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Detection of major mastitis pathogens by multiplex polymerase chain reaction assay in buffalo milk

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ABSTRACT

In the present study multiplex PCR assay was standardized for simultaneous detection of *Staphylococcus aureus*, *Streptococcus agalactiae*, *S. dysgalactiae* and *Escherichia coli* associated with mastitis. The target sequence 16S to 23S rRNA inter spacer regions was used. Primers used were chosen to have approximately same T_m value, common annealing temperature and easily differentiable specific amplified products. The performance of the assay was examined on 92 milk samples collected from subclinically and clinically infected buffaloes and the diagnostic sensitivity and specificity of multiplex PCR was compared with culture examination. Out of total milk samples, 16 were diagnosed for mixed infections of *Staphylococcus aureus* + *S. dysgalactiae* (43.75%), *S. aureus* + *S. agalactiae* (12.5%), *S. aureus* + *E. coli* (25%), *S. dysgalactiae* + *E. coli* (12.5%) and *S. aureus* + *S. dysgalactiae* + *E. coli* (6.25%). Multiplex PCR assay was more promising option than culture methods. Milk culture method is cumbersome and more time consuming and it may yield no bacteria due to the presence of very low number of pathogens or due to residual therapeutic antibiotics concentration in milk. The assay has an added advantage over simplex PCR that it can simultaneous detect and type different species of bacteria. Multiplex PCR assay is rapid, sensitive and specific assay which can be used as a routine diagnostic tool to detect major mastitis pathogens.

Key words: Mastitis, Multiplex PCR, Pathogens

Bovine mastitis is a multi-factorial disease characterized by physical, chemical, bacteriological changes in milk and pathological alterations in glandular tissues (Radostits *et al*. 2007) influencing milk quantity and quality. Most prevalent etiological agents of mastitis are *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Escherichia coli* (Pankaj *et al*. 2013). Economic losses due to mastitis have been estimated as ₹ 7,165.51 crore/ annum in India (Bansal and Gupta 2009). To minimize these losses, timely and early detection of single and multiple pathogens by a suitable test is essential for adoption of proper treatment and control measures. Polymerase chain reaction (PCR) assay is a better technique for detection of pathogens at sub optimum level and at a faster rate than conventional tests (Sindhu *et al*. 2010). Multiplex PCR being advantageous in detection of multiple pathogens at the same time with cost effectiveness, thus can be used for quick diagnosis.

Therefore the present study was planned to develop a multiplex PCR assay for simultaneous detection of *S. aureus*, *S. agalactiae*, *S. dysgalactiae* and *E. coli* from mastitic buffalo milk.

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MATERIALS AND METHODS

Bacteriological examination: The milk samples collected aseptically were shaken thoroughly and 0.01 ml was streaked on 5% sheep blood agar and MacConkey's lactose agar plates. The plates were incubated aerobically at 37°C for 24 to 48 h. Isolates were purified on blood agar plates and then identified on the basis of Gram's reaction, cultural morphology and colony characteristics.

The Gram-positive cocci were subjected to catalase test to differentiate between staphylococci and streptococci. On the basis of oxidase positive/negative test and catalase positive Gram-positive cocci were classified into micrococci and staphylococci. Staphylococci were further characterized into coagulase positive and coagulase negative types, on the basis of coagulase production.

The organisms which on preliminary examination were found to be streptococci, were further characterized for different species on the basis of CAMP test and latex agglutination test. Gram negative organisms like *E. coli* were identified on the basis of growth on Eosin Methylene Blue agar and biochemical tests.

Extraction of DNA from milk: DNA was extracted by SDS-phenol–chloroform isoamyl alcohol method (Phuektes *et al*. 2001) with some minor modifications. The concentration of DNA obtained directly from milk was measured using fluorometer.

