Full Length Research Paper

Shiga toxin-producing *Escherichia coli* O26 strains in bovine feces in East-Azarbaijan, Iran

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Shiga toxin-producing *Escherichia coli* (STEC) strains are a diverse group of organisms associated with severe gastrointestinal and systemic disease in human. Among non-O157 STEC family, *E. coli* O26 strains are the common serogroup causing disease such as HUS in human. Cattle are thought to be the main source of this microorganism. We collected 211 samples of bovine feces from different area in East-Azarbaijan province and cultured them in Rhamnose–MacConkey agar supplemented with Cefixime and potassium-tellurite, (CT- RMAC agar). Colorless colonies in CT-RMAC agar medium confirmed by multiplex PCR assay using primer specific for genes encoding biosynthesis of the O antigens (O-antigen flippase and O-antigen polymerase) and shiga toxins (stx 1 and stx 2). There were colorless colonies in CT-RMAC agar medium of 18 samples (8.53 %) that 17 samples (8.05 %) of them were positive for the presence of the respective genes. Relatively high number of STEC O26 positive cattle found in our region can be a potential vehicle for transmission of this pathogen directly or indirectly (from food) to human.

**Key words:** Shiga toxin-producing *E. coli* O26, Bovine, HUS, CT-RMAC agar, multiplex PCR.

INTRODUCTION

*Escherichia coli* is the common enteric commensal in animal species and part of strains of this microorganism is an established diarrhoeal pathogen and associated with human disease (Duffy, 2003). It is well documented that *E. coli* O157 strain, is the most significant group capable of producing cytotoxic factors to African green monkey kidney (Vero) cells and hence has been named Verotoxin or Shigatoxin, because of similarity to shigatoxin produced by *shigella dysenteriae* type1 (Meng et al., 2007).

Among the non-O157 shigatoxin-producing *E. coli* (STEC) *E. coli* O26 strains are thought to be the common serogroup, composing 18% of the total number of STEC isolates from 1997 to 1999 according to the reports of infections Agents surveillance center-National institute of infectious Disease (Hiramatsu et al., 2002 and DebRoy et al., 2004). STEC O26 associated with human disease syndromes including diarrhoea and Hemolytic Uremic Syndrome (HUS) and has been isolated from the feces of cattle, lambs, goats and pigs. Therefore, animals can be route of transmission of this pathogen directly or indirectly to human (Catarame et al., 2003; Van Diemen et al., 2005; Jenkins et al., 2008).

The carbohydrate-fermenting of *E. coli* O26 has been investigated previously and MacConkey – Rhamnose medium supplemented with Cefixime and Tellurite (CT-RMAC agar) has been developed (Cobbold and Desmarchelier, 2000 and Hiramatsu et al., 2002). Also, PCR based assays for detecting *E. coli* O26 were developed by targeting the *wzx* (O-antigen flippase) and *wzy* (O-antigen polymerase) genes that are specific for the respective serogroup (DebRoy et al., 2004).

The data available suggest that there are geographical
differences in the frequency of O157 and non-O157 STEC and in some countries non-O157 infections have been reported more frequently than with O157 STEC infections (Jenkins et al., 2008). The aim of this study was to determine the prevalence of E. Coli O26 strains in cattle feces in East-Azarbaijan, Iran.

MATERIALS AND METHODS

Sample collection

The study was carried out on Cattle feces obtained from different area of East-Azarbaijan, Iran between 24th April and 28th July 2009. Altogether 211 samples of feces were collected. All samples were packed in separate sterile plastic bags and transported to the laboratory immediately after sampling and were analyzed in the laboratory.

Enrichment

Twenty five grams of each sample were added in 225 ml of Buffered Peptone Water and incubated at 41°C for 18 hours, after homogenization for one minute.

Selective Plating

Each enrichment culture was serially diluted 10-fold up to $10^{-5}$ in 0.1% peptone water and 100 µl volumes of the $10^{-4}$ and $10^{-5}$ dilutions were spread onto Rhamnose–MacConkey agar (The medium was prepared by replacement of Lactose in MacConkey agar medium with 10 g/L of Rhamnose sugar) supplemented with 0.05 mg/L Cefixime and 2.5 mg/L potassium-tellurite (CT- RMAC agar). The plates were incubated at 37°C for 18 to 24 h (Hiramatsu et al., 2002).

