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Bacteriological and Molecular Detection with Antimicrobial Resistance Pattern of Major *Streptococcus* spp. Isolated from Bovine Mastitis

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A B S T R A C T

Mastitis in bovine cause various clinical consequence with much losses in milk quality. Inflammatory infection caused by various bacteria damage the mammary gland and sometimes leads irreversible damage to the udder tissue. Looking to above problem the current study was performed to know the prevalence of major *Streptococcus* spp. with its timely detection by molecular methods and its antimicrobial resistance patterns. Total 390 bovine milk samples (180 clinically mastitic and 210 from apparently healthy) were studied. Initially samples were screened for major *Streptococcus* spp. by conventional bacteriological methods of identification. The molecular detection of *Streptococcus* genus and its major *species Viz; Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis* was carried out by polymerase chain reaction by targeting Tu (*tuf)* gene, *sip* gene, *16 S rRNA* gene and *pau* gene respectively. Based on these overall 16.67 per cent of prevalence was observed for *Streptococci*. Among the major species, prevalence of *S. agalactiae, S. dysgalactiae* and *S. uberis* were found 13.59, 2.31 and 0.77 per cent respectively. These isolates showed highest antimicrobial resistance against amoxicillin- sulbactam (72.25%) followed by ceftriaxone (66.19%) and oxytetracycline (53.12%) while the gentamicin (8.38%) were found least resistant.

Introduction

Dairying the major connected activity associated with agriculture has now grown to the industrial scale contributing to the livelihood of millions of farmers in the country. Many elements including poor quality housing, inadequate nutrition, poor management practices and intra mammary infection are mostly responsible for decreased the production as well changed in quality of milk. Intramammary bacterial infection by bacteria damages the mammary gland and cause mastitis in bovine. It happens in various forms like mild sub clinical as well as clinical forms. Subclinical mastitis (SCM) occur

without any physical changes to milk and udder tissue but cause more financial losses to dairy industries (Shaheen *et al.,* 2016). So far approximately over 200 microbial species, sub species and subtypes have been isolated from bovine mammary gland (Mallikarjunaswamy and Krishnamurthy, 1997) and identified as causative agents of bovine mastitis. From all the aetiologies about 95 per cent of the identified cases are caused by *Streptococcus agalactiae, Staphylococcus aureus* and *Streptococcus dysgalactiae*, *Streptococcus uberis* and *Escherichia coli* (Jain *et al.,* 2012; Kandemir *et al.,* 2013). These predominant *Streptococcus* spp. play very important role in causing bovine mastitis and usually require special care for its isolation and identification. Antibiotic resistance patterns of these bacteria are helpful in treating the cases with best available antibiotics. Molecular techniques of diagnosis are widely used nowadays to reduce time and labour involved in conventional approaches. So keeping in views this study was undertaken to know prevalence of major *Streptococcus* spp. as an etiological agents of bovine mastitis with conventional and molecular methods its antimicrobial resistance patterns.

Materials and Methods

The present study was carried out from bovine clinical mastitic milk samples brought at Teaching Veterinary Clinical Complex as well as milks samples collected from apparently health bovine livestock. The Media, chemicals and reagents used in this study were of analytical and molecular grade. During the study 390 bovine milk samples including 180 from clinical mastitis cases and 210 from apparently healthy bovine were subjected for investigation. The somatic cell count (SCC) value > 5,00,000 cells/mL of milk (Hegde *et al.,* 2013) was considered the milk/animal as subclinically mastitic/infected. Accordingly, 72 samples with SCC value > 5,00,000 cells/mL along with 180 clinical samples were subjected for cultural isolation. So finally 252 milk samples were processed for primary cultural isolation of major *Strepotococcus* spp. as an etiological agent of bovine mastitis. Initially milk samples were enriched in *Streptococcus* selection broth, with 5-10 per cent CO_2 tension for 6 hours (hr.) at 37^{o}C and then streaked onto blood agar plates. The plates were incubated at 37^{o}C for 48 hr. After reading haemolysis in patterns and colony morphology, the colonies were again streaked onto blood agar plates and incubated further at 37^{o}C for 48 hr. to obtain pure culture.

Gram staining was performed as per standard protocol and the pure cultures were subjected for biochemical tests viz: Catalase test, Oxidase test, Esculin Hydrolysis test and Christie, Atkins, Munch-Petersen (CAMP) test (Collee *et al.,* 2008, Sandholm *et al.,* 1995).

