

DIAGNOSIS OF STAPHYLOCOCCAL MASTITIS DIRECTLY FROM MILK OF MURRAH BUFFALOES AND CROSS BRED COWS BY 16S-23S RIBOSOMAL RNA INTERGENIC SPACER PCR ANALYSIS

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ABSTRACT

In the present study, genus specific primers based on 16S to 23S rRNA intergenic spacer region were standardized for detection of *Staphylococcus* spp. directly from mastitic milk. By standardized optimum concentrations of magnesium chloride, *Taq* DNA polymerase, primers, optimum annealing temperature and number of cycles in thermocycler, PCR assay yielded amplified product sizes of 200bp and 298bp. Out of 228 and 367 milk samples from *Murrah* buffaloes and crossbred cows, 15 (6.58%) and 83 (22.61%) milk samples respectively revealed detection of *Staphylococcus* spp. by genus specific PCR, while conventional microbiological methods could detect 13 (5.70%) and 78 (21.25 %) samples respectively to be positive for staphylococcal mastitis. All the culturally positive milk samples were confirmed as belonging to genus *Staphylococcus* by PCR. As many as 60 and 67.95% of staphylococcal samples from *Murrah* buffaloes and crossbred cows were found positive by PCR using primers specific for detection of *Staphylococcus aureus* indicating public health significance. This assay was found to be rapid, sensitive and specific which could also detect staphylococci from milk samples of antibiotic treated animals. PCR assay can serve as a valuable tool in screening of large herds for staphylococcal mastitis.

Keywords: Mastitis, *Staphylococcus* spp., 16S-23S rRNA interspacers region, PCR

INTRODUCTION

Mastitis continues to be a major challenge to dairy industry worldwide despite the widespread implementation of mastitis control strategies. About 200 bacterial species, sub-species and serovarieties have been isolated from bovine mammary glands affected with mastitis (1, 2). In India, *Staphylococci* (*Staphylococcus aureus* and coagulase-negative staphylococci) have been reported as the most common pathogens associated with mastitis (3, 4, 5, 6, 7). *Staphylococcus aureus* infections are being contagious to other cows within a herd and occasionally to other herds and the cause of both clinical and subclinical infections. Coagulase-negative staphylococci (CNS) are environmental pathogens part of the normal skin microflora of dairy ruminants particularly in prepartum heifers and primiparous cows (8) causing subclinical infections of udders characterized by an elevated somatic cell count in milk samples and decreased milk production (9). Presence of *Staphylococcus aureus* in milk may lead to high degree of risk to consumers since they produce a battery of enzymes/toxins viz. catalase, coagulase and hemolysins. Due to production

of hyaluronidase, they are highly invasive organism, form abscesses which may result in fibrosis. Because of intracellular nature and development of the L-forms, staphylococci strains often resist phagocytosis and develop antibiotic resistance. Therefore timely detection of staphylococci by rapid, sensitive and specific technique is imperative. The present study was planned to standardize 16S to 23SrRNA intergenic spacer based polymerase chain reaction assay for detection of *Staphylococcus* spp. from mastitic milk of crossbred cows.

MATERIALS AND METHODS

Collection of Milk samples:

A total of 228 milk samples from 57 *Murrah* buffaloes and 367 milk samples from 92 crossbred cows (Hariana X Holstein Friesian with mixed crosses of Jersey, Sahiwal and Brown Swiss) were received in the Veterinary College Central laboratory, College of Veterinary Sciences, CCS, Haryana Agricultural University, Hisar from established farms and included in the present study. Milk samples were collected aseptically and were kept at 4°C until bacteriological

examinations were performed. A portion of the milk sample to be tested was collected into sterilized tubes and frozen at -20°C until tested for PCR.

Bacteriological examination

Ten microlitres of milk from each sample was streaked on 5 % sheep blood agar plates and MacConkey's lactose agar plates respectively. After incubation for 24 hours at 37°C, the resulting growth from respective plates of media was examined for colony characteristics, morphology, gram's reaction and haemolysis patterns. All gram positive, catalase positive and oxidase negative isolates were confirmed as belonging to genus *Staphylococcus*. Staphylococcal isolates were classified as coagulase positive and coagulase negative on basis of the citrate rabbit plasma coagulase test. Further characterization was done on the basis of identification schemes as described earlier (10). Coagulase positive isolates were confirmed as *Staphylococcus aureus* based on thermostable nuclease test (6), latex agglutination test (Staph latex test kit, HiMedia, Mumbai) and utilization of mannitol and maltose. Coagulase negative staphylococci were further characterized on basis of different biochemical reactions such as urease test, DNase test, phosphatase test and utilization of various sugars as described earlier (11).

