

## MULTIPLEX PCR ASSAY FOR DETECTING COMMON BACTERIAL PATHOGENS OF MASTITIS IN MILK AND TISSUE SAMPLES OF BUFFALOES

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**Abstract:** The present study was undertaken to identify the seven common bacterial pathogens of mastitis viz., *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* (*S. epidermidis*), *Staphylococcus chromogenes* (*S. chromogenes*), *Streptococcus agalactiae* (*S. agalactiae*), *Streptococcus dysgalactiae* (*S. dysgalactiae*), *Streptococcus uberis* (*S. uberis*) and *Escherichia coli* (*E. coli*) simultaneously in the milk and tissue samples of mastitis affected buffaloes collected from Kolar and Chikkaballapur districts, Karnataka, India by Multiplex PCR (m-PCR) based microorganism detection technique. A total of 65 milk (50 SCM, 15 CM) and 61 tissue (36 SCM, 25 CM) samples were collected from mastitis affected buffaloes were used to identify seven common bacterial pathogens by m-PCR. Milk samples were screened for subclinical mastitis (SCM) by California mastitis test (CMT). Based on m-PCR results, *S. aureus* was found to be the predominant bacteria detected from milk and tissue samples followed by *E. coli*, *S. agalactiae*, *S. epidermidis*, *S. chromogenes*, *S. dysgalactiae* and *S. uberis*. Eight milk and seven tissue samples were negative for seven targeted bacterial species and also 10 milk and 9 tissues were positive for mixed infection consisting of more than one bacterial species. Present study supported the fact that m-PCR based identification of mastitis causing pathogens from milk is a rapid and reliable method to reveal the exact bacterial etiology of mastitis.

**Key words:** Subclinical mastitis, Bacterial pathogens, Buffaloes milk

### INTRODUCTION

Mastitis is the most common infectious disease affecting dairy cows. It is the most important cause of economic losses to the dairy industry in India and throughout the world [1]. The losses are due to reduced milk production, production of low quality milk, cost of drugs and veterinary services, increased culling rate and reduced reproductive efficiency [2].

The major cause of bovine mastitis is the infection

of the udder by pathogenic bacteria. At present it is established that mastitis is caused by over 250 different contagious, environmental and miscellaneous microorganisms [3], of these the most common pathogens associated with mastitis occurrences are *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* (*S. epidermidis*), *Streptococcus agalactiae* (*S. agalactiae*), *Streptococcus dysgalactiae* (*S. dysgalactiae*) and *E. coli* [4]. The pathogens responsible for the mastitis must be identified rapidly and accurately in order to monitor

and control the infections in the dairy herd. Although conventional bacterial and biochemical tests could identify the microbial pathogens responsible for mastitis; they are time consuming, laborious and not highly specific [5].

Due to the limitations of cultural methods, the development of PCR-based methods provides a promising option for the rapid identification of bacteria. With these methods, identification of bacterial pathogens can be made in hours, rather than the days required for conventional cultural methods. PCR can also improve the level of detection due to its high 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 numbers of bacteria in milk may be very low [6]. The aim of this study was to develop Multiplex PCR assay for the simultaneous detection of seven common bacterial pathogens in the milk and tissues samples of mastitis affected buffaloes.

## MATERIALS AND METHODS

**Collection of Samples:** Milk samples were collected aseptically from a total of 65 mastitis affected buffaloes from seven taluks of Kolar and Chikkaballapur districts viz., Srinivasapur, Kolar, Mulbagal, Malur, Bangarpet, Chintamani and Sidlaghatta. Before sample collection udder and teats were washed with 0.1 per cent potassium permanganate solution, teat and teat orifices wiped with tissue paper and scrubbed with 70 per cent ethanol. Approximately 30 ml fore milk sample was collected from each quarter in sterile crew-capped plastic container and transported to the laboratory on ice. Tissue samples were collected aseptically from a total of 61 mastitis affected buffaloes from Kolar and Bengaluru slaughter houses. Milk samples collected from buffaloes were screened for SCM by CMT.

