Characterization of *Staphylococcus aureus* isolates from mastitic milk, udder surfaces and milkers’ hands from arid and semi arid regions of India for capsular (*cap*5K and *cap*8K) and collagen adhesin (*cna*) genes

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Received: 15 February 2019; Accepted: 28 March 2019

ABSTRACT

*Staphylococcus aureus* (*S. aureus*) is an important cause of bovine mastitis. The antiphagocytic capsule of bacteria promotes colonization and persistence on mucosal surfaces while the presence of collagen adhesin (*cna* gene) is associated with bacterial adhesion in mammary tissues; hence both are regarded as important determinants of virulence. In the present study, 197 samples consisting of mastitic milk, milkers’ hand swabs and udder surface swabs were collected which yielded 107 *S. aureus* isolates. These *S. aureus* isolates (107) from different sources of sampling were characterized for their capsular types targeting *cap*5K and *cap*8K genes in a duplex PCR along with amplification of *cna* gene. Majority of the isolates (67.6%) possessed *cap*5K gene with a lower percentage (22.9%) of isolates carrying *cap*8K gene and 9.5% carrying both the genes. Moreover, *cap*5K gene was predominant in isolates from milkers’ hands (85.7%) while *cap*8K gene was more common in bovine udder isolates (41.2%). *cna* gene was observed in 27.6% isolates with highest occurrence in milk isolates (44.8%) compared to udder (37.9%) and milkers’ hand (17.2%). *cna* positive isolates carried *cap*8K gene (66.7%) and were significantly associated with both *cap*5K and *cap*8K genes. In conclusion, the majority of the *S. aureus* isolates of mastitis obtained from arid and semi arid zones of India possessed the genes for capsule production, and *cap*5K was the predominant gene. The *cna* gene for collagen adhesion was observed in fewer isolates with significant association with *cap*8K gene.

Key words: Capsular types, *cna* gene, Duplex PCR, Mastitis, *Staphylococcus aureus*

*Staphylococcus aureus* is one of the leading causes of contagious bovine mastitis. It elaborates diverse range of virulence factors in the pathogenesis of intra-mammary infections (Schmidt et al. 2017). Majority of the *S. aureus* strains isolated from bovine mastitis are encapsulated (Tollersrud et al. 2000). Out of the 11 capsular polysaccharide serotypes that have been identified worldwide in *S. aureus* isolates, only types 5 and 8 are prevalent in *S. aureus* isolated from human and bovine infections (O’Riordan and Lee 2004). Collagen adhesin, encoded by the *cna* gene, responsible for the ability of *S. aureus* to bind collagenous tissues (Nashev et al. 2004) has been considered as one of the important trait for the virulence action in bovine mammary tissue (Saei 2012) along with other adhesins belonging to MSCRAMMs (microbial surface components recognizing adhesive matrix molecules). However, little is known about the presence of *cna* gene in *S. aureus* isolates responsible for bovine mastitis in India. Hence the present study was undertaken to carry out capsular typing and detection of *cna* gene in *S. aureus* isolates from mastitic milk, udder surfaces and milkers’ hands from arid and semi arid regions of India.

MATERIALS AND METHODS

Ethical approval: This study was conducted following approval by the research committee and Institutional Animal Ethics Committee guidelines were followed.

Isolation, identification and genotypic confirmation of *S. aureus* isolates: In the present study, 197 samples including mastitic milk samples (80), udder surface swabs (66) and milkers’ hands swabs (51) were collected from seven different places in arid and semi-arid regions in India. Following primary identification by Gram staining, morphology, catalase test, and glucose and mannitol fermentation (Markey et al. 2013), 107 *S. aureus* isolates comprising 51 isolates from mastitic milk, 35 isolates from udder surfaces and 21 from milkers’ hands were further genotypically confirmed by 23S rRNA gene based ribotyping as per the method described by Straub et al. (1999) and *mec* gene amplification as per Brakstad et al. (1992) as described earlier (Bhati et al. 2018).

