Detection of shiga-toxigenic *Escherichia coli* (STEC) in diarrhoeagenic stool & meat samples in Mangalore, India

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**Background & objectives:** 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. Hence, isolation of STEC from diarrhoeagenic stool samples of patients and beef samples marketed through retail outlets was attempted in Mangalore, India.

**Methods:** Diarrhoeagenic stool samples (n = 192) and meat samples (n = 103) were screened for STEC, using conventional culture methods and polymerase chain reaction (PCR) from December 2003 to 2006 in the department of Microbiology, Kasturba Medical College, Mangalore. All the *E. coli* isolates were subjected to antibiotic susceptibility testing and serotyping.

**Results:** Of the 40 *eae* positive *E. coli* isolates from meat sample, one was positive for all the STEC genes, namely *stx1*, *stx2*, *rfb* O157 and EHEC *hlyA*. This isolate belonged to O157 serogroup. Of the 110 *eae* positive *E. coli* isolated from stool samples, two were positive for EHEC *hlyA* and belonged to serogroup O8 and one was positive for *bfp* gene and found to be of O6 serogroup. Among the 192 stool enrichment broths tested, 160 were positive for *eae* gene, of which two were EHEC *hlyA* positive and one was *bfp* gene positive. Among the 103 meat enrichment cultures, 90 were positive for *eae* gene and one among them was positive for all the STEC genes.

**Interpretation & conclusion:** Our results showed a low incidence of STEC and high prevalence of *eae* positive *E. coli* other than STEC in stool and meat samples. A low positivity was observed for PCR performed directly on stool and meat samples. However, PCR on enrichment cultures gave better results. Since *E. coli* O157 was isolated and detected by PCR in one of the meat samples, this organism may be of public health significance. A study on a large sample may provide some answer.

**Key words** Antibiotic resistance - bovine meat - *eae* positive *E. coli* - PCR - shiga toxigenic *Escherichia coli* - stool

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 shiga toxin producing *E. coli* (STEC). EPEC, a common causative agent of infantile diarrhoea, is often grouped together as attaching and effacing *E. coli* (AEEC). In addition to their ability to induce attaching and effacing (AE) lesions, EPEC possesses large EPEC adherence
factor (EAF) plasmid and the cluster of genes that encodes bundle-forming pili (BFP)\(^3\). EPEC strains carrying eae gene but lacking the EAF plasmid and stx gene were described as atypical EPEC. Atypical EPEC are more closely related to STEC, and appear to be an emerging pathogen\(^4\).

STEC is increasingly recognized as the cause of severe diseases such as haemorrhagic colitis and haemolytic uraemic syndrome in addition to diarrhoea. Although O157:H7 serotype is the dominant STEC in many parts of the world, it is recognized that STEC strains belong to a very broad range of O:H serotypes\(^5\). STEC are typically characterized by the ability to cause the AE lesions, possession of 60 Mda plasmid encoding enterohaemolysin (hlyA) and production of shiga toxin (stx\(_1\) or stx\(_2\)). Cattle being the primary reservoir of STEC, humans may acquire STEC infections primarily from consumption of contaminated beef\(^6\). Other sources of infection include vegetables, fruits, contaminated drinking water, or direct contact with faeces of infected persons\(^7\).

In India, though STEC has not been identified as a major aetiologic agent of diarrhoea, it has been isolated from meat and fish samples\(^8,9\), suggesting that this enteropathogen may pose a public health problem. Hence, an attempt was made in the present study to isolate STEC from diarrhoeagenic stool samples of patients and also the fresh meat samples sold through retail outlets in Mangalore.

**Material & Methods**

**Samples:** Over a three year period (December 2003 to December 2006) stool samples (n = 192) were collected from children aged 6 months to 15 yr suffering from diarrhoea (more than 3 loose stool per day for 2-3 days), admitted to Kasturba Medical College Hospital-Attavar, Kasturba Medical College Hospital-Ambedkar Circle, Govt. Wenlock Hospital and Lady Goschen Hospital in Mangalore city, by random sampling method. Samples from patients on antibiotics were excluded from the study. Sterile wide mouth containers (Hi-Media, Mumbai) were used for sample collection and the collected stool samples were transported at room temperature (25-30°C) within 30 min to the department of Microbiology, Kasturba Medical College, Mangalore, for analysis.