**California Mastitis Test (CMT):** Mastitis reagent obtained from DeLaval Pvt. Ltd. Pune, Maharashtra, was used to perform CMT according to the procedure described by Schalm et al. [7]. An equal quantity of sample milk was mixed with the mastitis reagent in the wells of strip cup. The results were recorded as positive when it resulted in thickening/clot/ gel formation within 60 seconds.

**Reference strains:** Bacterial cultures of above pathogens as a control sample were procured from National Institute of Veterinary Epidemiology and Disease informatics, Bengaluru, Department of Veterinary Microbiology, KVAFSU, Veterinary College, Bengaluru and Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh.

**Isolation of Bacterial DNA:** Bacterial DNA were extracted from reference strains, milk and tissue samples using as per the standard protocol of “QIAamp DNA Minikit” procured from Qiagen, Inc., Chatsworth, CA. Extracted DNA from reference strains was used as a control template for standardization of m-PCR. The purity and concentration of the extracted genomic DNA was estimated by UV spectrophotometry. The bacterial preparation was checked for quality by running on 0.8 per cent agarose gel in 1.0X TAE (Tris 40 mM, Acetic acid 20 mM, EDTA 1 mM) buffer (pH 8.3).

### Standardization of three tube multiplex PCR:

The initial standardization of three tube m-PCR for simultaneous detection of seven common bovine mastitis causing bacterial pathogens were carried out using extracted DNA from reference strains. The PCR conditions and primers designed by Sundareshan [8]; Hegde and Isloor [9] were used for PCR studies. Tube-I containing specific primers *sip* and *pau A* to detect *S. agalactiae* and *S. uberis*, tube-II containing three specific primers 16S rRNA *dys*, *alr* and *nuc* to detect *S. dysgalactiae*, *E. coli* and *S. aureus* and tube-III containing specific primers *sod A* and *rpo B* to detect *S. chromogenes* and *S. epidermidis* respectively was used. The reaction mixture of 25  $\mu$ l prepared in 0.2 ml thin walled PCR tubes, on ice, in each of the three-tubes comprised of the following reagents:

A). Each 25  $\mu$ L of the PCR mixture in tube-I comprised of 2.5  $\mu$ L of 10X PCR *Taq* Buffer A, 1  $\mu$ L (100  $\mu$ M) of each dNTPs, 0.2  $\mu$ L (1U) of *Taq*, DNA polymerase, 0.25  $\mu$ L (5pm) each of Strep *sip*-F and Strep *sip*-R and 0.5  $\mu$ L each of Strep *pau*-F and Strep *pau*-R primers, 3  $\mu$ L each of *S. agalactiae* and *S. uberis* DNA template. NFW was added to make a final volume of 25  $\mu$ L.

B). Each 25  $\mu$ L of the PCR mixture in tube-II comprised of 2.5  $\mu$ L of 10X PCR *Taq* Buffer A, 1  $\mu$ L (100  $\mu$ M) of each dNTPs, 0.2  $\mu$ L (1U) of *Taq*,

DNA polymerase, 0.375  $\mu\text{L}$  (7.5pm) each of *alr*-F and *alr*-R primers; 0.5  $\mu\text{L}$  (10pm) each of *nuc*-F and *nuc*-R primers; 0.25  $\mu\text{L}$  (5pm) each of 16S *dys*-F and 16S *dys*-R primers and 3  $\mu\text{L}$  each of *E. coli*, *S. aureus* and *S. dysgalactiae* DNA templates. NFW was added to make a final volume of 25  $\mu\text{L}$ .

C). Each 25  $\mu\text{L}$  of the PCR mixture in tube-III comprised of 2.5  $\mu\text{L}$  of 10X PCR *Taq* Buffer A, 1  $\mu\text{L}$  (100  $\mu\text{M}$ ) of each dNTPs, 0.5  $\mu\text{L}$  (1U) of *Taq*, DNA polymerase, 1.0  $\mu\text{L}$  (10pm) each of Schrom-sodA-F and Schrom-sodA-R and 1.0  $\mu\text{L}$  each of Sepi-rpoB-F and Sepi-rpoB-R primers, 3  $\mu\text{L}$  each of *S. chromogenes* and *S. epidermidis* DNA template. NFW water was added to make a final volume of 25  $\mu\text{L}$ .

