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Capsular Typing of *Staphylococcus aureus* Isolated from Raw Milk Samples using Duplex Polymerase Chain Reaction

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A B S T R A C T

The present investigation was designed to determine the frequency of capsular polysaccharide genes (*cap5* & *cap8*) in *Staphylococcus aureus* strains isolated from raw milk samples of various regions of Jaipur, the capital city of Rajasthan state of India. In this study presence of *S. aureus* and *cap5*and *cap8* gene was evaluated by Polymerase Chain Reaction. A sum of 59 strains of *S. aureus* (confirmed by 23S rRNA amplification) were selected and screened for the presence of capsular genes. PCR amplification of the gene segment encoding *cap5* and *cap8* gene yielded product size of 361 and 173 bp, respectively. In the present study total 54.24% strains were found to carry *cap5* gene whereas*cap8* gene was found with 27.12% prevalence. Presence of both genes was found is 10.17% strains. Maximum prevalence (66.76%) of *cap5* gene was present in the strains of Amer and Bagru regions while lowest prevalence (37.50%) was found in the strains of Durgapuraregion of Jaipur city. Further, prevalence of *cap8* gene was maximum (42.86%) found in strains of Sodala area which is surprisingly as similar as *cap5* gene and was lowest in strains of Amer area (11.11%). 25% of the strains of Durgapura area were not typable. This study revealed that presence of *cap5* gene was higher than *cap8* gene and also provides an idea that infection of *S. aureus* may pose a potential risk to human health and these result may support the future strategies and actions related to milk safety programs.

Introduction

Milk is a great medium for the growth of many microorganisms and microbial contamination in milk can be largely happen from three fundamental sources viz. inside the udder, outside of the udder and through the surface of milk dealing equipments. The prevalent unhygienic conditions, poor commercialization of dairy sector and improper handling practices favors the entrance of these undesirable and infectious microorganisms into milk and affects the overall nutritional and market value of the product. As evidence from previous investigations, presence of such pathogenic bacteria poses serious health hazards to human and animal's health (Soomro *et al.*, 2003).

Staphylococcus aureus is an essential pathogen of nosocominal contaminations and it is liable for wide scope of human illnesses including skin disease, bone infection, food contamination, endocarditic, toxic shock syndrome etc. Remembering bovine mastitis for domesticated animals *S. aureus* is likewise the third most revealed reason for food borne diseases on the earth (Normanno *et al.*, 2005).

Different PCR based identification techniques were produced for *S. aureus* identification but most of them were not found satisfactorily or trustworthy to distinguish total strains of *Staphylococcus aureus*. Afterward, Straub et al.(1999) built up a Polymerase Chain Reaction (PCR) framework which depended on single primer pair focused against 23S rRNA based species targeted primer, permitting targeted identification of total strains of species. The aimed sequence 23S rRNA was picked on the grounds that it satisfied the necessity of phylogenetic marker having most reliability. This technique is presently being utilized broadly by numerous researchers all through the world for genotypic identification and authorization of *Staphylococcus aureus* among different type of contaminations (Khichar *et al.*, 2014).

Capsular polysaccharide enhances the bacterial virulence by rendering the bacteria resistant to phagocytosis. Although 11 capsular polysaccharide serotypes have been recognized in *Staphylococcus aureus* isolates, only types 5 and 8 are common in *Staphylococcus aureus* isolated from human and bovine infections.

The majority of *Staphylococcus aureus* strains isolated from bovine mastitis is encapsulated and the distribution of capsular serotypes among *Staphylococcus aureus* isolates from bovine mastitis from different countries shows great variability. Hence this study was designed to characterize *cap5* and *cap8* genes in *S. aureus* isolates from raw milk samples of different regions of Jaipur city of Rajasthan, India.

Materials and Methods

The current investigation was attempted to determine the predominance of *Staphylococcus aureus* as a developing food borne microbe considering its general wellbeing essentialness with the point of isolating and recognizing *S. aureus* from raw milk samples. The recouped isolates were confirmed based on their biochemical, morphological and cultural characteristics and further by Polymerase Chain Reaction (PCR) at species and genus level. Cultures identified as *S. aureus* were exposed to study for their properties corresponding to in vitro characterization for the presence of capsular polysaccharide (*cap5* & *cap8)* genes.

Isolation of S. aureus from milk samples

A sum of 790 raw milk samples were collected from 8 different regions of Jaipur city of Rajasthan, India. The samples were taken from street wanders and clinically healthy animals in sterile bottles. Initially samples were identified by conventional microbiological methods using phenotypic and biochemical characteristic. In this regard, the samples were poured onto *Staphylococcus aureus* specific chromogenic agar plates which contain polymyxin B (50 units/ml) (HiMedia Laboratories, India). After 24 hours of incubation at 37ºC, the colonies which produce greenish and bluish colour 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 clearly 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). By tube plasma agglutination test clustered colonies confirmed for coagulation activity were tested.

