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Research Article

DETECTION OF VIRULENCE GENE OF *STREPTOCOCCUS* SPP. ISOLATED FROM BOVINE MASTITIS

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Abstract: Mastitis occurs worldwide among dairy animals, and it has been described to have an extreme economic impact, despite the control strategies. This study aimed to investigate *Streptococcus* spp. Virulence gene found in clinical and subclinical mastitis milk samples from cows and buffaloes. The study was carried out on 285 milk samples collected from various area near to Banaskantha, Gujarat. Out of 285 milk samples (18 from subclinical and 95 from clinical cases) screened for *Streptococcus* spp. 17 isolates were obtained giving an overall incidence of 5.96 per cent. The incidence of *Streptococcus* spp. from subclinical mastitis was 2.63 per cent (5/190) and from clinical mastitis was 12.63 per cent (12/95). Species wise incidence was 8.10 per cent (6/74) in buffaloes and 5.21 per cent (11/211) in cows. All these 17 isolates were further confirmed as *Streptococcus* spp. using genus specific PCR All the 17 isolates were further confirmed using species-based PCR as *Streptococcus agalactiae, Streptococcus dysgalactiae* and *Streptococcus uberis* specific primers based Abstract gene amplification. In species specific PCR, *Streptococcus agalactiae* (n=4) (23.52%) produced 405 bp size amplicon, *Streptococcus dysgalactiae* (n= 6) (35.29%) produced 281 bp size amplicon whereas *Streptococcus uberis* (n=7) (41.17%) yielded 94 bp size amplicon. All the 17 isolates were further investigated for presence of virulence gene and out of 17 isolates of *Streptococcus* spp. 6 (35.29%) isolates were found positive for ScpB gene in which amplicon size of 255 bp while detected were other virulence gene *bca, Rib, cylE* found negative.

Keywords: *Mastitis, Gene amplification, Virulence*

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Introduction

Mastitis occurs worldwide among dairy animals and it has been described to have an extreme economic impact, despite the control strategies [1]. Mastitis contributes up to 70% of reduced milk production, 9% of discarded milk after treatment, 7% of the cost of veterinary services and 14% of premature culling [2]. The losses due to mastitis in monetary terms in India is reported INR1390 crore per lactation, among which around 49% is owing to loss of value from milk and 37% on account of veterinary expenses [3]. Mastitis occurs in clinical and subclinical forms and their average incidence was reported as 14.2% and 30%, respectively [4]. Furthermore, it has a serious zoonotic risk due to the presence of bacteria. Bovine mastitis is one of the most prevalent and costly diseases concerning the dairy industry worldwide [5]. *Streptococcus* spp. has become the major cause behind several mammalian infections [6], which can lead to streptococcal mastitis/endometritis in domestic mammals and skin lesions, meningitis, and bacteremia in humans. Besides, *Streptococcus* spp.[7]. However, the abuse of antibiotics leads to antimicrobial resistance among causative agents and causes reactions in humans allergic to antimicrobials [8]. This phenomenon is becoming more and more serious, which is coming to our notice. The virulence-associated determiners of *Streptococcus* spp. play a crucial role in the pathogenesis of the causative agents [9], including α-enolase, nephritis-associated gen-binding receptor, β- hemolysin, Lamining-binding Protein. But in fact, little is known about these act eristics about the *Streptococcus* spp. isolated from bovine mastitis in Gujarat. The aim of this work was to determine the incidence rate of *Streptococcus* spp. infection among mastitis cows and buffaloes among dairy farms based on both phenotypic and genotypic assays. Detection of some phenotypic virulence characteristics and some putative virulence associated genes in the isolated *Streptococcus* spp. strains towards a better understanding of their pathogenesis and epidemiology as mastitis causing pathogens.

Materials and Methods

Sample Collection

A total of 190 milk samples from animals comprising of 158 cows and 32 buffaloes belonging to Banaskantha, Mahesana, Sabarkantha, Patan districts were collected and screened for subclinical mastitis between 2018 and 2019. The incidence of bovine mastitis in cattle and buffalo ranges from 2% to 10%, and the parity of sick cows and buffaloes is about 4 to 5%. In India, the incidence is higher from March and April and from June to September, so the samples were mainly collected during this period.

