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ORIGINAL ARTICLE

A Duplex PCR for Detection of *S. aureus* and *Staphylococcus* spp. from Culture and Bovine Milk Samples

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SUMMARY

Mastitis is the most costly disease in dairy industry. *Staphylococcus* spp. is the most frequently isolated microorganisms and *Staphylococcus aureus* is one of the most important contagious mastitis agents in dairy cattle. The aim of this study is developing a duplex PCR technique for detection of *Staphylococcus* spp. and *S. aureus* from culture and milk samples. *S. aureus* (ATCC 25923) and *S. epidermidis* (ATCC 12228) DNAs were used as positive control. For the purpose of testing the developed technique, one coagulase-negative Staphylococcus and one *S. aureus* positive milk sample was used as a clinical sample that they were sent by Veterinary Practitioners. In this study, a duplex and rapid PCR protocol is developed for detecting and separating these organisms from culture and from milk samples. This procedure can be used as an alternative, reliable and fast detection and identification method for *Staphylococcus* spp. and *S. aureus* in few hours for deciding treating or culling the cows in farm base in clinical mastitis cases and can be a useful diagnostic method for subclinical mastitis cases too.

Key Words *Duplex PCR, Mastitis, Staphylococci, S. aureus*

Sığır Mastitis Süt Örneklerinden *Stafilokok* Türlerinin ve *S. aureus*'un Teşhisine Yönelik İkili bir PZR Tekniği

ÖZET

Mastitis süt sığırcılığı endüstrisinin en fazla ekonomik kayba neden olan problemidir. Stafilokok türleri en sık izole edilen etkenler olup, *Staphylococcus aureus* ise süt sığırlarında en önemli kontagiyöz etkendir. Bu çalışmanın amacı stafilokok türleri ve *S. aureus*'un kültür ve süt örneklerinden teşhisi için ikili bir PZR tekniğinin geliştirilmesidir. Çalışmada pozitif kontrol olarak, *S. aureus* (ATCC 25923) ve *S. epidermidis* (ATCC 12228) DNA'sı kullanıldı. Geliştirilen tekniğin denenmesi amacıyla Veteriner Hekimlerce laboratuvarımıza gönderilen örneklerden bir adet koagulaz negatif stafilokok bir adet de *S. aureus* pozitif süt örneği klinik örnek olarak kullanıldı. Çalışmada sütten ve kültür örneklerinden stafilokok ve *S. aureus'*un teşhisi ve ayrımı için ikili ve hızlı bir PZR tekniği geliştirildi. Geliştirilen bu tekniğin stafilokok türleri ve *S. aureus*'un bir kaç saat içinde hem süt hem de kültür örneklerinden tanısı ve ayrımı için alternatif, güvenilir ve hızlı bir tanı yöntemi olarak özellikle sonuçların çiftlik bazında kullanılmak üzere klinik ve subklinik olgularda ineğin tedavi edilmesi ya da kesime gönderilmesine karar verilmesi amacıyla kullanılabileceği ortaya konuldu.

Anahtar Kelimeler *Dubleks PZR, Mastitis, Stafilokok, S. aureus*

INTRODUCTION

Mastitis is one of the most important diseases in dairy industry and leads to high levels of economic losses (Friedman *et al*., 2004; Huijps *et al*., 2008). *Staphylococcus* spp., especially *Staphylococcus aureus* is the most frequently isolated microorganisms from mastitis in dairy cattle. It is considered that *Staphyloccoccus* spp. are environmental mastitis, but *S. aureus* is one of the most important contagious mastitis agents (NMC. 1996; Taponen *et al*., 2006). Due to the rapid transmission in the herd and development of the resistance to antibiotics, the treatment and control of these agents are very difficult (Belschner *et al*., 1996). And it is also known that *S. aureus* is often transmitted through the milk and milk products and causes public health problems (Bone *et al*., 1989; Wieneke *et al*., 1993). The rapid detection of these agents in mastitis is important to achieve the treatment and prognosis of the disease (Baştan, 2013). These bacteria grow easily in mediums and isolation and identification take 2-3 days in classical culture methods (Quinn *et al*., 1994). However, antibiotic residues, high somatic cell count and inflammation mediators may inhibit bacterial growth (Phuektes *et al*., 2001). The molecular based identification techniques are used in practice successfully for fast detection of different mastitis agents (Phuektes *et al*., 2001; Riffon *et al*., 2001).

The aim of this study is developing a duplex PCR technique for detection of *Staphylococcus* spp. and *S. aureus* from milk samples.

