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Detection of *ica*A and *ica*D genes in slime producing *Staphylococcus aureus* isolates from buffaloes with subclinical mastitis

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Abstract
One of the common cause of subclinical mastitis is *Staphylococcus aureus*. Slime production is an important virulence factor for *S. aureus*. In current study, total 84 milk samples pooled from all quarters were collected from buffaloes. Out of 84 samples, 62 milk samples had somatic cell count more than 0.25 million/ml and all of them were positive on bacteriological culture. *S. aureus* was isolated from only 41 samples and all 41 samples were genotypically confirmed by 23S rRNA based ribotyping method. All 41 isolates (100%) produced slime on Congo Red agar and both *ica*A and *ica*D genes were detected in all of them. The present study showed high percentage of slime producing *Staphylococcus aureus* isolates from subclinical mastitis in buffalo. Controlling of subclinical mastitis in buffalo can be challenging due to presence of such slime producing bacteria.

Keywords: Buffalo, *ica* genes, slime, *Staphylococcus aureus*, subclinical mastitis

Introduction
Staphylococcus aureus is a spherical, gram-positive, non-motile, non-spore forming and facultative anaerobic firmicute bacterium of family Staphylococcaceae. It is one of the most frequently isolated contagious mastitis pathogens that cause either clinical or subclinical mastitis in bovines. Various virulence factor such as protein A, hemolysin, clumping factor, fibronectin, coagulase, nucleases, exfoliative toxin and enterotoxins plays an important role in pathogenic properties of *S. aureus* (Peacock *et al*., 2002; Haveri *et al*., 2007; Fournier *et al*., 2008) [23, 14, 10].
Slime formation is a highly organized multicellular complex which not only associated with epithelium adhesion but also with evasion of host immune defense system (Melchior *et al*., 2009; Szweda *et al*., 2012) [16, 29]. Intercellular adhesion (*ica*) locus which consisting of *ica*ADB and C genes is required by *S. aureus* for Slime production. The protein encoded by this locas which facilitate synthesis of intercellular adhesin (Cramton *et al*., 1999) [7], of which *ica*A and *ica*D is important for slime or biofilm production (O'Gara 2007) [22].
The *ica*A gene synthesis N-acetyl glucosaminyl transferase, the enzyme which mediate synthesis of N- acetyl glucosamine oligomers from UDP-N-acetyl glucosamine (Arciola *et al*., 2001) [3]. Further, *ica*D has been known to play an crucial role in peak production of N-acetyl glucosaminyl transferase which leads to expression of the capsular polysaccharide (Gerke *et al*., 1998) [13]. So, this study was designed to detect the *ica*A and *ica*D gene in slime producing *S. aureus* isolated from buffalo with subclinical mastitis.

Materials and Methods
Sample collection
A total of 84 physically normal milk samples in 5-10 ml amount each, were collected from 84 buffaloes (pooled from all quarters) belonging to different farmers in and around Hanumangarh (Rajasthan).

The samples collected in sterilized test tubes were immediately transferred to the laboratory on ice for further processing.

Somatic cell count

The leukocyte count technique was used for total somatic cell count with little modification (Prescott and Breed, 1910) [24]. A 10 µl amount of pooled milk sample was taken with micropipette and was spread evenly in an area of 1 cm^2 on glass slide. After that slide was air dried. The fat globules were dissolved out by pouring a few drops of xylene for 1 minute. Methanol was then used for fixing slide. The slide was washed with distilled water and it was stained with methylene blue (aqueous) for 3-5 minutes. Twenty fields were randomly examined under oil immersion objective and total numbers of cells counted in 20 fields were multiplied by a common factor 15923.55 to determine the total somatic cell count per ml of milk sample.

