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PCR ASSAY WITH HOST SPECIFIC INTERNAL CONTROL FOR *STAPHYLOCOCCUS AUREUS* FROM BOVINE MILK SAMPLES

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ABSTRACT

Staphylococcus aureus is considered as one of the most important and common pathogens of bovine mastitis. Polymerase Chain Reaction is frequently proposed in the diagnosis of *S. aureus* directly from milk samples instead of classical culture. However, false-negative results may occur in the polymerase chain reaction analysis performed directly from clinical material. For the purpose of disclosing the false negative results, the use of internal amplification controls can be beneficial. Therefore, in this study a new polymerase chain reaction technique with host specific internal amplification control was developed by optimizing *S. aureus*-specific primers in combination with bovine specific primers. The effectiveness of the developed technique in this study was attempted in milk samples from bovine subclinical mastitis. This technique has the potential to detect *S. aureus* from bovine milk samples or dairy products.

Key words: bovine milk, internal control, polymerase chain reaction, *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus, one of the most prevalent pathogens of clinical and subclinical bovine mastitis, can spread rapidly throughout the herd if management precautions are not taken (1, 2). *S. aureus* is one of the most important and common pathogens of bovine mastitis. It causes significant economic losses in the dairy industry (3). *S. aureus* damages milk-producing tissue and significantly decreases milk yield. In the early stages of infection, damage may be reversible, but when diagnosis is delayed, tissue damage is excessive and irreversible (4). Therefore, a rapid and reliable method of identifying the bacteria responsible for mastitis is important for disease management in the herd (5). Moreover, *S. aureus* infections are considered a serious problem that may affect public health because they can be transmitted by milk and milk products (6).

While different methods have been used to detect these agents, bacterial culture is considered the gold standard method for identifying mastitis-causing microorganisms. However, conventional culture is slow, requiring 24–48 h, and needs labour intensive study for definitive identification (7, 8). Therefore, it is of commercial interest to accelerate this procedure by investigating alternative rapid DNA-based methods. PCR methods for detecting *S. aureus* in milk samples to identify cow mastitis have been proposed (9, 10, 11). However, PCR analyses from direct clinical material can result in false negative results caused by inhibitors in

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the clinical specimens (12, 13). Therefore the use of internal amplification controls with different strategies is recommended to determine false negative results of PCR analyses from direct clinical material or food samples (14, 15).

The aim of the present study was to develop PCR analyses with internal amplification control to detect *S. aureus*. A specific primer set for *S. aureus* was used and combined with bovine-specific primers.

Table 1. Properties of primers used in the study

Primer names	Target Gene	GC Contents (%)	Melting Temperature	Sequences of primers	Length of amplicons	References
Sau 327	23S rRNA	45	63 °C	5'-GGACGACATTAGACGAATCA-3'	1318 bp	10
Sau 1645		45	63 °C	5'-CGGGCACCTATTTTCTATCT-3'		
12SM-FW	12S rRNA gene sequences	39	65 °C	5'-CTAGAGGAGCCTGTTCTATAATCGATAA-3'	346 bp	17
12SBT-REV2		37	65 °C	5'-AAATAGGGTTAGATGCACTGAATCCAT-3'		

MATERIAL AND METHODS

Samples and Primers

Total of 50 milk samples from subclinical bovine mastitis were obtained from cattle farms in the Hatay region (The clinical samples were taken with permission with MKÜ Local Ethics committee; Meeting Date 17.06.2010: Meeting No: 2010/02: Decision No: 30) and nucleic acids were extracted from milk samples using the phenol–chloroform method. Extracted DNA was stored at -20°C (16).

Primers for *S. aureus* (Sau 327 and Sau 1645) and Bovine specific (12SM-FW and 12SBT-REV2) were used in this study. Simplex PCR protocols and procedures were carried out according to the recommendations in the literature for those primers. The properties of the primers used in this study are shown in Table 1.

Optimisation of internally controlled PCR mix

All procedures and protocols for multiplex PCR assays were optimized according to Henegariu et al., (18). The amplification mixture for multiplex PCR was carried out in a final volume of 25 µL. The mixture consisted of 200 ng of extracted DNA template, different amounts of 1.5U of *Taq*DNA polymerase (Vivantis Technologies), 2 µL of 10x PCR buffer (10X ViBuffer A without $MgCl_2$), 3 mM $MgCl_2$, 200 µM each of dNTPs (Vivantis Technologies), 20 pmol of primer pair (Sau 327 and Sau 1645) specific for *S. aureus*, and 5 pmol primers (12SM-FW and 12SBT-REV2) specific for bovine gene.

Amplification was carried out after an initial denaturation at 95°C for 3 min. The PCR protocol was: 60 s of template denaturation at 94°C, 60 s of different primer annealing temperatures at 54°C, and 90 s of primer extension at 72°C (total of 35 cycles), with a final extension at 72°C for 5 min. The amplified PCR products were electrophoresed in 1.5% agarose gel and stained with ethidium bromide (0.5 mg/mL), and the DNA bands were visualised under UV light.

