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Slime Production by *Staphylococcus aureus* isolated from raw milk samples of various regions of Jaipur city of Rajasthan state, India

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ABSTRACT

The present study was conducted to characterize the slime production activity of Staphylococcus aureus isolated from raw milk samples of various regions of Jaipur city of Rajasthan, India. Total 144 strains of S. aureus were confirmed by 23S rRNA amplification and 1250 bp product size on agarose gel confirmed S. aureus on molecular level. Slime production was determined by Congo red agar (CRA) method. Out of 144 S. aureus strains 134 (93.06%) produced slime and maximum slime production was found in Durgapura (100%) region followed by Mansarovar (95.65%) region and lowest slime production was found in Jhotwara area of Jaipur city of Rajasthan. This study provides an idea that infection of S. aureus may pose a potential risk to human health and these results may support the future actions related to milk safety programs.

KEY WORDS: *Slime production, Staphylococcus aureus, milk, biofilm*

INTRODUCTION

Milk is a principal territory for complex microbial ecosystems. *Staphylococcus aureus* is a primary pathogen of nosocominal infections and it is responsible for wide range of human diseases including skin infection, bone infection, food poisoning, endocarditis, toxic shock syndrome etc. including bovine mastitis in livestock (Oliveira et al., 2000). *S. aureus* is also the third most reported cause of food borne disease in the world (Normanno et al., 2005). Biofilm formation is one of the most important virulence factors. Bacterial adhesion genes have been shown to play important roles in the initiation of bacterial infection. Polysaccharide intercellular adhesin (PIA, also called biofilm) is one of the most important virulence determinants that facilitate to adherence and colonization of bacteria (Tojo et al., 1988). Biofilm gives special strength to *S. aureus* to contest wide range of adverse circumstances in the host and is considered a major virulence factor in the pathogenesis of mastitis, evade the host immune response and craft multidrug resistance (Brady et al., 2011; Kenar et al., 2012).

Biofilm or slime is composed of polymeric N-acetylglucosamine and its formation process includes initial attachment, cellular aggregation, clumping, exopolysaccharide production and detachment of planktonic cells (Melchior et al., 2006). The majority of *S. aureus* strains causing mastitis are surrounded by a slime layer, which helps in adherence and colonization of the organism on the mammary gland epithelium (Baselga et al., 1993; Aguilar et al., 2001). Biofilm

synthesis is closely related with the expression of intercellular adhesion genes (*icaA* and *icaD* genes). The objective of the present study was to determine the frequency of slime production by *S. aureus* strains isolated from various regions of Jaipur city of Rajasthan.

MATERIAL AND METHOD

Isolation of *S. aureus* from milk samples

Total 790 raw milk samples were collected from street wanders and clinically healthy animals in sterile bottles from 8 different regions of Jaipur city of Rajasthan, India. First of all samples were identified by microbiological methods using phenotypic and biochemical characteristic and poured onto *Staphylococcus aureus* specific chromogenic agar plates having polymyxin B (50 units/ml) (HiMedia Laboratories, India). After 24 hours of incubation at 37°C, the colonies which produce greenish and bluish color were transferred to a BHI broth medium. The color mixture in the center is specifically engraved by *Staphylococcus aureus* to produce bluish-greenish colonies, which can be seen undoubtedly against a dark background. For the further confirmation the cultures were suspended on Baird Parker Agar and Mannitol Salt Agar Plates (Himedia Laboratories). Subsequently, colonies were examined for morphology after Gram staining and confirmed using the API staph kit group (bioMeriux, Marcy-l'Etoile, France).

Genotypic confirmation of *S. aureus* (Ribotyping)

From the microbiologically confirmed cultures, the DNA was isolated by the method of Pospiech and Neumann 1995 and DNA quantification was done by spectrophotometer (Sambrook et al 1989). After that the DNA was diluted to concentration of 25 ng/µl in TE buffer and ribotyping based on 23S rRNA gene was done (Straub et al 1999) using species specific primers i.e. (Primer-1) 5'-CGGAGTTACAAAGGACGAC-3' and (Primer-2) 5'-AGCTCAGCCTTAACGAGTAC-3'. A 25 µl PCR cocktail was prepared which is composed of 12.5µl of 2X DreamTaq green PCR master mix (Thermo scientific, Mumbai, India), 0.5 µM of each primer and 1µl of template DNA was prepared and performed in a thermocycler system (Agilent Technologies, New Delhi, India). The programme used for PCR cycles was: 2 min at 95^0 C, followed by 35 cycles of 30s at 95^0 C, 45s at 57^0 C and 60s at 72^0 C with a final extension of 10 min at 72^0 C. 1 kb ladder was used as marker on 1.2% agarose gel. DNA band were visualized under Gel documentation system (Bio-Rad, USA).