During the same period, bovine meat samples (n = 103) were collected in sterile containers from randomly selected retail outlets located at four different areas in Mangalore, at regular fortnightly intervals, and transported at room temperature (25-30°C) to the laboratory within 1 h of collection.

**Isolation of Escherichia coli from stool and meat samples:** Stool samples were initially screened microscopically for pus cells, RBCs, ova and cysts of parasites. Culture media and antibiotic supplements used in the study were procured from Hi-Media Laboratories, Mumbai. All the samples were cultured to isolate *E. coli*. DNA was extracted directly from the samples, enrichment cultures as well as *E. coli* isolates for PCR. The protocol described by De Boer and Heuvelink\(^10\) was adopted for the isolation of STEC from stool samples. Briefly, 4-5 loopful of stool sample was added to 10 ml of modified tryptic soy broth (TSB containing 20 mg/l novobiocin) for enrichment culture and incubated for 6-8 h at 37°C. Enrichment culture in modified TSB was streaked onto sorbitol MacConkey’s agar (SMAC) and SMAC containing 0.05 mg/l of cefixime and 1 mg/l potassium tellurite (CTSMAC). The plates were incubated for 18-24 h at 37°C. Both sorbitol fermenting and non fermenting colonies were picked (5-10 from each plate) and identified biochemically. Apart from screening for STEC, the stool samples were also inoculated into Selenite F broth (SFB) and alkaline peptone water (APW) enrichment broths. These enrichment broths were subcultured onto MacConkey’s agar, deoxycholate citrate agar (DCA), thiosulphate citrate bile salt sucrose (TCBS) agar and examined for the presence of enteric pathogens like, *Salmonella* spp., *Shigella* spp. and *Vibrio* spp.

The same protocol, described above, with minor modifications was employed for the isolation of STEC from meat samples. Briefly, 25 g of meat sample was added to 225 ml of modified TSB and incubated at 37°C for 18-24 h. Modified TSB enrichment 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\(^11\), 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 in 20 per cent glycerol broth at -20°C for further characterization.

**Antibiotic susceptibility testing:** Muller-Hinton broth and Muller-Hinton agar were used and antibiotic susceptibility testing was performed by disk diffusion method\(^12\). Briefly, biochemically confirmed *E. coli* isolates were grown in Muller-Hinton broth for 6 h at
Laboratory Standards Institute (CLSI) standard and were measured and compared with Clinical and control strain. The zones of clearing around the disks were extracted by growing the isolates in 2 ml of Luria Bertani (LB) broth for 18 h at 37°C. LB broth was centrifuged at 10000 g for 10 min. The pellet was re-suspended in 200 µl of the DNA was used for PCR.

DNA extraction and polymerase chain reaction: PCR was performed to detect virulence genes of STEC in modified TSB enrichment broths, stool and meat samples, as well as in biochemically confirmed isolates obtained after selective plating. DNA from enrichment broth was extracted as described previously9. Briefly, 2 ml of enrichment culture was centrifuged at low speed (approx. 1000 g). The supernatant was transferred to a fresh tube and centrifuged at 10000 g. Phenol and chloroform were added in 1:1 ratio, vortexed and centrifuged at 5000 g. The aqueous layer was transferred to a fresh tube. This step was repeated twice and followed by extraction with chloroform. DNA was precipitated with 2 volumes of ethanol by keeping at -20°C for 1-2 h, centrifuged. The pellet was dried and re-suspended in sterile PCR grade water and 4 µl of the DNA was used for PCR.

DNA from stool samples were extracted by using QIAamp DNA stool mini kit (Qiagen, GmbH Hilden, Germany, marketed by Genetix Asia, Bangalore) as per the manufacturer’s instruction. DNA from meat samples were extracted by following the method adopted by Makino et al14. DNA from biochemically confirmed E. coli isolates were extracted by growing the isolates in 2 ml of Luria Bertani (LB) broth for 18 h at 37°C. LB broth was centrifuged at 10000 g for 10 min. The pellet was re-suspended in 200 µl distilled water and lysed by boiling for 10 min in a dry bath (Bangalore Genei, Bangalore) and 2 µl of the DNA was used for PCR.