A pre-PCR step at 94°C for 5 min was applied. A total of 30 PCR cycles were run under the following conditions: denaturation at 94°C for 30s, annealing 57°C for 30s and extension at 72°C for 30s. After the final cycle, the preparation was kept at 72°C for 10 min to complete the reaction. The PCR products were stored in the thermal cycler at 4°C until they were collected.

## RESULTS

The three-tube m-PCR was successfully standardized (Fig. 1) and the desired amplicons were obtained in all the three tubes. Positive reference cultures used in tube-1 yielded a band size of 266 bp and 439 bp amplicons specific for *S. agalactiae* and *S. uberis* respectively. The tube-2 yielded 366 bp, 181 bp and 549 bp amplicons specific for *E. coli*, *S. aureus* and *S. dysgalactiae* respectively. Similarly, the tube-3 yielded 303 bp and 466 bp amplicons specific for *S. chromogenes* and *S. epidermidis* respectively. All the bands were well differentiated on gel. However, when the ambiguity was noticed for any detected bacterial species with respect to the outcome of PCR, it was confirmed by repeating m-PCR.

Out of 65 mastitic milk samples collected, 50 were from subclinical and 15 were from clinical cases. Multiplex-PCR results of 50 subclinically mastitic milk samples revealed *S. aureus* (24%) to be the predominant bacteria, followed by mixed infection consisting of more than one bacteria (16%), *E. coli* (14%), *S. agalactiae* (10%), *S. epidermidis* (10%), *S. chromogenes* (6%), *S. dysgalactiae* (4%), *S. uberis*

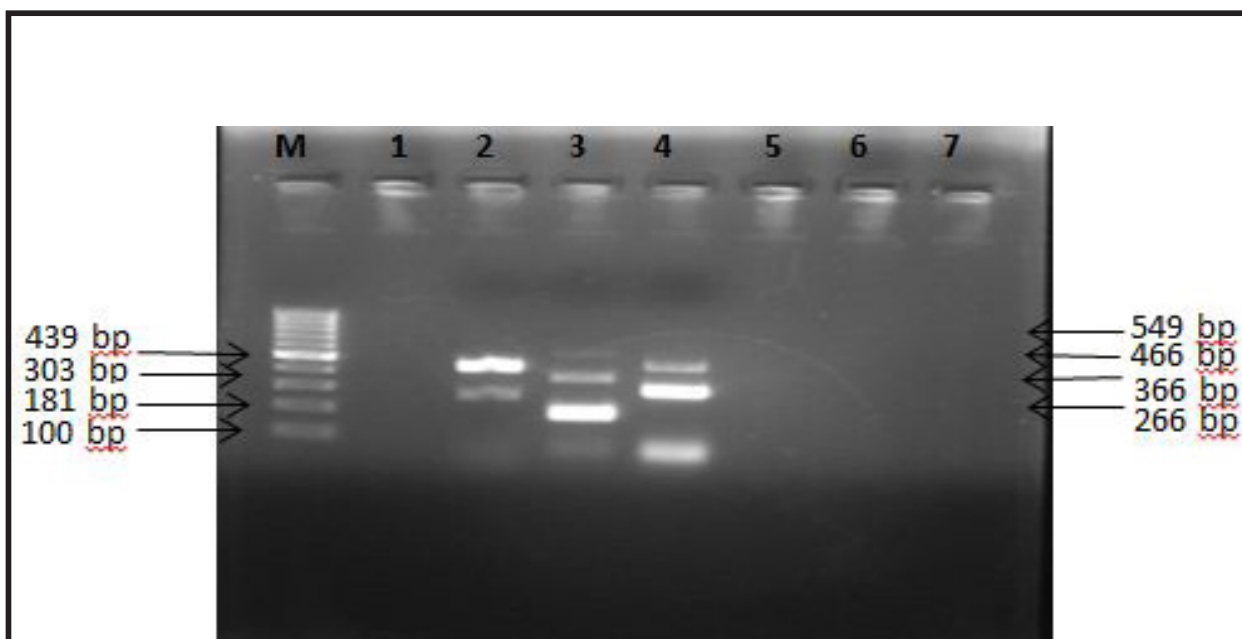
(4%) and no organisms were detected in 12% of the total samples screened. On the other hand, the m-PCR results of 15 milk samples of clinical cases also revealed *S. aureus* (20%) to be the predominant bacteria, which was followed by *S. agalactiae* (20%), mixed infection (13.34%), *E. coli* (13.33%), *S. epidermidis* (13.33%), *S. chromogenes* (6.67%) and 13.33% cases were found to be free from the targeted seven bacteria (Table 1 and Fig. 2).

