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## Detection of enterotoxigenic *E. coli* in neonatal diarrhoeic calves using PCR technique

**Satish Kumar, VS Rajora and Nidhi Arora**

### Abstract

*Escherichia coli* is incriminated as one of the important causal agent of mortality of new born dairy calves all over the world and reports from India indicate that this disease is prevalent even on organized dairy farms throughout the country. Diarrhoea producing *E. coli* possesses colonization antigens or adhesions that enable the bacteria to colonize the small intestines. The detection of *E. coli* enterotoxin provides the evidence that enterotoxin - producing *E. coli* (ETEC) has an etiological role, the expression of K99 fimbriae (or F5 ETEC) accounts for nearly all cases of ETEC infection found in newborn calves. In present study out of total 16 *E. coli* isolated samples, three isolates (18.75%) were found to be positive for the presence of lethal toxin (LT) gene, which PCR has shown 450bp amplified product confirming ETEC strain of *E. coli*.

**Keywords:** *Escherichia coli*, diarrhoea, PCR, lethal toxin

### 1. Introduction

Calf scour, the specific condition in which *E. coli* is incriminated as a causal agent, is the main cause of mortality of new born calves in dairy herds all over the world and reports from India indicate that this disease is prevalent even on organized dairy farms throughout the country. Enterotoxigenic *E. coli* (ETEC) infection is the most common type of colibacillosis of young animals primarily in pigs and calves, and it is a significant cause of diarrhoea among travellers and children in the developing world [1]. Neonatal calf diarrhoea remains an important cause of morbidity and mortality in young calves. *E. coli* was isolated from the faeces of neonates for the first time by Escherich as *Bacterium coli*. Molecular technique was used to investigate the prevalence of virulent diarrheic genes in pathogenic *Escherichia coli* and their antibiotic sensitivity patterns [2]. Diarrhoea producing *E. coli* possesses colonization antigens or adhesions that enable the bacteria to colonize the small intestines [3]. A number of diagnostic tests are currently available for detecting ETEC including: Double-antibody enzyme-linked immunosorbent assay (ELISA), DNA gene probes specific for genes encoding toxins and adhesions of ETEC, multiplex polymerase chain reaction (PCR) for the rapid screening of ETEC toxins, and monoclonal antibody-based co-agglutination test [4]. Pourtaghi *et al.* [5] estimated the prevalence of Enterotoxigenic *Escherichia coli* (ETEC) in calves' diarrhoea samples and detected 11% and 3.9% in diarrhoea samples by mPCR and ELISA methods respectively. The expression of K99 fimbriae (or F5 ETEC) accounts for nearly all cases of ETEC infection found in newborn calves [6]. The detection of *E. coli* enterotoxin provided the evidence that enterotoxin - producing *E. coli* (ETEC) had an etiological role in enteric colibacillosis. The present study was, thus undertaken with the objective of Isolation of *E. coli* and cultural examination and detection of enterotoxigenic *E. coli* in neonatal diarrhoeic calves using pcr technique in cases of neonatal diarrhoea at Instructional Dairy Farm of the university.

### 2. Materials and Methods

A total of 67 faecal samples collected from neonatal diarrhoeic calves were examined for the presence of *E. coli*. The samples were collected from the neonatal diarrhoeic calves of Instructional Dairy Farm of the GB Pant University of Agriculture and Technology, Pantnagar. Faecal material was picked up from the rectum with the gloved hands in the sterilized culture bottles. The faecal samples, collected in 1-2 gm quantities, were shifted to the laboratory for further processing. McConkey's lactose agar was used as differential media.

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### 2.1 Isolation of *E. coli*

MacConkey's lactose agar plates were directly streaked with the collected material and incubated at 37 °C for 24 hours. Colony and growth characteristics were studied. A single colony was picked up and inoculated into BHI (Brain Heart Infusion) broth for challenge studies. The suspected colonies were subjected to morphological and staining characters.

### 2.2 Morphology and staining characteristics

Gram's staining method was followed to study the morphology and the staining reactions. The culture was also confirmed by different biochemical test as described for *E. coli*.

### 2.3 PCR based Identification of *E. coli* by targeting LT gene

#### 2.3.1 Extraction of bacterial DNA

Bacterial isolates were grown in nutrient broth at 37 °C overnight. Exactly 1.5 ml of the culture was spin by centrifugation at 5000 rpm for 10 min. The bacterial pellet was lysed by boiling in 150 µl of distilled water in water bath. The lysate was centrifuged again as before and the supernatant was used directly as template for PCR.

