USE OF PCR TO DETECT CLASSICAL ENTEROTOXINS GENES (*ENT*) AND TOXIC SHOCK SYNDROME TOXIN-1 GENE (*TST*) IN *STAPHYLOCOCCUS AUREUS* ISOLATED FROM CRUDE MILK AND DETERMINATION OF TOXIN PRODUCTIVITIES OF *S. AUREUS* ISOLATES HARBORING THESE GENES

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ABSTRACT

During a 2-year period (2003-2004), 132 strains of *Staphylococcus aureus* isolated from crude milk (without thermal treatment) collected in different places in Piracicaba, São Paulo State, Brazil, were investigated for the presence of genes for enterotoxins (*ent*) and toxic shock syndrome toxin-1 (*tst*). Polymerase-chain reaction (PCR) was performed by using 6 pairs of relevant oligonucleotide primers. Ninety isolates (68.18%) were positive for (47 strains) or 2 (43 strains) toxin genes. The combination of *entA* and *tst* showed the highest prevalence (33 strains).The good correlation between PCR results and toxin protein detection and identification by optimum-sensitivity-plate (OSP) test was observed when 44.45% of strains showed positive for toxin production.

KEY WORDS: Enterotoxin, toxic shock syndrome toxin-1, crude milk, *Staphylococcus aureus*.

RESUMO

USO DA REAÇÃO DA POLIMERASE EM CADEIA (PCR) PARA DETECÇÃO DE GENES DE ENTEROTOXINA (*ENT*) E GENES DA TOXINA DA SÍNDROME DO CHOQUE TÓXICO (*TST*) EM *STAPHYLOCOCCUS AUREUS* ISOLADOS DO LEITE CRU E DETERMINAÇÃO DA PRODUÇÃO DE TOXINAS EM ISOLADOS PORTADORES DESTES GENES. Durante um período de 2 anos (2003-2004), 132 cepas de *Staphylococcus aureus* isoladas de leite cru foram coletadas de diferentes regiões de Piracicaba, no Estado de São Paulo. Foi investigada a presença dos genes de enterotoxinas (*ent*) e genes da Toxina-1 da Síndrome do Choque Tóxico (*tst*). A reação da polimerase em cadeia (PCR) foi executada usando 6 pares de oligonucleotídeos específicos para cada gene em questão. Noventa e quatro isolados (68,18%) se mostraram positivos para a presença de um (47 isolados) ou mais genes (43 isolados). A combinação da presença de *entA* e *tst* mostrou alta prevalência (33 isolados). Houve boa correlação entre a presença do gene e a produção / detecção da toxina, feita pelo teste da sensibilidade ótima em placas (OSP), que foi observada quando 44,44% dos isolados mostraram-se positivos para a produção de toxina.

PALAVRAS-CHAVE: Enterotoxina, toxina-1 da síndrome do choque tóxico, leite cru, *Staphylococcus aureus*.

INTRODUCTION

Staphylococcal enterotoxins (SEs) are emetic toxins and that cause of food poisoning in humans. SEs have been classified as members of the pyrogenic toxin superantigen family because of their biological activities and structural relatedness (Balaban & Rasooly, 2000; Dinges et al., 2000). The staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin-1 (TSST-1) are a group of low molecular weight proteins. SEs have been classified according to serological differences. They are designated SEA to SEE. Minor epitope differences in SEC group have resulted in a further subdivision into SEC-1, SEC-2 and SEC-3

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(ZSCHÖCK et al., 2000). TSST-1 commonly causes the toxic shock syndrome in humans and these exoproteins seem to have a variety of effects on cells of the immune system (WOOD et al., 1991).

Several reports have described the development of PCRs for the detection of *ent* and *tst* genes (BECKER et al., 1998; JARRAUD et al., 1999; MCLAUCHLIN et al., 2000; MEHROTRA et al., 2000; MONDAY & BOHACH, 1999, ZSCHÖCK et al., 2000; OMOE et al., 2002). However, it is noteworthy that the PCR is only able to demonstrate the existence of *ent* and *tst* genes in *Staphylococcus aureus* isolates and does not prove that the production os SEs proteins occurs. To demonstrate the ability of a strain to produce sufficient amount of SEs protein to induce disease, bioassays or immunological methods for the detection of SEs protein must be developed.

