



Characterization of biofilm and coagulase producing *Staphylococcus aureus* associated with bovine mastitis

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ABSTRACT

Present research was planned to study incidence, virulence factors viz. biofilm and coagulase production and antibiogram of *Staphylococcus aureus* associated with bovine mastitis. Milk samples (142) of clinical and subclinical bovine mastitis cases from Mumbai and around Mumbai region were processed. From the milk samples processed; 52 (36.61%) *Staphylococcus* spp. isolates were recovered. Incidence of *S. aureus* in bovine mastitis was 28.16% (in cows: 41.37% and in buffaloes: 24.77%). The incidence rate of *S. aureus* in subclinical and clinical mastitis was 17.24% and 30.97% respectively. The results of antibiotic resistance pattern of isolates indicated highest resistance to penicillin (100%) followed by vancomycin and oxacillin (77.5%) and gentamicin (15%). The least resistance was observed to chloramphenicol (12.5%), ceftriaxone (10%), methicillin and cephotoxim (7.5%). Out of 40 *S. aureus* isolates characterized for biofilm production, overall 32 (80%) isolates were biofilm producing which were confirmed using various methods, viz. CRA (45%), TCP (35%) and PCR (72.5%) of intercellular adhesin gene *ica* A and *ica* D. Of 32 biofilm producing *S. aureus*, 20 (62.50%) were coagulase producing strains and 65.62% of them also showed biofilm production. Out of 40 *S. aureus* isolates, 26 (65.00 %) were proved to be coagulase producing strains by tube method; whereas by *coa* PCR assay, 21 (52.5%) isolates showed presence of coagulase gene. Out of 21 *coa* exhibiting strains of *S. aureus*, 4 (10%) isolates were of *coa* genotype A with an amplicon size of 850 bp while 17 (42.5%) *S. aureus* isolates were of genotype B with an amplicon product of 600 bp. Out of 26 coagulase producing *S. aureus*, 21 (80.76%) were biofilm producing. Most of biofilm and coagulase producing strains displayed multiple drug resistant.

Key words: Biofilm, Bovine mastitis, Coagulase, *Staphylococcus aureus*

Mastitis alone causes approximately 70% of all avoidable losses incurred during milk production. The total economic losses per annum due to clinical and sub-clinical mastitis (SCM) in India are reported to be ₹ 6,038.7 and ₹ 2,345.5 million, respectively (Dua 2001).

While a several types of bacterial pathogens are responsible for this condition, *Staphylococcus aureus* is the principal cause accounting more than 19-40% cases (Kumar *et al.* 2004). The type of mastitis produced by *S. aureus* ranges from subclinical to peracute life threatening form, one of which is gangrenous mastitis.

S. aureus possesses a wide array of virulence factors, occurrence of Staphylococcal biofilms in the bovine udder was reported (Vasudevan *et al.* 2003, Cucarella *et al.* 2004, Fox *et al.* 2005), which raises concerns about the success of antibiotic therapy of persistent Staphylococcal infections by preventing penetration of many antibiotics (Thien and

O'toole 2001) and even antibodies and thus allowing bacterial survival at high antimicrobial concentrations (Oliveira *et al.* 2006). The intercellular adhesion (*ica*) locus consisting of the genes *ica* ADBC encodes the proteins mediating the synthesis of polysaccharide intercellular adhesin (PIA) and polysaccharide/adhesin (PS/A) in Staphylococcal species (Cramton *et al.* 1999, Borriello *et al.* 2005). Among *ica* genes, the *ica* A and *ica* D play a significant role in biofilm formation in *S. aureus* (Yazdani *et al.* 2006), *ica* A gene encodes N-acetylglucosaminyl transferase. Further, *ica* D plays an important role in expression of this enzyme (Arciola *et al.* 2001).

S. aureus exhibits virulence by producing coagulase enzyme that converts fibrinogen to fibrin and clots plasma around the cells which protect *S. aureus* from defence mechanisms, thereby helping in their survival and multiplication. Production of coagulase, a product of coagulase (*coa*) gene, is the principal criterion used in identification of pathogenic *S. aureus*. The amplification of the coagulase gene (*coa* gene) is considered as a simple and accurate method for identifying and discriminating *S. aureus* strains. Systematic study on incidence and characterization of *Staphylococcus aureus* strains associated

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with mastitis is planned in the present research.