#### 2.3.2 Detection of LT gene sequences by PCR

*Escherichia coli* isolates were subjected to PCR for the detection of LT genes. Primers for LT (450 bp) were 5'-GGCGACAGATTATACCGTGC -3' and 5'-CGGTCTCTATATCCCTGTT -3'. This primer pairs amplify LT toxins. Oligonucleotide primers were from ILS India. PCR assay was carried out in a 25µl reaction volume containing 0.25pmol each of LTr and LTf, PCR master mix (Genei) with 2.5 mmol of MgCl<sub>2</sub> and 5 µl of template DNA. The reaction mixtures were amplified with total of 40 cycles, denaturation at 95 °C for 5 min, annealing at 58 °C for 1 min, Extension at 72 °C for 2 min and final extension at 72 °C for 7 min in a (PCR System 9700 "Applied Biosystems"). Amplified PCR products were analysed by gel electrophoresis in 2% agarose containing ethidium bromide. The products were visualized with ultraviolet trans illuminator (UVtech® Genei) and imaged with gel documentation system (GelDoc system "Alphaimager® HP").

### 3. Results and Discussion

Enterotoxigenic *E. coli* (ETEC) is responsible for significant loss of neonatal animals (calves, lambs, suckling piglets and mithun calves) with huge mortality and morbidity [7]. A total

67 faecal samples from neonatal diarrhoeic calves (0-7 days of age) were screened for the presence of *E. coli* out of which 16 samples constituting 23.88% were accounted positive for *E. coli* infection (Fig 1).

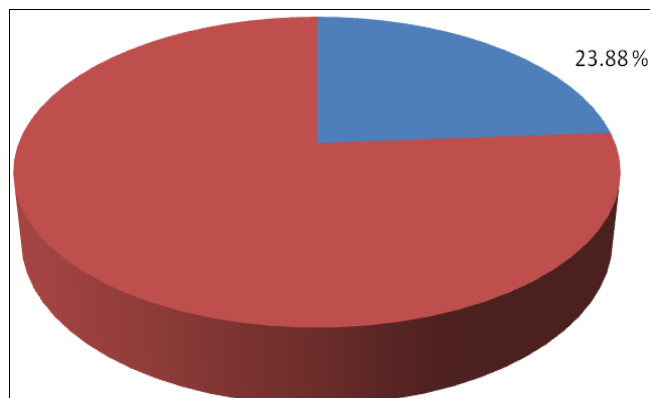


Fig 1: Percent isolation of *E. coli* from diarrhoeic samples

The colonies appeared after incubation for 24 hours at 37°C were observed to be of medium size and bright pink in colour. The smear stained using Gram's staining revealed Gram negative rods. Isolates, which showed metallic sheen on EMB plate (Fig 2) were further tested for IMViC pattern and found to be indole +ve, MR +ve, VP -ve and citrate -ve (Fig 3). The biochemical characteristics observed are presented in table.1.

