

Evaluation of *Streptococcus agalactiae* detection by PCR in Milk and its comparison to other microbiological methods

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Received: September 2009, Accepted: November 2009.

ABSTRACT

Background and objectives: The development of molecular biological techniques provide a method for direct detection and identification of pathogens. This study attempted to compare bacterial culture and PCR to detect *S. agalactiae* in cattle milk samples. The PCR method was based on using primers V1 and V2 derived from the *16s rRNA* gene.

Materials and Methods: One hundred milk samples were collected from individual cattle herds of Urmia, Iran. Direct bacterial DNA extraction was attempted from 100 milk samples. The PCR method was based on using primers V1 and V2 derived from the *16s rRNA* gene. In addition, two sets of positive control primer pairs (C1 and C2) were used to identify false negatives obtained with primers V1 and V2. In bacteriological culture, biochemical tests were carried out on suspected isolates.

Results: *S. agalactiae* was isolated from 8% of the 100 samples and 15% were positive in amplification of the *16S rRNA* gene and all of the positive samples gave the expected size fragment of approximately 120bp. All isolates which were diagnosed as *S. agalactiae* in microbiological methods were also positive with PCR detection.

Conclusion: Compared with culture, PCR is less time consuming. It takes less than 24h to complete, while identification of bacteria by conventional microbiological and biochemical methods requires more than 48h. The findings of our study revealed that PCR is more sensitive than culture for the detection of *S. agalactiae* in milk.

Key words: Cattle, PCR, *16S rRNA*, *Streptococcus agalactiae*, Iran.

INTRODUCTION

Bovine mastitis is considered the major cause of economic loss to the dairy industry through reduced milk yield and quality, cost of drugs and veterinary treatment, discarded milk and forced culling (1-3). Among the various pathogens causing mastitis, *Streptococcus agalactiae* is of particular importance because it is highly infectious and causes mainly subclinical infections which are not identified by the herdsmen (4). As a result, *S. agalactiae* can spread widely within a herd, causing immediate loss due to reduced milk yield, and eventual large losses as listed above, when it is finally recognized. For this reason, it is important to identify the presence of *S. agalactiae* in a herd with the appearance of the first infected animal. Because of its subclinical nature, such identification

must rely upon laboratory diagnosis. Current methods for identifying *S. agalactiae* are based on bacteriological examinations (1). Serological methods based on surface polysaccharide antigens are often used to confirm the biochemical identification (5).

Because of its subclinical manifestation and contagious nature, control of *S. agalactiae* requires early diagnostic identification of infected cows. At present, bacteriological screening of each milking cow is performed using standard methods. Clearly, this is not sufficient for early detection. In addition, the current bacteriological methods are labor intensive and take at least 2 to 3 days to yield a result. Thus, a less costly and more rapid diagnostic test for *S. agalactiae* is needed.

The development of molecular biological techniques provide a method for direct detection and identification of pathogens. PCR is one of these techniques which has high specificity, high sensitivity and can be carried out in less than 24h. PCR represents an innovative

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Table 1: Comparing results of the PCR and microbiological methods for detection of *S. agalactiae* from milk samples.

Number of samples	Positive in microbiological methods	Positive in PCR for <i>V1-V2</i> gene	Positive in PCR for <i>C1-C2</i> gene
100	8	15	100

diagnostic tool for the direct detection of *Streptococcus agalactiae* in milk.

MATERIALS AND METHODS

Collection of milk samples. A total of 100 milk samples were collected from individual cattle in industrial dairy herds of Urmia, Iran. Before sampling, the teat end was scrubbed with cotton soaked in 70% ethanol and the first squirt of milk was discarded. Approximately, 10 ml milk was collected from each teat in sterile tubes and then samples of one cow were mixed together and considered one sample (6).

DNA extraction from milk samples. DNA extraction was carried out as described by Meiri-Bendek *et al.* (2002) with minor modifications (4). 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 1 ml, 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 2 mg/ml and the sample was incubated 20 minutes at room temperature. After this time, Proteinase K (Fermentas, Germany) was added at concentration of 400µg/ml and the sample was incubated at 56°C for 1 hour. The sample was then boiled for 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, incubated at -20°C for 1 hour and centrifuged at 14000 rpm for 4 minutes. The DNA pellet was dissolved in 100 µl of distilled water for use in PCR.

