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Detection of *ica*A and *ica*D genes for slime production in *Staphylococcus aureus* isolates from bovine mastitic milk, udder surfaces and milkers' hands

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Abstract
Production of slime (biofilm) by *Staphylococcus aureus* is an important virulence factor in the pathogenesis of mastitis. In the present study 107 *S. aureus* isolates from bovine mastitic milk (n=51), udder surfaces (n=35) and milkers' hands (n=21) from different places were examined for *in vitro* slime production and for the presence of *ica* locus, *ica*A and *ica*D genes. On CRA, 104 (97.2%) isolates of *S. aureus* from various sources and places produced slime with 50 (98.0%), 34 (97.1%) and 20 (95.2%) isolates from mastitic milk samples, udder and milkers' hands, respectively producing slime. All the isolates carried *ica*D gene while a high number of tested strains, 94/107 (87.9%), carried *ica*A gene. The prevalence of *ica*A gene was 90.2%, 88.6% and 81% in isolates from mastitic milk, udder surfaces and milkers' hands, respectively. Hence a higher number of slime producing *S. aureus* isolates, both phenotypically and genotypically, of bovine origin were recovered in the present study. Further the presence of *ica* locus is not always associated with *in vitro* slime production.

Keywords: bovine, *ica* genes, mastitis, Slime, *Staphylococcus aureus*

1. Introduction
Staphylococcus aureus is the most prevalent contagious mastitis pathogen (Pereyra *et al.* 2016; Ganguly 2018) [1, 2] adversely affecting health of dairy animals. The majority of *S. aureus* strains causing mastitis are surrounded by a slime layer, which helps in adherence and colonization of the organism on to the mammary gland epithelium (Baselga *et al*., 1993; Aguilar *et al*., 2001) [3, 4]. Further production of slime enables the bacteria to survive host immune response as well as it leads to reduced susceptibility to antimicrobial by protecting pathogens from antimicrobial agents (Melchior *et al*., 2007; Szweda *et al*., 2012) [5, 6].
Slime production by *S. aureus* requires intercellular adhesion (*ica*) locus, consisting of *ica*ADB and C genes, which encode the proteins that mediate the synthesis of polysaccharide intercellular adhesin (Cramton *et al*., 1999) [7] of which *icaA* and *icaD* have been reported to play a significant role in biofilm or slime production (O'Gara 2007) [8]. As compared to phenotypic methods, molecular detection of *icaA* and *icaD* genes is reliable for determining the potential of *S. aureus* isolates to produce biofilms and may help in the rapid detection of biofilm-producing strains.
Slime producing *S. aureus* isolates have been reported both in animal and human infections but little information is available regarding genotypic characterization of *S. aureus* of animal and human clinical origin with reference to intercellular adhesion genes and its association with phenotypic characters of Indian isolates (Vasudevan *et al*., 2003, Dhanawade *et al*., 2010) [9, 10].
Therefore, the present study was undertaken to characterize *S. aureus* isolated from bovine mastitis and dairy workers for their slime formation trait by phenotypic and genotypic methods.

2. Material and Methods
Sample collection
In the present study, 197 samples were collected from seven different locations (five organized and one unorganized dairy farm in and around Bikaner, Rajasthan, India and one unorganized

dairy farm in Bhiwani, Haryana) in the morning hours and were immediately transported on ice to the laboratory for further processing. From each location, milk samples from cows with clinical mastitis, swabs from udder of infected cows and swabs of milkers' hands who were working in that farm or location, were collected. A total of 80 mastitic milk samples, 66 udder swabs and 51 swabs of milkers' hands collected from seven different places were included in the study.

Isolation and identification of bacteria

Bacterial identification was done by Gram staining, tube coagulase and catalase tests and mannitol salt agar plates as per the standard protocol (Quinn *et al*., 1994) [11]. Phenotypically identified isolates were further geno typically confirmed by *23S rRNA* species-specific polymerase chain reaction (Straub *et al*., 1999) [12]. The primer pairs are given in Table 1.

