Characterization of *Staphylococcus aureus* associated with bovine mastitis

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**Abstract** The present research was planned to study incidence, virulence properties and antibiogram of *Staphylococcus aureus* isolates recovered from bovine mastitis. A total 108 milk samples (48 cows and 60 buffaloes) from clinical and sub-clinical cases of bovine mastitis from different locations of Kolhapur and Sangali districts of Maharashtra state were collected during present investigation. Overall recovery of *Staphylococcus* spp. was 36 (33.33%) isolates (10 cows + 26 buffaloes) and 30 (27.77%) isolates were confirmed as *S. aureus* with an incidence rate of 16.66% in cows and 36.66% in buffaloes, whereas 6 (5.55%) isolates were other *Staphylococcus* spp. The incidence rate of *S. aureus* in subclinical and clinical mastitis was found to be 35.93% and 15.90% respectively. Conventional tube method for coagulase production, DNase test and studies on haemolysis production of *S. aureus* isolates resulted in 9 (30%) CPS, 23 (76.66%) DNase producing and 21 (70%) hemolytic strains. ABST of 30 *S. aureus* isolates by disc diffusion method indicated maximum resistance to vancomycin (90%) followed by ceftriaxone (80%), penicillin-G (73.34%), oxacillin (70%) and amoxicillin (60%) whereas, the least resistance was observed to methicillin (6.67%) and chloramphenicol (10%). Multiple drug resistance was observed in 26 (86.66%) isolates. The PCR amplification of Coagulase gene (coa) of *S. aureus* isolates yielded 14 (46.66%) coagulase positive isolates with amplification sizes ranging from 600 to 850 bp. Four different coa genotypes were generated based on the sizes of amplicons viz. A, B, C and D. Out of 14 coa exhibiting strains of *S. aureus*, Genotype A (850 bp) was observed in 7.14% isolates, whereas genotype B (750 bp) in 14.28% isolates, 50% isolates were of genotype C (700 bp) and 28.57% of genotype D (600 bp). Amongst 14 coapositive isolates, 11 were from subclinical mastitis origin and only 3 were from clinical mastitis cases. All coagulase producing strains of *S. aureus* were haemolytic and DNase producing with an exception of one DNase negative strain.

**Keywords**: Bovine mastitis, characterization, incidence, *Staphylococcus aureus*, virulence factors

**Introduction** Bovine mastitis is one of the main health issues and has been a major challenge to the worldwide dairy industry despite of the widespread implied control strategies (Gruet *et al.*, 2001; Bradley, 2002; Vigier *et al.*, 2009). Mastitis continues to be the most economically important disease of dairy cattle, accounting for 38% of the total direct costs of the common production diseases. *S. aureus* is reported as principal cause accounting for more than 19 to 40% of the cases of bovine mastitis (Kumar, 2004).

A wide array of virulence factors exhibited by *S. aureus* contributing to the virulence includes production of deoxyribonuclease (DNase), catalase, lipases and haemolysins (Gundogan *et al.*, 2006). Toxins produced by *S. aureus* includes extracellular cytolytic toxins like α-toxin, β-toxin, δ-toxin that acts on cell membranes. Enzymes like DNase and coagulase that facilitate in tissue colonization, immune evasion and tissue destruction as virulence factors.

Production of coagulase, a product of coagulase gene (coa gene), is the principal benchmark used in identification of pathogenic *S. aureus*. Coagulase test is a better measure of
the organism's ability to produce extracellular or free coagulase, however not to detect bound coagulase. Therefore, amplification of the coagulase gene (coa gene) is considered to be a simple and accurate method for detection of coagulase production and also for identification and discriminating the strains of *S. aureus*.

The development of antibiotic resistance during treatment with some beta-lactam antibiotics, e.g., Penicillin, is an additional reason for therapy failures (Petersson *et al.*, 1996). *S. aureus* is frequently resistant to antibiotic therapy due to its capacity to produce an exo-polysaccharide barrier and because of its location within micro-abscesses that limits the action of drugs (Gundogan *et al.*, 2006).

The knowledge of virulence factors and prevalence of different types of *S. aureus* strains associated with bovine mastitis cases of various regions of Maharashtra is crucial to develop suitable control strategies by finding out suitable antibiotics and understanding virulence factors, hence the present investigation was planned with a view to study incidence of mastitis due to *S. aureus* and its virulence factors.

## Materials and Methods

### Isolation and identification of *S. aureus*

The milk samples of clinical and subclinical mastitis cases from Kolhapur and Sangati district region of Maharashtra were collected aseptically in sterile screw-cap plastic vials, transported to the laboratory processed by warming to room temperature and homogenizing by vortexing for 1-2 min. A loopful of each sample was streaked on to *S. aureus* selective medium, Mannitol salt agar (MSA). Inoculated plates were incubated at 37°C for 24 hours and examined for appearance of typical growth suggestive of *S. aureus*.

