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Isolation, Antibiogram and Molecular Characterization of Group B Streptococci Isolates from Bovine Mastitis

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ABSTRACT

Background: Group B streptococcus (GBS) or *Streptococcus agalactiae* is an important pathogen associated with bovine mastitis. The organism is also of public health consequences and may cause variety of infections ranging from neonatal sepsis, pneumonia and meningitis to localized infections and urinary tract infection or arthritisin adult humans. Widespread use of antibiotics in veterinary medicine has led to development of resistance among the pathogens. So there is need for surveillance of antimicrobial resistance to ensure effective treatment.

Methods: Milk samples collected from mastitis affected animals were processed for isolation of *Streptococcus agalactiae*. The isolates were tested for antimicrobial susceptibility. Molecular characterisation was carried out by PCR to study the occurrence of resistance marker genes and virulence marker genes. RAPD was carried out to study genetic diversity among the isolates.

Result: Six isolates of *S. agalactiae* were obtained from 182 milk samples. Highest resistance was observed against co-trimoxazole and tetracycline followed by ampicillin. *tetM* gene and *tetO* genes could be amplified in four and three isolates, respectively. None of the isolates showed amplification for *ermA, ermB, mefA* and *mefE* genes. Three isolates were positive for the five virulence genes tested (*glnA, cfb, hylB, scaA* and *cyl*). RAPD analysis demonstrated great intraspecific genetic diversity among the streptococcal isolates.

Key words: Antibiotic resistance genes, Antibiotic sensitivity, Molecular characterization, PCR, RAPD analysis, *Streptococcus agalactiae*, Virulence genes.

INTRODUCTION

Mastitis is a disease of major economic importance in dairy herd, because of the reduction of farm profitability, decreased milk production, discarded milk, treatment costs, and culling. (Gröhn *et al.,* 2005). Group B streptococcus (GBS) also known as *Streptococcus agalactiae* is an important bovine pathogen and is frequently involved in clinical and subclinical mastitis. Infections due to *S. agalactiae* also have major public health consequences and may cause bacterial sepsis, pneumonia and meningitis in neonates and localized infections such as subcutaneous abscesses, urinary tract infection or arthritis in adults.

S. agalactiae is an obligate pathogen of the epithelium and tissues of ruminant mammary glands. Treatment with intra-mammary infusion of antibiotics is the main approach to deal with the infection and number of studies on *in vivo* and *in vitro* trials to assess the antibiotic sensitivity/resistant pattern has been documented. Frequent use/misuse of antibiotics in animals in general or for treatment of mastitis has resulted in development of resistance among the pathogens. Resistant determinants identified in *S. agalactiae* includes *mefA* and *ermB* genes encoding resistance to erythromycin (Marimón *et al.,* 2005), *tetM, tetO, tetL*, and *tetK* conferring resistance to tetracycline (Lopardo *et al.,* 2003) and *pbp2b* gene contributing to penicillin resistance (Charpentier and Tuomanen 2000).

A variety of virulence factors contribute to the pathogenicity of *S. agalactiae*. Several surface proteins and polysaccharide capsules have been identified within this species. *scpB* gene (encodes for surface enzyme) *ScpB* (C5a peptidase), the *bca* gene (a-protein), the *lmb* gene (laminine-binding protein) and *bac* (b-antigen), *cyl* (b-hemolysin), *glnA* (glutamine synthetase), *cfb* (Christie–Atkins-Munch-Peterson (CAMP) factor) and *scaA* (aggregation factor) (Dmitriev *et al.,* 2002).

Not much work has been reported from the region regarding occurrence of group B streptococci in bovine mastitis. Therefore, the present study was envisaged to study the phenotypic and genotypic antibiotic resistance patterns and genetic diversity among group B streptococci isolated from mastitis milk of bovines from the Punjab state.

MATERIALS AND METHODS

Milk samples were collected from 182 lactating bovines (130 cattle and 52 buffalo). The samples were streaked onto 5%

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sheep blood agar and incubated for at 37°C for 24-48 h. for isolation of *S. agalactiae.* The isolates were identified on the basis of cultural, morphological, biochemical characteristics and CAMP test.

