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Phenotypic and Genotypic Haemolysin Properties of *Staphylococcus aureus* Obtained from Milk of Cattle and Buffalo with Clinical Mastitis

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The qualitative and quantitative phenotypic expression of haemolysins along with presence of genes encoding α- and β-haemolysin were determined in 32 *Staphylococcus aureus* isolates from milk of cattle and buffalo with clinical mastitis. Overall haemolytic reactions on sheep blood agar revealed five (15.62%) isolates to show complete haemolysis, 20 (62.50%) isolates to show partial haemolysis, four (12.50%) isolates to show both complete and partial and three (9.37%) did not show any haemolysis. All the 32 (100%) isolates from both cattle (16) and buffalo (16) produced α-toxin, the maximum titre of which was 1: 5120. Whereas beta-toxin was produced by 11 (68.75%) cattle isolates and by seven (43.75%) buffalo isolates with maximum titre of 1:1280 and 1:240, respectively. Delta toxin was detected to be produced by only five (15.62%) isolates, two from cattle and three from buffalo. The genotypic characterization revealed an overall *hla* gene prevalence in 96.8% isolates from both cattle and buffalo wherein a single amplicons of 534 bp was produced while *hlb* gene was amplified by 84.3% (13 cattle and 14 buffalo isolates) producing single amplicon of 833 bp.

Key word: *Staphylococcus aureus*, Cattle, Buffalo, Mastitis, Haemolysis, *hla and hlb* gene.

Staphylococcus aureus is recognized worldwide as a major pathogen causing clinical intramammary infections in dairy cattle and buffalo. The disease is associated with reduced milk quality and high economic loss (Salasia *et al.*, 2004; Graber *et al.*, 2013), and is therefore a key problem for dairy industry. Three types of haemolysins namely α, β and d designated in the order of their discovery have been reported to be produced by *S. aureus* and are considered true virulent factors in causation of mastitis. Alpha-hemolysin (α-toxin) is considered a main pathogenicity factor because of its hemolytic, cytotoxic, dermonecrotic and neurotoxic effects on rabbit erythrocytes (Dinges, 2000; Aryanti *et al.*, 2011). Beta- hemolysin is a sphingo-myelinase that is highly active against sheep and bovine erythrocytes (Larsen *et al.*, 2002) while d-hemolysin as well as α-hemolysin induces pore formation perturbing the cell membrane permeability (Butt *et al.*, 1998). Though it has the ability to lyse erythrocytes and other cells of

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different animals species, it is more active against horse red blood cells (Quinn *et al.,* 1994).

The typing and titration of these haemolysins may well be an indicator of pathogenicity of these organisms in bovine clinical mastitis (Sanjiv and Kataria, 2007; Yang *et al.,* 2012). Several studies have been carried out to demonstrate hemolysin production by *S. aureus* obtained from bovine mastitis (Fitzgerald *et al.,* 1997; Dinges, 2000 and Larsen *et al.,* 2002). The gene *hla* responsible for α-haemolysin is able to dissolve many types of the cells in human and animals including monocytes, lymohocytes, red blood cells, platelets and endothelial cells. Whereas, cytotoxic effect of β-haemolysin (governed by *hlb* gene) suggesting that its primary virulent activity is to modulate host processes that effects pathogenesis rather than to directly kill the host cells. The gene *hlb* can promote the multiplication of *S. aureus* and increase the harm to bovine (Wang *et al.,* 2011). Many workers have carried out typing of *hla* and *hlb* genes (El-Sayed *et al.,* 2005; Haveri *et al.,* 2007; Sudagidan *et al.,* 2008; Coelho *et al.,* 2011; Salasia *et al.,* 2011). The present work elucidated various haemolysins produced by *S. aureus* in terms of qualitative and quantitative assays along with characterization of isolates for *hla* and *hlb* genes.

MATERIALS AND METHODS

Sample collection, Isolation and identification

The milk samples were collected in 5-10 ml amounts from cattle and buffalo affected with clinical mastitis. The sample phenotypically identified as per standard procedures (Quinn *et al.,* 1994). All phenotypically identified *Staphylococci* isolates were further confirmed to be *S. aureus* based on 23S rRNA gene ribotyping (Straub *et al.,* 1999).

Haemolytic properties and Haemolysin assays

The hemolytic activity was evaluated by plating staphylococci isolates on triplicate plates of blood agar base supplemented with 5% sheep, bovine and horse blood for alpha, beta and delta-hemolysin assays, respectively (Quinn *et al.,* 1994). Isolates were inoculated in the form of streaks on the surface of plates and incubated at 37°C for 24 and 48 h. The criteria for hemolysin identification were: complete lytic zone (transparent) with blurred edges for α-hemolysin on ovine and incomplete (non-transparent) lytic zone, which became complete with sharp edges after overnight incubation at 4°C on bovine blood agar, for beta-hemolysin. The delta-hemolysin production was determined as complete hemolytic zones on horse blood agar (Quinn *et al.,* 1994; Bedidi-Madani *et al.,* 1998; da Silva *et al.,* 2005). Qualitative and quantitative assays for haemolysins were done using rabbit, cattle and horse erythrocytes for α-, β- and d-haemolysin, respectively (Sanjiv and Kataria, 2007).

