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Detection of VTEC and simultaneous identification of associated virulent genes from animal clinical samples

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Abstract
E. coli is known to be one of the most ubiquitous organisms. Consequently, its pathogenesis is also seen quite frequent in wide range of hosts but the intensity may differ enormously regardless of its serotype, quantity, virulence, immunity of the host etc. Recently Verotoxic *E. coli* (VTEC) has created a great havoc in both livestock and mankind and emerged as one of the major foodborne zoonosis which causes hindrance in economy. Therefore, diagnosis of pathogenic *E. coli* has become an utmost important as this helps in early detection of the pathogen. Besides, molecular analysis of *E. coli* genes leads to more specific and confirmed detection of the bacteria. Among the 70 different clinical samples from animals tested for *E. coli*, 29 showed positive whereas 2 of them showed positive for VTEC. 1 of them having *stx*$_2$ gene and another 1 showed *stx*$_1$+*stx*$_2$+*hlyA* gene combination. As a result, prevention remains the best option to control and uproot the zoonosis.

Keywords: VTEC, *E. coli*, *stx*1, *stx*2, *hly*A

Introduction
Entero-Haemorrhagic *E. coli* has recently emerged as a major food-borne zoonotic pathogen acknowledged across the globe. Its transmission mainly occurs by consumption of contaminated food and during outbreaks, even person to person contact of the pathogen is also well documented. EHEC (also known as VTEC) exhibits pathogenesis radially by producing one or more exotoxins (VT-1, VT-2, VT-2 variant) having cytopathic effects [10]. Further, *E. coli* O157 (VTEC) inhibits protein synthesis, attachment of toxin to enterocytes and causes cell injury leading to Haemorrhagic Colitis (HC) and Haemolytic Uremic Syndrome (HUS) [3, 4]. Besides, virulence factors *viz*., shiga toxins (*stx*$_1$ and *stx*$_2$), enterohemolysin (*hly*A) and intimin (*eae*A) of *E. coli* O157 put a cherry on the cake as these are responsible for the development of STEC dependent diseases. Among these, stx$_2$ is 1,000 times more cytotoxic than stx$_1$. Therefore, stx$_2$ has more significant effect on the occurrence of HUS in humans [13, 9]. In this present study, detection of VTEC in various animal clinical samples been carried out and thereafter presence of virulent genes in the *E. coli* positive samples has been detected for more specific determination of the cause using molecular technique (PCR method). Hence, clear and prominent preventive and curative measures can be practiced to cease further promulgation of the disease.

Materials and Methods
Sampling
A total of 70 animal clinical samples (30 faeces, 20 pus and 20 urine samples) were collected aseptically for the detection of Verotoxic *E. coli*. Faecal and pus samples were taken sterile swab stick and carried in capped tubes having Trypticase Soy Broth (TSB) whereas urine samples in the centrifuge tube (15ml) and brought to the laboratory in ice. Further proceedings were carried out within 3 hours of collection.

Isolation and Identification of *E. coli* isolates
E. coli Isolation: For primary isolation of *E. coli*, swabs were enriched in TSB. The faecal samples were incubated at 37 °C for 6 hours and others at 37 °C for 16-24 hours. For selective plating, selective inoculum from this enrichment was streaked on McConckey Lactose Agar

and incubated at 37 °C for 24 hours. Lactose fermenting colonies on MLA were further streaked on Eosin Methylene Blue agar and colonies showing characteristic metallic sheen were considered as *E. coli*. All the isolates were stained by Gram's Method to identify morphological characteristics and purity of the isolates. The identification of bacteria was done according to Cruickshank *et al.* [5].

VTEC Identification: The purified cultures of *E. coli* were streaked on CT-SMAC and MUG sorbitol agar plates for primary detection of O157 serotype. The plates were incubated at 37°C for 24 hours and were examined for sorbitol fermentation and fluorescence under UV light.

Molecular Characterization: PCR analysis for the detection of virulence genes, *viz.*, *stx*$_1$, *stx*$_2$, *hlyA*, *eaeA* and *rfb*O157 was carried out as per the method described by Paton and Paton, [11]; Wang *et al.* [14]. For PCR assay, DNA was isolated by Bacterial Genomic DNA Purification kit (Hipura). Primers used for the detection of the selected genes are enlisted below.

Table 1: Details of primers for *stx*$_1$, *stx*$_2$, *eaeA* and *hlyA* genes.

