COAGULASE GENE BASED MOLECULAR DETECTION OF STAPHYLOCOCCUS AUREUS DIRECTLY FROM MASTITIC MILK SAMPLES OF MURRAH BUFFALO

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ABSTRACT

The study was conducted to detect Staphylococcus aureus directly in mastitic milk of Murrah buffaloes using coagulase gene based specific polymerase chain reaction assay. Out of 628 samples, a total of 140 samples were found positive with four amplified products of size 960 bp, 870 bp, 740 bp and 610 bp in 8.57 percent, 19.28 percent, 29.29 percent and 42.85 percent of the milk samples, respectively. On PCR examination of Staphylococcus aureus found positive by bacteriological examination and biochemical tests, similar amplified products were observed in 9.37 percent, 21.09 percent, 32.03 percent and 37.5 percent of culture isolates (n=128) respectively. Ubiquitous PCR assay with amplified product of size 108 bp was used as an internal control for detection of Staphylococcus aureus. By this assay, nonviable Staphylococcus aureus could also be detected in milk samples of animals treated with antibiotics. The study revealed that several coagulase gene types are responsible for genetic heterogeneity among Staphylococcus aureus isolated from mastitis cases in buffaloes and predominance of these amplified products shows significant variation over time paving way for understanding of epidemiology of mastitis in a particular location.

Keywords: mastitis, Staphylococcus aureus, coa gene, Murrah

INTRODUCTION

Buffaloes constitute about 35 percent of the bovine population but contribute more than 55 percent to the total milk production in India (Kumar et al., 2007). India has about 22 breeds of riverine buffaloes (Ahlawat et al., 2006) of which the Murrah breed, found most abundantly in Haryana State, is capable of milk yields as high as 35 litres a day (http://www.haryana-online.com/murrah.htm). Despite intense research and control programs, bovine mastitis has remained a major economic problem of the dairy industry (Dua, 2001; Sasidhar et al., 2002; Hillerton and Berry, 2005; Halasa et al., 2007; Huijps et al., 2008; Denis et al., 2009). In India, a plethora of bacteria have been isolated and designated as etiological agents of mastitis in buffaloes, but Staphylococcus aureus has been reported as the major pathogen (Dang et al., 2007; Sahay et al., 2007; Sharma and Sindhu, 2007; Sindhu et al., 2008). Polymerase chain reaction (PCR) amplification of the 3’ end coding variable region of coagulase gene has been considered a candidate for DNA diagnostic assay for identification of Staphylococcus aureus in cow mastitis (Aarestrup et al., 1995; Guler et al., 2005; Da Silva and Da

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Silva, 2005; Vimercati et al., 2006; Kalorey et al., 2007) but there are very few reports of this technique being used in buffaloes (Vieira-da-Motta et al., 2001). The study was carried out to detect Staphylococcus aureus directly from mastitic milk samples of Murrah buffaloes using coagulase gene based PCR assay and to compare the results with bacteriological examination.

MATERIALS AND METHODS

Collection of milk samples: A total of 628 milk samples collected from functional quarters of lactating Murrah buffaloes from organized farms and individual farmers brought from various parts of Haryana to the Veterinary College Central Laboratory, COVS, CCS Haryana Agricultural University, Hisar, were included in the present study.

Bacteriological examination: Ten microlitres of milk from each sample was streaked on five percent sheep blood agar plates and MacConkey’s lactose agar plates separately and incubated for 24 h 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 identified as Staphylococcus spp. Staphylococcal isolates were further classified as coagulase positive and coagulase negative on basis of standard citrate rabbit plasma coagulase test (Gibbs and Skinner, 1966). Coagulase positive isolates were further characterized biochemically by thermostable nuclease test (Faruki and Murray, 1986), latex agglutination test (Staph latex test kit, HiMedia, Mumbai) and mannitol fermentation.

DNA extraction directly from milk: DNA from milk samples was extracted by the method described by Phuektes et al. (2001) with some modifications. In brief, 1.5 ml of milk sample was centrifuged and the pellet suspended in 600 μl NTE buffer. After vortex, the suspension was treated with 100 μl of 24% sodium dodecyl sulphate and incubated in water-bath at 80°C for 10 minutes. The suspension was then digested using 12 μl of proteinase K (20 mg/ml, Fermentas, USA) and 2.5 μl of Ribonuclease A (Fermentas, USA) and incubated in a water-bath at 56°C for 2 h. 100 μl of 5M NaCl and 80 μl of CTAB-NaCl was then added and incubated in a water-bath at 65°C for 10 minutes. Then phenol:chloroform:isoamyl alcohol (PCI) and chloroform:isoamyl alcohol (CI) extraction was done until the interface was clear. 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 one hour for precipitation of the DNA. After centrifugation at 15000 g for 15 minutes at 4°C, ethanol was removed and washing of DNA pellet was done twice with 70% ethanol and then it was air-dried. Finally, the DNA was dissolved in 50 μl of TE buffer and stored at -20°C till further use. Purity and concentration of the DNA isolated was recorded using a biophotometer.

