Production of Verocytotoxin and Presence of \textit{stx} Gene in \textit{Escherichia coli} Strains from Diarrhoeic and Non-Diarrhoeic Cattle and their Handlers

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ABSTRACT

The present study was conducted to know the cytopathic effect of verotoxin producing \textit{E. coli} on the vero cells. A total of 230 samples from animal and animal handlers were processed for the isolation of \textit{E. coli}. Out of 121 \textit{E. coli} isolated from those samples, 77 were serogrouped into 25 serovars and all the 121 isolates were screened by PCR, where 52 (42.97%) were found possessing \textit{stx} genes, which comprised of 20.66% \textit{stx} \textit{1}, 16.52% \textit{stx} \textit{2} and 5.78% both \textit{stx} \textit{1} and \textit{stx} \textit{2}. The cell free supernatant of ten Verotoxin Producing \textit{E. coli} isolates (O15, O22, O55, O60, O71, O86, O102, O111, O116 and O139) possessing \textit{stx} genes were tested for cytotoxicity on vero cell lines. Microscopically undiluted culture filtrates of all the ten strains induced the cytopathic effect within 24 h. A dilution of 1/4, 1/16, 1/64 and 1/256 affected 50% of the vero cells on 1st, 2nd, 3rd and 4th day post-inoculation, respectively, in O15, O55, O60, O71, O86, O102, O111, O116 and O139 strains. The strain O22 affected 50% of the cells on day 3 and 4 with a dilution of 1/4 and 1/16, respectively. The cytopathic effect advanced with time, maximum effect was observed in 4 days and varied with strain dilution from 16 to 256.

Keywords: \textit{E. coli}, serogroups, verocytotoxicity, verotoxic \textit{E. coli}

Introduction

Production of cytotoxins active on Vero cells, termed Verocytotoxin/shigatoxin (VT/STX), has been detected in strain of \textit{E. coli} from cases of human and animal diseases (Konowalchuck et al., 1977). The verocytotoxin producing \textit{E. coli} (VTEC) also called shiga toxin producing \textit{E. coli} (STEC) of human and animal origin belong to a number of different ‘O’ serogroups. They have been found to be associated with cases of diarrhea, haemolytic uraemic syndrome (HUS) and haemorrhagic colitis (HC), (Karmali et al., 1983, Riley et al., 1983, Smith et al., 1987, Scotland et al., 1988). STEC strains of the bovine origin belonging to a large number of serogroups have been isolated from cases of diarrhoeic and non-diarrhoeic animals (Sherwood et al., 1985, Mohammad et al., 1986). The verotoxin positive O139 strains have been found to be associated with post weaning diarrhea and oedema disease in pigs (Blanco et al., 1983, Smith et al., 1983) and STX positive O55 and O111 strains were reported in number of HUS cases in humans (Griffin, 1995, Karmali, 1989, Paton and Paton, 1998).

To date, a majority of molecular epidemiological studies have mainly focused on the detection and characterization of VTEC/STEC, from calves and lambs (Wani et al., 2003, Latina et al., 2008). However, none of report describing occurrence and characterization of STEC simultaneously from food producing animals and their handlers in and around Jammu are available.

Considering the impact of occurrence of STEC in veterinary and human medicine as an emerging potential pathogen, the present study was conducted...
to characterize the isolates phenotypically and genotypically from human and animal samples.

**Materials and Methods**

A total of 230 samples from cattle and animal handlers (55 diarrhoeic, 65 non-diarrhoeic cattle, 50 diarrhoeic and 60 non-diarrhoeic animal handlers) of different age and sex were collected and processed for the isolation of *E. coli* as per standard bacteriological techniques (Quinn *et al.*, 1994).

**Serogrouping**

Out of 121, *E. coli* isolates, seventy seven were serogrouped from National *Salmonella* and *Escherichia* Centre, Central Research Institute, Kasauli-173204 (H.P), INDIA.

**Polymerase chain reaction (PCR) for detection of stx₁ and stx₂ genes**

Target gene specific polymerase chain reaction was performed for the molecular detection of *stx₁* and *stx₂* virulence genes of *E. coli* isolates. Primers used in the study are listed in Table 1.

**Template DNA preparation**

The template DNA was prepared as per the method of Blanco *et al.* (1996) with slight modifications. The *E. coli* isolates were cultured in brain heart infusion broth at 37°C for 4 h. One ml of the broth culture was taken in a microcentrifuge tube and centrifuged at 8000 rpm for 5 min to settle down the bacterial pellet. The supernatant was discarded and the pellet washed with NSS at 8000 rpm for 5 min. The pellet was mixed with 0.5 ml of nuclease free water and subjected to heat lysis by keeping in boiling water for 10 min. Then immediately placed at -20°C for 10 min and centrifuged again at 400 rpm for 4 min and 2.5 µl of supernatant was taken as template for PCR.