To determine the sensitivity and specificity of the PCR, a serial 10-fold dilutions of E. coli reference strain EDL 933 was inoculated onto tryptic soy agar plate to determine cfu/ml. Appropriate dilutions of this culture was inoculated to known aliquots of STEC negative fecal and meat samples. Two uninoculated meat and stool samples were used as negative controls. DNA from spiked stool and meat samples were extracted following the same procedure described earlier.

PCR primers and reaction condition: Based on the previously published reports for the detection of STEC virulence genes15-18, the following primers were selected.

**stx1** <F - ACACGTGGATGATCTCAGTG; R - CTGATCCCTCCCTCATTAGG>,

**stx2** <F - CAGAGCACAAGGACACGACGTT; R - CCTGCACTGACGACCACTTTG>,

**rfb** O157 <F - AAGATTGCGTAAAGCCTTTG; R - CATGGCATCCTGTTGAC>

EHEC hlyA <F - AGCATGTTGGTTATTCTGGA; R - CTTCACGTCACCATAcatAT>,

**eaeA** <F - GACCCGGCAACAAGCAAGC; R - CCACCTGCAAGCAACAGAGG>.

All the PCR primers and molecular reagents were procured from Bangalore Genei, Bangalore. Extracted DNA was amplified in a 30 µl reaction mixture containing 10x PCR buffer (100 mM Tris, 500 mM KCl, 15 mM MgCl2), 200 µM concentration of each of the 4-deoxynucleotide triphosphate (dNTPs), 30 picomoles of each primer and 1U Taq DNA polymerase. PCR was performed individually using the above primer pair for stx1, stx2, rfb O157, EHEC hlyA and eaeA genes.

The PCR reactions of 35 cycles were carried out in a thermocycler (Gene cycler, Bio Rad Inc., USA). The reaction conditions were: initial denaturation at 95°C for 5 min, denaturation at 95°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 1 min and final extension at 72°C for 5 min. The PCR products were separated on a 1.5 per cent agarose gel, stained with ethidium bromide19 and photographed using a gel documentation system. E. coli reference strain EDL 933 was used as positive control in all PCR reactions for STEC. Lysates that were positive for only stx1, stx2, rfb, eaeA and negative for other STEC genes were further tested for the presence of bfp gene by PCR using the primer pair20 <F - TCTTGCTTTGATCTGCA; R - GTAAAATCGTTAATTCGCA> and reaction condition was the same as earlier except for the annealing temperature of 56°C for 1 min. E. coli reference strain E2348/69 was used as positive control in all PCRs.

Results

Of the 192 diarrhoeal stools analyzed, 142 samples containing the pus cells yielded biochemically confirmed E. coli isolates by conventional culture
technique. *Shigella sonnei* and *S. flexneri* were isolated from eight samples each. Three samples showed the growth of *V. cholerae* and *Salmonella Typhimurium* was isolated from six of the stool samples. One stool sample each revealed microscopically the presence of ova of *Ascaris lumbricoides*, *Trichuris trichiura* and the larval forms of *Strongyloides stercoralis*.

Among the 103 meat samples processed, *E. coli* was isolated from 80 samples by conventional culture technique. Other bacterial pathogens were not isolated from these samples.

PCR performed on 142 biochemically confirmed *E. coli* isolates from stool for the detection of STEC virulence genes showed 110 to be positive for only *eae* gene. However, two of the *eae* positive *E. coli* stool isolates were also positive for EHEC *hlyA* gene. All *eae* positive *E. coli* isolates, that were negative for other STEC genes, were screened for the presence of *bfp* genes (197 bp) and one was found to be positive. Among the 80 biochemically confirmed *E. coli* isolates from meat samples, 40 were positive for *eae* gene. One of the *eae* positive meat isolate was also positive for all other STEC genes like *stx1* (614 bp), *stx2* (779 bp), *rfbO157* (497 bp), *eae* (384 bp) and EHEC *hlyA* (166 bp) by PCR (Fig.).

Out of 192 stool enrichment broths examined by PCR, 160 were positive for *eae* gene. Among these, two were also positive for EHEC *hlyA* gene and one was positive for *bfp* gene. All the stool enrichment broth lysates were negative for *stx* and *rfbO157* genes. Among the 103 meat enrichment lysates subjected to PCR for different virulence genes of STEC, 90 were positive for *eae* gene. Among these, one meat enrichment broth was also positive for all STEC genes (Table I).