Phenotypic screening for slime formation

Slime forming capability of the *S. aureus* strains was investigated by cultivation of the isolates on Congo Red Agar (CRA) at 37°C for 24 h. The strains producing black colonies were regarded as slime producers, whereas, strains producing red colonies were considered as slime non-producers.

Molecular detection of icaA and icaD genes

Polymerase chain reaction (PCR) was employed to detect *icaA* and *icaD* genes among all the isolates as given by Vasudevan *et al.* (2003) [9] using primer pairs given in table 1.The 25.0 µl master mix consisted of 5.0 µl 5X Go Taq® Flexi buffer, 3.0 µl $MgCl_2$ (25mM),1.0 µl dNTP mix (25mM each), 1.0 µl Forward Primer (10 pM/µl), 1.0 µl Reverse Primer (10 pM/µl), 0.2 µl *Taq* DNA polymerase (5U/µl), 3.0 µl DNA template (30 ng/µl) and 10.8 µl nuclease free water. 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 34 cycles of amplification (denaturation at 94°C for 60s, primer annealing at 49°C for both *icaA* and *icaD* for 60s and primer extension at 70°C for 60s), and final extension at 72°C for 10 min. The PCR products were resolved on 1.2% agarose gels prepared in 1.0X TBE buffer containing 0.5 µg/ml of ethidium bromide and 100 bp DNA ladder were used as molecular marker. The amplification products were electrophoresed for 50-60 min at 100 volts. The gels were then visualized under gel documentation system (ENDURO GDS).

Table 1: Primer pairs used for amplification of target genes

Target gene	Primers (5'-3')	Reference
23SrRNA	F-5'-ACG GAG TTA CAA AGG ACG AC-3' R-5'-AGC TCA GCC TTA ACG AGT AC-3'	[11]
icaA	F: CCT AAC TAA CGA AAG GTA G R: AAG ATA TAG CGA TAA GTG C	[8]
icaD	F: AAA CGT AAG AGA GGT GG R: GGC AATATG ATC AAG ATA C	[8]

3. Results

In the present study, 107 *S. aureus* isolates were presumptively identified from 197 samples which included 51 isolates from mastitic milk samples, 35 from udder and 21 from milkers' hands.

23S rRNA gene based confirmation- All the 107 isolates which were subjected to PCR amplification targeting 23S rRNA gene produced a species specific amplicon of 1250 bp size confirming them to be *S. aureus.*

Phenotypic detection of slime production by CRA method- Cultivation of the isolates on CRA showed that 104 (97.2%) isolates of *S. aureus* from various sources and places produced slime. Depending on the source of samples, 50 (98.0%), 34 (97.1%) and 20 (95.2%) *S. aureus* isolates from mastitic milk samples, udder and milkers' hands, respectively were found to be slime producers. Location wise, *S. aureus* isolates from four of the seven places of sampling were 100% slime producers while one isolate each from rest of the three places was slime non-producer (Table 2).

Molecular detection of icaA and icaD genes- The results of PCR amplification of *ica*A/D gene revealed that *ica*D gene was present in 107(100%) *S. aureus* isolates from all the sources and places of sampling.

Table 2: Detection of slime production among *S. aureus* isolates

Group No.	Place of sampling	Total isolates	Slime Production						Total (%)	
			Mastitic milk		Udder		Milkers' hands		P	N
			P	N	P	N	P	N		
1.	Bhiwani (Haryana)	08	03	00	02	01	02	00	07 (87.5)	01 (12.5)
2.	Phalodi	21	11	00	06	00	04	00	21 (100)	00 (0)
3.	LRS, Kodamdesar	14	04	00	04	00	06	00	14 (100)	0 (0)
4.	LRS, Beechwaal	11	04	01	03	00	03	00	10 (90.9)	01 (9.1)
5.	Sarvodya basti, Bikaner	34	19	00	13	00	02	00	34 (100)	00 (0)
6.	LRS, Chandan	10	05	00	03	00	02	00	10 (100)	00 (0)
7.	Local Dairy, Bikaner	09	04	00	03	00	01	01	08 (88.9)	01 (11.1)
Total (%)		107	50 (98.0)	01 (2.0)	34 (97.1)	01 (2.9)	20 (95.2)	01 (4.8)	104 (97.2)	03 (2.8)

