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BOVINE SUB-CLINICAL MASTITIS DETECTION BY TARGETING MAJOR PATHOGENS & BOVINE (GAPDH GENE, INTERNAL CONTROL) BY M-PCR

ABSTRACT

Mastitis is an inflammatory disease of mammary gland, caused by bacterial intramammary infection. It accounts for huge economic loss to the dairy industry. Detection of major pathogens in the udder during inflammation would help in saving the animal from clinical mastitis by avoiding permanent damage to the udder. Hence there is a requirement for rapid, sensitive and specific detection of the pathogens causing mastitis at an early stage of infection. Keeping this in mind we have standardized genus specific multiplex PCR (m-PCR) to detect major pathogens i.e. Staphylococcus aureus, Streptococcus spp. and Escherichia coli directly from milk samples. Method of DNA extraction directly from milk was standardized and milk from suspected cases and from the animals apparently looked healthy was subjected to the test. Genus specific primers were designed for these three genera involved in mastitis and tested in m-PCR using total DNA isolated from milk. To account for the efficiency of the DNA isolation method, we have included primers in one of the exon of the Glyceraldehyde phosphate dehydrogenase (gapdh gene) as internal control. The sensitivity and specificity of the test were evaluated using standard cultures. The test was compared with the conventional method of isolation and found to have a positive correlation. Of the total 147 samples tested 111 found to be positive for the presence of these pathogens. In our studies the test could detect the presence of a pathogen up to $1x10^5$ - $1x10^7$ CFU depends on the genus. Therefore the test may be considered as a rapid and efficient for diagnosis of bovine mastitis at subclinical level. The m-PCR test has several advantages like, avoids cumbersome and lengthy culturing steps, rapid, sensitive and specific and hence reliable for the detection of major pathogens causing bovine mastitis directly from milk. The results suggest that the assay could be used as an alternative method for routine detection of S. aureus, Streptococcus spp. and E. coli in milk samples in suspected cases of mastitis at subclinical levels before taking up antibiotic therapy.

KEY WORDS Bovine Mastitis, Staphylococcus, Streptococcus Spp, E. coli, m-PCR

INTRODUCTION

Bovine mastitis (BM) an inflammation of udder tissue due to a microbial infection (Watts, 1988) is responsible for huge economic losses to the tune of billions of dollars every year for the dairy industry. The losses are due to reduced milk quantity and quality. BM contributes a highest percentage of loss hence the major strategy to avoid this is by detecting the BM at an early stage of infection. When the infection is chronic, the udder loses its function permanently as the tissue becomes fibrosis. Hence it is important to identify quickly the new clinical cases in order to control infection in the herd. The causative bacteria can be classified as major or minor pathogens (Harmon, 1994) and the major pathogens responsible for BM can be further classified as environmental (Escherichia coli, Streptococcus dysgalactiae and Streptococcus uberis) or contagious (Staphylococcus aureus and Streptococcus agalactiae) depending on their primary reservoir (Bramley et al., 1996 & Riffon et al., 2001).

Since subclinical mastitis does not show visible changes in the milk or udder, it is detected by reduction in milk yield, altered milk composition and the presence of inflammatory components and bacteria in milk. Infection patterns of the major mastitis-causing pathogens using examples of E. coli, S. aureus and non-agalactiae streptococci. E. coli infections mainly cause clinical mastitis, that S. aureus infections cause sub-clinical mastitis and that non-agalactiae streptococci have an infection pattern with both subclinical and clinical appearances (Haas et al., 2005 & Yuan et al., 2011). Accordingly, most E. coli infections should be seen in clinical mastitis records, while S. aureus infections should be reflected in changes of Somatic Cell Count (SCC). Among the major pathogens, S.

aureus, Streptococcus agalacitae, Streptococcus dysgalacitae, Streptococcus uberis and E. coli had the highest prevalence. The main groups of environmental pathogens known to cause mastitis are Escherichia coli, Klebsiella pneumoniae, Streptococcus agalactiae, Streptococcus dysgalactiae and Streptococcus uberis (Todhunter et al., 1995 & Paulin et al., 2007 & Paulin et al., 2008).

Bacteria that commonly cause mastitis are generally classified as either 'contagious'or 'environmental' pathogens, depending on the likely source of the pathogen and mode of transmission. The main contagious pathogens are Staphylococcus aureus and Streptococcus agalactiae. These pathogens have adapted to survive within the mammary gland and are spread from cow to cow at or around the time of milking (Bradley 2002 & Hameed et al., 2008). Environmental pathogens are best described as opportunistic invaders. They are found within the cow's environment and are not thought to be adapted for survival within the mammary gland (Bradley 2002). Mastitis was mainly caused by infection by contagious pathogens. However, during the 1960s, the implementation of antibiotic dry cow therapy, post-milking teat disinfection, and routine maintenance of milking machines, reduced the incidence of mastitis caused by these bacteria (Bradley 2002). Although these control programs were efficient in reducing the problem of contagious pathogens, they were not as effective against environmental pathogens (Schukken et al., 1990 & Schukken et al., 2003).