DNA Extraction from milk

Mastitic milk (1.5 ml) was centrifuged and the upper layer of fat was removed. The pellet was then resuspended in 600µl NTE buffer (0.1M NaCl, 20mM Tris-HCl and 1mM EDTA). After vortex, the suspension was treated with 100µl of 24% sodium dodecyl sulphate and incubated in a water-bath at 80°C for 10 minutes. This was then digested using 12µl of proteinase K (20mg/ml) (Finnzymes) and 2.5µl of Ribonuclease A (Fermentas, USA) and incubated in water-bath at 56°C for 2 hours. 100µl of 5M NaCl and 80µl of CTAB-NaCl was then added and incubated in a water-bath at 65°C for 10 minutes. The resultant mixture was treated with an equal volume of saturated phenol:chloroform:isoamyl alcohol (PCI) mixture (25:24:1) The PCI extraction was repeated until the white interface was clear. The resultant aqueous solution was extracted with equal volume of chloroform:isoamyl alcohol mixture in the ratio of 24:1. The resultant aqueous phase was collected 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 minutes for precipitation of DNA. After centrifugation at 15000 g for 15 minutes at 4°C, the DNA pellet obtained was then washed twice with 70% ethanol and air-dried. Finally DNA was dissolved in 50µl of in TE buffer (10mM Tris HCl, 5mM EDTA) and stored at -20°C till further use. The purity and concentration of the DNA isolated was measured using a biophotometer (Eppendorf, Germany).

Standardization of PCR

Sequences of two genus specific oligonucleotide primers

of 21 and 19 bases GGAATAACGTGACATATTGTA (STA-I) and TTCACTCGGTTTGCTTGG (STA-II), were employed. For further differentiation of staphylococci into coagulase positive (*Staphylococcus aureus*) and coagulase negative staphylococci, primers specific to *Staphylococcus aureus* TCTTCAGAAGATGCGGAATA (STA-AuI) and TAAGTCAAACGTTAACATACG (STA-AuII) were taken. These primers were selected from published sequences (12) and synthesized from Operon Biotechnologies, Germany. PCR reactions were standardized using different magnesium chloride concentrations, *Taq* DNA polymerase concentrations, primer concentrations, annealing temperature and number of cycles in thermocycler (Bio-Rad iCycler, USA). Species specific primers for *Staphylococcus aureus* were optimized as per protocol described earlier (13). DNA isolated from pure bacterial culture of *Staphylococcus aureus* ATCC 25923 was taken as positive control and nuclease free water was taken as negative control.

Analysis of PCR products

PCR products were analyzed in two per cent agarose gel containing 0.2µg/ml ethidium bromide (Fermentas, USA) as markers. Products were visualized by ultraviolet light transillumination in Tris borate EDTA (TBE) electrophoresis buffer at 6.5 V/cm for one hour with 100 bp ladder. Sensitivity of PCR primers was evaluated by using different dilutions (CFU/ml) of bacteria. Specificity of PCR primers was checked with amplification of DNA isolated from milk samples infected with *E.coli*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis*.

RESULTS

The optimized PCR reaction mixture for species specific primers contained 200µm dNTP mix, 1X PCR buffer (with 10mM Tris-HCl, pH 8.8, 50mM KCl and 0.8% Nonidet P 40), 1.5 mM MgCl₂, 2.5 U *Taq* DNA polymerase, 10 pmoles of each primers, 200 ng of DNA extracted from milk and nuclease free water added to make reaction mixture volume of 25 µl. Optimized conditions for PCR amplification were obtained as follows: initial denaturation at 95°C for 5 minutes, 36 cycles each of denaturation at 95°C for 1 minute, annealing at 58°C for 30 seconds and extension at 72°C for 30 seconds followed by final extension at 72°C for 10 minutes. All the isolates belonging to genus *Staphylococcus* were subjected to PCR and two amplified products of approximately 200bp and 298bp were yielded at optimized conditions (Figure 1). Out of 228 milk samples from Murrah buffaloes and 367 milk samples from crossbred cows, 13 (5.70%) and 78 (21.25 %) milk samples respectively were identified to be positive for presence of *Staphylococcus* spp. on basis of catalase, oxidase, gram's reaction and haemolysis patterns. When these staphylococcal isolates were characterized further by biochemical tests, 7 (3.07%) samples from Murrah buffaloes and 47 (12.81%) milk samples from crossbred cows were identified as *Staphylococcus*

aureus. When samples were screened by PCR based on 16S-23S rRNA interspacer region, a total of 15 (6.58%) and 83 (22.61 %) samples were found positive by genus specific primers and 9 (3.95%) and 53 (14.44%) were found positive by species specific primers in samples from *Murrah* buffaloes and crossbred cows respectively. All culturally negative samples which were found positive for *Staphylococcus* spp. by PCR were further identified as *Staphylococcus aureus* by species specific primers. None of the milk samples positive for other mastitis pathogens viz. *Streptococcus dysgalactiae*, *Streptococcus agalactiae*, *Streptococcus uberis* and *E. coli* revealed amplification with PCR showing 100% specificity of the assay.

