Phenotypic and Genotypic Characterization of *Staphylococcus aureus* of Mastitic Milk Origin from Cattle and Buffalo for some Virulence Properties

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Mastitis is one of the most important diseases of dairy animals causing enormous financial loss and *Staphylococcus aureus* has been held the most common pathogen causing mastitis. The disease process is influenced by various virulence factors possessed by these organisms. In the present study 32 isolates from mastitic milk samples from cattle and buffalo were identified as *S. aureus* on the basis of their colony characteristics, biochemical tests and 23S rRNA ribotyping. All the isolates were subjected to different tests to detect production of virulence factors viz. coagulase, DNase, TNase and slime. In the present study production of coagulase, DNase, TNase and slime was shown by 100%, 93.7%, 100% and 96.87% isolates. The genotyping for capsular genes (*cap*5K and *cap*8K) revealed that 22 isolates (68.75%) were positive for *cap*5K and seven isolates (21.87%) were recorded positive for *cap*8K genes whereas three isolates (9.37%) were found non-typable for both *cap*5K and *cap*8K.

**Key words:** Buffalo, *cap*5 and *cap*8 genes, Cattle, Coagulase, DNase, mastitis, ribotyping, *Staphylococcus aureus*, slime, TNase.
Isolation and identification of S. aureus

In the present investigation, a total of 89 mastitic milk samples were collected (41 were from cattle and 48 were from buffalo) and processed for isolation and identification of S. aureus (Cowan and Steel 1974; Quinn et al., 1994). All phenotypically identified S. aureus were further confirmed by 23S rRNA gene ribotyping (Straub et al., 1999) with some modification using Primer 1: 5'-ACG GAG TTA CAA AGG ACG AC-3' and Primer 2: 5'-AGC TCA GCC TTA ACG AGT AC-3'. The reaction mixture of 30.0 µl volume was prepared by mixing 20.0 µl deionised water, 3.0 µl 10x Buffer, 1.8 µl MgCl₂, 1.0 µl Primer-1 (10 pM/µl), 1.0 µl Primer-2 (10 pM/µl), 0.6 µl dNTP-mix (10mM), 0.1 µl Taq DNA polymerase (5U/µl) and 2.5 µl template DNA (25ng/µl). Amplification was carried out in a Veriti thermal cycler (Applied biosystem) as follows: initial cycle at (denaturation at 94°C for 5 min, primer annealing at 55°C for 30 sec and primer extension at 72°C for 75 sec), 36 cycle of amplification (denaturation at 94°C for 30 sec and primer extension at 72°C for 60 sec), and final extension at 72°C for 5 min. The PCR products, after addition of 2 µl of tracking/ trekking dye were resolved in 1.2% agarose gels prepared in 1.0x TBE buffer containing 0.5 µg/ml of ethidium bromide. Specific DNA ladder (50bp, 100bp and 500bp) was used as molecular marker. The amplification products were electrophoresed for 50-60 min at 100 Volts and visualised under gel documentation system (ENDURO GDS).

RESULTS AND DISCUSSION

Although S. aureus can be identified through primary and secondary biochemical tests but often these tests are cumbersome and time consuming and sometimes we may get refractory results also. Hence, to overcome the limitations of cultural and biochemical methods, all the 32 isolates from cattle and buffalo, after their identification by conventional microbiological procedures were subjected to 23S rRNA based ribotyping for confirmation (Straub et al., 1999). In our study the ribotyping produced an amplicon of 1250 bp in all the isolates confirming them to be S. aureus (Fig. 1 and 2). Similar genotypic method of S. aureus identification have been used by Salasia et al. (2004); Sanjiv et al. (2008); Bhanderi et al. (2009) and Khichar and Kataria (2014) for S. aureus isolates.

In the present investigation all the 32 isolates produced coagulase on human plasma (Fig. 3). Coagulase production is an important phenotypic determinant of S. aureus which is associated with virulence as it resists phagocytosis and helps bacteria in virulence (Bhanderi et al., 2009). Our results are in complete agreement to
those of Arshad et al. (2006) who investigated 23 S. aureus isolates obtained from cattle and buffalo and found all the isolates to produce coagulase. Similarly, all the 71 isolates of S. aureus from cows suspected of mastitis were found to produce coagulase by Cousins et al. (1980). Though coagulase production has been considered to be important criterion in the identification of S. aureus but coagulase negative S. aureus isolates have also been identified and even confirmed genotypically. Singh et al. (2011) had reported coagulase production by only 78.5, 88.3 and 90.7% S. aureus isolates obtained from intramammary infections in Sahiwal cattle, Karan Fries cattle and Murrah buffalo, respectively. Similarly, coagulase negative S. aureus had also been reported by Citak et al. (2003), Turkyilmaz and Kaya (2005), Sanjiv et al. (2008) and Kateete et al. (2010). The present study suggested use of human plasma for the coagulase test for S. aureus. Kateete et al., 2010 also revealed that sensitivity of tube coagulase test with human plasma was more (91%) as compared to that with sheep plasma (81%) in identifying S. aureus.