Genotypic confirmation of *S. aureus* (Ribotyping)

The microbiologically confirmed cultures were allowed to grown overnight in incubator shaker in BHI broth and then DNA was isolated from these cultures individually by the method of Pospiech and Neumann (1995). DNA quantification was done by spectrophotometer (Sambrook *et al.,* 1989) and then 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'-AGCTCAGCCTTAACGAG TAC-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 95ºC, followed by 35 cycles of 30s at 95ºC, 45s at 57ºC and 60s at 72ºC with a final extension of 10 min at 72ºC. 1 kb ladder (Thermo scientific, Mumbai, India) was used as marker on 1.2% agarose gel.

Amplification of *cap5* and *cap8* genes

For the amplification of *cap5* and *cap8 g*enes the method of Verdier *et al.,* (2007) was followed. The primers sequence were (Primer-1) 5'-GTCAAAGATTATGTGATG CTACTGAG-3' and (Primer-2) 5'-ACTTCG AATATAAACTTGAATCAATGTTATACA G-3'used for the amplification of *cap5* gene and it were (Primer-1) 5'-GCCTTATGT TAGGTGATAAACC-3' and (Primer-2)5'-GGAAAAACACTATCATAGC AGG-3' used for the amplification of *cap8* gene. A 25 µl PCR cocktail was prepared as per quantity mention above. The programme used for *cap5*&*cap8*gene PCR cycles was: 2 min at 95ºC, followed by 35 cycles of 30s at 94ºC (denaturation), 50s at 55ºC (annealing) and 50s at 72ºC (elongation) with a final extension of 10 min at 72ºC. The PCR products, after addition of 2 µl of trekking dye were resolved in 1.2 % agarose gels prepared in 0.5 x TBE buffer containing 0.5 µg/ml of ethidium bromide. 100 bp DNA ladder (Thermo scientific, Mumbai, India) was used as marker. The amplified products were electrophoresed for 2 hours at 100 V. After that the gel was visualized under Gel Documentation system (Bio-Rad, USA).

Results and Discussion

Here at initial level, *S. aureus* isolates were identified by conventional microbiological procedures using biochemical and phenotypic characteristics and then final confirmation was done by 23S rRNA based ribotyping developed by Straub *et al.,* (1999) for genotypic confirmation. The PCR products having amplicon size of 1250bpwere confirmed as *S. aureus.* In the present investigation, out of 790 milk samples isolated from different regions of Jaipur city, only 144 isolates were confirmed on molecular level. Out of these 144 confirmed samples 59 isolates shows positivity towards coagulase producing gene (*coa* gene) and these samples having *coa* gene were selected for this study (Data related to *coa* gene is not mentioned here). 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. They did the same from milk samples of different geographical locations.

Genes for antiphagocytosis (*cap5* and *cap8*)

The PCR products 361 bp and 173 bp confirm the presence of *cap5* and *cap8* gene, respectively. A duplex PCR was carried out to detect both *cap5* and *cap8* (capsular genes) in single PCR reaction and also to detect variability in capsular genes (*cap5* and *cap8*) among 59 *S. aureus* isolates. Out of 59 strains, 32 isolates (54.24%) produced amplicon size of 361 bp confirming the presence of *cap5* gene and 16 isolates (27.12%) expressed presence of *cap8* gene with173 bp amplicon size (Table 1). Thus, the majority of the analyzed isolates were having*cap5*genes with compared to *cap8* gene. Both genes were detected in 06 isolates (10.17%). Variability in prevalence of capsular genes was observed in *S. aureus* isolates with respect to different places of sampling though *cap5* genotype predominated almost in all the regions except Sodala region as displayed in the form of figure (Fig. 1 and 2).

Table.1 Distribution of the *cap5* and *cap8* genes among *S. aureus* isolates obtained from different regions of Jaipur city

S. No.	Place of sampling	No. of samples	Total prevalence of capsular polysaccharides gene %			
			***cap5* (361bp)**	***cap8* (173bp)**	**Both**	**NT**
1.	Amer	9	6 (66.67%)	1 (11.11%)	2 (22.22%)	0 (0.00%)
2.	Bagru	6	4 (66.67%)	2 (33.33%)	0 (0.00%)	0 (0.00%)
3.	Durgapura	8	3 (37.50%)	2 (25.00%)	1 (12.50%)	2 (25.00%)
4.	Jhotwara	5	3 (60.00%)	2 (40.00%)	0 (0.00%)	0 (0.00%)
5.	Khatipura	8	4 (50.00%)	2 (25.00%)	1 (12.50%)	1 (12.50%)
6.	Mansarovar	10	6 (60.00%)	3 (30.00%)	0 (0.00%)	1 (10.00%)
7.	Sanganer	6	3 (50.00%)	1 (16.67%)	2 (33.33%)	0 (0.00%)
8.	Sodala	7	3 (42.86%)	3 (42.86%)	0 (0.00%)	1 (14.29%)
Total		**59**	**32 (54.24%)**	**16 (27.12%)**	**6 (10.17%)**	**5 (8.47%)**
***Abbreviations:- n= number of isolates; NT= non typable (negative for both *cap5* and *cap8*genes)**						

Fig.1 Distribution of *cap5*&*cap8* gene (%) in *Staphylococcus aureus* strains isolated from milk samples of various regions