Similarly, out of 61 collected mastitic tissue samples, 36 were from subclinical and 25 were from clinical cases. Multiplex-PCR results of 36 subclinically mastitic tissue samples revealed *S. aureus* (22.22%) to be the predominant bacteria identified, followed by *E. coli* (13.89%), mixed infection (13.9%), *S. agalactiae* (11.11%), *S. epidermidis* (11.11%), *S. chromogenes* (5.56%), *S. dysgalactiae* (5.56%), *S. uberis* (5.56%) and 13.9% cases were negative for the screened bacteria. On the other hand, the m-PCR results of 25 tissue samples of clinical cases revealed the presence of *S. aureus* in 24% cases followed by *E. coli* (16%), mixed infection (16%), *S. agalactiae* (12%), *S. epidermidis* (8%), *S. dysgalactiae* (8%), *S. chromogenes* (4%) and no organism was detected in 12% of the total samples screened (Table 1 and Fig. 3).

## DISCUSSION

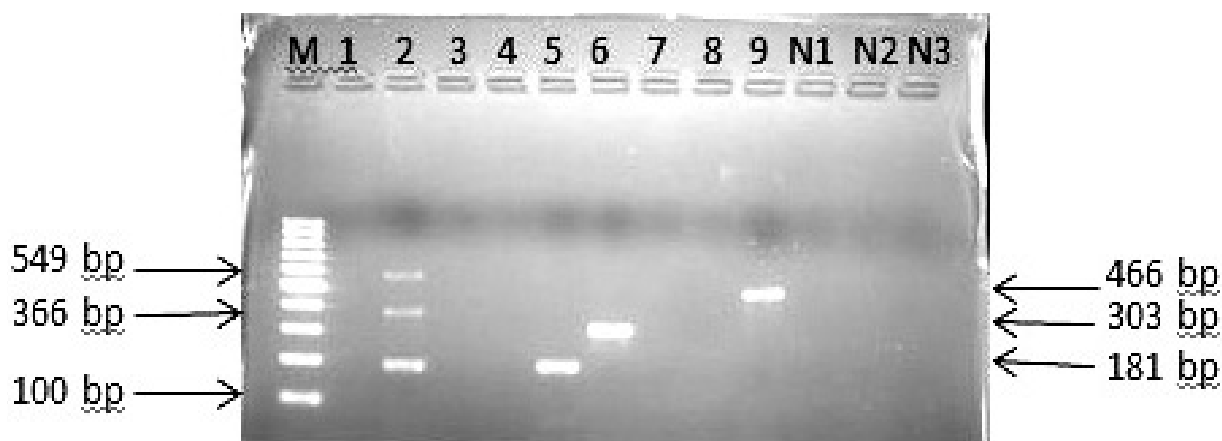
Prevention as a control measure of mastitis in dairy cattle needs sensitive, rapid and specific tests to identify the main bacteria that cause heavy losses in the dairy industry. Conventional procedures for the identification of mastitis pathogens are labor-intensive and most of the commercial identification systems are not designed to identify important veterinary pathogens [10,11]. The use of PCR detection and identification tests for mastitis pathogens that produced results in 1 day are specific, sensitive and cheap [12].

