POLYMERASE CHAIN REACTION ASSAY FOR DIAGNOSIS OF ESCHERICHIA COLI MASTITIS IN MURRAH BUFFALOES

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Abstract: Mastitis is a multietiological disease of lactating animals responsible for huge economic losses throughout the world. E. coli is the most common cause of severe clinical form of mastitis. Early and correct detection of E. coli infection by suitable test(s) is imperative for timely initiation of treatment. In the present study, PCR assay was standardized and applied on total of 202 quarters milk samples of 52 Murrah buffaloes for detection of E. coli. Results of PCR assay were compared with cultural examination. Sixty four samples showed presence of E. coli by using 16S to 23S rRNA specific primers whereas on culture examination only 57 samples were found positive for E. coli. This might be due treatment being going on or presence of very small number of organisms being present. The PCR assay has been found to be rapid, sensitive, reliable and less labour intensive. This method may be used as an alternative to culture examinations or can also be used in conjunction with cultural examination so that antimicrobial sensitivity can be carried out for initiation of treatment with suitable antibiotics.

Key words: Murrah buffaloes, Mastitis, E. coli

INTRODUCTION

Mastitis is considered to be economically the most important disease of dairy cattle [1] in India and worldwide [2]. It has been established that mastitis can be caused by about 200 microorganisms [3]. Mastitis pathogens can be divided into contagious and environmental. Studies conducted in different parts of India [4-6] have reported that E. coli to be the most prevalent among different environmental organisms causing mastitis. Mastitis triggered by E. coli is usually sporadic but most of the times it is acute or peracute showing clinical signs varying from very severe to fatal form even leading to death. E. coli is also capable of producing subclinical infections that persists for longer periods of time. These infections can also prove detrimental to the calf born to infected mother as can cause colibacillosis in suckling neonates [7]. Zoonotic importance of E. coli mastitis cannot be ignored because of potential health hazard and food toxin infections in human beings.

Bacteriological culturing of the milk can be used to detect E. coli. However, it is not uncommon to get no growth when culturing abnormal milk from coliform infections. Moreover, in severe clinical cases the results will not be known in time to affect the treatment. As culture-based methods are slow and labour intensive, they are not ideal for the analysis of the large numbers of samples that would be tested when possible environmental sources of an outbreak are being investigated. Molecular approaches for bacterial detection avoid the need for culture and can be designed to be specific

Timely detection of E. coli by rapid, sensitive and
specific technique is imperative. The objective of the present study was to standardize 16S to 23S rRNA intergenic spacer based polymerase chain reaction assay for diagnosis of E. coli directly from milk of Murrah buffaloes.

**MATERIAL AND METHODS**

**Sample collection** - A total of 202 clinical milk samples collected from functional quarters of 52 lactating Murrah buffaloes brought from various parts of Haryana and adjoining area of Rajasthan to the Veterinary College Central Laboratory, COVS, LUVAS, Hisar, were included in the present study.

**Bacteriological examination:** Ten microlitres of milk from each sample were inoculated on 5% defibrinated sheep blood agar and MacConkey’s lactose agar (MLA) and incubated for overnight at 37°C [8]. The bacterial isolates were purified by picking single colony and subculturing on fresh 5% sheep blood agar plates. On the basis of Gram’s staining and growth characteristics on MLA, E.M.B agar and T.S.I agar and biochemical reactions namely IMViC test, nitrate reduction, urease and Oxidative Fermentation test the isolates were identified as E. coli and the isolates were also confirmed by PCR assay.

