Typing of *Staphylococcus aureus* Obtained from Mastitic Milk of Cattle and Buffalo on the Basis of Coagulase (*coa*) Gene RFLP Patterns

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ABSTRACT

A typing procedure based on polymorphism of the coagulase gene (*coa*) was used to discriminate 32 *Staphylococcus aureus* isolates obtained from cattle (n=16) and buffalos' (n=16) mastitic milk. All the isolates showed coagulase production on plasma obtained from various species of animals and human. Amplification of *coa* PCR products (400bp, 510bp, 600bp and 650bp) were produced from cattle isolates and five different products (400bp, 510bp, 600bp, 650bp and 680bp) from buffalo isolates. From four coagulase types in cattle isolates five restriction fragment length polymorphism (RFLP) patterns were obtained and from five coagulase types in buffalo isolates six RFLP patterns were obtained. The *coa* gene amplicon of 600 bp was produced by the maximum number of isolates.

Keywords: *Staphylococcus aureus*; Cattle, Buffalo; Mastitis; *coa* gene; RFLP

INTRODUCTION

Mastitis causes considerable economic loss to the dairy industry. Although several bacterial pathogens can cause mastitis, *Staphylococcus aureus* is probably the most perilous agent as it causes chronic and deep infection in the mammary glands that are extremely difficult to cure (1, 2). Considerable genetic heterogeneity has been shown in natural populations of *S. aureus* isolates therefore, an exact identification of bacterial pathogens is necessarily for monitoring the spread of infection in animal populations (3, 4). Among numerous molecular techniques, coagulase (*coa*) gene typing is considered a simple and effective method for categorizing *S. aureus* isolates for epidemiological studies (5). Coagulase is an extracellular protein encoded by *coa* gene that possesses a conserved and a repeated polymorphic region that can be used to measure relatedness among *S. aureus* isolates (6, 7, 8).

The present study was designed to type *S. aureus* on the basis of polymerase chain reaction (PCR) and PCR-based restriction fragment length polymorphism (RFLP) analysis of the 3' end of the gene encoding staphylococcal coagulase.

MATERIAL METHODS

Samples were collected from cattle and buffalo without discriminating age, breed, calving and lactation yield from District Bikaner (28.0167°N, 73.3119°E), Rajasthan, India during December 2012 (Winter) to May 2013 (Summer). The only criteria were for sampled cattle and buffalo to be

suffering by clinical mastitis. All animal (cattle and buffalo) were Indian origin and reared at dairies and local farmers.

Sample collection, isolation and identification

In the present investigation a total of 89 mastitic milk samples (41 from cattle and 48 from buffalo) were processed for isolation and phenotypic identification (9). All the phenotypically identified *S. aureus* samples were further confirmed based on 23S rRNA gene ribotyping (10). The following sequences for the two primers were used: Primer 1 – 5' ACGGAGTTACAAAGGACGAC 3' Primer 2 – 5' AGCTCAGCCTTAACGAGTAC 3'.The reaction mixture of 30 µl was prepared by mixing 20 µl deionised water, 3.0 µl 10x DreamTaq Green buffer, 1.8 µl $MgCl_2$, 0.5 µl Primer-1 (10 pM/µl), 0.5 µl Primer-2 (10 pM/µl), 0.6 µl dNTP-mix (10mM), 0.1 µl DreamTaq DNA polymerase (5U/µl) and 2.5 µl template DNA (25ng/ µl). Primers and the PCR assay kit were obtained from Thermo Fisher Scientific, Waltham, MA, USA. Amplification was carried out in a Veriti thermal cycler (Applied Biosystem, Lincoln Centre Drive, Foster City, CA 94404, USA) as follows: initial cycle of amplification (denaturation at 94°C for 5 min, primer annealing at 55°C for 30 sec and primer extension at 70°C for 75 sec), 36 cycles of amplification (denaturation at 94°C for 40 sec, primer annealing at 57°C for 60 sec and primer extension at 70°C for 75 sec) and final cycle of amplification (denaturation at 94°C for 60 sec, primer annealing at 57°C for 60 sec and primer extension at 70°C for 3 min). The PCR products were resolved in 1.2% agarose gels.