Toxin Production

The test culture suspension (about 1-2 ml, 24h old) was poured, spread well onto surface of semisolid nutrient agar plate and then plates were incubated at 37°C in an atmosphere of 20% carbon dioxide tension for 48 h. Following incubation, the agar medium was sliced into small pieces and the plates were then transferred to deep freezer at -20°C for 30 min. Alternate freezing and thawing was carried out to obtain the fluid from culture. It was then centifuged at 4000 rpm in refrigerated centrifuge machine for 45 min. The supernatant having toxin was collected in screw capped plastic test tubes and was stored at -20°C in deep freezer till use for titration of haemolysins.

Titration of haemolysins

The preparation of erythrocytes and titration of haemolysins were done as per method described by (Sanjiv and Kataria, 2007).

Amplification *hla* and *hlb* gene

Amplification of these gene was carried out as described by Booth *et al.* (2001) using forward primer 5'GGTTTAGCCTGGCCTTC3' and reverse primer 5'CATCACGAACTCGTTCG3' for *hla* gene and forward primer 5'GCCAAAGC CGAATCTAAG3' and reverse primer 5'CGCAT ATACATCCCATGGC3' for *hlb* gene. Briefly, the reaction mixture of 30 µl was prepared by mixing 19.4 µl deionised water, 2.5 µl10x Buffer, 1.8 µl $MgCl_2$, 1.5 µl Primer-1 (10 pM/µl), 1.5 µl Primer-2 (10 pM/ µl), 0.6 µl dNTP-mix (10mM), 0.2 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 30 cycle of amplification (denaturation at 94°C for 30 sec, primer annealing at 53°C for 60 sec and primer extension at 72°C for 30 sec) and final extension at 10°C for 2 min. The PCR products, after addition of

2 µl of trekking dye were resolved in 1.2% agarose gels prepared in 1.0 x TBE buffer containing 0.5µg/ml of ethidium bromide and 500 bp DNA ladder was used as molecular marker. The amplification products were electrophoresed for 50-60 min at 100 V. The gel was then visualized under gel documentation system (ENDURO GDS).

RESULTS AND DISCUSSION

The ribotyping produced an amplicon of 1250 bp in all the 32 isolates confirming them to be *Staphylococcus aureus.* Of the 16 cattle isolates five (31.20%) isolates exhibited complete haemolysis, nine (56.20%) isolates exhibited incomplete/partial haemolysis of which seven isolate showed phenomenon of hot-cold lysis, one (6.25%) isolate showed both complete and partial haemolysis and one (6.25%) isolate was recorded not to produce any haemolysis and was considered as ahaemolytic on sheep blood agar. Of the 16 buffalo isolates 11 (68.75%) showed partial haemolysis of which one showed hot-cold lysis, three (18.75%) showed both complete and partial haemolysis and two (12.5%) did not show haemolysis on sheep blood agar. In the present study the overall haemolytic reactions on sheep blood agar revealed five (15.62%) isolates to show complete haemolysis, 20 (62.50%) isolates to show partial haemolysis, four (12.50%) isolates to show both complete and partial and three (9.37%) did not show any haemolysis.

The observation about haemolytic *S. aureus* in the present study was similar to that reported by Jasper *et al.* (1985) who recorded 99% of the isolates to produce haemolysins. Our results in regards to partial haemolysis by 62.5% *S. aureus* isolates support earlier observations of Matsunaga *et al.* (1993) who recorded 65.5% *S. aureus* from bovine mastitic milk and Morandi *et al.* (2009) who recorded 62% of the isolates from various cow dairy products showing incomplete haemolysis. Likewise, Aarestrup *et al.* (1999) recorded 72% *S. aureus* of bovine mastitic origin to produce incomplete haemolysis. However, our results are in contrast to observation of Boerlin *et al.* (2003) who did not detect incomplete haemolysis on blood agar plate by *S. aureus* isolates. Similar to our observations Islam *et al.* (2007) also recorded more 86.3% *S. aureus* from cattle showing incomplete haemolysis. Our results on haemolysis are also similar to observation of Annemuller *et al.* (1999) who recorded production of complete haemolysis by eight isolates and partial haemolysis