Isolation of *Staphylococcus aureus*

Isolation and identification of *S. aureus* was done according to Cowan and Steel (1975) [6] and Quinn *et al*. (1994) [25]. Each sample of milk was swabbed on to a nutrient agar medium and later incubated overnight at 37 °C. After 24 h different bacterial colonies were observed for their colour, consistency and morphology. Gram's staining was used as a primary identification test and suspected colonies were streaked on mannitol salt agar in primary, secondary and tertiary fashion and incubated for 24 h at 37 °C. The smears prepared from each of the sub-cultured colonies were fixed by gentle heat, stained by Gram's- method and visualized under oil immersion in order to check the purity of the cultures. After confirmation of the pure growth, the bacterial isolates were allocated code numbers accordingly and the colonies were transferred to paired nutrient slants and were kept under refrigeration at 4 °C.

Assessment of slime production in *S. aureus*

S. aureus isolates that produced slime were assessed as per method of Freeman *et al*. (1989) [11]. bacterial isolates were streaked on Congo Red agar (CRA) and incubated at 37°C for 24h. Slime producing isolates produced black coloured colony and the colour of medium also changed from red to black.

Genotypic confirmation of *Staphylococcus aureus*

Isolation of bacterial DNA was done as per the method by Nachimuttu *et al.* (2001) [18] with little modifications. The genotypic confirmation was done through 23S rRNA ribotyping as per the method by Straub *et al.* (1999) [28] using the primers as mentioned in table-1. The master mix was prepared by mixing GENETAQ Green Master Mix (2X) 12.5 µl, primers (25 pM/µl) 0.5 µl each, DNA template 3.0 µl and Nuclease free water to make 25.0 µl .The PCR cycle included pre denaturation at 95 °C for 5 min, 37 cycle of three steps (denaturation at 94 °C for 40s, annealing at 64 °C for 60s and extension at 72 °C for 75s) and final extension at 72 °C for 7 min.

Amplification of *ica*A and *ica*D gene

The method of Vasudevan *et al.* (2003) [31] was used for the amplification of *ica*A and *ica*D gene. The sequences for two primers used for *ica*A and *ica*D genes are given in table-1. The master mixture was prepared same as done in genotypic confirmation. The PCR cycle included pre denaturation at 95 °C for 5 min, 31 cycle of three steps (denaturation at 94 °C for 60s, annealing at 49 °C for 60s and extension at 72 °C for 60s) and final extension at 72 °C for 5 min.

Table 1: Primers used for detection of genes in *S. aureus* isolates from buffaloes with subclinical mastitis

S No.	Target Gene	Primer sequence	Size (bp)	Reference
1	23S rRNA	F: 5´-ACGGAGTTACAAAGGACGAC-3´	1250	(Straub *et al.*, 1999) [28]
		R: 5´-AGCTCAGCCTTAACGAGTAC-3´		
2	*ica*A	F: 5´-CCTAACTAACGAAAGGTAG-3´	1315	(Vasudevan *et al.*, 2003) [31]
		R: 5´-AAGATATAGCGATAAGTGC-3´		
3	*ica*D	F: 5´-AAACGTAAGAGAGGTGG-3´	381	(Vasudevan *et al*., 2003) [31]
		R: 5´-GGCAATATGATCAAGATAC-3´		

Results and Discussion

A total of 84 milk samples were collected of which 62 milk samples had somatic cell count more than 0.25 million/ml and all of them were positive on bacteriological culture but *S. aureus* was isolated from only 41 samples and all 41 samples were genotypically confirmed by 23S rRNA based ribotyping method. Similar genotypic method of *S. aureus* identification have been employed by many workers (Sanjiv *et al*., 2008; Upadhyay *et al*., 2010; Rathore and Kataria (2012); Nathawat *et al*., 2013; Yadav *et al*., 2015; Bhati *et al*., 2016; Gaurav *et al.*, 2019) [27, 30, 26, 19, 32, 4, 12] with the same primers as described by Straub *et al*., (1999) [28].

The overall incidence of *S. aureus* induced subclinical mastitis in buffalo was 48.80 percent.