The merit of any detection method for routine diagnosis depends on factors, such as specificity, sensitivity, expense, time involved, and apply to large numbers of milk samples. The most common but unspecific method to identify potential chronic infections is an SCC though it is not very specific. Early detection procedures have been shown to enhance cure rates and reduce the time required to return to normal milk when coupled with appropriate antimicrobial therapy (Milner et al., 1997). It is important to identify the pathogen not only for antimicrobial therapy purposes but also to monitor and control the rate of infection at the farm level. Some of the tests have been applied to pathogens of bovine origin, such as the Minitek Gram- Positive test for Streptococcus (Watts, 1989) but with no success because of a lack of information on veterinary pathogens in the database. An enzyme-linked immunosorbent assay method has been developed for the detection of S. aureus in BM cases (Bourry & Poutrel, 1996) but there was no correlation between antibody titer and bacterial load (Heisick et al., 1989 & Bourry & Poutrel, 1996). Other enzyme-linked immunosorbent assays were developed to screen milk for contamination with Listeria organisms (Adams et al., 1988 & Bourry et al., 1997). Nucleic acid based PCR methods have been developed for detecting the presence microorganisms in milk or in other organic samples however these tests needed a step to enrich the bacteria in culture media (Andrews, 1983 & Thomas et al., 1991 & Wernars et al., 1991 & Lorenz et al., 1998; Queipo-Ortuno et al., 1999 & Reale et al. 1999; Khaled et al., 2010) and are therefore, time-consuming. It has been previously shown that milk samples could serve as a source for the amplification of specific DNA sequences using PCR (Lipkin et al., 1993 & Berri et al., 2000). Since majority of BM cases is due to three major pathogens i.e. Staphylococcus sp, Streptococcus sp and E. coli the diagnostic test should target these organisms.

The diagnosis of Staphylococcus aureus mastitis and its potential antibiotic resistance in dairy cattle, a multiplex polymerase chain reaction (PCR) assay was developed for simultaneous species identification and detection of penicillin, erythromycin, and tetracycline resistance genes (Jian et al., 2011). Based on this presumption we developed a m-PCR technique with four sets of primers, three sets are specific for the major pathogen species while one is an internal control for identifying BM pathogens from milk samples, as the test is specific, sensitive and rapid. To take care of the experimental error in terms of DNA isolation or PCR reaction we have included primers for GAPDH that amplifies one of the exon regions of the gene from somatic cell DNA as internal control.

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MATERIALS AND METHODS

Bacteriological Examination

The milk sample was streaked on a blood agar plate and incubated for 24 to 48 h at 37°C. After incubation, the plate was read for primary isolation of mastitis pathogens. A milk sample was considered contaminated when three or more different colony types of bacteria were detected. Then, a single colony on the blood agar was inoculated into a broth and incubated for 18 to 24 h at 37°C. Further identification of specific bacterial species such as Staphylococci, Streptococci, and gram-negative bacteria were performed according to the methods described by the National Mastitis Council.

Bacterial Strains and Growth Conditions

The reference Streptococci viz., Streptococcus agalactiae (HM 355961), Streptococcus dysgalactiae (HC 359248) and Streptococcus uberis (HC 355971 and HC 355972) procured from Project Directorate on_Animal Disease Monitoring And Surveillance (PD_ADMAS), Bangalore and E. coli DH5α available at IVRI, Bangalore**,** Staphylococcus aureus maintained in the Department of Veterinary Microbiology, Veterinary College, Bangalore were used

Isolation of DNA from Milk Samples

The total DNA was isolated from milk as per the method (Cremonesi et al., 2006) with modification. Milk samples were centrifuged at 4 °C at 14 000 g for 5 min and the fat layer that formed on the top was removed. An aliquot of 200 µl of the centrifuged milk was taken into a, mixed with 200 µl phosphate buffer saline (PBS) and 400 µl methanol and the mixture was re- centrifuged at 4 °C at 14 000 g for 5 min. The pellet containing the cells was collected mixed with 100µl isoamyl alcohol, centrifuge for 5min and the pellet was re-suspended in 100µl of 0.9% NaCl. The above steps was repeated once. Lysis buffer (400µl) (3M guanidine thiocyanate, 20mM EDTA, 10mM Tris-HCl (pH 6.8), 40mg/ml, Triton X-100, 10mg/ml dithiothreitol) was added and incubate at room temperature for 5 min. The contents were centrifuged for 2 min at 14,000 g and the supernatant was passed through the silica column. The adsorbed DNA was treated once again with lysis buffer and centrifuged for 2 min. Wash buffer (25% absolute ethanol, 25% isopropanol, 100 mM NaCl, 10mM Tris HCl, pH 8) was added to the column and centrifuged for 2 min. The DNA bound to the column was once washed with absolute alcohol and eluted with sterile distilled water. DNA concentration was estimated using a Nanodrop spectrophotometer.