Duplex PCR for *cap*5K and *cap*8K genes: The 107 geno-typically confirmed isolates were subjected to amplification...
of capsular genes in duplex PCR using primer sets of Verdier et al. (2007). The sequences of primers used were 5'-GTCAAAAGATTGTGGTACTGAG-3' (Primer 1) and 5'-ACTTGAATA-TAAACTT GAATCAATGTTA- TACG-3' (Primer 2) for amplification of cap5K gene, and 5'-GCCTTTAGTTTAGTAAACC-3' (Primer 1) and 5'-GGAAAAACACTATCATA CGAG-3' (Primer 2) for amplification of cap8K gene. The PCR amplification was carried out in a Veriti thermal cycler (Applied biosystem) and consisted of PCR cycle of pre-denaturation at 94°C for 5 min, followed by 30 cycles of amplification (denaturation at 94°C for 60 sec, primer annealing at 55°C for 60 sec and primer extension at 70°C for 60 sec), and final extension at 72°C for 7 min. The PCR products (361 bp and 173 bp amplicons for cap5K and cap8K genes, respectively) were resolved on 1.2% agarose gels using 100 bp DNA ladder as molecular marker.

**Molecular detection of cna gene:** The PCR amplification of cna gene was carried out as described by van Leeuwen et al. (2005) using forward primer as 5'-AGT GGT TA TTG GTT TA-3' and reverse primer as 5'-CAG GAT AG TAA TAC TG-3' and molecular detection of cna gene. The PCR cycle consisted of pre-denaturation at 94°C for 5 min, followed by 34 cycles of amplification (denaturation at 94°C for 60 sec, primer annealing at 55°C for 60 sec and primer extension at 70°C for 60 sec), and final extension at 72°C for 10 min. The PCR products (1722/1122 bp) were resolved on 0.8% agarose gels and 1 kbp DNA ladder was used as molecular marker.

**Statistical analysis:** For statistical analysis, SPSS 16.0 software was used for analysing differences in the prevalence of cap5K and cap8K genes of S. aureus isolates from different sources using the chi-square test for each gene. A P value of <0.05 was considered as statistically significant. Nonparametric Kruskale Wallis test was used to analyze the association between cna and cap genes.

**RESULTS AND DISCUSSION**

In the present study, 107 genotypically confirmed S. aureus isolates obtained from mastitic milk, udder surfaces and milker’s hands from different farms were subjected to capsular typing (cap5K and cap8K genes) and detection of cna gene.

**Capsular typing:** Out of 107 S. aureus isolates, 105 isolates showed positive PCR reaction with specific primers for loci cap5 or cap8. Out of 105 strains, 71 isolates (67.6%) produced amplicon of 361 bp indicating presence of cap5K gene and 24 isolates (22.9%) showed presence of 173 bp amplicon indicating presence of cap8K gene. Both genes were detected in ten isolates (9.5%). Thus, the majority of the isolates analysed were of the cap5K genotype. Similar higher proportion of cap5 isolates has also been reported by Salasia et al. (2004) from Indonesia; Reinoso et al. (2008) and Camussone et al. (2012) from Argentina; Salimena et al. (2016) from Brazil, and Kumar et al. (2011) and Krithiga et al. (2018) from India. The present findings are also in agreement with previous studies from the same area reporting higher prevalence of cap5K gene, viz. 60% (Upadhyay et al. 2010); 92.86% (Khichar and Kataria 2014); 68.75% (Yadav et al. 2015); 68.38% (Nathawat et al. 2015) and 52.94% (Sharma et al. 2016) in comparison to cap8K gene in S. aureus isolates from different sources.

In our study, 85.7% isolates from milkers’ hands carried cap5K gene while a higher number of bovine udder isolates (41.2%) were positive for cap8K gene as compared to other sources of samples (Table 1) and a significant difference (P<0.05) was observed between different sources (Table 1). Also the presence of both genes was higher in isolates from udder (17.1%) than from mastitic milk (6.0%) and milkers’ hands (4.8%). Reinoso et al. (2008) in a similar study observed higher percentage of cap5K genotype in human isolates (11 out of 45) than bovine strains (9 out of 45). All bovine strains were negative with cap8K gene which is in contrast to present study. Other workers have reported a higher proportion of cap5K than cap5K gene contrary to present findings. Salasia et al. (2004) observed that most of the strains (12 out of 19) isolated in Hesse, Germany harboured the gene cap8 while Ikawaty et al. (2010) detected cap8 in 73 isolates and cap5 in three isolates from Netherlands. Some workers did not record any of the isolates harbouring cap8 gene (Proietti et al. 2010) or carrying cap5 (Soares et al. 2017).

**Detection of cna gene:** The present investigation revealed a low prevalence of cna gene being detected in 29 (27.6%) isolates with amplicon size either of 1722 bp (majority of isolates) or 1122 bp. The highest occurrence of cna gene was observed in milk isolates (44.8%) compared to udder (37.9%) and milkers’ hand (17.2%) (Table 2). Moreover most of the cna positive isolates carried cap8K gene. The observed low occurrence of cna genes in S. aureus isolates indicated that it may not play an important role in the pathogenesis of bovine mastitis due to S. aureus (Ahangari et al. 2017), although it is one of the major virulence factors.