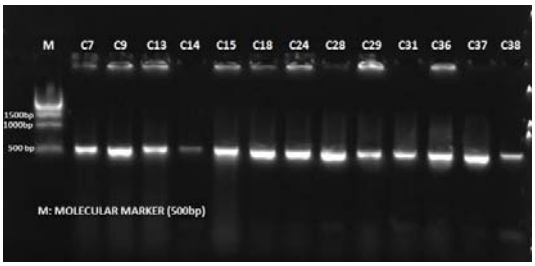

Fig. 1. Agarose gel electrophoresis of amplicons of *hla* gene of *S. aureus* isolates obtained from cattle with clinical mastitis

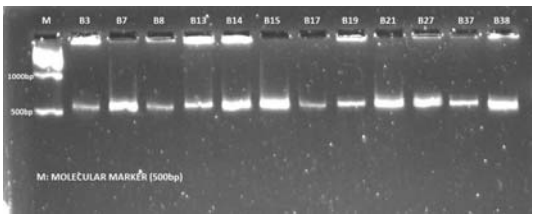

Fig. 2. Agarose gel electrophoresis of amplicons of *hla* gene of *S. aureus* isolates obtained from buffalo with clinical mastitis

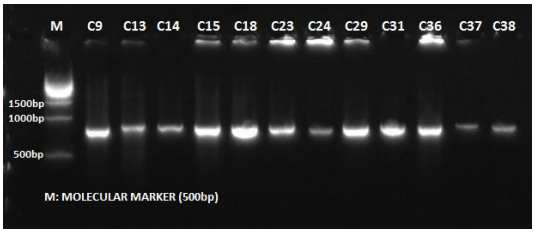

Fig. 3. Agarose gel electrophoresis of amplicons of *hlb* gene of *S. aureus* isolates obtained from cattle with clinical mastitis

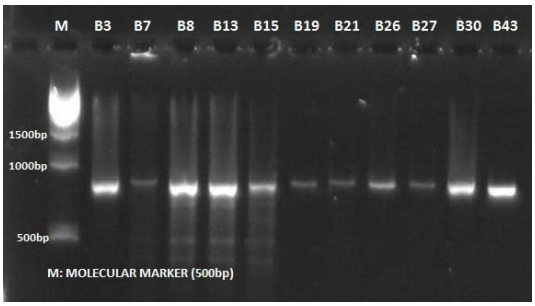

Fig. 4. Agarose gel electrophoresis of amplicons of *hlb* gene of *S. aureus* isolates obtained from buffalo with clinical mastitis

by 13 out of 25 *S. aureus* isolates of bovine mastitis. Similar to our observation Sharma *et al.* (2013) also reported that 12 out of 15 isolates showed partial haemolysis of which four isolates later showed hot-cold lysis whereas three isolated showed complete haemolysis.

In the present study 9.37% of the isolates were ahaemolytic. Graber *et al.* (2013) also recorded very low percentage (0-2%) of non-haemolytic *S. aureus* in their study. Likewise, Sanjiv and Kataria (2007) and Upadhyay and Kataria (2010) did not record presence of ahaemolytic *S. aureus* isolates from the present study area. Similar to our observations of obtaining ahaemolytic isolates, Salasia *et al.* (2004) also reported 10 non-haemolytic isolates out of 35 *S. aureus* isolates from bovine subclinical mastitis. The study of Ariyanti *et al.* (2011), the types of haemolysins of *S. aureus* on the sheep blood agar plate, revealed complete haemolysis for two isolates (18.18%), partial haemolysis for three isolates (27.27%) and no haemolysis for six isolates (54.55%). Production of delta (d) haemolysin is also an important property of *S. aureus* recorded as complete haemolysis on horse blood agar (Quinn *et al.*, 1994). In the present investigation, production of delta haemolysin was shown by nine (28.12%) isolates only. Our observations are contrary to findings of Garcia *et al.* (1980) who found delta-haemolysin production by 47 (82.45%) out of the 57 strains. Likewise, da Silva *et al.* (2005) reported 83.3% isolates of clinical and subclinical caprine mastitic origin to produce delta (d) haemolysin in combination with other haemolysins but not alone. Similarly, Ebrahimi and Taheri (2009) reported production of delta toxin by 62.5% of *S. aureus* isolates from clinical and subclinical mastitis of cow in combination with alpha, beta toxins. Chu *et al.* (2013) also reported 100% isolates to produce complete haemolysis on sheep and horse blood agar.

Toxin assay

Toxin production is considered related to pathogenicity of *S. aureus.* To study the qualitative and quantitative production of toxins, all the isolates were subjected to haemolytic assays using erythrocytes from rabbit, cattle and horse for alpha-, beta- and delta-toxins, respectively**.**

Qualitative Assay

In the present investigation, all the 32 (100%) isolates from both cattle and buffalo haemolysed rabbit erythrocytes indicating presence of alpha-toxin whereas beta-toxin was produced by 11 (68.75%) cattle isolates and by seven (43.75%) buffalo isolates whereas five (15.62%) isolates, two from cattle and three from buffalo were detected as delta-toxin producer. Similar to present observation Upadhyay and Kataria (2010) also reported production of ±-toxin by all the isolates from bovine and goat mastitic milk. Likewise, Jasper *et al.* (1985) also observed alpha and beta toxin by 99% of the isolates and Kenny *et al.* (1992) detected 94.3% of *S. aureus* from bovine mammary glands to produce alpha-haemolysin and suggested alpha-haemolysin production is a feature of bovine mammary isolates.

