

## ASSESSMENT OF THE RISK OF RAW MILK CONSUMPTION RELATED TO STAPHYLOCOCCAL FOOD POISONING

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### ABSTRACT

This study aimed to identify the risks of staphylococcal food poisoning due to the consumption of raw milk. Fifty-one farms in Londrina (PR) and 50 in Pelotas (RS) were analyzed, to determine the population of coagulase-positive staphylococci (UFC/mL), as well as to verify the ability of producing Staphylococcal Enterotoxin A (SEA) by immunodiffusion (OSP), the presence of the gene for the production of SEA (PCR) in the cultures, and the research of enterotoxin (SEA to SEE) in milk samples using ELISA commercial kit. Considering the 101 farms analyzed, 19 (18.8%) presented coagulase-positive staphylococci count above 10<sup>5</sup> UFC/mL. For the evaluation of the enterotoxigenic ability (SEA) by the OSP technique, six cultures coagulase-positive (5.5%) were positive to the test and identified as *S. aureus*. From the coagulase-negative sample, one (5.5%) was OSP positive. For the evaluation of the presence of the gene for EEA synthesis, 51 cultures of staphylococci were tested. From this total, 14 (27.45%)

presented the gene, and from that, only 5 (9.81%) cultures were capable of expressing it in the technique of the OSP. The morphologic characteristic of the evaluated cultures that had enterotoxigenic capacity, from the 14 (33,3%) cultures that presented the gene for EEA production, 05 (11.9%) were characterized as typical cultures of *S. aureus* in Baird Parker agar. All the 12 milk samples studied for the presence of EEA to EEE in milk were negative. Thus, it can be concluded that there is extensive contamination of raw milk for staphylococci coagulase, however, most of the isolated strains were not enterotoxigenic or did not express such a characteristic. Only 9.81% of the tested colonies expressed the gene and effectively produced SEA. None of the samples had sufficient counts to produce detectable amounts of SEA. The milk samples did not present risk to cause staphylococcal food poisoning if consumed *in natura* until the collection moment.

KEYWORDS: Enterotoxin, milk, quality, staphylococci.

### RESUMO

#### DETERMINAÇÃO DO RISCO DE CONSUMO DE LEITE CRU RELACIONADO À INTOXICAÇÃO ESTAFILOCÓCICA

Este trabalho teve como objetivo identificar os riscos de intoxicação estafilocócica pelo consumo de leite cru. Foram analisadas amostras de 51 propriedades em Londrina (PR) e 50 em Pelotas (RS), com o objetivo de determinar a população de estafilococos coagulase-positivos (UFC/mL) em leite, verificar a capacidade de produzir enterotoxina estafilocócica A (EEA)

através do teste de imunodifusão (OSP), a presença do gene para produção EEA (PCR) nas culturas isoladas e a pesquisa de enterotoxina (EEA a EEE) nas amostras de leite através do *kit* comercial ELISA. Das 101 amostras, 19 (18,8%) apresentaram contagens de estafilococos coagulase positivos (ECP) acima de 10<sup>5</sup> UFC/mL. Quanto à capacidade enterotoxigênica das culturas isoladas (OSP),

seis culturas produtoras de coagulase (5,5%) foram positivas no teste e identificadas como *S. aureus*. Das amostras coagulase negativas, 1 foi positiva no OSP (5,5%). Quanto à presença do gene para síntese de EEA, das 51 culturas de estafilococos testadas, 14 (27,45%) apresentaram o gene, sendo que, destas, somente 5 (9,81%) culturas foram capazes de expressá-lo na técnica do OSP. Quanto à característica morfológica das culturas avaliadas para a capacidade enterotoxigênica, 14 (33,3%) culturas que apresentaram o gene para produção de EEA foram caracterizadas como culturas típicas de *S. aureus* no ágar Baird Parker. Das amostras

(12) utilizadas para pesquisa de EEA a EEE no leite, em nenhuma se detectou a presença de EE. Pode-se concluir que há extensa contaminação do leite cru por estafilococos coagulase-positivos, embora a maioria das colônias isoladas não fosse enterotoxigênica ou não expressasse esta característica. Somente 9,81% das colônias testadas expressaram o gene e efetivamente produziram EEA. Nenhuma das amostras apresentou enterotoxina detectável no leite. As amostras de leite testadas não apresentaram risco de causar intoxicação estafilocócica (EEA) se consumidas *in natura* até o momento da coleta.