MATERIALS AND METHODS

Isolation and identification of S. aureus

Milk samples (142) from clinical and sub-clinical cases of bovine mastitis cases from organized farm as well as unorganized sectors located in and around Mumbai were processed for isolation on *S. aureus* selective medium i.e. mannitol salt agar (MSA). Inoculated plates were incubated at 37°C for 24 h and examined for appearance of typical growth suggestive of *S. aureus*.

Isolates which grew on MSA showing mannitol fermentation were further inoculated on nutrient agar (NA) Baird Parker agar (BPA) and blood agar. Isolates showing golden pigment production on NA and black, shiny, convex colonies of 1-5 mm in diameter with a narrow, white edge surrounded by a clear zone 2-5 mm wide. Opaque rings within the clear zones appeared after 48 h of incubation on BP and hemolytic colonies on blood agar. Isolates showing above typical colony characters of *Staphylococcus* spp. were confirmed for morphology of being Gram positive cocci arranged in grape like clusters and were further subjected to different biochemical tests for confirmation as *S. aureus* (Cowan and Steel 1970).

In vitro antibiotic sensitivity test

All isolates of *S. aureus* were subjected to *in vitro* antibiotic sensitivity test by single disc diffusion method (Bauer *et al.* 1966). Commercially available discs of 8 suitable antibiotics methicillin, oxacillin, penicillin-G, ceftriaxone, cephotaxime, gentamicin chloramphenicol and vancomycin were selected to test the sensitivity of 40 *S. aureus* isolates.

Detection of biofilm production by S. aureus

Phenotypic detection of biofilm production of *S. aureus* was carried out by following 2 conventional methods, viz. Congo red agar (CRA) method and tissue culture plate (TCP) assay.

Congo red agar (CRA) method: Qualitative detection of biofilm production by *S. aureus* was carried out by using the Congo red agar (CRA) method (Vasudevan *et al.* 2003). CRA plates were inoculated with *S. aureus* isolates and incubated aerobically for 24 h at 37°C followed by subsequent storage at room temperature for 24 h. A positive result was indicated by black colonies with a dry crystalline consistency. Non-slime producers remained red with smooth or dry crystalline colonial morphology. The cultures were tested thrice on CRA for biofilm production and results were taken.

Tissue culture plate (TCP) assay: The detection of biofilm production by TCP assay was performed as per Mathur (2006). *S. aureus* strains were grown overnight in TSB (trypticase soy broth) with 1% glucose at 37°C. The cultures were diluted 1:100 in TSB with 1% glucose and 200 µl of this cell suspension was inoculated in wells of sterile 96-well polystyrene microtiter plate in triplicate.

Table 1. Classification of bacterial adherence by TCP method

Mean OD Value	Adherence	Biofilm formation
<0.12	Non	Weak or Non
0.12 – 0.24	Moderately	Moderate
> 0.24	Strong	High

After 24 h incubation, the wells were gently washed 3 times with 200 µl of sterile PBS, dried in an inverted position and stained with 0.1% safranin for 30 sec. Wells were rinsed again for 3 times with sterile distilled water, and the absorbance (OD) was taken at 490 nm. Readings were taken as per Table 1 and isolates showing the strong and moderate adherence/biofilm production were taken as positive.

Genotypic characterization of S. aureus for biofilm production

DNA extraction: Chromosomal DNA of *S. aureus* was extracted as per Wilson (1987) with some modifications. Overnight grown broth culture of *S. aureus* was centrifuged at 12,000 rpm for 10 min. The pellets were dissolved in each of 200 µl TE buffer and 50 µl of 50 mg/ml stock solution of lysozyme. Vortexed and incubated for 2 h at 37°C. Sodium dodecyl sulphate (SDS 10%) in 30 µl quantity and 80 µl of 20 µg/ml stock proteinase K was added, vortexed and incubated for 1 h at 65°C. NaCl 5M in 100 µl quantity was added, vortexed and 80 µl of C-TAB/NaCl solution (10% cetyl-tri-methyl ammonium bromide in 0.7 NaCl) was added, mixed properly and incubated at 65°C for 30 min. Equal amount of phenol:chloroform (1:1) was added, vortexed for 2-3 min and centrifuged at 12,000 rpm for 10 min. Upper aqueous phase was transferred to sterile microcentrifuge tube to which equal amount of chloroform:isoamyl alcohol (24:1) was added, mixed and centrifuged at 12,000 rpm for 10 min. then transferred to sterile microcentrifuge tube. Precipitation of DNA from upper aqueous phase was done by using one-third volume of 7.5 M ammonium acetate and double volume of chilled absolute ethanol. The samples after storage at –20°C for overnight were centrifuged; the pellets were washed with 70% ethanol and air-dried. The pellets were resuspended in 30 µl of sterile nuclease free water (NFW) and incubated in water bath at 65°C for 2 h. The preparation was stored at –20°C and used for PCR.