The molecular detection of *Streptococcus* genus and major *Streptococcus* spp. *Viz; Streptococcus agalactiae, Streptococcus dysgalactiae* and *Streptococcus uberis* was carried out by polymerase chain reaction(PCR). The genomic DNA from bacterial isolates was extracted by column based method of purification by using Nucleo-pore gDNA Fungal/Bacterial Mini Kit (Genetix brand). The concentration and purity of the purified genomic DNA was measured by using µDrop™ Plate in uDrop plate reader (Thermo Scientific). Further, the purity of the DNA sample was checked by electrophoresis on 0.8 per cent of the agarose gel. All the oligonucleotide primers were synthesized and obtained from Eurofins India, Bangore.

The Genus specific primers targeting the elongation factor Tu (*tuf)* gene of *Streptococci* was used for amplification of

Streptococcus genus specific sequence. The primers targeting the Surface immunogenic protein (*sip)* gene of *S. agalactiae* was used for amplification of *S. agalactiae* specific sequence.

The primers targeting *16S rRNA* gene of *S. dysgalactiae was* used for amplification of *S. dysgalactiae* species specific sequence while the primers targeting the Plasminogen activator gene (*pau)* gene of *S. uberis* was used for amplification of *S. uberis* specific sequence (Nithinprabhu *et al.,* 2013, Preethirani *et al.,* 2015). The details of the primer sequences and its product size are depicted in table 1.

The PCR was performed in final reaction volume of 25 µl in thermal cycler (Verity, Applied Biosystems by life technology, Singapore). The compositions of reaction mixture was prepared by using12.5 ul of 2X PCR mastermix (Fermentas), 1 ul of each forward and reverse primers (10 pmol/ul), 3 ul (1 µg) of genomic DNA template in a total reaction volume of 25 µL with addition of nuclease free water. The cycling conditions and annealing temperature optimized are shown in Table 2.The final PCR products were observed and visualized by agarose gel electrophoresis.

All the *Streptococci* isolates obtained were subjected to *in vitro* antibiotic sensitivity test as per the Kirby-Bauer method (Bauer *et al.,* 1966) with commercially available discs(Table 3) in market on Mueller Hinton Agar medium (Himedia Lab.) with 5 per cent sheep blood.

The in-house reference strain previously characterized as *S. dysgalactiae* and *S. agalactiae* along with *Streptococcus uberis* (ATCC 700407) were used in this study as positive reference cultures. During the study *Staphylococcus aureus (*ATCC 43300) and *Escherichia coli* (MTCC 722) were used as negative controls.

Results and Discussion

Bovine mastitis is the single most important factor contributing to the economic losses to the dairy industry, resulting in reduction in milk yield and quality of the milk. Out of the total 390 sample included in this study, 210 milk samples collected from apparently healthy bovine were subjected for SCC. A result of the SCC of 210 milk samples collected from bovine revealed overall 34.29 per cent (72/210) incidence of SCM. These 72 samples along with another 180 samples collected from clinical mastitis cases of bovine were included for cultural isolation predominant *Streptococcus spp*. In the cultural isolation, 235 samples were found positive for at least one species of bacteria. The isolated bacteria were identified up to genus level based on colony characteristics of individual primary isolate. The minute, dew drop colonies on blood agar (Figure 1) and Gram positive cocci in chain was primarily considered as *Streptococcus* spp. Further, these isolates were subjected to biochemical tests *viz;* catalase, oxidase, esculin hydrolysis and CAMP test**.** Accordingly 16.67 per cent (65/390) prevalence was observed for major *Streptococcus* spp. All the Streptococci isolates were negative for catalase and oxidase. Variability in biochemical profile was observed among different species. The overall and species wise prevalence of predominant *Streptococcus* species are highlighted in table 4.

Genus specific confirmation of the *Streptococcus* isolates was done by using primers targeting *tuf* gene. All the 65 major Streptococcal isolates yielded 110 bp amplicon. The reference cultures *S. uberis* (ATCC 700407) used as positive control also amplified similar size product while *E. coli*

(MTCC 722) used as negative controls did not amplify the target gene (Figure 2).