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MATERIALS and METHODS

Samples and DNA Extraction

S. aureus (ATCC 25923) and *S. epidermidis* (ATCC 12228) DNAs were used as positive control. One Coagulase Negative Staphylococci (CNS) and one *S. aureus* positive milk samples were used as clinical samples in this study. Primarily, milk samples were washed three times with PBS and in the last step the pellet was resuspended in sterile distilled water for elimination of calcium ions and other inhibitors for PCR (Riffon *et al*., 2001). Then, the phenol-chloroform extraction method was used for isolation of genomic DNA from positive control strains and clinical samples (Sambrok *et al*., 1989).

PCR analyses

Staphylococcus spp. and *S. aureus* primers were derived from published sequences. Individual PCR assays were performed according to the original published protocols. And properties of primer pairs were shown in Table 1.

Table 1. Properties of the primers used in this study.

Target gene	Primer name	Primer sequence	Length of amplification products	References
16s rDNA for *Staphylococcus* spp.	16s 1	5'- CAGCTCGTGTCGTGAGATGT -3'	420 bp	Strommenger *et al*., 2003
	16s 2	5'- AATCATTTGTCCCACCTTCG-3'		
Coa gen for S. *aureus*	Coa 1	5'- GCTTCTCAATATGGTCCGAG-3'	131 bp	Schmitz FJ, *et al*., 1997
	Coa 1	5'- CTTGTTGAATCTTGGTCTCGC-3'		

Then, duplex PCR protocol was developed with some modifications (Henegariu *et al*., 2003). Duplex PCR reaction was carried out in a final volume of 25 µl. The mixture was consisted of 2 µl of extracted DNA template, 1 U of *Taq* DNA polymerase (Vivantis Technologies), 2,5 µl of 10x PCR buffer (10X ViBuffer A, without $MgCl_2$), 3 mM $MgCl_2$, and 200 µM each of dNTPs (VivantisTechnologies). Primers for PCR mixture were added 10 pmol primer each 16s primer and 20 pmol each of Coa primers. A pre-PCR step at 94°C for 3 minutes was applied. A total of 35 PCR cycles were run under the following conditions: denaturation at 94°C for 45 seconds, annealing 55°C for 1 minute, and extension at 72°C for 2 minutes. As a final step sample was kept for 7 minutes at 72°C. After the thermal cycling step, ten microliters of the PCR-amplified product were analyzed by electrophoresis on a 1.5% agarose gel stained with 0.5 mg of ethidium bromide/ml. The molecular size marker, a 100-bp plus, (Vivantis Technologies) was run concurrently. Gels were visualized under UV illumination and photographed.

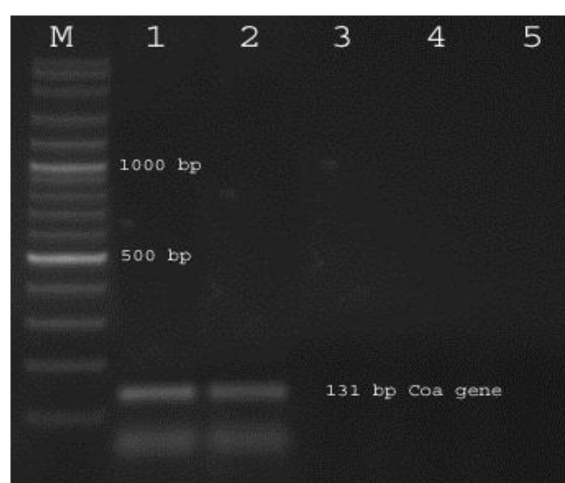

Figure 2. The amplification products of Coa primers specific for *S. aureus.* M:Marker 100-bp plus, (Vivantis Technologies), Lane 1 *S. aureus* (ATCC 25923), Lane 2 clinical specimens, Lane 3 *S. epidermidis* (ATCC 12228), and Lane 4 Clinical Specimen (Known CNS positive) and Lane 5 Negative Control (Distilled Water)

RESULTS

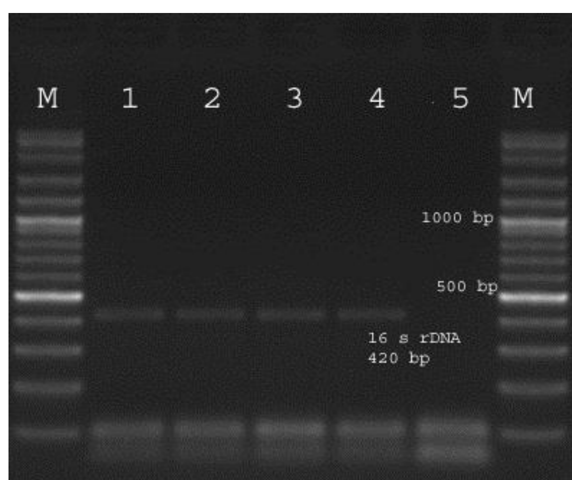

Figure 1. The amplification products of 16s primers specific for *Staphylococcus* spp. M:Marker 100-bp plus, (Vivantis Technologies), Lane 1 shows PCR products from *S. aureus* (ATCC 25923), Lane 2 shows *S. epidermidis* (ATCC 12228), Lane 3 and Lane 4 show PCR products from clinical specimens, and Lane 5 Negative Control (Distilled Water).

