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. 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

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 organisms which on preliminary examination were found to be streptococci, were further characterized into coagulase positive and coagulase negative types, on the basis of coagulase production.

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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Oligonucleotide primers: All oligonucleotide primers (desalted) used for PCR were selected from published sequences targeting 16S - 23S rRNA interspace region and procured from Integrated DNA technology (IDT) (Table 1). Primers used in multiplex PCR assay were chosen to have approximately similar melting temperatures (Tm). 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 1x 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](image-url)
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.

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

<table>
<thead>
<tr>
<th>Combination of organisms isolated</th>
<th>Number of mixed infection</th>
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<tbody>
<tr>
<td><em>S. aureus</em> + <em>S. dysgalactiae</em></td>
<td>7 (43.75%)</td>
</tr>
<tr>
<td><em>S. aureus</em> + <em>S. agalactiae</em></td>
<td>2 (12.5%)</td>
</tr>
<tr>
<td><em>S. aureus</em> + <em>E. coli</em></td>
<td>4 (25%)</td>
</tr>
<tr>
<td><em>S. dysgalactiae</em> + <em>E. coli</em></td>
<td>2 (12.5%)</td>
</tr>
<tr>
<td><em>S. aureus</em> + <em>S. dysgalactiae</em> + <em>E. coli</em></td>
<td>1 (6.25%)</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
</tr>
</tbody>
</table>

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. dysgalactiae*, 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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REFERENCES


