A polymerase chain reaction based study on the subclinical mastitis caused by *Streptococcus agalactiae*, *S. dysgalactiae* and *S. uberis* in cattle in Ahvaz

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Summary

Streptococcus agalactiae, *S. dysgalactiae* and *S. uberis* are Gram-positive bacterial pathogens that affect cows in dairy herds. These are considered as the major causes of economic losses of dairy producers without a control program. To reveal the role of these agents in inducing subclinical mastitis in dairy cattle in Ahvaz, 100 CMT-positive milk samples and 20 CMT-negative milk samples were collected and examined by polymerase chain reaction (PCR). The results were compared with the conventional bacterial culture. Based on the PCR results, *S. agalactiae*, *S. dysgalactiae* and *S. uberis* were causative agents in 20, 12.5 and 0.83% of samples, respectively. There was not any culture-positive samples being negative in PCR, but there were 10 PCR positives being negative in culture method. Based on McNemar test, there was 91.6% agreement between PCR and culture methods for the diagnosis of streptococcal mastitis. There were 14 and 7 CMT-negative samples, being positive by PCR and culture methods, respectively. The results of this study showed that subclinical infections from these bacteria are prevalent in dairy cattle of Ahvaz and regular testing and control programs should be planned to control the infection. CMT has not sufficient sensitivity for detection of subclinical mastitis due to these agents.

Key words: *Streptococcus* species, PCR, Cattle, Mastitis

Introduction

Mastitis, the most common infectious disease of dairy cows, is the most economically-important disease of dairy industries around the world. The major cause of bovine mastitis is the infection of the udder by pathogenic bacteria among which *Streptococcus agalactiae*, *S. dysgalactiae* and *S. uberis* are the most common pathogens.

S. agalactiae, an obligate bacteria in the bovine mammary gland, is susceptible to treatment with a variety of antibiotics (Keefe, 1997). Despite this fact, its prevalence in herd ranges from 11 to 47% (Radostits *et al*., 2000). It is highly contagious and causes mainly subclinical infections, which are not identified by the herdsman (Meiri-Bendek *et al*., 2002).

S. uberis and *S. dysgalactiae* are the most commonly isolated streptococci from intramammary infections. Both pathogens are widespread in the animal's environment and on the skin of the teats and are a leading cause of both subclinical and clinical mastitis in dairy cattle worldwide. *S. uberis* is now a common cause of intramammary infection occurring during the dry period, with most clinical cases occurring during the first part of lactation (Radostits *et al*., 2000).

Identification of bacterial pathogens in milk from cows with mastitis is regarded as the definite diagnosis of mastitis and is important for disease control and epidemiological studies. In most clinical laboratories, identification methods are based on bacterial culture of milk and

biochemical tests on the isolated bacteria. Advantages of culture are that the causative bacteria can be identified and tested for sensitivity to antibiotics. However, there are several disadvantages associated with bacterial culture. It is limited by the dynamic nature of infections. Subclinically infected cows are intermittent shedders of organisms and may cycle through low and high shedding patterns during lactation. Milk culture may yield no bacteria from truly subclinically infected glands due to the presence of very low number of pathogens, when samples are collected. Negative cultures may also be due to the presence in submitted samples of therapeutic levels of residual antibiotics. Furthermore, culture method cannot be performed on the preservative containing samples (Pinnow *et al*., 2001). The presence of leukocytes in milk samples from cases of clinical mastitis and in milk samples with high somatic cell counts may also potentially inhibit growth of bacteria (Phuektes *et al*., 2001). Moreover, microbiological culture of milk is time consuming. Species identification by standard biochemical methods requires more than 48 hrs.

Due to the limitations of cultural methods, polymerase chain reaction (PCR) has been developed to identify various mastitis pathogens (Forsman *et al*., 1997; Ghadersohi *et al*., 1997; Meiri-Bendek *et al*., 2002). The development of PCR-based methods provides a promising option for the rapid identification of bacteria. With this method, identification of bacterial pathogens can be made in hours, rather than the days required for the conventional culture methods. PCR can also improve the level of detection due to its high sensitivity. Theoretically, only a few numbers of the pathogens are necessary to yield a positive diagnosis. The presence of pathogens thus can be shown at earlier stages of infection and in carrier animals, when the number of bacteria in milk may be very low. Moreover, PCR can detect bacteria in the presence of residual antibiotics or preservatives in milk.