PALAVRAS-CHAVE: Enterotoxina, leite, qualidade, estafilococos.

## INTRODUCTION

Bacteria are the main cause of foodborne diseases (FBD) in the majority of the countries, causing 2/3 of food poisoning outbreaks (LOIR et al., 2003). According to the Toxicological Attendance Center (CEATOX- Campina Grande), the foodborne diseases represented 11.2 % from all the registered cases in 2005. As for foodborne cases, for which a diagnosis was possible, the symptoms were unspecific and common to other diseases. In many cases, the agent is not identified because there is not the laboratorial diagnostic (ALMEIDA et al., 2008).

Among the microorganisms associated to foodborne diseases, *Staphylococcus aureus* represented annually 4.5% of the food poisoning in the USA, i.e. almost 185.000 illnesses, 1753 hospitalizations and two deaths (MEAD et al., 1999). In Brazil, according to PEREIRA et al. (1994), the staphylococcal food poisoning are more common in the country, but the majority of the cases are not notified nor investigated. According to SILVA et al. (2008), in cases of food poisoning in Juiz de Fora-MG in the period of 2005/2006, coagulase-positive staphylococci were present in 44.5% of the analyzed cases. *S. aureus* was the bacterial agent involved in 41.2% of the cases of foodborne diseases outbreaks that happened in the State of Paraná, from 1978 to 2000 (AMSON et al., 2006).

The symptoms appear quickly, last for a short time, are self-limiting, and seldom cause death. Thus, people rarely look for physicians, what generates sub notifications and few data concerning the frequency of intoxications caused by such microorganism (SU & WONG, 1997).

Men, women and animals are the main *S. aureus* reservoirs. Nasal carriers and food manipulators who have hands or arms with wounds infected by the microorganism are important sources of food contamination (FRANCO & LANDGRAF, 2005). The high frequency of *S. aureus* as an infection agent of the mammary glands of milk-producing cows is another important factor in the epidemiology of this pathogen (LANCETTE & TATINI, 1992; SILVA et al., 2000; AKINEDEN et al., 2001; WOLTER & ZSCHOCK, 2001; ZECCONI & HAHN, 2001; WONG & BERGDOLL, 2002; CENCI-GOGA et al. 2003; KARAMA et al., 2003; FREITAS et al., 2008), considered the most frequent pathogen isolated in raw milk (ZECCONI & HAHN, 2001) and in mastitis cases (SILVA et al., 2000).

The main food involved in staphylococci food poisoning outbreaks are milk and dairy products: *in natura* milk, cream, cream pies, potato salad, tuna fish, chicken ham, cooked meats, and egg products (BREWER, 1991; LANCETTE & TATINI, 1992; FRANCO & LANDGRAF, 1996; LOIR et al., 2003).

Coagulase and thermonuclease (Tnase) are enzymes produced by staphylococci and are the most accepted indicators of the probable evidence of their enterotoxigenic property (WONG & BERGDOLL, 2002).

The minimum count of *S. aureus* for the production of a detectable amount of enterotoxin in food varies between  $10^5$  and  $10^6$  UFC / g or mL of food (LANCETTE & TATINI, 1992; PARK et al., 1992; WONG & BERGDOLL, 2002), and the minimum dose considered to cause staphylococcal food poisoning is 100 ng of SE (KOKAN & BERGDOLL, 1987; FDA, 1992; LANCETTE & TATINI, 1992).

Until now, 19 different enterotoxins and related toxins have been described in *S. aureus* with some differences in structure and biological activity (THOMAS et al., 2007). Initially, SEA, SEB, SEC1, SEC2, SEC3 and SED were identified, followed by SEG- SER and SEU. The relation of the last thirteen ones with food poisoning outbreaks is not very clear (JORGENSEN et al., 2005).

In Brazil there are few epidemiological data in relation to foodborne diseases, and the scientific publications are rare. The state of Paraná is one of the few that has available data, although sub notification is also part of its reality (ZOLI et al., 2002). According to PEREIRA et al. (1994), staphylococcal food poisoning is very common in the country and the majority of the cases are neither investigated nor notified. This study investigated the presence of *Staphylococcus* spp. with gene for the production of staphylococcal enterotoxin A and able to express this gene (*sea*), the direct detection of the toxin in the milk (SEA to SEE), generating information that enables to determine the danger consumers are exposed to when ingesting raw milk.