Phenotypic characterization of coagulase production

The genotypically confirmed 32 (16 from cattle and 16 from buffalo) *S. aureus* isolates were tested for coagulase production (9) using plasma from different animal species *viz*. buffalo, sheep, goat, dog, chicken, camel and pig, and humans. The varying degrees of plasma clotting reactions were observed at the interval of 1, 3, 5 and 24 hours after incubation in water bath at 37°C.

Amplification and RFLP of *coa* gene

Amplification of the *coa* gene was carried out as described by Hookey *et al.* (11) with some modification using forward primer 5' ATAGAGATGCTGGTACAGG 3' and reverse primer 5' GCTTCCGATTGTTCGATGC 3'. The reaction mixture of 30 µl was prepared by mixing 20 µl deionised water, 2.5 µl 10x DreamTaq Green buffer, 2.5 µl $MgCl_2$, 0.5 µl Primer-1 (10 pM/µl), 0.5 µl Primer-2 (10 pM/µl), 1.0 µl dNTP-mix (10mM), 0.5 µl DreamTaq DNA polymerase (5U/µl) and 2.5 µl template DNA (25ng/ µl). Primers and the PCR assay kit were obtained from Thermo Fisher Scientific, Waltham, MA, USA. Amplification was carried out in a Veriti thermal cycler (Applied Biosystem, Lincoln Centre Drive, Foster City, CA 94404, USA) as follows: initial cycle of amplification (denaturation at 94°C for 45 sec, primer annealing at 57°C for 15 sec and primer extension at 70°C for 15 sec), 28 cycles of amplification (denaturation at 94°C for 20 sec, primer annealing at 57°C for 15 sec and primer extension at 70°C for 15 sec) and final cycle of amplification (denaturation at 94°C for 45 sec, primer annealing at 57°C for 15 sec and primer extension at 70°C for 2 min). The PCR products were resolved in 1.2% agarose gels.

For restriction fragment length polymorphism of PCR *coa* gene products digestion by *Alu* I (restriction enzyme from Thermo Fisher Scientific, Waltham, MA, USA) was carried out (11). The PCR product (10 µl) was added with 5 µl nuclease free water, 2 µl 10x Buffer Tango and 2U *Alu*I (5 U/µl), mixed gently and incubated at 37°C for 3 h and the digests were resolved in 2% agarose gels.

RESULTS AND DISCUSSION

The phenotypically identified isolates were further confirmed by ribotyping (10) where an amplicon of 1250 bp was obtained in the 32 isolates. On phenotypic characterization of the 32 isolates for production of coagulase revealed that all the isolates produced coagulase. Our results are in agreement to those of Arshad *et al.* (12) who investigated 23 *S. aureus* isolates obtained from cattle and buffalo. Some of the isolates showed weak coagulation reaction even after 5 h of incubation however they all showed a strong reaction at the 24 h reading. Rayman *et al.* (13) and Turkyilmaz and Kaya (14) also reported the coagulation of plasma after 24 h. In the present study human plasma showed the best coagulation reaction followed by plasma from pig, dog, poultry, sheep, goat, camel and buffalo in decreasing order for isolates from both species. The results suggest that the use of human plasma for the coagulase test for *S. aureus* was preferable.

Since the coagulation reactions were carried out under similar atmospheric conditions the analysis suggested that this reaction is more dependent on the source of the plasma

than the isolates. Our results are in complete agreement to those of Kateete *et al.* (15), where human plasma was recorded to give the best coagulation results. Though most of the workers have defined the coagulase reaction in terms of firmness of the clot and superiority of plasma for a particular species, Turner and Schwartz (16) suggested that any degree of clotting in the coagulase plasma should be considered as a positive reaction.

Amplification of *coa* gene

It has been described that varying numbers (3 to 9) of 81-bp tandem repeats in the *coa* gene in *S. aureus* determine the sequence analysis (3, 17). The PCR amplification of this particular region produces DNA fragments of different sizes which can be further discriminated by restriction fragment length polymorphism (RFLP) after digestion with *Alu*I restriction enzyme (18). In the present investigation, all the *S. aureus* isolates from cattle origin were grouped into four coagulase types based on the size of *coa* amplicons obtained where 10 isolates produced amplicon of 600 bp, four produced amplicons of 650 bp and amplicons of 510 bp and 400 bp were produced by one isolate each (Figure 1).

