Isolation of *Staphylococcus* from minas frescal type cheese and detection of enterotoxin genes

Isolamento de *Staphylococcus* de queijo minas frescal e detecção de genes de enterotoxinas

RIALA6/1462

Juliana de Castro Beltrão da COSTA*, Elaine Ibrahim de FREITAS, Anderson Almeida de LEMOS, Carla de Oliveira ROSAS, Valéria de Mello MEDEIROS, Márcia Barbosa WARNKEN, Neide Hiromi Tokumaru MIYAZAKI[in memoriam], Victor Augustus MARIN

*Endereço para correspondência: Departamento de Microbiologia, Instituto Nacional de Controle de Qualidade em Saúde (INCQS), Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Brasil. Tel/Fax: (21) 3865-6151 / 2290-0915. E-mail: juliana.costa@incqs.fiocruz.br

Recebido: 27.03.2011- Aceito para publicação: 08.06.2012

RESUMO

Neste trabalho foi realizado o isolamento de *Staphylococcus* spp. de queijo minas frescal**,** e para esta finalidade foi padronizado um protocolo multiplex PCR (M-PCR) para efetuar a detecção de genes de enterotoxinas clássicas (*sea*, *seb*, *sec*, *sed* e *see*) de *Staphylococcus aureus*, usando-se o gene *femA* como controle positivo para cepas de *S. aureus*. Foi testada a detecção direta de bactérias de amostras de queijo minas frescal e do alimento artificialmente contaminado. Cento e onze colônias (104 coagulase-positivas e sete coagulase-negativas) foram selecionadas e analisadas por M-PCR e, dentre essas, 34 colônias (30.62%) foram positivas para pelo menos um dos cinco genes de enterotoxinas analisados. Os genes que codificam as enterotoxinas *sea* e *seb* foram os mais frequentemente detectados. O estudo de isolamento de estafilococos coagulase-positivos revelaram que 40% das amostras demonstraram contagens bacterianas acima do limite considerado como aceitável pela legislação brasileira.

Palavras chave. PCR, *Staphylococcus*, queijo.

ABSTRACT

This work aimed at isolating *Staphylococcus spp.* from minas frescal type cheese, and for this purpose a Multiplex PCR (M-PCR) was standardized for detecting the classical *Staphylococcus aureus* enterotoxin genes (sea, seb, sec, sed and see), using the *femA* gene as a positive control for *S. aureus* strains. Bacterial detection directly from the minas frescal type cheese and from artificially contaminated food was tested. One hundred eleven colonies (104 coagulase-positive and seven coagulase-negative) were selected for performing M-PCR assay. Thirty-four colonies (30.62%) were positive for at least one of the five enterotoxin genes analyzed; enterotoxins *sea* and *seb* were the most frequently detected. The study on Staphylococci coagulase-positive samples revealed that 40% of the samples showed bacterial counts above the limit established by Brazilian legislation.

Keywords. PCR, *Staphylococcus*, cheese.

INTRODUCTION

Foodborne illnesses are defined as diseases, usually either infectious or toxic in nature, caused by agents that enter the body through the ingestion of contaminated food. Foodborne diseases are a widespread and growing public health problem, both in developed and developing countries. Indeed, food contamination creates an enormous social and economic burden on communities and their health systems[1].

The minas frescal type cheese is a fresh and soft white cheese produced by the enzymatic coagulation of pasteurized milk, with high pH, a minimum of 55% moisture content and a low percentage of salt (1.4-1.6%). The cheese is produced industrially by three different processes: the traditional one by the addition of a lactic acid bacteria, that by the addition of lactic acid-direct acidification, and that by the use of ultrafiltration, using the method developed by Maubois, Mocquot and Vassal[2] by direct coagulation of the liquid pre-cheese with the addition of rennet in its final commercial packaging[3].

There are many enteropathogenic species that have been found in milk and cheese stored at low temperatures. Post-pasteurization contamination, during production, manipulation, from equipment, and inadequate cooling during transportation and storage can result in high levels of pathogenic microorganisms and enterotoxins in cheese[4,5]. The contamination, linked to changes arising in only a few days, makes the cheese unacceptable or even unfit for consumption.

The *Staphylococcus aureus* count is used as an indicator of post-processing contamination of dairy products, aiding in the hygienic control of these products. Studies have shown that minas frescal cheese and other dairy products have been involved in several staphylococcal food intoxication outbreaks in Brazil and other countries[5,6]. The Collegiate Directory Resolution nº 12 (published on January 2nd, 2001 by Brazil's public health agency, The National Health Surveillance Agency of Brazil (ANVISA)) establishes the Technical Regulation about Food Microbiological Standards and states that high humidity cheese, at ± 55%, including minas frescal type cheese and others, must be investigated for the following microorganisms groups: coliforms at 45 °C (the maximum permissible 5 x 10^2 CFU/g), coagulase-positive staphylococci (the maximum permissible 5 x 10^2 CFU/g), *Salmonella* spp. (absent in 25 g) and *Listeria monocytogenes* (absent in 25 g)[7].