For devising an m-PCR assay, it was essential that the amplicons besides being specific should differ in length by at least 40–50 bp from each other so that they are clearly distinguishable after agarose gel electrophoresis. It was not possible to identify suitable primers for all seven species in a single PCR reaction and so a three-tube format was chosen. The evaluation of primers with reference strains, unambiguously confirmed the accuracy of



**Fig. 1:** Standardized three tube multiplex PCR.

Lane M: 100 bp DNA ladder, Lane 2: Amplification of *sip* (266 bp) and *pau A* (439 bp)-Tube 1, Lane 3: Amplification of *nuc* (181 bp), *alr* (366 bp) and 16S rRNA dys (549 bp)-Tube 2, Lane 4: Amplification of *Sepi-rpoB* (466 bp) and *Schrom-sodA* (303 bp)-Tube 3.



**Fig. 2:** Screening of milk samples processed for isolation by m-PCR.

Lane M: 100 bp DNA ladder, Lane 1, 3, 4, 7 and 8: Negative for targeted seven bacteria, Lane 2: positive for *S. aureus* (181 bp), *E. coli* (366 bp) and *S. dysgalactiae* (549 bp), Lane 5: positive for *S. aureus* (181 bp), Lane 6: positive for *S. chromogenes* (303 bp), Lane 9: positive for *S. epidermidis* (466 bp), Lane N1: No template control for tube-1, Lane N2: No template control for tube-2 and Lane N3: No template control for tube-3.

**Table 1:** Number of bacterial pathogen identified from clinical and subclinical mastitis affected buffaloes by multiplex-PCR. Note: Figures in the parenthesis are the percentage of occurrence of bovine mastitis causing pathogens.

Sl. No	Species	No. of positive species detected by m-PCR					
		MILK samples			TISSUE samples		
		SCM (n=50)	CM (n=15)	Total (n=65)	SCM (n=36)	CM (n=25)	Total (n=61)
1.	<i>S.agalactiae</i>	5 (10.00%)	3 (20.00%)	8(12.30%)	4 (11.11%)	3 (12.00%)	7(11.48%)
2.	<i>S.uberis</i>	2 (4.00%)	-	2(3.08%)	2 (5.56%)	-	2(3.28%)
3.	<i>E.coli</i>	7 (14.00%)	2 (13.33%)	9(13.85%)	5 (13.89%)	4 (16.00%)	9(14.75%)
4.	<i>S.aureus</i>	12 (24.00%)	3 (20.00%)	15(23.08%)	8 (22.22%)	6 (24.00%)	14(22.95%)
5.	<i>S.dysgalactiae</i>	2 (4.00%)	-	2(3.08%)	2 (5.56%)	2 (8.00%)	4(6.56%)
6.	<i>S. chromogenes</i>	3 (6.00%)	1 (6.67%)	4(6.15%)	2 (5.56%)	1 (4.00%)	3(4.92%)
7.	<i>S.epidermidis</i>	5 (10.00%)	2 (13.33%)	7(10.77%)	4 (11.11%)	2 (8.00%)	6 (9.84%)
8.	<i>S.agalactiae + S.uberis</i>	1 (2.00%)	1 (6.67%)	2(3.08%)	1 (2.78%)	-	1(1.64%)
9.	<i>E.coli +S.dysgalactiae</i>	2 (4.00%)	-	2(3.08%)	-	2 (8.00%)	2(3.28%)
10.	<i>S.dysgalactiae+ S.aureus</i>	1 (2.00%)	-	1(1.54%)	-	1 (4.00%)	1 (1.64%)
11.	<i>S.epidermidis+ S.agalactiae</i>	-	1 (6.67%)	1(1.54%)	2 (5.56%)	-	2(3.28%)
12.	<i>S.epidermidis+ S.uberis + S.agalactiae</i>	1 (2.00%)	-	1(1.54%)	-	-	-
13.	<i>S.chromogenes+S.aureus + E. coli</i>	2 (4.00%)	-	2(3.08%)	2 (5.56%)	-	2(3.28%)
14.	<i>S.chromogenes+ E.coli + S.dysgalactiae+ S.aureus</i>	1 (2.00%)	-	1(1.54%)	-	1 (4.00%)	1(1.64%)
15.	No bacteria	6 (12.00%)	2 (13.33%)	8(12.30%)	4 (11.11%)	3 (12.00%)	7(11.48%)
Total		50	15	65	36	25	61

