Detection and virulence gene characterization of Shiga toxigenic *E. coli* from buffalo meat samples

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**ABSTRACT**

Shiga-toxigenic *Escherichia coli* (STEC) are causative agents of bloody diarrhoea, haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). Humans acquire infections primarily through contaminated beef. In India, STEC has not been implicated as a major cause of diarrhoea. The study was carried out to know the prevalence of shiga toxin (ST) producing *Escherichia coli* (STEC) and virulence genes characterization from buffalo meat samples sold at various retail meat shops at Anand, India. In the present investigation 70 isolates of *Escherichia coli* isolated from 150 retail market buffalo meat samples, were screened for STEC, using conventional culture methods, serotyping and polymerase chain reaction (PCR). Out of 70 *E. coli* isolates 11 different ‘O’ serogroups were recorded in 44 isolates. While 19 isolates were untypable and seven were rough isolates. Out of the 70 *E. coli* tested 56 isolates (80%) were positive for *stx* genes: of which 51 (72.85%) harboured *stx*2 genes and 2 isolates (2.85%) were positive for *stx*1 gene only. Moreover, 3 *E. coli* isolates (4.28%) harboured both *stx*1 and *stx*2. About 66 (94.28%) isolates were positive for *eaeA* gene. While out of 70 *E. coli* isolates tested, 3 (4.28%) were found to be positive for *rfb* O157 gene. Presence of STEC and other virulence factors in buffalo meat samples appeared to be matter of concern and threat to public health.

**Key words**: Buffalo meat, PCR, Shiga toxigenic *E. coli* (STEC), Serotyping.

**INTRODUCTION**

Acute diarrhoea is a leading cause of mortality in developing countries. Diarrhoeagenic *Escherichia coli* include enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC) and shigatoxin producing *E. coli* (STEC). Shiga toxin-producing *E. coli* (STEC) cause diarrhoea, haemorrhagic colitis, and haemolytic uremic syndrome (HUS) (Nataro and Kaper, 1998). The morbidity and mortality associated with recent large outbreaks of gastrointestinal disease caused by Shiga toxin-producing *Escherichia coli* (STEC) has highlighted the threat of these organisms to public health (Paton and Paton, 1998; Renter et al., 2008). All STEC produce one or both of two Shiga toxins, *Stx*1 and *Stx*2. STEC strains that produce *Stx*2 are more strongly associated with HUS than strains that produce *Stx*1 or both *Stx*1 and *Stx*2. Epidemiologic evidences indicate a recent increase in the rate of HUS among STEC outbreaks. The increasing rate of HUS could be explained by a shift in the toxin profiles of STEC strains. During the past two decades, an increasing number of human foodborne illness outbreaks have been traced to consumption of undercooked ground beef and other beef products contaminated with Shiga toxin-producing *Escherichia coli* O157 (STEC). STEC, also referred to as Verocytotoxin-producing *E. coli* (VTEC) cause major, potentially fatal, zoonotic food-borne illness whose clinical spectrum includes diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (Karmali, 2003).

STEC infections are considered a public health problem in both developed and developing countries due to the severity of the disease they cause and the global nature of the food supply (Brando, 2008). But in India though, STEC has not been identified as a major aetiologic agent of diarrhoea, it has been isolated from meat and fish samples, suggesting that this enteropathogen may pose a public health problem.

Hence, an attempt was made in the present study to isolate STEC from raw buffalo meat sold at various retail meat shops at Anand, India.

**MATERIALS AND METHODS**

**Samples**: Altogether 150 raw buffalo meat samples comprising of ground beef (keema), muscle, intestine, liver and lung (30 each) were collected in sterilized polyethylene...
Isolation of Escherichia coli: The standard protocol described Bacteriological Analytical Manual (BAM), U.S. Food and Drug Administration (USFDA) method (Hitchins et al., 2001) was adopted for the isolation of STEC from Buffalo meat samples. Briefly, 25 g of each type of sample was thoroughly triturated in a sterile mortar and pestle and transferred to 225 ml Mc conkey broth incubated at 37°C for 18-24 h. The enrichments cultures were streaked on Eosin Methylene Blue agar (EMB) and MacConkey agar medium

Isolation of Escherichia coli O157: Briefly, 25 g of each type of sample was thoroughly triturated in a sterile mortar and pestle and transferred to Modified EC Broth with novobiocin (MEC+n) (enrichment broth) incubated at 37°C for 18-24 h. The enrichments cultures were streaked on both SMAC and CTSMAC.

These agar plates were incubated at 37°C for 18-24 h. Both sorbitol fermenting and non fermenting colonies were picked and identified by standard biochemical tests viz., catalase, oxidase, fermentation of lactose and glucose using triple sugar iron agar, decarboxylation of lysine using lysine iron agar, production of indole, methyl red test, Voges Proskauer test and utilization of citrate. The colonies identified as E. coli were preserved and used for serotyping and virulence gene characterization by PCR.

