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Rapid detection of virulence associated genes in Streptococcal isolates from bovine mastitis

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In the present study, 15 *S. agalactiae* out of 56 streptococcal isolates recovered from 98 milk samples collected from clinical cases, one organized farm and two unorganized sectors in and around Bangalore. All the streptococcal isolates were confirmed at genus level using genus specific primers targeting *tuf* gene of *Streptococcus*. Species level identification for *S. agalactiae*, *S. dysgalactiae* and *S. uberis* was done using 16S rRNA. Primers were designed for targeting *cfb* gene of *S. agalactiae*, *mig* gene of *S. dysgalactiae*, whereas for targeting *sip*, *hyl* gene of *S. agalactiae* and *skc*, *pauA* gene of *S. uberis* either published or designed earlier were used to screen for virulence genes of streptococcal isolates and reference strains. Desired amplicons for the virulence genes were obtained. All the *S. agalactiae* isolates were also screened for CAMP factor phenotypically by employing CAMP test which was demonstrable in fourteen isolates but *cfb* gene encoding for CAMP factor was detectable by PCR in all the isolates. The study ultimately helps us to understand the virulence characteristics and mechanisms behind emergence of new strains or shifts in mastitis epidemiology in response to control measures, including antibiotic treatment and vaccination.

Key words: Streptococci, virulence factors, bovine mastitis, CAMP factor.

INTRODUCTION

Bovine mastitis is one of the most problematic diseases and continues to have major economic impact on the dairy industry throughout the world. Several bacterial genera and species capable of causing mastitis are widespread in the environment of dairy cows. *Streptococcus* species are one of the most important causative agents of mastitis. Usually the mastitis caused by Streptococci is of the

subclinical type, so early detection of such mastitis cases is of paramount importance. Among *Streptococcus* species, *S. agalactiae*, *S. dysgalactiae* and *S. uberis* are the predominant group of organisms isolated from mastitis next to *Staphylococcus* species. In spite of its high prevalence of Streptococci in both clinical and subclinical bovine mastitis, little is known about factors that contribute

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to the virulence of *Streptococcus* species. The ability of an invading pathogen to initiate growth *in vivo* and stably infect a host requires acquisition of virulence factors capable of neutralizing the mechanisms of the host's defense. These factors include structural components, toxins and enzymes that serve to overcome the otherwise effective nonspecific defensive measures of the host (Brubaker, 1985). These factors can exert a direct effect on stromal cells while others can thwart one or more host defense mechanisms to allow for survival and persistence of the pathogen in the invaded tissue (Woolcock, 1988). Several cell-associated and extracellular factors of *Streptococcus* species have been identified during last decade and Streptococci can interact with several plasma and extracellular host derived protein such as immunoglobulin G, fibrinogen, vitronectin, collagen, plasminogen and α_2 – macroglobulin. These interactions are mediated by bacterial virulence factors such as pore forming protein, surface expressed Mig protein, hyaluronidase and fibrinolysin which are involved in promoting dissemination of organism into the host. Yet, the relative importance of these factors in the transmission and pathogenesis of mastitis caused by Streptococci has not been understood (Calvinho et al., 1998).

The identification and characterization of virulence factors of Streptococci causing bovine mastitis will enhance our understanding of the pathogenesis of intra-mammary infection. In addition, the antibiogram of Streptococci needs to be studied which would indicate the pattern of resistance to various antibacterials contributing to their virulence properties. These may in turn contribute to the development of methods to minimize the production losses due to mastitis. Further, the study of evolution of strain-specific transmission and virulence characteristics including antibiotic resistance in Streptococci isolated from bovine mastitis may help us to understand mechanisms behind emergence of new strains or shifts in mastitis epidemiology in response to control measures, including antibiotic treatment and vaccination.

MATERIALS AND METHODS

Isolation and biochemical characterization

A total of 72 subclinical milk samples based on Electrical Conductivity (EC) using Oriental Instruments, Japan and somatic cell count (SCC) using ChemoMetec, Denmark were subjected for bacteriological examination. About 0.1 ml of milk sample having SCC more than 5,00,000 cells/ml and EC more than 6.5 mS/cm were inoculated in *Streptococcus* selection broth, with 10% CO₂ tension for 6 h to obtain sufficient growth of the organisms. Then the growth from *Streptococcus* selection broth was streaked on to blood agar plates (M/s. Hi-Media, Mumbai), incubated at 37°C for 48 h under 10% CO₂ tension to obtain pure culture. These pure cultures were again streaked onto secondary blood agar plates and then onto BHI agar (M/s. Hi-Media, Mumbai) for further identification procedures. Pure cultures thus obtained were subjected for the primary test like catalase test. Further, all the streptococcal isolates which are negative for catalase test were subjected for biochemical tests such as Voges Proskauer test, esculin hydrolysis, hippurate hy-

drolisis, sugar fermentation and PYR test according to the method described by Collee et al., 1996.

