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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 fators *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.

Mastitis is a well known problem among cattle and buffalo all over the world as it causes huge economic loss to dairy sector. The overall losses due to mastitis have been estimated to be 71655.1 million rupees (Hase *et al*., 2013). The prevalence of bovine mastitis ranges from 29.34% to 78.54% in cows and 27.36% to 70.32% in buffalo (Beheshti *et al*., 2011). *Staphylococcus aureus* is recognized as a major bovine mastitis pathogen worldwide and as one of the significant causes of udder infections in dairy animals (Haveri *et al*., 2007). It has a capacity to produce a large number of accepted virulence factors (Salasia *et al*., 2004). In the present study we investigated virulence factors including production of coagulase, slime, DNase and TNase to determine virulence of this organism.

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MATERIALS AND METHODS

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 $MgCl_2$, 1.0µl Primer-1 (10 pM/µl), 1.0 µl Primer-2 (10 pM/µl), 0.6µl 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 40 sec, primer annealing at 55°C for 60 sec and primer extension at 72°C for 75 sec), and final extension at amplification (denaturation at 94°C for 60 sec, primer annealing at 55°C for 60 sec and primer extension at 72°C for 3 min).

Phenotypic characterization of some virulence factors

Coagulase activity of each isolate was tested by tube coagulase test using sterile human plasma (Quinn *et al*., 1994). DNase activity was demonstrated by culturing organisms on DNase agar with toludine blue dye (Kateete *et al*., 2010). Thermostable nuclease activity was demonstrated by heating the organism in suspension to about 60°C and then putting this suspension on DNase agar with toludine blue dye, which changes color in the presence of the degraded DNA (Sperber and Tatini, 1975). Slime production by the organisms was determined by cultivation on Congo Red Agar (Vasudevan *et al*., 2003).

Capsular genotyping

Amplification of the cap5K and cap8K gene was done as described by Verdier *et al*. (2007) with some modification using primer for cap5K gene Primer1: 5'-GTC AAA GAT TAT GTG ATG CTA CTG AG-3', Primer 2: 5'-ACT TCG AAT ATA AAC TTG AAT CAA TGT TAT ACA G-3' and for cap8K gene Primer 1: 5'-GCC TTA TGT TAG GTG ATA AAC C-3', Primer 2: 5'-GGA AAA ACA CTA TCA TAG CAG G-3'. The reaction mixture of 30.0 µl was prepared by mixing 17.6 µl deionised water, 5.0 µl10x Buffer+$MgCl_2$, 1.5 µl Primer-1 (10 pM/µl), 1.5 µl Primer-2 (10 pM/µl), 1.2 µl dNTP-mix (10mM), 0.2 µl Taq DNA polymerase (5U/µl) and 3.0 µl template DNA (25ng/ µl). Amplification was carried out in a Veriti thermal cycler (Applied biosystem) as follows: initial cycle of amplification at 94°C for 5 min, 25 cycle at (denaturation at 94°C for 30 sec, primer annealing at 55°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%

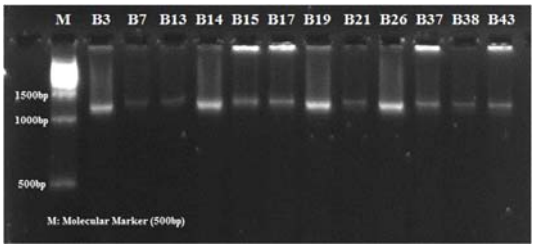

Fig. 1. Agarose gel electrophoresis of amplicons of 23S rRNA gene of *S. aureus* isolates obtained from buffalo mastitic milk

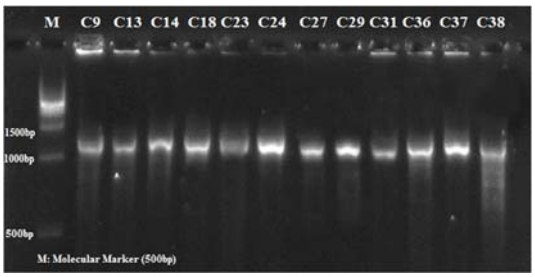

Fig. 2. Agarose gel electrophoresis of amplicons of 23S rRNA gene of *S. aureus* isolates obtained from cattle mastitic milk

Fig. 3. Tube coagulse production shown by *S. aureus* isolates from mastitic milk of cattle and buffalo on human plasma

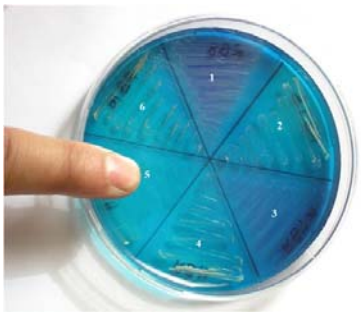

Fig. 4. DNase activity shown by *S. aureus* isolates from mastitic milk of cattle and buffalo species (area 1 and 3)

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 Garcia *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 Karan 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, Karan fries cattle and Murrah buffalo, 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 (76.8%) 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.