Our findings are contrary for delta toxin to those of Sanjiv and Kataria (2007) who did not record production of delta-toxin by *S. aureus* isolates obtained from H-F crossbred and Rathi cattle with clinical mastitis. Our results are in partial agreement to those of Upadhyay and Kataria (2010) who observed the production of d-toxin by all of the isolates of *S. aureus* from mastitic milk.

Quantitative Assay

In the present investigation all the 32 isolates produced alpha-toxin of which eight cattle isolates and seven buffalo isolates produced titre of 1:2560 and eight cattle and nine buffalo isolates produced the titre of 1: 5120. The production of beta-toxin was shown by lesser number of isolates where 11 cattle and seven buffalo isolates produced beta-toxin. The titres of beta-toxin were also much less than that for alpha-toxin ranging between 1:5 and 1:1280. Most of the isolates produced lower titres. In this study buffalo isolates produced lower beta-toxin titres than the cattle isolates. In our investigation delta toxin was detected to be produced by only five isolates, two from cattle and three from buffalo. The overall analysis of the haemolysis assay revealed that there was no difference in the qualitative and quantitative production of toxins by the isolates from cattle and from buffalo. Our results were in complete agreement to those of Sanjiv and Kataria (2007) and Upadhyay and Kataria (2010) who also reported production of alpha-haemolysin by all the isolates and with reported high alpha-toxin titres. The lower titres of ²-toxin than that of ±-toxin in the present investigation is in complete agreement to the findings of Sanjiv and Kataria (2007);

Upadhyay and Kataria (2010) who also recorded comparatively lower titres of ²-toxin in their studies.

***hla* and *hlb* genotyping**

The pathogenicity of *S.aureus* is related to the production of a wide variety of exoproteins including alpha and beta haemolysins which contribute to its ability to cause diseases in many mammalian species (da Silva *et al.*, 2005). Alpha-haemolysin or alpha-toxin is considered a main pathogenicity factor because of its haemolytic, dermonecrotic and neurotoxic effects and it is governed by *hla* gene. Beta-haemolysin contains sphingomyelinase that is more active against sheep and bovine erythrocytes (da Silva *et al.*, 2005; Dinges *et al.*, 2000; Larsen *et al.*, 2002) and is governed by *hlb* gene. In the present study all the isolates from cattle except one (C26) amplified *hla* gene producing amplicons of 534 bp (Figure 1). Similar amplicons were produced by all buffalo isolates (Figure 2). The overall *hla* gene prevalence was recorded as 96.8%. The absence of *hla* gene in C26 isolates was well correlated with absence of haemolysis on sheep blood agar.

The *hlb* gene was amplified by 13 cattle and 14 buffalo isolates producing single amplicon of 833 bp in each (Figure 3 & 4). The overall prevalence of *hlb* gene was recorded as 84.3% which was lower than that of *hla* gene. The prevalence of *hla* and *hlb* recorded in the present study was almost similar to observations of Haveri *et al.*, (2007) who recorded the prevalence of 97.4% and 76.7% of the 116 strains for the *hla* and *hlb* genes, respectively. Salasia *et al.*, (2011) also recorded the prevalence of 81.81% isolates for *hla* gene with amplicons size of 534 bp. Likewise, Yang *et al.*, (2012) recorded the prevalence of *hla* and *hlb* gene as 85% and 82%, respectively. However, Booth *et al.*, (2001) observed only 38% (77/200) of the isolates to possess hlb gene. Wang *et al.*, (2011) also recorded that 47 (34.88%) of the *S. aureus* isolates possessed *hla* gene. Similarly, Coelho *et al.*, (2011) also reported that only 24 and 16% of the isolates were positive for the *hla* and *hlb* genes, respectively.

The present investigation was in complete agreement with the findings of the Sudagidan *et al.*, (2008), Salasia *et al.*, (2011), Ariyanti *et al.*, (2011) and Memon *et al.*, (2013) who also found similar amplified product size of the *hla* and *hlb* gene. However, El-Sayed *et al.*, (2005) detected *hlb* gene with a size of approximately 840 bp in all 24 *S. aureus* isolates (100%) obtained from clinical mastitis and in 13 isolates (81.3%) from subclinical mastitis whereas gene *hla* of 550 bp was found in all the *S. aureus* isolates.

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