**Standardization of PCR protocol for stx₁ and stx₂ genes**

A total of 121 *E. coli* isolates were screened for *stx* genes by the polymerase chain reaction as per the method of Paton and Paton (1998) with suitable modification. Duplex PCR for detecting both *stx₁* and *stx₂* virulence genes was set up in 25 µl of reaction mixture containing 2.5 µl of 10x *Taq* buffer, 2.5 µl of dNTP, 2.5 µl MgCl₂ (25 mM), 1.0 µl of each forward and reverse primers (10 pmol each), 1 unit of *Taq* DNA polymerase and 2.5 µl of template DNA. The PCR protocol was standardized by optimizing the concentration of the components of the reaction mixture and by varying the annealing temperature and cycling conditions.

The cycling parameters used were initial denaturation at 94°C for 4 min, 35 amplification cycles each of 1 min denaturation at 94°C, annealing at 55°C for 1 min and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. Amplified products were analyzed by electrophoresis on 1.5% agarose gel (containing 0.5 µg per ml ethidium bromide) using molecular weight marker (100 bp DNA ladder) (Bangalore Genei).

**Cytotoxicity assay**

Preparation of cell free supernatant (CFS)

The *E. coli* isolates were grown in trypticase soya broth as per the method described by Konowalchuk

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Primers* used</th>
<th>Sequence (5’–3’)</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.</td>
<td><em>stx₁</em>-R</td>
<td>CCCCCTCACTGCTAATA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.</td>
<td><em>stx₂</em>-R</td>
<td>GGATGCATCTCTGTCATTG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Sigma Aldrich Chemicals Pvt. Ltd.*
et al. (1977) with slight modifications. The *E. coli* isolates from diarrhoeic cattle belonging to serogroups O15, O22, O55, O60, O71, O111, O116 and O139 and from diarrhoeic animal handlers 086 and 0102 were inoculated into 250 ml Erlenmeyer flasks containing 20 ml of trypticase soya broth incubated at 37°C in an incubator. After 24 h the cultures were centrifuged at 17000g for 30 min. The supernatants were filtered through 0.22 µm membrane filters (Millipore Pvt. Ltd.) and stored at 4°C until assayed. Filtrate dilutions were made in phosphate buffer saline (PBS), pH 7.0.

**Cell culture assay**

Cytotoxic effects of the VTEC were tested on Vero cell lines as per the method of Konowalchuk et al. (1977) with minor modifications. The cell lines were procured from Biological Products Division, IVRI Izatnagar, Bareilly grown as monolayers in 96 well microtitre plates. Serial dilutions of sterile culture filtrates prepared in PBS were added to the cell lines. For assay of toxin 10 µl of toxin filtrate was added into 100 µl of seeded Vero cells in microtitre plates. The plates were incubated at 37°C for 1-4 days, and observed under inverted light microscope (Nikon, Japan) daily for recording the observations. The medium was decanted daily from one plate and tissue culture plates were fixed with 95% methanol, followed by staining with 1% crystal violet and examined for any cytotoxic effect. The toxin titres were the highest dilution giving a cytotoxic effect on Vero cells after 4 days incubation (Smith et al., 1988). The morphological effects were recorded as 1, 2, 3 or 4 ratings corresponding to roughly 25%, 50%, 75%, or 90% of the cells affected. One 50% cytotoxic dose (CD$_{50}$ unit) was defined as the amount of the stx activity that caused cytopathic effect in 50% of a Vero monolayer. Each preparation was tested in triplicate. In each plate cell free supernatant of known Verotoxin producing *E. coli* and a preparation of BHI broth (Hi-Media) were included as positive and negative control, respectively.

**Results and Discussion**

Of the 230 samples analysed from animals and animal handlers, 121 (52.60%) yielded biochemically confirmed *E. coli* isolates. The 77 strains (32 from diarrhoeic and 25 from non-diarrhoeic cattle, 15 from diarrhoeic and 5 from non-diarrhoeic animal handlers) (Table 2), in this study the serogroups isolated both from cattle and animal handlers were 02, 062, 080, 0101, 0139 and some untypeable strains. The serogroups O55, and O111 VTEC recognized for a number of years causing human diseases ranging from sporadic to major outbreaks of mild diarrhea to life threatening haemolytic uremic syndrome (HUS), thrombotic thrombocytopenic purpura, (Karmali, 1989, Griffin, 1995, Paton and Paton, 1998, Hussein and Bollinger, 2005) were isolated from diarrhoeic cattle and not from animal handlers. In the present investigation VTEC human and animal origin belonged to a number of different ‘O’ serogroups. They were found to be associated with cases of diarrhea, HUS and HC (Karmali et al., 1983, Riley et al., 1983, Scotland et al., 1988, Smith et al., 1987, Dhanashree and Shrikar, 2008).

**Detection of verotoxin genes (stx$_1$ and stx$_2$)**

Screening of samples for the presence of *stx* gene was done by PCR for amplification of PCR products of 350 bp (*stx$_1$*) and 478 bp (*stx$_2$*) genes. The template prepared from brain heart infusion broth showed amplification of *stx$_1$* and *stx$_2$* genes (Fig.1). Fifty two (22.60%) samples from cattle and animal handlers were positive for STEC which included 47.27% from diarrhoeic and 21.53% from healthy cattle. Whereas, Das et al. (2005) reported STEC from 19% of faecal samples from healthy cattle, which is in agreement to our findings. But Chattopadhyay et al. (2003) found 22.03% of diarrhoeic cattle samples possessing STEC. The prevalence of STEC in diarrhoeic animal handlers was 24% in this study. However, the overall prevalence of STEC from cattle (diarrhoeic and non-diarrhoeic) and animal handlers (diarrhoeic and non-diarrhoeic) was 22.60%. The percentage of different *stx* genes among the *E. coli* isolates is given in Table 3.

