Haryana Vet. (Dec., 2014) **53 (2)**, 121-123

Research Article

PCR ASSAY FOR THE DETECTION OF STREPTOCOCCUS SPP. IN BUFFALO MILK

RAJENDRA YADAV[1*], ASHOK KUMAR[1], ANSHU SHARMA[2], PARVEEN GOEL[1], NEELESH SINDHU[1] and RICKY JHAMBH[1]

[1]Department of Veterinary Medicine; [2]College Central Laboratory, College of Veterinary Sciences
Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar–125 004, India

Received: 05.09.2014; Accepted: 15.11.2014

ABSTRACT

Clinical and sub clinical mastitis exerts a negative impact on milk quality, quantity and animal health along with profits. *Streptococcus* spp. is one of the main causative agents responsible for mastitis. Therefore, a suitable and specific test is required for the rapid diagnosis of these organisms. For definitive diagnosis of *Streptococcus* spp. in mastitic milk, polymerase chain reaction assay was optimized using target sequence of 16S to 23S rRNA spacer region. This is a highly sensitive and specific assay which can be performed within hours and avoids cumbersome and lengthy steps involved in microbiological culture of milk and biochemical tests. This assay can be used as a screening test for a large herd to detect *Streptococcus* spp. in milk.

Key words: PCR, *Streptococcus* spp., mastitis.

Mastitis is a multiple etiological disease, being more prevalent in high yielding dairy cattle, buffaloes, goats and sheep throughout the world and is caused primarily by microbial infection (Blowey and Edmondson, 1995; Watts, 1988). *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus dysagalactiae, Streptococcus bovis, Corynebacterium pyogenes, Pseudomonas auroginosa* and *Escherichia coli* have been considered as the major mastitogens (Radostits *et al.*, 2007). However, in the Indian subcontinent, the disease is mainly attributed to infection with *Staphylococcus* and *Streptococcus* spp. and their sero-variants (Kalorey *et al.*, 1983; Sharma and Kapur, 2000; Sharma and Sindhu, 2007). Incidence of streptococcal mastitis is reported to be higher than staphylococcal mastitis in buffaloes (Singh *et al.*, 2006). Streptococcal mastitis is highly contagious and causes mainly sub clinical infections, which are not identified by the farmers. So, the early detection of *Streptococcus* spp. is very important to institute proper treatment and control measures in buffaloes. Therefore, efforts were made to standardize a polymerase chain reaction assay to detect *Streptococcus* spp. in buffalo milk.

MATERIALS AND METHODS

Collection of Milk Samples: Milk samples were collected by standard aseptic methods and transported on ice to College Central Laboratory of the University and were kept at 4°C until subjected to cultural examination and conventional diagnostic tests like somatic cell count (SCC). A portion of milk sample was collected in a sterile vial and frozen at -20°C for PCR analysis.

DNA Extraction: SDS-phenol-chloroform-isoamyl alcohol method (Phuektes *et al.*, 2001) with some modifications was used for DNA extraction. Only those milk samples were subjected to PCR assay that were identified as being infected with *Streptococcus* spp. on the basis of routine cultural examination and conventional tests. Milk sample (1.5 ml) was centrifuged at 13,000 RPM for 10 min in a microcentrifuge tube and the upper layer of fat was removed by sterile cotton swab. The liquid portion was also discarded and the pellet was resuspended in 600µl NTE buffer [0.1M NaCl, 20mM Tris-HCl [pH7.4] and 1mM EDTA (pH 7.5)] containing SDS (0.5%) and proteinase K (100µg). After shaking vigorously, the suspension was treated with 100µl of 24% SDS and incubated in a water bath at 80°C for 10 min. It was then digested with 12µl of proteinase K (20mg/ml) (Finnzymes) and 2.5µl of RNase A (Fermentas) and incubated again in a water bath at 56°C for two hrs. 100µl of 5M NaCl and 80µl of CTAB-NaCl were then added and each tube was carefully vortexed in order to mix the contents of the tubes evenly and again incubated in a water bath at 65°C for 10 min. Equal volumes of saturated phenol: chloroform: isoamyl alcohol (PCI) mixture (25:24:1) was added after cooling to room temperature. It was then vortexed and centrifuged at

*Corresponding author: drrajendrayadav@gmail.com

12,000 g for 10 min. The upper aqueous layer was carefully transferred to a fresh microcentrifuge tube without disturbing the interface. The PCI extraction was repeated until the interface was clear. The resultant aqueous solution was extracted with equal volume of chloroform: isoamyl alcohol (CI) (Amersco) mixture in the ratio of 24:1. The aqueous solution obtained after centrifugation at 12,000 g for 10 min was then transferred to a fresh tube and one-tenth volume of 3M sodium acetate (pH 5.2) and two volumes of chilled 100% ethanol were added and kept at -20°C for 45 min. After centrifugation at 15,000 g for 15 min at 4°C, ethanol was removed by carefully inverting the tubes without disturbing the pelleted DNA. The DNA pellet was then washed twice with 70% ethanol and air dried. Finally the pellet was dissolved in 50µl of TE buffer [10mM Tris HCl, 5mM EDTA (pH8.0)] and stored at -20°C till further use.

