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Isolation and Molecular Characterization of Verotoxin Producing *E. coli* from Diarrhoeic Goats of Jabalpur Region

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ABSTRACT

Background: Verotoxin producing *Escherichia coli* (VTEC) such as O26, O103, O111, O145 and O157: H 7 are an important human zoonotic pathogen. Small ruminants such as goats are important reservoir of VTEC. The detection of VTEC and virulence genes in goats has not been studied in the Jabalpur region of Madhya Pradesh. The present study was carried out to determine the occurrence of verotoxin producing *E. coli* in goats in and around Jabalpur.

Methods: During the study period (July 2019 to February 2020), a total of 50 rectal swabs were collected from diarrhoeic goats. All the samples were processed for bacteriological isolation, molecular confirmation of *E. coli* and presence of the VT1 gene in *E. coli*.

Result: On cultural isolation, *E. coli* isolates were found in 52.00 per cent (26/50) samples. Out of them, 21 isolates confirmed as *E. coli* by PCR. Detection of verotoxin producing *E. coli* (VTEC) by slide agglutination test showed 20.00 per cent (10/50) verotoxin producing *E. coli* serogroups including O26, O103, O111, O145 and O157. A total of 10 VTEC isolates were subjected to molecular analysis, Polymerase chain reaction determined VT1 gene in 10.00 per cent of goats positive for verotoxin producing *E. coli* indicating the goats as a potential source for direct transmission of VTEC to humans.

Key words: Goats, Polymerase chain reaction, Verotoxin producing *Eshecrichia coli*, VT1 gene.

INTRODUCTION

Escherichia coli (*E. coli*) are a group or strains of bacteria found as a normal commensal in the intestinal micro flora of healthy animals and humans and excreted in the feces. Not all the strains are harmless and some strains can cause debilitating and sometimes fatal diseases in both animals and humans, therefore playing a significant role in the epidemiology of human infections (Heller and Chigerwe, 2018). The organisms enter into the food chain *via* faecal contamination of milk, contamination of meat with intestinal contents during slaughter or contaminated feed and water. Infection can also arise from animal to human contact, as well as human-to-human contact by both direct and indirect ways (Yim *et al*., 2010).

There are six recognized enteric pathotypes of *E. coli*, enterotoxigenic (ETEC), enteropathogenic (EPEC), diffusely adherent (DAEC), enteroaggregative (EAEC), enteroinvasive and verotoxigenic (VTEC) are also known as shiga toxigenic, STEC or enterohemorrhagic *E. coli* (Pal and Ayele, 2017).

Domestic ruminants, including goats, carry genes which produce verotoxins or Shiga-like toxins without having Shiga toxin receptors (Globotriaosylceramide). Therefore, they can act as asymptomatic carrier by STEC bacterium in faeces (Ashraf, 2016). The pathogenicity of VTEC is associated with phage-mediated genes VT1 and VT2 (Parul *et al*., 2016).

Moreover, verotoxigenic *E.coli* (VTEC) such as O157: H7 are not pathogenic to ruminants, but they cause serious diseases in humans worldwide, including diarrhoea, hemorrhagic colitis, hemolytic-uremic syndrome, thrombocytopenia purpura and sometimes death (Chileshe and Ateba, 2013).

Keeping in view the above facts in present study, *E. coli* was isolated from rectal swabs of goats and confirmed by a biochemical test, which was further subjected to molecular confirmation of *E. coli* along with the presence of VT1 gene in *E. coli*.

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MATERIALS AND METHODS

A total of 50 rectal swabs samples were collected aseptically from the diarrhoeic goats (irrespective of age, sex and breeds) of organized and unorganized farms in and around Jabalpur (Table 1). All the samples were processed for bacteriological isolation, molecular confirmation of *E. coli* and presence of the VT1 gene in *E. coli*.

Each swab was separately inoculated into nutrient broth and incubated at 37°C for 4 hours. A loopful inoculum was streaked on MacConkey agar plate and incubated at 37ºC for 24 hours. Organisms producing pink coloured colonies (lactose positive) on MacConkey agar plate were further streaked on to Eosin Methylene Blue (EMB) agar and incubated at 37°C for 18-24 hours. The colonies showing

characteristic metallic sheen on EMB agar were considered as presumptive of *E. coli* and subjected to Gram's staining for identification as per the standard procedure (Quinn *et al.*, 2004). *E. coli* isolates were further confirmed by biochemical tests *viz*. Indole, Methyl red, Voges-Proskauer, citrate utilization and carbohydrate utilization in HiIMViC biochemical kit (HiMedia KB001).

