Molecular Detection of *Escherichia coli* from Mastitic Milk of Crossbred Cows

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**Introduction**
Mastitis is considered to be the costliest disease of the dairy industry throughout the world. Mastitis pathogens have been divided into contagious and environmental organisms. *E. coli* is considered to be opportunistic pathogen and originate from cow’s faecally contaminated environment and infect the udder via teat canal (4). The public health importance of presence of *E. coli* cannot be undermined since several virulence factors of *E. coli* causes urinary tract infections, diarrhea, septicemia and meningitis in humans and animals (3). Clinical microbiologists have traditionally been involved in isolation of bacteria in pure culture and performing biochemical or immunological tests for detection of *E. coli*. These tests are labour intensive and time consuming and may give false negative results due to presence of residual antibiotics Also, the presence of leucocytes in milk samples with high somatic cell count also potentially inhibit growth of bacteria (5). The application of molecular diagnostic technique such as Polymerase chain reaction assay (PCR) is based on unique signature sequences in the nucleic acid of pathogen and subject to less variability providing rapid, sensitive and specific diagnosis of pathogens (2). The present study was planned to standardize polymerase chain reaction assay for detection of *E. coli* from mastitic milk of crossbred cows directly.

**Materials and Methods**
A total of 113 milk samples collected from functional quarters of crossbred cows (Hariana X Holstein Friesian with mixed crosses of Jersey, Sahiwal and Brown Swiss) received in College Central laboratory, College of Veterinary Sciences, CCS Haryana Agricultural University, Hisar were included in the present study. A portion of milk sample to be tested was collected into sterilized tubes and frozen at -20°C until tested for PCR. Isolates obtained on cultural examination of milk samples were further confirmed as *E. coli* for colony characteristics, morphology, Gram’s reaction and haemolysis patterns.

**DNA Extraction from milk:** DNA was extracted by SDS-Phenol–chloroform isoamyl alcohol method (5) with some modifications. Mastitic milk (1.5 ml) was centrifuged and the upper layer of fat was removed. Pellet was resuspended in 600µl NTE buffer (0.1M NaCl, 20mM Tris-HCl and 1mM EDTA). After vortex, the suspension was treated with 100µl of 24% SDS and incubated in water-bath at 80°C for 10 minutes. This was then digested using 12µl of proteinase K (20 mg/ml) (Finnzymes) and 2.5µl of RNase A (Fermentas, USA) and incubated in water-bath at 56°C for 2 hours. 100µl of 5M NaCl and 80µl of CTAB-NaCl was then added and incubated in water-bath at 65°C for 10 minutes. The resultant was treated with an equal volume of saturated phenol:chloroform:isoamyl alcohol (PCI) mixture (25:24:1) The PCI extraction was repeated until the interface was clear. The resultant aqueous solution was extracted with equal volume of chloroform:isoamyl alcohol mixture in the ratio of 24:1. The resultant aqueous phase was collected and one-tenth volume of 3M Sodium acetate (pH 5.2) and two volumes of chilled 100% ethanol were added and kept at -20°C for 45 minutes for precipitation of DNA. After centrifugation at 15000 g for 15 minutes at 4°C, the DNA pellet obtained was then washed twice with 70% ethanol and air-dried. Finally DNA was dissolved in 50µl of in TE buffer (10mM Tris HCl, 5mM EDTA) and stored at -20°C till further use. The purity and concentration of the DNA isolated was measured using biophotometer (Eppendorf, Germany).

