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APPLICATION OF PCR BASED TECHNIQUE FOR DETECTION OF COMMON MASTITIS PATHOGENS IN MILK SAMPLES OF HF CROSSBRED CATTLE

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ABSTRACT
Present study was undertaken to identify the incidence rate for five major pathogens *viz*., *Staphylococcus aureus* (*S. aureus*), *Staphylococcus epidermidis* (*S. epidermidis*), *Streptococcus agalactiae* (*S. agalactiae*), *Streptococcus dysgalactiae* (*S. dysgalactiae*) and *Escherichia coli* (*E. coli*) in the milk samples collected from Bangalore and Kolar district by PCR based microorganism detection technique. A total of 214 composite milk samples were screened by CMT and then PCR based detection of pathogens for the samples was carried out. Results revealed presence of *S. aureus* in 28.5 per cent of the total animals screened, *S. epidermis* was identified in 15.42 per cent cases. *E. coli* followed and the incidence rate was 13.55 per cent with regard to *E. coli*. *S. agalactiae* and *S. dysgalactiae* were identified in 10.28 per cent and 3.74 per cent of the cases respectively. None of the pathogens considered in this study were detected in 117 milk samples, these samples were also observed to be normal for CMT. Present study supported the fact that PCR based identification of mastitis causing pathogens from milk is a rapid and reliable method to reveal the exact bacterial etiology of mastitis.

KEYWORDS: Mastitis, PCR, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Escherichia coli*

INTRODUCTION

Mastitis is the most imperative economic problem faced by dairy producers all over the world. Inflammation of the mammary gland is called as mastitis. It may be of infectious or non-infectious origin. At present it is established that mastitis is caused by over 250 different contagious, environmental and miscellaneous microorganisms (Bhuvana and Shome, 2013), 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* (Anon, 2011). In most clinical laboratories, identification methods are based on microbiological culture of milk. The method of culture examination is however less sensitive and time consuming (Hegde and Isloor, 2013). Thus, PCR based diagnostic method to identify various mastitis causing pathogens is a rapid sensitive and reliable method to resolve bacterial etiology of mastitis milk samples (Forsman *et al.*, 1997 and Khan *et al.*, 1998, Phuektes *et al*., 2001, Phuektes *et al*., 2003, Shome *et al*,. 2011, Shome *et al*,. 2012). With this background, present study was undertaken for PCR based detection of 5 common pathogens in the milk samples collected in the area around Bangalore and Kolar district.

MATERIALS AND METHODS

Collection of Samples

Milk samples were collected aseptically from a total of 215 animals from nine packages of areas around Bangalore *viz*., Adugodi, Rajankunthe, Shivajinagar, Thondebavi, Kengeri, Pantur, Frezer Town, Hessargatta and Kolar dist. 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 foremilk sample was collected from each quarter in sterile screw-capped plastic container and transported on ice to the laboratory to conduct bacteriological examination.

California Mastitis Test (CMT) examination of samples

After cleaning with alcohol small amount of fore milk (3 ml) was squirted from each quarter into the appropriate quadrant of the paddle. Equal ratio of reagent was mixed to milk and observations were recorded base on amount of reaction and gel formation.

Control strains

Bacterial cultures of above pathogens as a control sample were procured from Project Directorate on Animal Disease Monitoring and Surveillance (PD_ADMAS), Hebbal, Bangalore. DNA was extracted from the bacterial culture by AMpurE Bacterial Genomic DNA Mini isolation kit (supplied by Amnion Biosciences). Extracted DNA was used as a control template for conducting PCR.

Isolation of Bacterial DNA from Milk

In this methodology 30 ml of milk sample was strained to remove milk somatic cells and further centrifuged at 1000xg for 15 min to discard the fat layer. DNA from the pellet containing bacterial cells was extracted by modified Proteinase-K method (Tarate, 2012). The bacterial

preparation was checked for quality by running on 0.8 per cent agarose gel in 0.5X TBE (Tris 45 mM, Boric acid 45 mM, EDTA 1 mM) buffer (pH 8.0).