## MATERIAL AND METHODS

In order to conduct the research, 101 dairy farms were selected: 51 in Londrina (PR) and 50 in Pelotas (RS). These regions were selected because they represent two areas in the country with distinct climatic conditions where there is milk production. The farms were selected according to their representativeness concerning management practices and predominant facilities in each region, as well as due to their laboratorial support for the development of the research.

Refrigerated *in natura* milk samples were collected from expansion or immersion tanks by using sterile flasks, and immediately transported under refrigeration to the laboratories where the researches were being carried out. In Londrina the samples were analyzed at the State University of Londrina, in the Laboratory of Inspection of Animal-origin Products, and in the city of Pelotas the samples were processed at the Federal University of Pelotas, in the Department of Science and Agroindustrial Technology.

From the milk samples collected, the enumeration of *Staphylococcus* spp., the determination of the

enterotoxigenicity of isolated staphylococci and the research of SE in the milk samples were carried out.

The analyses were carried out according to the Methods of Food Microbiological Analysis, BRASIL (2003), using Baird-Parker Agar (Merck, Darmstadt, Germany) for surface plating. Serial decimal dilutions were made and, for the count, this study preferably selected the dishes of each dilution that contained between 10 and 150 colonies, so that, for the coagulase test, five typical and five atypical colonies were collected. When the number of colonies of the smallest dilution was below five, all the colonies were collected for coagulase test.

The result of the *Staphylococcus* spp. (UFC/mL) count was corrected in accordance with the dilution and the amount of inoculums utilized, considering the number of typical and atypical colonies counted and the percentage of confirmed coagulase-positive colonies (SILVA et al., 1997).

The selected colonies were transferred to BHI (Brain Heart Infusion) broth (Difco, Detroit, USA) and after 24 hours at 35-37°C, the coagulase test was conducted according to AOAC (1995).

Catalase test was used to distinguish between the genera *Staphylococcus* and *Streptococcus*. In order to distinguish coagulase-negative staphylococcus and *Micrococcus*, the sensitivity test to lysostaphine (FDA, 1995) was used. The species *S. aureus*, *S. intermedius* and *S. hyicus* were distinguished by evaluating the ability of anaerobic and aerobic fermentations of mannitol and aerobic fermentation of maltose (PHILLIPS & KLOOS, 1981; FDA, 1995).

To determine the ability to produce enterotoxin A, 109 cultures of coagulase-positive staphylococci (CPS) (48 from Pelotas and 61 from Londrina) and 18 cultures of coagulase-negative staphylococci (CNS) (12 from Pelotas and six from Londrina) were tested, isolated from 101 refrigerated raw milk samples collected from these two cities. The CPS cultures tested represented 40 milk samples and CNS cultures 15 milk samples. The studied samples were stored in nutrient agar (Difco, Detroit, USA) at mean temperature of 8°C and recovered in BHI broth (Difco, Detroit, USA) at 35-37°C/ 24 hours for the conduction of the analysis. The CPS samples selected were those biochemically identified in terms of species as *S. aureus*, *S. intermedius* and *S. hyicus*, in both maltose and mannitol test.

The CNS selection was randomly made, according to the analyses availability.

In order to verify the production of enterotoxin by staphylococci, *Cellophane-over-Agar* (JARWIS & LAWRENCE, 1970) was used on Petri dishes (60 X12 mm) with BHI agar (Difco, Detroit, USA) covered in sterile cellophane paper, 0.1mL of a staphylococci suspension in BHI broth (Difco, Detroit, USA) containing 1% of yeast extract was sowed and incubated for 24 hours at 35-37°C. The obtained culture was removed by using 1.5 mL of a sterile solution of NaH<sub>2</sub>PO<sub>4</sub> 0.01M, centrifuged for 3 minutes (Iwaki Microcentrifuger, 10,000 g), and the supernatant was collected and used for the immunodifusion test.

Polyclonal anti-sera for SEA were produced in the Laboratory of Food Science, Department of Food Technology and Medicine at the Centre of Agro-sciences of the State University of Londrina (OLIVEIRA & HIROOKA, 1999). The pure SEA toxin and standard lineages (FRI 722 and FRI A100) of SEA producing *S. aureus* were kindly provided from *Food Research Institute*, Wisconsin, USA.