The isolated DNA samples were subjected to electrophoresis in ethidium bromide stained agarose gel for assessing their integrity as per Brown (2007). The integrity of DNA was checked by visualization of the DNA using U V transilluminator.

Detection of biofilm producing intercellular adhesion genes (ica) by PCR

PCR was carried out for detection of intercellular adhesion genes responsible for biofilm production, viz. *ica A* and *ica D* as per Vasudevan *et al.* (2003). The primers used for PCR of the intercellular adhesion genes (*ica A* and

Table 2. Primer sequences for amplification of *ica A* and *ica D* genes of *S. aureus*

Primers		Sequence (5'-3')	Product size	Reference
<i>Ica A</i>	Forward	CCT AAC TAA CGA AAG GTG G	1315 bp	Vasudevan <i>et al.</i> (2003)
	Reverse	AAG ATA TAG CGA TAA GTG C		
<i>Ica D</i>	Forward	AAA CGT AAG AGA GGT GG	381 bp	Vasudevan <i>et al.</i> (2003)
	Reverse	GGC AAT ATG ATC AAG ATA C		

ica D) were synthesized and procured commercially (Table 2).

Reaction mixture and cycling conditions: The reaction mixture set in total 25 µl quantity and the PCR mixtures were spinned briefly in PCR tubes (0.2 ml) and set into an automated thermal cycler. The cycling conditions included an initial denaturation at 94°C for 3 min followed by 40 cycles each of denaturation at 92°C for 45 sec, annealing at 49°C for 45 sec and extension at 72°C for 1 min, followed by final extension of 7 min at 72°C. The PCR products were further analysed for their molecular weight by agar gel electrophoresis (AGE).

Phenotypic and genotypic characterization of *S. aureus* for coagulase production

Coagulase test: Phenotypic characterization by tube coagulase test was carried out as per Sperber (1975) with slight modification. Each fresh overnight culture of *S. aureus* isolate prepared in brain heart infusion broth (BHI) was inoculated in 0.2 ml quantity separately in 0.5 ml of rabbit plasma and incubated at 37°C. The tubes were examined for the formation of clots at 2, 4 and 24 h intervals. The reactions were interpreted as 4+ reaction where very firm, opaque clot was formed and remained in place when the tube was tipped on its side. The typical 1+, 2+ and 3+ reactions were not as opaque as the 4+ reaction and were surrounded by clear plasma.

PCR of *coa* gene of *S. aureus*: Chromosomal DNA of *S. aureus* was extracted as per Wilson (1987) with some modifications as mentioned under biofilm production.

PCR amplification of coagulase gene (*coa*) of *S. aureus*

Table 3. Primer sequences used for amplification of *coa* gene

Primers		Sequence (5'-3')	Reference
<i>coa</i>	Forward	ATA GAG ATG CTG	Hookey <i>et al.</i> (1998)
		GTA CAG G	
	Reverse	GCT TCC GAT TGT	
		TCG ATG C	

Table 4. Identification of *S. aureus* isolates by conventional methods

Sr. No.1.	Growth on MSA (+)	Golden colonies on NA (+)	Biochemical test				
			Catalase (+)	Oxidase test (-)	VP test (+)	Sugar fermentation	
						Maltose (+)	Trehalose (+)
No. of samples showing above identification criteria	52	40	52	52	50	40	40

isolates was done as per Hookey *et al.* (1998). The primers used for the *coagulase gene (coa)* PCR (Table 3) were synthesized and procured commercially.

