

For *S. aureus* enumeration (Lancette and Bennett, 2001), serial dilutions of milk homogenates were plated on Baird Parker agar (Oxoid) with 5% egg yolk tellurite emulsion (Oxoid) and incubated at 35 °C for 48 h. Characteristic colonies were tested for catalase and coagulase production using the Staphytect Plus Dry Spot Kit (Oxoid). The two species positive for clumping were submitted to the Voges-Proskauer (VP) test to discriminate *S. aureus* (positive) from *S. intermedius* (negative).

2.3. PCR testing for genes encoding staphylococcal enterotoxins

For DNA isolation, a commercial kit (GFX; GE Healtcare) was used according to the supplier's instructions. The primers used for the detection of SE genes are listed in Table 1.

Each polymerase chain reaction (PCR) contained 2.5 μl PCR Buffer 10× (Invitrogen), 1.0 μM $MgCl_2$ (Invitrogen), 200 μM dNTP (Invitrogen), 1 U Taq DNA Polymerase (Invitrogen), 10 pmol of each primer, and 3 μl DNA. The final volume was adjusted to 25 μl by adding sterile ultrapure water.

DNA amplification was performed in a PTC-100 thermal cycler (MJ Research, Inc.) using the following conditions: initial denaturation for 5 min at 94 °C

Table 1
Primers and temperature used for the detection of *Staphylococcus aureus* SE genes

Gene	Primer	Sequence	Base pair	Annealing temperature (°C)	Reference
Sea	SEA-1 SEA-2	ttggaaacggttaaaacgaa gaaccttcccatcaaaaaca	120	50	Johnson et al. (1991)
Seb	SEB-1 SEB-2	tcgcatcaaactgacaaacg gcaggtactctataagtgcc	478	50	Johnson et al. (1991)
Sec	SEC-1 SEC-2	gacataaaagctaggaattt aaatcggattaacattatcc	257	50	Johnson et al. (1991)
Sed	SED-1 SED-2	ctagtttggtaatatctcct taatgctatatcttataggg	317	50	Johnson et al. (1991)
See	SEE-1 SEE-2	aggtttttttcacaggtcatcc ctttttttttcttcggtcaatc	209	50	Mehrotra et al. (2000)
Seg	SEG-1 SEG-2	aagtagacatttttggcgttcc agaaccatcaaactcgtatagc	287	55	Omoe et al. (2002)
Seh	SHE-1 SHE-2	gtctatatggaggtacaacact gacctttacttatttcgctgtc	213	46.4	Omoe et al. (2002)
Sei	SIE-1 SIE-2	ggtgatattggtgtaggtaac atccatattctttgcctttaccag	454	50	Omoe et al. (2002)
Sej	SEJ-1 SEJ-2	catcagaactgttgttccgctag ctgaattttaccatcaaaggtac	142	50	Nashev et al. (2004)

followed by 30 cycles of denaturation (94 °C for 2 min), annealing, and extension (72 °C for 1 min). Different annealing temperatures were tested, as shown in Table 1. A final extension step (72 °C for 5 min) was performed after the completion of the cycles.

As positive controls, PCRs containing template DNA extracted from the standard strains *S. aureus* ATCC 13565 (SEA), ATCC 14458 (SEB), ATCC 19095 (SEC), FRI 361 (SED, SEG, SEI and SEJ), ATCC 27664 (SEE) and FRI 137 (SEH) were carried out in parallel. Some PCRs received ultrapure water instead of template DNA to provide negative controls.

Aliquots of the PCR products, along with a 50 bp DNA ladder (Amersham-Pharmacia Biotech), were loaded into 1.5% agarose gel (Sigma–Aldrich) containing ethidium bromide (0.5 mg/ml-Invitrogen) and submitted to electrophoresis in TBE buffer (0.09 M Tris–HCl, 0.09 M boric acid, 2 mM EDTA, pH 8.3) for 30 min at 125 V (Electrophoresis Power Supply Model EPD 600; Amersham-Pharmacia Biotech, Inc.). The amplified DNA fragments were visualized with an image analyzer (AlphaImager, Alpha Innotech Corporation), using Alpha Ease FC Software.

