

## Detection by multiplex PCR of *Staphylococcus aureus*, *S. intermedius* and *S. hyicus* in artificially contaminated milk

### Detecção de *Staphylococcus aureus*, *S. intermedius* e *S. hyicus* em leite contaminado artificialmente por multiplex PCR

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#### ABSTRACT

This research aimed to detect coagulase-positive *Staphylococcus* (CPS) directly in samples of artificially contaminated milk, using multiplex PCR (mPCR). Standard and isolated bacterial strains of *S. aureus*, *S. epidermidis*, *S. hyicus*, *S. intermedius*, *Listeria monocytogenes* and *Escherichia coli* species were used, evaluating the specificity and detection limit of mPCR, for artificially contaminated UHT milk. Primers specific for the nuc gene (NUC1-NUC2 were used for *S. aureus*, NUC3-NUC4 for *S. hyicus* and NUC5-NUC6 for *S. intermedius*). It was possible to detect the three target species by mPCR, directly from bovine whole milk, with adequate specificity and acceptable detection limit for identification of coagulase-positive *Staphylococcus* (CPS) in foods. The specificity was determined by the amplification of species-specific fragments, and the detection limit was assessed by the detection thresholds obtained for the three species ( $10^3$  CFU mL<sup>-1</sup>). From these results, the mPCR described, with the proposed set of primers, has the potential for use in precise identification and differentiation between CPSs in milk samples.

**Key words:** *Staphylococcus aureus*, *Staphylococcus hyicus*, *Staphylococcus intermedius*, Multiplex PCR, nuc gene.

#### RESUMO

Esta pesquisa teve como objetivo detectar diretamente em amostras de leite contaminado artificialmente *Staphylococcus* coagulase positiva (ECP) por multiplex PCR (mPCR). Cepas padrão e isolados de *S. aureus*, *S. epidermidis*, *S. hyicus*, *S. intermedius*, *Listeria monocytogenes* e *Escherichia coli* foram utilizados no estudo. Foram utilizados primers específicos para o gene nuc (NUC1-NUC2 para o *S. aureus*, NUC3-NUC4 para o *S. hyicus* e NUC5-NUC6 para o *S. intermedius*). Foi possível

detectar as três espécies-alvo por mPCR, formar diretamente nas amostras de leite integral bovino, com especificidade adequada e limite de detecção aceitável para identificação de espécies de *Staphylococcus* coagulase positiva (ECP) em alimentos. A especificidade foi determinada por meio da amplificação de fragmentos específicos das espécies e o limite de detecção foi avaliado pelos limiares de detecção obtidos para as três espécies ( $10^3$  UFC mL<sup>-1</sup> para as espécies presentes nas amostras de leite contaminadas artificialmente).

**Palavras-chave:** *Staphylococcus aureus*, *Staphylococcus hyicus*, *Staphylococcus intermedius*, Multiplex PCR, nuc gene.

#### INTRODUCTION

Three species of coagulase-positive *Staphylococcus* (CPS), *S. aureus*, *S. hyicus* and *S. intermedius*, have already been involved in a number of food-poisoning outbreaks and are capable of producing enterotoxins and coagulase enzyme. These three species showed extremely similar morphologic characteristics, as well as biochemical properties, which makes difficult to differentiate and identify them in food through microbiological analyses.

For the identification of CPS, in the laboratory routine, enzyme production tests are used, such as coagulase and thermonuclease. However, these enzyme tests may not be enough to detect *S. aureus*, *S. hyicus* and *S. intermedius* in foods.

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(DOWNES & ITO, 2001). A viable alternative to phenotypic tests, such as enzyme production tests, are the molecular methods based on PCR. These methods have already been used with success for species identification through specific sequences of the microbial genome; for example, the *nuc* gene has been used for the correct identification of CPS in previous studies. (SILVA et al., 2003; BARON et al., 2004; BECKER et al., 2005; YANG et al., 2007). The *nuc* gene codes for the production of a thermostable endonuclease enzyme (TNase). *S. aureus*, *S. hyicus* and *S. intermedius* are TNase producers and therefore carriers of this gene. (BARKSI et al., 1996).

