



Characterization of *Staphylococcus aureus* isolates from mastitic milk, udder surfaces and milkers' hands from arid and semi arid regions of India for capsular (*cap5K* and *cap8K*) and collagen adhesin (*cna*) genes

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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 *cap5K* and *cap8K* genes in a duplex PCR along with amplification of *cna* gene. Majority of the isolates (67.6%) possessed *cap5K* gene with a lower percentage (22.9%) of isolates carrying *cap8K* gene and 9.5% carrying both the genes. Moreover, *cap5K* gene was predominant in isolates from milkers' hands (85.7%) while *cap8K* 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 *cap8K* gene (66.7%) and were significantly associated with both *cap5k* and *cap8k* 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 *cap5K* was the predominant gene. The *cna* gene for collagen adhesion was observed in fewer isolates with significant association with *cap8K* 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 *nuc* gene amplification as per Brakstad *et al.* (1992) as described earlier (Bhati *et al.* 2018).

Duplex PCR for *cap5K* and *cap8K* genes: The 107 genotypically confirmed isolates were subjected to amplification

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of capsular genes in duplex PCR using primer sets of Verdier *et al.* (2007). The sequences of primers used were 5'-GTCAAAGATTATGTGATGCTACTGAG-3' (Primer 1) and 5'-ACTTCGAATA-TAACTT GAATCAATGTTA-TACAG-3' (Primer 2) for amplification of *cap5K* gene, and 5'-GCCTTATGTTAGGTGATAAACC-3' (Primer 1) and 5'-GGAAAAACACTATCATAGCAGG-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 TAC TAA TAC TG-3' and reverse primer as 5'-CAG GAT AGA TTG GTT TA-3'. The PCR cycle consisted of predenaturation at 94°C for 5 min, followed by 34 cycles of amplification (denaturation at 94°C for 60 sec, primer annealing at 45°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 Kruskal 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 *cap8K* 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* gene (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	<i>cap5K</i>	<i>cap8K</i>	Both	Total	P value
Mastitic milk	39 (78.0)	08 (16.0)	03 (6.0)	50	<0.005
Udder	14 (41.2)	14 (41.2)	06 (17.6)	34	>0.10
Milkers' hand	18 (85.7)	02 (9.5)	01 (4.8)	21	<0.005
Total	71 (67.6)	24 (22.9)	10 (9.5)	105	<0.005

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

Source of sample	<i>cap5K</i>	<i>cap8K</i>	Both <i>cap5K</i> and <i>cap8K</i>	<i>cna</i> gene
Mastitic milk	04	07	02	13 (44.8)
Udder	02	08	01	11 (37.9)
Milkers' 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
<i>cap5K</i>	09 (12.7)	62 (87.3)	71	<0.00
<i>cap8K</i>	16 (66.7)	08 (33.3)	24	<0.00
Both <i>cap5K</i> and <i>cap8K</i>	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, Zeconi *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. Aslanta^o 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 (Zeconi 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.

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