One sample of each of the *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei*, and *sej* amplicons were sequenced, and the partial sequences were confirmed to correspond to GenBank accessions M18970, M11118, X05815, M28521, M21319, AY920261, U11702, AY920268, and AB075606, respectively.

3. Results

Staphylococcus aureus was observed in 38 (70.4%) out of 54 raw milk samples at concentrations of up to 8.9×10^5 CFU/ml. This microorganism occurred in 8 (14.7%) and 11 (20.4%) pasteurized milk samples analyzed prior to and on the expiration date, respectively, reaching concentrations of up to 8.7×10^3 CFU/ml. The presence of this microorganism after pasteurization can be attributed to inefficacy of the thermal process, since 18 (33.3%) out of 54 raw milk samples exhibited thermotolerant coliforms (data not shown).

Table 2 shows the results of molecular tests for the detection of genes encoding the toxins SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, and SEJ. Of the 57 strains of *S. aureus* tested, 39 (68.4%) were positive

Table 2
Genotypic profile of *S. aureus* strains, isolated from raw and pasteurized milk, according to *sea*, *seb*, *sec*, *sed*, *see*, *seg*, *seh*, *sei* and *sej* genes

Genotypic profile	Raw ($n = 38$)	Pasteurized ($n = 8$)	Validity date ($n = 11$)
a	8	2	2
b	2	–	–
c	3	1	2
d	1	–	1
e	2	–	–
g	1	–	–
g + i	4	2	1
d + j	2	–	–
a + c + h	2	–	–
a + g + i + j	1	–	–
a + d + g + i	–	–	1
b + g + h + i	1	–	–

for one or more SE genes, and 12 different genotypes were observed. Of these positive strains, 38 had been isolated from raw milk, and 27 (71.1%) carried genes encoding at least one toxin. The percentages of strains positive for SE genes were 62.5% (5 of 8 isolates) and 63.6% (7 of 11 isolates) in pasteurized milk tested before and on the expiration date, respectively.

Independent of the origin of the milk samples, 25 strains (64.1%) exhibited only one enterotoxin gene; 9 (23.1%) carried genes coding for two enterotoxins, and 2 (5.1%) were positive for three genes (*sea + sec + seh)*. Genotypes encoding four enterotoxins (*sea + seg + sei + sej*, *sea + sed + seg + sei* and *seb + seg + seh + sei*) were detected in 3 strains (7.7%). Genes encoding the enterotoxins SEH, SEI and SEJ were not observed separately.

Among the genes that code for classic enterotoxins (SEA-SEE), *sea* was the most frequent; it was found in 16 isolates (41%), followed by *sec* in 8 (20.5%), *sed* in 5 (12.8%), *seb* in 3 (7.7%) and *see* in 2 (5.1%) strains. Regarding the other enterotoxins, *seg* was the most frequently observed (11 strains, 28.2%), followed by *sei* in 10 (25.6%) and *seh* and *sej* in three strains each (7.7%).

4. Discussion

Although Brazilian law does not establish limits for the amount of *S. aureus* in milk, it is known that the amount of enterotoxins produced by enterotoxigenic strains achieve levels that are sufficient enough to bring about symptoms of foodborne disease when *S. aureus* concentration exceeds 10^5 CFU/ml (Tranter, 1996); that was the case for 24 (44.4%) out of the 38 raw milk samples.

With regard to the genes encoding enterotoxins, 39 (68.4%) out of 57 strains of *S. aureus* were positive for at least one enterotoxin gene. The most frequently observed gene was *sea*, observed in 16 (41%) isolates, followed by *sec* (8 strains, 20.5%), *sed* (5 strains, 12.8%), *seb* (3 strains, 7.7%), and *see* (2 strains, 5.1%).

In spite of the great discrepancy in data concerning the prevalence of enterotoxigenic *S. aureus* isolates found in the literature, which is attributable to different types of foods and biovars involved (Mathieu et al., 1991), SEA is the most frequently observed enterotoxin in enterotoxigenic strains of *S. aureus* (Normanno et al., 2005).

