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Comparison of PCR and bacterial culture methods for diagnosis of dairy cattle's subclinical mastitis caused by *Staphylococcus aureus*

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Abstract: **To compare PCR and bacterial culture methods for diagnosis of subclinical mastitis caused by *Staphylococcus aureus*, 100 milk samples from cattle with subclinical mastitis and 20 samples from healthy cattle were collected and tested. The samples were cultured on selective blood agar and bacteria were identified by standard methods. DNA extracted from samples was subjected to PCR reaction with species specific primers and PCR products were analyzed by agarose gel electrophoresis. Based on the PCR results the prevalence of subclinical mastitis due to *S. aureus* was 25%. In the bacteriological culture of single milk sampling, *S. aureus* was isolated from the same samples being positive in PCR. A correlation of 100% was found between PCR and single milk sampling culture method by Mc Nemar test. All of the CMT negative samples were also negative in culture and PCR methods. The results of this study indicate that the PCR reaction is sensitive and specific for diagnosis of *S. aureus* in subclinical mastitis and can detect this pathogen in milk samples at species level in few hours.**

Key words: *Staphylococcus aureus*, mastitis, bacterial culture, PCR.

Introduction

Mastitis, the most common infectious disease of dairy cattle, is the most economically important disease of dairy industries around the world (7, 11, 13) through reduced milk yield and quality, cost of drugs and veterinary treatment, discarded milk, and forced culling.

Staphylococcus aureus is one of the most common causes of contagious bovine mastitis in dairy cattle worldwide and 50-100 % of herds may be infected with this pathogen (16). The majority of intramammary infections due to *S. aureus* are subclinical and hence the response of this infection to treatment is comparatively poor and it causes premature culling and elimination of animals from herd (16, 22). Furthermore, the presence of *S. aureus* in milk may present a degree of risk to the consumer because of the organism's capacity to produce enterotoxins and a toxic shock syndrome toxin-1 (TSST-1) which causes serious food poisoning (16).

Concerning the importance of *S. aureus* mastitis, monitoring of herd for diagnosis of the first cases of infection is necessary for prevention of infection spreading. Although the culture of milk is considered as a gold standard test for mastitis diagnosis, there are several disadvantages associated with bacterial culture, including no bacterial yielding from truly subclinically infected cows due to presence of high number of leukocytes or presence of preservatives or residual therapeutic antibiotics in submitted samples, intermittent shedding of organisms and cycling through low and high shedding patterns during

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lactation (11, 13, 14). Moreover, microbiological culture of milk is time consuming and species identification by standard biochemical methods requires more than 48 hours to complete.

Due to the above-mentioned limitations of cultural methods, PCR has been developed to identify various mastitis pathogens (3, 10, 11, 23). The development of PCR based methods provides a promising option for the rapid identification of bacteria. With this method, identification of bacterial species can be made in hours, rather than the days required for conventional culture methods. PCR has high sensitivity and specificity and can improve the level of detection. Theoretically, only a few numbers of the pathogens are necessary to yield a positive PCR diagnosis; so with this method the presence of pathogens can be shown at earlier stages of infection and in carrier animals, when the numbers of bacteria in milk may be very low. Moreover, PCR can detect bacteria in the presence of residua of therapeutic antibiotics and preservatives in milk and therefore there won't be false negative results because of lack of bacterial growth.

The aim of this study was to investigate the applicability of PCR reaction for diagnosis of bovine mastitis in condition of Iran and to compare PCR and bacterial culture methods for diagnosis of subclinical mastitis caused by *Staphylococcus aureus*.

Material and Methods

Collection of Milk Samples: A total of 120 milk samples were collected from individual cows in industrial dairy herds of Ahvaz. Based on California Mastitis Test (CMT), 100 samples were collected from cows with subclinical mastitis and 20 samples from CMT negative cows. Before sampling, the teat end was scrubbed with cotton soaked in 70% ethanol and the first squirt of milk was discarded. Approximately, 5ml milk was collected from each teat in sterile tubes and then the samples of one cow with score of 1+ or more in CMT test were mixed together and considered as a one sample. The samples were transferred immediately to laboratory and were kept frozen at -20°C until be tested.

DNA Extraction from Milk Samples

DNA extraction was carried out as described by Meiri *et al*., (2002) with minor modifications. The modifications were doubling the time of centrifugation, the amount of enzymes and addition a final step for DNA precipitation by ethanol. Briefly, 1 ml of each sample was transferred to a microtube and centrifuged at 14000 rpm for 4 minutes. The supernatant was discarded and the pellet was resuspended and washed 2-3 times with Tris-EDTA buffer (Tris-HCL 10mM, EDTA 1mM, pH 8.8) until a clear solution was obtained. The pellet was washed with PCR buffer (Buffer 10X: Tris-HCL 100mM, KCL 500mM, pH 8.8) and finally resuspended in 100µl of PCR buffer. Thereafter, lysozyme (Merck, Germany) was added to each sample at the concentration of 2mg/ml and the sample was incubated 20 minutes at room temperature. After this time, Proteinase K (Fermentas, USA) was added at the concentration of 400µg/ml and the sample was incubated at 56°C for 1 hour. The sample was then boiled 15 minutes and centrifuged at 14000 rpm for 45 seconds. The supernatant was transferred to a new tube and DNA was precipitated by addition of 2.5 volumes of cold ethanol, incubation at -20°C for 1 hour and centrifugation at 14000 rpm for 4 minutes. The DNA pellet was dissolved in 100µl of distilled water for using in PCR.