Standardization of PCR

PCR primers were designed from highly divergent and species specific regions of the DNA coding for 16S–23S ISR rRNA, 16S r RNA and traT gene based on previously published sequence entries available in the NCBI – Gen Bank database for the detection of Staphylococcus, Streptococcus spp. and E. coli respectively. The primers were synthesized by M/S BIOSERVE India Ltd. (Hyderabad) and procured as lyophilized powders. The primers were reconstituted in TE buffer to a final concentration of 1000 pm/ µl and stored at -70ºC as stock. The working concentration of 20 pm/µl was prepared with sterile filtered distilled water, aliquoted and stored at -20ºC.

PCR was performed in a Gene Amp PCR System 2400 (Biometra) in a final volume of 25µl. Consisting of 5 pmol each of the primer pair, 1 U of Taq DNA polymerase (Chromous Biotech Ltd, India), 25 mM $MgCl_2$, and 100µM each of the four deoxyribonucleotide triphosphates (Fermentas, USA) in 25mM Tris-HCl, pH 8.3. Initial denaturation was done at 95°C for 2 min before applying 35

cycles, each cycle with denaturation at 95°C for 45 s, annealing 50°C for 1 min, and extension at 72°C for 30 Sec. After the final cycle, one cycle of extension at 72°C for 5 min was carried out to complete the reaction. Initially PCR assay was performed for each pathogen using known standard DNA and with 5pmol each of the specific primer pair. Subsequently PCR was performed with a mixture of standard DNAs with various combinations of templates and primers so as to rule out no specific amplification or internal primer-primer hybridization.

Detection of PCR products

Ten microliters of the PCR product was analyzed by 2% agarose gel electrophoresis in Tris-borate buffer using standard molecular size markers. Bands were visualized under ultraviolet (UV) transillumination (312 nm) and documented.

m-PCR for the Detection of Major pathogens

For multiplexing, all the four pairs of the primers (three for pathogens and one for internal control) were mixed at 5 pmol concentration of each primer and used for the PCR reaction in the presence of 5µl of the total DNA solution. The reaction conditions were same as shown before.

Table 1. Primers used in the m-PCR

Primer name Specificity	Target gene Amplicon Size (bp)	Sequence (5'–3')
SU-F Staphylococcus aureus	16s–23s ISR rRNA	TTC GTA CCA GCC AGA GGT GGA
SU-R Staphylococcus aureus	16s–23s ISR rRNA 229	TCT TCA GCG CAT CAC CAA TGC C
ST-F Streptococcus spp.	16s rRNA	GAT ACA TAG CCG ACC TGA GA
ST-R Streptococcus spp.	16s rRNA 561	AGG GCC TAA CAC CTA GCA CT
ECO-F E. coli	(tra T) gene	TCT GCG GGA GTC TCA GGG ATG GCT G
ECO-R E. coli	(tra T) gene 313	GTA TTT ATG CTG GTT ACC TGT TT
Bta-F Bos taurus (somatic cell)	GAPDH	ACC ACC GTC CAC GCC ATC AC
Bta-R Bos. taurus (somatic cell)	GAPDH 152	CCG TTG AGC TCA GGG ATG A

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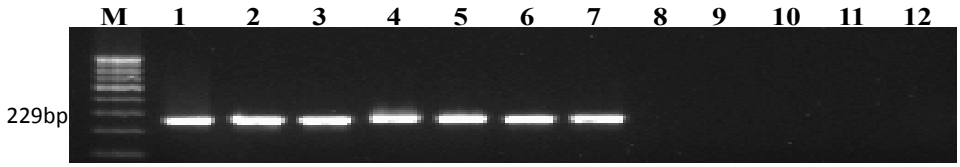

Figure 1. PCR amplification of 229bp S. aureus analyzed by electrophoresis on a 2% agarose gel, Lane M: 100 bp DNA ladder, Lane1 -7: PCR amplified 229bp product Reference Positive control (S. aureus), Lane 8-11 : Negative controls Streptococcus agalactiae, S. dysgalactiae, S. uberis and E. coli, Lane 12: Negative controls (NTC).