Phenotypic characterization

Determination of CAMP reaction

For this purpose, the test culture was streaked horizontally on a blood agar plate and a known β -hemolytic *S. aureus* was streaked vertically 3 to 5 mm above the test culture streaking. A positive reaction was recorded after incubation for 18 to 24 h at 37°C which results in half moon shaped zone of complete hemolysis in the zone of incomplete staphylococcal- β - hemolysis.

Determination of streptokinase activity

For this, about 0.5 ml of *S. uberis* (the reference strain AD2 and AD6) supernatant, a known producer of streptokinase enzyme was suspended with equal volume of 1:5 diluted rabbit plasma along with known coagulase producer such as *S. aureus* supernatant, incubated at 37°C and results were recorded at hourly intervals for 6 h. Absence of coagulation indicated positive reaction. Paralleling, supernatant of reference strain was added in equal quantity to the coagulated plasma produced by a known Coagulase producer such as *S. aureus*. Fibrinolysis as indicated by the dissolution of the clot was recorded as positive reaction.

Bacterial strains

Reference Streptococci namely, *S. agalactiae* (AD1) Genbank accession no. HM355961, *S. dysgalactiae* (AD3), HC359248 and *S. uberis* (AD2) HC355971 and (AD6) HC355972 procured from Project Directorate on Animal Disease Monitoring and Surveillance (PD AADMAS), Bangalore, and *E. coli* Genbank accession no. JF926686, *S. aureus* Genbank accession no. JN247783.1 maintained in the Department of Veterinary Microbiology, Veterinary College, Bangalore were used.

Preparation of bacterial DNA

Bacterial DNA was purified using the "QIAamp DNA Mini and Blood mini kit" as per the manufacturer's instructions.

Designing of virulent gene primers

The Genus specific (*tuf* gene) and species specific (16S rRNA gene) primers for *S. agalactiae*, *S. dysgalactiae* and *S. uberis*, primer targeting surface immunogenic protein (*sip*) and Plasminogen activator (*pauA*) gene for *S. agalactiae* and *S. uberis* respectively were designed at the Department of Veterinary Microbiology under NAIP scheme and were used for screening of *Streptococcus* isolates. Further, CAMP factor (*cfb*), surface-expressed mig protein (*mig*) gene based primers were designed using "Lasergene DNA STAR" software for *S. agalactiae* and *S. dysgalactiae*, respectively; streptokinase (*skc*) gene based primers for *S. uberis* designed at Molecular Virology Laboratory, IVRI, Bangalore under NAIP scheme; hyaluronidase (*hyl*) gene based published primers (Sukhnanand et al., 2005) for *S. agalactiae* were used for molecular studies and the working concentration of the primers for PCR was 20 pmol/ μ L. The primer sequences and the lengths of the amplified products are detailed in Table 1.

PCR amplification

The PCR reaction mixture contained 2.5 μ L of 10X PCR *Taq* Buffer A, 1 μ L (20 pmol) of each *Saga sip* F & R/ *Saga* CAMP F & R/ *Saga*

Table 1. Nucleotide sequences and product length of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* virulence gene specific primers.

Name of the primer	Sequence 5'– 3'	Product length (bp)	Reference
VM <i>Saga sip</i> - F VM <i>Saga sip</i> - R	ACTATTGACATCGACAATGGCAGC GTTACTGTCAAGTGTCTCAGGA	266	Nithinprabhu et al. (2010)
VM <i>Saga</i> CAMP- F VM <i>Saga</i> CAMP- R	CAAAGATAATGTTTCAGGGAACAGATTATG CTTTTGTCTAATGCCTTTACATCGTT	320	-
VM <i>Saga hyl</i> -F VM <i>Saga hyl</i> -R	CATACC TTAACAAAGATATATAACAA AGATTTTTTAGAGAATGAGAAGTTTTTT	950	Sukhnanand et al. (2005)
VM <i>Sdys mig</i> F VM <i>Sdys mig</i> R	CGTTTTTAGTTTCGGGAGCA TGCCTTCAATTGAGTCTGCTG	188	-
VM <i>Sub pauA</i> -F VM <i>Sub pauA</i> -R	TGCTACTCAACCATCAAAGGTTGC TAGCAGTCTCAGTAGGATGAGTGA	439	Nithinprabhu et al. (2010)
VM <i>Sub skc</i> -F VM <i>Sub skc</i> -R	TCCGGATTTTGGGTCTTAGCCA AGTCGACTTTGCGCCTGATGCAC	475	-

hyl F & R/ *Sdys mig* F & R/ *Sub pauA* F & R and *Sub skc* F & R primers and 1 µL (100 µM) of each dNTPs, 3 µL (150 ng) of streptococcal DNA and filtered quartz water was added to make a final volume of 25 µL. The amplification reactions were carried out in 0.2 ml micro centrifuge tubes using a programmable thermal cycler (Master Cycler pro, M/s Eppendorf, Germany). The amplification was programmed for 30 cycles with temperature cycles of denaturation at 94°C for 30 s annealing at 52, 55, 55, 52, 54 and 52°C, respectively for 30 s and extension at 72°C for 30 s. An additional cycle with an extension step of 10 min was included to complete the synthesis of unfinished products. After the completion of the reaction, PCR products were electrophoresed on a 1.8% agarose gel and the images were captured (Gel Doc XR, M/s, BioRad., U.S.A).