Out of 192 stool samples tested directly by PCR, 120 stool samples were found to be positive for *eae* gene and two among them were positive for EHEC *hlyA* and one for *bfp* gene. Among the 103 meat samples directly tested by PCR, 65 were *eae* positive and one was positive for all the STEC genes. All the samples that showed positive result when PCR was performed directly had grown *E. coli* by conventional method and these isolates were also found to be positive for respective genes.

Table I. Detection of STEC virulence genes by PCR

<table>
<thead>
<tr>
<th>Description</th>
<th>Source</th>
<th>STEC virulence gene detection*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>eae</td>
<td>EHEC hlyA</td>
</tr>
<tr>
<td>Direct PCR</td>
<td>Stool</td>
<td>120</td>
</tr>
<tr>
<td>on samples</td>
<td>Meat</td>
<td>65</td>
</tr>
<tr>
<td>PCR on</td>
<td>Stool</td>
<td>160</td>
</tr>
<tr>
<td>enrichment</td>
<td>cultures</td>
<td></td>
</tr>
<tr>
<td>PCR on <em>E.coli</em></td>
<td>isolates</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>Meat</td>
<td>40</td>
</tr>
</tbody>
</table>

*One stool sample, enrichment culture and an isolate from the same sample were positive for both *eae* & *bfp* genes.

stool samples n=192; meat samples n=103

(A)

![Agarose gel showing the amplification product of PCR performed on *E. coli* isolate for the detection of EHEC *hlyA*, *rfbO157*:H7 genes](<image_url>)

(B)

![Agarose gel showing the amplification product of PCR performed on *E. coli* isolate for the detection of EHEC *hlyA*, *rfbO157*:H7 genes](<image_url>)

Fig. Agarose gel showing the amplification product of PCR performed on *E. coli* isolate for the detection of EHEC *hlyA*, *rfbO157*:H7 genes (A) and *stx* genes (B). Lanes 1 & 4: isolate from meat sample positive for EHEC *hlyA* and *rfbO157*:H7 genes respectively. Lanes 8 & 11: isolate from meat sample positive for *stx1* and *stx2* genes respectively. Lanes 2, 5, 9 & 12: positive control (*E. coli* EDL 933), Lanes 3, 6, 10 & 13: negative control (*E. coli* ATCC 25922), Lane 7 & 14: molecular weight marker.
All the eae positive E. coli isolates from stool and meat samples were serotyped at National Salmonella and Escherichia Centre, Kasauli, India. Clinical isolates belonged to 34 different serogroups and meat isolates belonged to 13 different serogroups. The two isolates positive for both eae and EHEC hlyA genes belonged to serogroup O8 whereas, the isolate positive for both eae and bfp genes of E. coli belonged to serogroup O6. Rest of the 107 eae positive clinical isolates belonged to different serogroups, viz., O1, O2, O3, O4, O6, O10, O12, O15, O18, O20, O21, O23, O25, O48, O60, O73, O84, O86, O101, O111, O138, O139, O140, O148, O152, O153, O156, O158, O159, O160, O168, O169, and O172. One of the isolates from meat sample, which was positive for STEC genes, belonged to serogroup O157. The serogroups of rest of the eae positive meat isolates were O6, O10, O18, O20, O21, O73, O100, O101, O111, O117, O138 and O139. The antibiotic susceptibility pattern of eae positive E. coli isolates from stool and meat samples is shown in Table II.

Discussion

The epidemiological significance of each E. coli category in childhood diarrhoea may vary with the geographical area. In the present study, eae positive E. coli isolates that were negative for virulence markers of STEC and EPEC were found to be predominantly isolated from stool (75.35%) and meat (48.75%) samples. Earlier studies refer EPEC strains carrying eae gene, but lacking bfp and stx genes, as atypical EPEC and E. coli isolates carrying both eae and bfp genes as typical EPEC. Similar strains have been reported to be associated with diarrhoea, food borne and water borne outbreaks. Like STEC, these atypical EPEC isolated by us may be emerging pathogens. However, eae positive atypical E. coli requires further study with regard to their virulence and epidemiologic significance.