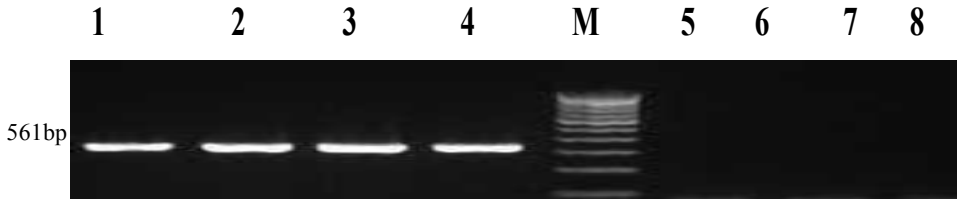

Figure 2. PCR amplification of 541bp Streptococcus spp. analyzed by electrophoresis on a 2% agarose gel, Lane 1-4: Streptococcus spp. Reference Positive Control, Lane M: 100 bp DNA ladder, Lane 6: Negative controls S. aureus , Lane 7:Negative controls E. coli , Lane 8: Negative controls NTC.

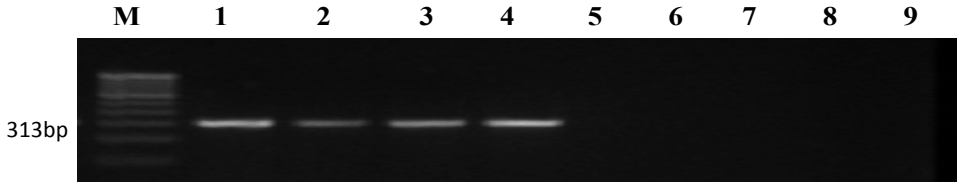

Figure 3. PCR amplification of 313bp traT gene of E. coli analyzed by electrophoresis on a 2% agarose gel, Lane M: 100 bp DNA ladder, Lane1-4: PCR amplified 313bp product of E. coli Positive control, Lane 5-8:Negative controls (S. aureus , Streptococcus agalactiae, S. dysgalactiae, S. uberis), Lane 9, Negative controls NTC.

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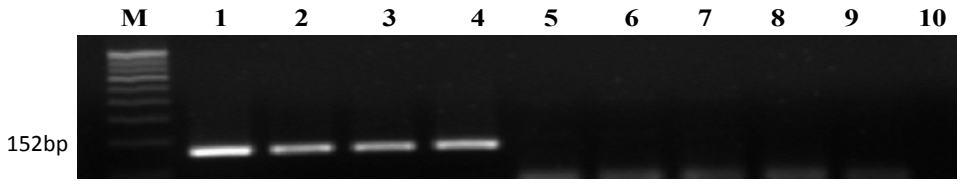

Figure 4. PCR amplification of 152bp GADPH gene of Bos taurus analyzed by electrophoresis on a 2% agarose gel, Lane M: 100 bp DNA ladder, Lane1-4: PCR amplified 313bp product of E. coli Positive control, Lane 5:Negative controls S. aureus , Lane 6-8:Negative controls Streptococcus agalactiae, S. dysgalactiae, S. uberis), Lane 6-8:Negative controls E. coli, Lane 10:Negative controls NTC .

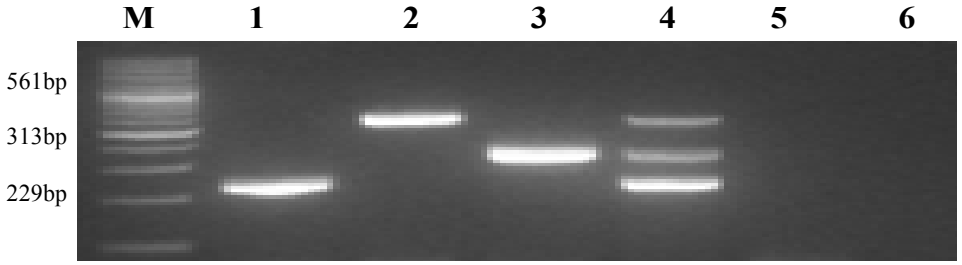

Figure 5. Amplification products of the different primer combinations were analyzed by electrophoresis on a 2% agarose gel. Lane M: 100-bp DNA ladder , Lane 1:S. aureus, Lane 2: Streptococcus spp., lane 3: E. coli and Lane 4: Mixture of these 3 primer pairs amplified specific genes from the DNAs from the 3 organisms.

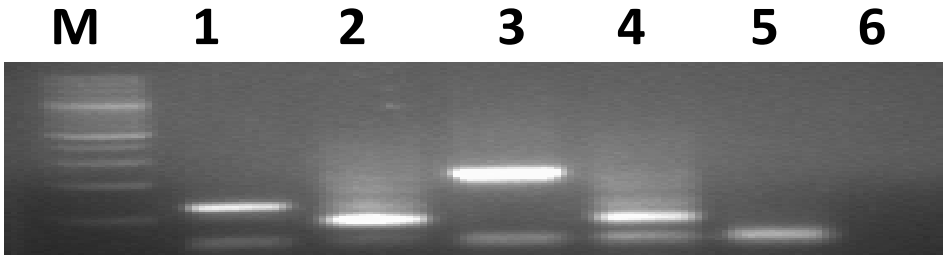

Figure 6. Amplification products of the different primer combinations were analyzed by electrophoresis on a 2% agarose gel. Lane M:100-bp DNA ladder , Lane 1: E.coli and Bos taurus ,Lane 2 and 4: S. aureus and Bos taurus ,lane 3: Streptococcus spp.,and Bos taurus Lane 5: Mixture of these 3 primers pairs amplified specific genes from the DNAs from healthy milk sample, Lane 6: Negative controls NTC.

