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Studies on prevalence and hemolytic activity of verocytotoxic *Escherichia coli* (VTEC) isolated from sheep

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Abstract
The present study was planned to detection of VTEC genes in fecal sample of Sheep due to its highly public health significance. The study was undertaken to detection of virulence genes like *stx1*, *stx2*, *eaeA* & *hlyA* using polymerase chain reaction (PCR) and to finding their hemolytic property. In present study, a total of 230 samples were processed for isolation of verocytotoxic *E. coli* (VTEC). All samples were positive for *Escherichia coli*. Out of 230 *Escherichia coli*, 21 samples were found positive for VTEC having one or more genes in different combinations. A total of 8 isolates (38.09%) were positive on sheep blood agar and produced the desired clear zone of alpha hemolysis.

Keywords: sheep feces, verocytotoxic *E. coli*, PCR, sheep blood

Introduction
Verocytotoxic *E. coli* (VTEC) was first identified as a distinct group of *E. coli* named as Verocytotoxic *E. coli* (VTEC), which has the ability to produce toxins with profound and irreversible effect on vero cells. *E. coli* is the most commonly observed gastrointestinal flora of animals and environmental contaminant considered as important food borne pathogen causing serious complications in man and animals (Malik *et al*., 2013; Dhama *et al.*, 2013) [14, 6]. VTEC is also termed as shiga-like toxin producing *E. coli* (SLTEC) or shiga toxin producing *E. coli* or STEC. Acronym STEC is derived from the fact that the toxins are shiga like that is similar to those produced by *Shigella dysenteriae* type1 (Brien *et al.*, 1987) [4]. The EHEC belong to the Verocytotoxin producing *E. coli* (VTEC). VTEC always do not induce clinical signs and are not enterohaemorrhagic until addition virulence factor are present like enterohemolysin and adherence factors (intimin). The adherence factor(s) enables the organism to attach to and colonize intestinal mucosal cells (Hiruta *et al.*, 2001) [11]. Among VTEC, serotype O157:H7 has been closely associated with the sporadic and clinical outbreaks of hemorrhagic colitis (HC), haemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) in human beings (Croxen and Finlay, 2010; Gyles and Fairbrother, 2010; Sanchez *et al.*, 2013) [5, 10, 22]. Healthy domestic ruminants are recognised as the main natural reservoir of VTEC and large game animal maybe healthy carriers of STEC (Diaz *et al.*, 2011; Sanchez *et al.*, 2010) [7, 20]. Keeping in view the importance of this organism, the present study was planned to reveal the genotypic study of VTEC in fecal samples of Sheep.

Materials and Methods
Sampling and isolation of *E. coli*
In a present study a total of 230 fecal samples of Sheep were collected from different villages of Mathura district (table-1). The samples were collected aseptically in UV sterile polythene bags (fisher scientific, UK) and immediately transported to the laboratory under chilled conditions for microbiological analysis. For primary isolation of *E. coli* (VTEC), 10gm of fecal sample were enriched in 90 ml modified trypticase soya broth (mTSB) (Hi-media, Mumbai) containing acriflavine (10 mg/L) to reduce the growth of gram positive organism. The method used for collection of materials, isolation and identification techniques were made as per the lines suggested by World organization for Animal Health (OIE, 2004) [18]. These samples were incubated at 37 ^{0}C for 6 h. MacConkey's Agar (MCA) used as differential media, while eosin ethylene blue (EMB) agar (Hi-Media, Mumbai) used as selective media. Suspected *E. coli* strains were subjected to morphological, cultural and biochemical characterization as per the standard method (Ewing, 1986) [9].