DNA extraction from bacterial culture isolates: For extraction of DNA from bacterial culture isolates and standard strains, the rapid boiling method was followed. In brief, a single colony from overnight grown culture was inoculated in 25 μl of TE buffer and boiled at 99°C for 15 minutes and then cooled immediately by putting on ice. The resultant template DNA was stored at -20°C and 5 μl of each sample was used for PCR analysis.

Polymerase chain reaction assay (PCR): 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). 5’-3’ sequences of oligonucleotide primers taken were F: ACCACAAGGTACTGAATCAACG and R: TGCTTTGATTGTTGCAG. These were taken from a report published earlier (Aarestrup et al., 1995). For internal control, ubiquitous PCR assay as described by Martineau et al. (1998) was performed. 5’-3’ sequences of primers taken for ubiquitous PCR assay were F: AATCTTGTCGGTACACGATATTCTTCACG and R: CGTAATGAGATTTCAGTAGAATAATACAAACA. The PCR reaction was performed in a thermocycler with a reaction volume of 25 μl. DNA isolated from pure bacterial culture of Staphylococcus aureus ATCC 25923 was taken as positive control, nuclease free water was taken as negative control, and PCR mixture without template was taken as PCR control to check for the possibility of contamination.

**Analysis of PCR Products:** After amplification, five microlitres of amplified products were subjected to electrophoresis in 2% agarose gel prepared using 0.5X Tris Borate EDTA (Amresco) containing ethidium bromide at a concentration of 0.2 μg/ml. A 100 bp gene ruler (Fermentas) was used as marker. Electrophoresis was carried out at 6.5 V/cm of gel in 0.5X TBE running buffer in submarine electrophoresis apparatus, using a power supply (Amersham Pharmacia Biotech) for one hour. The gel was visualized using UV transilluminator (Biovis Gel V4). Sensitivity of PCR primers was evaluated by using different dilutions (CFU/ml) of bacteria. Specificity of PCR primers was checked with milk samples inoculated with Streptococcus dysgalactiae, Streptococcus agalactiae, Streptococcus uberis and E. coli.

**RESULTS AND DISCUSSION**

The optimized reaction mixture for coa gene based assay contained 200 μm dNTP mix, 1X PCR buffer (with 10 mM Tris-HCl, pH 8.8, 50 mM KCl and 0.8% Nonidet P 40), 1.5 mM MgCl$_2$, 20 pmol of each primer, 2.5 U Taq DNA polymerase, and 200 ng of DNA extracted from milk and DEPC treated nuclease free water added to make reaction mixture 25 μl. PCR amplification was done with initial denaturation at 95°C for 5 minutes, 36 cycles each of denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 1 minute followed by final extension at 72°C for 10 minutes. The optimized PCR protocol for ubiquitous PCR assay was similar to coa gene based assay except that concentration of MgCl$_2$ was found to be 2.5 mM. None of the streptococcal and E. coli isolates were found positive with PCR when amplified with primers encoding coa gene, showing 100 percent specificity of primers.

On bacteriological examination, out of 628 milk samples, a total of 238 samples (37.89 percent) were found culturally positive. Further characterization on basis of colony characteristics, morphology, Gram’s reaction and haemolysis patterns revealed 156 staphylococci (65.55 percent), 72 streptococci (46.15 percent) and E. coli (6.41 percent). Staphylococcal culture isolates were further differentiated into 128 coagulase positive (53.78 percent) and 28 coagulase negative staphylococci (11.76 percent) on basis of standard citrate rabbit plasma coagulase test. All coagulase positive isolates were identified biochemically as Staphylococcus aureus, and no isolate of other coagulase positive Staphylococci (viz. Staphylococcus intermedius and Staphylococcus hyicus) was found. When these Staphylococcus aureus culture isolates were screened by coa gene based PCR under optimized conditions, all isolates were found positive revealing 100 percent sensitivity.
Figure 1. *Coa* gene amplification of *Staphylococcus aureus*.
Lane 1, 9, 11, 12: 610 bp; Lane 5, 6, 8, 10: 740 bp;
Lane 2, 7: 870 bp; Lane 3: 960 bp;
Lane 4: *coa* negative; Lane 13: Negative control;
Lane L: 100bp Ladder

Figure 2. Ubiquitous PCR assay for *Staphylococcus aureus*.
Lane 1-4: *Staphylococcus aureus* Positive samples;
Lane 5: *Staphylococcus aureus* ATCC 25923;
Lane 6: Negative control with Nuclease free water;
Lane L: 100bp Ladder
Extensive polymorphism with four amplified products (Figure 1.) of size 960 bp, 870 bp, 740 bp and 610 bp was observed in 12 (9.37 percent), 27 (21.09 percent), 41 (32.03 percent) and 48 (37.5 percent) respectively. When these isolates were screened by ubiquitous PCR assay, all the *Staphylococcus aureus* isolates revealed amplified product of size 108 bp. (Figure 2).