PCR performed on enrichment cultures of stool and meat samples is found to be more efficient in detecting pathogenic E. coli when compared to conventional techniques. Colony characters in selective media are based on biochemical characters and it is not unusual to find bacteria closely resembling E. coli in both stool and meat samples. Moreover, performing PCR on the lysates obtained from enrichment broth has the advantage that it dilutes the inhibitors of PCR, detects live bacteria and saves time needed for the isolation and identification.

The lower positivity of PCR performed directly on samples when compared to enrichment culture PCR could be due to the presence of inhibitors in these stool and meat samples. Hence, better DNA extraction methods are necessary to overcome inhibitors that will avoid the false negative results. To some extent the inhibitors were overcome by extraction of DNA after enrichment of the samples.

Schmidt et al. reported that, although some STEC strains may lack the EHEC hlyA gene, but its presence would be a good marker for STEC. Two EHEC hlyA and eae positive isolates in our study belonged to O8 serogroup and were negative for rfb O157 gene by PCR. Hence, these two isolates can be considered as STEC other than E. coli O157. However, the absence of stx genes in these isolates could be due to the fact that stx gene is bacteriophage coded and the isolate would have lost the same during preservation. The isolation of E. coli harbouring the EHEC hlyA gene is significant, as there is increasing evidence of haemolysin gene being the marker for shiga toxin-producing E. coli.

Therapeutic options vary depending on the type of E. coli infection. Antimicrobials used in this study are generally recommended for the treatment of diseases due to E. coli other than STEC. Among the eae positive

<table>
<thead>
<tr>
<th>Antibiotics tested (µg)</th>
<th>Stool samples (n = 110)</th>
<th>Meat samples (n = 40)</th>
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<tbody>
<tr>
<td></td>
<td>S (%)</td>
<td>I (%)</td>
</tr>
<tr>
<td>Amoxicillin (10)</td>
<td>35 (31.8)</td>
<td>9 (8.2)</td>
</tr>
<tr>
<td>Cefazidime (30)</td>
<td>75 (68.2)</td>
<td>4 (3.6)</td>
</tr>
<tr>
<td>Cephotaxime (30)</td>
<td>70 (63.6)</td>
<td>4 (3.6)</td>
</tr>
<tr>
<td>Ciprofloxacin (5)</td>
<td>44 (40.0)</td>
<td>4 (3.6)</td>
</tr>
<tr>
<td>Ceftriaxone (30)</td>
<td>66 (60.0)</td>
<td>4 (3.6)</td>
</tr>
<tr>
<td>Cefuroxime (30)</td>
<td>62 (56.4)</td>
<td>4 (3.6)</td>
</tr>
<tr>
<td>Cephotaxitin (30)</td>
<td>62 (56.4)</td>
<td>2 (1.8)</td>
</tr>
<tr>
<td>Gentamyacin (10)</td>
<td>75 (68.2)</td>
<td>3 (2.7)</td>
</tr>
</tbody>
</table>

S, sensitive; R, resistant; I, intermediate
E. coli isolates tested for the susceptibility pattern, approximately more than half displayed resistance to one or more antimicrobials. These data are in accordance with earlier reports. Increasing antimicrobial resistant phenotypes seen in human diarrhoea could be due to indiscriminate use of antimicrobials in clinical practice. However, STEC isolated from meat sample, EHEC hlyA and bfp positive isolates from stool samples were sensitive to all the antibiotics tested.

Earlier studies showed that serogroups O101, O48, O23, O73 have been isolated from human diarrhoea, HUS and serogroup O6 from HUS and cattle. So far, serogroups of STEC like O10, O21, O18, O84, O86, O138 and O139 have been reported to be isolated only from animals. Interestingly in the present study, most of these E. coli serogroups have been isolated from diarrhoeagenic stool and meat samples. Earlier reports from India showed a very low incidence of EHEC or STEC in humans. Our results are in agreement with this observation and showed a very low prevalence rate of STEC (two out of 142 from stool and one out of 103 from meat) and a high isolation rate of E. coli resembling atypical EPEC.

In conclusion, STEC could not be implicated as a major causal agent of diarrhoea. The presence of E. coli O157 in the meat samples of Mangalore suggests that this enteropathogen may be of public health importance. Hence, routine screening of diarrhoeagenic stool samples for STEC may be useful. Moreover, atypical EPEC found in most of the diarrhoeal stool and meat samples processed, belonging to common serogroup, could be implicated as a causative agent of diarrhoea in this part of the country. However, 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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