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Original article

Characterization of toxin from Verocytotoxigenic *Ecscherichia coli* (VTEC) strains isolated from neonatal calves in India

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Abstract

Objective: The present study has characterized dialyzed toxin from non-O157 VTEC *E. coli* isolates by vero cell toxicity assay and pathogenecity in mice model. **Methods**: Toxins from non-O157 verocytotoxic *Escherichia coli* isolated from neonatal calves were characterized. Dialyzed toxin from *E. coli* O26, O111 and O103 serotypes were prepared and characterized by verocell toxicity assay and pathogenicity in mice model. *E. coli* O157:H7 considered as positive control for this study. **Results**: Cytopathic effects in vero cell line first rounding of vero cells, followed by clumping of cells and finally disintegrated, blackened, shriveled cell line within 16 to 72 hrs. Phenotypic markers such as hind limb paralysis and reddening of tail were prominent in all the toxicated mice. Extensive histopathological study was conducted for multiple organ involvement. **Conclusion**: Several methods for toxin assay were developed based on biological, immunological and detection of virulence genes related to toxin production but each test has draw back. Therefore, it is likely that future effort will be focused on the development of assay, which is fast, reliable, specific and sensitive methods based on mice model.

Keywords: *Escherichia coli*; Non-O157 verocytotoxic *Escherichia coli*; Verocell cytotoxicity assay; Mice lethality test

INTRODUCTION

Verocytotoxigenic *Escherichia coli*(*E. coli*) belong to a large group of pathogenic strains that are characterized by their ability to produce verotoxins, also called shiga toxins, known as shiga toxin- producing *E. coli* that cause disease in humans. Verocytotoxigenic *E. coli* strains have been found as causative agent of worldwide outbreaks of fatal gastrointestinal diseases and haemolytic uraemic syndrome in humans, with outbreaks increasing in occurrence in recent years[1]. Cattle are known major reservoir of O157:H7 as well as non-O157 VTEC[2]. Human infection can occur through direct contact, but other routes of transmission have been reported; faeco-oral route, human-human transmission, contaminated food chains[3]. Transmission can also occur through animal to animal and via contaminated dairy water. Recently, many other serogroups mainly O26, O91, O103, O104, O111, O113, O117, O118, O121, O128 and O145, have also been recognized as VTEC serogroups. Moreover, O26, O111 and O103 have been found verotoxigenic (EHEC) for both humans and animals[4].

In this study, we characterized dialyzed toxin from non-O157 VTEC *E. coli* isolates by vero cell toxicity assay and pathogenecity in mice model.

MATERIALS AND METHODS

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Source of *E. coli* isolates

The non-O157 VTEC *E. coli* isolates namely O26, O111, O103 (proven positive for the presence of VT1 and VT2 gene, supplied from the department of microbiology and isolated from neonatal calves) and *E. coli* O157:H7 [procured from National Escherichia and Salmonella Center, Kasauli (H. P.) India] considered as positive control for this study.

Production, purification by dialyzation of crude toxins from Verotoxic *E. coli* toxin

E. coli of O26, O111, O103, O157:H7 (as reference strain) toxins was prepared from O26, O111 and O157 as per method of Yamada et al[5]. Strains were inoculated into TSB (500mL) and incubated for 18h with constant shaking at 37℃. Following incubation, TSB culture was centrifuged at 3 500 rpm for 30min at 4℃ and than filtered through 0.22 μm pore size membrane filter for collection of supernatant.

Filtrated material was mixed with solid ammonium sulfate to 70% saturation level. After overnight incubation at 4℃, the precipitate was collected following centrifugation at 2 500 rpm for 30 min. at 4℃. The precipitate was suspended in 0.05 M phosphate buffer (pH 6) and dialyzed against the same buffer for 48 hrs at 4℃. Finally, dialyzed toxins were stored at −20 ℃ for further use.