Antibiotic sensitivity test

All the confirmed *S. agalactiae* isolates were subjected to antibiotic sensitivity test by disc diffusion method using antibiotic discs (HiMedia Laboratories Ltd. Mumbai) against 11 antibiotics *viz*. ampicillin (10mcg), ceftriaxone (30mcg), ciprofloxacin (5mcg), co-trimoxazole (25mcg), erythromycin (15mcg), gentamicin (10mcg), meropenem (10mcg), ofloxacin (5mcg), tetracycline (30mcg), penicillin (10mcg) and vancomycin (30mcg). Zone of inhibition was interpreted as sensitive, intermediate and resistant as per the CLSI 2016 guidelines.

Molecular characterization of *S. agalactiae*
Confirmation of *S. agalactiae* isolates by PCR

DNA was extracted from the *S. agalactiae* spp. isolates using HiPurA bacterial genomic DNA purification kit (Himedia, Mumbai) as per manufacturer instructions.Confirmation of *S. agalactiae* isolates was done by PCR using the primers (STR-AG-I5-TAGTTTTGAGAGGTCTTGTGG-3 and STR-AG-II 5-ATATTCACAGCGTTTTCG-3') as described by Goli *et al.,* 2012. Amplification reaction mixture was prepared in a total volume of 25µL consisting of Green Taq master mix (12.5µL), forward primer (20 pmol/µl), reverse primer (20 pmol/µl), template DNA (3µL) and nuclease free water (7.5µL). PCR was performed in a thermo cycler with the conditions**:** initial denaturation (94°C for 1 min) followed by 30 cycles each of denaturation (94°C for 1 min), annealing (54.2°C for 1min)and extension (72°C for 1 min) and one cycle of final extension (72°C for 10 min).

Molecular detection of antibiotic resistance genes

Primers used to amplify *ermA, ermB, mefA* and *mefE* genes are listed in Table 1. PCR was performed with the conditions: initial denaturation (94°C for 10min) followed by 35 cycles, each of denaturation at 94°C for 1 min, Annealing temperature (as per Table 1) and extension at 72°C for 1 min and final extension at 72°C for 10 min.

Detection of virulence genes by PCR

Primer sets used to amplify five major virulence genes (*cyl, glnA, cfb, hylB,* and *scaA*) genes are listed in Table 1. PCR was performed with the conditions**:** initial denaturation (5 min at 94°C) followed by 35 cycles each of denaturation (94°C for 30 sec), annealing (52°C for 30sec) and extension (72°C for 90 sec) and a final extension (72°C for 10 min).

Analysis of PCR product

Ampliûed products were separated by electrophoresis at 70V for 1h in a 1.5% agarose gel and visualized by UV transillumination. A 100bp DNA ladder (Gene ruler, MBI fermentas) was used in as molecular size standards for each of the reactions described above. A negative control, consisting of the same reaction mixture but with water instead of template DNA was included in each run.

Characterization of *Streptococcus agalactiae* strains by randomly amplified polymorphic DNA analysis

Random amplified polymorphic DNA (RAPD) is easy and sensitive technique to characterize the genotypic diversity of *Streptococcus* spp. isolates (Zadoks *et al.,* 2003). In this technique arbitrary primers are used to amplify polymorphic segments of DNA to evaluating the genotypic diversity of *S.agalactiae* (Martinez *et al.,* 2000). The genetic diversity of the six *S. agalactiae* isolates was carried out as per protocol described by Chatellier *et al.,* 1997. Eight primers were selected with the properties that they are 9 or 10 nucleotides in length, between 40 and 77% GC in composition and contained no palindromic sequence. The sequences of primers used for RAPD are indicated in Table 2.

DNA extracted from *S. agalactiae* isolates using HiPurA bacterial genomic DNA purification kit (Himedia Mumbai) was used in the reaction. Amplification reaction mixture was prepared in a total volume of 25µL consisting of Green Taq

Table 1: Details of PCR primers used to amplify virulence genes and antibiotic resistance genes.