The buffalo isolates were divisible into five groups, where six produced amplicon of 600 bp, five produced amplicons of 680 bp, two produced amplicons of 650 bp and two of 510bp and one isolate produced 400bp amplicon. The overall results revealed that 16, 6, 5, 3 and 2 isolates produced amplicons of 600, 650, 680, 510 and 400 bp, respectively (Figure 2).

The present study was in agreement with the findings of Sanjiv *et al.* (19) and Upadhyay *et al.* (23) for *coa* amplicons (650 and 680 bp) of *S. aureus* in bovine and caprine mastitic milk. In the present study 50% of the isolates produced amplicons of similar size (600bp) which is in accordance with the observation of da Silva and da Silva (20); Aslantas *et al.* (4) and Saei *et al.* (8) demonstrating that there may be variety of coagulase types but only few types predominate in a particular area. The recovery of 600 bp amplicons in the highest number of isolates is in accordance to the observation of Salasia *et al.* (1), Khichar *et al.* (21) and Marques *et al.* (22). The amplicons of 400, 510 and 600 bp obtained in the present study were earlier reported by previous workers from the same laboratory (19, 23, 21) and also from elsewhere (5, 7, 1). In the present investigation the range of molecular size of *coa* amplicons obtained was narrower (400-680 bp). Similar observations were made by Sanjiv *et al.* (19) and Upadhyay *et al.* (23) who obtained three types of amplicons from the same study area. Similarly, Stephan *et al.* (24) recorded only two types of *coa* amplicons from 34 *S. aureus* isolates. However, a wide range of *coa* gene amplicons have been reported by various workers *viz.* 579 to 1442 bp (20); 730-1050 bp (4); 710 to 1456 bp (25); 610 to 960 bp (26) and 400 to 800 bp (27).

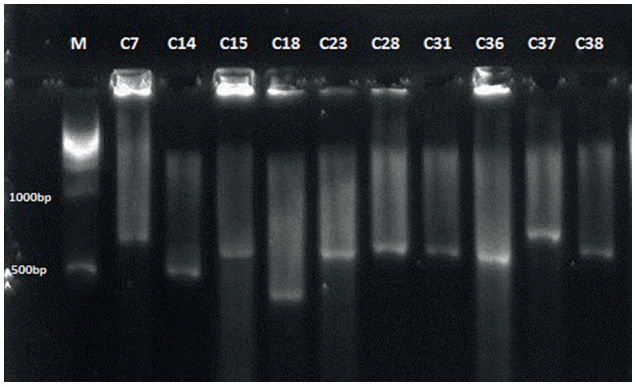

Figure 1: Agarose gel electrophoresis of amplicons of *coa* gene of *S. aureus* isolates obtained from cattle with clinical mastitis.

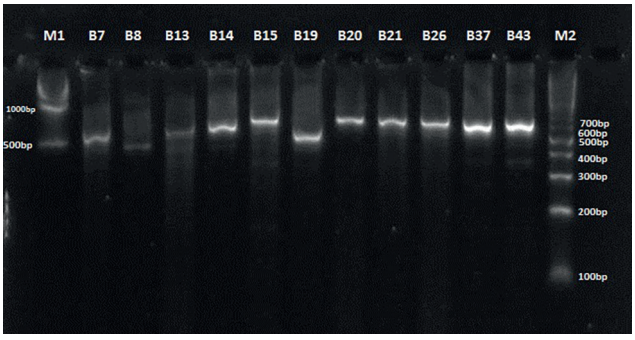

Figure 2: Agarose gel electrophoresis of amplicons of *coa* gene of *S. aureus* isolates obtained from buffalo with clinical mastitis.

Analysis of RFLP of *coa* gene products

From four coagulase types in cattle isolates, five RFLP patterns were obtained (Figure 3) and from five coagulase types in buffalo isolates, six RFLP patterns were obtained (Figure 4). The pattern of RFLP was similar for 400, 600, 650 bp amplicons for isolates from both cattle and buffalo. However, 510 bp amplicon was digested into 300 and 210 bp fragments in cattle isolates and into 400 and 110 bp fragments in buffalo isolates. The 400 bp *coa* gene amplicon remained undigested whereas amplicon of 600 bp showed two patterns. Out of the 16 isolates producing amplicons of 600 bp, 12 isolates showed a pattern wherein 300 bp fragments were obtained