The intoxication caused by *S. aureus* is manifested soon after the ingestion of food contaminated with pre-formed enterotoxin. The amount of enterotoxin required to cause disease is not well established, but it is known that susceptibility depends on the individual, body weight and especially the health status of the affected person. To form enterotoxins in sufficient quantity to cause intoxication, 10^5 to 10^6 *S. aureus* cells are required per gram of food[8]. The growth of *S. aureus* and production of enterotoxins are affected by physical and chemical parameters such as temperature, pH, water activity, salt concentration and the availability of oxygen[9].

Some species of coagulase-negative staphylococci (CoNS) can present a medical risk[10]. Staphylococci species used in cheese-making are novobiocin-resistant, coagulase-negative and are not usually identified at species level by routine laboratories. The high prevalence of CoNS and their pathogenic potential (existence of enterotoxigenic and multidrug-resistant strains) have not been clearly identified, but this is increasingly necessary for assessing the safety status of the CoNS utilized in fermented dairy foods[11]. In a study by Veras et al.[2] identifying enterotoxin genes from isolates of CoNS and coagulase-positive staphylococci obtained from dairy products, CoNS were responsible for 16 outbreaks of food poisoning in Minas Gerais, Brazil and showed the ability to produce active toxin.

The staphylococcal enterotoxins are a group of proteins, with low molecular weight (26 to 30 KDalton) that are produced during all stages of bacterial growth in culture, but mainly during the middle and end of the exponential growth phase[13]. They were named with alphabetical characters according to the chronological order in which they were discovered[9].

S. aureus produces a wide variety of toxins including staphylococcal enterotoxins (SEs; SEA to SEE, SEG to SEI, SER to SET) with demonstrated emetic activity, and staphylococcal-like (SE/) proteins, which are not emetic in a primate model (SE/L and SE/Q) or have yet to be tested (SE/J, SE/K, SE/M to SE/P, SE/U, SE/U2 and SE/V)[14]. SEs and SE/s have been traditionally subdivided into classical (SEA to SEE) and new (SEG to SE/U2) types. All have superantigenic activity and are encoded by accessory genetic elements, including plasmids, prophages, pathogenicity islands, genes located next to the staphylococcal cassette chromosome (SCC) implicated in methicillin resistance, or by vSa genomic islands (vSa refers to non-phage and non-

SCC genomic islands)[14]. SEA is the most common cause of staphylococcal food poisoning worldwide, but the involvement of other classical SEs has also been demonstrated. Of the new SE/SE/s, only SEH has clearly been associated with food poisoning. However, genes encoding novel SEs as well as SE/s with untested emetic activity are widely represented in *S. aureus*, and their role in pathogenesis may be underestimated[14].

Bacterial detection methods based on PCR can be utilized to characterize strains that are isolated and purified by traditional cultivation methods. They aid in the conventional final stage methods such as the biochemical and serological confirmations. PCR can also be utilized to detect food bacteria by applying it directly to food products, pre-enrichment broth or in the selective process containing mixed cultivation. It can be applied after a short step of sample preparation involving bacterial concentration and rupture[15].

The goal of this work was to quantify the presence of coagulase-positive *Staphylococcus* in minas frescal cheese acquired from supermarkets in Rio de Janeiro and to use a Multiplex-PCR (M-PCR) protocol for the detection of genes that code for the staphylococcal enterotoxins *sea, seb, sec, sed* and *see* in coagulase-positive and coagulase-negative staphylococci (using the *femA* gene as a positive-control for *S. aureus*).

MATERIALS AND METHODS

Minas frescal type cheese sample gathering

Thirty samples of minas frescal type cheese, weighting at least 200 g each and obtained from 10 units of 3 different commercial brands, were purchased at supermarkets from the State of Rio de Janeiro, Brazil. All cheese was made with pasteurized milk, inspected by the Brazilian sanitization authority and kept under refrigeration in the supermarkets. Of those, 10 samples were commercialized in plastic containers, 10 in vacuum-sealed plastic containers (both types of samples were produced by lactic acid-direct acidification), and 10 in low density vacuum sealed polyethylene containers (cheese made by the ultrafiltration process). The temperature of the supermarket distribution freezers was analyzed, and the average temperature measured at the time of sample collection was 8 °C. The samples were transported on ice to the laboratory of the National Institute for Quality Control in Health (INCQS, Brazil) and were analyzed before their expiration date.