Target Gene	Forward (5'-3')	Reverse (5'-3')	Annealing temp. (°C)	Amplicon (bp)	Reference
Virulence genes					
scaA	ACGGTATCAACCTTGAAACTGG	TCAGTGTTGATTTCCCAGATGTA	52	256	Dmitriev
glnA	ACGTATGAACAGAGTTGGCTATAA	TCCTCTGATAATTGCATTCCAC	52	471	*et al.* (2002)
cfb	ATGGGATTTGGGATAACTAAGCTAG	AGCGTGTATTCCAGATTTCCTTAT	52	193	
scaA	ACGGTATCAACCTTGAAACTGG	TCAGTGTTGATTTCCCAGATGTA	52	256	
hylB	ACAAATGGAACGACGTGACTAT	CACCAATTGGCAGAGCCT	52	346	
Antibiotic resistance genes					
ermB	GAAAAGGTACTCAACCAAATA	AGTAACGGTACTTAAATTGTTTAC	55	640	Sutcliffe
ermA	TCTAAAAAGCATGTAAAAGA	CTTCGATAGTTTATTAATATT AGT	52	640	*et al.,* 1996
mefA	AGTATCATTAATCACTAGTGC	TTCTTCTGGTACTAAAAGTGG	52	348	
mefE	CGTAGCATTGGAACAGC	TCGAAGCCCCCTAATCTT	52	515	Lopardo
tetO	AACTTAGGCATTCTGGCTCAC	CGGCGGGGTTGGCAAATA	55	519	*et al.,* 2003
tetM	TTATCAACGGTTTATCAGG	CGTATATATGCAAGACG	46	397	

master mix (12.5µL), primer 1µL (20pmol/µL), DNA 1µL (25ng) of extracted DNA) and 10.5µL nuclease free water. PCR was performed in a thermocycler (ABI thermo) with the conditions: initial denaturation (4min at 94°C) followed by 45 cycles each of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 1 min and final extension at 72°C for 10 min. Amplified products were separated by electrophoresis at 50V for 4h in a 1.2% agarose gel and visualized by UV transillumination. A 1-kb DNA ladder (Gene ruler, MBI fermentas) was used as molecular size standards. RAPD banding pattern was analyzed and phylogenetic tree constructed by PyElph software (Pavel and Vasile, 2012).

RESULTS AND DISCUSSION

A total of 182 (130 cattle and 52 buffalo) milk samples were subjected to CMT, of which 134 (112 cattle and 22 buffalo) were found positive for mastitis and were inoculated on 5% sheep blood agar. All organisms were presumptively identified as streptococci by colony morphology and Gram staining. Among a total of 8 streptococcal isolates, 6 strains were confirmed as *S.agalactiae* as they gave positive results as indicated by production of arrow head hemolytic pattern on CAMP test. CAMP test has been routinely used for confirmation of *S. agalactiae* by Ahmadi *et al.* (2010) and Amosun *et al.* (2010).

Antibiotic resistance among *S. agalactiae* isolates

All the isolates were sensitive to meropenem, vancomycin, ofloxacin, ciprofloxacin and erythromycin. Highest resistance was found for co-trimoxazole (66.66%), tetracycline (66.66%) and ampicillin (50%) followed by gentamycin and ceftriaxone (16.66% each). High degree (72.5% and 82.6%) of resistance to tetracycline among *S. agalactiae* isolates has also been reported by Gao *et al.,* (2012) and Gizachew *et al.* (2019), respectively. In present study, 33.33% of the isolates were resistant to penicillin, whereas Nakamura *et al.,* (2011) observed all isolates were susceptible to penicillin and Gizachew *et al.,* (2019) reported 33.6% resistance to penicillin among *S.agalactiae*. Although penicillin serves as a primary antimicrobial drug for clinical mastitis and has been used for decades in veterinary clinics, the results indicate that penicillin should be used discretely in the treatment of bovine *S.agalactiae* infection. Besides beta-lactams, erythromycin seemed to be the most active antimicrobial agent since all the isolates were sensitive to erythromycin. This finding is similar to those of previous reports from Sarah and Salah (2014). Masoud *et al.,* (2016) found 35.5% resistance for erythromycin in *S. agalactiae.*

Table 2: List of primers tested by RAPD for study of *S. agalactiae* field isolates.