Serotyping of E. coli isolates: Cultures identified as E. coli were serotyped at National Salmonella and Escherchia Centre (NSEC), Central Research Institute (CRI), Kasauli (Himachal Pradesh, India).

DNA extraction and polymerase chain reaction: The DNA of isolates of E. coli was prepared by bacterial lysis method. Approximately loopful of culture was taken in microcentrifuge in 100 µl of sterilized DNAs and RNase-free milliQ water. Then vortexed and samples were heated at 95°C for 10 min, cell debris was removed by centrifugation and 3 µl of the supernatant was used as a DNA template in PCR reaction mixture.

PCR primers and reaction condition: All the E. coli isolates were first screened for the presence or absence of virulence associated genes by using the PCR protocols separately standardized for the detection of different genes. The PCR was standardized for the detection of four genes viz. stx1, stx2, rfbO157 and eaeA following the methodology as described by (Hazarika et al., 2007) and Dhanashee and Mallaya, (2008) with suitable modifications. Standardization of PCR was done by using standard strain of (STEC) E. coli O157:H7. PCR was performed with four sets of primer pairs specific for the stx1, stx2, rfbO157 and eaeA gene as per the details given in Table 1.

The reaction was standardized in thin walled PCR tubes in 25 µl reaction volume with different concentration of reactants under different annealing temperatures and cycling conditions. Finally, the reaction mixture was optimized to contain 12.5 µl 2X PCR master mix, 10 pmol of each forward and reverse primer, 7.5 µl nuclease free distilled water and 3 µl of DNA template. The reaction was performed in the thermal cycler with pre-heated lid (Lid temp. 105°C). Reaction conditions employed were: initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min. A final extension of 7 min at 72°C was employed. Reaction condition was the same as earlier except for the annealing temperature for respective primer were used (Table 1).

On completion of the reaction the amplified products were analyzed on agarose gel electrophoresis through 2% agarose gel stained with 5 µg/ml of ethidium bromide with a 100 bp DNA ladder as molecular weight marker, visualized under UV light and results were noted.

<table>
<thead>
<tr>
<th>Primer pair target</th>
<th>Primer sequence (5′→3′)*</th>
<th>Annealing Temperature</th>
<th>Size of Amplified Product (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>stx1</td>
<td>F: CAGTTAATGTGGTGCGAG</td>
<td>55°C</td>
<td>894 bp</td>
<td>Hazarda et al., 2007</td>
</tr>
<tr>
<td></td>
<td>R: CTCGCTATATAGTCTGCGAC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>stx2</td>
<td>F: CTTCCGTATCTCTATCCCGG</td>
<td>55°C</td>
<td>478 bp</td>
<td>Hazarda et al., 2007</td>
</tr>
<tr>
<td></td>
<td>R: GGATGATCCCTCTGGTCTATG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rfbO157</td>
<td>F: AAGATTGCCTTAGACGCTTTG</td>
<td>58°C</td>
<td>497 bp</td>
<td>Dhanashee and Mallaya, 2008</td>
</tr>
<tr>
<td></td>
<td>R: CATTGCGATCGTGGGAGGC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eaeA</td>
<td>F: GACCCGCGACAGCATGAAAGC</td>
<td>58°C</td>
<td>384 bp</td>
<td>Dhanashee and Mallaya, 2008</td>
</tr>
<tr>
<td></td>
<td>R: CCCACCTGCAGCAAAAGAG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

During the present study 70 E. coli isolates were obtained from 150 buffalo meat samples. Out of these 70 E. coli isolates, 56 were confirmed as STEC and 14 EPEC. Interestingly 53 were confirmed as non O157 STEC and 3 isolates were E. coli O157. All the isolates revealed characteristic features of E. coli, which were Gram negative bacilli, produced pink lactose fermenting colonies on MacConkey agar and characteristic greenish metallic sheen on Eosin Methylene Blue agar (EMB). While E. coli O157 revealed colorless colonies on Sorbitol Mc Conkey (SMAC) and Sorbitol McConkey Agar supplemented with cefexime and potassium tellurite (CT- SMAC). On preliminary biochemical characterization they revealed characteristic IMViC pattern (+ + - -).

All the 70 positive E. coli isolates from Buffalo meat samples were serotyped at National Salmonella and Escherichia Centre, Kasauli, India. In the present study, out of 70 E. coli isolates 11 different ‘O’ serogroups were recorded in 44 isolates. While 19 isolates were untypable and seven were rough isolates. Among the 44 isolates the different serogroups detected in decreasing frequency were 18 isolates (25.71%) O84, 6 isolates (8.57%) O68, 4 isolates (5.71%) O60, 3 isolates (4.28%) each of O5, O157, O168, 2 isolates (2.86%) each of O59, O23 and 1 isolate (1.43%) each of O100, O22, O8. The serotypes like O5, O8, O22, O23, O59, O60, O68, O84 O100, O157 and O168 which were recovered during the present investigation have also been isolated frequently from infantile diarrhoea among neonates, adult patients suffering from gastroenteritis. Earlier studies showed that, among the isolates the serogroups viz. O5, O22, O23, O68 and O84 have been also reported by Dhanashree and Mallaya, (2008) from animals.