PCR amplification. To apply the PCR test for detection of *Streptococcus agalactiae* from milk samples, DNA was extracted from all milk samples. PCR was carried out using V1 and V2 primers derived from the 16S rRNA gene (4).

In addition, two sets of positive control primer pairs (C1 and C2) were used to identify false negatives obtained with primers V1 and V2. These are intended to react with the *Streptococcus* spp. which are normally present in raw milk.

The two sets of primer pairs are shown below.

V1: 5'-TTTGGTGTTCACACTAGACTG-3'

V2: 5'-TGTGTTAATTACTCTTATGCG-3'

C1: 5'-GCGTGCCTAATACATGCAA-3'

C2: 5'-TACAACGCAGGTCCATCT-3'

The PCR reaction mixture contained 2.5µl of 10× Tag polymerase buffer (1.5mM MgCl₂); 1.0µl of forward primer(10µM); 1.0µl of reverse primer(10µM); 0.2µl of dNTP (25 mM), 0.1 µl of Tag polymerase(0.25U); 5µl of DNA(50-100 ng/µl); add ddH₂O (sterile) to total volume 25 µl. The reaction was carried out in a PCR thermal cycler (Corbett, Australia), as follows: 94°C for 4 min; five cycles of 94°C, Tm°C and 72°C for 45 s each step; 20 cycles of 94°C, (Tm-4) °C, 72°C for 45s each step; and a step of 72°C for 5 min, at the end of the reaction. PCR products were run on agarose gel (1.8 to 2.0 %) and visualized by 0.005% EtBr (4).

Bacteriological Culture. For bacteriological culture, 300 microlitres from each sample was streaked onto 5% sheep blood agar. The plates were incubated for 24h at 37°C. Isolates were identified as *Streptococcus* if they were gram-positive cocci with a negative catalase reaction. Biochemical tests and CAMP test were carried out on suspected isolates (7).

RESULTS

All milk samples were positive in amplification with primer pairs C1 and C2 for *Streptococcus* spp. and gave the expected sized fragment of approximately 207 bps (Fig. 1). *S. agalactiae* was isolated from 8% of 100 samples and 15% were positive in amplification of the 16S rRNA gene with the expected sizes for amplicons of *S. agalactiae* (Fig. 2). Also, using V1 and V2 primers, no sample yielded any false negative result in comparison with amplification of C1 and C2 primers. Comparing of the PCR results using C1-C2 and V1-V2 genes and microbiological methods are shown in Table 1.

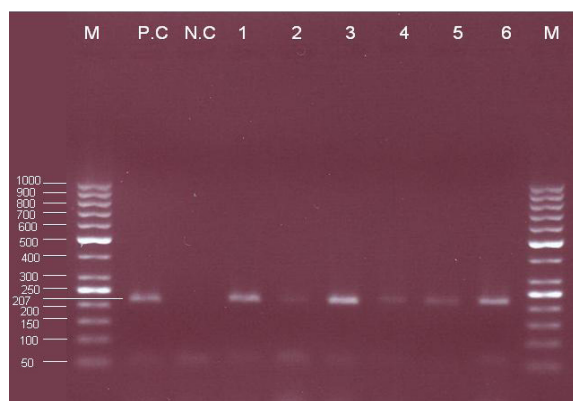


Fig. 1. PCR of raw milk samples for *Cl-C2* gene. Lanes: M; the 50bp marker (Fermentas), P-C; *S. agalactiae* reference strain ATCC 9808 (positive control), N-C; negative control containing water, 1-6: Positive milk samples.

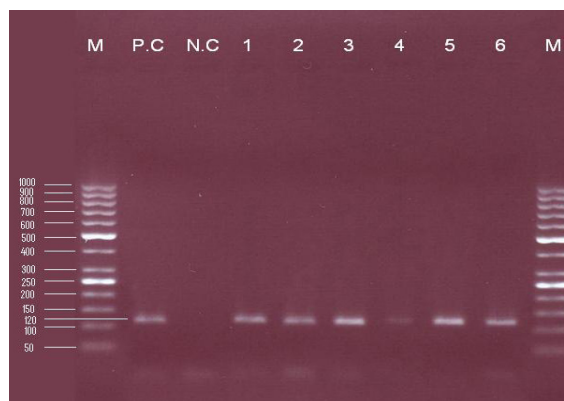


Fig. 2. PCR of raw milk samples for *V1-V2* gene. Lanes: M; the 50bp marker (Fermentas), P-C; *S. agalactiae* reference strain ATCC 9808 (positive control), N-C; negative control containing water, 1-6; Positive milk samples

All isolates with diagnosis of *S.agalactiae* in microbiological methods were also positive in PCR using *V1* and *V2* primers. Some samples were also positive in PCR while *S. agalactiae* was not isolated from it.