The Subclinical mastitis and clinical mastitis were confirmed by Electrical conductivity meter (Draminski 4Q MAST) and California mastitis test (CMT). All these farms employed veterinarians who had received professional training on sampling procedures and aseptic techniques for collecting samples. After sampling, the veterinarians disinfected the breasts of dairy cows with 75% ethanol and milk samples were transported to the laboratory at 4 °C for microbiological culture. The sampling process was like normal commercial milking and met the requirements of animal welfare. This study does not involve animal experiments therefore ethical approval for this study was not needed.

Microbiological Culture and Identification

The samples were processed to isolate and identify *S. aureus* as per the standard methods described in Bacteriological Analytical Manual (BAM) (FDA and USDA, 2016) and Peacock (2010) with certain modification. Each sample were inoculated with the aid of a sterile wire loop was Fig on sheep blood agar (Himedia, India) at 37°C aerobically for 24 to 48 h. It was considered as cultured positive if 1 or more colonies were observed. Milk samples with 3 or more species were considered contaminated, unless *Streptococcus* spp. were isolated. After growth, every single colony with different morphology was sub-cultured on blood agar.

Table-1 Oligonucleotide primers of Streptococcus spp. used in PCR.

SN	Primer name	Code		Product size (bp)	Reference
1	*Streptococcus Spp.*	*Str-I* *Str-II*	F-TGTTTAGTTTTGAGAGGT CTTG R- CGTGGAATTTGATATAGATATTC	150-210	[10]
2	*Streptococcus agalactiae*	*Sag 40* *Sag 445*	F-CGCTGAGGTTTGGTGTTTACA R- CACTCCTACCAACGTTCTTC	405	[11]
3	*Streptococcus dysgalactiae*	*Sdy 105* *Sdy 386*	F-AAAGGTGCAACTGCATCACTA R-GTCACATGGTGGATTTTC CA	281	
4	*Streptococcus uberis*	*Sub 302* *Sub 396*	F-CGAAGTGGGACATAAAGTTA R-CTGCTAGGGCTAAAGTCAAT	94	

Table-2 Oligonucleotide primers of some selected genes used in PCR

SN	Primer Name	Sequence	Target gene	Product size (bp)	Reference
1	*bca*	F-TAACAGTTATGATACTTCACAGAC R-ACGACTTTCTTCCGTCCACTTAGG	*bca*	183	[12]
2	*ScpB*	F-ACAACGGAAGGCGCTACTGTTC R-ACCTGGTGTTTGACCTGAACTA	*sepB*	255	[13]
3	*Rib*	F-CAGGAAGTGCTGTTACGTTAAAC R-CGTCCCATTTAGGGTTCTTCC	*rib*	369	
4	*cylE*	F-TGACATTTACAAGTGACGAAG R-TTGCCAGGAGGAGAATAGGA	*cylE*	248	[14]

Table-3 Cycling conditions of the different primers during PCR

SN	Initial denaturation	Denaturation	Annealing	Extension	Final extension	No. of cycles
Str	95°C 6 min.	95°C 40 sec.	44°C 35 sec.	72°C 45 sec.	72°C 9 min.	30
Sag	94°C 5 min.	94°C 1 min.	60 °C 1 min.	72°C 1 min.	72°C 5 min.	30
Sdy	94°C 5 min.	94°C 45 sec	47 °C 1 min	72°C 45 sec	72°C 5 min	35
Sub	94°C 5 min.	94°C 45 sec.	47 °C 1 min.	72°C 45 sec.	72°C 5 min.	35
Bca	94°C5 min.	94°C 30sec.	46 °C 30 sec.	72°C 2 min.	72°C 10 min.	35
ScpB	94°C 1 min.	94°C 1 min.	47 °C 1 min	72°C 1 min	72°C 10 min	35
Rib	95°C 5 min.	94°C 30 sec.	51 °C 30 sec.	72°C 2 min.	72°C 10 min.	35
cylE	95°C 5 min.	95°C 1 min.	47 °C 1 min	72°C 2 min	72°C 10 min	35

Table-4 Prevalence and distribution of Streptococcus isolates among the subclinical and clinical mastitis dairy animals

Collected milk samples			*Streptococcus* isolates	
Cases	Examined animals	No. of samples	No.	%
Subclinical mastitis	Cattle	158	3	19
	Buffaloes	32	2	6.25
Clinical mstitis	Cattle	53	8	15
	Buffaloes	42	4	10
Total		285		

Another optional sub-culture was conducted if different morphological colonies grew on the same Fig. A single colony was enrichment cultured in nutrient broth at 37°C for 24 to 48 h and stored with 15% glycerol at -80°C.