To visualize the ability of producing SEA of the 109 selected cultures, OSP (*Optimum Sensitivity Plate*) immunodifusion method was used (ROBBINS et al., 1974). The immunodifusion medium was prepared with 8.5g of NaCl, 5.0 mL of mertiolate solution at 2% and 12.0 g of high purity agar dissolved in 1000 mL of Tris buffer 0.05M, pH 7.4. The mix was heated till complete dissolution of the agar and filtered while hot by paper filter.

Petri dishes (50x12mm) were covered with agar-agar 0.2% (Biotec, Suffolk, England), dried and added to 4mL of melted immunodifusion medium. After solidification and rest for 24 hours under refrigeration, six holes were made, following the diameter and distance described by ROBBINS et al. (1974). In the central hole of the dish, 75µl of standard antiserum, and in the superior and inferior smaller holes, 50µl of the respective standard toxin were inserted. In the lateral holes 75µl of the supernatant of the cultures to be tested were added. The dishes were incubated in a wet chamber at 35-37° C/24h, and the reading was conducted by adding phosphoric acid solution 0.1M for better visualization of the precipitation lines.

To determine the gene *sea*, Polymerase Chain Reaction (PCR) technique was performed in the Labo-

ratory of Microbiology of the Institute of Bioscience at UNESP, Botucatu, SP, Brazil.

Fifty-one cultures stored in nutrient agar (Difco, Detroit, USA) at mean temperature of 8°C were evaluated in relation to their ability to produce enterotoxin A. Thus, in this study, at least one typical and one atypical culture of the coagulase-positive *Staphylococcus* spp. were selected, tested by the immunodifusion technique (OSP), what allowed the recovery of the culture in BHI broth (Difco, Detroit, USA).

Total nucleic acid was extracted from the *Staphylococcus* spp. lineages inoculated individually in 10 mL of Brain Heart Infusion broth (Difco) and incubated at 35-37°C/24 h. For extraction, the Kit DNazol (GibcoBRL, Grand Island, NY, 14072 USA) was used, which consisted in the initial digestion of the staphylococci cells with lysozime (10 mg/ml in 10mM Tris HCl pH 8.0) and proteinase K (20 mg/ml in 10mM Tris HCl pH 8.0). After that, 500 µL of the extraction solution (DNazol) were added to the mix and the DNA was precipitated with 250 µL of ethanol 100 %. After 10 minutes the mix was centrifuged at 4000 g/2 minutes at 4°C. Later, the nucleic acid was washed twice with ethanol 95%, placed to dry at 50° C, resuspended in 200 µL of NaOH 8 mM and the pH was neutralized with Hepes 1M.

The PCR reactions were conducted in microcentrifuger tubes of 0,5 mL in total volumes of 25 µL containing 10 pmol of each *primer* (Invitrogen) (Chart 1), 2.5 U of Taq DNA polymerase (Invitrogen), 200 µM of triphosphate desoxiribonucleotides, 20 mM of TrisNHCl (pH 8.4), 0.75 mM of MgCl<sub>2</sub> and 5 µL of the sample. In all the reactions carried out, a negative control was used by means of the substitution of the nucleic acid by sterile milli-q water and for positive control, the culture FRI 722, SEA producer. The reaction was conducted in Thermocycler PTCN100 MJ *Research* by using the parameters described by JOHNSON et al. (1991) with modifications proposed by CUNHA et al. (2001a,b), which consisted of: denaturation at 94°C for 2 min, annealing temperature of the *primers* at 55°C and extension to 72°C for one minute and 30 seconds, followed by a second cycle of denaturation at 94°C for 2 min, annealing temperature of the *primers* at 53°C and extension to 72°C for one minute and 30 seconds. At the third cycle, the annealing temperature was reduced to 51°C followed by more 37 denaturation

cycles at 94°C for 2 min, annealing temperature of the primers at 51°C and extension to 72°C for one minute and 30 seconds. After completing the 40 cycles, the

tubes were incubated at 72°C for seven minutes before cooling at 4°C.

**CHART 1.** Oligonucleotides for the detection of staphylococcal enterotoxin genes

Function	Gene	Primer	Sequence of nucleotides 5' to 3'	Location	Size of the amplified product (bp)
Primer	<i>eea</i>	<i>Sea1</i>	TTG GAA ACG GTT AAA ACG AA	490-509	120
Primer	<i>eea</i>	<i>Sea2</i>	GAA CCT TCC CAT CAA AAA CA	591-610	120

Source: JOHNSON et al., 1991.

