Verotoxic *Escherichia coli* (STEC) from beef and its products

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In the present investigation, out of 27 (24.10%) strains of *Escherichia coli* isolated from 112 beef samples comprising raw meat (45), kabab (36) and kofta (31), 9 (33.33%) belonging to 7 different serotypes were verotoxic as tested by vero cell cytotoxic assay. Serotype O145 was the predominant STEC in raw meat. Interestingly, one STEC-O157 strain was also detected. All the STEC strains were positive for Stx genes by polymerase chain reaction showing stx2 (77.78%) to be most prominent followed by stx1 (22.22%). Phenotypic enterohaemolysin production on washed sheep blood agar supplemented with CaCl2 revealed 6 (66.67%) STEC strains to be positive. Presence of STEC in cooked beef products, viz., kabab and kofta appeared to be a matter of concern and potential threat to public health.

**Keywords**: Animal products, Beef, *Escherichia coli*, STEC, Verotoxin

Verotoxin-producing *Escherichia coli* (STEC) strains have been associated with human diseases ranging from symptom-free carriage to uncomplicated as well as severe bloody diarrhoea, haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). STEC is a serologically diverse group of zoonotically important food-borne pathogens, of which serotypes O157:H7 and other non-O157:H7 have been epidemiologically significant worldwide because of their notoriety of being associated with life-threatening diseases1,2. Bovine is a major reservoir of STEC, while other animals such as sheep, swine, deer, horses, dogs and birds have also been implicated as the natural reservoir and a major risk factor3. Most of the cases have shown to be associated with products of bovine origin, including ground beef and unpasteurized milk4.

Ability of STEC to cause illness in humans is related to production of one or more types of related verocytotoxins (Stx) of which Stx1, Stx2 and variants of Stx2 are important virulence factors. These Stxs inhibit protein synthesis and thus lead to cell death5,6. Stx1 and Stx2 are encoded by stx1 and stx2 genes, respectively, and are present in the genomes of temperate, lambdoid bacteriophage to regulate Stx expression as part of their lytic switch7. In addition to Stxs, some other factors including enterohaemolysin (E-hly) and intimin encoded by eae gene also contribute to the pathogenicity8. Identification of animal products’ contamination by potentially pathogenic STEC strains is of importance for public health. Thus, there is a need to detect different STEC serotypes in beef and its products for efficient quality control and food safety.

The present study was undertaken to detect STEC from beef and its products by Vero cell cytotoxicity assay (VCA) as well as by polymerase chain reaction (PCR) and enterohaemolysin (E-hly) production.

**Materials and Methods**

Samples (112) comprising raw beef (45), kabab (36) and kofta (31) were collected aseptically and randomly from local retail meat shops, and apparently healthy animals. Meat samples (approximately 50 g) and their products were collected in UV radiation sterilized polyethylene sachets, sealed and brought to the laboratory under ice for bacteriological examination. Isolation of *E. coli* was carried out from these samples using MacConkey broth (Hi-Media) for enrichment, and MacConkey agar as well as EMB agar (Hi-Media) for plating. Suspected *E. coli* isolates were confirmed on the basis of biochemical reactions9.

VCA of 27 strains of *E. coli*, isolated from raw beef and its products including kabab and kofta, was performed as per standard method10. The Vero cell
lines, procured from Centre for Animal Disease Research and Diagnosis (CADRAD), IVRI, India were propagated and maintained in Glasgow minimum essential medium (GMEM; Sigma) supplemented with 10 per cent new borne calf serum (NBCS).

Each of the \textit{E. coli} isolates was inoculated in 5 ml of tryptic soy broth (TSB; Difco) and incubated at 37°C for 24 h. The broth culture (2 ml) was reinoculated into 20 ml TSB and incubated at 37°C for 18-20 h in a low speed shaking water bath. The bacterial cells were pelleted by centrifugation (10,000 \texttimes  g) at 4°C for 30 min in refrigerated centrifuge machine (Remi) and the supernatant was filtered through 0.22 µm pore size membrane filter (Sartorius). The sterility of the cell free culture supernatant (CFCS) was checked by streaking onto tryptic soy agar (TSA; Difco) and incubated at 37°C for 24 h. The sterile CFCS were then stored at –20°C until used.

The toxin assay was carried out in a 96-well microtitre tissue culture plates (Tarsons). \textit{Vero} cell culture was seeded in each well and allowed to grow until used.

For 24 h. The sterile CFCS were then stored at –20°C respectively. The tissue culture plates were incubated in a 5 per cent CO2 atmosphere. The medium was then discarded and monolayers were washed twice with Hank’s balanced salt solution (HBSS). CFCS (20 µl) was inoculated into each well in triplicate and 200 µl of the GMEM salt solution (HBSS). CFCS (20 µl) was inoculated by streaking onto tryptic soy agar (TSA; Difco) and incubated at 37°C for 24 h. The sterile CFCS were then stored at –20°C until used.