Molecular characterization

Multiplex polymerase chain reaction (pcr) was used for detection of virulent genes (*stx*$_1$, *stx*$_2$, *eaeA* and *hlyA*) of VTEC. All the *E. coli* isolates were subjected to genomic DNA isolation. The bacterial growth in TSB broth (HiMedia) was centrifuged at 3000 rpm for 15 min to make the pellet of bacterial cells. These cells were washed twice with PBS (ph 7.4) to remove any impurity of broth media. Bacterial DNA was extracted by using DNA extraction kit (Genei, Bangalore) as per the manufacturer's protocol. For the PCR reaction, PCR Master Mix solution (Genei, Bangalore) was used. To amplify DNA targeted to virulent genes (*stx*$_1$, *stx*$_2$, *eaeA* and *hlyA*) of VTEC by using primers on 3µl of DNA template in 25µl reaction mixture (Paton and Paton, 1998) [19]. After an initial denaturation step at 95 ºC for 4 min, 30 amplification cycles were performed, each consisting of 94 ^{0}C for 2 min., 65 ^{0}C for 2 min. and 72 ^{0}C for 1.5 min and followed by a final extension step at 72 ºC for 2.5 min. After the amplification, amplicons were separated in 1.5% gel in tris acetate EDTA (TAE) buffer at 60 volt for 80 min, stained with 0.5% ethidium bromide solution and visualized under ultraviolet light.

Hemolysis assay

The ability of certain *E. coli.* strains to lyse erythrocytes of mammalian species is termed as hemolysis (Beutin, 1991) [3]. Four different types of hemolysins viz alpha, beta, gamma and enterohemolysin (E-hly) are produced by different patho groups of *E. coli.* Alpha hemolysin (Hly), considered as a prototype of a large family of pore-forming toxins, named repeat in toxin (RTX), is a cell free factor which causes clear zone around colonies on blood agar plates within 4-8 hrs of incubation (Sanchez *et al.*, 2011). To assess the haemolytic properties of VTEC isolates, all were streaked on 5% washed sheep blood agar supplemented with 10 mM $CaCl_2$. Positive isolates produced clear zone of haemolysis.

Result

All the strains of *E. coli* were screened for the detection of VTEC genes using multiplex PCR (figure-1). An overall presence of VTEC in Sheep was found to be 9.13% (21/230). Out of these findings, 9 VTEC 42.85% was found to be positive for *stx*$_1$ gene (180 bp), and 8 VTEC 38.09% was found positive for both genes *stx*$_1$ and s*tx*$_2$ and 4 VTEC 19.05% positive for *stx*$_1$, *stx*$_2$ with *hlyA* (534bp). To assess the in-vitro pathogenicity, all VTEC isolates were subjected to haemolysis assay (Figure-2). A total of 8 isolates were positive on sheep blood agar and produced the desired clear zone of alpha haemolysis. The percent positivity of haemolysis assay were 38.09%.

Table 1: Area and prevalence of VTEC in fecal sample of sheep

Place of collection	Number of sample collected	No. of VTEC isolates	Percentage of VTEC (%)
DDD farm DUVASU, Mathura	60	7	11.67
Sheep farm, farah	45	5	11.12
Aurangabad	50	3	06.00
Goverdhan	50	4	08.00
Raya	25	2	08.00
Total	230	21	9.13

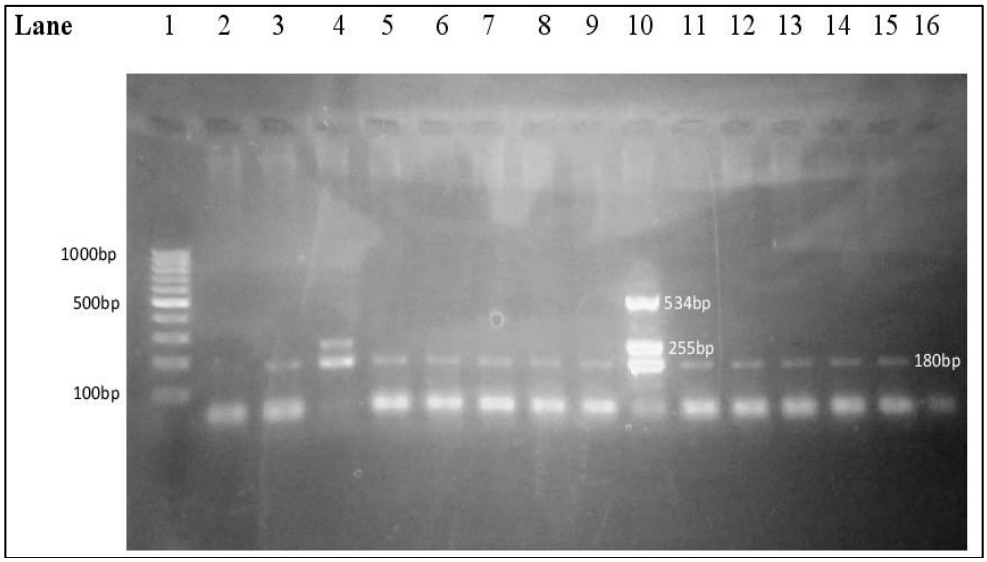

Fig 1: Agarose gel showing PCR amplified product for VTEC genes isolates from Sheep faeces