The products obtained in the amplification by the techniques used were analyzed on agarose gel at 1.5% prepared in 1 X TBE buffer (Tris-Boric Acid -EDTA pH 8.0), stained with ethidium bromide in the concentration 0.5 µg/mL. After that, they were compared with DNA markers of 50 and 100 bp (Amersham Biosciences) with the help of an ultraviolet transilluminator. The study considered positive the ones that presented bands with specific sizes and were compatible with the product amplified by each reaction. The amplified products (120 bp) were compared with the standard of 50 and 100 bp and photodocumented with camera Sony Cybershot F717.

The 101 milk samples collected in this study were frozen and, later, those that presented positive staphylococci for the production of SEA in the immunodiffusion and gene *sea* in the PCR were selected, as well as the samples that presented counts equal to or higher than 10<sup>5</sup> UFC of *S. aureus*/ mL.

For the research of staphylococcal enterotoxin in the raw milk samples, the ELISA technique was used through the commercial kit Tecra® Staphylococcal Enterotoxina (SET) Visual Imunoassay (VIA™), researching qualitatively the presence of the enterotoxins A, B, C<sub>1</sub>, C<sub>2</sub>, C<sub>3</sub>, D and E, with sensitivity of 1 ng de EE/ mL of food (PARK et al., 1996).

## RESULTS AND DISCUSSION

Considering the 101 farms analyzed in this experiment, all the milk samples presented counts of coagulase-positive staphylococci. Out of those, 17 (16.8%) presented counts of coagulase-positive

staphylococci between 10<sup>5</sup>- 10<sup>6</sup> UFC/mL and 2 (2%) above 10<sup>6</sup> UFC/mL, in the refrigerated raw milk (Table 1). JORGENSEN et al. (2005) found *S. aureus* in 124 of the 165 milk samples from tanks in the farms analyzed, with an average count of 40 UFC of *S. aureus*/mL. Out of that total, only 18.2% of the samples were above such average, and only 0.9% above 2 x 10<sup>3</sup> UFC/mL. In the samples of dairy products produced with raw milk analyzed by the authors, the average counts of *S. aureus* were 6.8 x 10<sup>6</sup> UFC/mL. BORGES et al. (2008) researched the staphylococcal presence in 25 raw milk samples utilized for coalho cheese production. The results showed that 100% of the samples had the microorganisms, with counts between 3.3 x 10<sup>4</sup> and 1.5 x 10<sup>7</sup> UFC/ mL of *Staphylococcus* sp. And from 8.0 x 10<sup>3</sup> to 5.0 x 10<sup>6</sup> UFC mL of coagulase-positive staphylococci.

As for the identification of the staphylococci, out of the total number of 256 coagulase-positive colonies (CPS) isolated in the raw milk samples from Londrina and Pelotas, 159 CPS were conclusively and biochemically identified by both mannitol and maltose, totaling 138 *S. aureus* cultures, 06 *S. intermedius* cultures and 15 *S. hyicus* cultures.

For the evaluation of the enterotoxigenic ability (SEA) by means of the OSP technique, 109 coagulase-positive cultures were tested, namely 94 *S. aureus* cultures, 11 *S. hyicus* cultures, 04 *S. intermedius* cultures and 18 coagulase-negative. Out of this total (109), only six (5.5%) cultures were able to produce SEA, two of which isolated in Pelotas (RS) and four in Londrina (PR), all of them identified as *S. aureus* (Table 2).

CARDOSO et al. (2000), also using the OSP technique, determined the enterotoxigenic characteristic of the *S. aureus* strains isolated from mastitis cases and obtained 83 (65%) out of the 127 samples producers of at least one of the types of enterotoxins tested (TSST-1, SEA, SEB, SEC and SEE). In relation to SEA, about 3% of

the samples were positive, which is a similar result to the one found in this study. As for the coagulase-negative staphylococci tested for the ability to produce SEA, one culture (5.55%) was positive, which is the same percentage found among the CPS (Table 2).