Isolates grown on MSA showing yellow colonies with change of media colour from pink to yellow (plate 1) were further identified by conventional methods based on morphology, colony characteristics (plate 2) and biochemical tests described by Cowan and Steel (1970) and confirmation of *S. aureus* spp. was done.

Conventional characterization of *S. aureus*

*In vitro* antibiotic sensitivity test of all isolates was carried out by single disc diffusion method (Bauer *et al.*, 1966), using seven suitable (commonly used) commercially available antibiotic discs (HiMedia).

Coagulase production was detected as per the method described by Sperber (1975). All the isolates of *S. aureus* were tested for the hemolysis pattern on five per cent sheep blood agar (SBA) and DNAse production was detected on DNAse test agar with Toluidine blue as an indicator as per the method described by Cruikshank *et al.* (1975).

### Detection of Coa gene by PCR

The PCR amplification of coagulase gene (coa gene) of *S. aureus* isolates was done as per the method described by Hookey *et al.* (1998). The primers used for the coagulase gene (coa gene) PCR were synthesized and supplied by M/s Sigma Aldrich Chemical Pvt. Ltd (US).

### Results and Discussion

#### Incidence of *S. aureus* in mastitis

In the present study, 108 (48 cow + 60 buffalo) mastitic milk samples were processed for detection of *Staphylococcal mastitis*. The results indicated recovery of 36 (33.33%) isolates of *Staphylococcus spp.* in bovine mastitis cases. The incidence of *Staphylococcus spp.* observed in the present study laid in the near range of the incidence rate observed by El-seedy (2010) and Mankar (2010) who reported incidence of 30.2% and 36.61% of *Staphylococcus spp.* in bovine mastitis respectively in different regions. However, higher percentage of incidence of *Staphylococcus spp.* from bovine mastitic milk viz 41.00%, 43.47%, 42 %, 40% and have been reported by Dhote *et al.* (1999), Khesar (2006), Chavan (2007) and Sumathy *et al.* (2008) respectively.

In the present study, *S. aureus* was isolated from 27.78% cases of mastitic milk samples. The similar findings of recovery of *S. aureus* from mastitic milk samples were observed by various authors. Arslan *et al.* (2009) recovered 25.66% *S. aureus* isolates. Mankar (2010) and Ranjan *et al.* (2011) reported 28.16% and 27.37% incidence of *S. aureus* respectively.

#### In-vitro antibiotic susceptibility test

The results of antibiotic susceptibility test of *S. aureus* to various antibiotics indicated maximum resistance to Vancomycin (90%) followed by Ceftriaxone (80%), Penicillin-G (73.34%), Oxacillin (70%) and Amoxicillin (60%) whereas, least resistance was observed to Methicillin (6.67%) and Chloramphenicol (10%). Multiple drug resistance was observed in 86.66% *S. aureus* isolates (Plate 3).
The similar result of high resistance pattern to Vancomycin, Oxacillin, Penicillin and Ceftriaxone antibiotics was observed by Deshmukh and Karpe (2005), Abera et al. (2010) and Mankar (2010).

The result of least resistance to Methicillin and Chloramphenicol i.e. 6.67% and 10% respectively were in agreement with the findings of Mankar (2010) i.e. 7.5% and 12.5% resistance to Methicillin and Chloramphenicol respectively. Moreover, Younis et al. (2010) observed 0% resistance to Methicillin, whereas Khesar (2006) reported 4.35% resistance to Chloramphenicol. Therefore, all above authors suggested Methicillin and Chloramphenicol as useful drugs in Staphylococcal bovine mastitis treatment.

Characterization of *S. aureus* for virulence factors by conventional methods

Coagulase production (tube method)

In the present study, out of 30 *S. aureus* isolates, 9 (30%) were found to be coagulase producing strains (CPS) by tube method. However, higher percentage of coagulase production by conventional method was reported by various authors. Boerlin et al. (2003) recovered 50%, Hussain et al. (2003) 38.33%, Khesar (2006) 84.78%, Chavan(2007) 47.01%, Pawar (2009) 95.12%, Mankar (2010) 65% and Mosafeiri et al. (2012) 56.96%. CPS of *S. aureus*.
Haemolysin and DNase production

Out of 30 *S. aureus* isolates, 21 (70%) isolates were found to be haemolytic in nature (Plate 4). The result of present study for haemolysin production was in accordance with the finding of Akineden *et al.* (2001) who reported 72.81%, haemolytic isolates.

