DETECTION OF VIRULENCE ASSOCIATED FACTORS FROM *STAPHYLOCOCCUS AUREUS* ISOLATED FROM BOVINE MASTITIS

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ABSTRACT

A total of 53 bacterial isolates obtained from bovine mastitis and positive to catalase, and 23S rRNA ribotyping tests were analyzed for production virulence factor viz. mannitol fermentation, Coagulase production, lipase activity, DNase activity, slime production assay and haemolysin production. Out of 53 isolates, 53 (100%), 49 (92.45%), 29 (54.72%), 44 (83.02%) and 42 (79.25%) isolates were found positive for mannitol fermentation, Coagulase production, lipase activity, DNase activity and slime production respectively. Out of 53 isolates, alpha, beta, gamma and alpha-beta haemolysin production were observed in 18 (33.96%), 26 (49.06%), 4 (7.55%) and 5 (9.43%) isolates respectively. Further the isolates were subjected to genotypic evaluation of virulence associated *Coa* and spa genes. Polymorphism was recorded in *Coa* and *spa* genes. Amplification of the *spa* gene revealed 200, 270 and 296 bp size amplicons while 723, 812 and 1000 bp amplicons found in *Coa* gene amplification. Out of 53 isolates, 36 (67.92%) and 32 (60.38%) isolates were found positive for *spa* and *Coa* genes respectively.

Keywords: *Staphylococcus aureus*, mastitis, virulence factor, PCR

INTRODUCTION

Buffalo and cattle are mostly reared for milk production, and the disease mastitis renders them useless for this purpose. Bovine mastitis is a major disease that affects dairy industry and *Staphylococcal* mastitis is a major concern in dairy farming and critical sources of subclinical and clinical intramammary infection in dairy animals leading to severe economic losses to the dairy industry worldwide (Hussain *et al*., 2012). *Staphylococcus aureus* produces a variety of extracellular and cell wall associated virulence factors which are involved in the pathogenesis of mastitis (Momtaz *et al*., 2010). Virulence associated factors like, surface proteins that promote colonization of host tissues, invasions that promote bacterial spread in tissues (leukocidin, kinases, hyaluronidase), surface factors that inhibit phagocytic engulfment (capsule, Protein A), biochemical properties that enhance their survival in phagocytes (catalase production), immunological disguises (Protein A, Coagulase, clotting factor), inherent and acquired resistance to antimicrobial agents and membrane damaging toxins like haemolysins that lyse eukaryotic cell membranes (Todar, 2005).

Staphylococcus aureus can be identified by conventional methods but it has been noted

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that this organism shows variations in phenotypic expressions (Ariyanti *et al*., 2011). In such situations, molecular typing approaches have been reported to be of great advantageous in identifying and monitoring the local and international spread of *S. aureus* strains (Diep *et al*., 2003). The identification of strain is also important to confirm the epidemiological relationships among them. Different workers have studied the phenotypic and genotypic properties to differentiate *S. aureus* isolates (Sanjiv *et al*., 2008).

Production of Coagulase is an important phenotypic feature used worldwide for the identification of *Staphylococcus aureus*. Analysis of Coagulase-encoding *S. aureus* DNA (*Coa*) genes has demonstrated variable sequences in the 3'-end coding region (Goh *et al*., 1992). This region contains a polymorphic repeat region that can be used to differentiate *S. aureus* isolates. This characteristic has been used to type *S. aureus* isolates of human and bovine origin (Schlegelova *et al*., 2003). Protein-A of *Staphylococcus aureus* encoded by *spa* gene is considered as one of the important virulence factors in the development and severity of mastitis (Akinden *et al*., 2001). *Staphylococcal* protein A is a bacterial cell wall product that binds immunoglobulin G and impairs opsonization by serum complement and phagocytosis by polymorohonuclear leukocytes (Gao and Stewart, 2004). The decrease of protein A on the cell surface of *S. aureus* resulted in a greater number of free receptor sites for complement C3b and an increase in phagocytosis (Gemmell and O'Dowd, 1983). The gene encoding protein A (*spa*) is composed of some functionally distinct regions: IgG Fc binding region (*spa*-IgG), X-region (*spa*-X) and at C terminus, a sequence required for cell wall attachment. The repetitive region X of the *spa* gene includes a variable number of 24-bp repeats. The number and sequence of individual repeats may differ among strains (Frenay *et al*., 1996). Strains with more than seven repeats in the X region tended to be epidemic and with seven or fewer repeat units as non-epidemic isolate (Frenay *et al*., 1994). The present study was carried out for the detection of virulence associated factors of *Staphylococcus aureus viz.,* mannitol fermentation, Coagulase production, haemolysin properties, DNase activity, slime production and lipase activity and further the genotypic evaluation of virulence associated *Coa* and *spa* genes in *Staphylococcus aureus* isolates.

