RESEARCH ARTICLE Asian Journal of Dairy and Food Research, Volume 43 Issue 3: 418-424 (September 2024)

Molecular Detection of Some Most Important Pathogenic Bacteria by Multiplex PCR for Diagnosis of Subclinical Mastitis in Bovine

Sudeep Solanki[1], Durga Devi[1]

10.18805/ajdfr.DR-2141

ABSTRACT

Background: The dairy sector faces formidable obstacles in the early detection of the bacteria causing subclinical mastitis and in the proactive treatment of those cases. The objective of the study was to access multiplex PCR assays (MPCR) and conventional methods for the concurrent identification of significant bacteria that cause sub-clinical mastitis and to compare these methods. 200 samples of pooled milk from cows and buffaloes in Rajasthan were gathered between 2020 and 2021.

Methods: The primary bacterial pathogens in milk samples were identified using conventional methods such multiplex PCR, biochemical testing and culture.

Result: From 200 combined samples of cow and buffalo milk, the traditional approach was able to extract 97 different strains. *Staphylococcus aureus* 54 (27%), Streptococcus spp. 30 (15%) and *E. coli* 13 (6.5%) were shown to be prevalent as single or mixed infections, respectively. Staphylococcus was the main pathogen that was discovered. concurrently, *S. aureus*, Streptococcus and *E. coli.* Direct detection of Staphylococcus 65 (32.5%), Streptococcus 37 (18.5%), *E. coli* and 16 (8%) by multiplex PCR was found in milk samples. The analysis revealed that because multiplex PCR assays have higher specificity and sensitivity than conventional procedures, they are more reliable. The multiplex PCR method employed in the current study was a simple and quick technique to identify the major pathogens and it has the potential to be a very helpful tool for determining the pathogens that cause environmental mastitis and evaluating the health of the herd.

Key words: Conventional, Mastitis, Multiplex PCR, Prevalence.

Abbreviation: CMT- California mastitis test, MPCR- Multiplex PCR, PCR- Polymerase chain reaction, SCC- Somatic cell count, SCM- Subclinical mastitis.

INTRODUCTION

Subclinical mastitis is a severe disorder of the dairy sector because there are no obvious alterations in the udder or glandular tissues, subclinical mastitis is a severe problem of the dairy industry. It serves as a constant source of infection for the other herd members. Etiological agents might differ from location to location and case to case depending on the climatic conditions, animal type and disease management techniques. Consequently, mastitis control and prevention remain difficult despite ongoing efforts, which results in significant financial losses for the dairy industry. Controlling the clinical and SCM of dairy cows will benefit from early identification of mastitis with low-cost and quick screening at the field level, sanitary farm management, biosecurity and awareness raising among farmers (Kabir *et al*., 2017). Since the frequency of sub-clinical mastitis is significantly higher in a dairy herd than that of clinical mastitis, it continues to be a mysterious and latent form of the disease that poses a greater economic risk to the dairy livestock industry (Shaheen *et al*., 2016).

Many mastitis-causing organisms have genome sequences that can be used to create nucleic acid-based testing procedures. Compared to other procedures, nucleic acid testing by PCR is excessively sensitive and specific. By amplifying a target gene that is highly conserved within the species but variable between species, bacteria can be identified by PCR at the species level (Hoque *et al*., 2018).

Considering the limits of conventional methods, various molecular typing techniques have been employed to identify bovine mastitis, with DNA-based assessment now enjoying the greatest level of popularity. The main benefit of DNA-based diagnostic analysis is that it focuses on the unique nucleic acid composition of the bacterial genome as opposed to different phenotypic expressions of by-products that encode nucleic acids. The adoption of PCR-based methods provides a favourable alternative for

[1]Department of Veterinary Microbiology, College of Veterinary and Animal Science, Udaipur-313 601, Rajasthan, India.
[2]Department of Livestock Product Technology, College of Veterinary and Animal Science, Udaipur-313 601, Rajasthan, India.