The DNase and thermostable nuclease activity (TNase) tests are being used for presumptive identification and appear to be a consistent property of S. aureus. The TNase activity is considered important in the identification of S. aureus along with tube coagulase test. In present investigation 30 isolates (93.7%) showed DNase activity by giving the characteristic pink color on the DNase test agar (Fig. 4). Our investigation was in complete agreement with Zarzour et al. (1978) and Citak et al. (2003) who recorded DNase activity in 99.3% and 93.6% of isolates from milk samples. Kateete et al. (2010) revealed that DNase test had a sensitivity of 75% and a specificity of 96% which is similar to our study too. Other researchers (Arshad et al., 2006; Graber et al., 2013; Gundogan et al., 2013) also found more than 90% specificity and sensitivity towards positive DNase activity. Similarly, Singh et al. (2011) reported DNase activity in 86.90%, 87.50% and 86.40% S. aureus isolated from intramammary infections in Sahiwal, Karan Fries cattle and Murrah buffalo, respectively. However, Marques et al. (2013) recorded only 36.84%
coagulase positive *S. aureus* with DNase activity which was much lower than our findings.

In the present investigation thermonuclease activity was shown by all the isolates from cattle and buffalo origin (Fig. 5). Similar to our observation García *et al.* (1980) and Berke and Tilton (1986) found almost 100% of the isolates to show TNase activity in their studies. However, lesser TNase positive *S. aureus* were also reported by some other workers. Sindhu *et al.* (2008) reported thermostable nuclease activity in 87.30% *S. aureus* isolated from mastitic cattle and buffaloes. Singh *et al.* (2011) reported the activity in 60.20% and 65.30% isolates from Kuran Fries cattle and Murrah buffaloes. Likewise, Kumar *et al.* (2011) reported TNase activity in 57% *S. aureus* isolates from mastitic Sahiwal cattle.

Slime production is an important trait of *S. aureus* associated with virulence. In the present study out of 32 isolates, 31 (96.87%) isolates were slime producer as they showed characteristic black color colonies on congo red agar (Fig. 6). Similarly, Vasudevan *et al.* (2003) also recorded 91.4% isolates obtained *S. aureus* from bovine mastitis to be slime producer. Singh *et al.* (2011) reported slime production in 65.4%, 83.6% and 81.4% *S. aureus* isolates from Sahiwal cattle, Kuran fries cattle and Murrah buffaloes, respectively with intramammary infections and Melo *et al.* (2013) reported 85% of *S. aureus* from bovine subclinical mastitis to produce slime. Similarly, Marques *et al.* (2013) also recorded that 176 out of 250 (70.8%) isolates from bovine mastitic milk were slime producers. Many researchers also found slime producing *S. aureus* in their studies but prevalence was much lower than that obtained in our study. In a study by Turkyilmaz and Kaya (2005), out of 180 strains of *S. aureus*, 77.8% coagulase positive *S. aureus* showed positive slime production activity. Liberto *et al.* (2009) recorded 55.2% isolates positive to the CRA test by giving characteristic black color colony. In an investigation by Citak *et al.* (2003) using congo red agar method, slime production was detected positive in only 36 out of 704 isolates (5.1%) of *S. aureus* from raw milk.

![Fig. 5. TNase activity shown by *S. aureus* isolates from mastitic milk of buffalo](image5)

![Fig. 6. Slime production activity shown by *S. aureus* isolates from mastitic milk of cattle and buffalo on congo agar plate by producing typical black color colonies](image6)

![Fig. 7. Agarose gel electrophoresis of amplicons of *cap5K* and *cap8K* gene of *S. aureus* isolates obtained from cattle mastitic milk](image7)

![Fig. 8. Agarose gel electrophoresis of amplicons of *cap5K* and *cap8K* gene of *S. aureus* isolates obtained from buffalo mastitic milk](image8)
Among 11 different capsular serotypes CP5 and CP8 are the predominant among S. aureus strains from ruminants (Tollersrud et al. 2000). In the present study 32 isolates from cattle and buffalo were subjected to amplification for cap5K and cap8K genes responsible for coding of CP5 and CP8, respectively. The amplification of both capsular genes revealed that 22 isolates (68.75%) were positive for cap5K by producing amplicon size of 361bp and seven isolates (21.87%) were positive for cap8K by producing amplicon size of 173bp from both species. However, three isolates (9.37%) were non-typable to both CP5 and CP8 (Fig. 7). While of the buffalo isolates seven (43.75%) were CP5 positive, six isolates (37.50%) were CP8 positive, and three isolates (9.37%) were non-typable for both cap5K and cap8K. Among all cattle isolates 15 (93.75%) were CP8 positive and three isolates (9.37%) were CP5 positive and one (6.25%) was CP8 positive (Fig. 7). When of the buffalo isolates seven (43.75%) were CP5 positive, six isolates (37.50%) were CP8 positive and three isolates (9.37%) were non-typable to both CP5 and CP8 (Fig. 8). The amplicon sizes obtained in the present study are similar to those obtained by (Verdier et al., 2007). Our observations were in complete agreement to those obtained by (Verdier et al., 2007). The presence of a DNase activity is often used as a substitute marker for the identification of coagulate positive staphylococi and particularly of S. aureus in milk samples (Marques et al., 2013).

Staphylococcus aureus can be easily identified by conventional methods but this organism shows variations in phenotypic expressions (Salasia et al., 2004; Sanjiv et al., 2008). Hence, molecular typing approaches have been reported to be of great advantages in identifying and monitoring the local and international spread of S. aureus strains (Sindhu et al., 2008).

REFERENCES