Oligonucleotide Primers: The sequences of *S. aureus* oligonucleotide primers have been published by Forsman *et al*., (1997). The designed primers were complementary to the 16s - 23s rRNA intergenic spacer region of the rRNA operon, which has been proven useful for identification of bacteria at the species level (1, 6, 9).

In addition of the primers used for *S. aureus* diagnosis, a set of positive control primers, specific for the bovine mitochondrial cytochrome B gene (Meiri *et al*., 2002) was also used. These control primers are intended to react with bovine somatic cells that are normally present in milk and if there were some faults in the amplification reaction, the positive control would also not give any amplification product (11). The sequence of all

Table.1. Sequence of oligonucleotide primers for identification of *S. aureus* and cow mitochondrial cytochrome B gene (Forsman *et al.*, 1997).

Target	oligonucleotide	Sequence (5 - 3)	Mgcl2 (Mm)	PCR product size (bp)
S. aureus	STAA- AUI STAA-AUII	TCTTCAGAAGATGCGGAATA TAAGTCAAACGTTAACATACG	2.0	420
Cytochrome B	BMC I BMCII	CGATACATACACGCAAACG TGTTGGGTTGTTGGAGCC	1.5	389

primers is shown in Table 1.

The PCR assay: The PCR reaction mixture contained 2.5U Taq DNA polymerase (Cinnagen, Iran), 0.4mM dNTPs, 50ρmol of forward and reverse primers, 5µl of 10X PCR buffer, 5µl of extracted DNA, 2mM $MgCl_2$ and PCR grade sterile water up to 50µl. These components were mixed in a 0.2ml PCR microtube and the reaction was carried out in a PCR thermocycler (Corbet Research, Australia). Amplification was performed through 40 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. Predenaturation was at 94°C for 2 minutes and final extension at 72°C for 5 minutes (3). Beside each set of PCR reaction, a positive control, with primers specific for mitochondrial cytochrome B gene and a negative control (water instead of extracted DNA) was prepared and tested. PCR products were electrophoresed in a 1.5% agarose gel containing 0.5 µg/ml of ethidium bromide and visualized by ultraviolet light transillumination. It was expected a DNA bond of 420bp be amplified from the positive samples.

Bacteriological Culture: Ten µl of each sample was streaked on to a 5% selective sheep blood agar containing 15mg/lit of nalidixic acid and 10mg/lit of colistin (15). The plates were incubated for 48 hours at 37°C. After this time a smear was prepared from colonies and stained by Gram staining method. Colonies that had Gram- positive cocci were examined in catalase test were followed for *S. aureus* diagnosis. The bacteria were cultured on Baired - Parker, and Manitol salt agar medium and the final and definite diagnosis was based on the following criteria:

Hemolysin, Coagulase and DNase production, manitol fermentation, production of black colonies on Baired-Parker medium and resistance to polymyxin B (15).

The bacterial culture was performed without any previous information from PCR results.

Results

Among the total of 120-tested milk samples, 25 samples, positive in CMT resulted in isolation of *S. aureus* in milk culture, and amplification of the expected 420bp PCR product. In the other words, 25% of subclinically infected dairy cows were infected with *S. aureus*. There wasn't any culture positive sample, being negative in PCR. Mc Nemar test revealed a 100% agreement between PCR and single sampling bacterial culture for *S. aureus* diagnosis. The modification we made in the extraction of DNA, also improved the results of PCR (Fig. 1).

Discussion

According to the results of this research and the previous studies on bovine mastitis in Iran (2, 4, 5, 17, 19, 21), *S. aureus* mastitis has a high prevalence in dairy cattle and remains as a main problem in herds. Recently, molecular methods, like PCR have been used successfully for the identification of mastitis pathogens (3, 10, 11, 14, 18, 23). The purpose of this study was an attempt to diagnosis *S. aureus* intramammary natural infections by PCR analysis in condition of Iran and to compare the results with those of routine bacterial culture. The used PCR method was able to identify all *S. aureus* strains isolated from intramammary infections. Khan *et al.*, (1998) showed that PCR assay had 100% sensitivity and specificity in comparison to bacterial culture method for detection of *S. aureus* in sheep milk samples.

