Characterization of *Staphylococcus aureus* of Goat Mastitis Milk Origin for cap and clfA Genes

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*Staphylococcus aureus* is major pathogen causing mastitis in dairy milk animals including goats. The present study was carried out on 27 *S. aureus* isolates of milk origin from goats with clinical mastitis which included characterization of isolates for two important virulence associated genes namely clfA, responsible for clumping factor and cap, responsible for capsule. All the isolates were genotypically identified by 23S rRNA ribotyping in which a species-specific amplicon of 1250 bp was obtained. Of the 27 isolates 17 (68.38%) showed single amplicon of approximately 350 bp representing presence of cap5K gene responsible for capsular type 5 whereas nine (34.61%) of the isolates produced amplicons of approximately 200bp representing presence of cap8K genes responsible for production of capsular type 8, whereas one isolate was non-typable for any of the two genes. In the present investigation each of the 25 isolates produced single amplicon of 1050 bp indicating no polymorphism in clfA gene while two isolates did not produce any PCR amplicon. Epidemiological investigation of these genes is significant in terms of vaccine design and control of mastitis from the study area.

Key words: *Staphylococcus aureus*, cap, clfA, goat, mastitis.
pathogenesis of Staphylococcal infections. Of the many capsular types cap5 and cap8 have been considered important in pathogenesis and hence in vaccine development (Risley et al., 2007; Tuchscherr et al., 2008). These capsular polysaccharides are surface bound and promote bacterial colonization and persistence on mucosal surfaces. Clumping factor A (ClfA) is a cell wall-anchored S. aureus surface protein that has been shown to enhance staphylococcal virulence in animal infection models (Moreillon et al., 1995; Wolz et al., 2002) and has been suggested as a vaccine potential candidate (Josefsson et al., 2001; Nour El-Din et al., 2006) and as a target for passive immunization approaches (Domanski et al., 2005; Patti, 2004; Vernachio et al., 2003).

Both of the virulence factors have also been suggested to be potential vaccine candidates (Risley et al., 2007). In one study, Tuchscherr et al. (2008) showed that antibodies to clfA enhanced the protection against infection provided by capsular polysaccharides antibodies. Caprine mastitis has been recorded in different countries by different authors (Moroni et al., 2005; Ayedin et al., 2009) but data from India are scarce. The present paper reports characterization of S. aureus of goat milk origin for capsular and clumping factor genes.

MATERIALS AND METHODS

Isolation and identification of bacteria

Isolation of S. aureus was carried out from milk samples of Marwari goats native to Bikaner with clinical mastitis. The organisms were isolated and identified as per the methods described by Cowan and Steel, (1975). Further they were genotypically identified by ribotyping based on 23S rRNA gene (Straub et al., 1999) using species-specific primer-1 (5'-ACGGAGTTAC AAAGGACGC-3') and primer-2 (5'-AGCTCTAGGTAACGC-3'). The amplification products were electrophoresed for 1.5 h at 100 V and then gel was visualized using a UV transilluminator.

cap gene amplification:

The method of Verdier et al. (2007) was used for the amplification of cap5Kand cap8K genes using the primer sets: The sequences for two primers used for cap5K gene were: Primer-1: 5'GTCAGGAGTTATGCTGACCTGAG3' and Primer-2: 5'ACTTCTGATATATCCTGAAT CAAATCTACAG 3', the sequences for two primers used for cap8K gene were: Primer 1: 5' GCCCTGTAGTGGATACCA 3' Primer 2: 5' GAAAGAATACATCATAGCAGG 3'. The PCR mixture (total volume 30 µl) was prepared by mixing 1.0 µl of primer-1 (75 pmol/µl), 1.0 µl of primer-2 (75 pmol/µl), 3.5 µl of 10x Taq buffer A (containing 15mM MgCl2), 1 unit of Taq polymerase (5 U/µl), 2.0 µl of dNTP mix (10 mM/µl), 21.0 µl of deionised water, and 2.5 µl of DNA template (25 ng/µl). The PCR was performed using following cycling parameters: Cycle 1: Denaturation at 94°C for 5 min, Primer annealing at 55°C for 30 s, Primer extension at 72°C for 1 min; Cycle 2-25: Denaturation at 94°C for 30 s, Primer annealing at 55°C for 30 s, Primer extension at 72°C for 1 min; Cycle 26: Denaturation at 94°C for 30 s, Primer annealing at 55°C for 30 s, Primer extension at 72°C for 5 min.

clfA gene amplification

The method of Stephan et al. (2001) was used for the amplification of clfA gene using the primers set (Primer 1: 5'GGCCTCATGTGGAGTGTG-3'; Primer 2: 5'TTTTCAGGG TCAATAAGGC-3'). The PCR mixture (total volume 30 µl) was prepared by mixing 1.25 µl of primer-1 (75 pmol/µl), 1.25 µl of primer-2 (75 pmol/ µl), 2.5µl of 10x Taq buffercontaining MgCl2 (25mM), 0.3 µl of Taq polymerase (5 U/µl), 2.5 µl of dNTP mix (10 mM/µl), 17.20 µl of deionised water and 2.5 µl of DNA template (25 ng/µl). The PCR was performed in Palmcycler (Corbett Research, Australia) using following cycling parameters:- Cycle 1: Preheating at 94°C for 4 min; Cycle 2-36: Denaturation at 94°C for 1 min, Primer annealing at 57°C for 1 min, Primer extension at 72°C for 1 min; final extension at 72°C for 5 min.