Corresponding Author: Sudeep Solanki, Department of Veterinary Microbiology, College of Veterinary and Animal Science, Udaipur-313 601, Rajasthan, India. Email: sudeepdrsolanki@gmail.com

How to cite this article: Solanki, S. and Devi, D. (2024). Molecular Detection of Some Most Important Pathogenic Bacteria by Multiplex PCR for Diagnosis of Subclinical Mastitis in Bovine. Asian Journal of Dairy and Food Research. 43(3): 418-424. doi: 10.18805/ajdfr.DR-2141.

Submitted: 13-07-2023 **Accepted:** 29-01-2024 **Online:** 12-04-2024

the quick identification of bacteria. Rapid identification PCR protocols have been developed with this technique to recognize several mastitis pathogens (Ajitkumar *et al*., 2012; Ashraf *et al*., 2018; Kurjogi *et al*., 2018; Vatalia *et al*., 2021).

Rapid identification of the pathogen responsible for animal mastitis can support management decisions. The polymerase chain reaction has become available to identify mastitis-causing organisms, offering a rapid and sensitive test (Galvan, 2016).

The polymerase chain reaction has several advantages over phenotypic methods, including direct detection and identification without culturing, rapid turnaround time, high throughput capability, accuracy, reproducibility and sensitivity and/or specificity. However, this method is labor-intensive and it is much more expensive to do a different PCR test for every possible mastitis pathogen. But multiplex PCR using endpoint and real-time methodologies, alone or in combination with other techniques; have been applied to identify multiple pathogens. Hence, the significance of multiplex PCR tests is of interest, in which several pathogens can be tested simultaneously, thereby saving precious time and cost (Elsayed *et al*., 2015; Gaddi *et al*., 2016; Kalin *et al*., 2017; and Machado *et al*., 2018).

Development of multiplex PCR

Multiplex PCR can also improve the detection level due to its higher sensitivity. Theoretically, only a few cells of the pathogen are necessary to yield a positive diagnosis. The presence of pathogens may be detected at earlier stages of infection and in carrier animals when the number of bacteria in milk may be exceptionally low. Multiplex PCR detection and identification tests for subclinical bovine mastitis pathogens that produce results in a day is specific, sensitive and cheap.

The present investigation focused on developing an expeditious detection method, a multiplex Polymerase Chain reaction was designed to allow the concurrent detection of different pathogenic bacteria of mastitis in a single reaction with minimum reagents and limited time than simplex PCR. The multiplex PCR assay proved to be a handy tool to sort the bacterial aetiology of mastitis milk samples simultaneously, like earlier reports by (Phuektes *et al*., 2001; Gillespie *et al*., 2005). The specific Oligonucleotide primers were designed based on a species-specific gene to amplify and identify the bacteria causing mastitis.

Here an effort was made to standardize multiplex polymerase chain reaction for early detection of major bacterial pathogens and timely treatment of disease. Similar types of efforts were also made by multiple scientists across the globe for the detection of bacterial pathogens causing mastitis Dmitriev *et al*., 2006; Shome *et al*., 2011; Hegde *et al*., 2013 and Preethirani *et al*., 2015). Many polymerase inhibitors have been found in milk samples, unlike the PCR investigation performed using DNA extracted method from pure cultures and researchers have initiated several protocols to remove these PCR inhibitors as devised by (Cremonesi *et al*., 2006 and Kim *et al*., 2001). In the present study, to increase PCR sensitivity, the multiplex PCR systems and DNA extraction procedure from milk samples were further optimized.

In the current study, a one-tube multiplex-polymerase chain reaction was standardized to detect major bacterial mastitis pathogens, such as *S. aureus,* Streptococcus and *E. coli.* The one-tube multiplex PCR allowed the simultaneous detection of major bacterial mastitis pathogens using small amounts of reagents and limited time to set up and examine than uniplex PCR, thus making it more acceptable for routine diagnostic uses.

MATERIALS AND METHODS

About two hundred milk samples were collected under aseptic conditions from domesticated dairy Cattle and buffaloes (5-8-year age group) from organized and unorganized dairy farms. Out of these 200 milk samples, 100 milk samples were collected from cows and 100 milk samples were collected from buffaloes. The animal was examined Clinically and the samples of milk were taken following standardized aseptic procedures. These samples were kept on ice and transferred immediately to the laboratory.