For the preparation of medium 0.4 g Congo red dye, 10 g Glucose/Dextrose and 40 g Blood Agar Base were dissolved in 800 ml of double distilled water. The medium pH was adjusted to 7.2 and 2 % type 1 agar-agar was added. After that volume was make up to one litre and then autoclaved at 121^0C for 15 min. After sterilization, the medium was allowed to cool down to 45^0C in a water bath before transferring into the sterile petriplates.

RESULT AND DISCUSSION

Microbiological identification was done by pacing the samples on specific chromogenic agar plates having polymyxin B and bluish-greenish colonies confirmed the presence of *S. aureus*. Genotypic confirmation was done by 23S rRNA based ribotyping developed by Straub *et al.* (1999). The PCR product with amplicon size of 1250 bp confirms the presence of *S. aureus*. In this study total 144 isolates were confirmed on molecular level and the same method of genotypic confirmation has been used by Yang *et al.* (2012); Parth *et al.* (2016); Hamid *et al.* (2017) and Choudhary *et al.* (2018). They obtained species-specific amplicon of 1250 bp which confirm genotypic identification of this organism from milk samples of different geographical locations.

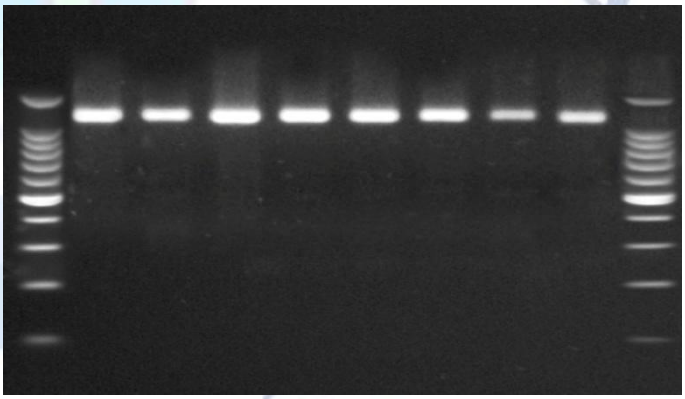

Fig. 1: Molecular identification of *S. aureus* on agarose gel. Amplicon having 1250 bp product size confirms the presence of *S. aureus*.

In the present study, all the 144 *S. aureus* isolates were examined for biofilm production using Congo Red Agar (CRA) method and 132 of the 144 (93.06%) isolates of *S. aureus* from various sources and places produced slime on Congo red agar by formation of rough black color colonies. Only ten isolates (6.94%) were found to be negative for slime production as described in table 1. The strains of

Durgapura are was found to be 100% slime producers while the strain of Mansarovar regions produces 95.65 % slime production. Minimum slime production (87.50%) was found in the stains of Jhotwara region. *S. aureus* isolates from all area's milk samples were found to be slime producers. Similar to the present findings, Vasudevan et al. (2003) also obtained *S. aureus* from bovine mastitis and found 91.40% isolates to be slime producer on Congo red agar. With contrary to this study Fox et al. (2005) reported 41.40% *S. aureus* from milk samples as biofilm producers, as compared to 24.70% and 14.70% of the isolates collected from skin and liners and suggested that biofilm production was a risk factor for infection.

Table 1: Detection of slime production among *S. aureus* isolates from various places of Jaipur city of Rajasthan.

