International Journal of Veterinary Sciences and Animal Husbandry

ISSN: 2456-2912
VET 2023; SP-8(4): 28-31
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www.veterinarypaper.com
Received: 03-05-2023
Accepted: 05-06-2023

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Isolation and identification of streptococcus organisms in mastitis milk

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DOI: https://doi.org/10.22271/veterinary.2023.v8.i4Sa.628

Abstract
The study focused on isolating and identifying *Streptococcus*, a mastitis-causing organism, from raw milk samples obtained from different dairy farms. A total of 100 milk samples were collected, and microbiological testing was conducted to analyse the raw milk. Results showed that 27 samples (27%) tested positive for *Streptococcus* through cultural methods, while 33 samples (33%) tested positive using PCR assay. Further analysis of the samples revealed that out of 40 milk samples from organized dairy farms, 10 (25%) tested positive for *Streptococcus* through cultural methods. Among the 40 samples from unorganized dairy farms, 15 (37.5%) were positive for *Streptococcus*. For the 20 samples from individual farmers, 2 (10%) tested positive through cultural methods. Similarly, using PCR, 12 (30%) samples from organized dairy farms, 18 (45%) samples from unorganized dairy farms, and 3 (15%) samples from individual farmers tested positive for *Streptococcus*. Conducting microbiological testing is crucial in identifying the causative agents of mastitis and implementing appropriate control measures to eliminate the infection.

Keywords: *Streptococcus*, Milk, Mastitis, Microbial testing, PCR.

Introduction

Mastitis is a significant economic concern for dairy farmers, as it can lead to substantial losses in milk production and, consequently, impact the farmer's income. It has been estimated that mastitis alone causes a 70% reduction in farm production [1]. Mastitis is characterized by inflammation of the mammary gland [2]. This inflammatory condition leads to abnormalities in milk, such as increased somatic cells, particularly leukocytes, and pathological changes in the mammary tissue. Additionally, clinical signs of mastitis include heat, redness, swelling, hardness, and pain [3]. Mastitis exists in both clinical and subclinical forms, with clinical mastitis exhibiting detectable changes in mammary tissue, while subclinical mastitis is mainly recognized through the somatic cell count in milk [4]. The development of mastitis is influenced by multiple factors due to its complex nature. The udder of a cow provides an ideal environment for microbial growth, and when optimal conditions in terms of temperature, nutrition, and lack of external influences are present, harmful organisms can proliferate rapidly, leading to udder damage and the development of mastitis [5].

Materials and Methods

Sample Collection

Milk samples were aseptically collected from various dairy farms in and around Hyderabad, known to have a high incidence of mastitis. A total of 100 milk samples were collected using sterile vials and stored at 4°C until further processing.

Californian Mastitis Test (CMT)

The CMT was performed to detect subclinical mastitis. Teats were disinfected using alcohol before collecting individual 20 ml half-udder milk samples under sterile conditions. These samples were kept at 4 °C until bacterial cultural examination within 24 hours. CMT scores ranged from (-) for negative results to (+ + +) for different degrees of positive reactions [6]. A commercial CMT reagent was used, and the CMT results were observed. (Fig 01).

Fig 1: CMT Result

Sample Processing

The milk samples were centrifuged at 2000 rpm for 15 minutes, and the supernatant was discarded. The remaining sediment was resuspended in a small volume (0.05 ml) of saline solution [7]. Diluted milk samples were then aseptically inoculated into nutrient broth, followed by incubation at 37 °C for 24 hours. After the incubation period, Gram staining and morphological analysis were performed. *Streptococcus* microorganisms were isolated using blood agar. All media were purchased from Hi Media, Mumbai and prepared in the laboratory following standard procedures.

Isolate Identification

The obtained pure cultures were subjected to primary tests, such as the catalase test. Catalase-negative cultures were streaked onto nutrient agar slants and preserved at 4 °C. Pure cultures from the slants were further tested using various biochemical tests following standard procedures.

a) Hemolytic pattern: On five percent sheep blood agar plates, β-hemolysis (clear zone of clearance) and α-hemolysis (greenish discoloration around and beneath the colonies) were observed (Fig: 02).