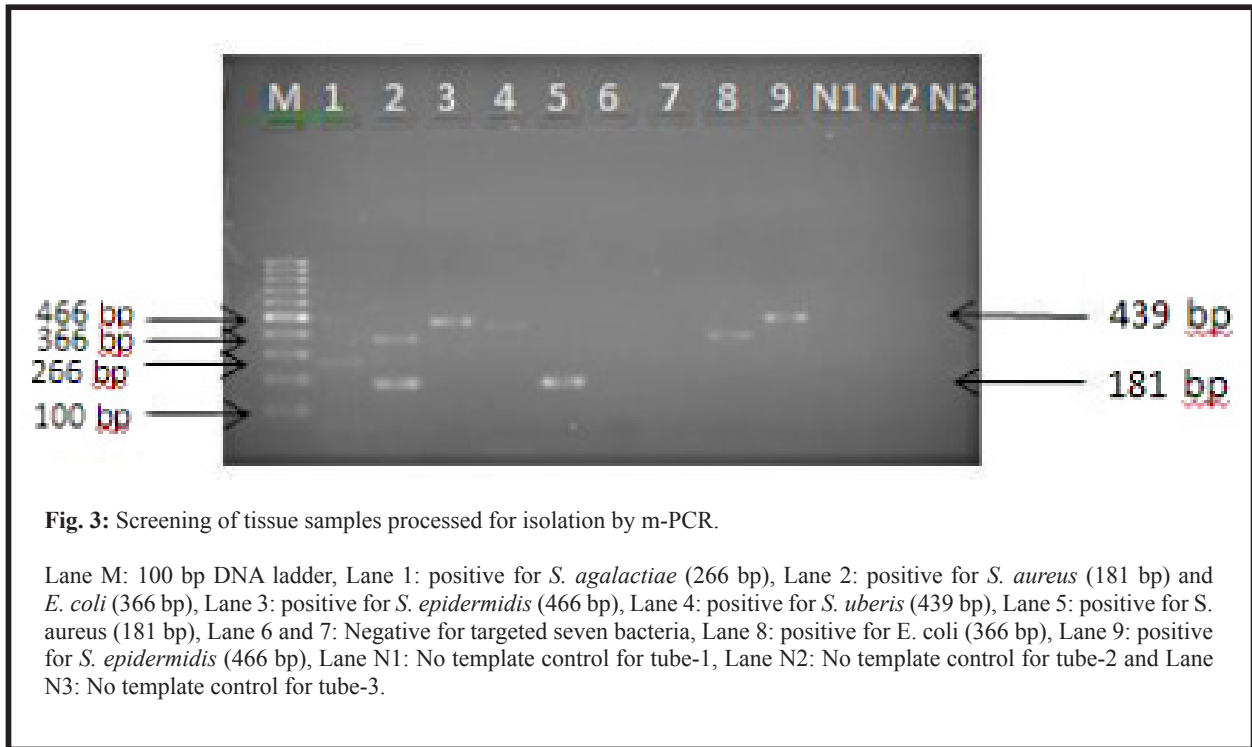
the primers employed in the assay, thus reducing the likelihood of any kind of misidentification [13].

*S. aureus* and *S. agalactiae* were encountered in a higher percentage in the examined milk and tissue samples. This finding is similar to Lafi et al. [14]. However, it disagrees with Hillerton et al. [15] and Sargeant et al. [16], who mentioned that these two bacteria are currently classified as causes of subclinical rather than clinical mastitis. This disagreement can be attributed to the poor mastitis control measures applied in some dairy farms. Nevertheless, *S. aureus* and *S. agalactiae* are considered as significant organisms associated with

clinical and subclinical bovine mastitis worldwide [17,18] due to persistent cow to cow spread, possibly via milking machines and perhaps by the hands of milkers [19]. Their main reservoirs are infected quarters [20]. In addition, *S. aureus* can also be isolated from the skin of the udder and teats and from many other sites in dairy cows as well as feed and caretakers [21].