**Assay for cytotoxicity**

The cell free supernatant (CFS) of ten STEC isolates O15, O22, O55, O60, O71, O86, O102, O111, O116 and O139 positive for *stx* gene were tested for their toxicity on Vero cell lines, which were examined 1-4 days post inoculation. The results of cytotoxicity
Table 2. Distribution of *Escherichia coli* serogroups in diarrhoeic and non-diarrhoeic cattle and animal handlers.

<table>
<thead>
<tr>
<th>Types of Sample analysed</th>
<th>E. coli isolates serogrouped</th>
<th>Serogroups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cattle (D*)</td>
<td>32</td>
<td>02 (1), 014 (2), 015 (1), 022 (1), 050 (1), 052 (1), 055 (1), 059 (2), 0 (60) (4), 062 (1), 071 (1), 076 (1), 087 (1), 095 (1), 0100 (1), 0101 (1), 0111 (1), 0116 (2), 0123 (1) 0139 (2), UT* (5)</td>
</tr>
<tr>
<td>Cattle (ND*)</td>
<td>25</td>
<td>014 (1), 022 (1), 052 (1), 059 (1), 060 (4), 076 (1), 080 (2), 084 (1), 095 (1), 0116 (2), 0123 (3), 0139 (1), 0173 (1), UT* (5)</td>
</tr>
<tr>
<td>AH*(D*)</td>
<td>15</td>
<td>02 (1), 062 (1), 080 (1), 086 (1) 0101 (1), 0102 (1), 0139 (1) R* (3), UT* (5)</td>
</tr>
<tr>
<td>AH*(ND*)</td>
<td>5</td>
<td>R* (1), UT* (4)</td>
</tr>
<tr>
<td>Total</td>
<td>77</td>
<td>02 (2), 014 (3), 015 (1), 022 (2), 050 (1), 052 (2), 055 (1), 059 (3), 0 (60) (8), 062 (2), 071 (1), 076 (2), 080 (3), 084 (1), 086 (1) 087 (1), 095 (2), 0100 (1), 0101 (2), 0102 (1), 0111 (1), 0116 (4), 0123 (4) 0139 (4), 0173 (1), rough (4), untypeable (19)</td>
</tr>
</tbody>
</table>

*AH = Animal handlers, D = diarrhoeic, ND = non-diarrhoeic, UT = untypeable, R = Rough, Figures in parenthesis indicates total isolates.

Table 3. Prevalence of different stx genes among *Escherichia coli* isolates from animals and animal handlers

<table>
<thead>
<tr>
<th>Types of samples analysed</th>
<th>No. of isolates screened</th>
<th>No. of different types of genes</th>
<th>Total stx positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>stx1</td>
<td>stx2</td>
</tr>
<tr>
<td>Cattle (D*)</td>
<td>36</td>
<td>12 (33.33)</td>
<td>11 (30.55)</td>
</tr>
<tr>
<td>Cattle (ND*)</td>
<td>37</td>
<td>7 (18.91)</td>
<td>5 (13.51)</td>
</tr>
<tr>
<td>AH*(D*)</td>
<td>32</td>
<td>6 (18.75)</td>
<td>4 (12.5)</td>
</tr>
<tr>
<td>AH*(ND*)</td>
<td>16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>121</td>
<td>25 (20.66)</td>
<td>20 (16.52)</td>
</tr>
</tbody>
</table>

*D = diarrhoeic, ND = non-diarrhoeic, AH=animal handlers, Figures in parenthesis indicates percentages, tests of the isolates were enumerated in Table 4. Undiluted culture filtrates induced the cytopathic effects within 24 h. A dilution of 1/4 1/16, 1/64 and 1/256 affected 50% of the vero cells on 1<sup>st</sup>, 2<sup>nd</sup>, 3<sup>rd</sup> and 4<sup>th</sup> day of post-inoculation, respectively, in O15, O55, O60, O71, O86, O102, O111, O116, and O139 strains. Whereas the strain O22 affected 50% cells on day 3<sup>rd</sup> and 4<sup>th</sup> with a dilution of 1/4 and 1/16, respectively (Table 4). The cytopathic effect advanced with time and varied with strain dilution. The cellular changes produced by different dilutions varied and included detachment of cells with rounding, shrinkage, sloughing, degeneration and homogenization of cells.
This study demonstrates that cattle are important reservoirs of STEC and their routine handling can lead to human infections. The undiluted culture filtrate of STEC affected the Vero cell monolayers within 24 h. Screening of a larger bovine and human population in rural areas should be carried out to further explore the information on epidemiology and zoonotic potential of STEC as well as presence of different strains.

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References