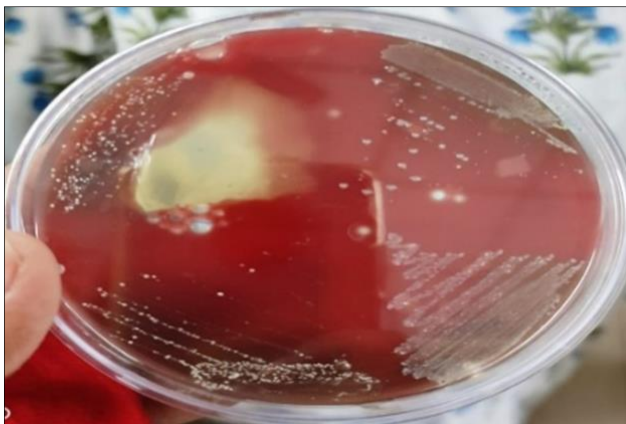

Fig 2: Haemolytic pattern of *Streptococcus*

b) Gram staining: Gram's staining kit from M/s. Hi-Media, Mumbai, containing crystal violet, Gram's iodine, decolorizer, and safranin, was used to stain all the pure culture isolates (Fig 03).

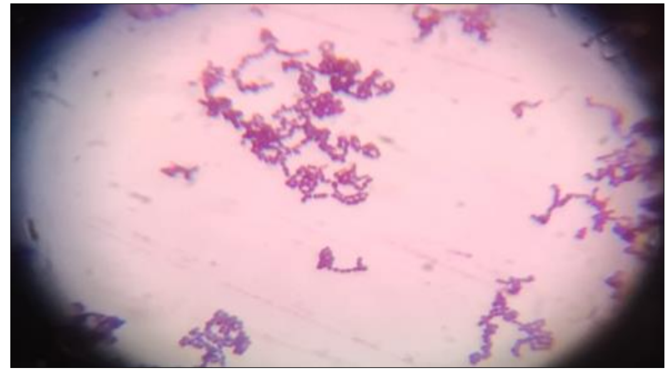

Fig 3: Gram staining

PCR assay

Extraction of Genomic DNA from isolated *Streptococcus*

Genomic DNA was extracted from the isolated *Streptococcus* strains. The pure isolates were cultured in 3 ml of Luria Bertani (LB) broth for 24 hours at 37 °C. Genomic extraction was performed using the Maricon DNA Bacterial plus kit, following these steps:

1. A highly turbid LB broth culture (1 ml) was transferred to a microcentrifuge tube and centrifuged at 10,000 rpm for 10 minutes to collect the bacterial cell pellet.
2. The supernatant was carefully removed to avoid disturbing the pellet.
3. Rapid lysis buffer (200µL) was added to the tube containing bacterial pellets, which were then resuspended by vortexing for 5 minutes.
4. The tube was heated at 100 °C for 5 minutes using a rotary thermal block set, followed by snap chilling for 10 minutes.
5. After centrifugation at 10,000 rpm for 10 minutes, the supernatant (150µl) was transferred to a new microcentrifuge tube. A portion of this supernatant was used for quality, purity, and concentration analysis and stored at -20 °C for up to three weeks.
6. The aliquot containing genomic DNA was used in PCR reactions for further genomic studies.

Estimation of Quality, Purity and Concentration of DNA

The quality, purity, and concentration of isolated DNA were determined using agarose gel electrophoresis and Nano Drop™ 2000/2000c spectrophotometers. Gel electrophoresis showed that the extracted genomic DNA did not exhibit any shearing, indicating its high quality. The DNA samples were then analysed by measuring their absorbance at 260 and 280 nm. All samples exhibited an A_{260}/A_{280} ratio higher than 1.8, confirming their suitability for PCR amplification.

Molecular Detection of *Streptococcus* Genus

To confirm the presence of *Streptococcus spp*., PCR technique was employed for all previously phenotypically confirmed isolates. Specific *Str* gene primers synthesized by Eurofins Genomics Pvt Ltd, Bangalore, India were used. Details regarding primer sequences, amplicon sizes, reaction mixture composition, and cycling conditions can be found in Table 01, 02, and 03 respectively.