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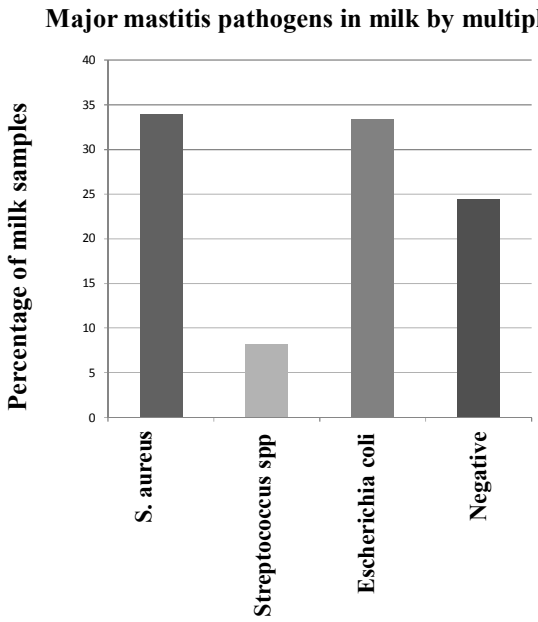

Figure 7. Milk samples with mastitis cases: 147 samples tested by the standardized Multiplex-PCR, Frequently present in milk was S. aureus being founded in 50 (34.013%) samples , E. coli in 49 (33.333%) samples, Streptococcus spp in 12 (8.163%) samples, 36 (24.489%) -negative for all the bacterial species.

RESULTS

Specificity of the Primers

S. aureus Specific PCR: S. aureus specific primers targeting 16s–23s ISR rRNA were designed and employed for the specific confirmation of the S. aureus DNA. The isolates yielded a single DNA band of 229 bp amplicon of 16S–23S ISR rRNA (Fig. 1). No other nonspecific amplification was seen either with DNA from reference cultures (lane 1, 2, 3, 4) or from positive milk samples (lane 5, 6, 7) indicating the primers are specific. On the other hand the DNAs from the reference cultures of Streptococcus spp. and E. coli did not show any amplification indicating the PCR is specific for S. aureus 16-23S ISR of rRNA genes (lane 8, 9, 10, 11). There was no amplified product seen in the case of No Template Control (NTC) indicating the amplified product is specific (lane 12).

Streptococcus spp. Specific PCR: A house keeping 16S rRNA gene was targeted in the case of Streptococcus spp. Upon amplification of the genus specific 16S rRNA gene with designed primers using the DNA isolated from the reference culture, as expected a single 561 bp DNA band was seen in the lane of 16S rRNA (Fig. 2, lane 1, 2) indicating the amplicon of 561 bp is specific for 16 S RNA gene of Streptococcus spp. A few positive samples which were confirmed by culturing had also shown the similar amplification fragment (lane 3, 4). No amplification was seen with the DNAs isolated from reference cultures of S. aureus and E. coli indicating the primers are specific for 16 S RNA gene of Streptococcus spp (lane 5,6). Similarly there was no amplification in case of no NT indicating there was no primer-primer annealing (lane 7,8).

E. coli specific PCR: Primers were designed for traT gene of E. coli which is supposed to be conserved in >90% of all E. coli strains. The DNA from standard culture showed an amplified band of 313 bp (Fig. 3 lane 1, 2) which is absent with the DNAs of the standard cultures of S. aureus and Streptococcus spp (lane 5, 6, 7, 8) indicating that the gene is specifically for E. coli. Similar size band was also seen in the reference cultures (lane 3, 4) which were used as positive controls. No amplification was observed in the case of NTC as well (lane 9).

Bos taurus Specific PCR: Primers targeting one of the exons of the GAPDH gene of Bos taurus were designed and employed for the specific amplification of the gene from the somatic cell DNA present in the total DNA isolated from the milk. As expected 152 bp amplicon was observed in the case of DNA isolated from milk samples (Fig. 4 lane 1, 2, 3, 4) which is absent in the case of DNA isolated from bacterial cultures (lane 5, 6, 7, 8, 9, 10) indicating the amplicon was specific for somatic cell DNA.