RESULTS

Simplex PCR reaction results

In the PCR analyses of milk samples, 3 of 50 samples were found to be *S. aureus* bysimplex PCR and the specific amplification products (1318 bp). They are shown in Figure 1. *S. aureus*–specific bands were amplified very effectively with simplex PCR.

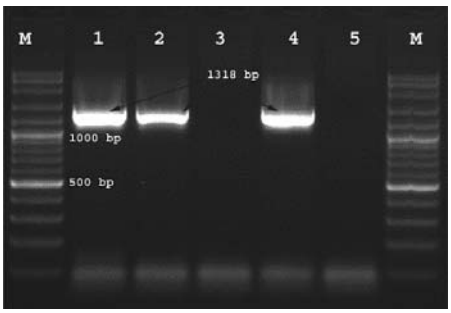

Figure 1. Results of simplex PCR from milk samples with *S. aureus* specific primers. M: 100bp plus marker. Lanes 1, 2, and 4: *S. aureus*-specific bands with Sau 327 and Sau 1645 primers (1318 bp). Lane 5: Distilled Water (Negative Control)

Multiplex PCR reaction results

In the PCR analysis results, specific bands for bovine gene as an internal control and specific bands for *S. aureus* were amplified effectively in the same PCR mixture; the amplification products are shown in Figure 2.

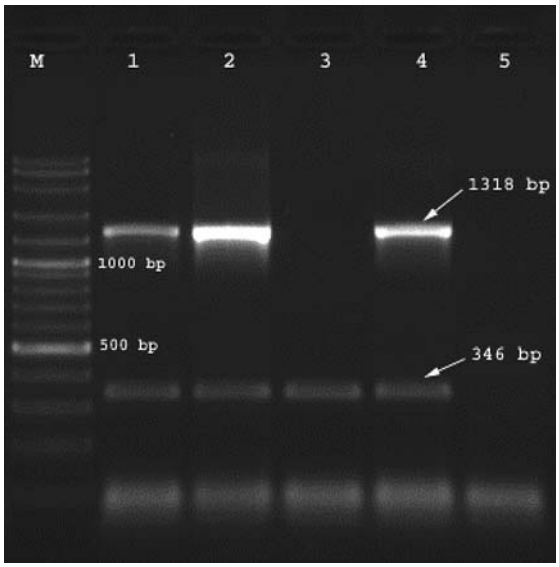

Figure 2. Results of multiplex PCR from milk samples. M: 100bp plus marker. 1–4: bovine-specific bands with 12SM-FW and 12SBT-REV2 primers (346 bp); 1, 2, and 4: *S. aureus*–specific bands with Sau 327 and Sau 1645 primers (1318 bp); 5: Distilled Water (Negative Control)

DISCUSSION

Staphylococcus aureus is considered an important and common pathogen causing bovine mastitis that can also be dangerous to human health. There are some studies including PCR detection of *S. aureus* from bovine milk samples (9, 10, 11). However, in the PCR analyses from clinical material false negative results can be encountered due to inhibitory substances. In order to avoid these false negative results, using internal control was recommended (12, 13). Particularly, using of host specific internal control can be useful to determine false negative results due to mistakes in sampling, DNA extraction or preparing PCR mixtures and thermal cycler failures (14, 15, 19). Therefore, a PCR technique with host specific internal control was developed in this study by combining *S. aureus* specific primers and cow specific primers.

In this study a preliminary type, a specific primer set for *S. aureus,* was combined with specific bovine gene primers to develop an easy and rapid multiplex PCR system with high specificity. Using this optimised combination, a PCR analysis with an internal control specific for bovine genes was developed to detect *S. aureus*. The primer sets successfully amplified the target genes in the multiplex PCR without nonspecific or additional bands. There was no difficulty in discriminating between each band for the target strain (1318 bp) and the internal control (346 bp). Despite the low numbers of positive samples used in this study, the authors are confident that the developed technique will be reproducible in larger experiments.

At the end of the optimisation experiments, the best amplification results were obtained by decreasing 10x PCR buffer (2 µL) and host-specific primers 12SM-FW and 12SBT-REV2 (internal control, 5 pmol) and increasing to 20 pmol primers (Sau 327 and Sau 1645) specific for *S. aureus*. While low and high concentrations of $MgCl_2$ negatively affected the multiplex PCR, the best results were obtained with 3 mM of $MgCl_2$ concentration. The other important parameter was the annealing temperature: the best results were obtained at 54°C. The effects of these parameters were found to be in agreement with the recommendations of Henegariu et al. (18) and Phuektes et al. (20).

Despite the small number of samples analysed, the results of this preliminary study showed that this method can be used as a reliable and effective method for the diagnosis of *S. aureus* in the individual bovine milk or bulk milk samples. This technique should be tried for *S. aureus* detection from bovine milk products

In conclusion, conventional PCR has already become widespread and is less expensive than other molecular techniques. The developed technique in this study might have the potential to detect *S. aureus* from bovine clinical samples. This method can be used for detecting *S. aureus* in the bovine milk or milk products. The technique can also be extended to detect other contagious mastitis pathogens, such as *Streptococcus agalactiae* and *Trueperella pyogenes*.

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