The toxin assay was carried out in a 96-well microtitre tissue culture plates (Tarsons). \textit{Vero} cell culture was seeded in each well and allowed to grow as confluent monolayers at 37°C under 5 per cent CO2 atmosphere. The medium was then discarded and monolayers were washed twice with Hank’s balanced salt solution (HBSS). CFCS (20 µl) was inoculated into each well in triplicate and 200 µl of the GMEM with 10 per cent NBCS was then added into each well. Culture supernatant of \textit{E. coli} O157:H7 and non-toxic \textit{E. coli} O11:H, procured from National Institute of Cholera and Enteric Diseases (NICED), Kolkata were used as positive and negative control, respectively. The tissue culture plates were incubated at 37°C under 5 per cent CO2 atmosphere and examined daily up to 4 days for characteristic cytopathic effect (CPE). The supernatant of \textit{E. coli} isolates causing CPE in more than 50 per cent \textit{Vero} cells were regarded as positive for verocytotoxicity.

Serotyping of \textit{E. coli} isolates was done at National Salmonella and Escherichia Centre, Central Research Institute, Kasauli, India.

PCR was performed to detect virulent genes of STEC by 3 different primers\textsuperscript{11} specific to \textit{stx1}, \textit{stx2} and \textit{eae} genes (Table 1). Genotypic detection of \textit{E-hly} could not be carried out.

Strains of \textit{E. coli} showing CPE on \textit{Vero} cell and 10 randomly selected CPE negative strains were grown in BHI broth (Hi-media) overnight at 37°C. \textit{E. coli} O157:H7 was used as positive control. One milliliter of culture was taken in microfuge tube and heated for 10 min in boiling water. The microfuge tube was transferred immediately to ice and used for PCR.

Amplification of bacterial DNA for detection of \textit{Stx1}, \textit{Stx2} and \textit{eae} was performed in a thermal cycler (Mastercycler gradient, Germany) in 50 µl volume containing 10 µl of the prepared sample, 20 pmol of oligonucleotide primers, 0.2 mM each of dATP, dGTP and dCTP, 10 mM of MgCl\textsubscript{2} and 1.5 U of Taq DNA polymerase (Genei, Bangalore). The conditions for PCR were 94°C for 2 min for initial denaturation of DNA within the sample followed by 30 cycles of 94°C for 1 min (denaturation), 55°C for 1 min (primer annealing) and 72°C for 1 min (DNA synthesis). The amplified products of desired size were visualized by submarine gel electrophoresis using 10 µl of PCR product on 1.5 per cent agarose gel in Tris-borate EDTA buffer for 45 min at 120 v. The amplified DNA fragments of specific sizes were located by UV fluorescence after staining with ethidium bromide (0.5 µg/ml). Molecular size markers DNA Ladder marker, (Genei, Bangalore) were included in each gel.

All the strains of STEC and 10 randomly selected non-verotoxic strains isolated from raw buffalo meat and its products were also tested for haemolysin production on washed sheep blood agar (w-SBA) plates supplemented with calcium chloride\textsuperscript{8}. Streaked plates were incubated at 37°C and examined at 4 and 24 h intervals. Strains of \textit{E. coli}, which produced haemolytic reaction after 4 h and a clear zone after 24 h, were considered as alpha-haemolytic, whereas strains producing small turbid haemolytic zone around the streaking line after 18-20 h were considered as enterohaemolytic.

### Results

The VCA of 27 \textit{E. coli} isolated from raw beef and its products including kabab and kofta revealed 9 (33.33\%) as STEC belonging to 7 different ‘O’

<table>
<thead>
<tr>
<th>Primers</th>
<th>Oligonucleotide sequence (5’-3’)</th>
<th>Product size</th>
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<tbody>
<tr>
<td>\textit{Stx1a}</td>
<td>CAGTTAATGTTGGTGCGAAG</td>
<td>894</td>
</tr>
<tr>
<td>\textit{Stx1b}</td>
<td>CTGCTAATAGTTGCGATC</td>
<td>847</td>
</tr>
<tr>
<td>\textit{Stx2a}</td>
<td>CTTGGAGATCATATGGCGG</td>
<td>478</td>
</tr>
<tr>
<td>\textit{Stx2b}</td>
<td>GGAATGATCTCTGATTG</td>
<td>718</td>
</tr>
<tr>
<td>\textit{eae-1}</td>
<td>ACGTTCAGCATGGGTAACTC</td>
<td>815</td>
</tr>
<tr>
<td>\textit{eae-2}</td>
<td>GATCGGCAACAGTTCCACTG</td>
<td>815</td>
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serogroups. Using microscopic visualization, verotoxin (Stx) affected cells appeared round and shriveled. A total of 7 STEC isolates was detected from raw beef belonging to 6 different serotypes. STEC O145 (28.57%) was identified as the predominant serotype. The only strain of *E. coli* O157 isolated from raw beef was also positive for Stx production. Detection of STEC from kabab and kofta depicted 1 isolate each, which belonged to O153 and O8, respectively (Table 2).