Count of coagulase-positive *Staphylococcus*

Staphylococci isolation and counts of the coagulase-positive *Staphylococcus* were conducted according to the American Public Health Association[16] and Food and Drug Administration[17]. Twenty-five grams of each cheese were homogenized in 225 mL of Butterfield's phosphate buffer in a Stomacher (Seward Medical, Blend 400). The sample dilutions were plated on Baird Parker agar and incubated at 35 °C for 45-48 h.

For the counts performed in this study, only coagulase-positive *Staphylococcus* isolates were used, according to the Collegiate Directory Resolution nº 12[7], and Baird-Parker agar (Oxoid) plates with the same dilution were selected, which presented 20 to 200 dark gray to black circular, smooth, humid colonies, surrounded or not by a transparent, double or opaque halo. During counting, two or more types of colonies were classified, listed and sent for identification separately, with verification of morphological characteristics using Gram staining and tests of catalase activity, coagulase production, thermonuclease production, lysostaphin sensitivity and anaerobic utilization of glucose and mannitol.

Table 1 shows counts for 24 samples, since 6 of them were not tested for the evidence of coagulase due to timing issues. Therefore these samples (samples 12, 13, 15, 23, 24 and 26) were excluded from the count of *Staphylococcus* coagulase-positive.

Table 1. Count of *Staphylococcus* coagulase-positive

Staphylococci coagulase-positive/g isolate from minas frescal cheese (CFU/g)	Number of samples with this count	%
$<10^2$	10	33,34
$> 10^0 / 10^2$	2	6,67
$> 5 \times 10^2 / 10^3$	4	13,33
$> 10^3 / 10^4$	5	16,66
$> 10^5$	3	10
Not tested	6	20
Total	30	100

Total number of 142 staphylococci coagulase-positive colonies isolated from 20 samples of minas frescal cheese

M-PCR to detect enterotoxin genes from isolates of minas frescal cheese

One hundred eleven colonies (104 coagulase-positive and seven CoNS) were selected for M-PCR in order to detect the genes that code for the A, B, C, D and

E enterotoxins and the *femA* gene. Those colonies were isolated from the minas frescal type cheese samples and randomly selected in a way that the different batches were analyzed: sample 2 (14 colonies analyzed), sample 3 (15 colonies analyzed), sample 5 (15 colonies analyzed), sample 11 (13 colonies analyzed), sample 21 (7 colonies analyzed), sample 22 (6 colonies analyzed), sample 25 (9 colonies analyzed), sample 27 (15 colonies analyzed), sample 28 (8 colonies analyzed) and sample 30 (9 colonies analyzed).

Reference strains

S. aureus ATCC 13565 (*sea*), ATCC 14458 (*seb*), ATCC 19095 (*sec*), ATCC 23235 (*sed*) and ATCC 27664 (*see*) strains were analyzed by M-PCR. All strains possess the *femA* gene, wich encodes a precursor protein that plays a role in peptidoglycan biosynthesis in *S. aureus* and is also considered as a factor influencing the level of methicillin resistance[12,18]. As an internal positive control for each reaction, we incorporated primers specifically designed to amplify *femA*, which have been reported by Mehrotra et al.[19] to be specific for *S. aureus*.

Artificial food contamination

The protocol of artificial food contamination was designed in this study, based in the protocol designed by Wang et al.[20] e Mehrotra et al[19].

The *sea, seb, sec* and *sed* enterotoxin-producing reference strains were inoculated in 5 mL Nutrient Broth, and the *see* enterotoxin-producing strain was inoculated in 5 mL Brain Hearth Infusion broth (BHI). The cultures were incubated for 16-24 h at 35 ± 2 °C with agitation. From these cultures, serial dilutions to 10^{-9} were made, using BHI as diluent. Next, two steps were performed:

1) For each of the 10^{-6} to 10^{-9} dilutions, aliquots of 0.3, 0.3 and 0.4 mL (total of 1 mL) were spread on the surface of 12 nutrient agar plates. The inoculums were spread with the help of a Drigalsky spatel. The plates were then incubated at 35 ± 2 °C for 48 h, in an inverted position. Plates of the same dilution were selected for colony counting, which presented from 30 to 300 circular, smooth, humid, light colored colonies. The count of viable cells of pure cultures inoculated on nutrient agar plates were on the order of 10^2 CFU/mL from the 10^{-6} dilution, 10^1 to 10^2 CFU/mL from the 10^{-7} dilution, and 1 to 10^1 CFU/mL from the 10^{-8} and 10^{-9} dilutions.