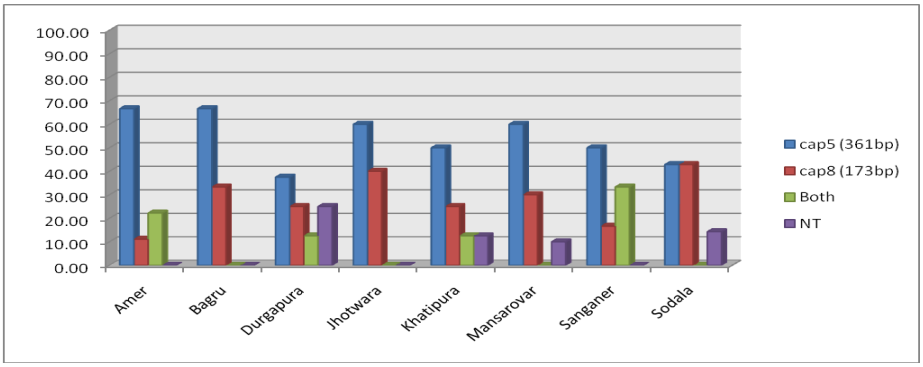
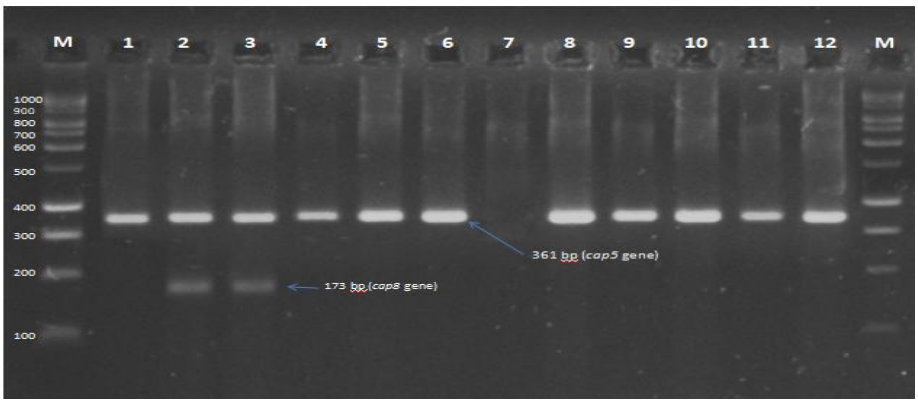


Fig.2 Agarose gel electrophoresis of the*cap5*&*cap8* genes present in various strains of *Staphylococcus aureus.* M=Marker (100 bp ladder)



The present study support the previous studies reporting higher prevalence of *cap5* gene (in comparison to *cap8* gene) in *S. aureus* isolates from different sources viz. 60% *cap5* gene by Upadhyay *et al.,* (2010).Similarly higher proportion of *cap5* isolates has also been published from Indonesia by Salasia *et al.,* (2004), Reinoso *et al.,* (2008); Camussone *et al.,* (2012) from Argentina; Salimena *et al.,* (2016) from Brazil, Kumar *et al.,* (2011) and Krithiga *et al.,* (2018) from India. In a similar study observed by Reinoso *et al.,* (2008), the higher percentage of *cap5* genotype in human isolates (11out of 45) than bovine strains (09 out of 45). All bovine strains were negative with *cap8* gene which is in dissimilar to present study.

Maximum prevalence (66.76%) of *cap5* gene was present in the stains of Amer and Bagru regions while lowest prevalence (37.50%) was found in the strains of Durgapura region of Jaipur city. Further, prevalence of *cap8* gene was maximum found (42.86%) in the strains of Sodala area which is surprisingly as similar as *cap5* gene and it was lowest in the strains of Amer area (11.11%). 25% of the strains of Durgapura area were not typable. Actually such kinds of studies was not done previously in these particular regions so here we can only analyze the same data in cumulative manner with others studies.

In contrary to present study, Tollersrud *et al.,* (2000) reported a greater variation in the distribution of capsular serotypes among isolates from cows of various geographical regions and reported a higher proportion of *cap8* than *cap5* gene. Salasia *et al.,* (2004) observed that majority 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 didn't record any of the isolates harbouring*cap8* gene (Proietti *et al.,* 2010) or carrying *cap5* gene (Soares *et al.,* 2017). Non-typable strains as observed in this study were also reported by Upadhyay *et al.,* (2010); Yadav *et al.,* (2015) from the same laboratory and locations. Contrary to the above findings very low (14%) percentage of *cap5* and *cap8S. aureus* isolates were detected by Sordelli *et al.,* (2000) as 7.10% serotype 5 and 6.60% serotype 8 and very high percentage (86.20%) of non-typable isolates by serotyping.

In conclusion the overall, 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.* By PCR based method, all the isolates were typed by targeting specific genes (*cap5*&*cap8*) with the predominance of

*cap5*gene.This study will be helpful to obtain a better knowledge on the distribution of capsular polysaccharides among *Staphylococcus aureus* present in milk samples of various regions of Jaipur, the capital of Rajasthan and might be helpful to formulate future strategies to control the bacterial contamination in milk.

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