Vero cell cytotoxicity assay

Cytotoxicity of verotoxigenic *E. coli* strains (VTEC) in verocell was done according to OIE manual[4]. Briefly, each of the *E. coli* isolates was inoculated in 5 mL of tryptic soy broth (TSB) and incubated at 37℃ for 24 h. The 2mL of broth culture was then reinoculated into 50mL conical flask containing 20mL TSB and incubated at 37℃ for 18-20h in a low speed shaking water bath. The bacterial cells were pelleted by centrifugation at 10 000 r. p. m for 30 min at 4℃ and the supernatant was filtered through 0.22 m pore size membrane filter. The sterility of the cell free culture supernatant was checked by streaking onto tryptic soy agar, which was incubated at 37℃ for 24h. The sterile culture supernatants were then stored at −20℃ till used. Culture supernatant of *E. coli* O157:H7 [procured from National Escherichia and Salmonella Center, Kasauli (H. P.) India] was used as positive control and non-toxic *E. coli* O4 as negative control. The tissue culture plates were incubated at 37℃ under 5% CO_2 atmosphere and examined daily (6hrs interval) under the inverted microscope up to 3 days for characteristic morphological cytopathethic changes. Supernatant of *E. coli* strain causing CPE in more than 50% vero cells were regarded as positive for verotoxin.

Mice lethality assay and histopathology study

Dialyzed verotoxin preparations from enterohaemolysin positive *E. coli* of 026, 0111, O103, 0157:H7 (as reference strain) serogroups were made and subjected to mice lethality test. Mouse lethality test was conducted as described by Vernon et al[6] with some modification. Two separate groups of 6-8 week old albino mice (6 mice / verotoxin sample and 6 as control) were taken. Mice were injected were 0.5 mL dialyzed verotoxin intraperitonially and control group was given only PBS (0.5mL) I/p. Inoculated mice were observed daily upto 3 days and morbidity and mortality were noted. If not died, they were sacrificed and visceral organs (intestine, kidney, Lung, and liver) were examined for gross lesions and collected in 10% neutral formalin for histopathological studies.

RESULTS

All the three dialyzed toxin preparations exhibited cytotoxigenicity. The changes in monolayer cell sheet of vero cell culture were first rounding of vero cells, followed by clumping of cells and finally disintegrated, blackened, shriveled cell line within 16 to 72 hrs (Fig. 2, 3, 4, 5, 6). The mice inoculated with toxin showed characteristic clinical symptoms such as paralysis of hind limbs (Fig. 7) and reddening of tail due to engorgement of tail vein confirmed the presence of verotoxin (Fig. 8). All the mice were examined for presence of gross lesions in kidney, lung, liver and intestine and histopathological studies. Gross changes included swelling of kidney and liver, congestion and hemorrhages in the lung and small intestine.

Histopathological changes were also studied. In kidney, mainly convoluted tubules exhibited degenerative changes and presence of pinkish mass in tubular lumen (Fig. 9). Lungs of mice showed vascular congestion and focal haemorrhage. Bronchial epi-

thelial lining showed elongation and small projection into lumen, desquamated cells presented into the lumen. Alveolar septa were also thickened due to increased cellularity. Presence of mononuclear cells and few neutrophils were found. Bronchi showed activation, proliferation and desquamation of lining cells (Fig. 10). Liver showed focal degenerative changes, small fatty vacuoles were seen in hepatocytes at various places in parenchyma, mild to moderate accumulation of mononuclear cells around central vein blood vessels (Fig. 11). Intestinal mucosa showed necrosis and sloughing of lining epithelial cells, submucosa showed edematous changes (Fig. 12).

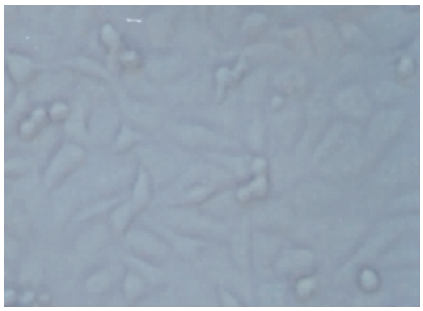

Figure 1 Normal monolayer of Verocell line.