2) Ten Erlenmeyer flasks containing 220 mL of tryptic soy broth with 0.6% yeast extract (TSBYE)[20] and 25 grams of frescal minas cheese were used in this step for each reference strain. Two flasks were inoculated with 1 mL of the 10^{-6} dilution, two flasks with 10^{-7} dilution, two flasks with 10^{-8} dilution, and two flasks with 10^{-9} dilution of each reference strain, respectively. Two flasks were not inoculated.

The flasks were incubated for 16-24 h at 35 ± 2 °C with agitation (Wrist Action® Skaker. Model 75 – Burrell – Burrell Corporation Pittsburgh, P.A., USA). After this period, 100 µL aliquots were removed from each flask and added to tubes containing 10 mL of TSBYE broth. The tubes were incubated for 16-24 h at 35 ± 2 °C with agitation.

After the incubation, 100 µL aliquots were removed from each tube and added to tubes containing 10 mL of TSBYE broth. The tubes were incubated for 16-24 h at 35 ± 2 °C with agitation. At the end of the incubation, DNA was extracted from the cultures and M-PCR was performed.

Bacterial detection directly from the minas frescal type cheese

This procedure was originally designed in this study. It differs from the previously procedure explained, since this time, this procedure simulates the detection of the microorganisms directly from the minas frescal type cheese, without passing through any step of microbial growth.

Six Erlenmeyer flasks were prepared: *1 and 2*: containing 220 mL of TSBYE broth, 25 g of minas frescal type cheese and 1 mL of each of the 24 h bacterial cultures of the *sea, seb, sec* and *sed* enterotoxin-producing reference strains inoculated previously in 5 mL Nutrient Broth, and the *see* enterotoxin-producing strain previously inoculated in 5 mL Brain Hearth Infusion broth (BHI) (total 5 mL). *3 and 4* containing: 220 mL TSBYE broth and 25 g of minas frescal type cheese; and *5 and 6* containing 220 mL of TSBYE broth and 1 mL of the bacterial cultures of each of the reference strains (total 5 mL). Next, one procedure was performed:

1) Aliquots of 0.5 mL and of 1 mL were removed from flasks after 24 and 48 h of incubation, for subsequent DNA extraction and M-PCR.

RESULTS

Count of coagulase-positive *Staphylococcus*

Thirty samples of minas frescal type cheese were analyzed of three different brands. Ten cheese samples

Table 2. Primer sequences, gene location and amplicon size

Gene	Sequence (5'/ 3')	Gene location	Amplicon size (bp)*
sea	GGTTATCAATGTGCGGGTGG	349/368	102
	CGGCACTTTTTTCTCTTCGG	431/450	
seb	GTATGGTGGTGTAACTGAGC	666/685	164
	CCAAATAGTGACGAGTTAGG	810/829	
sec	AGATGAAGTAGTTGATGTGTATGG	432/514	451
	CACACTTTTAGAATCAACCG	863/882	
sed	CCAATAATAGGAGAAAATAAAAG	492/514	278
	ATTGGTATTTTTTTTCGTTC	750/769	
see	AGGTTTTTTCACAGGTCATCC	237/257	209
	CTTTTTTTTCTTCGGTCAATC	425/445	
femA	AAAAAAGCACATAACAAGCG	1444/1463	132
	GATAAAGAAGAAACCAGCAG	1556/1575	

* bp = base pairs / Nucleotide sequences and location genes for sea were obtained from Betley & Mekalanos (1988), for seb from Jones & Khan (1986), for sec from Bohach & Schlievert (1987), for sed from Bayles & Iandolo (1989), for see from Couch, Soltis & Betley (1988) and for femA from Berger-Bachi et al.(1989).

(33.34%, numbered 6, 7, 8, 9, 10, 16, 17, 18, 19 and 20) showed counts of coagulase-positive *Staphylococcus* lower than 10^2 CFU/g, and were from vacuum sealed plastic containers. Six samples (20% of the total of samples) were not tested for counts of coagulase-positive *Staphylococcus* as explained above in the materials and methods.

The samples of minas frescal type cheese number 11 and 15 (6.67%) had coagulase-positive *Staphylococcus* counts of 5 x 10^2 CFU/g in accordance to the limits established by RDC 12[7]. However, 40% of the samples were in violation of the limit established by legislation, which, according to Jablonski et al.[8], is enough for enterotoxin production. The results of count of *Staphylococcus* coagulase-positive in the minas frescal type cheese samples are showed in Table 2.

M-PCR to detect enterotoxin genes from isolates of minas frescal type Cheese

Of the 111 colonies that were isolated and selected for M-PCR for genes of the *sea, seb, sec, sed, see* enterotoxins and *femA*, 104 were coagulase-positive and seven were CoNS.

