

PCR-RFLP of *Staphylococcus aureus* Coagulase Gene Isolated from Bovine Subclinical Mastitis

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The present study was carried out to characterize *Staphylococcus aureus* isolated from subclinical cases of mastitis in cattle by PCR-RFLP using coagulase (*coa*) gene. In our study, 38 isolates of *S. aureus* recovered from Holstein-Friesian (H-F) crossbred and Rathi (a native breed) cattle with subclinical mastitis were subjected to *coa* gene amplification. Out of 38 isolates, 35 strains produced single amplicon of either 400, 490, 510, 550, 600, 710, 760, 810 or 850 bp while three isolates did not produce any *coa* amplicon. Subsequently, the products were digested with restriction enzyme *AluI* for typing of *coa* gene. Results showed higher *coa* gene polymorphism of *S. aureus* isolated from native breed (eight *coa* types) as compared to that in H-F crossbred cattle (three *coa* types). The *AluI* restriction endonuclease generated four and seven RFLP patterns with isolates from H-F crossbred and Rathi cattle respectively. The RFLP patterns obtained from similar amplicons in isolates from these two breeds did not show any variation.

Key words. *Staphylococcus aureus*, Mastitis, *coa* gene, PCR, RFLP.

Mastitis is a well known challenge to dairy sector in India caused mostly by *Staphylococcus aureus*. This bacterium is associated with various forms of mastitis of which subclinical mastitis has unique importance as they go unnoticed and continue to affect production performance of animal [Suleiman *et al.*, 2012]. More than 50% cases of subclinical mastitis caused by *S. aureus* have been reported to turn into clinical mastitis resulting in serious consequences [Pearson and Mackie, 1979]. Further milk from animals with subclinical mastitis may lead to different subsequent infections or food poisoning as such in the consumers [Abdel *et al.*, 2009; Oliviera *et al.*, 2011].

The organism produces a variety of extracellular and cell wall associated virulence factors which are involved in the pathogenesis of mastitis [Momtaz *et al.*, 2010]. Coagulase, a collagen binding protein encoded by *coa* gene, is directly related with bovine mastitis [Momtaz *et al.*, 2010]. The *coa* gene amplification has been considered a simple and accurate method for typing of *S. aureus* [El-Jakee *et al.*, 2010]. The *coa* gene has a polymorphic repeat region comprising of 81bp tandem short sequence repeats (SSRs) that can be used for differentiating *S. aureus* isolates [Van Belkum *et al.*, 1998].

In the present study *S. aureus* isolates obtained from H-F crossbred and Rathi (a native breed) were investigated for polymorphism in *coa* gene by PCR-RFLP in order to determine variations among isolates for epidemiological typing.

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MATERIALS AND METHODS

Sampling

Eighty five milk samples were collected during early morning hours in sterilized test tubes from Holstein–Friesian (H-F) crossbred and Rathi cattle from different locations in Bikaner (Rajasthan, India). The samples were immediately taken to the laboratory for further processing on ice.

Somatic cell counting (SCC)

A 0.1ml amount from each properly shaken milk samples was withdrawn with Pasteur pipette and spread evenly on a glass slide to count the somatic cell count as per the method described earlier [Prescott and Breed, 1910].

Isolation and Identification of *S. aureus*

All the milk samples which showed SCC corresponding to subclinical mastitis were processed for isolation of *S. aureus*. Phenotypic and biochemical identification of isolates was done as per the standard protocol [Quinn *et al.*, 1994]. The isolates were further genotypically confirmed by 23S rRNA species specific PCR using forward primer-1 (5'-ACGGAGTTACAAAGGACGAC-3') and reverse primer-2 (5'-AGCTCAGCCTT AACGAGTAC-3') [Straub *et al.*, 1999].

Amplification of *coa* gene

Amplification of *coa* gene was done as per the previously described method [Hookey *et al.*, 1998]. Primers used in the study included forward primer-1 (5' - ATAGAGATGCT GGTACAGG-3') and reverse primer-2 (5'-GCTTCCG ATTGTTTCGATGC-3').

RFLP of *coa* gene products

Restriction fragment length polymorphism of PCR amplified *coa* gene products were digested with *AluI* as per the protocol

previously described [Hookey *et al.*, 1998]. The PCR product (10 μ l) was added with nuclease-free water (5 μ l), 10 \times buffer Tango (2 μ l), and *AluI* (2 U, concentration of stock enzyme was 5 U/ μ l), mixed gently and incubated at 37°C for 3 h. The digests were resolved by electrophoresis as described above for *coa* amplicons, except that 2% agarose gels was used instead of 1.2%.

RESULTS AND DISCUSSION

Out of the 85 milk samples, 38 milk samples showed somatic cell count in the range of 200 \times 10³ to 500 \times 10³ cells/ml corresponding to subclinical cases of mastitis as per the IDF (2005) criterion. The SCC has been detected to be the most reliable test and closest to the bacteriological results for SCM in dairy cows by Sharma *et al.* (2010). A total of 38 isolates of *S. aureus* were isolated from these samples and identified on the basis of cultural and biochemical properties. All of the 38 isolates produced an amplicon of 1,250 bp in species specific PCR targeting 23S rRNA gene. Out of 38 isolates, 16 were isolated from H-F crossbred cattle while 22 isolates were from native Rathi cattle.