RESULTS

A total of 147 bacterial isolates were recovered from 86 milk samples including 14 clinical and 72 subclinical milk samples. Of these, majority of the isolates recovered were *S. aureus* (45), CoNS (23), Streptococci (56) followed by *E. coli* (23). For identifying *Streptococcus* isolates, *tuf* gene based primer was used at genus level with an amplicons size of 110 bp (Figure 3) and 16S rRNA based primer was used at species level with an amplicons size of 329 bp (Figure 4), 549 bp (Figure 5) and 854 bp (Figure 6), to identify *S. agalactiae*, *S. dysgalactiae* and *S. uberis*, respectively. Out of 56 *Streptococcus* isolates, only fifteen isolates (Genbank accession no. JN998527.1), and reference *S. agalactiae* (AD1) yielded 329 bp amplicon of 16S rRNA gene confirming them as *S. agalactiae*, none of the isolates yielded 549 bp amplicon which would confirm them as *S. dysgalactiae* and none of the isolates yielded 854 bp amplicon confirming it

to be *S. uberis*. However, the reference culture yielded the desirable amplicons. All the fifteen isolates and the reference strain (AD1) yielded specific amplicon of 266 (Figure 7), 320 (Figure 8) and 950 bp (Figure 9) confirming the presence of *sip*, *cfb* (Genbank accession no. JN657311.1, JN657312.1, JN378717.1) and *hyl* gene (Genbank accession no. JN247792.1, JN247784.1, JN247791.1, JN256018.1 and JN120257.1), respectively. The screening of *S. dysgalactiae* reference strain (AD3) revealed the presence of *mig* gene which yielded specific amplicon of 188 bp (Figure 10). Reference strains of *S. uberis* (AD2 and AD6) were also screened for the virulence gene *pauA* and *skc* by earlier designed primers. The screening of reference strains revealed the presence of *pauA* and *skc* gene in both the reference strain AD2 and AD6 yielded specific amplicons of 439 (Figure 11) and 475 bp (Figure 12), respectively.

Majority of the isolates in the present study could not be speciated based on biochemical tests; they were neither *S. uberis* nor *S. dysgalactiae*. Sequence specific primer for identification of these isolates was designed at the Lead centre, PD_ADMAS Bangalore and used for PCR amplification. The amplified products were sequenced and NCBI BLAST results indicated that these isolates belong to *S. bovis-equinus* complex.

The reference cultures were used as the positive controls whereas; *S. aureus* and *E. coli* were used as negative controls. The PCR amplified products were then sequenced and the primer specificity was confirmed by sequence BLAST analysis. Furthermore, these sequences were aligned by Clustal V method using MegAlign program of the same software with sequences available in NCBI and phylogenetic analysis revealed the genetic

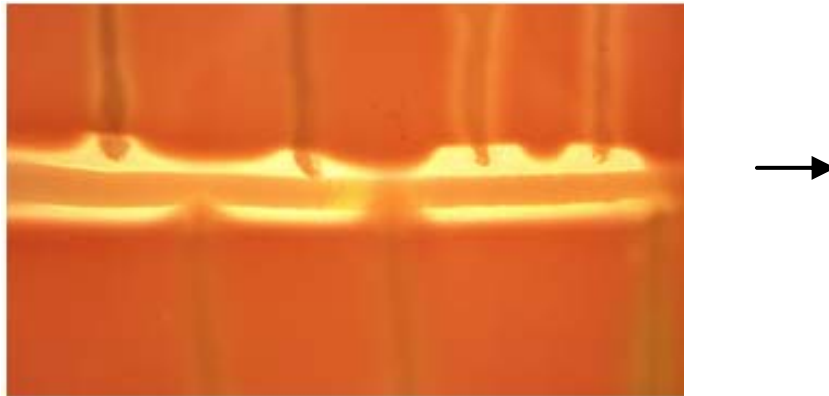


Figure 1. CAMP test showing half-moon shaped zone of complete hemolysis on a blood agar by *S. agalactiae* along with *S. aureus*.