Screening for SCM by modified california mastitis test

Screening of the SCM was conducted by modifying the California mastitis test. The CMT was performed and interpreted as described by the previous method. The udders were tested for SCM using the Modified California Mastitis Test and only those milk samples which were found positive for mastitis were used in the study. Somatic cell counts were determined by a Lactoscan milk analyzer (Belgium) according to the technique described by the manufacturer. The SCC value>5,00,000 cells/mL (Hegde *et al*., 2013) milk was taken as a criterion to declare milk/ animal as sub-clinical mastitis/infected and these milk samples were subjected to cultural isolation.

Isolation and biochemical characterization

A total of 74 milk samples based on CMT and SCC were subjected to bacteriological examination for the isolation and identification of bacterial species in the milk samples, the techniques as per standard procedures by (Markey *et al*., 2013) were implemented.

Identification and biochemical analysis

Pure culture isolates were subjected to initial examinations, gram staining and further catalase and oxidase testing. The catalase-positive cultures were streaked on nutrient agar obliques and kept at 4°C for preservation. After that, various secondary biochemical assays were performed on the pure cultures in accordance with industry standards (Markey *et al*., 2013). Using the colony traits of individual primary isolates, the isolated bacteria were identified up to the species level.

Single-tube multiplex PCR

DNA isolation from milk sample

Bacterial genomic DNA was directly isolated from milk samples based on the protocol described by (Phuektes *et al*., 2001) with some modifications in the initial steps. To determine the integrity of the samples of isolated DNA, they were electrophoresed in agarose gel containing ethidium bromide.

The genus-specific genes were chosen as the target for the desired organism for multiplexing-based organism detection. As previously employed by (Hegde, 2011) oligonucleotide primer sequences and matching amplicon sizes were used to identify Staphylococcus at the genus level by targeting the *tuf* gene, Streptococci at the genus level by targeting the *tuf* gene and *E. coli* by targeting the *alr* gene. The particulars of the primers enlisted with their base sequences are given in Table 1. The PCR reaction mixture was prepared and spined briefly and set into an automated thermal cycler (Applied Biosystems). The cycling condition employed was as shown in Table 2.

RESULTS AND DISSCUSSION

Although the frequency of sub-clinical mastitis in dairy herds is far higher than that of clinical mastitis, this form of the disease continues to go undiagnosed and unreported and it represents a more serious financial threat to the dairy livestock business (Shaheen *et al*., 2016).

Even though it is very difficult to assess the cost of SCM, most experts agree that it is more expensive for the average dairy farmer than clinical mastitis. Because DNA-based recognition assays are more accurate and produce results with less variability, they can help solve some of the problems with traditional microbiological methods. As a result, in addition to the conventional approaches like CMT, SCC, isolation and biochemical characterization, this expertise was applied in the current inquiry. Use of more expedient, timely methods of detection, the accuracy and sensitivity of multiplex PCR and polymerase chain reaction were evaluated.

Screening of the milk samples by CMT

A total of 200 milk samples collected from cows and buffaloes from Sirohi district of Southern Rajasthan were subjected to California Mastitis Test (CMT) for screening for subclinical bovine mastitis. Among the 200-milk sample, CMT was found to be positive in 45% (n=90/200) samples.

Screening of milk samples by SCC and incidence of sub clinical mastitis

The collected milk samples were subjected to measurement of SCC for evaluation of subclinical mastitis. According to the result of SCC, milk samples were grouped into six different groups *viz*., 0-1 lakh, 1-2 lakh, 2-3 lakh, 3-4 lakh, 4-5 lakh and >5 lakh cells/mL Overall 20 milk samples showed SCC value of <1 lakh cells/mL, 22 showed 1-2 lakhs cells/mL, 35 showed 2-3 lakhs cells/mL, 28 showed 3-4 lakhs cells/mL, 21 showed 4-5 lakhs cells/mL and 74 milks samples showed SCC >5 lakhs cells/mL SCC values of cows and buffaloes milks samples given separately. A result of the SCC of 200 milk samples indicated SCM at 37% since, 74 out of 200 samples were positive for SCM.

