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...
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 was carried out by using *COAG2 Forward 5’ CGAGACCAAGATTCAACAAG3’,  COAG3...
Reverse 5′AAAGAAACCACTCACAATC3′ and Forward 5′CAAGCACACAAAGAGGAA-3′ Reverse 5′CACCAGGTCTAAGCAT-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 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 Staphylococcus 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.
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 Al-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, Bhandari (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 9 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.

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REFERENCES


Suleiman, A.B., J.K.P. Kwaga, V.J. Umoh, E.C. Okolocha, M. Muhammed, C. Lammler,