The objective of this study was to compare PCR as a molecular diagnostic method with the conventional bacterial culture in diagnosis of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* infections in mastitis cases of subclinically infected dairy cattle in Ahvaz.

Materials and Methods

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 CMT-positive cows and 20 samples were taken 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 50-ml milk samples were collected in sterile 50-ml tubes and transferred immediately to microbiology laboratory of Ahvaz School of Veterinary Medicine. The samples were kept frozen at -20°C until be tested.

DNA extraction from milk samples

DNA extraction was carried out as described by Meiri-Bendek *et al*. (2002) with minor modifications. One ml of milk was transferred in a 1.5-ml microtube and centrifuged at 14000 rpm for 4 min. The supernatant was discarded and the pellet was resuspended and washed 2-3 times with tris-EDTA buffer until a clear solution was obtained. The pellet was washed once with PCR buffer and finally resuspended in 100 µl of PCR buffer. Thereafter, lysozyme (Merck, Germany) was added to each sample at the concentration of 2 mg/ml. The sample was incubated 15-20 min at room temperature. After this time, proteinase K (Fermentas, USA) was added at the concentration of 400 µg/ml and the sample was incubated at 56°C for 1 hr. The sample was then boiled 15 min and centrifuged at 14000 rpm for 45 sec. The supernatant was transferred to a new tube and DNA was precipitated by 2.5 volumes of cold ethanol at -20°C for 1 hr. After centrifugation at 14000 rpm for 4 min, the pellet was dissolved in 100 µl of distilled water for using in PCR.

Oligonucleotides primers

The sequences of all oligonucleotide primers have been published by Forsman *et al*. (1997). The designed primers were

complementary to the 16S to 23S rRNA intergenic spacer regions of the rRNA operon, which has been proven useful for identification of bacteria at the species level (Barry *et al*., 1991; Jensen *et al*., 1993; Gurtler and Stanisich, 1996).

A set of positive control primers was also used. The control primers were specific to the bovine mitochondrial cytochrome B gene (Meiri-Bendek *et al*., 2002). The control primers are intended to react with bovine somatic cells that are normally present in milk. If there was some fault in the amplification reaction, the positive control would also not give an amplification product (Meiri-Bendek *et al*., 2002). The sequence of all primers and their optimal $MgCl_2$ concentrations are shown in Table 1.

The PCR assay

The PCR reaction mixture contained 2.5 U Taq DNA polymerase, 0.4 mM dNTPs, 50 pmol of each primer, 5 µl of 10× PCR buffer (500 mM KCl, 200 mM tris-HCl, pH = 8.4), 5 µl of extracted DNA, optimal $MgCl_2$ concentration for each primer pair and PCR grade sterile water up to 50 µl. The reaction was carried out in a PCR thermocycler (Corbet research, Australia) as follow: a predenaturation at 94°C for 2 min, 40 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec and a final step of 5 min at 72°C (Forsman *et al*., 1997). Besides, each set of PCR reaction, a positive control (with primers specific for bovine mitochondrial cytochrome B gene) and a negative control (water instead of extracted DNA) were prepared and tested.

To analyse the PCR products, 10 µl of each PCR product was electrophorezed on a 1.5% agarose gel containing 0.5 µg/ml of ethidium bromide and visualized by ultraviolet light transillumination.

Bacteriological culture

Milk samples were thawed and 10 µl from each was streaked onto 5% selective sheep blood agar containing 15 mg/L of nalidixic acid and 10 mg/L of colistin (Quinn *et al*., 1994). The plates were incubated for 24 hr at 37°C. The bacteria were identified by standard laboratory methods. Isolates were identified as *S. agalactiae* based on the positive CAMP test with *Staphylococcus aureus* and negative esculin hydrolysis. *S. dysgalactiae* were identified based on negative CAMP test with *Staphylococcus aureus* and a negative esculin hydrolysis and *S. uberis* were identified on the basis of a negative CAMP test with *S. aureus* and a positive esculin hydrolysis (Quinn *et al*., 1994).

Statistical analysis

The PCR and bacterial culture results were compared with McNemar test.

Results

PCR results

Out of 120 tested milk samples, *S. agalactiae* was detected by PCR in 24 (20%) samples of which 16 were CMT-positive and eight were CMT-negative.