### Table 1. Distribution of the capsular polysaccharide genotypes among S. aureus isolates obtained from different sources of sampling

| Source of sample | cap5K | cap8K | Both | Total | P value |
|------------------|-------|-------|------|-------|---------|
| Mastitic milk    | 39    | 08    | 03   | 50    | <0.005  |
| Udder            | 14    | 21    | 06   | 34    | >0.10   |
| Milker’s hand    | 18    | 02    | 01   | 21    | <0.005  |
| Total            | 71    | 24    | 10   | 105   | <0.005  |

### Table 2. Distribution of the cna gene among S. aureus isolates obtained from different sources of sampling

| Source of sample | cap5K | cap8K | Both | Total | cap5K and cap8K |
|------------------|-------|-------|------|-------|----------------|
| Mastitic milk    | 04    | 07    | 02   | 13    | (44.8) |
| Udder            | 02    | 08    | 01   | 11    | (37.9) |
| Milker’s hand    | 03    | 01    | 01   | 05    | (17.2) |
| Total            | 09    | 16    | 04   | 29    |     |
Table 3. Association of presence of cna gene with cap5K and cap8K genes

| Source of sample | Positive | Negative | Total | P value |
|------------------|----------|----------|-------|---------|
| cap5K            | 09 (12.7) | 62 (87.3) | 71    | <0.00   |
| cap8K            | 16 (66.7) | 08 (33.3) | 24    | <0.00   |
| Both cap5K and cap8K | 04 (40.0) | 06 (60.0) | 10    | <0.00   |
| Grand total      | 29 (27.6) | 76 (72.4) | 105   |         |

In staphylococcal diseases. In addition, this adhesin protein gene was not present in all S. aureus strains and might; therefore, be of importance for the virulence of single strains of this species. Results from previous studies have shown that the frequency of isolates harbouring cna gene both in human (Nashev et al. 2004) and in animal isolates (van Leeuwen et al. 2005, Zeconci et al. 2006, Reinoso et al. 2008) was lower.

In the present study, highest prevalence of cna gene was observed in mastitic milk isolates (44.8%) which may suggest that the presence of cna gene and expression of collagen adhesin may influence the virulence action in bovine mammary tissue. Similar low prevalence of cna gene in mastitis isolates was also reported by other workers, viz. El-Sayed et al. (2006), Kumar et al. (2011) and Memon et al. (2013) while Ahangari et al. (2017) reported higher prevalence of cna gene (65.3%) among bovine clinical and subclinical mastitis milk samples. Aslantaş and Demir (2016) found 87.5% isolates positive for cna gene which is in contrast to present study. The study by van Leeuwen et al. (2005) showed that the cna gene was not equally distributed among the different lineages of S. aureus which may explain differences in the ability of the strains to spread. Saei (2012) suggested that different types of S. aureus may use different adhesion mechanisms, some mediated by collagen binding, others independent of collagen binding such as fibronectin binding proteins (FnBP) A and B, fibrinogen binding proteins called clumping factors (Clf A and B that contribute to initiate infection (Zecconi and Scali 2013) and biofilm formation which is associated not only with epithelial adhesion but also with evasion of host immune defence (Melchior et al. 2009). The presence of several genes involved in adhesion and biofilm production in S. aureus strains from intramammary infections have been studied (Haveri et al. 2007, Veh et al. 2015) but neither presence of genes associated with adherence and biofilm formation and in vitro biofilm-forming ability nor internalization capacity of S. aureus were related to strain clinical origin (Pereyra et al. 2016).

The present study also revealed significant association between presence of cna gene with cap5K and cap8K genes in S. aureus isolates from different sources (Table 3). Similar strong association between the presence of cna and cap8 was also reported by Ryding et al. (1997), Booth et al. (2001) and El-Sayed et al. (2006) which corroborated with the findings of present study. A comparable relation could be demonstrated for the cna positive strains in the present study as majority of the cna positive strains (66.7%) were of cap8K genotype.

In conclusion, the majority of the S. aureus isolates of mastitis obtained from arid and semi arid zones of India possessed the genes for capsule production and cap5K was the predominant gene. The cna gene for collagen adhesion was observed in fewer isolates with significant association with cap8K gene.

ACKNOWLEDGEMENTS

The authors are thankful to Dean, College of Veterinary and Animal Science, Bikaner for the facilities provided to accomplish the work.

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