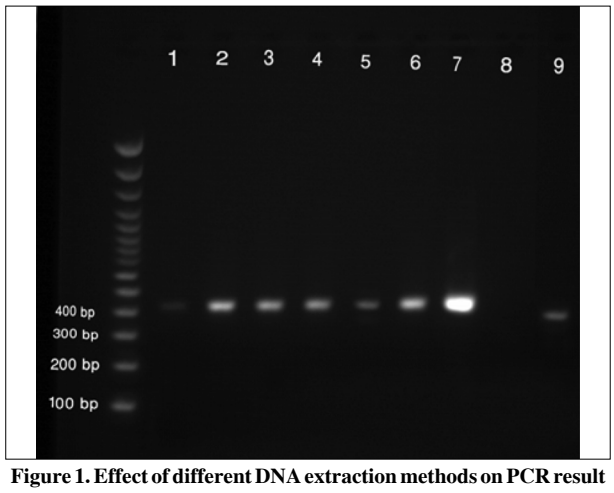

Figure 1. Effect of different DNA extraction methods on PCR result for diagnosis of *S. aureus*.
1-DNA extraction based on Meiri *et al.*, (2002) method
2-Doubling centrifugation time (14000 rpm for 4 minutes)
3-Doublimg lysozyme and proteinase K concentration (2mg/ml and 400μg/ml respectively)
4- DNA precipitation with ethanol
5- Doubling centrifugation time and enzyme concentration
6- Doubling centrifugation time and DNA precipitation with ethanol
7- Doubling centrifugation time and enzyme concentration and DNA precipitation with ethanol
8- Negative control
9-Positive control with mitochondrial cytochrome B primers (389bp)

Their result is in agreement with the result of the present study.

Riffon *et al*., (2001) designed a PCR assay based on the 23s rRNA gene sequence with two different DNA extraction methods to investigate *S. aureus* in milk samples. Their results showed that the detection limit of the assay increased by addition of a pre-PCR enzymatic step for *S. aureus* DNA extraction. They also determined that the sensitivity of the assay would be significantly increased if the calcium ions be eliminated with several washing during DNA extraction. The results of the present study also showed that doubling of centrifugation time and the amount of enzymes during DNA extraction step will increase the sensitivity of the DNA extraction method, described by Meiri *et al*., (2002).

According to our results, the CMT has enough sensitivity as a screening test for detection of subclinically affected quarters by *S. aureus*. This finding is in contrast with the results of Middleton *et al*., (2004); Janosi and Baltay (2004); Sargeant *et al*., (2001) and Ghargouzlu *et al*., (2003). This discrepancy might be related to the fact that in other studies CMT has been used for diagnosis of all types of subclinical mastitis.

In conclusion, in condition of Iran, PCR assay can be used as a rapid diagnostic method with high sensitivity for diagnosis of *S. aureus* mastitis and it can be easily used for designing of monthly or even weekly control and preventive care programs in dairy herds to determine and control the first cases of infection.

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مقالیسه دو روش PCR و کشت باکتریایی جهت تشخیص ورم پستان تحت بالینی ناشی از استافیلوکوکوس آرئوس در گاو

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به منظور PCR و روش کشت باکتریایی برای تشخیص پستان تحت بالینی از استافیلوکوکوس آرئوس در گاو از ۱۰۰ راس گاو شیری مبتلا به ورم پستان تحت بالینی و ۲۰ راس گاو شیری سالم نمونه شیر گرفته شد. نمونه های شیر به روش استاندارد در محیط آگار خون انتخابی جهت جداسازی استافیلوکوکوس آرئوس کشت گردیدند. از نمونه ها DNA نیز استخراج گردید و واکنش PCR با استفاده از پرایمرهای اختصاصی باکتری استافیلوکوکوس آرئوس انجام شد و فرآورده PCR از طریق الکتروفورز در ژل آگارز مورد آنالیز قرار گرفت. براساس واکنش PCR میزان آلودگی به باکتری استافیلوکوکوس آرئوس در گامهای مبتلا به ورم پستان تحت بالینی ۲۵ در صد تعیین گردید. براساس یک نوبت کشت نمونه های شیر نیز از ۲۵ نمونه شیری که در PCR و مثبت بودند این باکتری جدا و تعیین هویت گردید. آزمون مک نمار تطابق دو روش PCR و کشت باکتریایی جهت تشخیص ورم پستان حاصل از استافیلوکوکوس آرئوس ۱۰۰ در صد برآورد گردید. نتیجه PCR و کشت باکتریایی هیچ یک از گاوهای CMT منفی مثبت نشد. واکنش PCR حساسیت و ویژگی لازم جهت تشخیص استافیلوکوکوس آرئوس در نمونه های شیر را دارا بوده و در طی چند ساعت می تواند تشخیص قطعی در حد گونه فراهم آورد.

واژه‌های کلیدی: استافیلوکوکوس آرئوس، ورم پستان، کشت باکتریایی، PCR.

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