MATERIALS AND METHODS

Milk samples from suspected cases of subclinical and clinical mastitis in cows and buffaloes belonging to various places of Banaskantha district were collected aseptically in sterilized vials. A total of 185 milk samples from animals belonging to 161 cows and 24 buffaloes were collected and screened for subclinical mastitis. Samples from subclinical cases were first processed for detection of subclinical mastitis by indirect tests viz. Electrical conductivity meter (Draminski 4Q MAST) and California Mastitis Test (CMT) using Standard protocol as per the manufacturer's instructions. A total of 70 milk samples from clinical cases of mastitis were also collected from cattle and buffaloes (42 from cattle and 28 from buffaloes). Isolation and Identification of *Staphylococcus aureus* was done as per the methods described by Buchanan and Gibson (1974); Cowan and Steel (1974).

Virulence associated factors of *Staphylococcus aureus*

Mannitol fermentation

The isolates of *Staphylococcus aureus,* were inoculated on the Mannitol Salt Agar and incubated at 37°C for 48 h. The yellow coloration of colony along with media considered as mannitol fementers.

Coagulase production

Rabbit plasma (B. D., USA.) was used for production of Coagulase test. The contents of one vial were aseptically rehydrated with 3 ml of sterile distilled water and 0.5 ml of rehydrated plasma was added in a tube. To this 2 to 3 pure colonies picked from agar plate was added. After gentle mixing, the tubes were incubated at 37°C in incubator for upto 4 h. Any degree of clotting within 4 h was considered as positive results.

Haemolysin production

Haemolytic activity of the *Staphylococci* was detected on 5% sheep blood agar. All the isolates were streaked on the medium and incubated aerobically overnight at 37°C for 24 h. Results were interpreted after keeping all the plates at 4°C for 1 to 2 h. Isolates showing Haemolytic zones were taken as positive. Interpretation was made as per Quinn *et al*. (1994). Strains, which showed a wide zone of complete haemolysis with blurred edges, were considered as Alpha haemolysis. Strains, which showed a wide zone of incomplete haemolysis with sharp edges were considered as Beta haemolysis. No haemolysis was considered as Gamma haemolysis.

DNase production

For DNase production, the test was carried out using Tolluidine Blue DNase agar medium. The isolates were streaked on the DNase agar and the plates were incubated at 37°C for 4 days. After incubation, 1N HCL was poured in the plates. Clearing zone around the bacterial growth was evaluated as positive (Deighton *et al*., 1988).

Slime production assay

For slime production assay, each isolate was streaked on the Congo red agar medium and incubated aerobically at 37°C for 24 h followed by storage at room temperature for 48 h. The production of rough black colonies by the isolates indicated production of slime (Freeman *et al*., 1989).

Lipase activity

Lipase activity was detected by adding 5% Egg Yolk Emulsion (Hi-Media Pvt. Ltd., Mumbai) in a Mannitol Salt Agar. All the isolates were streaked on the medium and incubated aerobically at 37°C for 72 h. A yellow opaque zone around colonies was indicative of lipase activity produced by *Staphylococcus aureus* (Gunn *et al*., 1972) and considered as positive for Lipase activity.

Molecular characterization of *staphylococcus aureus* by *coa* and *spa* gene

The DNA extraction was carried out as per the protocol outlined in the manufacturer's manual using Genpro ™ 3-in-1 DNA isolation kit (GeNei, MERCK). The quality and purity of DNA were checked by Agarose Gel Electrophoresis using 0.8% agarose and by Picodrop. (Picodrop, U.K.) The amplification of *Coa* and X region of spa gene spa gene was carried out by using *COA*G2 Forward 5'CGAGACCAAGATTCAACAAG3', *COA*G3

Reverse 5'AAAGAAAACCACTCACATCA3' and Forward 5′CAAGCACCAAAAGAGGAA-3′ Reverse 5′CACCAGGTTTAACGACAT-3′ respectively. PCR was carried out in final reaction volume of 25 µl in a thin walled 200 µl PCR tubes using a Nexus Mastercycler (Eppendorf). Cycling condition of PCR for detection of *Coa* and X-region of Spa gene of *Staphylococcus aureus* isolates was 94°C for 5 minutes, 94°C for 1 minute, 60°C for 1 minute, 72°C for 1 minute, 72°C for 5 minutes, 94°C for 5 minutes, 95°C for 30 seconds, 55°C for 45 seconds ,72°C for 2 minutes, 72°C for 7 minutes respectively.