Once hundred and eleven colonies isolated from cheese samples were analyzed by M-PCR, and 34 (30.62%) were positive for at least one of the five enterotoxin genes (Table 3).

From seven CoNS analyzed by M-PCR, four were negative for all genes analyzed, one was positive for *femA* and *sed*, and two were positive only for *sed*.

None of the strains contained the *sec* and *see* genes.

Table 3. *Staphylococcus* spp. enterotoxin genes from 111 isolated colonies and selected for Multiplex-PCR

Gene	Number of colonies	%
femA	39	35,14
femA + sea+ seb	15	13,51
Sea	5	4,50
sea + seb	9	8,11
Sed	2	1,80
femA + sed	3	2,70
Neither	38	34,24
Total	111	100

Artificial Food Contamination

In the second stage of the artificial contamination, in which approximately 10 to 100 CFU/mL of each of the strains were inoculated to samples of minas frescal type cheese, and there were two incubation periods with TSBYE, the M-PCR was negative. Of the six target genes, only the enterotoxin D gene was amplified; the viable cell count was approximately 2.0 to 2.7 x 10^2 CFU/mL. Therefore, the sensitivity of M-PCR for this gene was from 10 to 100 cells.

DNA was extracted from cultures using a Qiagen kit and the protocol of Wang et al.[19] The best method of extraction after artificial contamination was with the Qiagen kit, because it facilitated standardization.

Bacteria detection directly from the minas frescal type cheese

The amplification products of M-PCR for direct detection of bacteria on minas frescal type cheese were ineffective in the amplification of all target genes (*sea*,

seb, *sec*, *sed*, *see* and *femA*). These data showed that for minas frescal type cheese it is necessary to first isolate coagulase-positive *Staphylococcus* colonies and then proceed to DNA extraction and M-PCR.

DISCUSSION

From the 30 minas frescal type cheese samples analyzed, 12 (40%) showed *Staphylococcus* coagulase-positive counts above the maximum limits (5 x 10^2 CFU/g) allowed by ANVISA[7]. These counts are similar to those found by Carmo et al.[22], Almeida Filho et al.[4], Loguercio and Aleixo[23] and Araújo et al.[5] Furthermore, 10% of the samples showed values that are near the zone of enterotoxin production that initiates staphylococcal food poisoning. Carvalho et al.[3] evaluated the influence of three different cheese-making processes on the physicochemical characteristics and the microbiological quality of Brazilian minas frescal type cheese. For coagulase-positive staphylococcal strains, the cheeses produced by the traditional addition of a lactic culture represented the worst case, with 12.9% of the samples showing more than 10^3 CFU/g (the maximum acceptable limit established by the Brazilian regulations for the process of addition of a lactic acid culture[7]) followed by the cheeses made by direct acidification (9.7% above the acceptable limit of 5×10^2 CFU/g).

In Carvalho et al.[3], only the cheeses produced by the use of ultrafiltration showed negative results, probably due to the closed system process with no handling after pasteurization. In our study, we found similar results; samples from vacuum-sealed plastic containers, which are also produced by the ultrafiltration process, were negative for staphylococcal contamination. The contamination of post-pasteurization, production, manipulation, and equipment, as well as inadequate temperature during the transportation and storage of cheese and the use of raw milk are all possible causes of the presence of staphylococcal contamination in the minas frescal type cheeses[3].

Two protocols were utilized for the DNA extraction, one using a Qiagen Kit and the other according to Wang et al.[20] Both methods were efficient, but the chosen method was the Qiagen Kit because it facilitated standardization.

The results of the artificial contamination and the bacterial detection directly from the minas frescal type cheese showed that it is necessary to isolate the coagulase-positive staphylococcal colonies before DNA extraction for this food matrix. In the Artificial Food Contamination experiments (Sensibility Test and Live Bacteria Detection), the M-PCRs were negative, and nonspecific amplicons were found in the bacteria detection directly from the minas frescal type cheese. In the artificial contamination and bacteria detection directly from the minas frescal type cheese, the five strains were inoculated together in the TSBYE broth. The M-PCR results were negative, and this could be due to growth interference among the strains.

This result is in disagreement with Ercolini et al.[24] who detected the enterotoxin SEG and SEH genes by PCR directly from artificially contaminated raw milk. They also tested samples (with PCR) of artificially contaminated raw milk that were incubated for 24 to 48 h for culture enrichment. The results showed that after 48 h enrichment, all target genes (*sec*, *seg*, *seh* and *sei*) were amplified. However, results similar to those of our study were found by McLauchlin et al.[25] for artificially contaminated samples of canned mushroom soup, cream, cottage cheese, mozzarella cheese, fat cheese and sliced ham. In that study, the detection of enterotoxin genes was weak or was not obtained in the four dairy products (different types of cheese and cream).