Isolation and characterization of most prevalent microorganism in sub clinical mastitis

In the present study out of 200 milk samples 74 milk samples which showed SCC value >5 lakh cells/mL were cultured for primary isolation of predominant *Staphylococcus aureus*, Streptococci and *E. coli* found positive for presence of bacteria. Out of these 74 positive samples for SCC, 72 samples had bacterial growth and while in 02 samples there was absence of bacterial growth. Out of the 72 samples that showed bacterial colonies, only 40 had single bacterial growth whereas rest of the 32 samples had mixed growth. A total of 97 isolates were

Table 1: Oligonucleotide primer sequences and amplicon sizes.

Bacterial species and their gene	Oligonucleotide primer sequence (5′-3′)	Amplicon size (bp)	Reference
Staphylococcus (Tuf)	GAA GAA TTA TTA GAA TTA GT		
	GTG ATT GAG AAT ACG TCC TCA AC	235	(Hegde, 2011)
Streptococcus (tuf)	CAA CTT GAC GAA GGT CCT GCA		
	TGG GTT GAT TGA ACC TGG TTT A	110	(Hegde, 2011)
E. coli (alr)	CTG GAA GAG GCT AGC CTG GAC GAG		
	AAA ATC GCC ACC GGT GGA GCG ATC	366	(Hegde, 2011)

Table 2: One tube Multiplex PCR for the identification of pathogens.

Stage	Step	Temperature (°C)	Duration	No. of cycles
1.	Initial denaturation	94	5 min	1
2.	Denaturation	94	45s	35
	Annealing	55	1 min	
	Extension	72	1 min	
3.	Final extension	72	10 min	1

recovered from these milk samples. The prevalence of mastitis caused by *Staphylococcus aureus,* (54/200, 27%), *Streptococcus* spp. (30/200, 15%) and *E. coli* (13/200, 6.5%) respectively either as single and or as mixed infections. *S. aureus* (54 isolates), Streptococci spp. (30 isolates, including 21 *S. agalactiae* isolates and 9 additional Streptococcal spp.) and *E. coli* (13 isolates) were phenotypically identified as the 97 recovered isolates from these samples. Although a clear diagnosis of an intra mammary infection can be made by finding a bacterial pathogen in milk from a cow with mastitis using standard microbiological techniques, the procedure is substantially more time-consuming. Usually, it takes longer than 48 hours or more to finish. To get around this, tactful and Sensitive multiplex PCR was used to precisely and quickly identify the primary bacterial pathogens that cause SCM.

Development of multiplex PCR

Multiplex PCR was used to assess the microorganism species using all the genus- and species-specific primers (Table 1). Amplification products and the size of each gene were displayed in (Fig 1a-d). The simultaneous identification of Staphylococcus 65 (32.5%), Streptococcus 37 (18.5%) and *E. coli* 16 (8%) was made possible by multiplexing. However, Choudhary, (2017). evaluated nine different microorganism species using multiplex PCR and found that *S. aureus, S. hyicus, S. agalactiae, S. uberis, E. coli, Klebsiella pneumoniae, Listeria monocytogenus, Mycoplasma bovis* and *Pseudomonas aeruginosa* were all currently detectable. *S. aureus* was found to be present in 62. Similar to this, Shome *et al*. (2011) concurrently identified 10 bacterial strains using multiplex PCR.

Major mastitis pathogens found in several nations were consequently, the standardisation of multiplex polymerase chain reaction assay for side-by-side identification of these pathogens overcomes the cost-effectiveness of conventional uniplex PCR. These pathogens include *S. agalactiae, S. aureus* (contagious pathogens), *S. dysgalactiae, S. uberis* and *E. coli* (environmental pathogen). For each reaction diagnosis, it uses fewer chemicals, such as DNA polymerase. It also requires less setup and research time than other techniques that employ a large number of simplex PCR tubes. By detecting the isolates obtained from the Sub Clinical Mastitis cases in the current experiment, the methodology was approved after standardising the multiplex-polymerase chain reaction utilising reference strains.