Reaction mixture and cycling condition: The PCR was set in total 25 µl reaction volume and PCR mixtures were spinned briefly in PCR tubes (0.2 ml) and set into an automated thermal cycler. The cycling condition included an initial denaturation at 94°C for 5 min followed by 30 cycles each of 40 sec denaturation at 94°C, 1 min annealing at 58°C and 1 min extension at 72°C, followed by final extension of 10 min at 72°C. The PCR products were further analysed for their molecular weight.

Amplified products of *coa* genes were evaluated by agarose gel electrophoresis was carried out in submarine electrophoresis apparatus at 12 V/cm and the product was visualized and documented using automatic computerized gel documentation and analysis system and the size of PCR product generated was determined.

RESULTS AND DISCUSSION

In the present research work, isolation of *Staphylococcus aureus* from clinical and sub-clinical cases of bovine mastitis and their characterization for coagulase and biofilm production was carried out using conventional bacteriological and molecular techniques.

Isolation of *Staphylococcus* spp. was carried out on mannitol salt agar and identification of *S. aureus* isolates recovered from milk samples was done using conventional bacteriological techniques like growth characteristics on various media (Table 4), morphology and staining characters, biochemical and sugar fermentation tests.

Incidence of S. aureus in bovine mastitis

Bovine mastitis is responsible to cause economic loss to the dairy industry world over. Although, a number of microbial species are known to be associated with this condition, *Staphylococcus aureus* is the principal reported cause of mastitis.

Out of 142 (29 cows and 113 buffaloes) milk samples

from the cases of clinical and subclinical bovine mastitis processed, 52 (36.61%) isolates of *Staphylococcus* spp were recovered with an incidence rate of 16 (55.17%) and 36 (31.85%) *Staphylococcus* spp. from cows and buffaloes respectively.

Out of 52 *Staphylococcal* isolates recovered, 40 isolates were confirmed as *S. aureus* based on various identification criteria, while remaining 12 were proved to be the other *Staphylococcus* species. Whereas, from 29 samples of cows and 113 samples from buffaloes, 12 (41.37%) and 28 (24.77%) were confirmed as *S. aureus* respectively (Table 5). The incidence of *S. aureus* in clinical and subclinical mastitis was 30.97 and 17.24% respectively.

Rate of incidence of *Staphylococcus* spp observed in the present study laid between the incidence rate observed by Rao *et al.* (1989) wherein they reported 30.3% of *Staphylococcus* spp. in bovine mastitis, whereas Dhote *et al.* (1999), Khesar (2006) and Chavan (2007) reported 41, 43.47 and 42% incidence of *Staphylococcus* spp. respectively from bovine mastitic milk.

In the present study *S. aureus* was recovered from 28.16% cases of mastitic milk samples. The similar ranges, i.e 26.20 and 26.24% of recovery of *S. aureus* from mastitic milk samples was reported by Takeshinge *et al.* (1983) and Kalorey *et al.* (2007) respectively. Higher rate, i.e. 82.1% was reported by Roberson *et al.* (1996), whereas lower incidences of *S. aureus* in bovine mastitis, i.e. 19 and 15.67% were reported by Njau and Kundy (1985) and Saei *et al.* (2009) respectively. The rate of incidence found in present study laid between the ranges of incidence observed by various authors.

Incidence of *S. aureus* in the present study of clinical and subclinical mastitis was found to be 30.97 and 20.68% respectively. The results of incidence of clinical mastitis due to *S. aureus* in present study laid between incidence reported by Wani and Salmon (2003) and Sumathi *et al.* (2008) who observed 45 and 24% incidence of *S. aureus* respectively from cows with clinical mastitis. Whereas, Getahun *et al.* (2007) observed higher (42.6%) prevalence of *S. aureus* in sub-clinical mastitis. The observations of higher incidence rate of *S. aureus* in clinical than in subclinical mastitis of present study are in correlation with Cheng *et al.* (2010) who reported that *S. aureus* was more (41%) frequently associated with clinical mastitis than sub-clinical case.