Confirmation tests

Rhamnose-non fermenting colonies that were colorless, (up to 10 per sample) were selected for verification. These colonies were tested for catalase and oxidase activity and catalase positive and oxidase negative colonies were confirmed to be E. coli , using Indol test with SIM medium (Merk, Germany) and citrate utilization test with Simmon’s citrate agar (Merk, Germany) and the Methyl red and Vogens-proskauer tests using MR-VP medium (Merk, Germany) (Harrigan, 1998).

PCR test

Presumptive colonies and E. coli O26 used as positive control (H31b, WHO) were grown in 5 ml of Brain Heart Infusion (BHI) broth for 18 h at 36°C and then the DNA was extracted according to the protocol below: (a) Centrifuge 1.5 ml of prepared broth in Eppendorf tube at 10000 rpm for 5 min and discard the supernatant. (b) Add 800 µl of lyses buffer (containing in 5 mol NaCl, 100 mM Tris-base, 20 mM EDTA-Na2 and CTAB 20% ) and homogenize the sample with shaker. (c) Incubate the tube at 65°C for 10 - 20 min and centrifuge again at 12000 rpm for 10 min., (d) Add equal volume of chloroflor – isoamylalcohol (24:1) to the supernatant. (e) Centrifuge the tube at 12000 rpm for 10 min and transfer the supernatant to a new tube (f) Add equal volume of –20°C isopropanol and keep the sample at -20°C for 30 min. (g) Centrifuge the tube at 12000 rpm for 10 min and discard the supernatant. (h) Add 250 µl of 4°C ethanol (70 %) and centrifuge at 10000 rpm for 5 min. (i) Discard the supernatant and let the pellet to be dry at room temperature. (j) Dissolve the dried pellet in 50 µl of sterile distilled water.

The oligonucleotide primers (MWG, Germany) used in this study are listed in Table 1. PCR assays were performed in 25 µl volume containing 50 ng of extracted DNA, 12.5 µl PCR primer master kit (Cinagene,Iran cat.number PR8250C), 0.4 µM of each primer and 9.5 µl distilled water. The reaction were carried out with amplification thermal cycler (Eppendorf model 22331, Germany). The procedure consisted of initial denaturation at 94°C for 3 min, followed by 35 cycles of template denaturation at 94°C for 40 s for assay1 and 30 s for assays 2 and 3, primer annealing at 52°C for 1 min for assay1 and 62°C for 30 s for assays 2 and 3 and extension at 72°C for 20 s for assay1 and 72°C for 75 s for assays 2 and 3. The final cycle was followed by incubation of the reaction mixture for 10 min at 72°C.

PCR products were analyzed by electrophoresis with 1.5% agarose gels containing 0.5 mg of ethidium bromide per ml. These were visualized with UV illumination and photographed. DNA standard molecular weight size marker (ladder) was included in each agarose gel electrophoresis run.

RESULTS

Results of analysis of feces are presented in Table 2. There were colorless colonies in CT-RMAC agar plates of 18 samples and from these, 17 samples confirmed to be E. coli using IMVIC tests.

As shown in Figures 1 and 2, presence of bands of expected sizes for E. coli O26 wxz (O- antigen flippase), wxy (O- antigen polymerase) , stx 1 and stx 2 genes compared to those of positive O26 control strain for all these presumptive isolates were visualized by agarose gel electrophoresis.

DISCUSSION

The increased incidence of cases of diarrhoea and HUS caused by STEC O26 in human renewed interest and attention in this E. coli serogroup (Hiruta et al., 2001; Goldwater and Bettelheim, 2002; Weber et al., 2002). Hence in many countries there is no standard method for detection of non-O157 STEC and routine selective medium for isolation of O157 STEC, (Sorbitol-Mackonkey agar supplemented with Cefixime and Tellurite) is not able to separate O26 strain (E. coli O26 strains generally ferment the sorbitol), so the reported incidence of STEC O26 associated disease is probably underestimated, but in countries where tests for STEC include serogroups other than O157, the incidence of STEC O26 is even similar to that of O157(Hiramatsu et al., 2002; Jenkins et al., 2008).