**Extraction of DNA from milk:** DNA was extracted by SDS-Phenol–chloroform isoamyl alcohol method [9] with some minor modifications. In this study milk sample (1.5 ml) was centrifuged at 12000 g for 30 minutes at 4°C and the upper layer of fat was removed by sterile cotton swab. Pellet was resuspended in 600µl NTE buffer. After shaking vigorously, the suspension was treated with 100µl of 24% SDS (final concentration) and incubated in water-bath at 80°C for 10 minutes. Then digestion with 12µl of proteinase K (20mg/ml) (Finnzymes) and 2.5µl of RNase A (Fermentas) followed by incubation at 56°C for two hours was done. After that 100µl of 5M NaCl and 80µl of CTAB-NaCl was added and each tube was vortexed in order to mix the contents of the tubes evenly and incubated in water-bath at 65°C for 10 minutes. Then digestion with 12µl of proteinase K (20mg/ml) (Finnzymes) and 2.5µl of RNase A (Fermentas) followed by incubation at 56°C for two hours was done. After that 100µl of 5M NaCl and 80µl of CTAB-NaCl was added and each tube was vortexed in order to mix the contents of the tubes evenly and incubated in water-bath at 65°C for 10 minutes. Equal volume of saturated phenol: chloroform: isoamyl alcohol (PCI) mixture (25:24:1) was added after cooling to room temperature. Tube was then vortexed and centrifuged at 12,000 g for 10 min using refrigerated centrifuge. The upper aqueous layer was carefully transferred to a fresh sterile micro centrifuge tube without disturbing the interface. The PCI extraction was repeated until the interface was clear. The resultant aqueous solution was extracted with equal volume of chloroform: isoamyl alcohol (CI) (Amersco) mixture in the ratio of 24:1. The aqueous solution obtained after centrifugation at 12,000 g for 10 minutes was then transferred to a fresh micro centrifuge tube and one-tenth volume of 3M sodium acetate (pH 5.2) and two volumes of chilled absolute ethanol were added and kept at -20°C for 45 min for precipitation of DNA. After centrifugation at 15,000 g for 15 min at 4°C, ethanol was removed by carefully inverting the tubes without disturbing the pelleted DNA. The DNA pellet was then washed twice with 70% ethanol and air-dried. Finally pellet was dissolved in 50µl of TE buffer (10mM Tris HCl, 5mM EDTA [pH8.0]) and stored at -20°C till further use. The concentration the DNA obtained directly from milk was measured using Quibit 2.0 fluorometer (Invitrogen).

**DNA extraction from bacterial culture isolates:** For extraction of DNA from bacterial culture isolates, the rapid boiling method was followed. In brief, a single colony from overnight grown culture was inoculated in 25µl of TE buffer and boiled at 99°C for 15 minutes and then cooled immediately by putting on ice. The resultant template DNA was stored at -20°C and 5µl of each sample was used for PCR analysis.

**Primers:** PCR primers were designed from highly divergent and species specific regions of the DNA coding for 16S and 23S rRNA (16S or 23S rRNA). Genes encoding rRNA were used as target sequences rather than genes encoding mRNA because of the signal enhancement due to the presence of several copies of genes encoding rRNA in the genome. The sequences, specificities, and G+C contents are summarized in Table 1. These primers were synthesized by Integrated DNA Technology.

**Polymerase chain reaction (PCR) assay:** Assay was standardized using master mix (Qiagen) for 50 µL reaction using gradient PCR. The amplification was performed in a thermal cycler PCR was performed in a 50 µl volume containing 25 µl of Top Taq master mix (Qiagen), 1 µl of each primer (10 µM), 5 µl of extracted DNA and 18 µl of DNAse free water. Appropriate positive and negative controls were kept in the PCR. The negative control
Fig. 1: Conventional PCR assay showing 662bp band specific to 16S to 23S rRNA using ECO-2083 and ECO-2745 Primers

Fig. 2: Conventional PCR assay showing 232bp band specific to 16S to 23S rRNA using ECO-223 and ECO-455 Primers
consisted of nuclease free water instead of DNA template while positive control consisted of DNA extracted from bacterial culture.

**Analysis:** After amplification, 10 µL of the reaction mixture was electrophoretically analysed in a 1.5% agarose gel at 90 volts for 60 min in 1X TAE buffer using mini gel electrophoresis assembly, having ethidium bromide (0.5 µg/mL). The amplified product was visualized as a band of expected size under UV light and documented by a gel documentation system (BioRad).

**Specificity:** Specificity of PCR primers was checked with amplification of DNA isolated from milk samples infected with *E. coli*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Staphylococcus aureus*.