In the present investigation 70 isolates of Escherichia coli isolated from 150 retail market buffalo meat samples, subjected to virulence genes detection by PCR. In this study, out of 70 E. coli isolates 56 isolates (80%) were positive for stx genes: of which 51 (72.85%) harboured stx2 genes and only 2 isolates (2.85%) were positive for stx1 gene (Figure 1 & 2). While 3 E. coli isolates (4.28%) harboured both stx1 and stx2. About 66 (94.28%) isolates were positive for eaeA (figure 3). Similar results for high per cent (40 per cent or more) of stx gene positive E. coli isolates have been reported by Kobayashi et al., (2001); Irino et al., (2005) and
TABLE 2: Detection of STEC virulence genes by PCR

<table>
<thead>
<tr>
<th>Source of Sample</th>
<th>No. of samples analyzed</th>
<th>Total No. of positive samples</th>
<th>No. of positive strains for stx1</th>
<th>No. of positive strains for stx2</th>
<th>No. of positive strains for stx1 &amp; stx2</th>
<th>eaeA</th>
<th>rfb O157</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ground Beef</td>
<td>30</td>
<td>27</td>
<td>2</td>
<td>17</td>
<td>1</td>
<td>27</td>
<td>1</td>
</tr>
<tr>
<td>Muscle</td>
<td>30</td>
<td>14</td>
<td>0</td>
<td>11</td>
<td>0</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Intestine</td>
<td>30</td>
<td>24</td>
<td>0</td>
<td>18</td>
<td>2</td>
<td>22</td>
<td>2</td>
</tr>
<tr>
<td>Liver</td>
<td>30</td>
<td>03</td>
<td>0</td>
<td>03</td>
<td>0</td>
<td>03</td>
<td>0</td>
</tr>
<tr>
<td>Lung</td>
<td>30</td>
<td>02</td>
<td>0</td>
<td>02</td>
<td>0</td>
<td>02</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>150</td>
<td>70</td>
<td>2</td>
<td>51</td>
<td>3</td>
<td>66</td>
<td>0</td>
</tr>
</tbody>
</table>

Zweifel et al., (2005). However, lesser isolation rate for stx positive E. coli isolates has been reported by Rahman (2002) and Chattopadhyay et al., (2003). Detailed result is given in Table 2.

Out of 70 E. coli isolates tested, about 66 (94.28%) isolates were positive for eaeA. Among these 70 E. coli isolates, 14 (20.00%) isolates possess only eaeA and 52 (74.28%) isolates possess eaeA in combination of stx gene. So those isolate which possess only eaeA are Enteropathogenic E. coli (EPEC) while those isolates in which eaeA is present in combination with stx gene are Shiga toxicigenic E. coli (STEC). So in present study intimin gene has been reported in (74.28 %) STEC isolates. Similar findings have been reported by Galland et al., (2001); Wani et al., (2003) and Osek (2004) which is in contrast to lower prevalence as reported by Blanco et al., (2004); Mora et al., (2007) and Arya et al., (2008).

In the present study 51 (7.85%) isolates harboured stx2 genes and 2 isolates (2.85%) were positive for stx1 gene only. Similar results for predominance of stx2 producing strains were reported by Wani et al., (2003); Blanco et al., (2004); Osek (2004); Hazarika et al., (2007); Milnes et al., (2008); and Salnec et al., (2009). In this study lower prevalence of stx1 is reported which is similar to finding reported by Galland et al., (2001); Blanco et al., (2004); Osek (2004); Arya et al., (2008) and Milnes et al., (2008).

In the present investigation, one of the most important serogroup O157 (3 isolates) known to cause certain life-threatening infections in humans and animals was isolated (Figure 4). Isolation of O157 is also reported by Wani et al., (2003); Mora et al., (2007) and Dhanashree and Mallya, (2008).

Prevalence of verocytotoxin genes among non-O157 strains could be detected by PCR amplification technique. In the present study, 53 E. coli isolates were non-O157 isolates. Similar prevalence of verocytotoxin genes among non-O157 were reported by Blanco et al., (2003).

In conclusion, the presence of E. coli O157 in the buffalo meat samples of Anand suggests that this enteropathogen may be of public health importance. Hence, routine screening of larger number of buffalo meat shops for the presence of STEC may be useful. However, atypical EPEC found in most of the meat samples processed, belonging to common serogroup, further characterization of these isolates from a large population of diarrhoeagenic individuals and healthy controls is necessary to know their role as emerging pathogens.

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REFERENCES