Table 5. The organisms isolated from bovine mastitic milk samples

Milk samples	Number of milk samples processed	Bacterial isolates recovered	
		<i>Staphylococcus</i> genus	
		<i>S. aureus</i>	Other <i>Staphylococcus</i> spp
Cow	29	12 (41.37 %)	4 (13.79%)
Buffalo	113	28 (24.77%)	8 (7.07%)
Total	142	40 (28.16%)	12 (8.45%)

In vitro antibiotic sensitivity test

All organisms vary in their susceptibility to antibiotics therefore antibiogram typing, which involves determining susceptibility to battery of antimicrobials is a widely used phenotypic method for characterization of *S. aureus* associated with bovine mastitis (Myllys *et al.* 1998, Osteras *et al.* 2006). The method can be useful for initial screening of multiresistant strain and assist in application of suitable antibiotics leading to proper treatment.

Out of 40 isolates of *S. aureus*, 3 (7.5 %) isolates were resistant to methicillin (M) and cefotaxime (CE), 4 (10%) to ceftriaxone (CI), 5 (12.5%) to chloramphenicol (C), 6 (15 %) to gentamycin (GM), 31 (80 %) to oxacillin (OX) and vancomycin (VA), whereas all isolates (100%) were resistant to penicillin (P) (Fig. 1).

The similar results of high (100%) resistance pattern to penicillin group was observed by Turatoglu and Mudul (2003), Guler *et al.* (2005), Ahmadi *et al.* (2008) and Shi

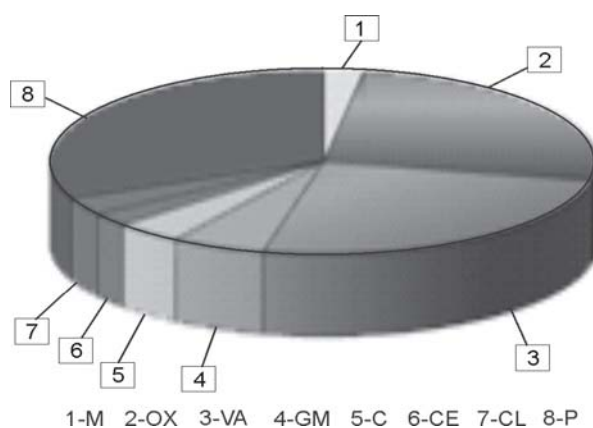


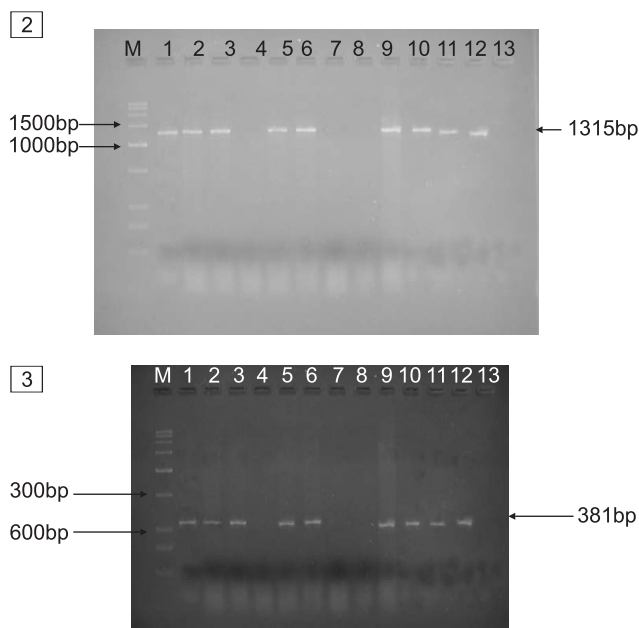
Fig. 1. Resistance pattern of *S. aureus* isolates to different antibiotics used.

et al. (2010). The resistance patterns to methiciline 7.5%. obtained was totally in accordance with Chavan (2007). The least resistance to gentamycin and chloramphenicol, i.e. 15 and 12.5%, respectively correlated with the findings of Khesar (2006), Ahmadi *et al.* (2008) and Sumathi *et al.* (2008) who observed gentamicin and chloramphenicol as useful drug against *S. aureus*. The cephotaxim and ceftriaxone were found to be most effective antibiotics in the present study (Amorena 1999).

Multiple drug resistance was observed in all isolates of cows and buffaloes except in 5 isolates of buffaloes. Out of 3 methicillin resistance strains one was from cow and 2 were from buffaloes which were also resistant to other penicillin group, i.e. VA, OX and P. All 3 methicillin resistance strains were strong biofilm producers whereas, one was coagulase positive (CPS) and 2 were coagulase negative (CNS).