For the quicker and simultaneous detection of more than one bacterial species of food pathogens PCR reactions with more than one pair of primers have been proposed. This technique is known as multiplex PCR (mPCR) (TAMARAPU et al., 2001; YANG et al., 2007). It is common to use five or six pairs of primers in the same reaction, including for *Staphylococcus* species. For example, OKOLIE et al. (2015a; 2015b) simultaneously used primers for sequences of 16S rRNA for eubacteria, *tuf* gene for *Staphylococcus* genus and three genes for *Staphylococcus aureus*, *spa*, *mecA* and *vanA*, in the same PCR assay in two different studies. In this context, the objective of this research was to detect *Staphylococcus aureus*, *S. intermedius* and *S. hyicus* in artificially contaminated milk, using mPCR, with specific sequences for the *nuc* gene.

## MATERIALS AND METHODS

### Bacterial strains

For *S. aureus* standard strains ATCC 29213, ATCC 10832 and FRI-100 were used. For *S. intermedius* and *S. hyicus* clinical isolates were used (previously identified at species level through biochemical and molecular tests by SILVA et al., 2003). As controls standard strains of *Listeria monocytogenes*, *Escherichia coli* and *Staphylococcus epidermidis* were used (ATCC 764, ATCC 11229 and ATCC 14990, respectively).

### DNA extraction

One-milliliter samples of UHT (*Ultra High Temperature* thermal process) whole milk were used. For the mPCR specificity evaluation, these samples were artificially and separately inoculated with a loopful of culture from Baird-Parker Agar (strains identified as CPS) incubated at 37°C for 48h. DNA extraction was performed immediately after inoculation.

For the detection limit experiment, samples were contaminated with three CPS species, with 100µL of bacterial suspension containing 50, 10<sup>2</sup>, 10<sup>3</sup>, 10<sup>4</sup> and 10<sup>5</sup>UFC mL<sup>-1</sup>. One mL of contaminated milk samples were submitted to centrifugation at 14,000 × g for two min, and the supernatant was discarded. Then the pellet was washed, with centrifugation at 10,000 × g for 15min at 4°C, and resuspended with 100µL Tris-Hcl buffer solution until a clear solution was obtained (MATTHEWS et al., 1997; MEIRI-BENDEK et al., 2002). After this point, DNA extraction was carried out exactly in accordance with MATTHEWS et al. (1997). The DNA extraction of each strain was performed at least twice with distinct cultures. All DNA samples were submitted to mPCR amplification.

### Primer design

The primer design was based on complete sequences of *nuc* gene obtained from GenBank/NCBI. These sequences were aligned among themselves and with the other species' genes using the software Blast (GenBank/NCBI) in order to verify the homology among the three genes. The sequences were specific for *S. aureus*, *S. intermedius* and *S. hyicus* (accession numbers NC\_002745, X67678 and L23973, respectively). Primers were designed to nest in the non-homologous regions, to certify specificity and allow species-specific amplifications.

### Multiplex PCR

Reaction solutions were prepared with 1µL of DNA from each bacterium (20ng) to verify specificity, or 1µL of DNA solution extracted from 5 series of concentrations in TSB (50, 10<sup>2</sup>, 10<sup>3</sup>, 10<sup>4</sup> and 10<sup>5</sup>UFC mL<sup>-1</sup>) from each one of the microbial species, for the experiment that was to evaluate the detection limit technique. Next, 1µM of each primer, using three pairs of primers in all the reactions (mPCR), 200µM of each desoxynucleotide triphosphate (dNTP, Invitrogen), 1U of Taq Polymerase (5UµL<sup>-1</sup>), 2.0mM of MgCl<sub>2</sub> and 4µL of 10X buffer were added, making a total volume of 40µL. The PCR program used was adapted from the methodology of MOTTA et al. (2001). This consisted of 2min at 94°C, 2min at 55°C and 3min at 72°C for 40, cycles. For the separation of the amplified products, electrophoresis was performed (150V, 80mA, 50min) in agarose gel 1% (p/v) TBE buffer, pH 8.4, stained 0.5µg mL<sup>-1</sup> of ethidium bromide. Together with the PCR products, a molecular weight pattern was applied to the gel (2Log DNA Ladder or 100bp DNA Ladder).