S.No	Place of sampling	Total no. of samples	Total slime production (%)	
			P	N
	Amer	22	20 (90.91%)	2 (9.09%)
	Bagru	14	13 (92.86%)	1 (7.14%)
	Durgapura	19	19 (100%)	0 (0.00%)
	Jhotawara	16	14 (87.50%)	2 (12.50%)
	Khatipura	15	14 (93.33%)	1 (6.67%)
	Mansarovar	23	22 (95.65%)	1 (4.35%)
	Sanganer	17	15 (88.24%)	2 (11.76%)
	Sodala	18	17 (94.44%)	1 (5.56%)
	Total	**144**	**134** (93.06%)	**10** (6.94%)

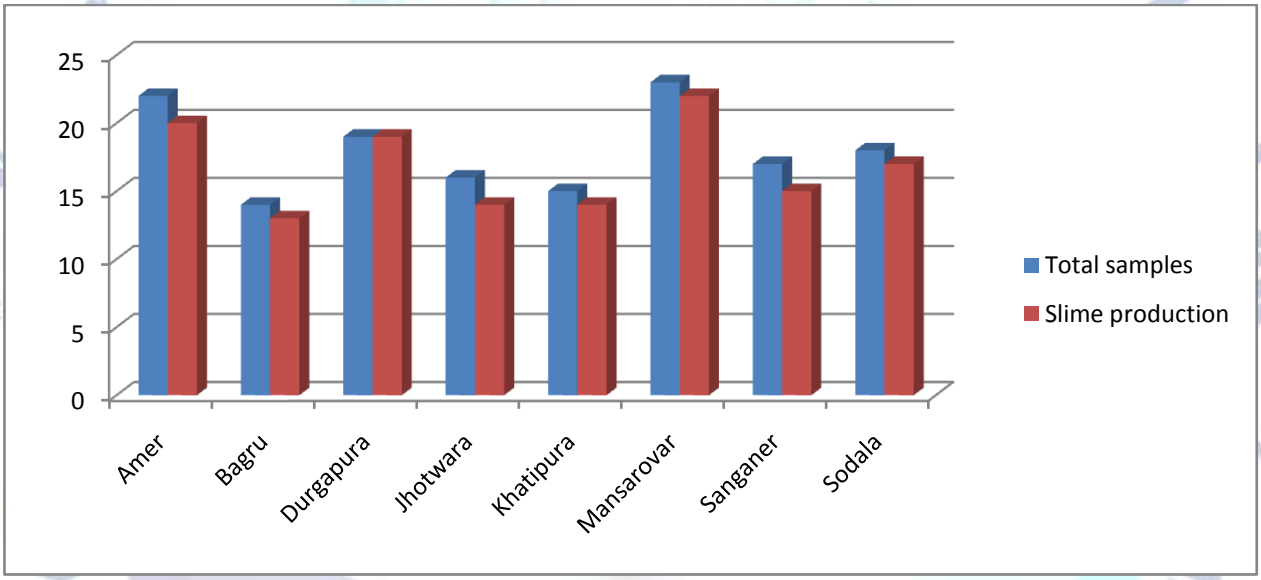

Fig. 2: Slime production by *Staphylococcus aureus* with comparison to total number of milk samples isolated from various regions of Jaipur city of Rajasthan.

Our observations of 93.06% slime producing *S. aureus* isolates from mastitic milk samples is very close to the findings of Singh et al. (2011) 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 which is contrary to present findings.

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); 78.4% strains by He et al. (2014); 55.5% by Castelani et al. (2015); 96.87% by Yadav et al. (2015) and 94.17% by Al-Rubaye et al. (2016). Contrary to the present study, Baloch et al. (2018) reported 96 isolates of *S. aureus* from mastitic milk samples from two different farms producing biofilm with 8.3%, 70.8%, and 18.8% of the isolates demonstrating weak, moderate and strong biofilm formation, respectively.

Many researchers have also found slime producer *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) while Ciftci et al. (2009) observed 37.2% *S. aureus* strains isolated from bovine mastitis producing black colonies on CRA. Dubravka et al. (2010) found only eight (11.42%) out of 70 S. *aureus* isolates from bovine mastitis to be slime producers on CRA.

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. Further, the source and geographic location of *S. aureus* isolates may be responsible for the variations in the slime production ability.

CONCLUSION:

The ability of *S. aureus* to form biofilm or slime *in vivo* is considered to be a major virulence factor influencing its pathogenesis. The formation of biofilm or slime protects bacteria from host immune defense mechanism often resulting in persistent and difficult-to-treat infections. Overall, the outcome of this study revealed that bovine raw milk samples collected from the various regions of Jaipur city of Rajasthan were contaminated with *S. aureus*. This study will be helpful to obtain a better knowledge on the prevalence of slime production among *Staphylococcus aureus* present in milk samples and might be helpful to formulate future strategies to control the bacterial contamination in milk.

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