Standardization of PCR Assay: PCR reactions were optimized using different concentrations of magnesium chloride ($MgCl_2$), *Taq* DNA polymerase, primers, annealing temperature and number of cycles in a thermalcycler (Bio-Rad icycler, USA). Oligonucleotide primers were synthesized from published sequences (Forsman *et al.*, 1997) and these were:

5'TGTTTAGTTTTGAGAGGTCTTG3' (STR-I; F) and 5'CGTGGAATTTGATATAGATATTC3' (STR-II; R).

Analysis of PCR Products: Amplified PCR products were electrophoresed in 2% agarose gel containing 0.2µg/ml ethidium bromide in Tris borate EDTA (TBE) buffer. A 100 bp ladder (Fermentas, USA) was used as a marker. Finally, the products were visualized by ultraviolet light transillumination.

Sensitivity and Specificity: Sensitivity of the PCR primers was evaluated using different dilutions (CFU/ml) of bacteria. Specificity of PCR primers was checked with milk samples infected with other mastitis causing organisms such as *Staphylococcus* spp. and *E. coli.*

RESULTS AND DISCUSSION

In the present study, primers encoding 16S-23S rRNA interspacer region were optimized as per the method of Phuektes *et al.* (2001). The optimized reaction mixture contained 200µm dNTP mix, 1X PCR buffer, 1.5 mM $MgCl_2$, 2.5 U *Taq* DNA polymerase, 10 pmol of each primers, and 300 ng of DNA extracted from milk and nuclease free water added to make reaction mixture 25 µl. PCR amplification was done with initial denaturation at 95°C for 5 min, 36 cycles each of denaturation at 95°C for one min, annealing at 58°C for 45 sec and extension at 72°C for one min followed by a step of final extension at 72°C for 7 min. The sensitivity of PCR assay was found to be 2.7 x 10^2 CFU/ml and the assay was found to be specific for *Streptococcus* spp. PCR amplification at optimized conditions yielded a product of approx. 154 bp (Fig 1). Phuektes *et al.* (2001) reported optimum concentration of $MgCl_2$ as 2.0-2.5 mM but in our study, 1.5 mM $MgCl_2$ was found to be optimum.

Khan *et al.* (1998), Kim *et al.* (2001) and Phuektes *et al.* (2001) reported the presence of PCR inhibiting substances in the milk samples despite the use of DNA purification methods. In relation to annealing temperature, Phuektes *et al.* (2001) reported optimal annealing temperature to be 59°C for 30 sec but the optimized value for annealing temperature in present study was 58°C for 45 sec.

Out of 767 Streptococci-positive milk samples, 706 (92%) samples were found to be positive for *Streptococcus* spp. by PCR assay. The sensitivity of PCR assay for primers encoding 16S-23S rRNA region in the present study i.e. 2.7 x 10^2 CFU/ml was higher than that reported by Phuektes *et al.* (2001). The higher sensitivity may be due to repeated extraction of bacterial DNA by PCI to remove possible PCR inhibitors and increasing concentration of *Taq* DNA polymerase (upto 2.5 units). The presence of inhibitors in milk and a variety of other clinical samples including urine, blood and faeces has been reported previously by Higuchi

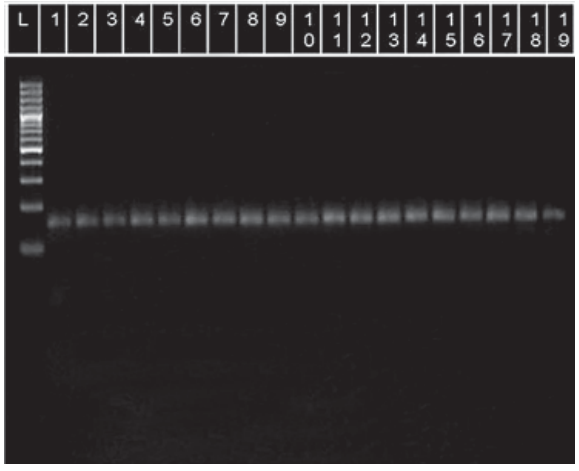

Fig 1. Amplification by STR-I and STR-II Primers Lane 1-19: Milk samples positive for *Streptococcus* spp. Lane L: 100 bp Ladder

(1989) and Toye *et al.* (1998). The primers were tested for specificity and yielded no band on gel electrophoresis with DNA of other bacterial species such as *S. aureus* and *E. coli* which resembles to the findings of Phuektes *et al.* (2001).