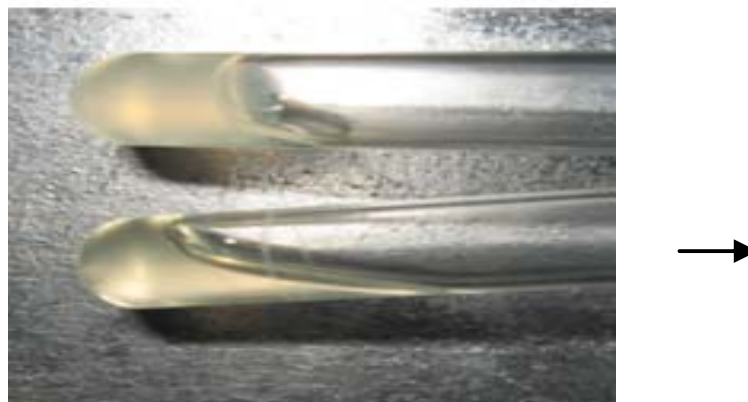


Figure 2. Streptokinase assay showing absence of coagulation or fibrinolysis as indicated by the dissolution of the clot produced by known coagulase producer *S. aureus*.

diversity among the isolates.

Of the fifteen *S. agalactiae* isolates tested, only fourteen isolates were phenotypically positive for CAMP factor as detected by CAMP test (Figure 1); whereas, all the isolates were positive genotypically as detected by PCR by targeting *cfb* (CAMP factor) gene.

DISCUSSION

In India, improvisation in quality and quantity of milk produced is a prerequisite for export of milk and milk products. However, it is threatened by mastitis which continues to be a cause of significant economic loss to the dairy industry not only in India, but also internationally. In the present study, the highest prevalence of SCM at 82% was observed in organized sector, which comprised of crossbred animals. The observations made in this study, despite thorough biochemical characterization of streptococcal isolates, could not lead us to precise

identification of these isolates up to the species level due to variability in their biochemical profiles; hence, the findings emphasize the need for development of molecular methods for precise identification of streptococci as this is one of the most useful tools applied to the revision of the bacterial classification system (Facklam, 2002). Rapid nucleic acid amplification and detection technologies are quickly displacing the traditional assays based on pathogen phenotype rather than genotype. The development of the PCR based methods provides a promising tool for the rapid identification of bacteria. The *tuf* gene provided a better discrimination over the 16S rRNA at the streptococcal genus level, which is particularly useful for the identification of very closely related species. Thus, this peculiarity of the streptococcal *tuf* gene was used in the present study. Interestingly, nine isolates were obtained from fifteen clinical cases (60%) which signify their role in clinical mastitis. Streptococcal isolates detected in the present study were either from the clinical or subclinical cases which indicated their potential to cause the

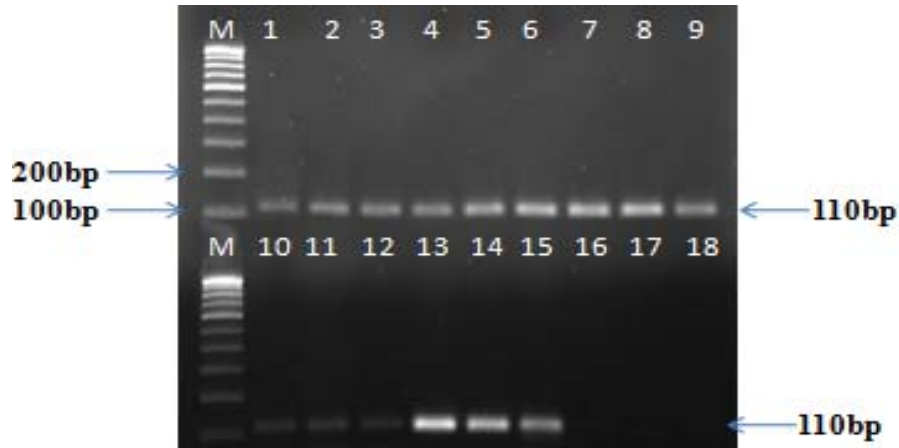


Figure 3. PCR amplification of 110 bp *tuf* gene of *Streptococci* isolated from bovine mastitis cases, Lane M: 100 bp DNA ladder, Lanes 1-12: *Streptococci* isolate No. 41 to 52, Lanes 13-15: Positive control (*S. agalactiae* AD1, *S. dysgalactiae* AD3, *S. uberis* AD6 respectively), Lanes 16-18: Negative controls (*S. aureus*, *E. coli*, NTC respectively).