## RESULTS AND DISCUSSION

Primers NUC1 and NUC2 presented specificity for *S. aureus*, obtaining the amplification only of the fragment estimated (458pb) from DNA extracted from the milk that had been artificially contaminated with this species, even in the presence of the other primers used in mPCR (Figure 1).

The primers NUC3 and NUC4 also presented specificity. However, this was for *S. hyicus* alone, because the estimated fragment (270pb) was obtained only when the DNA extracted from the milk contaminated with this microorganism was used (Figure 1).

For *S. intermedius*, likewise, the estimated fragment was obtained (106pb) always when the DNA extracted from milk contaminated with this microbial species was submitted to amplification (Figure 1).

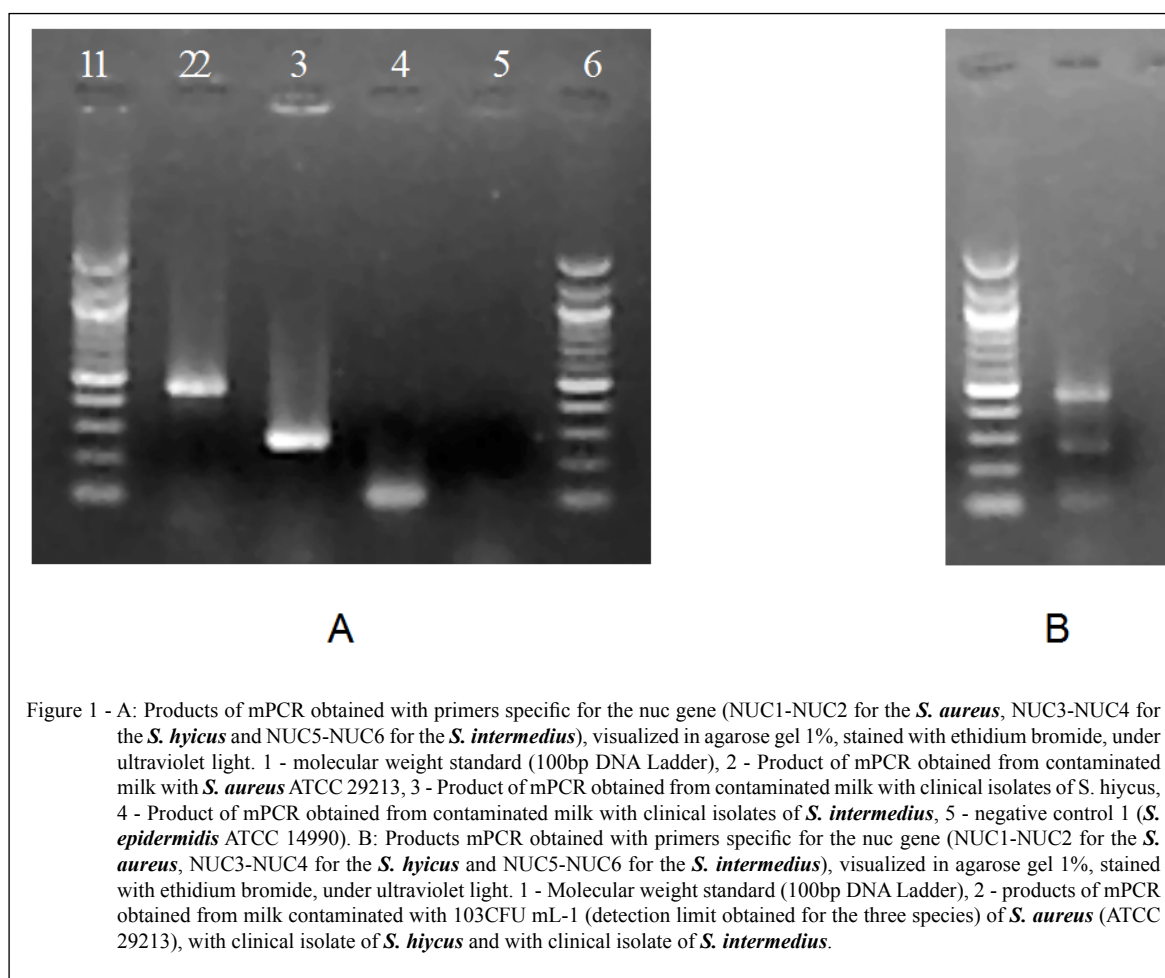
For *S. aureus* it was possible to amplify concentrations of up to  $10^2$ UFC mL<sup>-1</sup> (corresponding to a DNA concentration of 1.63µg mL<sup>-1</sup>) showing that