PCR amplification for identification of specific region of bacteria

Primers reported by Shome *et al*., 2011 were used to identify and amplify the region of interest for 5 bacterial species viz. *Staphylococcus aureus*, *Staphylococcus epidermis*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Escherichia coli*. PCR was performed in a Master Cycler (Eppendorf). All reactions were carried out in a final reaction volume of 25 µl. Volumes of 2 µl of extracted DNA template *i.e.,* bacterial preparation, 10 pmol primer, 1 U of *Taq* DNA polymerase (supplied by Amnion Biosciences), PCR buffer (10X) with $MgCl_2$ (Amnion Biosciences) and all four deoxynucleotide triphosphates (supplied by Sigma) were added to a 0.2 ml microcentrifuge tube. A pre-PCR step at 94°C for 5 min was applied. A total of 35 PCR cycles were run under the following conditions: denaturation at 94°C for 45 s, annealing (at the temperature in table 1) for 1 min, and extension at 72°C for 1 min. After the final cycle, the preparation was kept at 72°C for 5 min to complete the reaction. The PCR products were stored in the thermal cycler at 4°C until they were collected.

TABLE 1. PCR thermal cycle protocols for PCR amplification and identification of bacteria in milk

Primer	Annealing	Amplicon size (bp)
Staphylococcus aureus	59.7°C	894
Staphylococcus epidermis	59°C	130
Streptococcus agalactiae	58.3°C	317
Streptococcus	57°C	572
Escherichia coli	58°C	468

RESULTS & DISCUSSION

A total of 214 milk samples from nine packages of areas in Bangalore and Kolar districts were screened by CMT and further PCR based microorganism detection for the samples was carried out. CMT results revealed that 118 of the total samples screened were normal, 94 were sub-clinically affected and 2 were clinical. All the samples (bacterial preparations of all the samples) were further subjected to PCR as indicated above. Every time a control template sample was included for PCR amplification along with the case samples. PCR bases technique revealed presence of any of the five pathogens in 97 milk samples and no organism was detected in 117 of the total samples screened. This proves that PCR is an accurate method for mastitis screening of animals. However its accuracy to identify exact organism have been reported by many researchers (Phuektes *et al*., 2003, Shome *et al.,* 2011, Shome *et al.,* 2012) and hence was not tested in the present study as no culture examination of the samples was carried out to tally the results.

An amplicon of 894 bp of *23S rRNA* gene (loction 678-1571 within the gene) of *S. aureus* was amplified in a thermal cycler using sequence specific primers. Fig.1 demonstrates the PCR product on the agarose gel. Out of total 215 animals screened, 61 samples (28.50 per cent) were found to be *Staphylococcus aureus* positive. CMT (California Mastitis Test) for all these 61 samples screened displayed that the samples were either sub-clinically or clinically infected. Sindhu *et al*., 2001 used PCR based technique for detection of *S. aureus* from the Murrah buffalo milk samples in Haryana and found that 10.24 per cent of the total samples screened were positive for *S. aureus*.

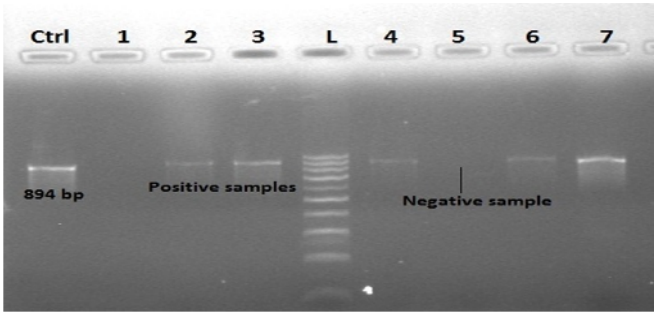

FIGURE 1. PCR product of 894 bp of *23S rRNA* gene of *S. aureus* in lane 1- positive control 2-7 PCR product for samples; L- 1000bp ladder

For *S. epidermis*, an amplicon of 130 bp from *rdr* gene (location 400016 bp to 400145 bp) was amplified and checked on 2 per cent agarose gel in 0.5X TBE (fig 2). *S. epidermis* was identified in 33 samples (15.42 per cent). CMT however revealed that 1 sample among the 33 samples (found positive for *S. epidermis* by PCR based identification) was normal, 1 sample was clinically affected and 31 were subclinical samples. The sample was detected as normal in CMT because the cow may be suffering from latent infection where in the mammary cells and the leucocytes shaded in the milk were reduced but the microorganism was still present in the milk.