Application of Primers in m-PCR

After confirming the specificity of each primer pair with the DNAs isolated from the reference cultures obtained from ATCC, the PCR was conducted on mixed DNAs of S. aureus, Streptococcus spp. and E. coli with primers specific for S. aureus. The amplification of a single 229bp amplicon corresponding to S. aureus 16S–23S ISR rRNA was seen no other amplified product was observed (Fig. 5, lane 1). Similarly primers specific for Streptococcus 16SrRNA showed specific amplification of 561 bp only with Streptococcus (Fig. 5, lane 2) but not with S. aureus or E. coli. Similarly primers for E. coli traT gene amplified specific product with E. coli DNA. No amplified product was seen with the DNAs isolated from Staphylococcus spp or Streptococcus spp (Fig. 5, lane 3) indicating they are genus specific. However the mixture of the all the 3 primer pairs amplified the corresponding genes from the mixed DNAs. (Fig. 5, lane 4). This indicates that the primers are compatible and can amplify the specific genes even when used in m-PCR.

Encouraged with the specificity of the primers, the m-PCR test was conducted on the DNAs isolated from milk samples (Fig.6). Milk samples were collected from organized farms which were reported to have the clinical cases of mastitis. Of the 147 samples tested by the standardized Multiplex-PCR, 36 (24.489%) were negative for all the five bacterial species screened. The species most frequently present in milk was S. aureus, being founded in 50 (34.013%) samples followed by E. coli in 49 (33.333%) samples. Streptococcus spp. was detected in 12 (8.163%) samples respectively (Fig.7).

DISCUSSION

Bovine mastitis is one of the most economically important diseases that affect the dairy industry. Billions of dollars are lost throughout the world because of reduced quality and quantity of milk produced. Though the mastitis is of multi-etiological in nature, the high percentage of loss is due to the three major bacterial pathogens, Staphylococcus, Streptococcus and Escherichia coli. Mastitis, if detected at subclinical levels can be controlled by antibiotic therapy. Detection of subclinical mastitis and the causative agent is the prerequisite for undertaking therapeutic measures. Conventional procedures for identification of pathogens of BM are labor-intensive, and most of the commercial tests are not designed to identify important veterinary pathogens (Watts, 1989 & Jayarao et al., 1991). Detection of mastitis at sub-clinical stage needs tests that are highly sensitive and PCR based tests can meet this requirement. However some of these available methods still need enrichment of the culture, which is time consuming. Hence the detection of pathogen directly from milk is ideal. Also to detect the pathogen the PCR reactions are to be carried separately for different pathogens. Multiplexing is alternative method however, the compatibility of the primer pairs is important for the reaction to work efficiently with high sensitivity of detection. Presence of PCR inhibitors may sometimes show false negative results. This can be detected by including an internal control.In this investigation the efforts are made to develop modified m-PCR to overcome these limitations and developed sub-clinical mastitis detection test using the milk sample. The test is based on m-PCR that can identify major pathogens. The test which can detect the BM within 4 hours did not require a culture step is specific, sensitive and cheap. PCR methods are very efficient for the detection of pathogens at very low concentration. Methods involving ribotyping are useful for

pathogen detection. Therefore PCR based detection of 16S rRNA or 23S rRNA regions have been successfully applied for the identification of many bacteria (Bentley & Leigh 1995 & Forsman et al., 1997; Saruta et al., 1997 & Mendoza et al., 1998 & Straub et al., 1999 & Bes et al., 2000 & Sabat et al., 2000 & Whitehead & Cotta, 2000). The major advantage of PCR is its sensitivity as a few picogram quantities of nucleic acid is enough to detect the presence of the organism, allowing the elimination of the culture. Therefore, we selected m-PCR amplification of DNA regions coding for rRNA and ISR because of the presence of genus specific hypervariable regions.

Moreover, rRNA is present in many copies, which permits signal enhancement (Bentley & Leigh 1995). Primers described here (Table 1) were proven to be specific since on agarose gel only one band was observed for each set of primers and no signal was detected with negative controls. As internal control to verify the PCR efficiency we have included the primers for amplifying DNA sequence coding for one of the exon of GAPDH of Bos taurus, were designed and included. The intensity of the bands varied among samples which may be due to the difference in the copy numbers of the coding regions for these particular (Watts, 1989). Even though the nucleotide sequence data comparison (BLASTN 2.0) showed that the four primers are conserved in all pathogens of BM. The test was standardized for direct detection of organism in milk samples without a culture step. As expected the m-PCR is specific for S. aureus, Streptococcus spp. and E. coli.

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- Dept. of Veterinary Microbiology, Veterinary College, KVAFSU, Hebbal, Bangalore

REFERENCE

1. Adams, DS, McDonald, JS, Hancock, D, & McGuire, TC (1988). Staphylococcus aureus antigens reactive with milk immunoglobulin G of naturally infected dairy cows. Journal of Clinical Microbiology 26 1175–1180.