Table 1. Details of oligonucleotide primers used

Organism	Primer	Sequence (5' to 3')	Product size	T_m value (°C)	Reference
S. aureus	STAA-AuI and STAA-AuII	F: TCTTCAGAAGATGCGGAATA R: TAAGTCAAACGTTAACATACG	420bp	F:58R:55	Phuektes *et al.* 2001
S. agalactiae	STR-AG-I and STR-AG-II	F: TAGTTTTGAGAGGTCTTGTGG R: ATATTCACAGCGTTTTCG	206bp	F:59R:55	Goli *et al*. 2012
S. dysgalactiae	STRD-DyI and STRD-DyII	F: GAACACGTTAGGGTCGTC R: AGTATATCTTAACTAGAAAAACTATTG	264bp	F:60R:54	Phuektes *et al.* 2001
E. coli	Eco 2083 and Eco 2745	F: GCTTGACACTGAACATTGAG R: GCACTTATCTCTTCCGCATT	662bp	F:59R:60	Riffon *et al.* 2001

Oligonucleotide primers: All oligonucleotide primers (desalted) used for PCR were selected from published sequences targeting 16S - 23S rRNA interspacer region and procured from Integrated DNA technology (IDT) (Table 1). Primers used in multiplex PCR assay were chosen to have approximately similar melting temperatures (T_m). Nucleotide blast was performed to determine specificity of primers and to rule out primer dimer formation.

Optimization of individual PCR assays: The PCR for *S. aureus*, *S. agalactiae*, *S. dysgalactiae* and *E. coli* from milk samples of mastitis were optimized separately. Reactions were standardized using different Top Taq master mix volume, primer concentrations, annealing temperature and number of cycles in thermocycler. Preliminary trials with Top Taq master mix volume (12.5 µl and 25 µl) and primer concentrations (0.2, 0.5, 1 and 2 µM) were performed to define the optimal PCR conditions for each individual PCR assay.

Optimization of multiplex PCR assay: Trials with different volumes of 2x multiplex PCR mix (12.5 and 25 µl), and primer concentrations with different combinations in the range of 0.2 to 0.5 µM were performed to define the optimal conditions. PCR was carried out with an initial denaturation at 95°C for 15 min, followed by 35 cycles of 94°C for 1 min, annealing temperature of 54°C for 1 min, and 72°C for 1 min, then final extension at 72°C for 10 min. Initial denaturation of 15 min was done for activation of HotStar Taq DNA polymerase present in multiplex PCR master mix.

Specificity of oligonucleotide primers: Specificity of oligonucleotide primers was also determined by using known bacterial spiked milk samples and their corresponding negative controls.

Analysis of PCR amplified products: Amplified products were electrophoresed in 1.5% agarose containing ethidium bromide at a concentration of 0.2 µg/ml for 60 min at 90 volts in 1× TAE buffer using mini gel electrophoresis assembly and bands were visualized under ultraviolet light.

RESULTS AND DISCUSSION

Out of 92 milk samples, 104 isolates were obtained bacteriologically of which 80 organisms were isolated individually whereas 24 were from mixed infection with 2 organisms. Isolates were characterized to genus *Staphylococcus*, *Streptococcus* and *Escherichia* on the basis of colony characteristics, morphology, Gram's reaction and haemolysis patterns. Further these isolates were characterized up to species level by biochemical tests and latex agglutination kit as *S. aureus* (40.38%), *S. dysgalactiae* (32.69%), *S. agalactiae* (5.76%), and *E. coli* (21.15%).

Optimization of individual PCR assays for S. aureus, S. dysgalactiae, S. agalactiae *and* E. coli*:* The optimized PCR reaction mixture contained 25µl of 2x Top Taq master mix, 1µl of each primer of 10µM concentration, 200 ng of DNA extracted from milk and nuclease free water added to make final volume of reaction mixture 50 µl. Amplification was done with initial denaturation at 94°C for 1 min, 30 cycles each of denaturation at 94°C for one min, annealing temperature in a gradient of 50°C to 56°C for 60 sec and extension at 72°C for one min followed by final extension at 72°C for 10 min. The optimal annealing temperature for *S. aureus* was 53.9°C, *S. dysgalactiae* was in between 53 to 54°C, *S. agalactiae* was 54.2°C and *E. coli* was 53.5°C (Fig. 1–4).