Identification of bacterial pathogens in milk from animals with mastitis is regarded as the definite diagnosis of mastitis and is important for disease control and epidemiological studies. In most of the clinical laboratories, identification methods are based on cultural examination 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 different antimicrobials. However, there are several disadvantages associated with bacterial culture. Subclinically infected animals 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 (Pankaj *et al*., 2013). 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. DNA-based diagnostic assays do not depend on phenotypic expression of products encoded by nucleic acids but focus on the nucleic acid composition of the bacterial genome. Therefore, DNA-based identification assays are subject to less variability as compared with diagnostic methods based on phenotypic characterization (Gillespie and Oliver, 2005). The presence of pathogens may thus be detected at earlier stages of infection and that in carrier animals, when the numbers of bacteria in milk may be very low. The 16S to 23S rRNA intergenic spacer of the rRNA operon has been proved useful for identification of bacteria at the species level and sequence variation in this spacer region has been used in simplex PCR assays for the identification of various pathogens at species level (Forsman *et al.* 1997).

The present study revealed PCR assay to be a rapid and specific test for the identification of *Streptococcus* spp. in mastitic buffaloes.

ACKNOWLEDGEMENT

Authors are very thankful to the Dean, College of Veterinary Sciences for providing the necessary facilities.

REFERENCES

Forsman, P., Tilsalatimisjarvi, A. and Alatossava, T. (1997). Identification of Staphylococcal and Streptococcal causes of bovine mastitis using 16S–23S rRNA spacer regions. *Microbiol.* **143:** 3491–3500.

Gillespie, B.E. and Oliver, S.P. (2005). Simultaneous detection of mastitis pathogens, *Staphylococcus aureus*, *Streptococcus uberis*, and *Streptococcus agalactiae* by multiplex real-time polymerase chain reaction. *J. Dairy Sci.* **88:** 3510-3518.

Higuchi, R. (1989). Simple and Rapid Preparation of Samples For PCR. In: PCR Technology: Principles and Applications for DNA Amplification. H.A. Ehrlich (edt.). Stock Press, New York.

Kalorey, D.R., Purohit, J.H. and Dholakia, P.M. (1983). Studies on incidence of sub-clinical mastitis, its etiology and *in vitro* sensitivity of isolates. *Indian J. Anim. Sci.* **53:** 961-963.

Khan, M.A., Kim, C.H., Kakoma, I., Morin, E., Hansen, R.D., Hurley, W.L., Tripathy, D.N. and Baek, B.K. (1998). Detection of *Staphylococcus aureus* in milk by use of polymerase chain reaction analysis. *American J. Vet. Res.* **59:** 80–813.

Kim, C.H., Khan, M., Morin, D.E., Hurley, W.L., Tripathy, D.N., Kehrli, M., Oluoch, A.O. and Kakoma, I. (2001). Optimization of the PCR for detection of *Staphylococcus aureus* nuc gene in bovine milk. *J. Dairy Sci.* **84:** 74-83.

Pankaj, Sharma, A., Chhabra, R. and Sindhu, N. (2013). Subclinical mastitis in Murrah buffaloes with special reference to prevalence, etiology and antibiogram. *Buff. Bull.* **32:** 107-115.

Phuektes, P., Mansell, P.D. and Browning, G.F. (2001). Multiplex polymerase chain reaction assay for simultaneous detection of *Staphylococcus aureus* and streptococcal causes of bovine mastitis. *J. Dairy Sci.* **84:** 1140-1148.

Radostits, O.M., Gay, C.C., Hinchcliff, K.W. and Constable, P.D. (2007). Veterinary Medicine (10^{th} edn.), W.B. Saunders. Philadelphia.

Ramachandraiah, K., Kumar, K.S. and Sreemannarayana, O. (1990). Survey of mastitis in a pure Jersey herd. *Indian Vet. J.* **67:** 103-106.

Sharma, A. and Kapur, M.P. (2000). Prevalence of sub clinical mastitis in cows at an organized farm. *Haryana Vet.* **39:** 13-16.

Sharma, A. and Sindhu, N. (2007). Occurrence of clinical and subclinical mastitis in buffaloes in state of Haryana (India). *Italian J. Anim. Sci.* **6:** 965-967.

Singh, S., Jaiswal, S., Singh, D.D. and Singh, H. (2006). Bovine Mastitis and Udder Affections. (1^{st} edn.), International Book Distributing Co., Lucknow.

Toye, B., Woods, W., Bobrowska, M. and Ramotar, K. (1998). Inhibition of PCR in genital and urine specimens submitted for *Chlamydia trachomatis* testing. *J. Clin. Microbiol.* **36:** 2356-2358.