Asao et al. (2003) reported an outbreak of foodborne disease in Kansai, Japan, where 13,420 people were affected after ingesting skim milk and yogurt (prepared with powdered milk) contaminated with 0.38 ng/ml and 3.7 ng/g of SEA, respectively. According to Tranter (1996), the minimal amount of enterotoxin that is required to cause the disease is not known, but the ingestion of at least 1 g of toxin per 100 g of food is enough to induce the symptoms.

Scherrer et al. (2004), analyzed 172 samples of goat and sheep milk in Switzerland, and found that 65.2% of 296 strains were positive for the presence of genes that encode enterotoxins, a frequency very close to that observed in the present work (68.4%). In Japan, Katsuda et al. (2005) observed that 183 (67.8%) out of 270 *S. aureus* isolates were positive for the presence of

genes coding for one or more enterotoxins. In Italy, Morandi et al. (2007) also found a very similar frequency value: 67% of the *S. aureus* strains isolated from milk and dairy products were positive for the presence of toxin genes.

With the discovery of the new enterotoxins, the perceived percentage of enterotoxigenic, or potentially enterotoxigenic, *S. aureus* strains increased. In this study, 39 (68.4%) strains were positive for the presence of at least one SE gene; however, that number would drop to 31 (52.5%) if only the classic enterotoxins (*sea* to *see*) were considered. Rosec and Gigaud (2002) also observed the increase in the perceived number of enterotoxigenic strains as a consequence the discovery of the new SEs. In their study, 30% of the isolates had genes encoding the classic toxins; that frequency was found to be 57% when the new SEs were taken into account.

The *seg* gene was observed in 11 (28.2%) out of 39 samples of *S. aureus*; in 90.9% of the cases, it was associated with *sei*, which was present in 10 isolates (25.6%). Similar values were reported by Rosec and Gigaud (2002), who observed that *seg* and *sei* were associated in 80.6% of 155 strains. These genes are frequently found together because they are within the same cluster, in a 3.2 kb DNA fragment (Jarraud et al., 2001). The small percentage of strains carrying only one of these two enterotoxin genes could be explained by point mutations in *seg* or by variations in the cluster where these two genes are located (Jarraud et al., 2001). According to Zhang et al. (1998), *sed* e *sej* are carried by the same plasmid. As shown in Table 2, these two genes were found separately and, even though this is not common, Blaiotta et al. (2004) and Cremonesi et al. (2005) have also found single-positive results for *seg* and *sei*.

Zschöck et al. (2005) have observed the simultaneous occurrence of these two genes in *S. aureus* strains isolated from milk of cows with mastitis. Of the 61 strains analyzed in that study, 36 were shown to carry *seg* and 22 (61%) were found to be positive for *sei*. Lämmler et al. (2000) and Omoe et al. (2002) have also reported that *seg* and *sei* are frequently found together in cows with mastitis and in raw milk IBGE (in press).

In the present work, three isolates (7.7%) were simultaneously positive for genes encoding four enterotoxins. Their individual genotypes were *sea* + *seg* + *sei* + *sej*, *sea* + *sed* + *seg* + *sei* and *seb* + *seg* + *seh* + *sei*. Nashev et al. (2004) have identified genetic profiles comprising multiple genes in *S. aureus*. In that study, 9.1% and 4.5% of the tested strains were positive for four (*seb* + *seg* + *seh* + *sei*) and five (*sea* + *sed* + *seg* + *sei* + *sej*) enterotoxin genes, respectively.

In conclusion, we detected genes encoding the classic (SEA to SEE) and most recently described enterotoxins (SEG, SEH, SEI and SEJ) in *S. aureus* strains isolated from milk at different production stages. The recent identification of new SEs has considerably increased the perceived frequency of enterotoxigenic staphylococci isolates, indicating that the pathogenic potential of *S. aureus* may be greater than previously thought. Further studies are needed to confirm the expression of these new enterotoxins by *S. aureus*, and to assess their significance for foodborne disease.

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