Optimization of multiplex PCR assays for simultaneous detection of S. aureus, S. dysgalactiae, S. agalactiae *and* E. coli*:* The PCR reaction mixture was prepared with 1 µl of each forward and reverse primers of *S. aureus*, *S. agalactiae*, *S. dysgalactiae* and *E. coli*, 2x Multiplex PCR master mix (25µl), 200 ng template DNA and nuclease free water to make total volume of 50 µl. Thermal conditions were standardized under the following conditions: initial denaturation at 94°C for 15 min, 35 cycles each of

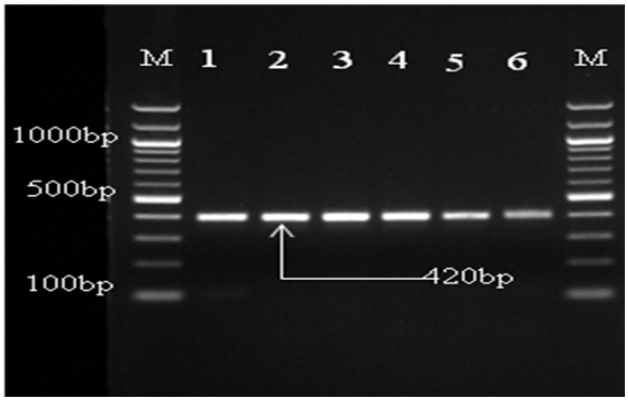

Fig. 1. Gradient PCR for *S. aureus*, annealing temperature from 52.5°C to 56°C. Lane M: 100bp ladder; lane 1: 56°C; lane 2: 55.2°C; lane 3: 54.6°C; lane 4: 53.9°C; lane 5: 53.2°C; lane 6: 52.5°C

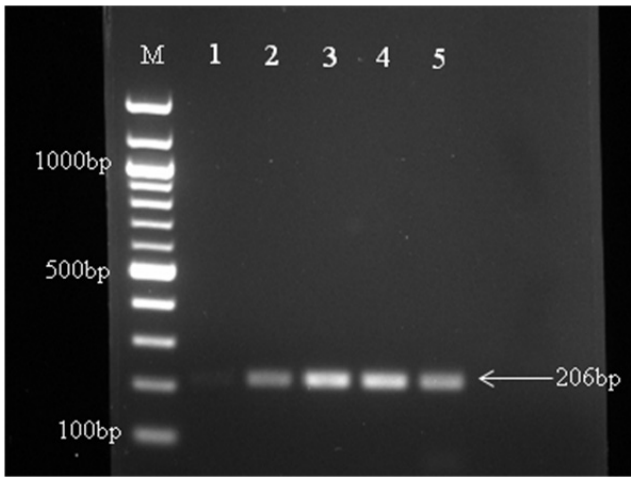

Fig. 2. Gradient PCR for *S. agalactiae,* annealing temperature from 52.5°C to 55°C. Lane M: 100bp; lane 1: 52.5°C; lane 2: 53°C; lane 3: 53.6°C; lane 4: 54.2°C; lane 5: 55°C

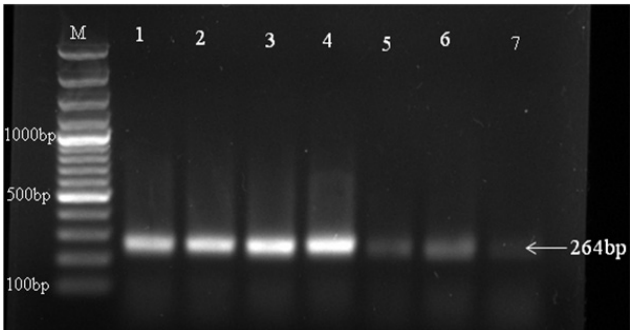

Fig 3. Gradient PCR for *S. dysgalactiae*, annealing temperature from 51°C to 56°C. Lane M: 100bp; lane 1: 51°C; lane 2: 51.8°C; lane 3: 52.9°C; lane 4: 54.3°C; lane 5: 54.9°C; lane 6: 55.5°C; lane 7: 56°C

denaturation at 94°C for 1 min, annealing at 54°C for 60 sec and extension at 72°C for one min followed by final extension at 72°C for 10 min. By multiplex PCR,amplified products of 206 bp, 264 bp, 420 bp and 662 bp corresponded to *S. agalactiae, S. dysgalactiae, S. aureus* and *E. coli*, respectively (Fig. 5). Mixed infections were also detected and relative frequency of different combinations of infections is shown in Table 2.