Because 21 other bacteria (Table 3) were likely missed by more conventional approaches due to their independent non-viability, multiplex PCR most likely found them. Additionally, the difference in the types of bacteria found in milk could encourage the growth of the dominant species while squelching the lesser ones. In addition, colonies were selected in isolation from other cultures based on phenotypic characteristics like size, colour and form.

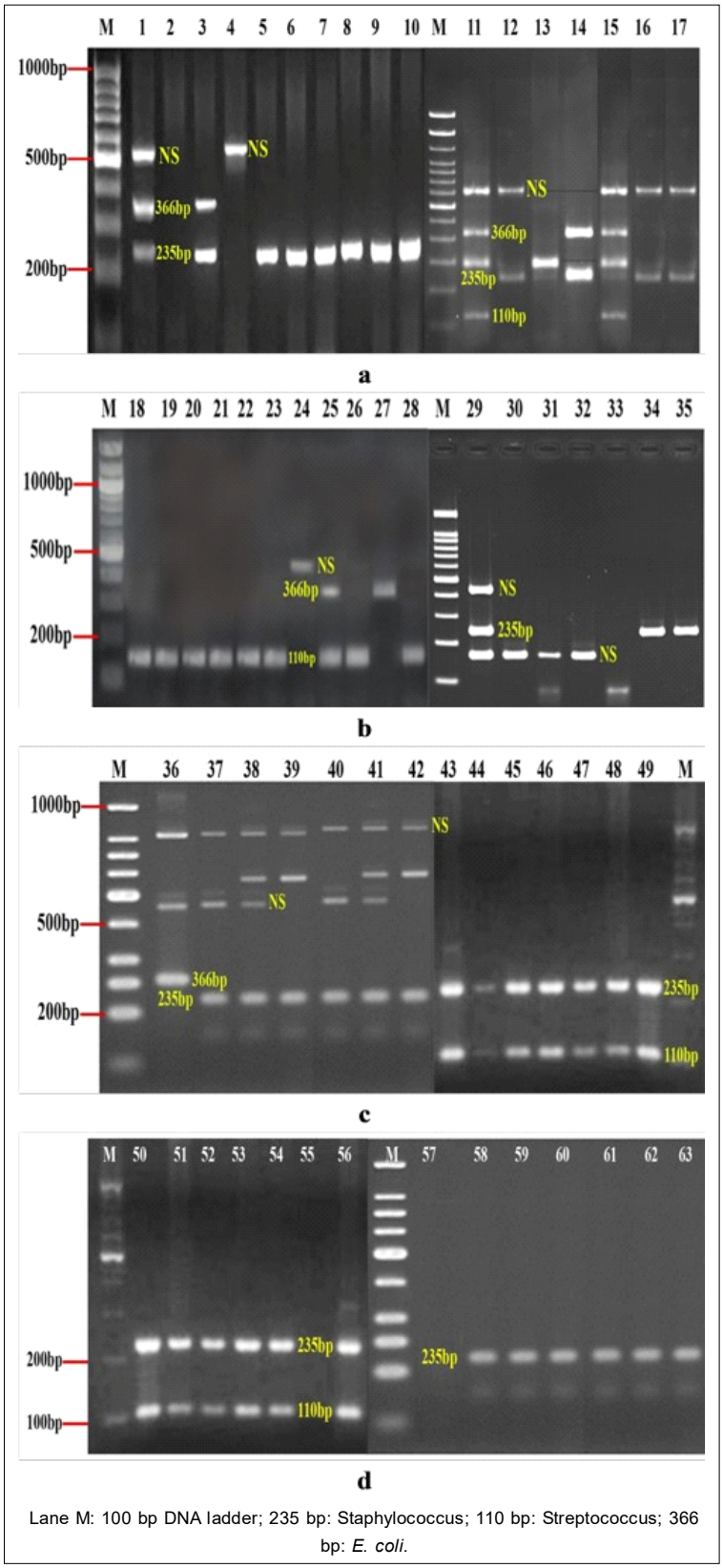
Out of the 200 milk samples utilised in the investigation, 117 isolates for the targeted bacteria were found using multiplex polymerase chain reaction, whereas 97 isolates were found and validated using conventional microbiological methods. The result suggests that the multiplex polymerase reaction is more sensitive than tried-and-true traditional methods. A summary comparison of multiplex PCR and traditional methods is produced and presented in Table 3. Similar multiplex PCR-based detection investigations to the current study have been described and verified by other researchers (Koskinen *et al*., 2010).