Lane 1:100bp DNA Ladder
Lane 3, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15: *stx*$_1$
Lane 4: *stx*$_1$ *stx*$_2$
Lane 10: *Stx*$_1$ *Stx*$_2$ and *hly*

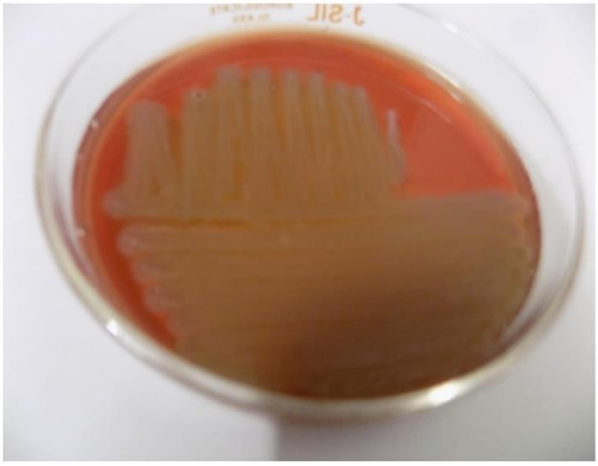

Fig 2: VTEC isolates of Sheep showing alpha hemolysis on washed sheep blood agar

Discussion

In this study, we detected VTEC in fecal samples of sheep, indicates that these represent an important natural reservoir of VTEC. In present study the prevalence of VTEC in Sheep was found to be 9.13%. Some lover finding of prevalence of VTEC 4.1% (Heuvelink *et al.*, 1998) and 8% reported by Mersha *et al.* (2010) [15]. However, prevalence of VTEC in higher level was reported by previous workers as 24.4% (Orden *et al.*, 2003) [17], 38% (Novotna *et al.*, 2005) [16] and (Sanchez *et al.*, 2010). VTEC genes in different combinations was isolated by Wani *et al.* (2003). The most common exotoxin produced by *E. coli* isolates that enhances virulence in a number of clinical infections of human and animals is a Alpha-haemolysin (Addison *et al.*, 2000) [1]. It is a cell free haemolytic factor which produces large clear zones of haemolysis often apparent after 4 hrs of incubation on washed sheep blood agar (Karch *et al.*, 1996). Human lymphocytes have more sensitive for the alpha-haemolysin (Bauer *et al.*, 1996) [2], so it is mainly involves to causing hemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS). Enterohemolysin (E-hly) generally produced by VTEC group of *E. coli* are pathogenic to Humans. In present study, 38.09% VTEC isolate sproduced alpha haemolysis. The findings were agreement with various worker (Elseisy *et al.*, 2010) [8] as 34%. Some lower finding of haemolytic activity observed by Shekh *et al.* (2013) [23] and Lorenz *et al.* (2013) [13] showed 2.8% haemolytic activity. The results of this study indicate that *stx* production and *hly* production are frequently associated in most of the VTEC. The close association of *stx* and *hly* production with *E. coli* strains, could indicate that these are genetically very closely related strains and phenotypically, is a suitable epidemiological marker for detection of some frequently occurring EHEC and VTEC serotypes in humans and animals.

Conclusion

Presence of VTEC genes in feces of a large population of Sheep in different domestics rearing place and due to its highly zoonotic importance, public health awareness including safe and hygienic practices while handling the Sheep would be paramount importance in reducing the VTEC infections in humans. Constant monitoring and surveillances programme to keep a record of the prevalence from time to time is needed to reduce the chance of infection.

Competing interests

The authors declare that they have no competing interests.

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