The PCR products, after addition of 2 µl of trekking dye were resolved in 1.2% agarose gels prepared in 1.0 x TBE buffer containing 0.5µg/ml of ethidium bromide and 100 bp DNA ladder was used as molecular marker. The amplification products were electrophoresed for 1 h 30 min at 100 V. The gel was then visualized under U.V. transilluminator.
RESULTS AND DISCUSSION

23S rRNA gene based confirmation

All the 27 isolates on 23S rRNA ribotyping produced a single PCR product of 1250 base pairs confirming them to be *S. aureus*. This method was found 100% specific in genotypic identification of *S. aureus*.

cap genotyping

Seventeen of the 27 (68.38%) isolates showed single amplicon of approximately 350 bp representing presence of cap5K gene responsible for capsular type 5 (fig. 1) whereas nine (34.61%) of the isolates produced amplicons of approximately 200bp representing presence of cap8K genes responsible for production of capsular type 8 (fig. 2). In our study one of the 27 isolates was nontypable for CP5 or CP8.

clf A genotyping

In the present investigation 25 isolates produced single amplicon in each isolate whereas two of the isolates did not produce any PCR amplicon. The size of the amplicon product obtained in the present study was 1050 bp indicating no polymorphism in clfAgene (fig. 3).

Mastitis is one of the most important diseases of dairy animals globally leading to severe financial losses to dairy industry. Among dairy animal species goat has its own important place in many of the countries. In India also goat rearing is practiced by marginal farmers for their livelihood. *Staphylococcus aureus* has been identified as the most important udder pathogen in small ruminants (Bergonier et al., 2003). This organism doesn’t demonstrate host specificity and is responsible for many different infections in animals as well as humans. Aires-de-Sousa et al. (2007) characterized *S. aureus* from buffalo, cattle, sheep and goat milk samples by phenotypic and genotypic methods and they concluded that a major clonal type is responsible for mastitis in different regions which does not demonstrate host specificity among the different animal species and seems to be capable of both infection and colonization. Mastitis caused by *S. aureus* should be eliminated because of the risk of contamination of milk products by the thermo-stable toxins (Contreras et al., 2007).

The identification of *S. aureus* strains is important to confirm the epidemiological relationships among them (Aarestrup et al., 1994). All the isolates in the present study were identified by genotypic method in which 23S rRNA gene was targeted using specific primers. This method is being used for confirmation of *S. aureus* from different sources worldwide (Akineden et al., 2001;
Momtaz et al., 2010; Proietti et al., 2010; Khichar et al., 2012; Suleiman et al., 2012).

Various workers have experimentally elucidated the antigenic properties and possible role of capsular polysaccharides in the generation of vaccines against S. aureus infections. The gene cluster for CP5 and CP8 type contains 16 open reading frames (cap5A through cap5P and cap8A through cap8P, respectively) the four of which are located in the central region (H - K) and are type specific. In the present study except one isolate (no.22) all other 26 isolates were typable for cap5K or cap8K gene fragments. Seventeen isolates showed presence of cap5K gene whereas nine showed presence of cap8K genes. The amplicon sizes obtained in the present study were almost similar to those obtained by Verdier et al. (2007), Upadhyay et al. (2010) and Khichar, (2011).

Of the 11 CP types CP5 and CP8 are produced by majority of the animal mastitic isolates (Poutrel et al., 1988; Naidu et al., 1991). Likewise, CP5 and CP8 have been reported to be produced by more than 75% of human isolates (Fatton et al., 1996; Lee et al., 1997). The cap genes are highly conserved and the capsular polysaccharides have got limited antigenic specificity (O’Riordan and Lee, 2004). Similarly, other studies have also shown that antibodies to capsular polysaccharides have some protective efficacies for preventing infections in experimental animal (Fatton et al., 1996; Lee et al., 1997). The occurrence of cap5K or cap8K varies in isolates from different geographical regions (Sordelli et al., 2000; Salasia et al., 2004).