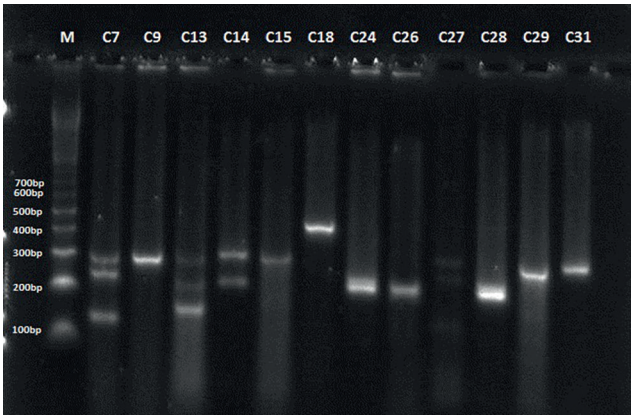

Figure 3: Agarose gel electrophoresis of amplicons of *Alu* I digests of *coa* gene amplicons (RFLP) of *S. aureus* isolates obtained from cattle with clinical mastitis.

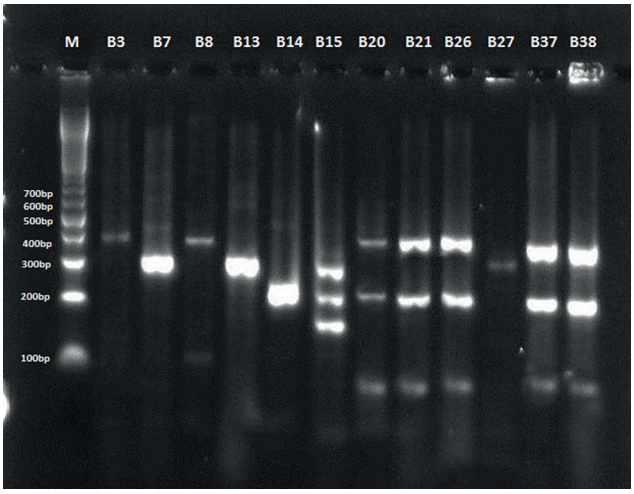

Figure 4: Agarose gel electrophoresis of amplicons of *Alu* I digests of *coa* gene amplicons (RFLP) of *S. aureus* isolates obtained from buffalo with clinical mastitis.

whereas four of the isolates with 600 bp *coa* gene amplicon produced fragment of 200 bp. The digestion of 650 bp *coa* gene amplicon in the isolates from both species of animals produced 300, 200 and 150 bp fragments. The amplicon of 680 bp obtained in buffalo isolates produced three fragments of 400, 200 and 80 bp. Out of the total isolates 12 isolates were similar as identified by RFLP patterns.

Our observations of seven RFLP patterns in the present study are similar to those of Himabindu *et al.* (28) who observed nine different fragment sizes which were similar to the fragment sizes obtained in the present study. A few of the RFLP fragments *viz*. 400, 300, 210 and 110 bp obtained in the present study were also reported by Sanjiv *et al*. (19), Upadhyay *et al*. (23) and Khichar *et al*. (21) from bovine and goat mastitis isolates from the same area of this study. Fragment of 300 bp was produced by the maximum of isolates (18) and fragment of 210 bp was produced by only one isolate in our study.

In the present investigation one coagulase amplicon of 400bp was not digested by *Alu*I. This observation is in agreement to findings of Lange *et al.* (3) who also did not observe digestion of three PCR products from *S. aureus* of bovine mastitis origin. Similarly, da Silva and da Silva (20) also observed non-digestion of some of the amplicons. In the present study similar *coa* amplicons produced different RFLP patterns. Similar to our observation, da Silva and da Silva, (20) and Moon *et al.* (5) also recorded that the isolates with similar *coa* amplicon have generated different RFLP patterns.

CONCLUSION

In the present investigation 32 genetically confirmed coagulase positive *S. aureus* isolates from cattle and buffalo mastitic mik were subjected to *coa* gene RFLP typing. Of the 16 cattle and 16 buffalo isolates five and six, respectively RFLP types were obtained. The study recorded RFLP polymorphism even in similar *coa* amplicons. The RFLP tying was found satisfactory to discriminate the *S. aureus* isolates.

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