From 38 isolates of *S. aureus*, 35 strains showed amplification of single amplicon of *coa* gene either of 400, 490, 510, 550, 600, 710, 760, 810 or 850 bp while 3 isolates did not show any amplified product corresponding to *coa* gene. Thus nine different PCR products of *coa* gene ranging from 400 to 850 bp were obtained which is in conformity to observations made by previous workers in India [Upadhyay *et al.*, 2012; Khichar *et al.*, 2012] and from abroad [Coelho *et al.*, 2009, Saei *et al.*, 2009]. However, a wider range of PCR amplicon products has also been reported by other

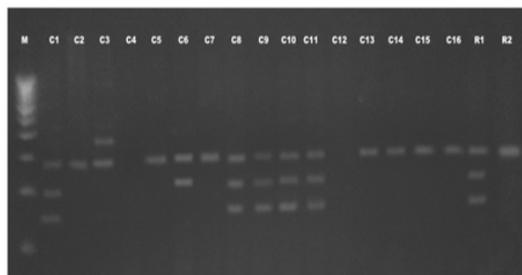


Fig. 1. Restricted fragment length polymorphism (RFLP) patterns of *S. aureus* isolates from H-F cattle (C1-C16) and Rathi cattle (R1-R2) with subclinical mastitis, M: 100bp DNA ladder.

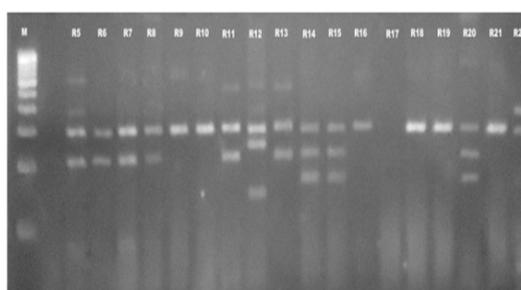


Fig. 2. Restricted fragment length polymorphism (RFLP) patterns of *S. aureus* isolates from Rathi cattle (R5-R22) with subclinical mastitis, M: 100bp DNA ladder.

workers viz. 579 to 1442 bp [Da Silva and Da Silva, 2005]; 484 to 1080 bp [Da Silva *et al.*, 2006] and 500 to 1400 bp [Karahan and Cetinkaya, 2007]. As opposed to the present observation for *coa* amplicons in the present investigation, Suleiman *et al.* (2012) obtained only one type of amplicons i.e. 500 bp in the *S. aureus* isolates from subclinical mastitis in Nigeria.

In the present investigation, more polymorphism of *coa* gene was recorded in isolates recovered from native breed Rathi wherein eight *coa* types were obtained as compared to isolates from isolated from H-F crossbred cattle where only three coagulase types were obtained. Two of the amplicons viz. 400 and 510 bp were common to isolates from both the cattle breeds whereas amplicon of 850 bp was obtained in isolates from H-F crossbred cattle only and other amplicons were recorded in isolates from Rathi cattle only. In the present investigation only single *coa* gene amplicon of variable size was obtained in each isolate. However, two bands of *coa* gene amplicons have also been reported by various authors [Karahan and Cetinkaya, 2007; Gharib *et al.*, 2013].

In the present investigation eight RFLP patterns of *coa* gene amplicon were generated using *AluI* restriction enzyme from nine different coagulase types (Fig. 1, 2). This is in agreement to Annemuller, (1999) who discriminated 25 *S. aureus* strains from bovine subclinical mastitis and obtained six types. RFLP analysis revealed four patterns from three coagulase types (850, 510 and 400 bp) of 16 isolates obtained from H-F cross bred. Some of the restriction fragments obtained in the present investigation were very similar to those reported by Annemuller *et al.* (1999). The eight coagulase types from Rathi cattle isolates were divisible into seven different RFLP patterns. In some of the Rathi isolates coagulase amplicons produced different RFLP patterns while in some strains, the pattern was similar even with different coagulase types. Similar observations have been reported by Schwarzkopf and Karch, (1994) who studied unrelated *S. aureus* strains and found that unrelated strains share identical *AluI* RFLP patterns. Their isolates with *coa* amplicon of 400 bp produced similar RFLP pattern, with two fragments of 300 and 210 bp.

The PCR-RFLP showed fragment of 300bp was produced by maximum number of isolates (20)

and fragments of 190 and 260 bp each were produced by only one isolate. Saei *et al.* (2009) also recorded the isolates with similar *coa* amplicon to have generated different RFLP pattern and isolates with different *coa* amplicons to have similar RFLP patterns. The presence of similar fragments obtained in RFLP demonstrated the clonal associations among the isolates, and variation in fragment size demonstrated variability in genetic composition of the isolates.

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