Phenotypic characterization of biofilm producing *S. aureus* strains

Congo red agar (CRA) method: All 40 *S. aureus* isolates screened thrice for detection of biofilm production on CRA.



Figs. 2–3. 2. PCR of *ica A* gene of *S. aureus*; LANE M: DNA Ladder, 1:C4, 2:C5, 3:C10, 4:C18, 5:C29, 6:B7, 7:B11, 8:B16, 9:B60, 10:B63, 11:B118, 12: Known Positive, 13: Known Negative; 3. PCR of *ica D* gene of *S. aureus*; LANE M: DNA Ladder, 1:C4, 2:C5, 3:C10, 4:C18, 5:C29, 6:B7, 7:B11, 8:B16, 9:B60, 10:B63, 11:B118, 12: Known Positive, 13: Known Negative

Out of 40 isolates, 18 (45%) showed black colonies with a dry crystalline consistency suggesting biofilm production. In cows 6 (50%) out of 12 and in buffaloes out of 28, 12 (42.85%) *S. aureus* isolates were biofilm producing. Out of total 18 positive isolates tested by CRA, 2 isolates were negative for *ica* gene.

The results of biofilm production by CRA method in 18 (45%) isolates of *S. aureus* laid between the ranges recorded by Oliveira *et al.* (2006) and Dhanavade *et al.* (2009), i.e. 37.5 and 48.05% respectively, although reports of higher percentage (91.42%) was also recorded by Vasudevan *et al.* (2003).

Tissue culture plate (TCP) method: Out of 40 isolates tested by tissue culture plate (TCP) method, 14 (35%) were biofilm producing. Out of total 14 positive isolates, 11 (78.57%) isolates gave strong biofilm formation (6 from cow and 5 from buffalo) whereas, 3 (21.42%) isolates were moderate biofilm producers (2 from cow and 1 from buffalo). Out of 14 positive isolates tested by TCP, 1 isolate was negative for *ica* gene.

The abundant studies on biofilm production were carried out more in medical fields than in veterinary and the reports of recovery of 57.10 and 57.80% of biofilm producing strains by Johannes *et al.* (2002) and Mathur *et al.* (2006), respectively, from human infections and medical devices are observed at higher range than the findings of biofilm formation in 14 (35%) isolates of present study. The results of above 2 phenotypic methods were correlated, although CRA was found slightly more sensitive than TCP method.

Genotypic characterization of biofilm producing *S. aureus* strains

The extracted DNA showing good integrity were subjected to PCR for amplification of *ica* gene, i.e. *ica A* and *ica D* genes. Out of 40 isolates of *S. aureus*, 29 (72.5%) were positive to *ica A* with an amplicon of 1315 bp (Fig. 2) and *ica D* gene with amplicon size of 381 bp (Fig. 3). The PCR method was more sensitive than CRA and TCP method for detection of biofilm producing strains (Table 6, Fig. 4).

By PCR method of intercellular adhesin gene amplification, 29 (72.5%) *S. aureus* isolates were positive for both *ica A* and *ica D* genes. Higher (100%) prevalence of intercellular adhesion genes namely *ica A* and *ica D* from *S. aureus* of bovine mastitic origin were reported by Vasudevan *et al.* (2003). The importance of intercellular adhesion genes (*ica AD*) in biofilm production were detected in *S. aureus* recovered from human medical devices by Cramton *et al.* (1999), Arciola *et al.* (2001) and Yazdani *et al.* (2006). This also indicated important role of biofilm in pathogenesis of infections caused by *S. aureus*.

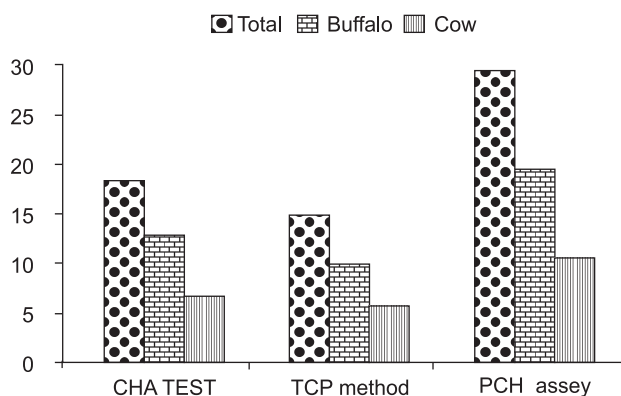


Fig 4. Comparative Efficacy of CRA, TCP and PCR Method for detection of Biofilm producing *S. aureus*