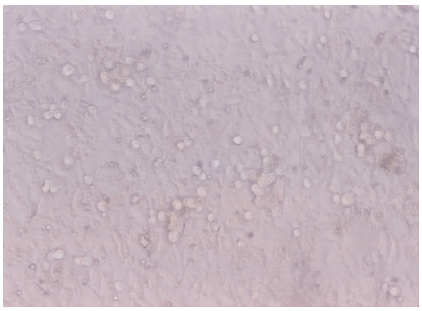

Figure 2 Showing rounding of Vero cells after 16hrs of intoxication of cell line with dialyzed toxin of non-O157 VTEC.

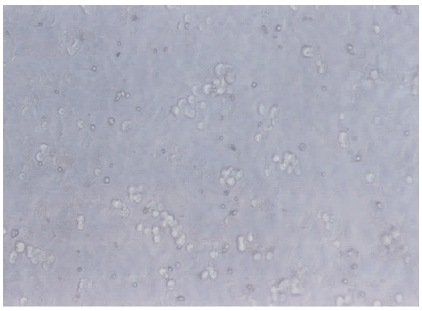

Figure 3 Showing clumping of Vero cells after 24 hrs of intoxication of cell line with dialyzed toxin of non-O157 VTEC.

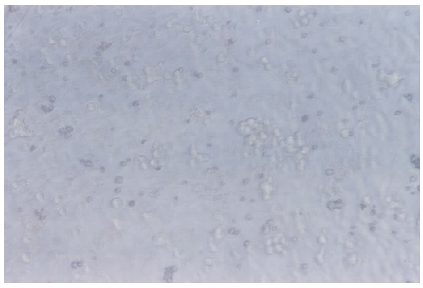

Figure 4 Showing clumping of Vero cells after 36 hrs of intoxication of cell line with dialyzed toxin of non-O157 VTEC.

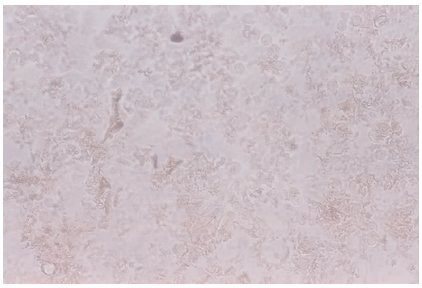

Figure 5 Showing degeneration and blackening of Vero cells after 48hrrs of intoxication of cell line with dialyzed toxin of non-O157 VTEC.

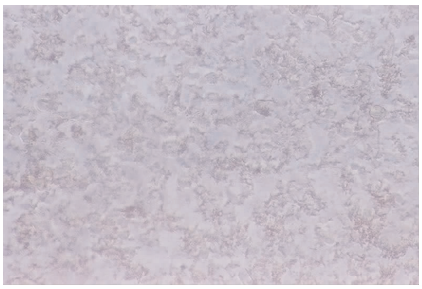

Figure 6 Showing complete degeneration of Vero cells after 72 hrs of intoxication of cell line with dialyzed toxin of non-O157 VTEC.

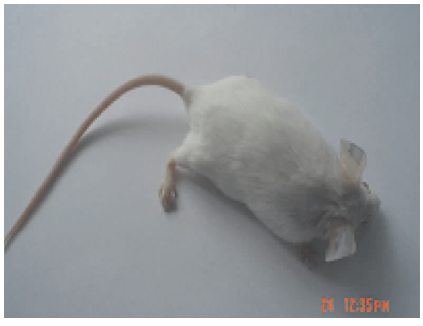

Figure 7 Mice intoxicated with Dialyzed toxin from *E. coli* O26. Showing paralysis of hind limbs.

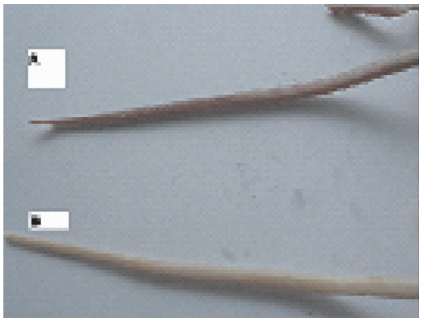

Figure 8 A. Reddening of tail of Mice intoxicated with Dialyzed toxin from non-O157 VTEC; B. Normal tail of Mice.