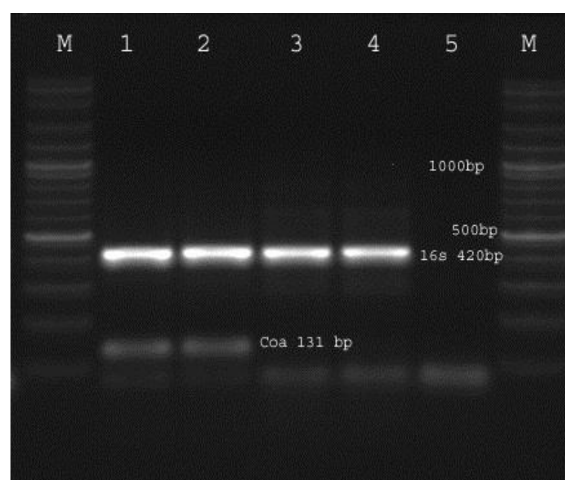

Figure 3. The amplification products of duplex PCR. M: Marker 100-bp plus, (Vivantis Technologies), Lane 1 *S. aureus* (ATCC 25923), Lane 2 clinical specimens, Lane 3 *S. epidermidis* (ATCC 12228), and Lane 4 Clinical Specimen (Known CNS positive) and Lane 5 Negative Control (Distilled Water)

In the result of simplex PCR assays, bands for 16s rDNA gene were showed in Fig 1 and bands for *Coa* gene were showed in Fig 2.

For the detection and separation of *Staphylococcus* spp. and *S. aureus* a duplex PCR analysis was performed and amplification products were showed in Fig 3.

DISCUSSION and CONCLUSION

The using of polymerase chain reaction analysis in mastitis has been suggested especially for culture negative milk samples. Because, it can be encountered with false negative results due to subclinical infections, antibiotic residues and leucocyte infiltrations (Phuektes *et al*., 2001). Similarly, Riffon *et al*., (2001) have suggested that two sets detection of major pathogens in mastitis. And these suggested techniques were used clinical and subclinical mastitis many times in field studies (Amin *et al*., 2011, Pradhan *et al*. 2011).

16s rDNA was used as a useful target gene for detection of the *Staphylococcus* spp. in different studies. This gene is often used as an internal positive control in PCR analyses for detecting characteristics of *Staphylococci* (Maes *et al*. 2002; Ardic *et al*. 2006). In this study, *Staphylococcus* spp., 16s rDNA was used effectively in control and clinical samples.

The first step in the *S. aureus* identification procedure is detecting the coagulase activity (Quinn *et al*. 1994). The expression of this property is not always detectable in vitro. Thus, the detection of *Coa* gene is important for determining the coagulase characteristic of *S. aureus* (Tiwari *et al*. 2008*)*. The determination of *Coa* gene was used for detection of *S. aureus* milk samples (Ahmadi *et al*. 2010) and especially, it was used for genotyping of *S. aureus* isolates (Goh *et al*., 1992; Hookey *et al*. 1998; Karahan and Cetinkaya, 2006).

Coa and 16s genes were combined for developing a duplex PCR procedure for detection of Staphylococci and *S. aureus* in this study. It was suggested that Coa and 16s genes combination can be used as a reliable set for PCR detection of these organisms (Fan *et al*. 2008). The best visual results in the gel were shown by using double amount of *Coa* gene in PCR mixture in this study. It is considered that these differences may cause from the number of copies of target genes or the effectiveness of the primers.

Staphylococcus spp., especially *S. aureus* causes serious and costly mastitis problems in dairy cows. There are different PCR assays for detection of these organisms from culture or milk samples. In this study, a duplex and rapid PCR protocol is developed for detecting and separating these organisms from culture and from milk samples. This procedure can be used as an alternative, reliable and fast detection and identification method for *Staphylococcus* spp. and *S. aureus* in few hours for deciding treating or culling the cows in farm base.

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