Bhati *et al.* (2016) [4] observed 44.70 percent incidence of *S. aureus* induced subclinical mastitis, which is similar to our study. Manasa *et al.* (2019) [15] reported a comparable 50 percent incidence of subclinical mastitis induced by *S. aureus* in buffalo and cows. In another study, Nigam *et al*. (2015) [20] found that the incidence of subclinical mastitis caused by *S. aureus* was 17.50 percent, which is significantly lower than the findings of present study.

Detection of slime production by CRA

In this investigation, all isolates (100%) showed production of slime. Our isolates producing slime on CRA. Similarly, our observations are similar to that of Melo *et al.* (2013) [17] who recorded 85% of 94 *S. aureus* strains from subclinical mastitis affected cows and Al-Iedani (2016) [1] who found 86.1% (31 out of 36) of *S. aureus* isolates to be slime producer.

Molecular detection of *ica*A and *ica*D genes

All 41 isolates found positive for *ica*A and *ica*D genes (Fig.1 and Fig.2, respectively). The detection of the *ica*A (1315 bp) and *ica*D (381 bp) genes in all the investigated staphylococci was done by the amplification of the corresponding fragments.

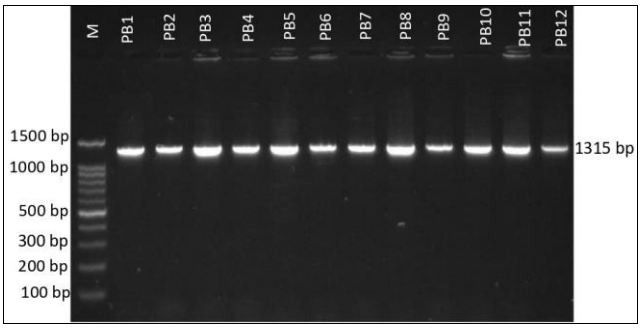

Fig 1: Agarose gel electrophoresis of amplicons of *ica*A gene *S. aureus* isolated from buffaloes with subclinical mastitis. M-Molecular marker (100 bp)

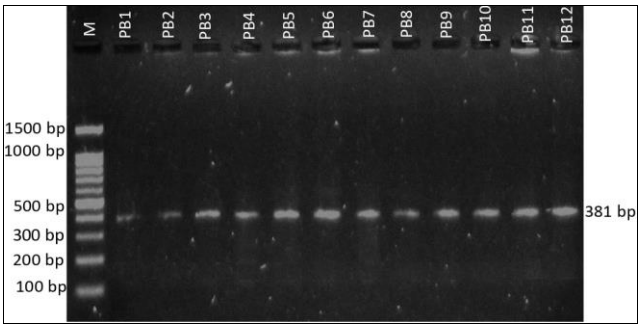

Fig 2: Agarose gel electrophoresis of amplicons of *ica*D gene *S.aureus* isolated from buffaloes with subclinical mastitis. M-Molecular marker (100 bp)

Our finding is in agreement with earlier studies of various researchers (Vasudevan *et al*., 2003; Felipe *et al*., 2017; Notcovich *et al.*, 2018) [31, 9, 21] where 100% *S. aureus* isolates from bovine mastitis were found positive for *ica*A and *ica*D genes. A similar result was reported by Bhati *et al. (*2018) [5] wherein they detect 87.9% *ica*A and 100% *ica*D in the mastitic milk sample.

Some other researcher also found slime producing *S. aureus* but their prevalence was lower the than our present investigation. Almeida *et al.* (2017) [2] reported none of the 32 *S. aureus* isolates from milkers' hands, milking machines and buffalo milk were positive for *ica*A while only seven for *ica*D gene. Low detection rate of *ica*A (15%) and *ica*D (62.5%) genes were observed by Darwish and Asfour (2013) [8] in bovine subclinical mastitis isolates.

Conclusion

Present study revealed that all *S. aureus* isolates from buffalo subclinical mastitis produced slime and all of them were positive for both *ica*A and *ica*D genes. Slime production pattern and detection of gene responsible for them, will help in understanding of virulence and antibiotic resistance property of *S. aurus* and further to control subclinical mastitis in buffaloes.

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