RESULTS AND DISCUSSION

Mastitis is an infectious disease of dairy ruminants that affects milk production and quality. This disease has been singled out as the most significant cause of economic losses to the dairy industry. *S. aureus* was considered to be the most common cause of subclinical and clinical bovine mastitis in most countries in the 1990's and remains a major mastitis pathogen despite use of various control measures (Booth, 1995).A total of 53 isolates were obtained from subclinical and clinical cases of mastitis. Overall incidence of *S. aureus* in clinical and subclinical mastitis was found to be 20.78% (53/255). Considering species wise incidence, 30.77% (16/52) buffaloes were found positive for *S. aureus* from the clinical and subclinical mastitic milk, where as 18.23% (37/203) cows were detected positive. From clinical cases, 54.29% of samples yielded *S. aureus* (cows 61.90% and buffaloes 42.85%). Similarly, in subclinical mastitis 8.11% samples detected positive for *S. aureus* (cows 6.83% and buffaloes 16.66%). In contrast to the present study, Bhanderi *et al*. (2009) observed higher rate of incidence for *S. aureus* mastitis. The possible reasons for the lower incidence of *Staphlylococcus aureus* responsible for clinical and subclinical mastitis compared to other reports may be attributed to the etiology of mastitis that the *Staphylococcus* may not be responsible for the mastitis or some other reasons like breed of the animal, seasons of the sampling, mastitic status of the animals etc.In Gram's stained culture smears under microscope, organisms revealed spherical and irregular clusters like bunch of grapes. The isolates were found Catalase, Maltose fermentation and Phosphatase test positive whereas found Oxidase negative.

Virulence factors of *Staphylococcus aureus*

Mannitol fermentation

In the present study all the 53 (100%) isolates were able to ferment mannitol on MSA. Similar findings were reported by Khichar (2011), Makwana *et al*. (2012) and Thaker *et al*. (2013) who observed percent mannitol fermentator *Staphylococcus aureus* while Bhanderi (2007) who found 74.41% mannitol fermentation by *Staphylococcus aureus* isolates.

Coagulase production

In the present study, all the 53 isolates of *S. aureus* were subjected to tube Coagulase test using rabbit plasma (B.D., U.S.A). Out of these, 49 (92.45%) isolates were found positive for Coagulase production and 4 isolates were found negative. These Coagulase negative isolates in further study were confirmed not to possess *Coa* gene but were *S. aureus* as confirmed by 23S rRNA gene ribotyping using species specific primers. Similar findings were reported by Niazi *et al*. (1987) and Pandya (1991) using rabbit plasma. Turutoglu

et al. (2005) reported 89.77% Coagulase *S. aureus* from cows with subclinical mastitis. Makwana *et al*. (2012) reported that among 100 *Staphylococcal* isolates, 94 isolates (94.00%) were positive for tube Coagulase test. Lower Coagulase activity of *Staphylococcus aureus* ranging from 31.30% to 53.48% from mastitic milk was also reported by Pankaj *et al*. (2013) and AI-Jumaily *et al*. (2014).

Haemolysin production

Out of total 53 isolates, number of isolates showing alpha, beta, gamma and alpha-beta haemolysin production were 18 (33.96%) (13 from cattle and 5 from buffaloes), 26 (49.06%) (18 from cattle and 8 from buffaloes), 4 (7.55%) (3 from cattle and 1 from buffaloes) and 5 (9.43%) (3 from cattle and 2 from buffaloes) on sheep blood agar, respectively. The present study is more or less similar to the findings of Pandya (1991), who observed alpha (11.71%), beta (54.68%) and alpha-beta (19.53%) haemolysin and Patel (2008) observed alpha (27.5%), beta (48.75%), alpha-beta (11.25%) and gamma (13.75%) haemolysin. In the present study, beta haemolysin producing *Staphylococcus aureus* were found predominant (49.06%) in the clinical and subclinical mastitis cases. Which support the views of Morandi *et al*. (2009) opined that beta haemolysin production is a characteristic of animal strains of *Staphylococcus aureus*. In contrast to these, Bhanderi (2007) found that 62.79% alpha hemolytic *Staphylococcus aureus* of animal origin. Fei *et al*. (2011) reported 43.4% alpha, 34.11% beta and 22.48% gamma haemolysis from bovine mastitis.