*E. coli* was also encountered in a higher percentage in the examined milk and tissue samples. This agrees with the findings of many investigators [22], who considered *E. coli* organisms as major etiological agents of clinical mastitis, opportunistic





**Fig. 3:** Screening of tissue samples processed for isolation by m-PCR.

Lane M: 100 bp DNA ladder, Lane 1: positive for *S. agalactiae* (266 bp), Lane 2: positive for *S. aureus* (181 bp) and *E. coli* (366 bp), Lane 3: positive for *S. epidermidis* (466 bp), Lane 4: positive for *S. uberis* (439 bp), Lane 5: positive for *S. aureus* (181 bp), Lane 6 and 7: Negative for targeted seven bacteria, Lane 8: positive for *E. coli* (366 bp), Lane 9: positive for *S. epidermidis* (466 bp), Lane N1: No template control for tube-1, Lane N2: No template control for tube-2 and Lane N3: No template control for tube-3.

environmental or enteric pathogens and the infected quarter could possibly serve as a reservoir for recurrent episodes of clinical mastitis [23].

The results of this study also showed *S. agalactiae* to be the predominant *Streptococci* among the generally identified *Streptococcal* species associated with bovine mastitis. Our findings are in line with the findings of Nithinprabhu et al. [24] who also reported higher prevalence of *S. agalactiae*, *S. dysgalactiae* and *S. uberis* in subclinical and clinical mastitis infected milk samples.

*S. epidermidis* was found to be the predominant bacteria identified among Coagulase-negative staphylococci (CoNS) followed by *S. chromogenes*, which is in accordance with the observation of Sundareshan et al. [25] and Shome et al. [13] who also identified highest prevalence of *S. epidermidis* and *S. chromogenes* in subclinical and clinical mastitis infected milk samples.

An m-PCR was used to allow the detection of multiple clinical pathogens in a single reaction using smaller amounts of reagents and less time [5] and also PCR detects living and dead organisms, since it is based on detection of organism DNA, while culture detects only living organisms. PCR assays could detect fewer numbers of organisms per

milliliter of milk than could be detected by direct culture [26].

One of the problems often encountered with multiplex PCR is a reduction in sensitivity than compared with simplex PCR. This may be due to the competition between individual reactions for dNTPs and Taq polymerase when multiplex primer sets are combined in a single reaction [27].

The correct species identification is important for mastitis treatment, prevention, control and in epidemiological investigations, as well as in understanding of the significance of infections caused by different bacterial species, this assay would prove to be an adequate tool for the identification of the most common mastitis pathogens, independent of their phenotypic characteristics for diagnosis of clinical mastitis as well as continuous monitoring of subclinical mastitis in dairy sector. In conclusion, the developed m-PCR assay can be used for rapid, sensitive, specific and reliable identification of the seven common mastitis pathogens from milk and tissues.