Recent improvements in the biochemical and molecular detection techniques help clarify the true incidence of STEC O26 (Zhang et al., 2000). Many of the isolates of non–O157 STEC from animals belonged to E. coli O26
Table 1. Primer sequences and predicted length of PCR amplification product.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Direction</th>
<th>Primer sequence (5′ - 3′)</th>
<th>size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>O26 wzx</td>
<td>Forward</td>
<td>GCGCTGCAATTGCTTATGTA TTTCCCGCAATTTATTCAG</td>
<td>152</td>
<td>DebRoy et al., 2004</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O26 wzy</td>
<td>Forward</td>
<td>TAAAATTGCGGGGAAAGAATG GACTTCATGGGTACCGCCTA</td>
<td>276</td>
<td>DebRoy et al., 2004</td>
</tr>
<tr>
<td></td>
<td>reverse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stx1</td>
<td>Forward</td>
<td>CATTGTCTGGTGACAGTAGCT CCGTAATTTGCAGCTCA</td>
<td>732</td>
<td>Gannon et al., 1997</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stx2</td>
<td>Forward</td>
<td>CCATGACAACGGACAGCAGTT CCGTAACCTGAGCTCA</td>
<td>779</td>
<td>Gannon et al., 1997</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Phenotypic / biochemical characteristics of isolates.

<table>
<thead>
<tr>
<th>Total sample</th>
<th>Colorless</th>
<th>Oxidase negative and catalase positive</th>
<th>Indol and MR positive and VP and citrate negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>211</td>
<td>18</td>
<td>18</td>
<td>17</td>
</tr>
</tbody>
</table>

Figure 1. Multiplex PCR assay with *E. coli* O26 wzx and wzy oligonucleotide primer pairs. Lanes M: 100 bp DNA size marker (fermentase, SN1143), Lane a: positive control, Lanes 1to17:17 presumptive *E. coli* O26 isolates.

strains (Orden et al., 1998; Cobbold and Desmarchelier, 2000; Geue et al., 2002; Leomil et al., 2005). These data together with similarity of *E. coli* O26 isolated from animal and human (Hiruta et al., 2001; Allerberger et al., 2003) and evidence on detection of this pathogen from beef and other foods of animal origin (Allerberger et al., 2003; Murphy et al., 2005) support the probability that animals are the main source of spreading of this serogroup. In the present study STEC O26 strains have been detected in relatively high number of cattle in our region (8.05%) and long term survival of *E. coli* O26 in bovine feces (Fukushima et al., 1999) rises the potential of transmission of this pathogen directly or indirectly (foods and environment) to human.

The ability of strains of STEC to cause disease in human and animal is associated with a number of virulence factors including the expression of shigatoxins (Obrien et al., 1992; Law, 2000; Meng et al., 2007). Studies on stx genotype of STEC O26 indicate that there has been a shift in the stx genotype of serogroup O26 from stx1 to stx2 or both stx1 and stx2 and it may have occurred because of change in prevalence of *E. coli* O26.
stx2 positive strains in the animal reservoir (2,23). As stx2 is more commonly associated with severe disease in human, it is possible that this genotype shift has caused an increase in case of HUS associated with STEC O26 (23Bonnet et al., 1998 and Zhang et al., 2000). In this work also, we found that all of E. coli O26 isolates were stx1 and stx2 positive as shown in Figure 2.

Another finding of this research is that 94.4% of Rhamnose non-fermenting colonies (17 of 18 samples), verified by PCR assays and as previously described (Hiramatsu et al., 2002), CT- RMAC agar has high specificity and is useful for isolation of E. coli O26 from fecal samples. Also, as shown in Figures 1 and 2, all isolates that carried wzx and wxy genes were positive for presence of stx1 and stx2 genes. So, The wzx and wxy genes maybe specific for shigatoxigenic E. coli O26. Although, further studies are necessary to evaluate the specificity of respective target genes for isolation of STEC O26.

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REFERENCES