In the case of *S. dysgalactiae*, PCR was found positive in 15 (12.5%) samples only nine of which were CMT-positive. In the PCR reaction for *S. uberis*, only one (0.83%) sample was positive in PCR. This sample had a positive reaction in CMT test.

In all sets of PCR reactions for the above

Table 1: Oligonucleotide primers for identification of *S. agalactiae, S. dysgalactiae, S. uberis* and mitochondrial cytochrome B gene (Forsman *et al*., 1997)

Target	Oligonucleotide	Sequence (5′-3′)	$MgCl_2$ (mM)	PCR product size (bp)
S. agalactiae	STRA-AgI	GGAAACCTGCCATTGCG	3.0	280
	STRA-AgII	TAACTTAACCTTATTAA CCTAG		
S. dysgalactiae	STRD-DyI	TGGAACACGTTAGGGTCG	3.0	270
	STRD-DyII	CTTTTACTAGTATATCTTAACTA		
S. uberis	STRU-UbI	TAAGGAACACGTTGGTTAAG	1.5	330
	STRU-UbII	TCCAGTCCTTAGACCTTCT		
Cytochrome B	BMCI	CGATACATACACGCAAACG	1.5	389
	BMCII	TGTTGGGTTGTTGGAGCC		

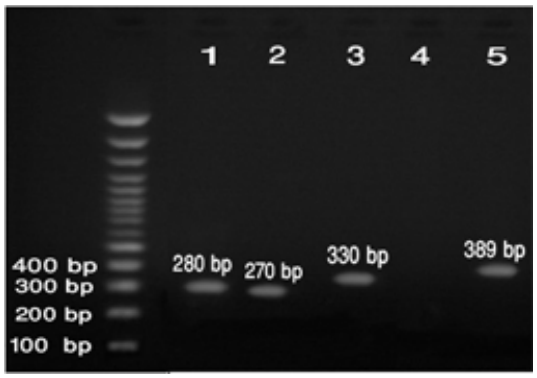

Fig. 1: PCR products electrophoresis. From left to right: DNA ladder, PCR products of *S. agalactiae*, *S. dysgalactiae*, *S. uberis*, negative control and positive control (mitochondrial cytochrome B gene)

bacteria, the positive and negative controls were accurate. The PCR products obtained for the bacteria and the bovine cytochrome B used as positive control are shown in Fig. 1.

Culture results

In bacteriological culture of milk samples, *S. agalactiae* isolated from 19 samples, 15 with positive CMT reaction, and four from CMT-negative samples. *S. dysgalactiae* isolated from 10 samples, seven of which were CMT-positive and three were CMT-negative. *S. uberis* isolated from one CMT-positive sample. There was not any culture-positive samples being negative in PCR. Based on the McNemar test, there was 91.6% agreement between the PCR and bacterial culture results for identification of these bacterial agents.

The results of PCR and culture of the samples are shown in Table 2.

Discussion

The most important streptococcal agents of bovine mastitis are *S. agalactiae*, *S. dysgalactiae* and *S. uberis*. *S. agalactiae* is a highly infectious pathogen that can rapidly spread among a herd from a single infected animal. The main source of the infection is the udder of infected cows. Although, when hygiene is poor, contamination of the environment may also provide an additional source (Radostits *et al*., 2000; Meiri-Bendek *et al*., 2002).

S. dysgalactiae and *S. uberis* are the most commonly isolated environmental streptococci of bovine mastitis. *S. uberis* has been isolated from bedding materials and lips and tonsils of cows and *S. dysgalactiae* can also be found in the environment of dairy cattle and has been isolated from the tonsils, mouth, vagina and the mammary glands (Radostits *et al*., 2000).

All of these bacteria are leading causes of both subclinical and clinical mastitis in dairy cattle worldwide. However, the importance of environmental pathogens has been raised relative to contagious pathogens (Radostits *et al*., 2000).

Good farming management and a high level of veterinary monitoring and treatment, may allow control of these pathogens in a herd. However, diagnosis is difficult, because of normally subclinical expression of these pathogens (Keefe, 1997). Therefore, early diagnosis of the presence of new infection in a herd is important for an effective control.

Based on the study of Forsman *et al*. (1997) the primers used in this PCR are able to result a PCR product by using as little as 50 pg of purified bacterial DNA, while DNA extraction procedure we used would result in 50-100 ng/µl DNA (Meiri-Bendek *et al*., 2002).