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### REFERENCES

- [1] Miller, R.H., Paape, M.J., Fulton, L.A. and Schutz, M.M.: J. Dairy Sci., 76: 728-733 (1993).
- [2] Barker, A.R., Schrick, F.N., Lewis, M.J., Dowlen, H.H. and Oliver, S.P.: J. Dairy Sci., 81: 1285-1290 (1998).
- [3] Bhuvana, M. and Shome, B. R.: Etiology of Bovine Mastitis. *Proceedings of Model training course on "Bovinemastitis: theoretical and practical consideration in management."* PD\_ADMAS. Bangalore, pp 7-10 (2013).
- [4] Anon: Veterinary investigation surveillance report. London: Veterinary Laboratories Agency (2011).
- [5] Phuektes, P., Mansell, P.D. and Browning, G.F.: J. Dairy Sci., 84: 1140-1148 (2001).
- [6] Brikenmeyer, L.G. and Mushahwar, I.K.: J. Virological Methods., 35: 117: 126 (1991).
- [7] Schalm, O.W., Carrol, E.J. and Jain, N.C.: Factors involved in infection and mastitis. In: *Bovine mastitis*. (Lee and Febiger, I. Eds.), Philadelphia, USA, pp 139 (1971).
- [8] Sundareshan, S.: *Studies on phenotypic and molecular characterization of coagulase negative Staphylococci isolated from clinical and subclinical cases of bovine mastitis*. Ph.D Thesis, Karnataka Veterinary, Animal and Fisheries Sciences, University, Bidar, Karnataka, India (2012).
- [9] Hegde, R. and Isloor, S.: Trends in diagnosis of bovine mastitis. *Proceedings of Model training course on "Bovine mastitis: theoretical and practical consideration in management."* PD\_ADMAS. Bangalore pp 15-24 (2013).
- [10] Watts, J.L.: J. Clin. Microbiol., 27: 1008-1010 (1989).
- [11] Jayarao, B.M., Dore, J.J., Baumbach, G.A., Matthews, K.R. and Oliver, S.P.: J. Clin. Microbiol., 29: 2774-2778 (1991).
- [12] Khan, M.A., Kim, A.C., Kakoma, I., Morin, E., Hansen, R.D., Hurley, W.L., Tripathy, D.N. and Baek, B.K.: Am. J. Vet. Res., 59: 807-813(1998).
- [13] Shome, B.R., Dasmitra, S., Bhuvana, M., Krithiga, N., Velu, D., Shome, R., Isloor, S., Barbuddhe, S.B. and Rahman, H.: J. Appl. Microbiol., 111: 1349-1356 (2011).
- [14] Lafi, S.Q., Al-Rawasheh, O.F., Ereifej, K.I. and Hailat, N.Q.: Vet. Med., 18: 89-98 (1994).
- [15] Hillerton, J.E., Bramley, A.J., Staker, R.T. and McKinnon, C.H.: J. Dairy Res., 62: 39-50 (1995).
- [16] Sargeant, J.M., Scott, H.M., Leslie, K.E., Ireland, M.J. and Bashirl, A.: Can. Vet. J., 39: 33-38 (1998).
- [17] Bramley, A.J.: Mastitis. In: Andrews A.H.R.W. Blowey, H. Boyd and R.G. Eddy (Eds.). *Bovine Medicine: Diseases and Husbandry of cattle*. Blackwell Scientific Publication, Boston (1992).
- [18] Su, C., Kanevsky, I., Jayarao, B.M. and Sordillo, L.M.: Phylogenetic relationships of *Staphylococcus aureus* from bovine mastitis based on coagulase gene polymorphism. Vet. Microbiol., 71: 53-58 (2000).
- [19] Myllys, V., Ridell, J., Bjorkroth, I., Biese. and Pyorala, S.H.K.: Vet. Microbiol., 51: 245-251 (1997).
- [20] Rivas, A.L., Gonzales, R.N., Wiedmann, M., Bruce, J.L., Cole, E.M., Bennett, G.J., Schulte, H.F., Wilson, D.J., Mohammed, H.O. and Batt, C.A.: Am. J. Vet. Res., 58: 482-487 (1997).
- [21] Larsen, H.D., Sloth, K.H., Elsberg, C., Enevoldson, C., Pedersen, L.H., Eriksen, N.H.R., Aarestrup, F.M. and Jensen, N.E.: Vet. Microbiol., 71: 89-101 (2000).
- [22] Dopfer, D., Barkema, H.W., Lam, G.M., Schukken, Y.H. and Gaastra, W.: J. Dairy Sci., 82: 80-85 (1999).
- [23] Dopfer, D., Almeida, R.A., Lam, G.M., Nederbragt, H., Oliver, S.P. and Gaastra, W.: Vet. Microbiol., 74: 331-343 (2000).
- [24] Nithinprabhu, K., Isloor, S., Hegde, R., Rathnamma, D., Veeregowda, B.M., Narasimhamurthy, H. N., Shome, R. and Suryanarayana, V.V.S.: Ind. J. Biotechnol., 12: 208-212 (2013).
- [25] Sundareshan, S., Isloor, S., Haribabu, Y., Awati, B., Hegde, R., Suguna, R., Waryah, C.B., Gogoi-tiwari, J., Mukkur, T.K. and Hegde, N.R.: Ind. J. Comp. Microbiol. Immunol. Infect. Dis., 35(2): 73-78 (2014).
- [26] Gopinath, S.M., Pranayapradhan, Krishankumar. and Joshi, R.R.: The Experiment., 4 (4): 253-264 (2012).
- [27] Madico, G., Quinn, T.C., Boman, J. and Gaydos, C.A.: J. Clin. Microbiol., 38: 1085-1093 (2000).