Subsequently, the Figs were examined for colony morphology, pigmentation and hemolytic characteristics after 24-48 h. Presumptive colonies of *Streptococcus* spp. were selected and streaked into a slant agar for 24 h for biochemical tests, and Gram staining. Catalase, Oxidase test, KOH string test, Hippurate Hydrolysis test performed to detect. Seventeen isolates were identified as *Streptococcus* spp. and stored at -20°C. The pure colony was cultured in 2 mL of Tryptone Soya broth (HiMedia, India) at 37 °C for 24-48 h. Then, the genomic DNA was extracted using the Bacterial DNA Kit (QIAGEN, Germany) following the manufacturer's protocol.

Molecular and Virulence Characterization of *Streptococcus* spp

For the molecular level identification, isolated colonies were cultured in BHI broth and incubated at 37ºC for 24 hrs. Then DNA of 24 h old cultures were extracted using the mericon DNA Bacteria plus Kit (Qiagen, Germany) with some modifications from the manufacturer's recommendations. PCR was applied on 8 selected different *Streptococcus* isolates to determine 4 genes included *Streptococcus* Spp. (Str), *Streptococcus agalactiae* (Sag), *Streptococcus dysgalactiae* (Sdy) and *Streptococcus uberis* (Sub) and 4 virulence genes (*Bca*, *ScpB*, *Rib* and *CylE*). The primer sequences, size of generated products and cycling conditions used in PCR amplification were illustrated in [Table-1,2 and 3].

Results

Prevalence and Distribution of *Streptococcus* isolates in Clinically Mastitis animals

Out of 190 collected milk samples (158 cows and 32 buffaloes) screened for subclinical mastitis, a total of *Streptococcus* s pp. was recovered with a total prevalence of 57.9%; distributed as 18 (9.47%) and species wise 14 Cows and 4 Buffaloes found positive for SCM (Draminski 4Q MAST) respectively. A total of 95 milk samples from clinical cases of mastitis were also collected from cattle and buffaloes (53 from cows and 42 from buffaloes) and were processed for isolation and identification of *Streptococcus* spp.

Fig-1 (*Streptococcus* on BHI)

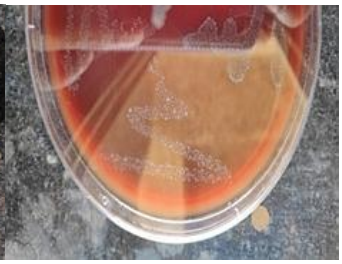

Fig-2 (*Streptococcus* on Sheep blood agar)

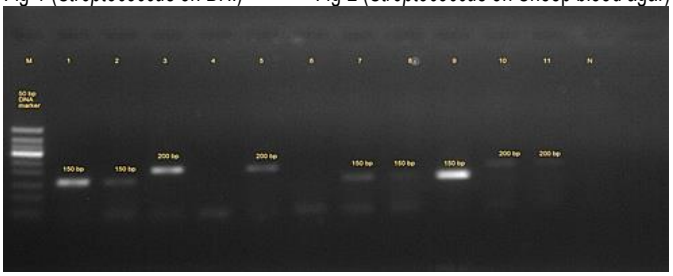

Fig-3 Genotypic confirmation of *Streptococcus* spp. by PCR Product size (150-200 bp)

Identification and Distribution of *Streptococcus* Isolates

Samples which were detected positive in subclinical mastitis (n=18) and clinical mastitis (n=95) were primarily inoculated on Brain and Heart infusion [Fig-1] and/or Blood agar for isolation of bacteria.