Primers	Sequences (5'-3')	Size of Amplified product (BP)
stx$_1$F	5'-ATAAATCGCCATTCGTTGACTAC-3'	180
stx$_1$ R	5'-AGAACGCCCACTGAGATCATC-3'	
stx$_2$ F	5'- GGCACTGTCTGAAACTGCTCC-3'	255
stx$_2$ R	5'-TCGCCAGTTATCTGACATTCTG-3'	
eaeA F	5'- GACCCGGCACAAGCATAAGC-3'	384
eaeA R	5'- CCACCTGCAGCAACAAGAGG-3'	
hlyA F	5'- GCATCATCAAGCGTACGTTCC-3'	534
hlyA R	5'- AATGAGCCAAGCTGGTTAAGCT-3'	
*rfb*O157 F	5'-CGGACATCCATGTGATATGG-3'	259
*rfb*O157 R	5'-TTGCCTATGTACAGCTAATCC-3'	

***Source*:** [11].

Results and Discussion

E. coli Isolation: Among all 70 animal samples, 29 (41.42%) clinical samples were found positive for *E. coli* in which 14 (48.28%) were detected from faeces, 4 (13.79%) from pus and 11 (37.93%) were acquired from urine samples. Higher prevalence from this outcome was noticed by Savita *et al.* [12] (65.22%), Wani *et al.* [15] (80.2%) and Doregiraee *et al.* [6] (88.8%) whereas a lesser positivity was revealed by Gyles, [8] as 36%.

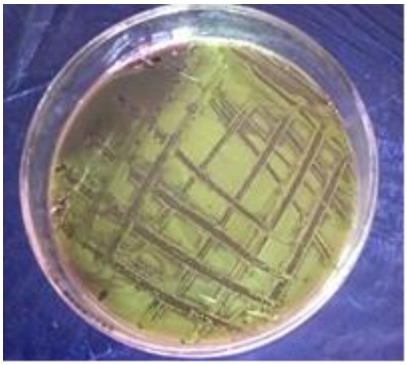

Fig 1: *E. coli* colonies with characteristic metallic sheen on EMB Agar

VTEC Identification: Among these *E. coli* samples, 2 (5.71%) were found positive for pathogenic *E. coli* (VTEC). Of these 2 samples, 1 of each VTEC were revealed in faeces and urine samples respectively which is lower than 7.4% by Cobbold *et al.*, [2]. Compared to the present study a lower detection of 4.81% was made by Chattopadhyay *et al.*, [1]. Variations in prevalence arise may be due to the presence of specific serotype and its role in disease production which in turn depends upon the health status of animals, climatic conditions, variation in feed and water supply along with geographical situation and management strategies.

Gene detection: On further screening of all these 29 positive *E. coli* isolates for detecting *stx*$_1$, *stx*$_2$, *eaeA*, *hlyA* and *rfbO157* genes using multiplex PCR, only 1 faecal sample isolate was found positive for *stx*$_2$ gene alone and 1 urine sample isolate for *stx*$_1$, *stx*$_2$ and *hlyA* gene combinations. But in contrast, all the samples were found negative for *eaeA*, *hlyA* and *rfbO157* genes. Similarly, Ghanbarpour *et al.* [7] in Iran detected 4.5% *stx*$_2$ gene from healthy broilers whereas Wani *et al.* [15] found no *stx* genes in their study.

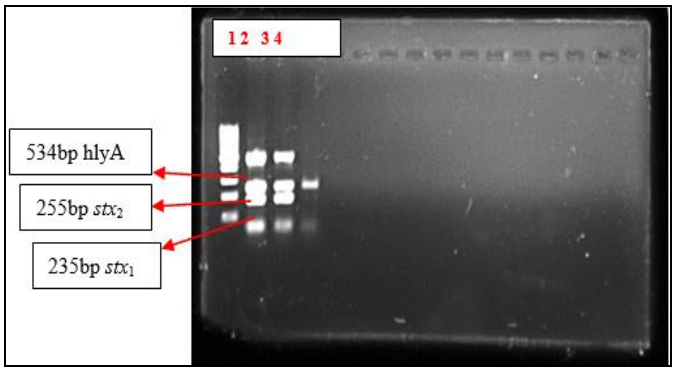

Fig 2: Agarose Gel showing PCR amplified product for *stx*$_1$, *stx*$_2$ and *hlyA*

Lane 1: 100 bp DNA ladder Lane 3: stx$_1$, stx$_2$ & hlyA
Lane 2: Positive control Lane 4: stx$_2$

Conclusion

In this study it is being observed that the positivity of *E. coli* is almost in half of the total sampling done as may be because of the ubiquitous nature of this pathogen. But the prevalence of verotoxic *E. coli* is quite low which may be due to the effect of treatment carried out in the affected animals. At the same time, it is also annoying that faecal and urine samples can be a potent hazard to both animal and human health as the virulent genes were detected from these clinical samples. As a result, emphasis must be given on proper hygienic disposal of clinical samples, appropriate treatment in order to arrest its spread, employing utmost care and management of the animals. Above all, preventive measures on evaluating the critical control points and analysing the hazards, should compulsorily be adopted to have a healthy livestock and safe public health.

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