The specificity of the primers has also

Table 2: Comparison of milk samples positive in PCR and culture

Number of samples		PCR positive			Culture positive		
		S. agalactiae	*S. dysgalactiae*	*S. uberis*	*S. agalactiae*	*S. dysgalactiae*	*S. uberis*
CMT positive	100	16	9	1	15	7	1
CMT negative	20	8	6	-	4	3	-
Total	120 (100%)	24 (20%)	15 (12.5%)	1 (0.83%)	19 (15.83%)	10 (8.33%)	1 (0.83%)

been tested with DNA from 31 different bacterial species (Forsman *et al*., 1997). Therefore, it is impossible that there were false positive results in our study. On the other hand, the absence of PCR product in our negative control shows that there was not any contaminations during the PCR assay and rejects any possible false positive results. In addition, amplification of the expected PCR product by the primers specific for the mitochondrial cytochrome B gene suggests that the DNA extraction has been successfully performed and there was not any faults in the amplification reaction.

The negative bacteriological culture in cases that PCR was positive may be due to the presence of a very low number of pathogens in samples or the presence of residual antibiotics in milk. (Radostits *et al*., 2000; Phuektes *et al*., 2001). The culture positivity of CMT-negative samples suggests that CMT is less sensitive than culture for screening of infected quarters. Similar results have also been reported by Middleton *et al*. (2004).

Meiri-Bendek *et al*. (2002) developed a PCR-based on the 16S rRNA gene sequence for diagnosis of *S. agalactiae* in cow's milk. Their results showed that this PCR assay can determine one infected quarter among 125 milk samples or one infected cow among 500 cows. Therefore, the PCR has enough sensitivity and specificity for the diagnosis of *S. agalactiae* in milk samples and is more sensitive than bacterial culture. They concluded that with this method, the definite diagnosis can be made in few hours and so enable us to do monthly or even weekly testing on bulk milk samples. In this way, the first cases of infection in herd can be diagnosed and with aggressive monitoring and treatment may be able to completely eradicate this pathogen from herds.

Phuektes *et al*. (2001) used a 16S-23S rRNA spacer region based multiplex PCR assay for the diagnosis of *S. aureus*, *S. agalactiae*, *S. dysgalactiae* and *S. uberis* from cases of bovine mastitis and compared the results with the conventional milk culture. They reported that PCR was more sensitive than bacterial culture for detection of *S. aureus* and *S. uberis* but, they did not found any significant differences between PCR and bacterial culture for *S. agalactiae* and *S. dysgalactiae*. Moreover, the sensitivity of multiplex PCR was lower than individual PCR for these bacteria.

Martinez *et al*. (2001) developed a PCR method based on the 16S rRNA gene for direct diagnosis of *S. agalactiae* in cow's milk. In this study, specific identification was proven on a collection of 147 *S. agalactiae* isolates of bovine and human origin. In addition, 17 strains belonging to different bacterial species that potentially can be found in milk samples also tested negative. These researchers suggest that PCR, which has high specificity and high sensitivity (100 CFU/ml) and can be carried out in less than 24 hrs, represents an innovative diagnostic tool for the detection of *S. agalactiae* in milk.

Riffon *et al*. (2001) developed a PCR method to identify with rapidity, sensitivity, and specificity the major pathogens involved in intramammary infections in cows including *S. agalactiae*, *S. dysgalactiae* and *S. uberis*. The primers used for detection of *S. agalactiae* and *S. dysgalactiae* were designed based on the 16S rRNA gene and that for *S. uberis* was based on the 23S rRNA gene. Their results showed that these specific primers can discriminate close phylogenetic bacterial species (*S. agalactiae* and *S. dysgalactiae*). Furthermore, when used with pre-PCR enzymatic lyses step, the limit of detection was 3.12×10^2 CFU/ml, while without the pre-PCR enzymatic lyses step the limit of detection was 5×10^3 CFU/ml. They concluded that these PCR tests could be readily implemented in clinical veterinary microbiological laboratories and be of great value for prompting prevention of bovine mastitis.

As we can see, the results of other researchers are in agreement with the results of this study and suggest that PCR, as a molecular diagnostic method, can be used as a rapid diagnostic method for bovine mastitis with high sensitivity and specificity.

The results of this study suggest that subclinical infections from the above bacteria are prevalent in dairy cattle of Ahvaz. Due to the highly contagious nature of *S. agalactiae*, authors suggest that regular testing and preventive programs must be planned to control the infection.

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