**TABLE 1.** Counts of coagulase-positive staphylococci (UFC/mL) in refrigerated raw milk samples, collected between April and December 2003, in 51 farms in Londrina (PR) and 50 in Pelotas (RS)

Coagulase-positive staphylococci (CFU/mL)	Londrina (PR) N* (%)	Pelotas (RS) N* (%)	Total N* (%)
10 <sup>1</sup> - 10 <sup>2</sup>	16 (31.4)	16 (32.0)	32 (31.7)
10 <sup>2</sup> - 10 <sup>3</sup>	--	2 (4.0)	2 (2.0)
10 <sup>3</sup> - 10 <sup>4</sup>	8 (15.7)	14 (28.0)	22 (21.8)
10 <sup>4</sup> - 10 <sup>5</sup>	12 (23.5)	14 (28.0)	26 (25.7)
10 <sup>5</sup> - 10 <sup>6</sup>	13 (25.5)	4 (8.0)	17 (16.8)
≥ 10 <sup>6</sup>	2 (3.9)	--	2 (2.0)
Total	51 (100)	50 (100)	101 (100)

N\*= sample numbers.

**TABLE 2.** Evaluation of the ability to produce SEA, by immunodiffusion technique (OSP), of coagulase positive and negative staphylococci cultures isolated from refrigerated raw milk samples in dairy farms at the regions of Londrina (PR) and Pelotas (RS), between April and December 2003

Total of colonies	Species	Origin of the samples	OSP positive colonies	Count of <i>staphylococci</i> in OSP positive milk samples
94	<i>S. aureus</i> *	Pelotas (43) Londrina (51)	Pelotas (02) Londrina (04)	10 <sup>3</sup> - 10 <sup>5</sup> UFC/ mL
11	<i>S. hyicus</i> *	Pelotas (5) Londrina (6)	0	----
04	<i>S. intermedius</i> *	Londrina (4)	0	-----
Subtotal	--	Pelotas (48) Londrina (61)	---	---
18	Coagulase-negative staphylococci**	Pelotas (12) Londrina (6)	Pelotas (01)	10 <sup>4</sup> UFC/mL
Total	--	Pelotas (60) Londrina (67)	---	---

\* The CPS cultures tested represented 40 milk samples. \*\* The CNS cultures tested represented 15 milk samples.

Some authors, using other techniques to research SEA in milk samples, found cultures of staphylococci producing little amount of SEA and cultures that did not produce such isolated toxin. OMBUI et al. (1992)

evaluated 97 *S. aureus* strains isolated from raw milk using latex agglutination and found 72 (74.2%) positive samples, from that three (4.17%) produced isolated SEA. JORGENSEN et al. (2005), also using latex

agglutination, found 36 (22.1%) positive samples, two of them (1.2%) produced SEA and SEC simultaneously, from the 163 isolated *S. aureus* strains. CENCI-GOGA et al., (2003) evaluated, by using a latex agglutination kit, 106 *S. aureus* strains and found that 14% of the samples had enterotoxigenic ability; however, none of the isolated strains was identified as SEA or SEB producer. HILL (1983) tested 24 isolated *S. aureus* strains by using microslide technique, and found that 8.3% (24) were SE producers and none positive for SEA.

The OSP (*Optimum Sensitive Plate*) technique is sensitive to detect minimum amounts of 0.5 ug/mL of enterotoxin, suitable for the majority of enterotoxigenic strains (ROBBINS et al., 1974; SU & WONG, 1997; PEREIRA et al, 2001; WONG & BERGDOLL, 2002), but it does not allow the detection of little produced lineages (BERGDOLL, 1990). The technique of production and concentration with *cellophane-over-agar* utilized in this study can increase the sensitivity to 0.1ug/mL (CUNHA et al., 1996; WONG & BERGDOLL, 2002). Thus, it is possible to say that, in the current study, only 5.5% of the tested coagulase-positive staphylococci cultures had the ability to produce at least 100 ng of SEA per mL of supernatant, and that there may be strains that are low SEA producers not identified by the test used.

For the evaluation of the presence of the gene for SEA synthesis through Polymerase Chain Reaction (PCR), 51 CPS cultures were also tested in the immunodiffusion (OSP). They were 42 *S. aureus*, eight *S. hyicus* and one *S. intermedius*, originated from 40 different milk samples. Out of this total, 14 (27.45%) cultures were positive for the presence of the gene *sea*. However, KATSUDA et al. (2005), analyzing 270 isolated *S. aureus* strains from milk-producing farms, found 67.8% of the samples positive for one or more enterotoxigenic genes tested, although no sample was positive for SEA, SEB or SEE. FREITAS et al. (2008) obtained 81 *Staphylococcus* spp. strains from milk samples from the cows and one of the isolates analyzed amplified from the classical *sea-see*, *tst*, *eta* and *etb* toxin genes. Sixty-five (80.2%) isolated agents amplified the *seg*, *seh*, *sei* and *sej* genes, whereas 16 (19.8%) amplified no toxin gene.