the detection limit for *S. aureus* was the same as for the isolate cultures in milk samples. However, for *S. hyicus* and *S. intermedius*, clear and defined bands were obtained until concentration  $10^3$  UFC mL<sup>-1</sup> (corresponding to DNA concentrations of 7.13µg mL<sup>-1</sup> and 7.63µg mL<sup>-1</sup>, respectively).

At a concentration of  $10^2$ UFC mL<sup>-1</sup> weak, not very clear bands were obtained. These were not reproduced in some repetitions; and it is therefore more appropriate to accept  $10^3$ UFC mL<sup>-1</sup> as the concentration limit.

In another study the threshold of detection was also considered to be in the range of  $10^2$ UFC mL<sup>-1</sup> (TAMARAPU et al., 2001). In this case, the mPCR was developed for detection of standard strains of *S. aureus* in artificially contaminated cheddar cheese and milk.

Other authors obtained a detection limit that was more effective than the one reported in this study for the detection of pathogens in food samples. YANG et al. (2007) carried out



amplification by a uniplex PCR of a 279pb *nuc* gene sequence from samples of milk and cheese artificially contaminated with *S. aureus*; they verified a detection limit of 10UFC mL<sup>-1</sup> for milk samples and 55UFC mL<sup>-1</sup> for cheese samples. COCOLIN et al. (2000) obtained a threshold of 10<sup>1</sup>UFC mL<sup>-1</sup> for detection of *E. coli* by mPCR in samples of artificially contaminated meat. These differences in the detection limit reported in experiments involving mPCR in food samples, as well as the absence of amplification in most of the cases at concentrations lower than 10<sup>3</sup> UFC mL<sup>-1</sup>, can be associated with DNA purity and/or the existence of inhibitors in food sources.

As noted by CRIBB et al. (2002), DNA concentrations equal or higher than 25ng are necessary so that there is detection in a gel stained with ethidium bromide, which represents 10<sup>11</sup> copies of a fragment with 200 base pairs. The same authors assert that through theoretical calculations it was demonstrated that when an amplification is carried out with 10 molecules of a DNA template, the global efficiency of this reaction after 35 cycles will have to be 90% to generate detectable fragments. They also highlighted the fact that this efficiency has been achieved in research; however, it is not commonly obtained in clinical diagnosis, due to the critical problem of the influence of PCR reaction inhibitors on clinical samples and on microorganism culture media, which could lead to false negative results.

In another study with mPCR, CREMONESI et al. (2005) detected simultaneously sequences of genes 23S of rRNA, of coagulase, of *nuc* and of enterotoxin A, B, C, D, G, H, I, J and L from a concentration of 1pg of DNA of *S. aureus* isolates, obtained from samples of milk and dairy products. In the same research, the global efficiency of the process was not calculated, but the quantity of DNA template obtained from microbial concentrations equal to or lower than 10<sup>2</sup>UFC mL<sup>-1</sup> was shown to vary between 0.33µg mL<sup>-1</sup> (extracted from the concentration of 50UFC mL<sup>-1</sup> in bovine whole milk artificially contaminated with *S. aureus*) and 1.63µg mL<sup>-1</sup> (extracted from concentration of 10<sup>2</sup> UFC mL<sup>-1</sup> in bovine whole milk artificially contaminated with *S. aureus*). This DNA quantity was higher than that reported by CRIBB et al. (2002) and by CREMONESI et al. (2005), a fact that makes it possible to infer that the probable cause of the non-appearance of amplified products from these DNA concentrations is related to DNA purity and

the presence of interferers, such as inhibitors of the amplification process.