PCR based analytical methods for ascertaining the occurrence of pathogenic or toxigenic microorganisms in food are widely recognized as capable of decreasing detection time and increasing specificity and sensitivity[24]. Unfortunately, large sample volumes (≥25 mL or g) compared with small amplification volumes (10–50 µL), residual food components that inhibit enzymatic reactions (e.g., calcium ions and protein[26], low levels of contaminating pathogens, and the presence of competitive microbiota that may interfere with amplification and detection reactions, have been consistent stumbling blocks to the widespread use of nucleic acid amplification for pathogen detection in food[27]). PCR has often been used for direct detection of *Staphylococcus aureus* in milk and cheeses[28,29], but none of these approaches are ideal, and in many cases, a technique optimized for one food system or microorganism is not readily adaptable to others[30,31].

The original protocol for M-PCR by Mehrotra et al.[19] has several alterable parameters (i.e., annealing temperature, extension time, dNTP concentration, primers, $MgCl_2$ and polymerase concentration). In this

work, the annealing temperature was decreased by 5 ºC to 52 °C for the amplification of the six bands. The same situation was reported by Nájera-Sánchez et al.[32] who tested various anneling temperatures. In that work they observed that low annealing temperatures (48 °C to 50 °C) resulted in reduced sensitivity and specificity of the target gene because of competition between nonspecific components of the reaction.

Regarding the M-PCR for the detection of enterotoxins in the 111 isolates from minas frescal type cheese, 34 (30.62%) were positive for at least one of the five enterotoxin genes studied. These results are in accordance with Veras et al.[12], in which 15 coagulase-positive and 15 CoNS isolates from food poisoning outbreaks in Minas Gerais, Brazil were tested by PCR to detect enterotoxin genes. Veras et al.[12] found that among the 30 selected isolates, 21 harbored enterotoxin genes. Within this group, 38% amplified only *sea*, 29% amplified only *seb*, and 24% amplified both *sea* and *seb*. Genes for *sec* and *sed* (either alone or concomitantly) were detected with low frequency.

Borges et al.[9] evaluated staphylococcal contamination and their enterotoxins in a coalho type cheese production line using an ATP bioluminescence assay. They used a *VIDAS® Kit Staph enterotoxin II (BioMérieux SA, Marcy-l'Etoile, France)* kit and mini VIDAS® equipament to detect enterotoxins in four samples of food, pasteurized milk, raw milk, curd and cheese. The staphylococcal enterotoxins were detected in 20% of the raw milk samples and therefore in pasteurized milk, curd and cheese.

Rall et al.[33] 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 or pasteurized bovine milk. In the 57 strains studied, the sea gene, coding for enterotoxin A, was the most frequently found (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). Rall et al.[33] showed that 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. Rall et al.[33], 39 (68.4%) strains were positive for the presence of at least one SE gene, but that number would drop to 31 (52.5%) if only the classical enterotoxins (*sea* to *see*) were considered. Regardless, in our study, the M-PCR performance for five staphylococcal enterotoxins detections (A, B, C, D, E) provided rapid results and detected the potential risk of staphylococcal food poisoning.

In this work, the complementary test used for *Staphylococcus* colony identification was the M-PCR with *S.aureus* specific *femA* gene amplification. This gene encodes an essential factor for the resistance to methicillin and is universally present in all strains of *S. aureus*[19]. Interestingly, *femA* appears to be a unique feature of *S. aureus*; it is not found in other *Staphylococcus* species[18,34], but in this study, we found one coagulase-negative colony that was positive for the *femA* and *sed* genes. In Veras et al.[12], 15 CoNS were examined by PCR for the presence of the *femA* and *coa* genes. Isolates that amplified *coa* were considered to be coagulase-positive and there were three CoNS isolates in wich the PCR detection of *femA* did occur.

Vannuffel et al.[18] showed that homologous genes for *femA* are present in *S. aureus* and in certain isolates of CoNS strains as well. Indeed, a *femA* homologous gene was characterized in *S. epidermidis*, entailing the possibility of *femA* phylogenetic conservation in staphylococcal species. The genomic organization of all these *femA* genes appeared highly conserved, with alternation of homologous and variable regions. Homology of the nucleic acid sequence in that study ranged from 75.1% to 78.3% among the *S. aureus* and CoNS analyzed[12]. In our study, the coagulase type of the isolates was determinated only by biochemical, not molecular, methods (i.e. detection of the *coa* gene).