When milk samples were subjected directly to PCR based on *coa* genes, a total of 140 (56 percent) samples were found to be positive with four amplified products of molecular weight 960 bp, 870 bp, 740 bp and 610 bp in 12 (8.57 percent), 27 (19.28 percent), 41 (29.29 percent) and 60 (42.85 percent) respectively. All the culturally negative twelve samples which were tested positive by direct PCR with amplified product of 610 bp were found to be taken from buffaloes with histories of prior administration of antibiotics for treatment of mastitis, and this may have inhibited the growth of bacteria. These “no-growth” samples pose a challenge for microbiological laboratories, veterinarians, and dairy producers. Failure of growth in as many as thirty percent of milk samples from clinical and subclinical bovine mastitis even after 48 h of conventional culture have been reported and identified by molecular analysis (Barlow et al., 2008; Sharma et al., 2009; Taponen et al., 2009).

The etiology of mastitis is diverse and varies significantly internationally as well as within regions within countries and between farms, and shows significant variation over time and between seasons on individual units (Bradley et al., 2007). Analysis of coagulase-encoding *Staphylococcus aureus* DNA (*coa*) genes has demonstrated variable sequences in the 32 -end coding region. This region contains a polymorphic repeat region that can be used to differentiate *Staphylococcus aureus* isolates (Goh et al., 1992; Guler et al., 2005). This genetic variability may contribute to the emergence of distinct epidemiological profiles which are dependent on predominant strains within a herd, suggesting the necessity to identify such strains or subtypes before applying specific measures of mastitis control (Zecconi and Piccinini, 1999).

On intensive review of literature, no study has been reported in India regarding PCR amplification of *coa* gene in Murrah buffaloes. In our study, four amplified products of sizes of approximately 610, 740, 870 and 960 bp were obtained, and this is in close agreement with the findings of Vieira-da-Motta et al. (2001) in buffaloes who reported similar fragment sizes of 612, 740, 870 and 964 bp in 8.6 percent, 29.7 percent, 19.5 percent and 42 percent samples, respectively. In a study in cows, Kalorey et al. (2007) obtained three different products of 627, 710 and 910 bp for 20, 10 and seven isolates respectively.

Studies carried out on PCR amplification of *coa* gene in different countries using the same primer pairs revealed extensive polymorphism with predominance of one or more of *coa* gene amplified products among *Staphylococcus aureus* responsible for mastitis in cows and buffaloes. Annemuller et al. (1999) obtained four PCR products of 990, 900, 800, and 740 bp, with 990 bp being the predominant product. Raimundo et al. (1999) reported 73.3 and 15.2 percent of isolates assigned to 1000- and 700- to 750- bp products, respectively. Lange et al. (1999) found seven PCR products ranging from 580 to 1060 bp. Schlegelova et al. (2003) identified three coagulase genotypes, and the 730 bp product was predominant in 83.3 percent of the isolates. Guler et al. (2005) obtained 1000-, 900-, 800-, and 700-bp PCR products in 60.8, 16.8, 12.8, and 9.6 percent of the isolates, respectively. Da Silva and Da Silva (2005) reported twenty-seven amplicons ranging from 579 bp to 1442 bp with 790, 759, 725 and 579 bp accounting for 52 percent of the isolates. Katsuda et al. (2005) found five types of amplified products ranging from 420 ± 20 bp to 820 ± 20 bp. Vimercati et al. (2006) observed amplified products of *coa*
gene ranging from 420 to 900 bp. Moon et al. (2007) reported amplified products between 620 to 809 bp in Staphylococcus aureus isolates. Saei et al. (2009) observed five different PCR products with molecular weight ranging from 490-850 bp in a study in nine dairy herds.

When pathogens of multiple genotypes infect a host, they compete for source of nutrition and transmission. In this condition, the genotype with greater virulence is competitive (Nowak and May, 1994). The predominance of one or more coa gene genotypes may be more beneficial in the control of Staphylococcus aureus mastitis since they were reported to be more resistant to neutrophil bactericidal activities than rare genotypes (Su et al., 1999). It also suggests a common source, host to host transmission i.e. contagious transmission, host adaptation of subsets of the population of Staphylococcus aureus strains. Also, differences in distribution of coagulase gene variants in Staphylococcus aureus may reflect presence of virulence factors responsible for suppressing host defence mechanisms (Goh et al., 1992).

In conclusion, profile of coa gene based genotypic PCR assay can be used as additional suitable identification criterion to differentiate among coagulase staphylococci for understanding the epidemiology of mastitis associated with high milk yielding Murrah buffaloes since it enables rapid and accurate diagnosis of Staphylococcus aureus within hours and paves the way for new approaches aimed at improvement in mastitis diagnosis, treatment, prevention and control.

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