DISCUSSION

Streptococcus agalactiae is a highly infectious bovine mastitis pathogen that can rapidly spread throughout a herd from a single infected animal. Consequently, early diagnosis of the infection in a herd is important for effective control. Good farming management, with high level of veterinary monitoring and treatment, may allow eradication of this pathogen from the herd. Diagnosis is difficult because of the normally subclinical expression of the pathogen. Current methods for diagnosis of *Streptococcus agalactiae* are based on biochemical characteristics of the organism, such as the CAMP or esculin tests, followed by serological procedures for definitive diagnosis (1).

The aim of this study was to use a PCR- based system for a highly sensitive, low cost, rapid and specific identification of *Streptococcus agalactiae* in milk. Our results show high sensitivity and specificity of *Streptococcus agalactiae* identification using primers *V1* and *V2* specific to 16S rRNA. All *Streptococcus agalactiae* isolates and all *Streptococcus agalactiae* sequences in the Genbank had identical *V1-V2* primer sequences (4).

In a field test of the PCR procedure on an outbreak of *S. agalactiae* occurring in a herd in and they study, the *V1-V2* primer pair was tested on a set of milk samples taken directly from known infected and uninfected cows. The PCR procedure did not give any false- positive or false- negative reactions. Thus the results of the PCR method were completely specific

and consistent with those of the classical bacteriological methods (4).

The suitability of a protocol for routine diagnosis depends on several factors such as specificity, sensitivity, time required and its applicability to large numbers of samples.

Inhibitors of PCR are common in clinical specimens. Milk, blood and other biological fluids contain a variety of substances that inhibit the polymerase chain reaction and they are the reason for false negative results (8-11). Bacterial cells and thermonuclease enzyme were found to be inhibitory factors in milk (12). False-negative results may have medical consequences. Increased levels of sensitivity by PCR are required for detection of *Streptococcus agalactiae* directly from milk samples when the number of target cells can be low. Sensitivity of PCR in diagnosis of *Streptococcus agalactiae* can be improved by choice of a suitable extraction method (8, 9, and 11), selection of DNA polymerase less sensitive to inhibitors and an internal amplification control included for each sample (8,11,13,14).

Identification of bacterial pathogens in raw milk is regarded as the definitive identification of food poisoning sources. It also provides information important for prevention and control of these poisonings. In most clinical laboratories, identification methods are based on microbiological culture of milk and biochemical tests on the bacteria isolated. There are several disadvantages associated with microbiological culture. It is limited by the dynamic nature of infections of the milk sample. Milk culture may yield no bacteria from truly infected milks due to the presence of very low numbers of bacteria in samples. Negative cultures

may also be due to the presence of residual therapeutic antibiotics that may inhibit bacterial growth in vitro. The presence of leukocytes in milk samples may also inhibit growth of bacteria. Moreover, microbiological culture of milk is time consuming. Species identification by standard biochemical methods requires more than 48 h to complete (15, 16). Due to the limitations of culture based methods, PCR has been developed to identify various bacteria in milk samples (15,17-19).

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 days required for conventional culture methods. PCR can also improve the level of detection due to its high sensitivity. Theoretically, only a few cells of pathogen are necessary to yield a positive diagnosis (15).

In conclusion, conventional procedures for the identification of *Streptococcus agalactiae* are labor-intensive, and most of the commercial identification systems are not designed to identify important veterinary pathogens. We suggest detection of V1-V2 genes in raw milk samples as a developed method for identification of *Streptococcus agalactiae* that produced results in 1 day, did not need a culture step, and was specific, sensitive, and economically feasible. Finally, PCR assay can be used as a rapid diagnostic method with high sensitivity for diagnosis of *Streptococcus agalactiae* in raw milk samples and it can be used for designing accurate pasteurization or sterilization methods of milk as a major food source.

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