Detection of verotoxin producing *E. coli* by slide agglutination test

E. coli 'O' pool antisera comprising of VTEC serogroup O26, O103, O111, O145, O157 was used for slide agglutination with live cultures to detect verotoxin producing *E. coli* and the positive reaction was recorded when visible agglutination reaction was seen within 5-10 seconds.

Molecular characterization of *Escherichia coli* by genus-specific primer using 16SrRNA gene

The extraction of genomic DNA from broth of overnight grown culture of *Escherichia coli* was carried out as per the method described by Wilson (1987) with a slight alteration. The Purity of DNA was checked and quantified in a Nanodrop spectrophotometer and stored at -20°C for further use. PCR was performed on extracted DNA samples for amplification 16SrRNA gene fragment specific to genus *Escherichia coli* by using already published *E. coli* genus-specific primer (Table 2) as described by Shrivastav (2016). A reaction mixture of 25 µl, was prepared consisting of 12.5 µl, mastermix, forward primer 1 µl, reverse primer 1 µl, 3 µl of template DNA, 7.5 µl nuclease free water. The PCR protocol was performed under the following thermal cyclic conditions. The PCR cycling conditions consisted of an initial denaturation at 95°C for 5 minutes followed by 30 denaturation cycles at 94°C for 30 seconds each, annealing for 30 seconds at 69°C and extension for 2 minutes at 72°C. This was followed for 10 minutes by a final extension at 72°C. PCR amplification was verified on 1.5 per cent agarose horizontal gel electrophoresis.

Molecular characterization of VT1 gene specific for verotoxin producing *E. coli*

To investigate the virulence potential of the *E. coli*, isolates were subjected to PCR methodology as described by Jackson *et al*. (1987) and Strockbine *et al*. (1988).

Characterized strain of *E. coli* was used as positive control and was obtained from Department of Veterinary Microbiology, Jabalpur. In the present study, PCR was optimized with primer targeting gene VT1 (Table 3). Following initial optimization trials, reactions mixtures was standardized in 25 µl volume containing 12.5 µl master mix, forward primer 1 µl, reverse primer 1 µl, 3 µl of template DNA and 7.5 µl nuclease free water. The standardized amplification reaction began with initial denaturation at 95°C for 5 minutes, followed by 25 cycles each with denaturation at 94°C for 1 minute, annealing at 54°C for 1.5 minute and extension at 72°C for 1 minute, with final extension for 5 minutes at 72°C. A nucleic acid blank negative control was taken at each screening along with isolates to check PCR quality control conditions. On completion of PCR amplified products were analysed by 1.5 per cent horizontal agarose gel electrophoresis and the amplified product visualized as a single compact fluorescent band of expected size under U-V light.

RESULTS AND DISCUSSION

Isolation and identification of *E. coli* organism

In the present study, 52.00 per cent (26/50) samples were positive for *E. coli*. On MacConkey agar, lactose fermenting *E. coli* showed smooth bright pink coloured colonies (Fig 1) and dark centered blue black colonies with metallic sheen (Fig 2) were observed on EMB agar. The bacterial smear prepared from EMB agar revealed pink coloured medium sized Gram negative rods with Gram's staining (Fig 3), which were morphologically identified as *E. coli*.

Characterization of *Escherichia coli* by various biochemical tests

All the isolates (26) were found positive for indole and methyl red tests whereas none were positive for citrate utilization and Voges-Proskauer test as shown in Fig 4.

Detection of verotoxin producing *E. coli*

Positive isolates of *E. coli* obtained from EMB agar was further tested for agglutination reaction with known positive *E. coli* 'O' pooled antisera against verotoxin producing *E. coli* (VTEC) serogroups (O26, O103, O111, O145, O157). Of them, 38.46 percent (10/26) isolates were positive for VTEC showing agglutination reaction in slide agglutination test (Fig 5). Isolation of caprine VTEC serogroups from rectal contents of diarrhoeic goats was also consistent with the

Table 1: Sample collection from diarrhoeic goats.