Abbreviations:- P- Positive, N- Negative; LRS- livestock research station

The overall prevalence of *ica*A gene was found to be 94/107 (87.9%) with 90.2%, 88.6% and 81% isolates from mastitic milk, udder surfaces and milkers' hands, respectively harbouring the *ica*A gene (Table 3). Hence a higher percentage of bovine strains were slime producers both phenotypically and genotypically. All the isolates (100%) from group nos. 4 and 6 and 13 out of 14 (92.9%) isolates of group no. 3 were positive for *ica*A gene. Lowest prevalence of *ica*A gene was 66.7% in group no. 2 isolates as presented in table 3. The presence of the *ica*D (381 bp) and *ica*A (1315 bp) genes in all the investigated staphylococci was shown by the amplification of the corresponding fragments (Figs. 1 and 2).

Table 3: Detection of *ica*A genes among *S. aureus* isolates

Group No.	Place of sampling	Total isolates	*ica*A gene (1315bp)						Total (%)	
			Mastitic milk		Udder		Milkers' hands		P	N
			P	N	P	N	P	N		
1.	Bhiwani (Haryana)	08	03	00	02	01	02	00	07 (87.5)	01 (12.5)
2.	Phalodi	21	08	03	05	01	01	03	14 (66.7)	07 (33.3)
3.	LRS, Kodamdesar	14	04	00	03	01	06	00	13 (92.9)	01 (7.1)
4.	LRS, Beechwaal	11	05	00	03	00	03	00	11 (100)	00 (0)
5.	Sarvodya basti, Bikaner	34	18	01	12	01	02	00	32 (94.1)	02 (5.9)
6.	LRS, Chandan	10	05	00	03	00	02	00	10 (100)	00 (0)
7.	Local Dairy, Bikaner	09	03	01	03	00	01	01	07 (77.8)	02 (22.2)
Total (%)		107	46 (90.2)	05 (9.8)	31 (88.6)	04 (11.4)	17 (81)	04 (19)	94 (87.9)	13 (12.1)

Abbreviations: P- Positive, N- Negative; LRS- livestock research station

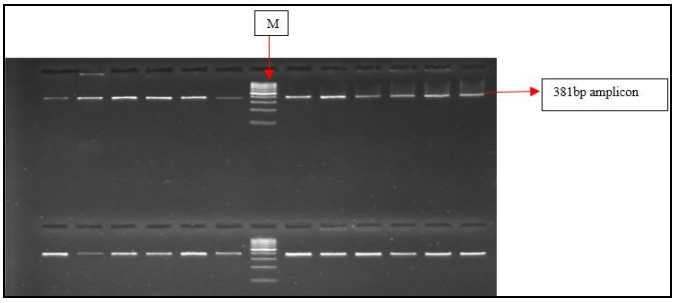

Fig 1: PCR results of *ica*D gene of *S. aureus* isolates; M: molecular marker (100bp)

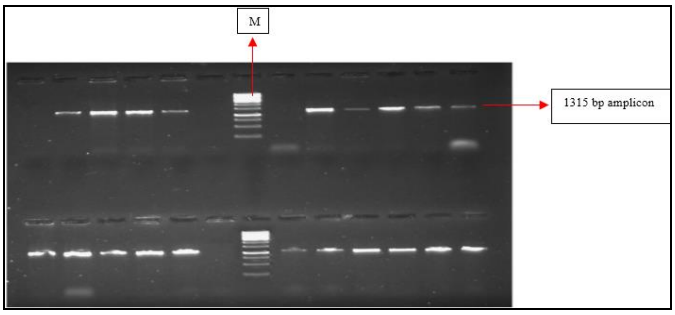

Fig. 2: PCR results of *ica*A gene of *S. aureus* isolates; M: molecular marker (1000bp)

4. Discussion

The first step in the pathogenesis of mastitis caused by *S. aureus* is the adherence and production of slime (biofilm) which enables adhesion of bacteria to the epithelium of mammary glands and is influenced by the presence of *ica* locus (*ica*A and *ica*D genes). Hence early detection and elimination of such slime forming bacteria is necessary to control mastitis.