Multiplex PCR assay can positively check mastitis milk samples which may not show any bacterial growth under conventional culture conditions (Taponen and Pyorala 2009) due to the presence of very low number of pathogens or

Table 2. Samples showing mixed infection and type of organisms detected using multiplex PCR assay

Combination of organisms isolated	Number of mixed infection
S. aureus + *S. dysgalactiae*	7 (43.75%)
S. aureus + *S. agalactiae*	2(12.5%)
S. aureus + *E. coli*	4 (25%)
S. dysgalactiae + *E. coli*	2 (12.5%)
S. aureus + *S. dysgalactiae* + *E. coli*	1(6.25%)
Total	16

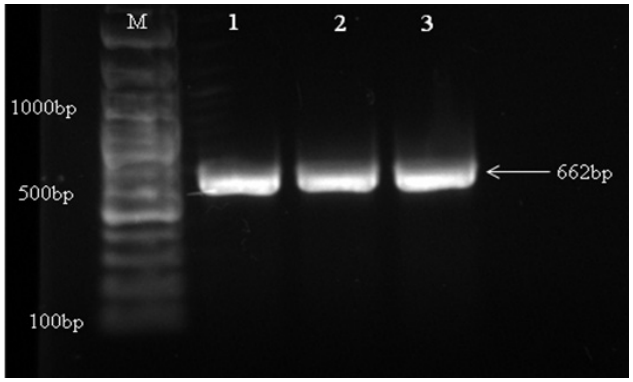

Fig. 4. Gradient PCR for *E. coli*, annealing temperature from 53.5°C to 56°C. Lane M: 100bp; lane 1: 53.5°C; lane 2: 54.8°C; lane 3: 56°C

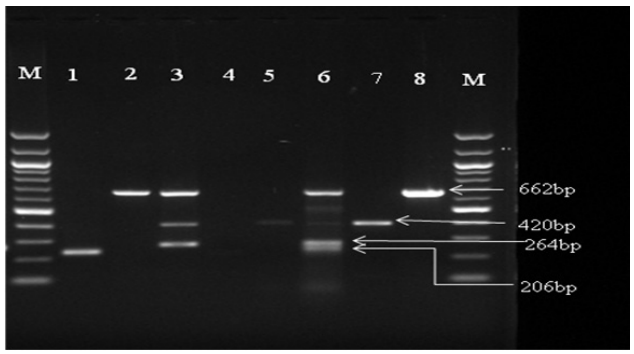

Fig. 5. Multiplex PCR for simultaneous detection of *S. aureus, S. agalactiae, S. dysgalactiae* and *E. coli.* lane M: 100bp ladder; lane 1: Sample positive for *S. agalactiae;* lane 2, 8: Sample positive for *E. coli*; lane 3: *s*ample positive for *S. aureus, E. coli* and *S. dysgalactiae;* lane 4: Negative control; lane 5, 7: samples positive for *S. aureus;* lane 6: Sample positive for *S. aureus, S. agalactiae*, *S. dysgalactiae* and *E. coli.*

residual therapeutic antibiotic concentration inhibiting growth of microbes.

Only few researchers have carried out multiplex PCR assay for diagnosis of mastitis pathogens in milk. None of these workers developed PCR assay for simultaneous detection of *S. aureus*, *S. agalactiae*, *S. dysgalactiae* and *E. coli* directly from buffalo milk. For differentiation of *S. agalactiae* and *S. dygalactiae*, Phuektes *et al*. (2001) used primers yielding products of 6 base pair difference only. In our study, we could more clearly differentiate these organisms by using specific primer sets whose amplified products varied in size more than 50bp. Pradhan *et al*. (2011) amplified traT gene for *E. coli*, 16S–23S rRNA for *S. aureus* and genus specific 16S rRNA for *Streptococcus* spp. Amin *et al*. (2011) carried out multiplex PCR assay for detection of *S. aureus*, *S. agalactiae* and *E. coli* directly from milk but did not consider *S. dysgalactiae* under their study. Shome *et al*. differentiated 5 species of *Staphylococcus* genus (2012a) and *S. agalactiae*, *S. uberis* and *S. dysgalactiae* (2012b) using multiplex PCR assay in bovine subclinical mastitis.

In conclusion, multiplex PCR assay standardized and

performed on buffalo milk samples was rapid, sensitive and specific assay for simultaneous detection of *S. agalactiae*, *S. dysgalactiae*, *S. aureus* and *E. coli*. Multiplex PCR assay can serve as a screening test for epidemiological study, for accurate and timely treatment of mastitis and for formulating the strategy for prevention and control of mastitis.

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