Month	0-6 months		>06 months and above		Total
	Male	Female	Male	Female	
Jamunapari	04	01	03	03	11
Sirohi	03	02	06	02	13
Black Bengal	06	05	09	06	26

Table 2: Detail of primer used for PCR reaction

Primer	Primer sequence	Product size
E. coli 16srRNA	F(5'-TGGGAACGGCGAGTCGGAATAC-3') R(5'-GGGCGCAGGGGATGAAACTCAAC-3')	1476bp

Table 3: Detail of primer used for PCR reaction.

Primer	Primer sequence	Product size
VT1	F (5'-CGC-TCT-GCA-ATA-GGT-ACT-CC-3') R (5'-CGC-TGT-TGT-ACC-TGG-AAA-GG-3')	256bp

findings of previous studies by Osman *et al*. (2013), Oluyege and Famurewa (2015) and (Tarabees *et al*., 2016).

Molecular detection of *E. coli* by genus-specific primer using 16SrRNA gene PCR

All positive *E. coli* isolates were used for molecular detection by targeting the 16SrRNA gene of *E. coli*. Among them, 21 isolates were found to be positive for *E. coli* by PCR (amplicon size 1476 bp) (Fig 6). Similar study was done by Begum *et al*. (2016) who confirmed *E. coli* by genus specific PCR targeting 16SrRNA gene of *E. coli* (amplicon size 585 bp). Molecular characterization of *E. coli* through genus specific PCR targeting 16SrRNA gene of 232 bp were also done by Kumar *et al*. (2019). Sabat *et al.* (2000) conducted genus specific PCR for *E. coli* isolates targeting 16SrRNA gene of 544 bp. Nguyen *et al.* (2016) confirmed the *E. coli* isolates by genus specific PCR targeting 16SrRNA gene of 654 bp.

Detection of virulence gene VT1 specific for verotoxin producing *E. coli* by PCR

A total of 10 VTEC isolates were used for the detection of VT1 virulence gene by amplifying product of size 256 bp for VT1 gene in 10.00 per cent (01/10) isolates (Fig 7). The findings are more or less similar with the earlier reports (Oluyege and Famurewa, 2015) and 11.11 per cent per cent from India (Neher *et al*., 2016). The present findings suggest that, isolates of *E. coli* which were found negative by PCR may contain another virulence gene except VT1 (VT2, eae and hlyA).

Similarly, higher prevalence of VT1 gene in VTEC isolates was reported in various countries as 69.80 per cent from Spain (Cortes *et al*., 2005), 63.70 per cent from Spain

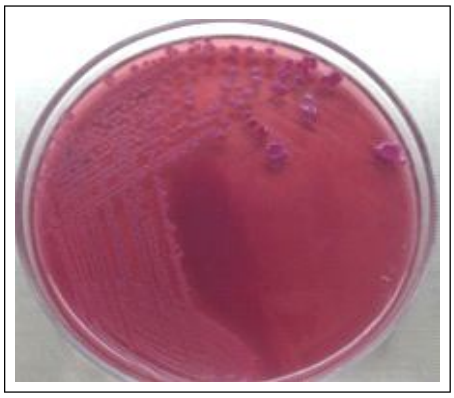

Fig 1: Bacterial growth on MacConkey agar medium showing bright pink coloured lactose fermenting colonies of *E. coli.*

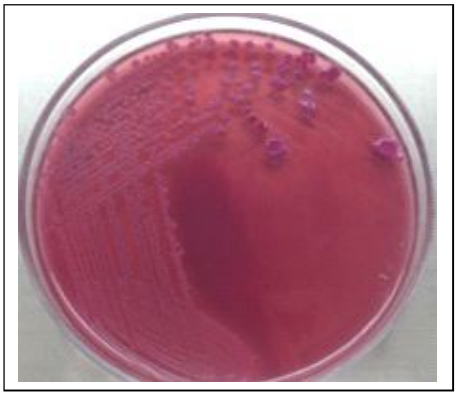

Fig 2: Bacterial growth of E.M.B agar plate showing blue-black colonies of *E. coli* with characteristic metallic sheen.

Fig 3: Gram negative bacilli. Gram's stain X1000.