2. Andrews AT. (1983).Proteinases in normal bovine milk and their action on caseins. Journal of Dairy Research 50 45–55.

3. Bentley RW & Leigh JA (1995).Determination of 16S ribosomal RNA gene copy number in Streptococcus uberis, S. agalactiae, S. dysgalactiae and S. parauberis.FEMS Immunology Medical Microbiolgy 12 1.

4. Bentley RW & Leigh JA (1995). Development of PCR-based hybridization protocol for identification of streptococcal species.Journal of Clinical Microbiology 33 1296–1301.

5. Berri M, Laroucau K & Rodolakis A (2000).The detection of Coxiella burnetii from ovine genital swabs, milk and fecal samples by the use of a single touchdown polymerase chain reaction. Veterinary Microbiology 72 285–293.

6. Bes M, Guerin-Faublee V, Meugnier H, Etienne J & Freney J(2000). Improvement of the identification of Staphylococci isolated from bovine mammary infections using molecular methods Veterinary Microbiology 71 287–294.

7. Bourry A & Poutrel B(1996). Bovine mastitis caused by Listeria monocytogenes: kinetics of antibody responses in serum and milk after experimental infection. Journal of Dairy Science 79 2189-2195.

8. Bourry A, Cochard T & Poutrel B (1997).Serological diagnosis of bovine, caprine, and ovine mastitis caused by Listeria monocytogenes by using an enzyme-linked immunosorbent assay. Journal of Clinical Microbiology 35 1606–1608.

9. Bradley AJ (2002). Bovine mastitis: An evolving disease. The veterinary journal 16 31-13.

10. Bramley AJ, Cullor JS, Erskine RJ, Fox LK, Harmon RJ, Hogan JS, Nickerson SC, Oliver SP, Smith KL and Sordillo LM (1996). Current Concepts of Bovine Mastitis, 4th edn. National Mastitis Council, Madison, WI.

11. Cremonesi, P, Castiglioni, B, Malferrari , G, Biunno, I, Vimercati, C, Moroni, P, Morandi, S, & Luzzana, M (2006). Technical Note: Improved method for rapid DNA extraction of mastitis pathogens directly from milk. Journal of Dairy Science 89163–169.

12. Haas, de Y, Barkema, HW, Schukken, YH & Veerkamp, RF (2005). Associations between somatic cell count patterns and the incidence of clinical mastitis. Preventive Veterinary Medicine 67 55-68.

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

14. Hameed, KGA, Sender, G & Kossakowska, AK (2008). An association of BoLA alleles DRB3.2 16and DRB3.2 23 with occurrence of mastitis caused by different bacterial species in two herds of dairy cows. Animal Science Papers and Reports 26 37-48.

15. Harmon, RJ (1994). Physiology of mastitis and factors affecting somatic cell counts.Journal of Dairy Science 77 2103.

16. Heisick, JE, Harrell, FM, Peterson, EH, McLaughlin, S, Wagner, DE, Wesley, IV& Bryner J (1989).Comparison of four procedures to detect Listeria spp. in foods. Journal of Food Protection 52 154–157.

17. Jayarao, B. M, Dore, Jr, JJ, Baumbach, GA, Matthews, KR & Oliver, SP (1991). Differentiation of Streptococcus uberis from Streptococcus parauberis by polymerase chain reaction and restriction fragment length polymorphism analysis of 16S ribosomal DNA.Journal of Clinical Microbiology 29 2774– 2778.

18. Jian, Gao, Miro, Ferreri, Xiu ,Q Liu, Li B Chen,Jing L & Su, Bo Han (2011).Development of multiplex polymerase chain reaction assay for rapid detection ofStaphylococcus aureus and selected antibiotic resistance genes in bovine mastitic milk samples. Journal of Veterinary Diagnostic Investigation 5 894-901.

19. Khaled,A, Abd, El-Razik, Khaled, A, Abdelrahman, Youssef, F, Ahmed, Alaa.M, Gomaa, Hazem A, & Eldebaky (2010). Direct Identification of Major Pathogens of the Bubaline Subclinical Mastitis in Egypt using PCR. Journal of American Science 6(10) 652-660

20. .

21. Lipkin E, Shalom A, Khatib H, Soller M & Friedmann. A. (1993).Milk as a source of deoxyribonucleic acid and as a substrate for the polymerase chain reaction. Journal of Dairy Science 76 2025–2032.

22. Lorenz H, Jager C, Willems H, & Baljer G (1998).PCR detection of Coxiella burnetii from different clinical specimens, especially bovine milk, on the basis of DNA preparation with a silica matrix. Applied and Environmental Microbiology 64 4234–4237.