Detection of virulence genes by PCR in all the 9 STEC isolates capable of producing CPE on Vero cells, consisting of 7 different serotypes from raw beef, kabab and kofta demonstrated that the majority possessed stx2 (77.78%). Notably, 2 strains of STEC O145 isolated from raw beef were found to bear stx1 and stx2 alone. The gene encoding Stx (stx1) was only detected in 2 STEC serotypes (O98 and O145) isolated from raw beef. Moreover, the only STEC O157 strain isolated from raw beef was recorded to bear stx2 genotype. STEC strains O153 and O8 isolated from kabab and kofta, respectively, were found to contain only stx2. None of the 9 STEC isolates was found to possess eae (Table 3).

Of the 9 STEC isolates of different serotypes from raw beef, kabab and kofta, 6 (66.67%) were positive for E-hly. Detection of E-hly production by STEC isolates from raw beef (85.71%) involved 5 different serotypes which included O25, O98, O113, O145 and O157. STEC O153 isolates from raw beef as well as kabab and kofta were found to be negative for E-hly production. None of the 10 non-STEC isolates was found to be capable of E-hly production.

**Discussion**

The prevalence of STEC worldwide in beef detected 14.10% in Netherlands, 17% in UK, 23-25% in USA, 15-40% in Canada. STEC from 9% market beef and 8-28% fresh beef was recorded in Thailand whereas only 3% STEC was reported in raw beef in Australia. Although there is paucity of information on STEC in India, 1.78% of the organisms have been reported in raw beef samples.

In the present investigation, O153 STEC was found in kabab besides raw beef, which indicated improper cooking, cross-contamination, or some degree of resistance in the isolates. On the other hand, the presence of STEC O8 was only detected in kofta suggesting cross-contamination at some point throughout processing or post-processing.

STEC isolates from beef and its products were positive for either stx1 or stx2. The predominant stx genotype has been reported to vary in different geographical locations. High detection rate of stx2 (52%) followed by stx1 (20%) in STEC has been reported earlier from bovine. Several workers have reported a similar distribution of stx genes in STEC from bovine sources. The predominance of stx2 either alone or in combination with stx1 has been highly associated with HUS and considered to be the most important virulence factor as compared to stx1.

Several studies have suggested that STEC carrying stx1 as well as eae genes might have a role in diarrhoea. However, STEC strains that did not bear eae have also been reported to cause HUS and HC in humans. The high and low prevalence of

<table>
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<tr>
<th>Sample(s)</th>
<th>STEC serotype(s) recovered</th>
<th>STEC isolates recovered Serotype-wise Total no. of isolate(s)</th>
<th>Type(s) of virulence genes produced</th>
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</thead>
<tbody>
<tr>
<td>Raw beef</td>
<td>O145</td>
<td>2</td>
<td>Stx1 Stx2 Stx1 &amp; Stx2 eae</td>
</tr>
<tr>
<td>Raw beef</td>
<td>O98</td>
<td>1</td>
<td>1 1 Nil Nil</td>
</tr>
<tr>
<td>Raw beef</td>
<td>O25, O113, O153, O157</td>
<td>1 each</td>
<td>4 Nil 4 Nil Nil</td>
</tr>
<tr>
<td>Kabab</td>
<td>O153</td>
<td>1</td>
<td>1 Nil 1 Nil Nil</td>
</tr>
<tr>
<td>Kofta</td>
<td>O8</td>
<td>1</td>
<td>1 Nil 1 Nil Nil</td>
</tr>
<tr>
<td>Total</td>
<td>-</td>
<td>9</td>
<td>2 7 Nil Nil</td>
</tr>
</tbody>
</table>

Table 2—Distribution of STEC serotype(s) in beef and its products

Table 3—Distribution of virulence gene(s) in STEC serotypes in beef and its products

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eae among STEC from diarrhoeic calves as well as non-diarrhoeic calves\textsuperscript{24,25}, and healthy cattle\textsuperscript{26}, respectively, have suggested eae to be a factor in the colonization of the calf intestine by bovine STEC. In another report, none of the 33 STEC isolates from healthy cattle was found positive for eae\textsuperscript{27}. In the present study, eae was not detected in any of the 9 STEC isolates. The beef samples during collection were available from apparently healthy cattle. No samples were available from calves. This might be the reason, why none of the 9 STEC isolates was positive for eae.

Phenotypic E-hly, produced by STEC has been associated with various serotypes\textsuperscript{28,29}. Most of the STEC serotypes produced E-hly except STEC O153 and O8 strains in the present study. Similar association of E-hly in STEC from animals, human clinical cases and foods of animal origin has been reported earlier\textsuperscript{30}. A close association between E-hly and STEC isolates from animals and humans regarded E-hly to be a virulence factor\textsuperscript{29}. The findings in the present study also appeared to bear an association between Stx as well as stx genotypes and E-hly production in STEC isolates. STEC O153 has been associated with cases of HUS\textsuperscript{31} and yet this strain does not possess E-hly. However, STEC O8 is not known to cause HUS or HC. The presence of E-hly has been correlated with eae negative as well as positive strains for differentiating the degree of virulence in STEC\textsuperscript{30}. Further studies of STEC isolates from calves, adult cattle and other food animal species are required to assess their role as human pathogen.

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