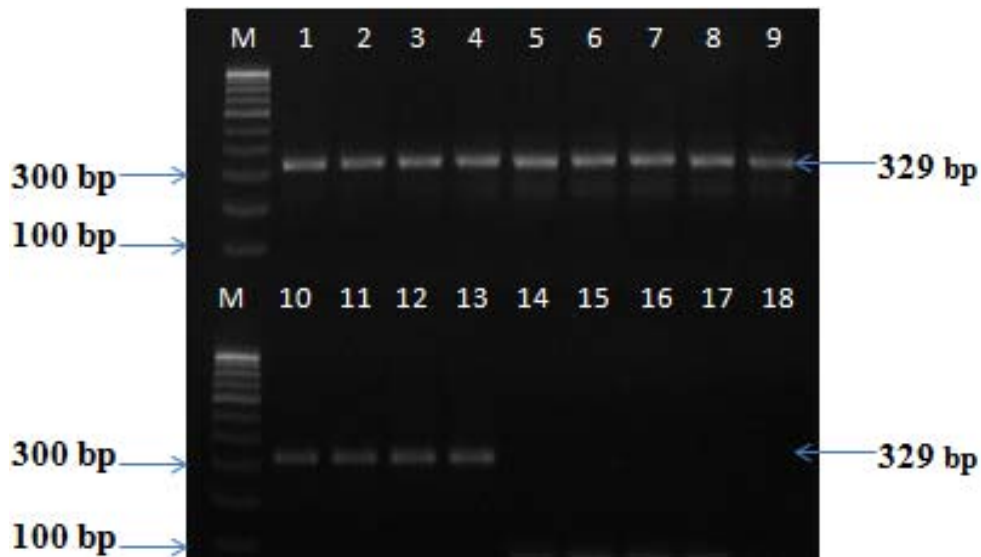


Figure 4. PCR amplification of 329 bp 16S rRNA gene of *S. agalactiae* isolated from bovine mastitis cases, Lane M: 100 bp DNA ladder, Lane 1: Positive control *S. agalactiae* AD1, Lanes 2-13: *S. agalactiae* isolate No. 55, 56, 57, 61, 86-92, 95, Lanes 14-18: Negative controls (*S. dysgalactiae* AD3, *S. uberis* AD6, *S. aureus*, *E. coli*, NTC respectively).

infection. *S. agalactiae* was the major species among streptococci reported even in previous studies (Mallikarjunaswamy and Murthy, 1997; Ross et al., 2001; Balakrishnan et al., 2004).

Sip (surface immunogenic protein) is an antigenic protein localized on the surface of *S. agalactiae* which is capable of raising an antibody response. It is also known that *sip* is highly conserved at the gene level. The *sip* gene based primers amplified all fifteen isolates of *S. agalactiae* and a reference *S. agalactiae* (AD1) precisely

without any ambiguity. Cell surface protein like pore forming protein encoded by CAMP factor/*cfb* gene, was found to produce a classical CAMP phenomenon with the typical half moon forming hemolytic zones on cattle or sheep blood agar plates by the influence of β -lysin of *S. aureus* and exosubstances of non-hemolytic streptococci (Christie et al., 1944). CAMP factor genes are described to be fairly widespread among streptococci, at least in serogroups A, B, C, G, M, P, R and U (Gase et al., 1999). The results of the present study are in accordance with

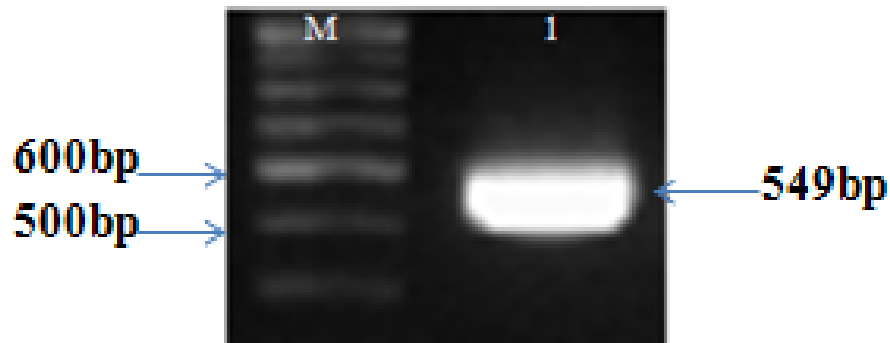


Figure 5. PCR amplification of 549 bp 16S rRNA gene of *S. dysgalactiae*, Lane M: 100 bp DNA ladder, Lane 1: Positive control *S. dysgalactiae* (AD3).