With respect to the detection of the *femA* gene, 57 (51%) of 111 colonies analyzed amplified this gene. The fact that 49% did not amplify *femA* may indicate that these 54 colonies are not *S. aureus*. Borges et al.[9] detected twelve *Staphylococcus* species within the selected 68 isolates, nine being negative and three positive for coagulase. In cheese, six species were identified among 16 isolates, with the prevalence of 37.5% for *S. epidermidis*, 25% for *S. xylosus*, 18.8% for *S. aureus*, 6.2% for *S. cohnii*, 6.2% for *S. haemolyticus* and 6.2% for *S. lentus*. There was a high frequency (81.3%) of CoNS and a low frequency (18.7%) of coagulase-positive species in the samples. *S. aureus* was found in 12% of the cheeses analyzed.

From seven CoNS analyzed by M-PCR, four were negative for all genes analyzed, one was positive for *femA*

and *sed* and two were positive only for *sed*. Indeed, several studies have shown that some CoNS species possess the genes for SE and can produce functional toxin[12]. In Veras et al.[12], five of the CoNS isolates were genotypically and phenotypically enterotoxigenic, and these isolates displayed the ability to produce active toxin capable of detection by immunological methods. The CoNS present in minas frescal type cheese have the potential to produce enterotoxins and because of this, it would be interesting to include these groups of microorganisms in the Collegiate Directory Resolution no. 12 for greater protection of consumers of this type of food.

ACKNOWLEDGEMENTS

The authors wish to thank the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support and the Instituto Nacional de Controle de Qualidade em Saúde (INCQS/Fiocruz) for operational support for this study.

REFERENCES

1. World Health Organization, 2007. Food Safety and Foodborne Ilness. 2007. [acesso 2010 mar 14]. Disponível em: [http:// www.who.int/mediacentre/factsheets/fs237/en/].
2. Maubois JL, Mocquot G. Application of membrane ultrafiltration to preparation of various types of cheeses. J Dairy Sci. 1975;58:1001-7.
3. Carvalho JDG, Viotto WH, Kuaye AY. The quality of Minas Frescal cheese produced by different technological processes. Food Control. 2007;18:262-7.
4. Almeida Filho ES, Nader Filho A. Ocorrência de *Staphylococcus aureus* em queijo tipo "frescal". Rev Saúde Pública. 2000;34:578-80.
5. Araújo VS, Pagliares V A, Queiroz M LP, Freitas-Almeida AC. Occurrence of *Staphylococcus* and enteropathogens in soft cheese commercialized in the city of Rio de Janeiro, Brazil. J Appl Microbiol. 2002;92:1172-7.
6. Ikeda T, Tamate N, Yamaguchi K, Nakino S-i. Mass outbreak of food poisoning disease caused by small amounts of staphylococcal enterotoxins A and H. Appl Environ Microbiol. 2005;71(5):2793-5.
7. Brasil. Ministério da Saúde. Resolução RDC n. 12, de 2 de janeiro de 2001. Aprova o Regulamento Técnico sobre Padrões Microbiológicos para Alimentos. Diário Oficial [da] Republica Federativa do Brasil, DF, 10 jan 2001. Seção 1, n. 7-E, p. 45-53.
8. Jablonski LM, Bohach GA. Staphylococcus aureus. *In*: L. Beuchat, M. Doyle & T. Montville (Eds). Fundamentals of Food Microbiology. Washington, DC: American Society for Microbiology; 2001. p. 410-34.
9. Borges MF, Arcuri EF, Pereira JL, Feitosa T, Kuaye AY. *Staphylococcus* enterotoxigênicos em leite e produtos lácteos, suas enterotoxinas e genes associados: revisão. Bol Centro Pesq Proc Alim. jan/jun 2008;26:71-86. Disponível em: [http://ojs.c3sl.ufpr.br/ojs2/index.php/alimentos/article/view/11794/8312].
10. Samelis J, Mataxopoulos J, Vlassi M, 1998. Stability and safety of traditional greek salami: a microbiology ecology study. Int J Food Microbiol. 1998;44:69-82.
11. Irlinger F. Safety assessment of dairy microorganisms: Coagulase-negative staphylococci. Int J Food Microbiol. 2008;126:302-10.
12. Veras JF, do Carmo LS, Tong LC, Shupp JW, Cummings C, dos Santos DA, et al. A study of the enterotoxigenicity of coagulase-negative and coagulase-positive staphylococcal isolates from food poisoning outbreaks in Minas Gerais, Brazil. Int J Infect Dis. 2008;12:410-5.
13. Soriano JM, Font G, Moltó C, Mañes J. Enterotoxigenic staphylococci and their toxins in restaurant foods. Trends Food Sci Technol. 2002;13:60-7.
14. Argudín MÁ, Mendoza MC, Rodicio MR. Food Poisoning and *Staphylococcus aureus* Enterotoxins. Toxins. 2010;2:1751-73.
15. Rijpens NP, Herman LMF. Molecular Methods for Identification and Detection of Bacterial Food Pathogens. J AOAC Int. 2002;85:984-95.
16. Lancette GA, Bennett RW. *Staphylococcus aureus*. *In*: Downes FP, Ito K, (Eds). Compendium of methods for the microbiological examination of foods. 4. ed. Washington, DC: APHA; 2001. p. 387-400.
17. Bennett RW, Lancette GA. *Staphylococcus aureus*. *In*: Food and Drug Administration. Bacteriological analytical manual (8ª ed., rev. A). Arlington: AOAC/FDA; 1998. p. 12.01-12.05.
18. 18- Vannuffel P, Heusterspreute M, Bouyer M, Vandercam B, Philippe M, Gala JL. Molecular characterization of *femA* from *Staphylococcus hominis* and *Staphylococcus saprophyticus*, and *femA*-based discrimination of staphylococcal species. Res Microbiol. 1999;150:129-41.
19. Mehrotra M, Wang G, Johnson WM. Multiplex PCR for Detection of Genes for *Staphylococcus aureus* Enterotoxins, Exfoliative Toxins, Toxic Shock Syndrome Toxin 1, and Methicillin Resistance. J Clin Microbiol. 2000;38:1032-35.
20. Wang RF, Cao WW, Cerniglia CE. A universal protocol for PCR detection of 13 species of foodborne pathogens in foods. J Appl Microbiol. 1997;83:727-36.
21. Sambrook J, Russel DW. Molecular Cloning: a laboratory manual. 3ª ed., vol. 3. Nova York: Cold Spring Harbor Laboratory Press; 2001.
22. Carmo LS, Dias RS, Linardi VR, Sena MJ, Santos DA, Faria ME, et al. Food Poisoning due to enterotoxigenic strains of *Staphylococcus* present in Minas cheese and raw milk in Brazil. Food Microbiol. 2002;19:9-14.
23. Loguercio AP, Aleixo JAG. Microbiologia de queijo minas frescal produzido artesanalmente. Cienc Rural. 2001;31:1063-67.
24. Ercolini D, Blaiotta G, Fusco V, Coppola S. PCR-based detection of enterotoxigenic *Staphylococcus aureus* in the early stages of raw milk cheese making. J Appl Microbiol. 2004;96:1090-8.
25. Mclauchlin J, Narayanan GL, Mithani V, O'Neill G. The detection of enterotoxins and Toxic Shock Syndrome toxin genes in *Staphylococcus aureus* by Polymerase Chain Reaction. J Food Prot. 2000;63:479-488.
26. Wilson IG. Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol. 1997;63:3741-51.