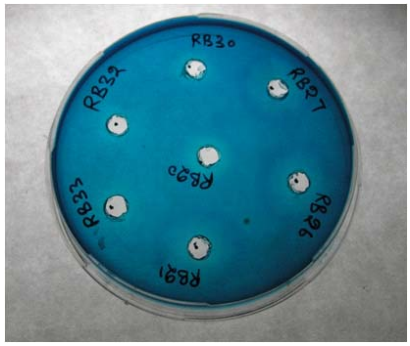

Fig. 5. TNase activity shown by *S. aureus* isolates from mastitic milk of buffalo

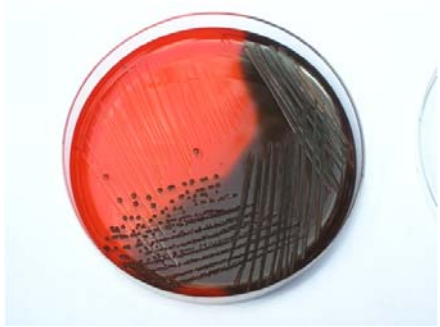

Fig. 6. Slime production activity shown by *S. aureus* isolates from mastitic milk of cattle and buffalo on congo agar plate by producing typical black color colonies

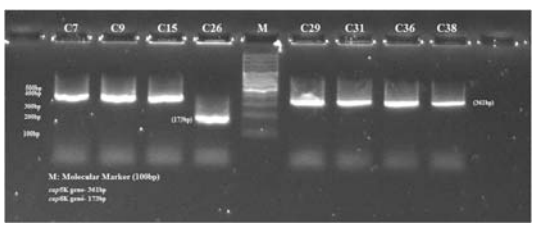

Fig. 7. Agarose gel electrophoresis of amplicons of *cap*5K and *cap*8K gene of *S. aureus* isolates obtained from cattle mastitic milk

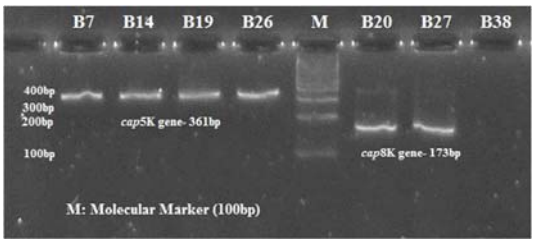

Fig. 8. Agarose gel electrophoresis of amplicons of *cap*5K and *cap*8K gene of *S. aureus* isolates obtained from buffalo mastitic milk

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 recorded positive for cap8K by producing amplicon size of 173bp from both species. However, three isolates (9.37%) were non-typable for both cap5K and cap8K. Among all cattle isolates 15 (93.75%) were CP5 positive and one (6.25%) was CP8 positive (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 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 Poutrel *et al*. (1988) who reported that cap5K and cap8K accounted for 69.4% of bovine isolates and cap5K was predominant in *S. aureus* strains from bovine sources (51.4%) in France. Guidry *et al*. (1998) evaluated the prevalence of serotype5 and 8 *S. aureus* strains in milk from bovines in US and Europe and found that 41% of the US isolates and 70% of the isolates from Europe were CP5 and CP8 positive. Verdier *et al*. (2007) reported 87% of strains (169 of 195) were positive for capsular typing of which 46% strains were the cap5 positive and rest 54% were cap8 positive. Similarly 70% *S. aureus* from bovine with mastitis were reported to possess CP5 or CP8 by Naidu *et al*. (1991). Contrary to the above findings very low (14%) prevalence of CP5 and 8 *S. aureus* isolates was reported by Sordelli *et al*. (2000) who recorded 7.1% serotype5 and 6.6% serotype 8 and very high percentage (86.2%) of non-typable isolates was detected by them by serotyping. Similarly, Sompolinsky *et al*. (1985) reported 17.5% of *S. aureus* isolates to possess CP5 or CP8 capsules. Havaei *et al*. (2013) also studied capsular typing in 193 *S. aureus* isolates and found (49%) were type 8, 46 (24%) type 5, and 53 (27%) were of other types.

Several laboratories in developing countries screen for presumptive *S. aureus* based on growth on mannitol salt agar (MSA), and/or DNase tests and confirmation is done by tube coagulase test and thus improves the efficacy of the tube coagulase test (Kateete *et al*., 2010). A combination of MSA/DNase results in specificity and sensitivity of 92% and 96%, respectively. Other than mannitol various sugars were also fermented by *S. aureus*. However, these tests are not specific for *S. aureus*, so this dual combination can be used along with coagulase for identification of *S. aureus* (Kateete *et al*., 2010; Gundogan *et al*., 2013). Deoxyribonuclease (DNase) is an enzyme that breaks down DNA while thermostable nuclease (TNase) is a specific, heat-stable DNase that breaks down DNA (Gerceker *et al*., 2009). The presence of a DNase activity is often used as a substitute marker for the identification of coagulase positive staphylococci 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).

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