Primers	Sequence (5' to 3')	% CG	Reference
OPS16	AGGGGGTTCC	70	Chatellier
AP42	AACGCGCAAC	60	*et al.,* 1997
A4	GCATCAATCT	40	
OPS11	AGTCGGGTGG	70	
OPA3	AGT CAG CCA C	60	Amal *et al.,*
OPA17	GAC CGC TTG T	60	2013
OPB17	AGGGAACGAG	60	Martinez
OPB18	CCACAGCAGT	60	*et al.,* 2000

Detection of tetracycline resistance genes

Four isolates out of 6 (66.66%) were positive for the presence of *tetM* gene (Fig 1). All the four isolates had been phenotypically observed as tetracycline resistant. Sarah and Salah (2014) reported high prevalence to the presence of the *tetM* gene (99%) in tetracycline resistant streptococci. Three out of the six tested isolates (50%) revealed amplification of *tetO* gene (Fig 2), of the four isolates showing phenotypic resistance to tetracycline, only three isolates harbor *tetO* gene whereas one isolate showing resistance to tetracycline, was negative for *tetO* gene. *tetM* gene is the most prevalent resistance determinant accounting for tetracycline resistance in Gram-positive bacteria (Roberts, 1996). We observed high rate of tetracycline resistance as was described in other study of Gao *et al.* (2012) reported 52.9% of *tetM* gene and 17.6% *tetO* gene. High rates of tetracycline resistance in tetracycline resistant isolates correlated with the presence of the *tetM* gene.

Detection of erythromycin resistance gene

Out of 6 isolates tested for identification of erythromycin resistance genes (*ermA, ermB, mefA* and *mefE*), none were

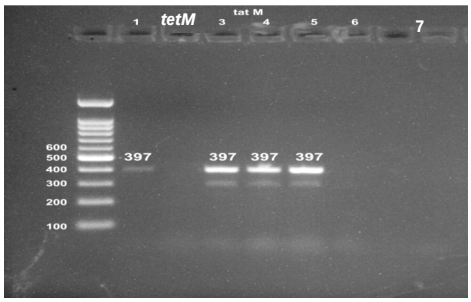


Fig 1: Agarose gel electrophoresis showing amplification of *tetM* gene of *S. agalactiae* isolates.

Lane 1-6: *S. agalactiae* isolates; Lane M: 100bp DNA ladder; Lane 7: Negative control.

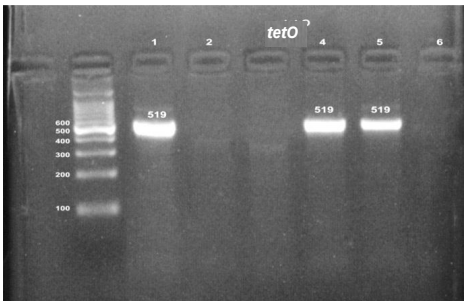


Fig 2: Agarose gel electrophoresis showing amplification of *tetO* gene of *S. agalactiae* isolates.

Lane 1-6: *S. agalactiae* isolates; Lane M: 100bp DNA ladder; Lane 7: Negative control.

found positive. However occurrence of erythromycin resistance genes in *S agalactiae* isolates have been reported by Boswihi *et al.* (2012) who reported *ermA* (5.5%), *mefA* (5.5%) and *mefE* (11%).

In present study, phenotypically all isolates were highly sensitive for erythromycin. It can be inferred that there was a definite pattern observed between the antibiotic resistance by the phenotypic and genotypic methods.

The details of occurrence of different antibiotic resistance genes in individual isolates have been shown in Table 3.