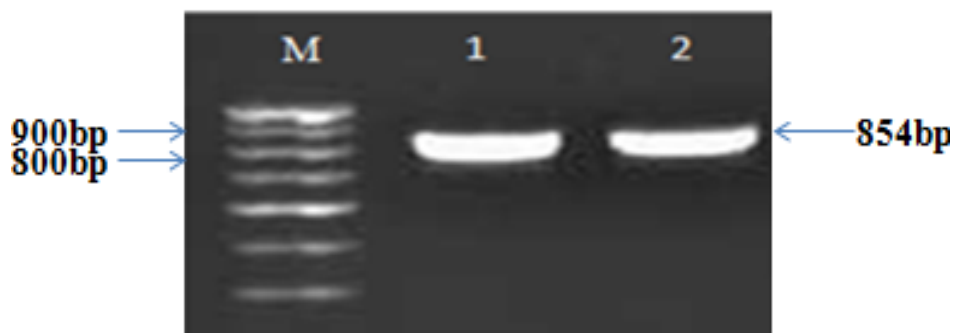


Figure 6. PCR amplification of 854 bp 16S rRNA gene of *S. uberis*, Lane M: 100 bp DNA ladder, Lane 1: Positive control *S. uberis* (AD2), Lane 2: Positive control *S. uberis* (AD6).

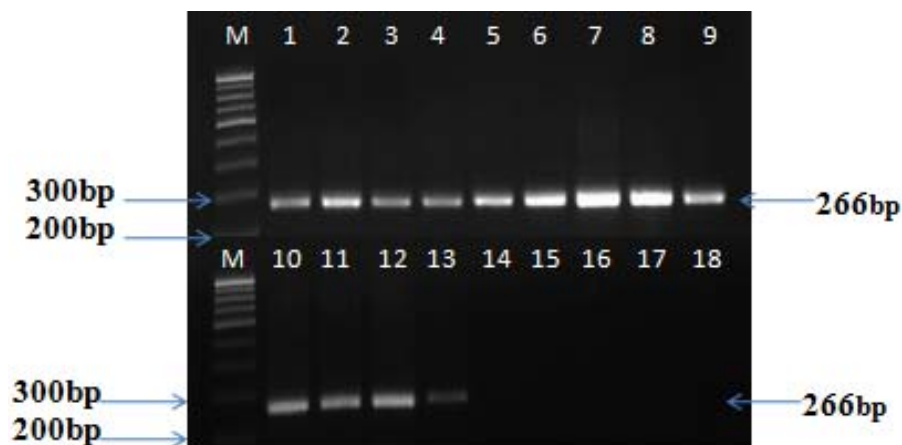


Figure 7. PCR amplification of 266 bp *sip* gene of *S. agalactiae* isolated from bovine mastitis cases, Lane M: 100 bp DNA ladder, Lane 1: Positive control (*S. agalactiae* AD1), Lanes 2-13: *S. agalactiae* isolate No. 55, 56, 57, 61, 86-92, 95, Lanes 14-18: Negative controls (*S. dysgalactiae* AD3, *S. uberis*, *S. aureus*, *E. coli*, NTC respectively).

the earlier reports confirming wide prevalence of CAMP factor possessing *S. agalactiae*. Phenotypic property was not demonstrable in one isolate out of 15, which may be

due to lack of expression of the gene. This could be due to the absence of complete open reading frame (ORF). Although, it is reported in earlier studies that the CAMP

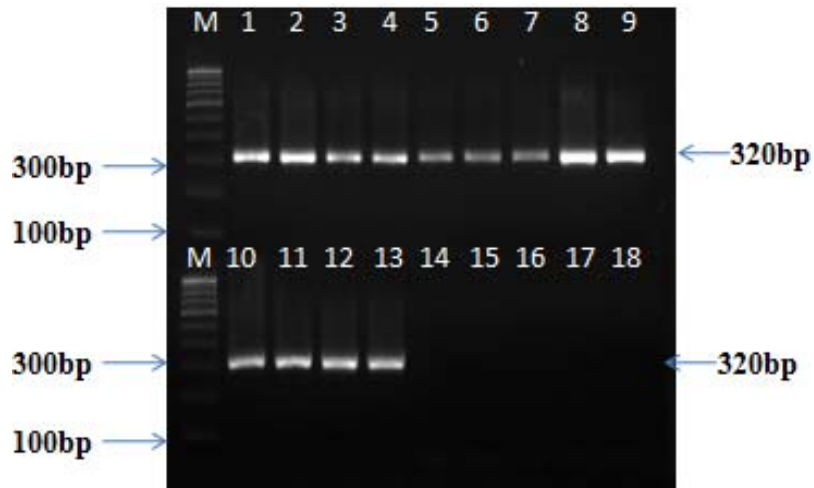


Figure 8. PCR amplification of 320 bp *cfb* gene of *S. agalactiae* isolated from bovine mastitis cases, Lane M: 100 bp DNA ladder, Lane 1: Positive control (*S. agalactiae* AD1), Lanes 2-13: *S. agalactiae* isolates No. 55, 56, 57, 61, 86-92, 95, Lanes 14-18: Negative controls (*S. dysgalactiae* AD3, *S. uberis* AD6, *S. aureus*, *E. coli*, NTC respectively).