In our study one of the 27 (3.7%) isolates was non-typable for cap5 or cap8 genes, which is in agreement to the observations of many workers who found a variable percentage of non-typable isolates for these genes. Upadhyay et al. (2010) had reported 50% caprine S. aureus isolates to be non typable whereas 30% showed presence cap5K gene and 20% possessed cap8K gene. They did not observe any polymorphism in cap5K and cap8K genes which is in complete agreement to the present study. However, a very high percentage (86.2%) of non-typable isolates was detected by Sordelli et al. (2000) by serotyping. Guidry et al.(1998) also recorded 59% of the US isolates and 30% of the isolates from Europe to be CP5 or CP8negative. Similarly, 30% of S. aureus from bovine mastitis were reported not to possess CP5 or CP8 by Naidu et al.(1991). This finding is almost similar to observations of Khichar, (2011) from the same study area who did not record any of the isolates without cap genes in his study involving bovine cattle mastitis isolates. Similarly Singh et al.(2011) recorded that only 86.8% (60.7% cap5K and 26.1% cap8K), 79.0% (56.3% cap5K and 22.7% cap8K) and 81.1% (22.2% cap5K and 18.9% cap8K) S. aureus isolates from intra-mammary infections in Sahiwal cattle, Karan-Fries cattle and Murrah buffaloes respectively were capsulated.

Khichar, (2011) had also reported capsular typing of cattle mastitic S. aureus isolates from the same laboratory and found 92.86% isolates to possess cap5K gene while 7.14% isolates to possess cap8K gene amplicon with no polymorphism in cap genes.

In the present study 68.38% isolates were detected with cap5K genes and 34.61% with cap8K genes among typeable isolates which is in conformity to observations of Upadhyay et al.(2010) who recorded that 60% of mastitis isolates from cattle and goats had cap5K genes and 20% had cap8K gene. Similarly Khichar, (2011) reported 92.86% isolates with presence of cap5K gene and 7.14% isolates with presence of cap8K gene. Contrarily, Alves et al.(2008) recorded CP type 8 with an overall prevalence of 83.1% in ovine and caprine isolates.

Clumping factor A (clfA) is a cell surface bound protein in S. aureus which is thought to be essential for colonization and establishment of infections. It is known to participate in the infection process by facilitating bacterial binding via soluble or immobilized fibrinogen (Karahan et al., 2011) and has also been suggested as a potential vaccine candidate. Said et al.(2010) also suggested clfA typing useful for revealing the clonal nature of mastitis isolate lineage. Risley et al.(2007) in a study found that capsular polysaccharide masks clfA mediated adherence of S. aureus to fibrinogen and platelets and suggested that these findings have important implications in vaccine development.

In the present investigation 25 isolates each produced single amplicon whereas two isolates (Moroni et al., 2005; Aires-de-Sousa et al., 2007) did not produce any PCR amplicon. The observation is in accordance to the finding of Salasia et al. (2004), Karahan et al. (2011) and
Momtaz et al. (2010) who suggested that clumping factor is present in majority of the isolates. The size of the amplicon product obtained in the present study was 1050 bp which is in complete agreement to report of Kalorey et al. (2007) who characterized 37 strains of *S. aureus* of bovine milk origin for *clf*Agene and obtained an amplicon of approximately 1042 base pairs in all the isolates. Moroni et al. (2005) characterized *S. aureus* isolated from milk from chronically infected Alpine dairy goats and obtained an amplicon of 1030 bp in all 28 isolates by amplification of *clf*Agene. Similarly, Akineden et al. (2001) investigated 103 *S. aureus* isolates from milk of mastitic cows for *clf*Agene and obtained a single amplicon with a size of approximately 1000 bp. Salasia et al. (2004) also carried out amplification of *clf*Agene and found a single amplicon with a size of approximately 1000 base pairs.

We did not observe any polymorphism in *clf*Agene which is in accordance with the observations of some other workers from various locations viz. Akineden et al. (2001) from Germany; Stephan et al. (2001) from North-east Switzerland; Salasia et al. (2004) from Germany and Momtaz et al. (2010) from Iran. Contrarily, polymorphism in *clf* gene was reported by Fitzgerald et al. (2000) who carried out genotyping of 102 Irish and 42 USA *S. aureus* strains of bovine intramammary infection origin and recorded the *clf*Agene-R amplicons to vary between 220 and 1180 bp in sizes. Similarly, Reinoso et al. (2008) on genotyping of *S. aureus* isolates from different sources for *clf*Agene revealed an amplicon size of 1000 bp for 40 *S. aureus* whereas four strains from bovine origin and one strain from food origin had an amplicon of 900 bp size. Likewise, Karahan et al. (2011) investigated *S. aureus* isolated from sub-clinical bovine mastitis in Turkey and found that 91.3% of the 92 coagulase positive *S. aureus* isolates carried *clf*Agene in which 68 isolates had *clf*Amplicon of 1000 bp and remaining 16 isolates had amplicon of 900 bp, the remaining eight isolates did not possess *clf* gene.

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