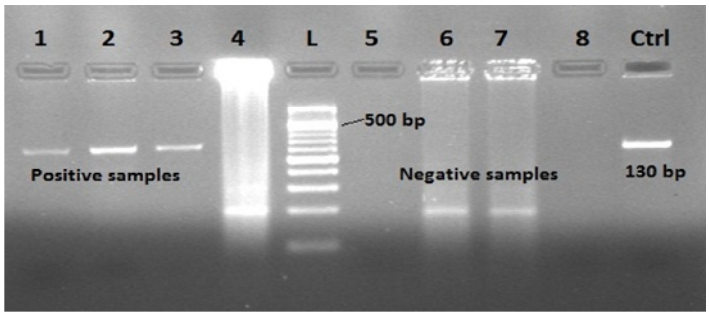

FIGURE 2. PCR product of 130 bp of *rdr* gene of *S. epidermis.* In lane 1- 8 PCR product for samples, 9- positive control; L- low molecular weight ladder

A region from 132bp to 448 bp of *16S rRNA* gene of *S. agalactiae* was amplified by region specific primers to obtain a PCR product of 317 bp (Fig 3). *Streptococcus agalactiae* was found in 29 samples i.e., 10.28 per cent of the total samples screened. CMT examination revealed that 28 samples were subclinical and 1 was clinical sample among these 29 samples found infected by *S. agalactiae*.

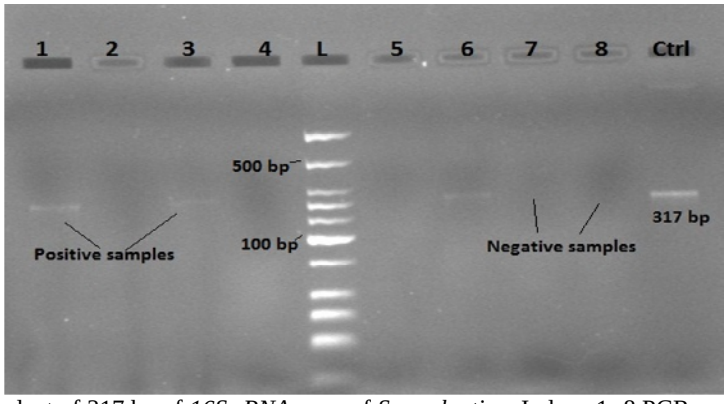

FIGURE 3. PCR product of 317 bp of *16S rRNA* gene of *S. agalactiae.* In lane 1- 8 PCR product for samples, 9- positive control; L- low molecular weight ladder

A 572 bp PCR product of 16S rRNA (459bp-1030bp) was amplified for specific identification of *S. dysgalactiae* (Fig 4). *S. dysgalactiae* was isolated from in 3.74% of the total samples screened and thus the number of cases infected with *S. dysgalactiae* was the least. All the eight samples detected positive for *S. dysgalactiae* by PCR based diagnosis method were also found to be sub-clinically infected for CMT.

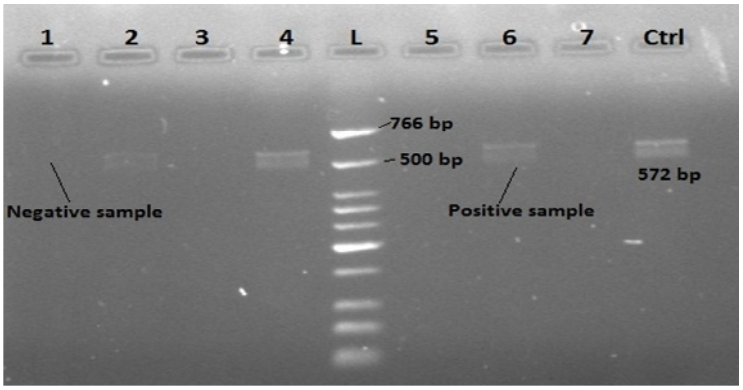

FIGURE 4. PCR product of 572 bp of *16S rRNA* gene of *S. dysgalactiae.* In lane 1- 7 PCR product for samples, 8- positive control; L- low molecular weight ladder

Escherichia coli were isolated in 29 samples (13.55 per cent). For identification of *E.coli*, *phoA* gene specific segment was amplified (location 433 bp to 900 bp) and amplicon of 468 bp was obtained (Fig 5). Out of the 29 samples detected positive for *E.coli* by PCR based microorganism detection, 27 were detected as sub-clinically and 2 were detected as clinically infected in CMT based diagnosis.

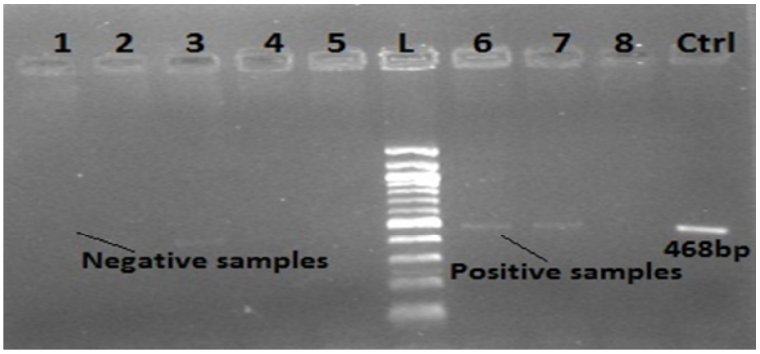

FIGURE 5. PCR product of 468 bp of *phoA* gene of *E.coli.* In lane 1- 8 PCR product for samples, 9- positive control; L- 1Kb ladder