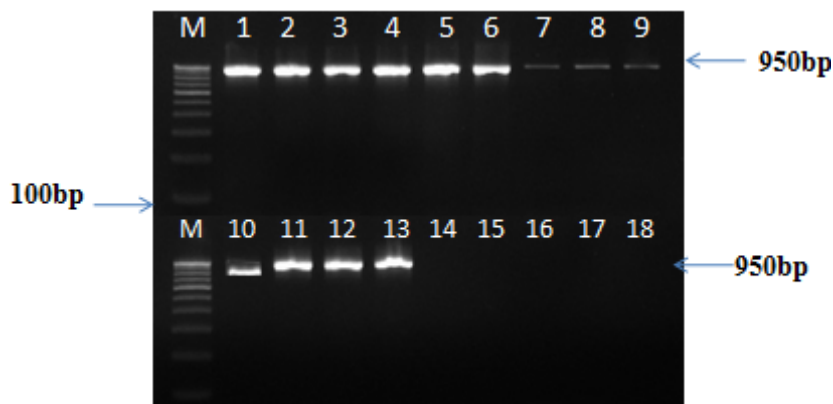


Figure 9. PCR amplification of 950 bp *hyl* gene of *S. agalactiae* isolated from bovine mastitis cases, Lane M: 100 bp DNA ladder, Lane 1: Positive control (*S. agalactiae* AD1), Lanes 2-13: *S. agalactiae* isolates No. 55, 56, 57, 61, 86-92, 95, Lanes 14-18: Negative controls (*S. dysgalactiae* AD3, *S. uberis* AD6, *S. aureus*, *E. coli*, NTC respectively).

factor is not essential for systemic virulence of GBS (Hensler et al., 2007), its phenotypic detection for presumptive identification of GBS in clinical laboratory is of immense diagnostic value. *S. agalactiae* *hylB* encodes hyaluronate lyase (hyaluronidase), a putative virulence factor facilitate the spreading of bacteria in host tissues (Akhtar and Bhakuni, 2004). The hyaluronidase activity in *S. agalactiae* is associated with host specificity (Lin et al., 1994). The secreted and putative virulence gene (*hylB*) was used as a target for DNA sequencing-based subtyping and often provided a higher discriminatory power and might provide insight into the evolution of virulence-

related characteristics (Cai et al., 2002). The results of the present study are in accordance with the earlier reports (Cai et al., 2002; Correa et al., 2010).

The study confirmed wide prevalence of three important virulence genes in *S. agalactiae* isolates obtained from both organized as well as unorganized sectors, including both subclinical and clinical cases of mastitis from different geographical locations. The findings of the study emphasize the role of virulent gene possessing *S. agalactiae* in causing clinical as well as subclinical cases of bovine mastitis. In continuation, it is necessary to scan *S. agalactiae* for other virulence genes and their possible



Figure 10. PCR amplification of 188 bp *mig* gene of *S. dysgalactiae*, Lane M: 100 bp DNA ladder, Lane 1: *S. dysgalactiae* (AD3), Lanes 2-6: Negative controls (*S. agalactiae* AD1, *S. uberis* AD6, *S. aureus*, *E. coli*, NTC respectively).

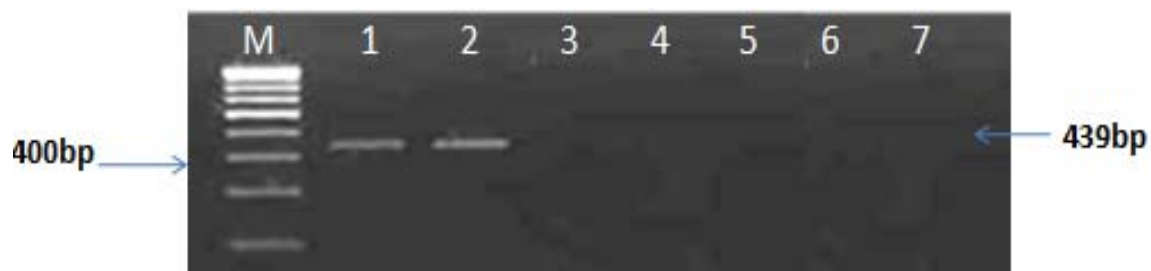


Figure 11. PCR amplification of 439 bp *pauA* gene of *S. uberis*, Lane M: 100 bp DNA ladder, Lanes 1 and 2: *S. uberis* AD2 and AD6, Lanes 3-7: Negative controls (*S. agalactiae* AD1, *S. dysgalactiae* AD3, *S. aureus*, *E. coli*, NTC respectively).