Detection of virulence associated genes

The PCR amplification results for the five virulence genes are depicted in (Fig 3) (*cyl, glnA*) and (Fig 4) (*cfb, scaA*, *hylB*) and Table 3. *cyl* gene of *S. agalactiae* is required for the production of hemolysin. It codes for beta-hemolysin, which is responsible for tissue injury and the systemic spread of the bacteria and lead to meningitis (Doran *et al.,* 2003).We found 83.33% isolates were positive for *cyl,* these results are in accordance with those of Dmitriev *et al.* (2002) they found *cyl* gene in all the isolates while Spellerberg *et al.* (2000) reported that 23% isolates were harbouring the *cyl* gene. Gene *glnA (*glutamine metabolism) have significant role in the virulence and involved in nutrition and metabolism of various bacterial pathogens (Hendriksen *et al.,* 2008). In present study, 66.66% isolates found to contain the *glnA* gene, these results are in accordance with those of Ding *et al.,*(2016), according to their study, gene *glnA* was discovered only in *S. agalactiae* at incidences of 46.9% whereas Dmitriev *et al.,*(2002) reported in all the isolates. The CAMP factor (*cfb*) is a pore-forming protein (protein B) secreted by *S.agalactiae* that potentiates the action of staphylococcal sphingomyelinase (beta toxin)(Jain *et al.,* 2012). In the present study *cfb* gene was discovered at 66.66%, which is similar to Shome *et al.,* (2012) reported 85.7% prevalence of *cfb* gene among *S. agalactiae* isolates. Still the significance of CAMP factor in pathogenicity of *S. agalactiae* is not properly know, hence it is not putative virulence factor (Lasagno *et al.,* 2011).The *hylB* gene codes for hyaluronate lyase which help to break hyaluronic acid, N-acetylglucosamine and glucuronic acid (components of extracellular matrix) It also known as spreading factor which helps to the host tissue invasive (Duran-Reynals, 1942). In present study we found 83.33% isolates were positive for *hyl*B, these results are in accordance with those of Gunther *et al.,* (1996), according to their 72% of the GBS were *hylB* positive whereas Dmitriev *et al.,* (2002) reported *hylB* gene in all the isolates studied. In the present study, 66.66% isolates were positive for *sca*A. Occurrence of *scaA* gene in 45.7 per cent and 100 percent of *S. agalactiae* isolates has been reported by Ding *et al.,* (2016) and Dmitriev *et al.,* (2002), respectively.

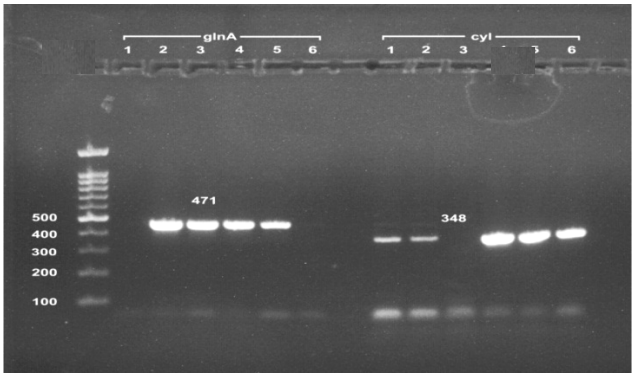


Fig 3: Agarose gel electrophoresis showing amplification of *cyl* and *glnA* genes of *S. agalactiae* isolates.

Lane 1-13: Two set of *S. agalactiae* isolates; Lane M: 100bp DNA ladder; Lane 14: Negative control