The results of present investigation demonstrated that *Staphylococcus aureus* was identified in 28.5 per cent of the total animals screened followed by *Staphylococcus epidermis* (15.42 per cent), *Escherichia coli* (13.55 per cent) and *Streptococcus agalactiae* (10.28 per cent). *Streptococcus dysgalactiae* was identified in 3.74 per cent of the cases (table 2).

TABLE 2. Detection of pathogens in milk samples by PCR

Organism	Positive	Negative	Total	Incidence
Staphylococcus aureus	61	153	214	28.50%
Staphylococcus epidermis	33	181	214	15.42%
Streptococcus agalactiae	22	192	214	10.28%
Streptococcus dysgalactiae	8	206	214	3.74%
Escherichia coli	29	185	214	13.55%

These results correlate with the results of Shome *et al*., 2011 where in PCR based detection of mastitis pathogens in bulk tank milk samples revealed highest incidence rate for *S. aureus* followed by *S. epidermidis* and *E. coli* in areas around Bangalore dist. Thus PCR based identification of mastitis causing pathogens from milk is a rapid and reliable method to detect presence of mastitis at the initial stage and reveal the exact bacterial etiology, furnish appropriate assistance and/or medical aid to the affected animals.

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REFERENCES

Anon (2011) Veterinary Investigation Surveillance Report. London: Veterinary Laboratories Agency.

Bhuvana, M. and Shome, B. R. (2013) Etiology of Bovine Mastitis. *Proceedings of Model training course on "Bovine mastitis: theoretical and practical consideration in management".PD_ADMAS. Bangalore*. p.7-10.

Forsman, P., Tilsal-Timisjarvi, A. and Alatossava, T. (1997) Identification of staphylococcal and streptococcal causes of bovine mastitis using 16S-23S rRNA spacer regions. *Microbiology.* **143,** 3491–3500.

Hegde, R. and Isloor, S. (2013) Trends in diagnosis of bovine mastitis. *Proceedings of Model training course on "Bovine mastitis: theoretical and practical consideration in management".PD_ADMAS. Bangalore*. p.15-24.

Khan, M. A., Kim, C.H., Kakoma, I., Morin, E., Hansen, R.D., Hurley, W.L., Tripathy, D.N. and Baek. B.K. (1998) Detection of *Staphylococcus aureus* in milk by use of polymerase chain reaction analysis. *Am. J. Vet. Res.* **59,** 807–813.

Phuektes, P., Browning, G. R., Anderson, G. and Mansell, P. D. (2003) Multiplex polymerase chain reaction as a mastitis screening test for *Staphy aureus, Strep agalactiae, Strep dysgalactiae* and *Strep uberis. J. Dairy Res.* **70,** 149–155.

Phuektes, P., Mansell, P. D. and Browning. G. F. (2001) Multiplex polymerase chain reaction assay for simultaneous detection of *Staphylococcus aureus* and *Streptococcal* causes of bovine mastitis. *J. Dairy Sci.* **84,** 1140–1148.

Shome, B.R., Das Mitra, S., Bhuvana, M., Krithiga, N., Shome, R., Velu, D. and Prabhudas, K. (2012) Multiplex PCR for the detection of five important *Staphylococcus* spp. in bovine subclinical mastitis milk. *Indian Journal of Animal Science*. **82** (1), 9-14.

Shome, B.R., Das Mitra, S., Bhuvana, M., Krithiga, N., Velu, D., Shome, R., Isloor, S., Barbuddhe, S.B. and Rahman, H. (2011) Multiplex PCR assay for species identification of bovine mastitis pathogens. *Journal of Applied Microbiology*. ISSN, 1364-5072.

Sindhu, N., Sharma, A. and Jain, V.K. (2011) Detection of Enterotoxin producing S. aureus from mastitis milk. *Indian Veterinary J*. **88,** 34-35.

Tarate, S.B., Jadhav, P.V., Ghadhigaonkar, D.D., Khade, P., Bhatt, V., Khan, A., Joshi, C.G. and Rank, D.N. (2012) Evaluation of breed- dependent variation in microbial load during scbclinical mastitis in cattle using Real Time PCR. *Indian J Dairy Sci*. **65**(1), 60-65.