Table 6. Biofilm producing *S. aureus* from bovine mastitis detected by phenotypic and genotypic (PCR) method

Bovine species	Total isolates	Phenotypic method		PCR assay of <i>ica</i> genes	
		CRA	TCPM	<i>ica A</i>	<i>ica D</i>
Cow	12	06 (50.00)	05 (41.66)	10 (83.33)	10 (83.33)
Buffalo	28	12 (42.85)	09 (32.14)	19 (67.85)	19 (67.85)
TOTAL	40	18 (45.00)	14 (35.00)	29 (72.50)	29 (72.50)

*Figures in parentheses indicate percentage.

Out of 32 biofilm producing isolates, 29 isolates possessed both *ica A* and *ica D* genes, whereas 3 isolates which were positive for biofilm production by CRA and TCP method did not possess *ica A* and *ica D* genes. Several workers have investigated the biofilm production in *S. aureus* and other *Staphylococci*, by using phenotypic and molecular techniques. Chaieb *et al.* (2005) investigated the biofilm production in *S. epidermidis* employing CRA and PCR of *ica A* and *ica D* genes and observed that one strain, which was negative for presence of *ica A* and *ica D* genes was phenotypically positive.

The findings of present study of biofilm producing ability of 2 isolates in absence of *ica A* and *ica D* genes is in agreement with that of Chaieb *et al.* (2005). Our results further supported by the observation of Liberto *et al.* (2006) who reported 79.3 % concordance between the slime production and presence of *ica A* and *ica D*. Dhanawade *et al.* (2009) studied biofilm production in *S. aureus* by different methods and observed greater numbers of isolates positive by Congo red agar than by PCR of *ica A* and *ica D* genes.

In present investigation, total 32 (80%) isolates proved to be biofilm producing by both phenotypic and genotypic methods of identification of biofilm producing *S. aureus*. Out of 32 biofilm producing *S. aureus*, 20 (62.50%) were coagulase producing strains and most (65.62%) of them displayed multiple drug resistance.

Phenotypic and genotypic characterization of coagulase producing *S. aureus* strains

Tube method

Out of 40 *S. aureus* isolates tested for coagulase production by tube method using rabbit plasma, 26 (65%) were proved to be positive for coagulase production while 14 (35%) turned out to be coagulase negative (Fig. 5). In cows out of 12 isolates, 11 (91.66%) were CPS, whereas in buffaloes out of 28 isolates, 15 (53.57%) were CPS.

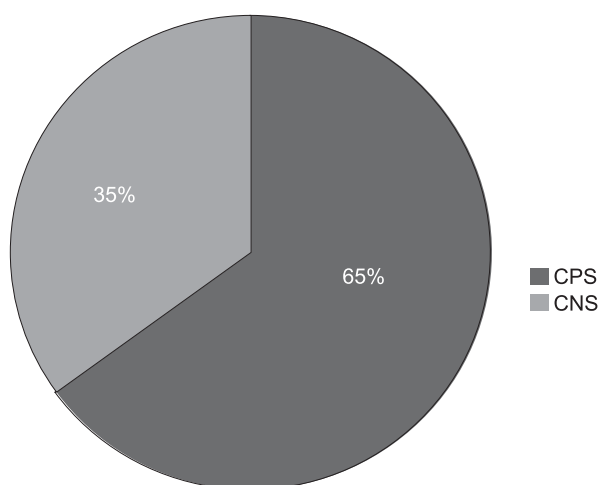


Fig. 5. Proportion of CPS and CNS amongst *S. aureus* isolates from bovine mastitis.