23. Mendoza, M, Meugnier, H, Bes, M, Etienne, J, & Freney, J (1998).Identification of Staphylococcus species by 16S–23S rDNA intergenic spacer PCR analysis. International Journal of Systematic Bacteriology 48 1049–1055.

24. Milner, P., Page, K. L. & Hillerton, J. E. (1997).The effects of early antibiotic treatment following diagnosis of mastitis detected by a change in the electrical conductivity of milk. Journal of Dairy Science 80 859–863.

25. Paulin-Curlee, G. G., Singer, R. S., Sreevatsan, S., Isaacson, R., Reneau, J., Foster, D. & Bey, R. (2007).Genetic Diversity of Mastitis-Associated Klebsiella pneumonia in Dairy Cows. Journal of Dairy Science 90 3681–3689.

26. Paulin-Curlee, GG, Sreevatsan, S, Singer, RS, Isaacson, R, Reneau, J, Bey, R & Foster, D (2008).Molecular Subtyping of Mastitis-Associated Klebsiella pneumonia Isolates Shows High Levels of Diversity Within and Between Dairy Herds. Journal of Dairy Science 91 554–563.

27. Queipo-Ortuno, M I., Garcia-Ordonez, MA, Colmenero, JD & Morata, P (1999) Hydrogen peroxide improves the efficiency of a peripheral blood PCR assay for diagnosis of human brucellosis. BioTechniques 27 248– 252.

28. Reale S., Maxia L, Vitale F, Glorioso NS, Caracappa S & Vesco G(1999).Detection of Leishmania infantum in dogs by PCR with lymph node aspirates and blood. Journal of Clinical Microbiology 37,2931–2935.

29. Riffon R, Sayasith K, Khalil HP, Dubreuil, P., Drolet M & Lagace, J (2001). Development of a Rapid and Sensitive Test for Identification of Major Pathogens in Bovine Mastitis by PCR. Journal of Clinical Microbiology 7 2584–2589.

30. Sabat, G, Rose, P, Hickey, WJ & Harkin, J. M. (2000).Selective and sensitive method for PCR amplification of Escherichia coli 16S rRNA genes in soil. Applied and Environmental Microbiology 66 844–849.

31. Saruta, K, Matsunaga, T, Kono, M, Hoshina, S, Ikawa, S, Sakai, O, & Machida, K(1997). Rapid identification and typing of Staphylococcus aureus by nested PCR amplified ribosomal DNA spacer region. FEMS Microbiology Letters 146 271–278.

32. Schukken, YH, Grommers, FJ, Van de Geer, D, Erb, HN & Brand, A (1990). Risk factors for clinical mastitis in herds with a low bulk milk somatic cell count. 1. Data and risk factors for all cases. Journal of Dairy Science 73 3463-3471.

33. Schukken, Y. H., Wilson, D. J., Welcome, F., Garrison-Tikofsky, L., & Gonzalez, R. N. (2003). Monitoring udder health and milk quality using somatic cell counts. Veterinary Research 34 579-596.

34. Straub, JA, Hertel, C, & Hammes, W. P. (1999).A 23S rDNA-targeted polymerase chain reaction-based system for detection of Staphylococcus aureus in meat starter cultures and dairy products. Journal of Food Protection 62 1150–1156.

35. Thomas EJ, King, RK, Burchak J & Gannon VP(1991).Sensitive and specific detection of Listeria monocytogenes in milk and ground beef with the polymerase chain reaction. Applied and Environmental Microbiology 57 2576–2580.

36. Todhunter DA, Smith KL, & Hogan JS (1995). Environmental streptococcal intramammary infections of the bovine mammary gland. Journal of Dairy Science 78 2366-2374.

37. Watts JL (1989) Evaluation of the Minitek Gram-Positive Set for identification of streptococci isolated from bovine mammary glands. Journal of Clinical Microbiology 27 1008–1010.

38. Watts, J. L., (1988) Etiological agents of bovine mastitis. Veterinary Microbiology 16 41–66.

39. Wernars, K., Heuvelman, C. J., Chakraborty, T. & Notermans, S. H. (1991). Use of the polymerase chain reaction for direct detection of Listeria monocytogenes in soft cheese. Journal of Applied Bacteriology 70 121–126.

40. Whitehead, T. R. & Cotta, M. A. (2000).Development of molecular methods for identification of Streptococcus bovis from human and ruminal origins. FEMS Microbiology Letters. 182, 237–240.

41. Yuan, Z. R., Li, J., Zhang, L. P., Zhang, L. M., Chen, C., Chen, X. J., Gao, X., Li, J.

42. Y.,Chen, J. B., Gao H. J. & Xu, S.Z. (2011). Novel SNPs polymorphism of bovine CACNA2D1 gene and their association with somatic cell score. African Journal of Biotechnology 10:1789-1793.

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