[Fig-2] Of these 106 samples yielded the growth of bacteria. Among these, 17 samples yielded small, translucent dew drop like colonies, which were tentatively identified as *Streptococcus* spp. *Streptococcus* isolates (n=17) were identified using traditional methods including morphological, colonial and biochemical characteristics

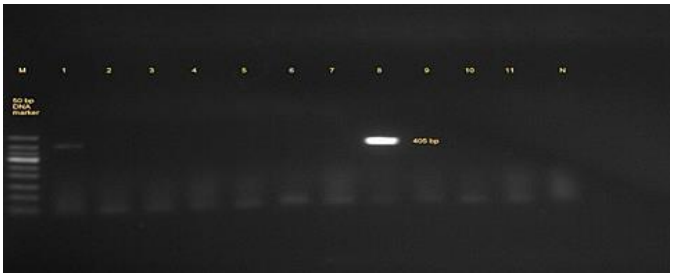

Fig-4 Confirmation of *Streptococcus aglactiae* by PCR Product size (405 bp)

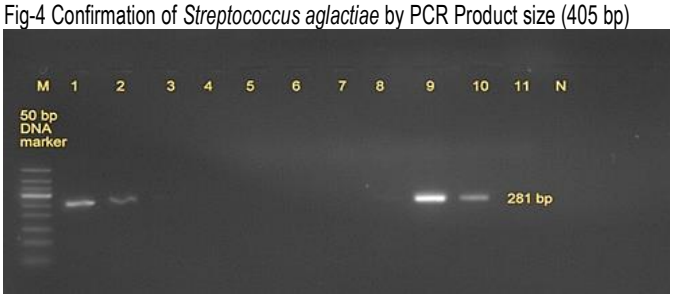

Fig-5 Confirmation of *Streptococcus dysgalactiae* by PCR product size (281 bp)

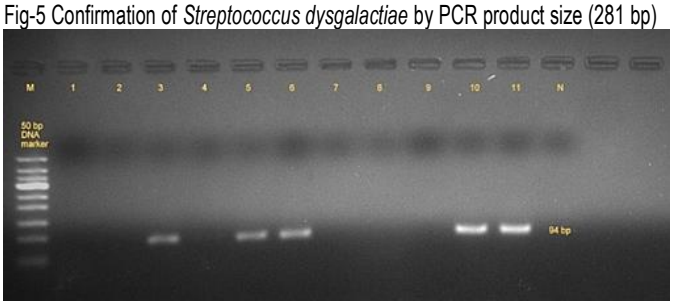

Fig-6 Confirmation of *Streptococcus uberis* by PCR product size (94 bp)

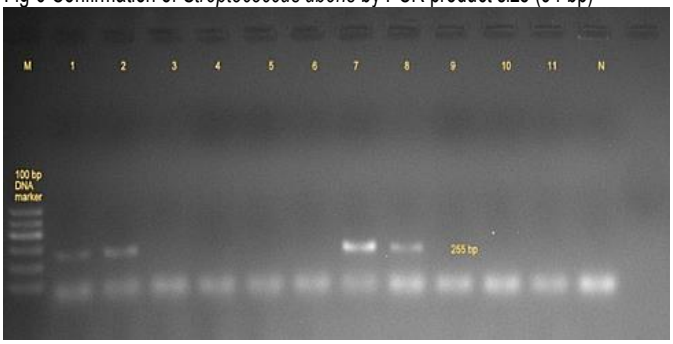

Fig-7 Confirmation of *ScpB* gene by PCR product size (255bp)

Prevalence and Distribution of *Streptococcus* Isolates among examined Animals

In addition to the biochemical tests, all the probable *Streptococcus* isolates were further identified for confirmation using *Streptococcus* specific primers. In genus specific PCR, all the 17 isolates tentatively identified as *Streptococcus* spp. [Fig-3] yielded 150-210 bp fragments which confirmed that all these isolates belonged to the genus *Streptococcus*. All the 17 isolates were further identified as *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis* specific primers-based gene amplification. In species specific PCR, *Streptococcus agalactiae* (n=4) (23.52%) [Fig-4] produced 405 bp size amplicon, *Streptococcus dysgalactiae* (n= 6) (35.29%) [Fig-5] produced 281 bp size amplicon whereas *Streptococcus uberis* (n=7) (41.17%) [Fig-6] yielded 94 bp size amplicon.