The purposes of this study were to analyze the distribution of genes *entA* to *entE* and *tst* in *S. aureus* isolated from crude milk, in Piracicaba, São Paulo State, Brazil and the *in vitro* production of SEs and TSST-1 by isolates harboring the respective genes.

MATERIALS AND METHODS

Sources and strains of *S. aureus*

One-hundred thirty-two (132) *S. aureus* strains were studied. Strains were isolated from crude milk originating from Piracicaba and surrounding localities, in São Paulo State, Brazil, colected in 2003 and 2004 using standard methods according to recommendations of the International Dairy Federation (IDF, 1981). Selection was carried out by sampling only one strain per milk sample. Additionally, six *S. aureus* isolates previously characterized as producing one or more toxins were used as reference strains. Three enterotoxin-negative *S. aureus* strains were used as negative controls. Samples were seeded on Baird-Parker Agar (BP). Agar plates were incubated at 37° C and read after 24 and 48h. Isolates were subcultured on nutrient agar (NA) and identified using the following criteria: Gram staining result, the presence of catalase-positive cocci in clumps, coagulase production, a characteristic haemolysis pattern when plated on sheep blood agar and characterization by using a commercial identification system.

DNA isolation

The DNA isolation method of DOYLE & DOYLE (1990) was modified in order to facilitate the extraction of *S. aureus* DNA. A volume of 2.5 mL was collected from 5 mL overnight cultures in BHI and centrifuged at 14.000 rpm (20.800 g) for 30 seconds. The supernatant was discarded and the pellet was resuspended in 700 mL extraction buffer (1.4 M NaCl, 100 mM Tris-HCl [pH 8.0], 200 mM EDTA [pH 8.0], PVP (polyvinylpyrrolidone) 40%, CTAB (cetyltrimethylammonium bromide) 2%, Proteinase K 20 mg / mL and β-Mercaptoethanol 0,2%). After mixing, the tube was incubated at 65°C for 30 minutes with occasional mixing every 10 minutes. Next, 650 µL of chloroform-isoamyl alcohol (24:1) were added and the solution was centrifuged at 14.000 rpm (20.800 g) for 7 mins. The upper aqueous phase was transferred to a 1.5-mL tube and 200 µL of extraction buffer without Proteinase K were added. The solution was gently mixed and 650 µL of chloroform-isoamyl alcohol (24:1) was added and centrifuged at 14.000 rpm (20.800 g) for 7 min. The upper aqueous phase was transferred to a new tube and the previous chloroform-isoamyl alcohol (24:1) extraction was performed twice more. The DNA was precipitated by adding an equal volume of isopropanol at room temperature, mixing and centrifugation at 14.000 rpm (20.800 g) for 7 min. The isopropanol was removed and the pellet was washed twice with 70 µL of 70% ethanol. The DNA pellet was dry and ressuspended in 40µL of TE buffer (10mM Tris-HCl [pH 8,0], 1mM EDTA [pH 8,0] and 10 mg / mL of RNAse) and incubated at 37°C for 30 minutes.

PCR primers

Primers for PCR were synthesized by Promicroä (São Paulo, Brazil) based on sequences published by Mehotra et al. (2000) for *entA* to *entE* and *tsst* genes (Table 1).

Polymerase-chain reaction (PCR)

PCR amplification was performed in 25 mL reaction mixture containing (20 to 90 ng/µL of DNA, 1X PCR-buffer, 3 mM MgCl2, 200µM dNTPs, 20 pmols of primers (40 pmol for *sed* gene) and 1.25 U of Taq DNA polymerase. The following amplification program was carried out at 96° C for 5min followed by 35 cycles of 2min at 94° C, 2min at 54° C and 1min at 72° C and a final extension at 72° C for 7min in a Gene Amp PCR System 9700 thermocycler. PCR products were visualized after eletrophoresis on 2% agarose gel stained with ethidium bromide and the product size estimated using a 100-bp DNA ladder.