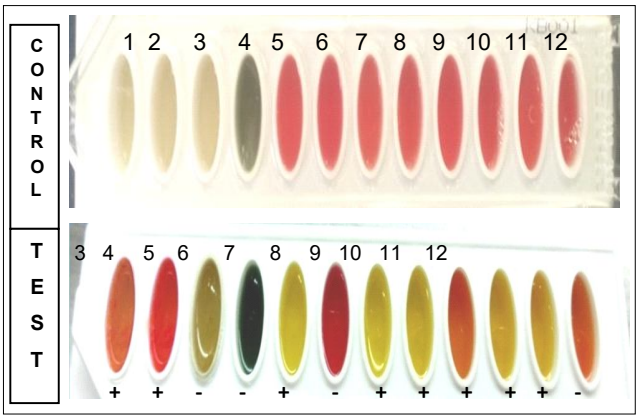

Fig 4: Biochemical characterization of culture from E.M.B agar plate showing positive for Indole test (1) Methyl red test (2) Glucose, Arabinose, Lactose, Mannitol, Rhamnose (5,7,8,10,11).

Fig 5: Slide agglutination test showing positive (+) and negative (-) reaction of *E. coli* culture with polyvalent antisera of VTEC serogroups.

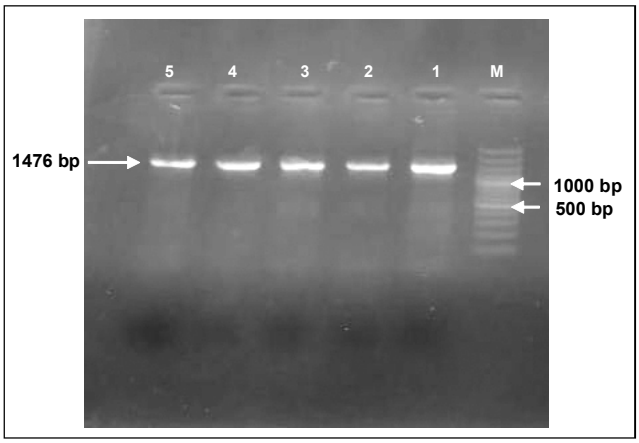

Fig 6: Agarose gel electrophoresis showing amplified product (1476 bp) of 16 SrRNA gene of *E. coli* isolates. Lane M: GeneRulerDNA ladder. Lane 1-5: Test samples.

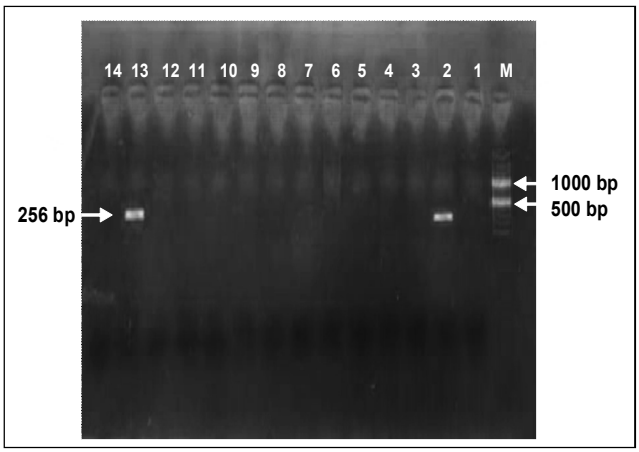

Fig 7: Agarose gel electrophoresis showing amplified product (256 bp) of VT1 gene of *E. coli* isolates.
Lane M: GeneRulerDNA ladder. Lane 1: Negative control. Lane 2: Positive control. Lane 3-14: Test samples.

(Orden *et al*., 2008), 67.40 per cent from USA (Jacob *et al*., 2013), 27.50 per cent from Southeast of Iran (Tagadoshi *et al*., 2018) and 22.20 per cent from turkey (Khider and Buyukcanga, 2018).

The wide variations in incidence of VT1 gene in goats could be attributed to epidemiological determinants such as herd size, age, sex and season, whereas sampling strategy, sample handling and laboratory methods might also have profound effect on prevalence values reported in different countries.

CONCLUSION

PCR determined VT1 gene in 08.33 per cent of goats positive for verotoxin producing *E. coli* indicating the goats as a source for direct transmission of VTEC to humans.

Conflict of interest: None.

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