Table 7. Results of coagulase production by *S. aureus* by tube method and molecular method

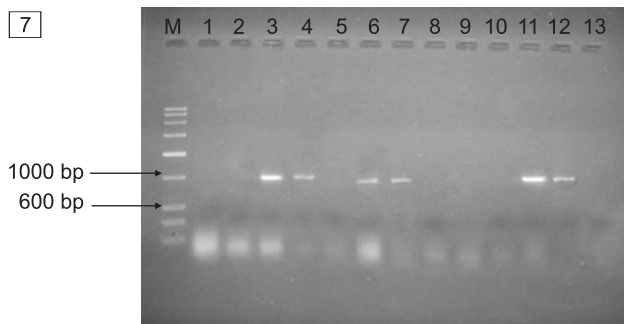
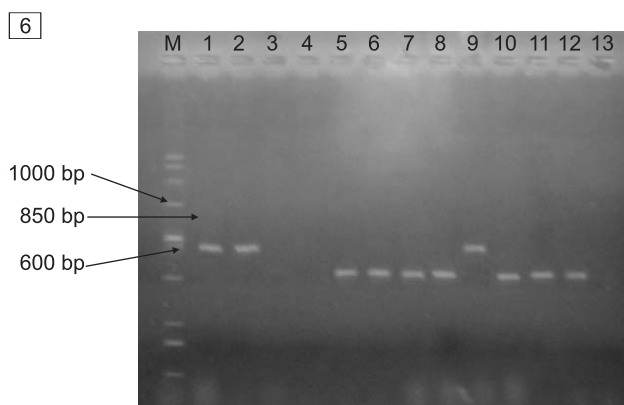
Bovine species	Total isolates	Coagulase production	
		Tube method	<i>coa</i> gene
Cow	12	11(91.66%)	10(83.33%)
Buffalo	28	15(53.57%)	11(39.28%)
Total	40	26 (65.00 %)	21 (52.5%)

Out of 26 isolates tested positive by tube method, 21 exhibited *coa* gene, whereas 5 isolates were lacking *coa* gene (Table 7). Out of 26 coagulase producing *S. aureus* 21 (80.76%) were biofilm producing and most (88.46%) of them displayed multiple drug resistant.

In present study out of 40 *S. aureus* isolates, 26 (65%) were coagulase producing strains (CPS) by tube method. Higher ranges of CPS strains were reported by Baker *et al.* (1985) and Khesar (2006), i.e. 92.5 and 84.78%, respectively, by similar method. Whereas, lower percentages, i.e. 57.14 and 47.05 % CPS isolates were also observed by Hussain *et al.* (2002) and Chavan (2007) respectively. The range of CPS recovered in present study laid between the ranges observed by the above authors.

Detection of coagulase gene (*coa*) by PCR

Out of 40 isolates of *S. aureus* subjected to PCR for amplification of *coa* gene, 21 (52.5%) possessed *coa* gene. Of 21 *S. aureus* isolates tested positive for *coa* gene, 4 (10%) were of genotype A, which generated an amplicon of 850



Figs 6–7. 6. PCR of *coa* gene of *S. aureus*. 7. PCR of *coa* gene of *S. aureus*.

Table 8. Coagulase gene (*coa*) genotypes of *Staphylococcus aureus*

<i>coa</i> Genotype	Amplicon size (bp)	No. of isolates	Percentage (%)
A	850	4	10
B	600	17	42.5

bp, whereas 17 (42.5%) *S. aureus* isolates yielded an amplification product of 600 bp suggestive of genotype B. Only 2 types of genotypes of *coa* gene were observed in 21 isolates originated from different locations in and around Mumbai (Table 8; Fig 7).

The findings of present study concerning existence of different *coa* genotypes among *S. aureus* isolates are in accordance with those of many other workers who have observed prevalence of multiple *coa* genotypes among the *S. aureus* strains. Hookey *et al.* (1998) observed 4 different *coa* PCR types among the *S. aureus* strains tested that yielded amplification products of 875, 660, 603 and 547 bp. Thus results of molecular characterization matched with the result of authors quoted by da Silva *et al.* (2005) who reported 10 different *coa* PCR types. The amplification products generated in their studies ranged from 484 to 1080 bp. Grzegorzczuk *et al.* (2006) while studying the *S. aureus* isolates from nasal mucosa of children encountered 4 different *coa* genotypes with amplicon sizes of 600, 700, 750 and 800 bp. Kalorey *et al.* (2007) while detecting genes encoding virulent determinants of *S. aureus* from sub-clinical mastitis recorded 3 *coa* PCR types generating amplification products of 627, 710 and 910 bp. Karahana and Cetinkaya (2007) reported PCR of *coa* gene produced amplicon ranging from 500 to 1400 bp.

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