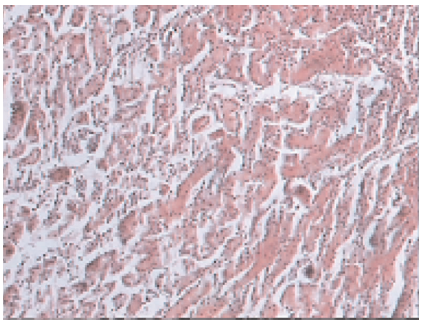

Figure 9 Mouse Kidney (H&E, 100×). Showing mild degeneration in convoluted tubules and presence of pinkish mass in tubular lumen.

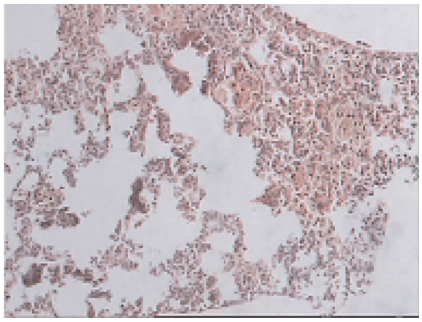

Figure 10 Mouse lung (H&E, 100 ×. Showing presence of mononuclear cells and few neutrophils. Proliferation, desquamation of bronchial epithelial lining and focal haemorrhages and vascular congestion.

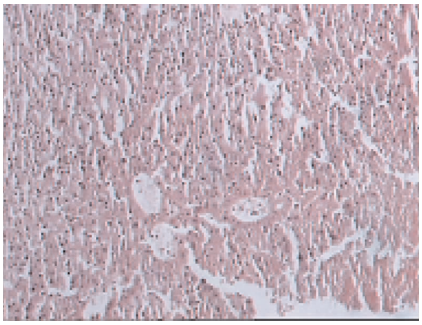

Figure 11 Mouse liver (H&E, 100 ×). Showing focal degeneration in centrilobular area and hepatocytes with vascular changes.

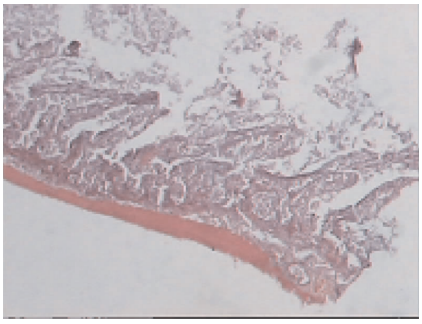

Figure 12 Mouse intestine (H&E, 100 ×). Intestinal mucosa showed necrosis and sloughing of lining epithelial cells and sub mucosa showed edematous changes.

DISCUSSION

Our study in accordance with Veron et al[6] who reported that the development of hind limb paralysis may require longer periods of exposure to sub lethal doses of verotoxins and histopathological examination of toxin injected mice showed damaged limited to a large extent to renal proximal tubule epithelial cells. But in our experiments we found multiple organ involvement including liver, lung and intestine. We have demonstrated that reddening of tail is marker clinical sign that is noted in all the intoxicated mice. This is needed further study. *E. coli* toxins are an important cause of a variety of human and animal diseases. Several methods for toxin assay were developed based on biological, immunological and detection of virulence genes related to toxin production but each test has draw back. Biological assay involve whole or part animal test, which are expensive and require days to complete. Immunological techniques do not give result in real time detection fashion. The poor stability of antibodies also limits their use in field application. Highly specific and sensitive DNA probes and PCR have been described which can detect potentially toxigenic strains of bacteria, but a positive result does not indicate that toxin gene was actually expressed. Therefore, it is likely that future effort will be focused on the development of assay, which is fast, reliable, specific and sensitive methods for the detection of bacterial toxins for use in clinical investigation.

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