Table 1: *Streptococcus* Genus specific *Str* gene primer

S. No	Target Genes	Primer sequence (5'→3')	Product Size	Reference
***Streptococcus* Genus**				
1	*Str*	F: GTA CAG TTG CTT CAG GAC GTA TC	197 bp	Picard *et al.*, (2004) [8]
		R: ACG TTC GAT TTC ATC ACG TTG		

Note: F: Forward, B: Backward

Table 2: Components of reaction mixture for *Streptococcus*

S. No.	Name of the Reagent	Quantity (µl)
1.	2X Master mix (Emerald)	12.5
2	Primer-F	1.25
3.	Primer-R	1.25
4.	DNA template	2.5
5.	Molecular grade water	7.5
7.	Total	25

Table 3: Cycling conditions used for *Streptococcus* primers

S. No	Step	*STR*
1.	Initial denaturation	94^0C, 2 min
2.	Final denaturation	94^0C, 45 Sec
3.	Annealing	60^0C, 1 min
4.	Initial extension	72^0C, 1 min
5.	Final extension	72^0C, 1 min
6.	Hold	4^0C

Following the completion of the PCR reaction, the amplified products were briefly stored at 4 °C before undergoing analysis using agarose gel electrophoresis.

Agarose Gel Electrophoresis for visualization of PCR Products

A 2% w/v agarose gel (Sigma, USA) was prepared by boiling in 0.5X tris-borate EDTA (TBE) buffer. Ethidium Bromide (EtBr) (Sigma, USA) was added at a final concentration of 0.5µg/ml to enable intercalation between DNA base pairs for visualization using UV light. The molten agarose, cooled to approximately 50 °C, was used for this purpose.

For gel electrophoresis, 5 µl of the targeted amplified PCR product from each PCR tube was mixed with 1 µl of 5X gel loading dye. Electrophoresis was conducted using 5 µl of DNA molecular weight marker (Gene RulerTM, 100 bp DNA ladder and O Gene Ruler 100 bp Plus DNA Ladder, Thermo Scientific). The initial voltage was set at 90V for 10 minutes, followed by 70V for one hour in 1X TBE buffer. The amplified PCR product was visualized as a single, compact band of the expected size under UV light for each primer. Gel documentation was performed using the Bio-Rad Gel DocTM XR+ Gel Documentation System (Sweden) with Lab image computer software.

Results and Discussion

4.5.2 Incidence of *Streptococcus* in Milk Samples

(Table 04) Presents the incidence of *Streptococcus* in the milk samples obtained in this study. Out of the 100 milk samples collected from various sources, 27 (27%) samples tested positive for *Streptococcus* using traditional cultural methods, while PCR assays detected the presence of *Streptococcus* in 33 (33%) samples. The cultural method exhibited an 81.8% efficiency when compared to the PCR assay.

Regarding the 40 milk samples from organized dairy farms, 10 (25%) were positive for *Streptococcus* according to the culture method, while the PCR method detected *Streptococcus* in 12 (30%) samples. The cultural method showed an 83.33% efficiency compared to the PCR assay. In the case of milk samples from unorganized dairy farms, 15 (37.5%) were positive for *Streptococcus* based on the culture method, and the PCR method identified *Streptococcus* in 18 (45%) samples. The cultural method demonstrated an 83.33% efficiency relative to the PCR assay.

Lastly, out of the 20 milk samples obtained from individual farmers, 2 (10%) samples were positive for *Streptococcus* using the culture method, while the PCR method detected *Streptococcus* in 3 (15%) samples. The cultural method exhibited a 66.66% efficiency compared to the PCR assay.

Table 4: Cultural and PCR results of mastitis milk from different farms for *Streptococcus*

S. No	Source	No of samples	Cultural method	PCR assay	% of cultural method compared to PCR assay
1	Organized dairy farm	40	10 (25%)	12 (30%)	83.33
2	Unorganized dairy farm	40	15 (37.5%)	18 (45%)	83.33
3	Individual farmers	20	2 (10%)	3 (15%)	66.66
Total		100	27 (27%)	33 (33%)	81.8

Incidence of *Streptococcus* in milk samples

In this study, 100 milk samples were collected from various sources. Traditional cultural methods identified *Streptococcus* in 27% of the samples, while PCR assays detected the bacterium in 33% of the samples. The cultural method showed an 81.8% efficiency compared to PCR. Hegde *et al.* (2013) [9] reported a higher incidence of 34.5% for *Streptococcus* in milk samples collected from different dairy farms using cultural methods, which was more than the 27% incidence observed in our study. Similarly, Elango *et al.* (2010) [10] reported a higher incidence (35.08%) in Namakkal milk samples using cultural methods. Conversely, a lower incidence (18.1%) was reported by Preethirani *et al.* (2015) [11] using PCR assay on mastitis milk from Bangalore.