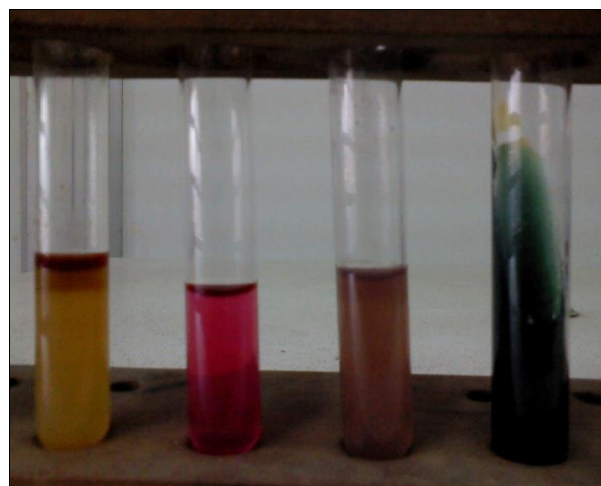


Fig 2: *E. coli* on Eosine Methylene Blue Plate

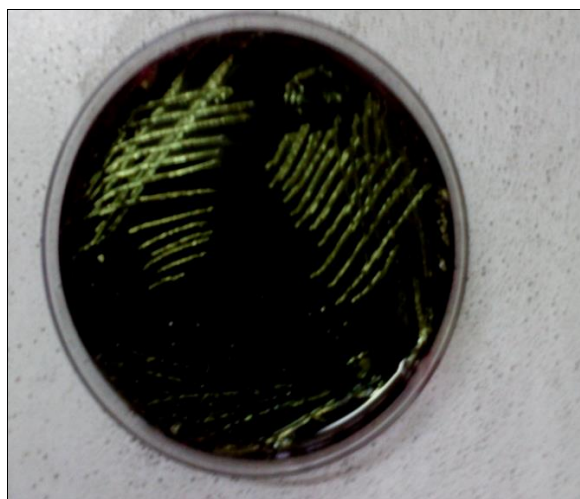


Fig 3: IMViC pattern of *E. coli*

**Table 1:** Biochemical characteristics of *E. coli*

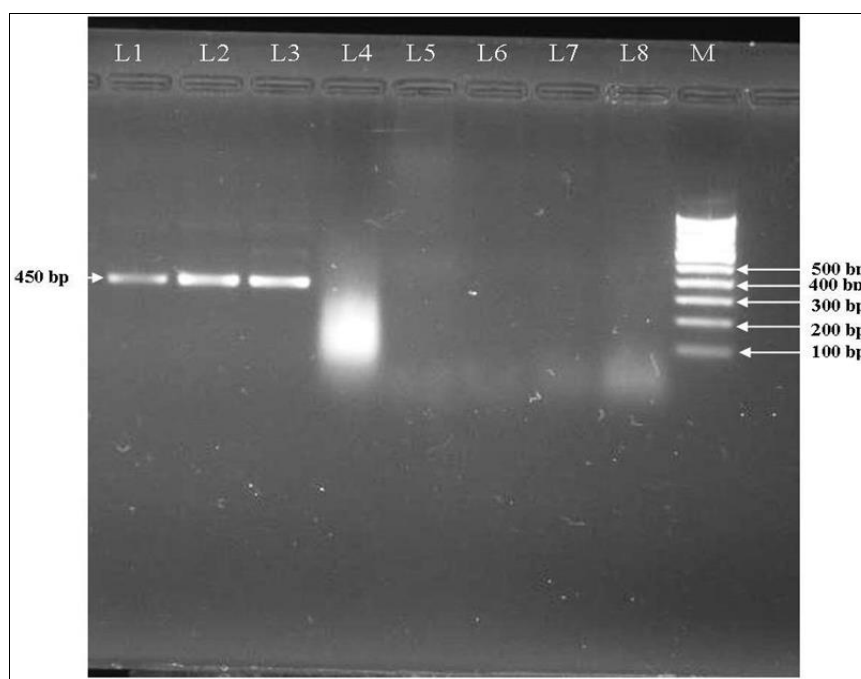
organism	Gas from								
	Indole	Methyl Red	Voges Proskauer	Simmon's citrate	H <sub>2</sub> s(TSI)	Urease	glucose	lactose	sucrose
<i>E. coli</i>	+	+	-	-	-	-	+	+	D

+ = positive, - = negative, D = Delayed positive

### 3.1 PCR based identification of *E. coli* isolates by targeting LT gene

In the present study out of the 16 *E. coli* isolates tested, three isolates (18.75 %) were found positive for the presence of LT encoding gene, which PCR has shown 450 bp amplified product hence confirming ETEC strain of *E. coli* (Fig 4). This finding is in agreement of Singh *et al.* [8] in buffalo calves (20%) and Acha *et al.* [9] in cow calves (16%). Whereas *E. coli* other than ETEC strain has not show targeted LT gene. The simplex PCR assays presented in this study correctly determined the presence of ETEC *E. coli* in all positive tested samples. Pourtaghi *et al.* [5] reported the prevalence of three virulence factors including K99 (F5), F41 and STa was similar to each other (11%) which was approximately corroborate with the result reported by Younis *et al.* [10] (10.36 %). On the contrary, lower prevalence 3.86% of ETEC K99 was recorded by Kanwar *et al.* [11]. The difference in the prevalence from those recorded might be possibly due to variations in region, managerial conditions and hygienic

measures. Rigobelo *et al.* [12] reported higher prevalence rate of *E. coli* carrying genes for ST (25.4%) and LT (13.2%) enterotoxins from diarrhoeic cow calves in Brazil. Salvadori *et al.* [13] also recorded 3.9% ETEC possessing ST (Heat stable) and LT enterotoxin genes from diarrhoeic calves by PCR in Brazil. Nishikawa *et al.* [14] isolated 67 strains of *E. coli* isolates from 66 specimens which were identified as putative diarrhoeic and causal agents. Among 67 strains of *E. coli*, four (6%) isolates were found to be ETEC positive with amplified product at 132 bp with PCR. Bandyopadhyay *et al.* [15] conducted a study on, 107 faecal swabs collected from diarrhoeic lambs, among them only 73% were found to possess *E. coli*. Total 234 *E. coli* were isolated and further subjected to multiplex PCR for the study of virulence gene(s) profile of STEC (Sheigella like enterotoxin producing *E. coli*) and ETEC. Out of the 234 isolated *E. coli*, 74 (32%) were found positive for STEC, and 22 (9%) were carrying virulent gene(s) for ETEC.



**Fig 4:** Simple x PCR Assay Targeting LT gene of *E. coli* from neonatal diarrhoeic samples lan 1 to 3 ETEC and lan 4 to 7 other *E. coli* and lan 8 nuclease free water as negative control, lan M, DNA size molecular markers (100-bp ladder).

### 4. Conclusion

In the present study, the detection of *E. coli* enterotoxin provides the evidence that enterotoxin - producing *E. coli* (ETEC) has an etiological role in neonatal diarrhea. Out of the 16 *E. coli* isolates tested, three (18.75%) isolates were found to be positive for the presence of lethal toxin (LT) gene through PCR technique which has shown 450bp amplified product confirming ETEC strain of *E. coli*.

### 5. Acknowledgements

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