27. Bej AK, Mahbubani MH. Detection of foodborne microbial pathogens. *In*: Griffin H, Griffin A (Eds). PCR Technology: Current Innovations. Boca Raton: CRC Press; 1994. p. 341-65.

28. Kim C-H, Khan M, Morin DE, Hurley WL, Tripathy DN, Kehrli MJ, et al. Optimisation of the PC for detection of *Staphylococcus aureus* nuc gene in bovine milk. J Dairy Sci. 2001;84:74-83.

29. Tamarapu S, McKillip JL, Drake M. Development of a multiplex polymerase chain reaction assay for detection and differentiation of *Staphylococcus aureus* in dairy products. J Food Prot. 2001;64:664-8.

30. Baruzzi F, Matarante A, Caputo L, Morea M. Development of a culture-independent polymerase chain reaction-based assay for the detection of Lactobacilli in stretched cheese. J Rapid Methods Autom Microbiol. 2005;13:177-192.

31. Cremonesi P, Perez G, Pisoni G, Moroni P, Morandi S, Luzzana M, et al. Detection of enterotoxigenic *Staphylococcus aureus* isolates in raw milk cheese. Lett Appl Microbiol. 2007;45:587-91.

32. Nájera-Sánchez G, Maldonado-Rodríguez R, Olvera PR, Garza LM. Development of two multiplex polymerase chain reactions for the detection of enterotoxigenic strains of *Staphylococcus aureus* isolated from foods. J Food Protect. 2003;66:1055-62.

33. Rall VLM, Vieira FP, Rall R, Vieitis RL, Fernandes Jr A, Candeias JMG., et al. PCR detection of staphylococcal enterotoxin genes in *Staphylococcus aureus* strains isolated from raw and pasteurized milk. Vet Microbiol. 2008;132:408-13.

34. Ünal S, Hoskins J, Flokowitsch JE, Wu CYE, Preston DA, Skatrud PL. Detection of methicillin-resistant staphylococci by using the polymerase chain reaction. J Clin Microbiol. 1992;30:1685-91.