For the 40 milk samples obtained from organized dairy farms, *Streptococcus* was detected in 25% and 30% of the samples using cultural and PCR assays, respectively. The cultural method exhibited an 83.33% efficiency compared to PCR. Hegde *et al.* (2013) [9] reported a higher incidence (36.5%) of S. *agalactiae* in milk samples from organized dairy farms, which was more than the 25% incidence found in our study. In contrast, Preethirani *et al.* (2015) [11] reported a lower incidence (21.8%) in organized dairy farms from Bangalore using PCR assay. Mekibib *et al.* (2010) [15] reported a lower incidence (7.2%) of S. agalactiae in mastitis milk from organized dairy farms in Ethiopia compared to our cultural method (25%).

For the 40 milk samples obtained from unorganized dairy farms, *Streptococcus* was detected in 37.5% and 45% of the samples using cultural and PCR assays, respectively. The cultural method exhibited an 83.33% efficiency compared to PCR. Hegde *et al.* (2013) [9] reported a lower incidence (32.8%) of *Streptococcus* in milk samples from unorganized dairy farms, which was less than the 37.5% incidence found

in our study. Similarly, Preethirani *et al.* (2015) [11] reported a lower incidence (16.6%) in unorganized dairy farms from Bangalore using PCR assay.

Comparing the milk samples from unorganized and organized dairy farms, our study found a slightly higher incidence (45%) of *Streptococcus* in unorganized farms compared to organized farms (30%). However, Hedge *et al.* (2013) [9] reported a higher incidence (36.5%) in organized dairy farms compared to the incidence (32.8%) in unorganized dairy farms.

For the 20 milk samples from individual farmers, 10% tested positive for *Streptococcus* using cultural methods, while 15% tested positive using PCR. The cultural method exhibited a 66.66% efficiency compared to PCR. The incidence in milk samples from individual farmers was lower compared to milk samples from both organized and unorganized dairy farms.

Standardization of PCR assay for *Streptococcus*

To standardize the PCR assay for *Streptococcus*, we optimized the annealing temperature, primer concentration, template volume, and cycling conditions. The specific PCR product of 197 bp (Fig: 04) for *Streptococcus* was found to be stable when stored at -20°C, as storing it at 4°C for a longer duration resulted in product degradation possibly due to the action of thermo-stable endogenous nucleases (Gibson and McKee, 1993) [16].

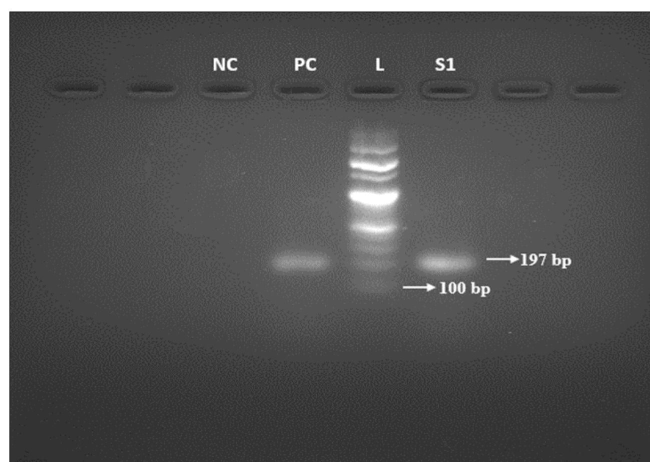

Fig 4: The specific PCR products of 197 bp for *Streptococcus*

NC: Negative Control
PC: Positive Control
L: Ladder (100bp)
S1: Sample 1

Conclusion

This study found that the incidence of *Streptococcus* was highest in unorganized dairy farms compared to both organized dairy farms and individual farmers. Additionally, the PCR method demonstrated higher efficiency in isolating the bacteria compared to the cultural method.

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