Species specific *sip* gene of *S. agalactiae*, *16S rRNA* of *S. dysgalactiae* and *pau* gene of *S. uberis* were targeted by the primer previously designed by Nithinprabhu *et al.*, 2013. Out of total 65 isolates of *Streptococcus* spp., 53 isolates were amplified the *sip* gene specific 266bp amplicon (Figure 3) while 9 and 6 isolates amplified *16S rRNA* and *pau* gene specific 549bp (Figure 4) and 439bp (Figure 5) amplicon respectively. The respective amplification was also seen in the reference cultures *S. agalactiae*, *S. dysgalactiae* and *S.uberis (*ATCC 700407) which were used as positive controls. *Staphylococcus aureus (*ATCC 43300) was used as negative controls and did not amplify the target gene.

Different level of antimicrobial-resistance were observed in Major *Streptococcus* spp. based on antibiotic susceptibility test. Here high level of antibiotic resistance was observed for amoxicillin- sulbactam (72.25%), ceftriaxone (66.19%) and oxytetracycline (53.12%). These isolates were less resistance to levofloxacin (10.14%) and gentamicin (8.38%) (Figure 6).

Table.1 The details of the primer sequences and its product size

Targeted Genus/Species	Primer sequence (5'- 3')	Target gene	Product size	Reference
Streptococcus	CAACTTGACGAAGGTCCTGCA	*tuf*	110bp	Nithinprabhu *et al.*, 2010, 2013, Preethirani *et al.*, 2015
	TGGGTTGATTGAACCTGGTTTA			
S. agalactiae	CTATTGACATCGACAATGGCAGC	*sip*	266 bp	
	GTTACTGTCAGTGTTGTCTCAGGA			
S. dysgalactiae	GGAGTGGAAAATCCACCAT	*16S rRNA*	549bp	
	CGGTCAGGAGGATGTCAAGAC			
S. uberis	TGCTACTCAACCATCAAAGGTTGC	*pau*	439bp	
	TAGCAGTCTCAGTAGGATGAGTGA			

Table.2 The cycling conditions and annealing temperature used in PCR

Cycling conditions for Genus *Streptococcus- Tuf* gene				
Initial denaturation	**Denaturation**	**Annealing**	**Extension**	Final extension
95°C for 5 min	94°C for 30 sec	50°C for 45 sec	72°C for 45 sec	72°C for 10 min
	Repeated for 35 cycles			
Cycling conditions for *S. agalactiae- sip* gene				
94°C for 5 min	94°C for 30 sec	53°C for 45 sec	72°C for 45 sec	72°C for 10 min
	Repeated for 40 cycles			
Cycling conditions for *S. dysgalactiae -16S rRNA* gene				
94°C for 5 min	94°C for 30 sec	51°C for 45 sec	72°C for 45 sec	72°C for 10 min
	Repeated for 35 cycles			
Cycling conditions for *S. uberis - pau*gene				
94°C for 5 min	94°C for 30 sec	55°C for 45 sec	72°C for 45 sec	72°C for 10 min
	Repeated for 38 cycles			

Table.3 Antimicrobial disc used during the study

Name of antibiotic with its Concentration	Break point to declare resistance
Amoxicillin/Salbactum(30/15µg)	31 mm
Ceftriaxone (30 µg)	24 mm
Gentamicin (10 µg)	12 mm
Levofloxacin (5 µg)	13 mm
Oxytetracycline(30 µg)	18 mm

Table.4 Prevalence of bovine mastitis by predominant Streptococcal species

Bacteria	Total no. of milk samples screened	No. of positive samples	Prevalence (%)
S. agalactiae,	390	53	13.59
S. dysgalactiae		9	2.31
S. uberis		3	0.77
Overall Streptococcal species		65	16.67