The results of present study revealed 97.2% isolates to be slime producers which is in agreement to that of Vasudevan *et al.* (2003) [9]. Who found 91.4% *S. aureus* isolates from bovine mastitis to be slime producer on Congo red agar. Fox *et al*. (2005) [13] in a similar study reported 41.4% *S. aureus* from milk samples as biofilm producers, as compared to 24.7 and 14.7% of the isolates collected from skin and liners and suggested that biofilm production was a risk factor for infection.

Our observations of 98% slime producing *S. aureus* isolates from mastitic milk samples is in conformity to that of Singh *et al.* (2011) [14] who reported slime production in 65.4%, 83.6% and 81.4% *S. aureus* isolates from Sahiwal cattle, Karan-fries cattle and Murrah buffalo, respectively with intramammary infections.

Other workers from different parts of the world have also reported a high occurrence of slime producing *S. aureus* isolates from bovine intramammary infections *i.e*. 85% by Melo *et al*. (2013) [15]; 78.4% strains by He *et al*. (2014) [16]; 55.5% by Castelani *et al.* (2015) [17] and 94.17% by Al-Rubaye *et al*. (2016) [18].

Many researchers have also found slime producing *S. aureus* in their studies but prevalence was lower than that obtained in our study. A lower (5.1%) percentage of isolates producing slime from raw milk samples by using Congo Red Agar method was studied by Citak *et al*. (2003) [19] while Ciftci *et al*. (2009) [20] observed 37.2% *S. aureus* strains isolated from bovine mastitis producing black colonies on CRA. Dubravka *et al.* (2010) [21] found only eight (11.42%) out of 70 *S. aureus* isolates from bovine mastitis to be slime producers on CRA.

High prevalence of *ica*A (87.9%) and *ica*D (100%) genes in *S. aureus* isolates, in present study, was found in agreement to earlier studies of Vasudevan *et al.* (2003) [9]; Szweda *et al.* (2012) [6]; Castelani *et al.* (2015) [17]; Felipe *et al.* (2017) [22]; Baloch *et al*. (2018) [23] and Notcovich *et al*. (2018) [24] where 100% *S. aureus* isolates from bovine mastitis were found positive for *ica*A and *ica*D genes. Kuler *et al.* (2013) [25] detected *ica*A gene in 23 out of 25 *S. aureus* isolates from clinical and subclinical mastitis which is in conformity to present results.

In contrast to present study, relatively lower percentage of *ica*A and *ica*D positive isolates were reported from bovine mastitis. de Almeida *et al*. (2017) [26] reported none of the 32 *S. aureus* strains isolated from buffalo milk, milking machines and milkers' hands positive for *ica*A; only seven were positive for *ica*D gene. The low positive rates of *ica*A (15%) and *ica*D (62.5%) genes were observed by Darwish and Asfour (2013) [27] in bovine subclinical mastitis isolates.

In this study, although 104 of 107 *S. aureus* isolates produced slime (biofilm) *in vitro* on Congo red agar, all the 107 isolates were found to carry *ica*D gene while 94 (87.9%) isolates were positive for *ica*A gene. Of the three non-biofilm forming isolates two did not carry *ica*A gene while the third isolate was positive for both *ica* genes. Similar results were reported by Vasudevan *et al.* (2003) [9] and Darwish and Asfour (2013) [27] concluding the fact that the presence of *ica* genes is not always associated with *in vitro* formation of slime or biofilm and the changed phenotype might be associated with the deletion of the entire *ica* locus.

The phenotypic expression of biofilm formation is highly susceptible to *in vitro* conditions hence a combination of phenotypic and genotypic methods should be employed for screening *S. aureus* isolates for biofilm formation.

5. Conclusion

A high percentage of *S. aureus* isolates from different sources

and places of sampling in the present investigation were slime producers as detected by CRA method and PCR amplification of *ica*A/D genes. The presence of *ica* genes is not always associated with *in vitro* formation of slime and other factors may be involved in slime formation. Further, the source, disease conditions, strain differences and geographic location of *S. aureus* isolates may be responsible for the variations in the slime production ability and variations observed in the prevalence of biofilm forming genes.

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