The findings showed that for all target species, Comparing the multiplex polymerase reaction methodology to bacterial culture and biochemical test methods, we find that the latter is less sensitive. It has been said that a polymerase chain reaction assay may successfully detect DNAs from both viable and non-viable bacteria, in contrast to a bacterial culture technique, which can only detect and identify viable bacteria. Bacterial DNA can frequently be detected in the udder even after the infection has been treated, which can produce false-positive PCR assay findings (Koskinen *et al*., 2010). The ability to detect *S. uberis* at concentrations below those connected to clinical mastitis highlighted the potential use of this technique for early infection detection or confirmation of effective therapeutic therapy for speedy results. Staphylococcus and other bacteria were identified at the genus level for the current investigation using PCR. Streptococcus. The *Tuf* gene encodes the elongation factor Tu (EF-Tu) (Martineau *et al*., 2001; Picard *et al*., 2004). Elongation factor-Tu is a GTP-binding protein required for protein synthesis. The tuf gene-based PCR was utilised to identify the Staphylococcus isolates at the genus level and all of the Staphylococcus isolates yielded a 235 bp product that was amplified.

The tuf-based PCR assay for staphylococcal species identification has previously shown to have excellent sensitivity and specificity and it can be modified for the direct

Table 3: Comparison of multiplex PCR (mPCR) with the conventional method.

Organism	Phenotypic determination		Genotypic determination	
	No of isolates	Prevalence	No of isolates	Prevalence
Staphylococcus	54	27%	65	32.5%
Streptococcus	30	15%	37	18.5%
E. coli	13	6.5%	16	8%
Total isolates	97	48.5%	118	59%
Sensitivity	Less		More	



Lane M: 100 bp DNA ladder; 235 bp: Staphylococcus; 110 bp: Streptococcus; 366 bp: *E. coli.*

Fig 1: Multiplex PCR for simultaneously detecting Staphylococcus, Streptococcus and *E. coli* directly from DNA isolated through bovine milk sample.

detection of staphylococci from contaminated blood or from clinical specimens that are typically sterile, like blood or urine (Martineau *et al*., 2001). The results of the current analysis confirm earlier findings that Staphylococcus may be recognised using *tuf* gene-based PCR at the genus level.

By boosting a 110 The tuf gene-based Polymerase chain reaction has been standardised for the identification of Streptococcus isolates at the level of the genus in the current experiment. bp product from each of the 30 Streptococcal isolates. Picard *et al*. (2004). drew the phylogenetic tree using the same gene.

CONCLUSION

Multiplex PCR assay is superior diagnostic tool for detection of major mastitic bacterial pathogens directly from milk samples as compared to conventional methods and mPCR has higher sensitivity and specificity with 100% negative predictive value which signifies its importance in epidemiological investigations with shorter test time frame. Early and accurate diagnosis using recent molecular methods like multiplex PCR can be of immense significance in framing the strategy for prevention and control of bovine mastitis in days to come.

ACKNOWLEDGEMENT

We are highly grateful to the Dean, College of Veterinary and Animal Sciences and Department of Veterinary Microbiology for the support provided during the whole investigation and study.

Author's contributions

Dr. Sudeep Solanki, participated in the Conceptualization, Formal analysis, Investigation, Dr. Durga Devi participated in the Formal analysis, Investigation and Writing-of the original draft of the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no competing interests.

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