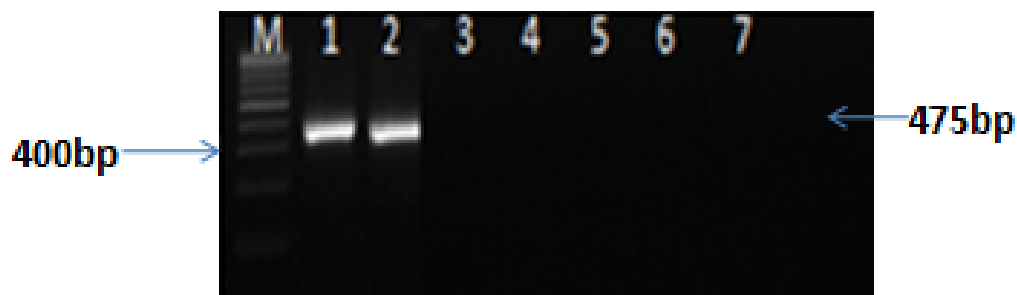


Figure 12. PCR amplification of 475 bp *skc* gene of *S. uberis*, Lane M: 100 bp DNA ladder, Lanes 1 and 2: *S. uberis* AD2 and AD6, Lanes 3-7: Negative controls (*S. agalactiae* AD1, *S. dysgalactiae* AD3, *S. aureus*, *E. coli*, NTC respectively).

role in causing mastitis. The Mig protein is involved in resisting phagocytosis by bovine neutrophils (PMNs) in the presence of bovine serum (Song et al., 2001). Thus, the Mig protein, an M-like protein, is considered as a potential virulence factor of *S. dysgalactiae*. This protein could act as the sensory component of a multiple component system, whereby, binding of IgG and or IgA to Mig could trigger a conformational change on this protein, resulting in the activation of secondary proteins with histidine-kinase activities that result in the modulation of

gene expression of factors involved in virulence. The DNA sequence encoding the α_2 -M receptor portion of the *mig* gene was different from other *Streptococcus* and which was highly specific to *S. dysgalactiae* (Jonsson et al., 1994). All of them possessed DNA fragments that hybridized to the IgG probe suggesting that the IgG-binding sequence of *mig* is highly conserved in these strains. Surprisingly, none of the streptococcal isolates obtained in our study were identified as *S. dysgalactiae* by PCR in contrast to the biochemical assays. Further, *mig* gene pri-

mer based PCR revalidated the earlier identification process by using 16S rRNA gene based PCR and it is a good tool to ascertain virulence properties of *S. dysgalactiae* with reference to Mig protein.

Streptokinase, a bacterial plasminogen activator is produced by a variety of pathogenic *Streptococcus* species and is needed for degradation of extracellular matrix proteins and subsequent colonization. Notably, streptokinases isolated from different strains of streptococci possess an intrinsic species specificity for their target plasminogen molecules that parallels the host range of the microorganisms (Mccoy et al., 1991). It has also been reported that the amino acid sequence of streptokinase gene (*skc*) of *S. uberis* was highly conserved within the species (Johnsen et al., 1999). However, *skc* gene based PCR standardized in this study is a useful assay for identification of virulent *S. uberis*. An effort was made to standardize the procedure for streptokinase assay using two reference strains of *S. uberis* (AD2 & AD6). This procedure could be used even for the clinical isolates to study their potential to produce the enzyme streptokinase. The ability of the streptokinase enzyme to cause fibrinolysis or prevent the formation of coagulation in rabbit plasma produced by a known coagulase producer such as *S. aureus* was tested. The reference strain of *S. uberis* used for the streptokinase assay was able to cause fibrinolysis as well as prevent the formation of coagulation. Streptokinase which activates bovine plasminogen might be an essential virulence factor of *S. uberis*, allowing its rapid growth in the bovine mammary gland (Leigh and Field, 1993). Hence, the phenotypic detection of streptokinase enzyme produced by streptococci could serve as an indicator of pathogenicity of the isolates under study (Figure 2).

The *pauA* is a putative virulence factor of *S. uberis* and encodes the plasminogen activator which converts plasminogen in blood plasma and tissues in cattle to plasmin (Leigh, 1999, 2000). However, *pauA* gene specific PCR provides useful supplementary data to differentiate *S. uberis* from closely related species. It was also shown that *pauA* gene based PCR (Zadoks et al., 2005) could be used for rapid species identification, since *pauA* is *S. uberis* species-specific and absent in other *Streptococcus* species or other bacteria commonly associated with bovine mastitis (Ward and Leigh, 2004). However, none of the streptococcal isolates obtained in our study were identified as *S. uberis* by PCR in contrast to the biochemical assays. To summarize, phylogenetic and sequence pair distance analysis revealed high genetic variation among the streptococci isolates with respect to the virulence genes as observed in the present study. This provides a virulence gene based tool to study the molecular epidemiology of streptococcal mastitis in bovines which would in turn help us to understand mechanisms behind emergence of new strains or shifts in mastitis epidemiology in response to control measures, including antibiotic treatment and vaccination.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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