**RESULTS AND DISCUSSION**

*E. coli* is one of the major infectious agents which can be transmitted by environment and then establishes clinical, detrimental and periodically reactivating infections in the host. In this study, *E. coli* was successfully detected in milk from mastitis cases. Partial sequences of 16S or 23S rRNA gene were successfully amplified by PCR assay in positive control and positive cases of mastitis having product of 662 bp and 232 bp respectively (Figs. 1,2). The Standardized cycling conditions consisted of an initial denaturation step at 94°C for 2 minutes, followed by 30 cycles of 94°C for 1 minute, 53°C for 1 min, 72°C for 1 min and a final extension at 72°C for 10 minutes (Tables 2,3). A total of 202 milk samples from 52 Murrah buffaloes were collected and processed. Out of these 64 samples were showing positive results for detection of *E. coli* by PCR assay with both the primers. Primers were also found to be specific for *E. coli* as no cross reaction was seen with primers coding for other bacteria. For Eco 223 and Eco 455 primer set optimum annealing temperature was 64°C by Riffon [10] while we observed best result in the range of 52-58°C. For both the primers, Riffon and co-workers [10] used 100µl reaction mixture with 5µM primer concentration and 35 cycles of PCR and got faint bands. In contrary to their study we got dark band using 50 µl reaction mixture, 1µM primer concentration and 30 cycles of PCR. By bacteriological examination 189 milk samples revealed isolation of 209 organisms of which 50 were positive for single *E. coli* infections and 7 samples were of mixed infections having one of the bacteria as *E. coli*. All the milk samples found positive by bacteriological examination were also showing positive reaction for detection of *E. coli* by PCR assay whereas 7 more samples which were negative by culture examination showed positive reaction by PCR assay. All the milk samples detected negative for *E. coli* with PCR assay were also found to be negative by culture examination. Results showed that PCR assay provides a useful approach for the detection of pathogens in milk samples. This method may be used as an alternative to culture examinations assay or can also be used in conjunction with cultural examination so that antimicrobial sensitivity can be carried out for initiation of treatment with suitable antibiotics.

The host defence of the bovine mammary gland has been shown to be efficient in controlling and eliminating *E. coli* infection [11]. Once coliform bacteria enter the mammary glands, they either multiply rapidly or remain dormant. In mammary gland, activation of host defence system results into phagocytosis and lysis of bacteria by neutrophils.

### Table 1: Showing sequence of Primers used with product size, published source and G+C content

<table>
<thead>
<tr>
<th>Primers* eco 2083 and eco 2745</th>
<th>Sequence</th>
<th>Product size</th>
<th>Published source</th>
<th>G+C content</th>
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<tbody>
<tr>
<td>Eco 2083 and Eco2745 F: GCTTGACACTGACATTGAG. R: GCA CTT ATC TCT TCC GCA TT</td>
<td>662bp</td>
<td>Rifton et al., 2001</td>
<td>52%</td>
<td></td>
</tr>
<tr>
<td>Eco 223 and Eco 455 F: ATCAACCGAGATTCCCCAGT R: TCATATCG GTCAGTCAGGAG</td>
<td>232bp</td>
<td>Rifton et al., 2001</td>
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### Table 2: Conditions for amplification of Eco 2083 and Eco 2745

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<th>Time (minutes)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>Initial denaturation</td>
<td>94</td>
<td>2</td>
</tr>
<tr>
<td>30</td>
<td>Denaturation</td>
<td>94</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Primer annealing</td>
<td>52-58</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Primer extension</td>
<td>72</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>Final extension</td>
<td>72</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>Final hold</td>
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### Table 3: Conditions for amplification of Eco 223 and Eco 455

<table>
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<th>Time (minutes)</th>
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<tr>
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<tr>
<td>30</td>
<td>Denaturation</td>
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</tr>
<tr>
<td>1</td>
<td>Final hold</td>
<td>4</td>
<td>∞</td>
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</table>
leading to release of endotoxin in milk. In these cases *E. coli* may not be isolated from milk by culture examination but can be detected by PCR assay. However, this host defence ability has been shown to be less effective during early lactation, due to deficiencies in neutrophil function and number [12]. In such cases *E. coli* can be detected by cultural examination.

A good management plan should always include bacteriologic culturing of the milk to help confirm the nature of the infection and thus the prevalence of specific organisms in the herd. Bacterial cultures are the single best means to accurately diagnose mastitis, and accurate diagnosis is very important in any management plan. But in case of *E. coli* mastitis PCR assay is better as it is less time consuming and can serve as a good screening test of large herd. PCR assay can also diagnosed negative samples by culture which might be due to treatment being going on and presence of dead microorganism [10].

**CONCLUSION**

Based on the results of the present study, it can be concluded that PCR assay being accurate, rapid, reliable, sensitive and less cumbersome can be used for diagnosis of mastitis caused by *E. coli*.

**REFERENCES**