Among the inhibitors that can be present in the bovine whole milk, lipids are the most important. According to TAMARAPU et al. (2001), the fat content is probably the main interfering factor in the PCR detection limit, making necessary to use a fat extractor (such as petroleum ether) before DNA extraction. This step was not performed in the present research.

An interesting fact highlighted by TAMARAPU et al. (2001) is that when the same primers are used in mPCR for the same milk samples, but in a uniplex PCR form for identification of *S. aureus*, the detection limit moved from 10<sup>2</sup> to 10<sup>1</sup>UFC mL<sup>-1</sup>. This decrease by one logarithmic unit in mPCR indicated that the detection limit can be associated with the reaction itself, because there is competition between the primers for different base pairs in the mPCR, which can contribute effectively to altering the detection limit.

RAMESH et al. (2002) verified the influence of the number of primers in the detection limit, as it directly affected milk samples. Primers were used that were specific for sequences of *nuc* gene of *S. aureus* and for sequences related to the adhesion and invasion of *Yersinia enterocolitica*. Individually, the detection limit for both species was 10<sup>3</sup>UFC mL<sup>-1</sup>; however, in a multiplex with all primers, the detection limit went to 10<sup>4</sup>UFC mL<sup>-1</sup>.

Another factor that could make the detection limit more effective in food samples is the use of a non-selective enrichment period prior to DNA extraction. However, one of the aims of this research was to verify the use of mPCR directly in milk, without the need for previous treatments, and therefore stages of previous enrichment were not performed. In addition, such steps would make difficult to control the number of microorganisms and, thus, the detection limit analysis.

Comparing the traditionally phenotypic techniques and the molecular technique proposed in this study, it was possible to infer that after establishing the laboratory structure for both techniques, the molecular technique needs less time. Using mPCR has the advantage that results can be obtained in 18 hours; however, other studies have proposed DNA extraction by chemical action associated with lysis by heating, allowing analysis based on PCR to be made in a period of 6 to 8 hours (RAMESH et al., 2002; TAMARAPU et al., 2001).

The phenotypic techniques commonly used require a longer time for analysis and, in

most cases, additional tests are necessary. For example, just for the characterization of CPS, without differentiation at species level, growth in selective differential Agar (Baird-Parker) needs to be carried out, followed by free coagulase tests and thermonuclease production, requiring at least 72 hours to obtain the result.

Another important consideration is that in phenotypic tests false-negative results can occur, reflecting the influence of environmental factors on gene expression (DOWNES & ITO, 2001). In contrast, the set of primers proposed here were showed to be specific, and they presented an acceptable detection limit for the identification of CPS in the food source used, in this case bovine whole milk. This took into consideration that the detection threshold verified was of  $10^3$ UFC mL<sup>-1</sup>.

It The minimum number of CPS capable of producing enterotoxins in food in quantities that are high enough to cause food poisoning is approximately  $10^6$ UFC (TAMARAPU et al., 2001). Besides, for the majority of foods the legislation in general demands that the allowed limit for CPS is  $10^3$ UFC mL<sup>-1</sup>, a fact that reaffirms the adequacy of the detection limit verified.

## CONCLUSION

Amplification by mPCR of *nuc* gene sequences using primers NUC1, NUC2, NUC3, NUC4, NUC5 and NUC6 allowed the detection of three species of CPS in bovine whole milk samples that had been artificially contaminated by these bacteria. The threshold of detection for the three species of coagulase-positive *staphylococcus* was  $10^3$ UFC mL<sup>-1</sup> in samples of artificially contaminated milk. From these results, the mPCR described, with the set of primers proposed, has the potential to provide precise identification and differentiation between CPS in milk samples.

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