Fig.1 *Streptococcus* spp. on 5 % blood agar after 48hr incubation at 37°C

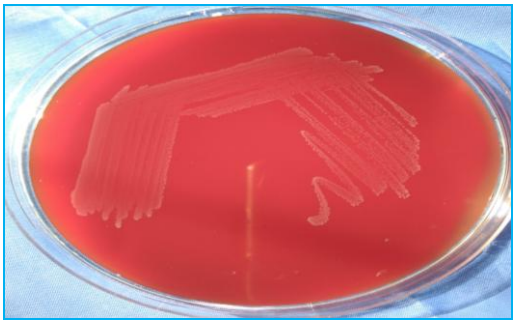
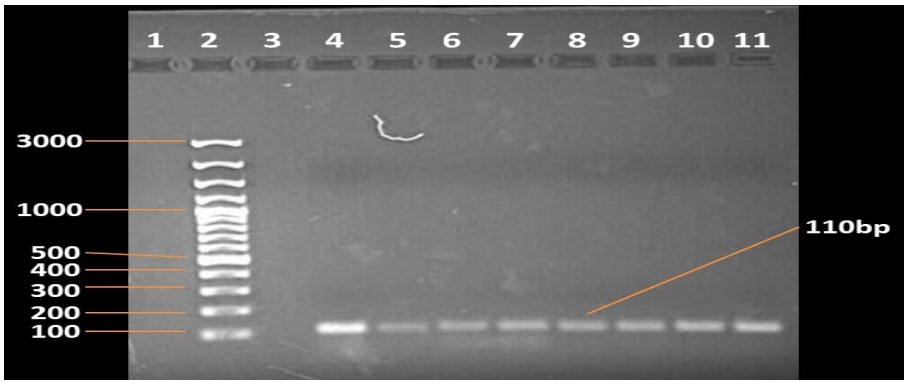
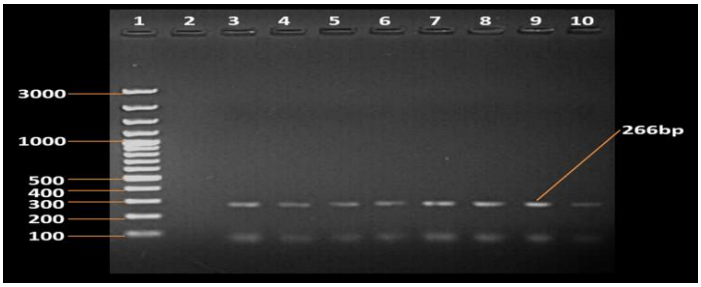


Fig.2 Genus specific PCR of *Streptococcus* spp. for *tuf* gene (110 bp)



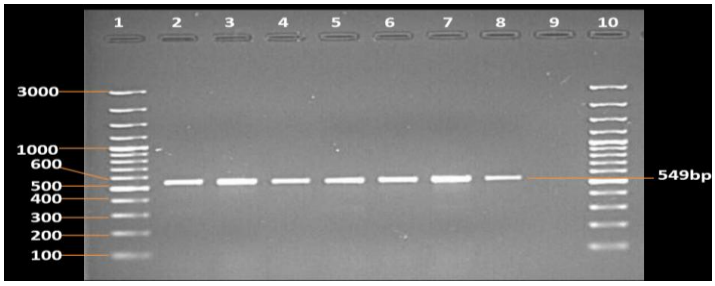
Lane 1: Negative template control, Lane 2: 100bp plus ladder, Lane 3: *E. coli* (MTCC 722) as negative control, Lane 4: *S. uberis* (ATCC 700407) as positive control, Lane 5 to 11: *Streptococcus* spp. isolates from bovine milk samples

Fig.3 Species specific PCR of *S. agalactiae* for *sip* gene



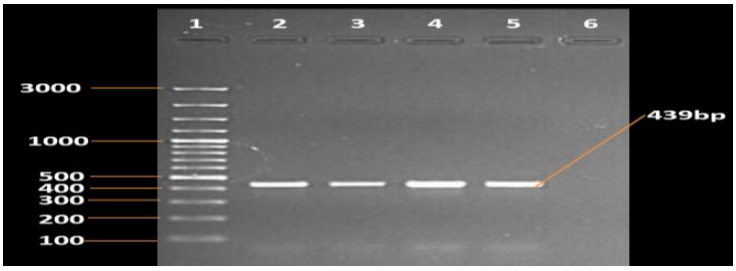
Lane 1: 100bp plus ladder, Lane 2: *S. aureus* (ATCC 43300) as negative control: Lane 3 *S. agalactiae* reference strain as positive control, Lane 4-10 *S. agalactiae* isolates from bovine milk samples

Fig.4 Species specific PCR of *S. dysgalactiae* for *16SrRNA* gene



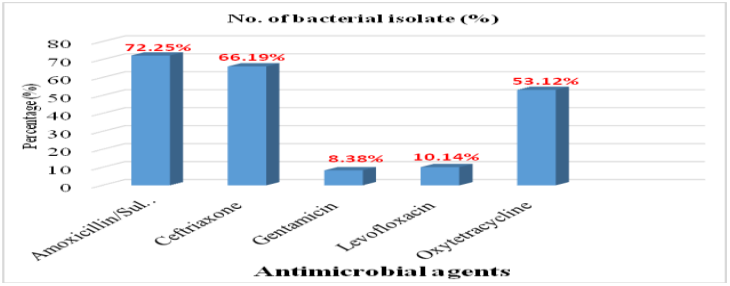
Lane 1: 100bp plus ladder, Lane 2: *S. dysgalactiae* as positive control, Lane 3 to 8: *S. dysgalactiae* isolates from bovine milk samples, Lane 9: *S. aureus* (ATCC 43300) as negative control, Lane 10: 100bp plus ladder