Production and detection of Enterotoxins (SEA-SEE) and TSST-1

All strains were tested for enterotoxins A to E (SEA-SEE) and TSST-1 by cellophane-over-agar method for enterotoxin production (HALLENDER, 1965; JARVIS et al., 1970; ROBBINS et al., 1974), and the optimum-sensitivity-palte (OSP) method for enterotoxin detection and identification (ROBBINS et al., 1974).

Table 1 – Sequences used for PCR enterotoxins genes (*ent*) and toxic shock syndrome toxin-1 gene (*tst*) detection.

Gene	Primer	Sequence	Location inside the gene	Amplicon size
entA	GSEAR-1	GGTTATCAATGTGCGGGTGG	349-368 431-450	102
	GSEAR-2	CGCCACTTTTTTCTCTTCGG		
entB	GSEBR-1	GTATGGTGGTGTAACTGAGC	666-685 810-829	164
	GSEBR-2	CCAAATAGTGACGAGTTAGG		
*entC*2	GSECR-1	AGATGAAGTAGTTGATGTGTATGG	432-455 863-882	451
	GSECR-2	CACACTTTTAGAATCAACCG		
entD	GSEDR-1	CCAATAATAGGAGAAAATAAAAG	492-514 750-769	278
	GSEDR-2	ATTGGTATTTTTTTTCGTTC		
entE	GSEER-1	AGGTTTTTTCACAGGTCATCC	237-257 425-445	209
	GSEER-2	CTTTTTTTTCTTCGGTCAATC		
tst	GTSSTR-1	ACCCCTGTTCCCTTATCATC	88-107 394-113	326
	GTSSTR-2	TTTTCAGTATTTGTAACGCC		

Table 2 – Genotypic (PCR) and phenotypic results for *S. aureus* toxins detection from crude milk, Piracicaba, São Paulo, Brazil (2003-2004).

Gene (genotype)	Isolates	Gene detection (%)	Toxin (phenotype)	Isolates	OSP Toxin detection method (%)
Total	90	68.18	Total	40	44.44
entA	61	67.78	SEA	14	35.00
entB	30	33.33	SEB	27	67.50
*entC*2	5	5.56	SEC2	5	12.50
entD	0	0.00	SED	0	0.00
entE	0	0.00	SEE	0	0.00
tst	38	42.22	TSST-1	13	32.50
entA + *tst*	33	36.66	SEA + TSST-1	10	25.00

RESULTS

The reaction with each individual primer pair resulted in amplification of single products when DNA from each reference strain was used as a template. The sizes of products obtained from control strains in PCR designs corresponded to the predicted sizes (Fig. 1). Reproducibility was observed in all tested strains.

Testing for the enterotoxins (*ent*) and TSST-1 (*tst*) genes was performed in all 132 isolates of *S. aureus* on this study and 90 isolates (68.18%) were positive for one or two toxin genes. Of these positive strains, 61 (67.78%) were positive for *entA*, 30, (33.33%) for *entB*, 5 (5.56%) for *entC*2 and 38 (42.22%) for *tst*. Also 33 strains (36.66%) co-amplified *entA* and *tst* genes. None of the 90 *S. aureus* strains carried the *entD* and *entE*-genes (Table 2).

The production of SE and TSST-1 was detected in 40 strains (44.44%): 14 (35%) for SEA, 27 (67.5%) for SEB, 5 (12.5%) for SEC2 and 13 (32.5%) for TSST-1 (Table 2).

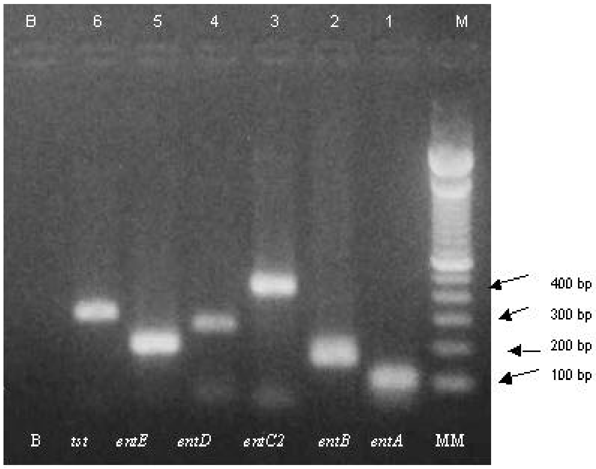

Fig. 1 - Electrophoresis of the PCR fragments generated using DNA extracted according DOYLE & DOYLE (1990) methodology and primers sets specific for the *S. aureus* enterotoxins genes. Lane M l HindIII molecular marker, 1 *entA* 102bp, 2 *entB* 164bp, 3 *entC2* 451bp, 4 *entD* 278bp, 5 *entE* 209bp, 6 *tst* 326bp and B negative control (no DNA).