Out of the total positive cultures in the PCR (14), only five (9.81%) were positive in the OSP technique

as well, expressing the gene and effectively producing SEA. Therefore, it is possible to say that only 9.81% of the tested colonies had the gene to synthesize SEA and expression ability according to the immunodiffusion methodology used. Those five *S. aureus* cultures were from three different milk samples out of the 101 evaluated.

As for the morphological characteristic of the cultures evaluated in relation to enterotoxigenic ability, the 14 cultures that presented the gene *sea* and five that expressed this gene were characterized as typical *S. aureus* cultures in Baird – Parker and identified as *S. aureus* by mannitol and maltose.

There were 12 milk samples that originated positive staphylococci cultures both in the immunodiffusion (OSP) and PCR, and/or presented counts equal to or higher than  $10^5$  UFC of *S. aureus*/ mL. Such samples were tested for the presence of SEA and SEE in the milk and all of them were negative. It is known that maintenance of food at low temperature can be used to control the production of enterotoxins, because bacterial multiplication diminishes and the synthesis of enterotoxins is almost completely inhibited at temperatures below 7°C (ASPERGER, 1995).

The risk of staphylococcal intoxication by the ingestion of milk requires the presence of four factors: the milk should contain staphylococci that carry the enterotoxin-producing gene; the staphylococci present in the milk should have the ability to express this gene; the counts of such staphylococci in the milk should be superior than  $10^5$  UFC/ mL; there should be conditions for the production of toxins in the food.

Therefore, it is possible to say that the studied milk samples present a small risk of staphylococcal intoxication until the moment of collection, as none of the samples presented the four factors simultaneously.

On the other hand, one should consider the fact that this milk may be used in the production of dairy products like cheese. This way, the possibility that this enterotoxigenic staphylococcal population grows and finds favorable conditions for the production of enterotoxin should be pondered, as much cheese in Brazil is homemade with the use of raw milk. FREITAS et al. (2009) founded coagulase- positive staphylococci counts between  $10^2$  a  $10^6$  UFC/g in coalho cheese. Still, the toxigenic genes *tst*, *sec*, *sed*, *seg*, *seh*, *sei* e *sej* were identified in 18 of the 20 *Staphylococcus* spp

cultures, with 5, 11, 9, 20, 16, 25 and 14%, respectively. According to the authors, the raised percentage of the cultures with different types of toxigenic genes is a reason to concern about the consumer's health.

Several authors report outbreaks of staphylococcal intoxication due to the ingestion of cheese contaminated by *S. aureus*. SABIONI et al. (1988) reported an intoxication outbreak in the city of Ouro Preto (MG), where the food presented *S. aureus* counts of  $9.3 \times 10^7$  UFC/ g and strains producers of SEA, SEB, SED and SEE. In a report of food poisoning outbreaks by staphylococcal enterotoxin occurred in the state of Minas Gerais between 1995 and March 2001, the cheese was responsible for 20.5% of the cases, affecting 660 people and causing one death (CARMO, 2002). According to the Pan-American Institute of Food Protection and Zoonose (INPPAZ, 2010), from 1993 to 2002 there were 18 notified staphylococcal food poisoning outbreaks involving dairy products. Different types of cheese were involved in 88.9% of such cases and milk in 11.1%. In Canada, in 1980, there were 62 cases of staphylococcal intoxication involving cheese and the presence of SEA and SEC (BUYSER et al., 2001).

## CONCLUSIONS

All the raw milk samples analyzed presented coagulase-positive staphylococci. However, most of the isolated strains were not enterotoxigenic or did not express such characteristic.

Only 9.81% of the tested colonies expressing the gene and effectively producing SEA and none of the samples had sufficient counts to produce detectable amounts of SE.

The milk samples evaluated did not present risk to cause food poisoning due to staphylococcal enterotoxin A if ingested *in natura* until the moment of collection.

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