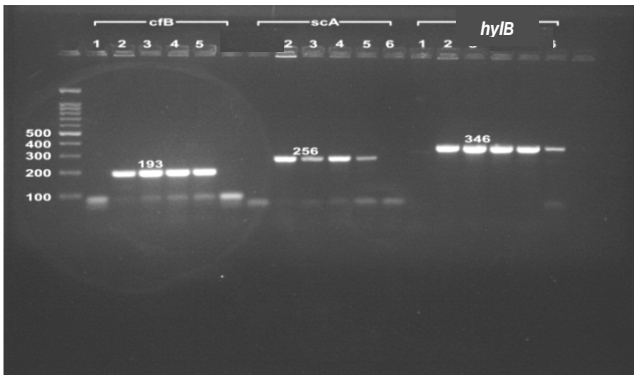


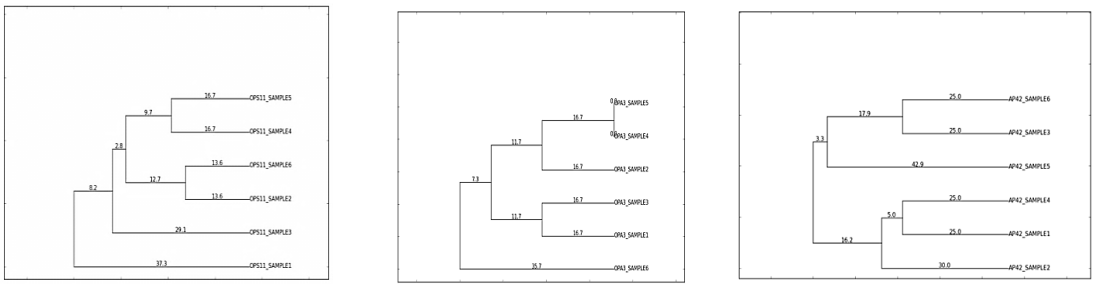
Fig 4: Agarose gel electrophoresis showing amplification of *cfb, scaA* and *hylB* genes of *S. agalactiae* isolates.

Lane 1-18: Three sets of *S. agalactiae* isolates Lane M: 100bp DNA ladder.

Lane 19: Negative control.

Table 3: Presence of Antibiotic resistance genes and virulence genes.

S. No.	AMR genes					Virulence genes				
	tetM	*tetO*	*ermA*	*ermB*	*mefA/E*	*cyl*	*glnA*	*cfb*	*hylB*	*scaA*
S1	+	+	_	_	_	+	_	_	_	-
S2	_	_	_	_	_	+	+	+	+	+
S3	+	_	_	_	_	_	+	+	+	+
S4	+	+	_	_	_	+	+	+	+	+
S5	+	+	_	_	_	+	+	+	+	+
S6	_	_	_	_	_	+	_	_	+	_



a) OPS11 b) OPA3 c) AP42

Fig 5: Dendrogram showing genetic similarity analysis using RAPD of *S. agalactiae* isolates using primer a) OPS11; b) OPA3 and c) AP42.

Molecular characterization of group B streptococci by random amplification of polymorphic dna (rapd)

Out of 8 primers tested, only 3 primers (OPS11, OPA3 and AP42) with GC content of 40 to 70% gave reproducible patterns comprising fragments with a large size range and a small number of low-intensity bands. They gave the best differentiation of the 6 isolated strains. The reproducibility of the RAPD patterns obtained with these three primers was verified by repeating experiments under the same conditions. Each strain was tested at least twice times.

Dendrogram of *S.agalatciae* by OPS11 (Fig 5a) revealed that isolates S5 and S4, S6 and S2 evaluate with similar distance but S3 forming near out group with all other isolates. Sample1 is evaluating totally different from other isolates. OPA3 revealed sample 5 and 4 showed same genetic distance from other isolates (Fig 5b). Isolates 5, 4, 2, 3, 1 formed two clades and sample 6 was having entirely different level of evolution. According to AP42 primer S6, S3, S5 forming one group and Samples 4, 1, 2 forming another group (Fig 5c). In this two groups sample 5 and 2 forming outgroup with other isolates. Primers AP42 and OPS11 yielded similar type of pattern but OPA3 primer giving different kind of banding pattern. The isolates of *S.agalactiae* showed a great intraspecific diversity; various workers also reported high genetic diversity among bovine isolates (Martinez *et al.,* 2000 and Baseggio *et al.,* 1997). RAPD is simple and fast technique which is used for characterization of *S. agalactiae* strains. In cluster analysis, RAPD method identifies the same virulent families and able to discriminate strains inside each cluster and thus is more sensitive for identifying intraspecific diversity among isolates (Wang *et al.,* 1993).

CONCLUSION

S.agalactiae (Group B streptococci) isolated from six out of a total of 182 milk samples indicated a prevalence of 3.3%. The antibiogram of six isolates revealed higher resistance against oxytetracycline (66.66%) and co-trimoxazole (66.66%) followed by ampicillin (50%), penicillin (33.33%). The antibiotic resistant isolates were carrying genes for tetracycline resistance (*tetM* and *tetO*). Two of the isolates carrying all five virulence associated genes *viz*. *cyl, scaA, hylB, glnA* and *cfb.* The isolates of *S. agalactiae* showed a great intraspecific diversity.

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