DNase production

Out of total 53 isolates, 44 (83.02%) (32 from cattle and 12 from buffaloes) isolates were revealed DNase production from clinical and subclinical cases of mastitis. Similar findings were obtained by El-Jakee *et al*. (2010) and Bhati (2013) who found 84.9 and 81.6 percent isolates producing DNase activity, respectively. Higher incidence (100%) of DNase producing *Staphylococcus aureus* has been reported Matsunaga *et al*. (1993) and Gundogan *et al*. (2006) whereas, lower incidence was reported by Kalorey *et al*. (2004) who observed 58.62% isolates producing DNase, respectively.

Slime production

A total 53 isolates were examined for slime production, out of these 42 (79.25%) (31 from cattle and 11 from buffaloes) isolates gave positive result. Similar to the present findings, Patel (2008); Darwish and Asfour (2013) also observed 82.5% and 70.4% isolates positive for *Staphylococcus aureus* for slime production on CRA medium, respectively.

Lipase production

Out of 53 isolates, 29 (54.72%) (20 from cattle and 09 from buffaloes) isolates showed Lipase activity. Similar findings have been reported by Bhanderi (2007) who observed 37.2% and Patel (2008) who observed 46.35% of isolates having lipase activity. However, lower percent isolates (26.47%) producing lipase was reported by Stephan *et al*. (2001) from mastitic milk samples.

Molecular characterization of *Staphylococcus aureus*

In the present investigation on the 53 isolates of *S. aureus*, 36 (67.92%) produced a single amplicon of spa gene for each strain of *S. aureus* and three different product size were amplified at 200, 270 and 296 bp indicating polymorphisms of this gene. Out of these 36 isolates, 7 (19.44%) (5

from cattle and 2 from buffaloes), 17 (47.22%) (12 from cattle and 5 from buffaloes) and 12 (33.33%) (8 from cattle and 4 from buffaloes) isolates were amplified at 200, 270 and 296 bp, respectively. The spa types in the present study corroborates the earlier observations of Karahan *et al*. (2011) who also carried out spa typing of *S. aureus* strains isolated from bovine subclinical mastitis and recorded nine spa types with amplicons ranging from 100 to 320 where most of the spa types were similar to that obtained in the present study. Contrary to the results in the present study, only uniform amplicon of 300 bp size were obtained by Suleiman *et al*. (2012) in 20 isolates of *S. aureus* from subclinical bovine mastitis. The absence of spa X-region gene has also been reported by Kalorey *et al*. (2007) in subclinical mastitis; Momtaz *et al*. (2010) from bovine clinical and subclinical mastitis.In the present study, out of 53 isolates, 32 (60.38%) isolates were found positive for *Coa* gene and showed three different product size viz., 723, 812 and 1000 bp. Of these 32 isolates, 6 (18.75%), 11 (34.38%) and 15 (46.88%) isolates were amplified at 723, 812 and 1000 bp, respectively. Among them, 1000-bp PCR product was the most predominant. Similar results were obtained by Himabindu *et al*. (2009) who showed that the sizes of PCR products obtained after amplification of *S. aureus* of human subjects range from 650 to 1000 bps. Schlegelova *et al*. (2003) also observed the range of 650 to 1050 bp size of *Coa* gene PCR product of *S. aureus* isolates from dairy cows and human. Reinoso *et al*. (2008) also detected that PCR amplification of the *Coa* gene of *S. aureus* isolated from human, bovine subclinical mastitis and food samples which yielded seven different *Coa* types from 45 *S. aureus* strains with amplicon sizes ranging from 400 to 1000 bp. Moreover, during the study one isolate detected negative in tube Coagulase test was also found positive in *Coa* gene based PCR. The same result was also obtained by Himabindu *et al*. (2009) and De Moura *et al*. (2012) who noted that the strains those were classified as Coagulase negative by tube Coagulase test were found to be positive with PCR amplification of the gene. So the correct amplification of all the isolates by PCR not only confirms the results of biochemical tests but is more accurate. Coagulase production is the principle criterion used by the clinical microbiology laboratories for the identification of *S. aureus*. Numerous allelic forms of *S. aureus* Coagulase exist, with each isolate producing one or more of these enzyme variants (Landolo, 1990). However, further studies employing a RFLP technique and nucleotide sequencing methods on a large collection of strains is warranted to determine the common characteristics of the predominant strains.

In conclusion, the high percentage of virulence factor producing strains obtained in this work; suggest an important role of virulence factors in the pathogenesis of bovine mastitis. The presence of two or more virulence factors could increase the pathogenic ability of isolates in relation to those that express only one virulence factor so well planned strategies should be adopted to combat bovine mastitis.

ACKNOWLEDGEMENTS

Authors are thankful to the Dean, College of Veterinary Science and A.H., SDAU, Sardarkrushinagar for providing the necessary facilities where this study was conducted. We also gratefully acknowledge the help of field Veterinarians for collection of milk samples.

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