**Standardization of PCR and analysis of PCR products:** Sequences of two oligonucleotide primers of 21 bases were as follows:

ATCAACCAGATTCCCCCAGT
And TCACTATCGTGAGTCAAGGAG

These primers were selected from published sequences (6) and synthesized from Operon Biotechnologies, Germany. PCR reactions were standardized using different magnesium chloride concentrations, *Taq* DNA polymerase concentrations, primer concentrations, annealing temperature and number of cycles in thermocycler (Bio-Rad icycler,USA). PCR products were analyzed in two per cent agarose gel containing 0.2µg/ml ethidium bromide in Tris borate EDTA (TBE) electrophoresis buffer at 6.5 V/cm for one hour with 100 bp ladder (Fermentas, USA) as marker. Products were visualized by ultraviolet light transillumination. Sensitivity of PCR primers was evaluated by using different dilutions (CFU/ml) of bacteria. Specificity of PCR primers was checked with amplification of DNA isolated from milk samples infected with *Staphylococcus aureus*, *Streptococcus agalactiae*, *Streptococcus dysgalactiae* and *Streptococcus uberis*. 

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Results and Discussion

PCR assay was standardized using different MgCl\(_2\) concentrations, Taq DNA polymerase concentrations, primer concentrations, annealing temperature and number of cycles in thermocycler. The optimized PCR reaction mixture contained 200\(\mu\)m dNTP mix, 1X PCR buffer (with 10mM Tris-HCl, pH 8.8, 50mM KCl and 0.8% Nonidet P 40), 1.5 mM MgCl\(_2\), 2.5 U Taq DNA polymerase, 20 picomoles of each primers, 200 ng of DNA extracted from milk and nuclease free water added to make reaction mixture 25 \(\mu\)l. Optimized conditions for PCR amplification obtained were as follows: initial denaturation at 95\(^{\circ}\)C for 5 minutes, 36 cycles each of denaturation at 95\(^{\circ}\)C for 1 minute, annealing at 64\(^{\circ}\)C for 45 seconds and extension at 72\(^{\circ}\)C for 7 minutes. PCR Amplification at optimized conditions yielded amplified product of approximately 232 bp (Fig. 1) which was similar as reported earlier (6). Out of 113 milk samples examined, 38 milk samples were identified to be positive for presence of \textit{E. coli} on basis of bacteriological examination, Gram’s reaction and haemolysis patterns. When samples were screened by PCR, 40 samples were found positive and none of the culturally positive milk sample was found negative. The two culturally negative samples found positive by PCR in present study were from animals which were treated with antimicrobials. In these cases, negative cultures might be due to presence of residual antibiotics that may render bacterial strains nonviable. Our study is in accordance to previous reports in which etiological agents of culturally negative samples were identified by molecular analysis (1, 7). Milk samples found positive for other mastitis pathogens like \textit{Staphylococcus aureus}, \textit{Streptococcus dysgalactiae}, \textit{Streptococcus agalactiae}, and \textit{Streptococcus uberis} revealed negative results with PCR assay showing 100 % specificity. In our study, amplification has been successfully done with DNA isolated from mastitic milk of crossbred cows directly. Initially the sensitivity was found to be quite low (10\(^4\) CFU/ml) possibly due to presence of PCR inhibitors present in milk (8) but high SDS concentration, repeated extraction of bacterial DNA by phenol chloroform isoamyl alcohol to remove possible PCR inhibitors and increasing concentration of Taq DNA polymerase concentration (upto 2.5 Units) attributed successful amplification of DNA isolated directly from mastitic milk with high sensitivity (10\(^5\) CFU/ml).

In conclusion, PCR was found to be rapid, specific and sensitive assay for detection of \textit{E.coli}. This test can be completed within hours and avoids cumbersome, time consuming and lengthy steps involved in microbiological culture of milk and biochemical tests and provides a promising option as a screening test for a large herd to detect \textit{E. coli} directly from mastitic milk of crossbred cows.

References

![Fig. 1 Ethidium bromide stained agarose gel showing specificity of PCR assay for detection of \textit{E.coli} from mastitic milk of crossbred cows](image)

L: 100 bp molecular marker
1: Control Positive, known culture of \textit{E.coli}
2: DNA isolated from mastitic milk positive for \textit{E.coli}
3: \textit{Staphylococcus aureus} ATCC 25923
4: \textit{Streptococcus agalactiae}
5: \textit{Streptococcus dysgalactiae}
6: \textit{Streptococcus uberis}
7: Control Negative with Nuclease free water