Results of PCR for Detection of Virulence Genes in *Streptococcus* Isolates

The results of PCR revealed that out of 17 isolates of *Streptococcus* spp. 6 (35.29%) isolates were found positive for *ScpB* gene in which amplicon size of 255 bp [Fig-7] were detected were other virulence gene *bca*, *Rib*, *cylE* found negative, respectively. At species level 04 isolates of *Streptococcus agalactiae* and 02 isolates of *Streptococcus dysgalactiae* showed the presence of *ScpB* gene.

Discussion

Streptococcus is a worldwide pathogen that causes intra- mammary infections in dairy animals. *Streptococcus* spp. has been described as an opportunistic pathogen that utilizes nutritional flexibility to adapt to a range of ecological positions, including the mammary gland [15]. *S. agalactiae* was first identified in 1887 as a pathogen causing the mastitis [16].

Results of the prevalence and distribution of *Streptococcus* isolates from subclinical and clinical mastitis animals presented that, out 17 of *Streptococcus* isolates; 3(19%) and 2(6.25%) subclinical and 8(15%) and 4(10%) clinical isolates were recovered from clinically mastitis cows and buffaloes, respectively. This prevalence differs from that obtained by [17] who reported clinical mastitis in cattle as (20%) compared to buffaloes (11%) and in line with [18,19] who reported a prevalence of 40% and 30.3% of clinical mastitis in buffaloes and cattle, respectively.

Such variation in the prevalence might be attributed to the difference in herd management and the level of hygiene. Several hygienic and management practices can decrease the incidence as teat dipping before and after milking, washing milkers' hands before and after milking, preparation of clean towel for each lactating cow, milking of infected cow lastly, using dry cow therapy method as well as treating clinical cases at early stage of infection [20]. The prevalence and distribution of *Streptococcus* spp. among examined animals cleared that, among the 17 Streptococci isolated in this study 4(23.52%), 6(35.29%) and 7(41.17%) were *S. agalactiae, S. dysgalactiae* and *S. uberis* respectively based on biochemical examination and confirmation by PCR.

The prevalence rate was much higher than the 72.3% isolation of *S. dysgalactiae* from milk samples obtained by [21]. The prevalence of *S. dysgalactiae* from cases of mastitis samples in our study was higher than those reported by [22], 10.5% from clinical mastitis in China, [23], 12.1% in Norway and [24] 4.0% in Thailand. Regarding, *Streptococcus uberis* [25]; reported 4.35%. Our results disagreed with the results of [26, 27,28] who reported that, the *S. uberis* strains were 48 out of 74 *Streptococcus* spp. (64.9%).

Also, our results of molecular detection of virulence genes in *Streptococcus* isolates cleared that, 17 different selected isolates of *streptococci*; 1 *S. agalactiae*, 3 *S. dysgalactiae*, 3 and *S. uberis* were screened by simplex PCR and the results showed that only 6 (35.29%) isolates were found positive for *ScpB* virulence gene. The isolates were negative for the virulence genes *bca, Rib and cylE*. Earlier molecular reports showed that most bovine isolates lack surface proteins-encoding genes *bca* and *Rib*, in contrast to isolates [9, 29] but that however they can be detected in some *S. agalactiae* bovine strains [30].

Conclusion

Streptococcus species was recovered in a percentage of 57.9% from totally examined mastitis milk samples. Out of them, S. *agalactiae* isolated in (13.7%) and (20.5%) from cows and buffaloes, *S. dysgalactiae* (6.8%) and (23.7%) and *S. uberis* (10.6%), respectively. All the 17 isolates were further investigated for presence of virulence gene and out of 17 isolates of *Streptococcus* spp. 6 (35.29%) isolates were found positive for *ScpB* gene in which amplicon size of 255 bp while detected were other virulence gene *bca*, *Rib*, *cylE* found negative.

Application of research: *Streptococcus* spp. one of the major contributor for the cause of mastitis. By identifying virulence gene of *Streptococcus* spp. helpful in understanding development of disease process and treating mastitis in animals.

Research Category: Veterinary Microbiology

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Author Contributions: All authors equally contributed

Author statement: All authors read, reviewed, agreed and approved the final manuscript. Note-All authors agreed that- Written informed consent was obtained from all participants prior to publish / enrolment

Study area / Sample Collection: Banaskantha, Mahesana, Sabarkantha and Patan districts of Gujarat

Breed name: Cows, Buffaloes

Conflict of Interest: None declared

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.
Ethical Committee Approval Number: Nil

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