DISCUSSION

Some *S. aureus* strains produce one or more enterotoxigenic toxins including SEA-SEE and TSST-1 and these toxins represent the main cause of staphylococcal food poisoning. It has been estimated that about 95% of these outbreaks were due to classical SEs, such as SEA to SEE (OMOE et al., 2002). The determination of staphylococci enterotoxins type has a long history of sucessful use in epidemiologic studies in both clinical and environmental microbiology studies. The limitation of all genotipic tests is that the presence of the gene does not always necessarily mean that the toxin will be produce. Some researches (NEILL et al., 1990) identified the presence on an *entC* gene in two strains which did not produce detectable levels of SEC toxin when they used the SET-RPLA assay. This situation above and many others may be due to low-level production of enterotoxin below the threshold of detection for the immunological assay. Since the production of enterotoxins by staphylococcal strains can be affected by the growth conditions used (inoculum level, temperature, pH, and water activity) (GENIGEORGIS, 1989), it is possible that for these particular isolates the standard culture conditions specified for the immunological assays are suboptimal for gene expression. Alternatively, the *ent* genes may not be expressed due to mutations either in the coding region or in aregulatory region (SHARMA et al., 2000). However, one major application of the immunological assays is the toxin typing of strains for epidemiological purposes it is no usually essential to know whether or not a gene is expressed. FUEYO et al. (2001) reported that up to 28% (62 out 224) of the *S. aureus* strains generated positive agglutination with one or two sera and all of them contained *ent* genes as determined by the PCR method.

In the report by OMOE et al. (2002), analysed 71 *S. aureus* isolates from various sources and 66 (93%) were found to be positive for one or more *ent* genes. SÁ et al. (2004) analysed 209 samples from bovine milk and found that 9 strains (4.39%) were enterotoxin producer being 1 (0.49%) for SED, 3 (1.46%) for SEE and 3 (1.46) for SEB production. ZSCHÖCK et al. (2000) performed test for enterotoxin genes in 94 field isolates and found 34 (36.2%) *S. aureus* isolates were toxin (*ent-tsst*)-gene positive by PCR. Three field strains (3.2%) were classified as carrier of *entA*-gene, 2 isolates (2.1%) had *entB*-genes and 22 (23.4%) were positive for *entC*-gene. The *entD*-gene was found in 4 (4.3%) isolates, *tst*-gene occurred as single gene (3 isolates, 3.2%), and in combination with *entC*-gene (15 field strains, 16%) or *entA*-gene (1 isolate, 1,1%). None of the 94 *S. aureus* strains carried *entE*-gene. Different works have report extremely variable results from the frequency of SEs among staphylococcal strains (SÁ, 2004; FUEYO et al., 2001; JARVIS et al., 1970) and from presence of *S. aureus* enterotoxin genes (BECKER et al., 1998; MCLAUCHLIN et al., 2000; MEHROTRA et al., 2000; SHARMA et al., 2000). Data from the present study reported variable results from frequency of enterotoxin genes and toxin productivies that showed a good relation between detection of SEs and their *ent* genes.

CONCLUSION

The fact that PCR technique allows detection of the genetic potential for enterotoxin production may make it useful as both a screening test and a confirmatory test for enterotoxins actually elicited, as detemined by immunological assays. The existence of *ent* genes in *S. aureus* isolates is necessary for these stains to cause food poisoning. However, it is debatable whether all *ent* gene-positive strains can cause disease. The combination of both methods is a guarantee for success in diagnostic analisys tests and can also be recommend PCR use a screening test for presence of enterotoxin genes.

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