Conflicting reports of both lower i.e. 28.30% (Boerlin *et al.*, 2003) and higher percentage 92.68% (Pawar, 2009) incidence of haemolytic strains of *S. aureus* were published. Out of 21 hemolytic isolates, 16 (53.33%) were alpha haemolytic and remaining 5 (16.66%) showed beta haemolysis. Thus indicating lower percentage of beta haemolytic strains than alpha haemolytic strains. Contrast reports of lower percentage of alpha and higher percentage of beta haemolytic strains was observed by Akineden *et al.* (2001) wherein he reported 24.27% alpha haemolytic and 48.54% of beta haemolytic strains. Similarly Younis *et al.* (2010) reported 4.20% of alpha-haemolytic and 33.61% beta haemolytic strains of *S. aureus* isolates.

From total 30 *S. aureus* isolates, 23 (76.66%) isolates were found to be DNase producing strains and remaining 7 (23.33%) were DNase negative (Plate 5). The above results of DNase production was in the near range of the finding reported by Younis *et al.* (2010) in which they found 83.3% DNase positive and 16.6% DNase negative isolates. The results of as high as 98.74% and 100% DNase positive isolates of *S. aureus* were also observed by Boerlin *et al.* (2003) and Nammeen and Jalade (2009) respectively.

All 9 coagulase positive *S. aureus* isolates tested by conventional tube method were also found to be haemolytic and 8 isolates were DNase producing strains. Out of 23 DNase positive isolates, 13 were found to be haemolytic in nature. Similar finding of correlation between above virulence factors was observed by Nammeen and Jalade. (2009) who detected all 52 isolates which were coagulase positive were also found to be haemolysin and DNase producing strains. However, Younis *et al.* (2010) observed no correlation between phenotypic characteristics like haemolysis and DNase activity. Menzies *et al.* (1977) compared the coagulase test with the deoxyribonuclease (DNase) test and observed contrast results, wherein 9 (1%) coagulase-positive isolates were DNase-negative and 56 (18%) coagulase negative isolates were DNase-positive.

Detection of coagulase (coa) gene by PCR

Molecular characterization of *S. aureus* isolates by PCR of coa gene revealed polymorphisms (plate 6) with four genotypes i.e. A, B, C and D with amplicon size of 850 bp, 750 bp, 700 bp and 600 bp respectively. Out of 14 isolates, one isolate was of genotype A (850 bp), 2 were of genotype B (750 bp), 7 were of genotype C (700 bp) and 4 were of genotype D (600 bp). Out of 14 coa gene positive isolates, only 9 were yielded coagulase positive results by conventional tube method, whereas additional 5 isolates which exhibited coa gene were negative in tube method. Fourteen coagene positive isolates were recovered from both Kolhapur and Sangali district. All 7 coagulase positive isolates were from Kolhapur region and were from sub-clinical mastitis cases. Whereas, coa positive isolates of Sangali region were from both subclinical and clinical mastitis cases.

The results of present study concerning existence of different coa genotypes among *S. aureus* isolates are in accordance with the findings of workers who observed prevalence of multiple coa genotypes amongst the *S. aureus* strains. Hookey *et al.* (1998) detected similar gene polymorphism with 4 coa PCR types with amplification products of 875, 660, 603 and 547 bp, Akineden *et al.* (2001) observed 7 PCR products with 600 bp size and 9 products with 840 bp size. Aslanta *et al.* (2007) detected 4 PCR products of coagulase gene with molecular sizes of 730 bp, 810 bp, 890 bp and 970 bp. Kalorey *et al.* (2007) recorded 3 coaPCR types generating amplification products of 627, 710 and 910 bp. Kumar *et al.* (2008) revealed three different types of coagene products (600, 680 and 850 bp). Moreover, the results of molecular characterization of coa gene of *S. aureus* in present study was highly equivalent to the result of Grzegorczyk *et al.* (2006) who obtained 4 different coageneotypes with amplicon sizes of 600, 700, 750 and 800 bp coa genes in *S. aureus* isolates.

In present study gene polymorphism was laid between the amplicon size range of 600 to 850 bp. Gene polymorphism with similar range of amplicon size was observed by Grzegorczyk *et al.* (2006) and Kumar *et al.* (2008) i.e. 600-800 bp and 600-850 bp respectively. The near range of coagene amplicon size as in present study was reported by Hookeyet al. (1998) i.e. 545-875 bp, Akineden *et al.* (2001) 600-840 bp, Aslanta *et al.* (2007) 730-970 bp, Kalorey *et al.* (2007) 627-910 bp, Ciftci *et al.* (2009) 636 - 809bp and Demir *et al.* (2011) 570 to 970bp.

Conclusions

*Staphylococcus aureus* was found to be one of the important bacterial species associated with bovine mastitis. *S. aureus* was found to be associated more with subclinical mastitis cases than clinical mastitis. Majority of isolates were found multiple drug resistant, possessing virulence factors. This is a main concern in public health hazards. The study indicates that still there is prevalence of mastitis, which can be reduced by following good managemental practices at farm, thereby contributing in clean milk production and overcoming the economic losses.
References