Fig.5 Species specific PCR of *S. uberis* for *pau* gene)



Lane 1: 100bp plus ladder, Lane 2: *S. uberis* (ATCC 700407) as positive control, Lane 3 to 5: *S. uberis* isolates from bovine milk samples, Lane 6: *S. aureus* (ATCC 43300) as negative control

Fig.6 Resistance patterns of Major *Streptococcus* spp.



A result of the SCC of 210 milk samples collected from bovine revealed overall 34.29 per cent (72/210) incidence of SCM. These findings were also supported by Nithinprabhu (2010) and Hegde *et al.,* (2013) who reported 47 per cent and 45 percent SCM respectively in bovine but it was in contrast to finding of Jena *et al.,* (2015) who reported 74.55 per cent case SCM. Increased SCC as an indicator of intra mammary infection has been described previously (Hayakawa *et al.,* 2000). In the present study 46.15 per cent (180/390) of milk samples collected from clinical mastitis cases were included for cultural isolation. This could be reason for observance high prevalence of bovine mastitis in current study. The comparable finding of high prevalence of bovine mastitis were also stated by Jena *et al.,* (2015), Preethirani *et al.,* (2015), Tesfaheywet and Gerema (2017).

The prevalence of mastitis caused by *S. agalactiae, S. dysgalactiae* and *S. uberis* was found 13.59% (53/390), 2.31% (9/390) and 0.77% (3/390) respectively. Accordingly 16.67 per cent (65/390) prevalence were observed for major *Streptococci*. Present study were very much in concordance with the earlier works who have isolated 18.51 per cent (Mohinikumari and Janakiramgupta, 2002), 14.01 per cent (Rajeev, 2006), 17.9 per cent (Feng-Li-Yang *et al.,* 2011) and 18.1% per cent (Preethirani *et al.,* 2015) prevalence of *Streptococci*. These studies in general indicated the high prevalence of *S. agalactiae* among *Streptococci* isolated from bovine mastitis cases based on their conventional studies. In the present study variability observed in biochemical differentiation of major *Streptococcal* spp. were confirmed by amplification of species specific gene which was found more suitable for detection. Gonano and Winter, 2008, Hegde *et al.,* 2013 also observed that a consistent biochemical differentiation of *Streptococcus* species was tough and conventional routine diagnostics have to be upgraded to give specific information on species level for creating farm specific mastitis prevention programs. Hence, these findings highlight the need to depend on molecular methods based on *16SrRNA*genefor accurate identification of Streptococci (Facklam, 2002). Quick nucleic acid amplification and detection tools are quickly transferring the traditional test which is based on phenotypic characteristic rather than its genotype. The PCR has increasingly been described as the latest gold standard for detecting mastitis pathogens by many scientists (Phuektes *et al.,* 2001, Shome *et al.,* 2011, Hegde *et al.,* 2013, Javia *et al.,* 2018, Ghodasara *et al*, 2018). Sequence analyses of conserved "housekeeping" genes such as the bacterial *16S rRNA* and other species specific are increasingly being used to identify bacterial species in clinical practice and scientific investigations (Petti *et al.,* 2005).

Streptococcal infections in both humans and bovines are treated by administration of antibiotics. Penicillin was the drug of choice for treatment of both human and bovine *S. agalactiae* infections (Schrag *et al.,* 2000). Antibiotic resistance against Amoxicillin/ Sulbactum was observed 72.25% in present study which may be due to extensive use of these antibiotics during treatment. Levofloxacin and Gentamicin has been found to be the most sensitive 89.86% and 91.62% respectively which was in accordance to some of the reports (Bhanot *et al.,* 2012 and Ranjan *et al.,* 2010). In this study, 53.12% isolates were resistant to oxytetracycline, which is in contrast to Duarte *et al.,* 2004 who observed 100% resistance to tetracycline. High degree of antimicrobial resistance suggests to improve judicious use of it for the treatment of bovine mastitis.

Bovine mastitis diagnosis and its treatment with most effective drug is an important factor for growth of animal husbandry sector

and timely detection of subclinical mastitis by molecular methods is a need of coming days.

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