DISCUSSION

The advent of nucleic acid amplification and detection has resulted in change from conventional laboratory methods that rely on phenotypic expression of antigens or biochemical products, to molecular methods for the rapid identification of a number of infectious agents (14). The 16S-23S rRNA intergenic spacer of the ribosomal RNA operon is considered non functional and is consequently argued to be under minimal selective pressure (15) which provides promising options for identification of various bacterial pathogens. In the present study, genus specific PCR assay based on 16S-23S rRNA interspacer region for detection of staphylococci yielded two amplified products of 200bp and 298bp. The possible explanation for more than one PCR product might be due to the fact that bacterial genome can contain several *rrn* operons and the weaker band may represent heteroduplex molecules resulting from cross hybridization of amplification products from different kinds of operons (16). The majority of culturally negative samples found positive by PCR in the present study were from animals which were treated with antimicrobials. In these cases, negative cultures might be due to presence of residual antibiotics that may render bacterial strains nonviable. Our study is in accordance to previous reports in which etiological agents of culturally negative samples were identified by molecular analysis (17, 18). Another explanation for negative culture results may be the presence of leucocytes in milk samples with high somatic cell count which may also potentially inhibit growth of bacteria (13). All the culturally negative and PCR positive samples were identified as *Staphylococcus aureus* by species specific PCR protocol based on 16S-23S rRNA interspacer region. The presence of high number of *Staphylococcus aureus* infections indicates a public health implication where strict management practices especially hygienic measures are imperative for the safe production of milk for human consumption. Earlier reports (12, 19, 20, 21, 22) have also used 16S-23S ribosomal RNA spacer based PCR assays for specific detection of staphylococci. However, all these studies reported isolation of DNA from cultural isolates for use in PCR amplification. In the present study, amplification has been successfully done

with DNA isolated from mastitic milk of crossbred cows directly. Initially the sensitivity was found to be quite low which may possibly be due to presence of PCR inhibitors present in milk (23). Other investigators also encountered similar problems in 'nuc' gene based PCR directly on milk samples (24, 25). They observed that the possible presence of PCR inhibiting substances in the milk samples inhibit the *Taq* DNA polymerase reaction increasing optimum concentration levels. In our study, repeated extraction of bacterial DNA by phenol chloroform isoamyl alcohol to remove possible PCR inhibitors and increasing concentration of *Taq* DNA polymerase concentration attributed the successful amplification of DNA isolated directly from mastitic milk.

Genus specific PCR based on 16S-23S rRNA interspacer region was found to be specific and sensitive assay for detection of *Staphylococcus* spp. which could be completed within 8 hours. The cumbersome, time consuming and lengthy steps involved in microbiological culture of milk and biochemical tests can be avoided by PCR assay for screening large herds to detect staphylococcal organisms directly from mastitis milk of *Murrah* buffaloes and crossbred cows to ensure early detection and rapid treatment measures.

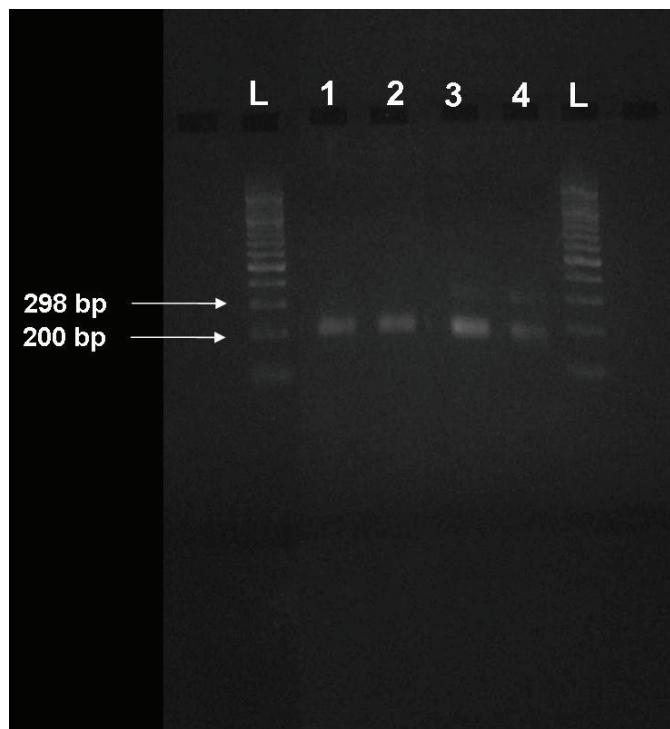


Fig. 1:
Polymorphism exhibited by Genus specific 16S-23S rRNA primers

Lane 1, 2: 200 bp;
Lane 3, 4: 200 bp and 298 bp;
Lane L: 100bp Ladder

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