Prevalence and distribution of the \textit{stx}_1, \textit{stx}_2 genes in Shiga toxin producing \textit{E. coli} (STEC) isolates from cattle

Tahamtan Y*, Hayati M, Namavar MM

Razi Vaccine and Serum Research institute Shiraz-Iran.

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ABSTRACT

Background and Objectives: Shiga toxin-producing \textit{Escherichia coli} (STEC) strains are human pathogens linked to hemorrhagic colitis and hemolytic uremic syndrome. Shiga toxins (Stx1 and Stx2) are the major virulence factors of these strains. The aim of this work was to study the prevalence and distribution of \textit{stx}_1 and \textit{stx}_2 gene in \textit{E. coli} O157:H7 and non-O157:H7 strains isolated from cattle in Shiraz, Iran.

Materials and Methods: Four hundred and twenty samples consisted of recto-anal mucosal swabs were collected from cattle. They were checked for the presence of the \textit{stx}_1 and \textit{stx}_2 gene using multiplex-PCR every 1 week over a 1-year period (2007-2008).

Results: A total of 146 strains carrying the \textit{stx}_1 and \textit{stx}_2 gene were isolated from 51 (12.14%) cattle. Overall, 15 (3.57%) were identified as O157:H7 and 131 (31.19%) revealed to be non-O157:H7. Both \textit{stx}_2 and \textit{stx}_1 genes were detected in 51 (34.93%) STEC isolates. Genotypes \textit{stx}_1 and \textit{stx}_2 were detected in 15 (10.27%) and 78 (53.42%) respectively. Seasonal distribution of \textit{stx} genes revealed high percentage of positive animals in warm seasons. The gene sequence similarity ranged from 94 to 100%.

Conclusion: Frequency of \textit{stx}_1 and \textit{stx}_2 in animals and its relation to human disease is not well understood in Iran. The high prevalence of STEC in cattle seems to parallel that which is usually observed in warm seasons and it also parallels occurrence of human STEC. The higher prevalence of the \textit{stx} gene than \textit{stx}_1 in strain populations isolated from cattle indicates a risk alert of \textit{E. coli} O157:H7 being shed by cattle in these populations. Appropriate measures are now needed to prevent the spread of this life-threatening foodborne disease in our country.

Keywords: STEC, \textit{stx}1, \textit{stx}2, cattle, Iran.

INTRODUCTION

The broad group of \textit{E. coli} are known as Enterohemorrhagic \textit{Escherichia coli} (EHEC), including \textit{E. coli} O157:H7 and non-O157 (1). EHEC refers to a subset of Shiga toxin-producing \textit{Escherichia coli} (STEC) strains found to cause human and sometimes animal disease (2). They are linked to development of hemorrhagic colitis (HC), hemolytic uremic syndrome (HUS) (3) and thrombotic thrombocytopenic purpura (TTP), which requires hospitalization and intensive care (4). STEC O157:H7 strains were first isolated from cattle in Argentina in 1977, although the strains were identified as such 10 years later (5).

Cattle are considered the primary reservoir of both O157:H7 and non-O157 STEC bacteria (2). Cattle frequently excrete the bacteria in their feces (6). The illness is often linked to the consumption of contaminated and undercooked ground beef. Although other means of transmission (7, 8) have been reported (8).

Several virulence factors have gained importance for the pathogenesis of the STEC infections (9). However, the complete list of bacterial virulence determinants necessary for STEC to cause EHEC-related disease is not known. Shiga toxin is the key...
factor in STEC pathogenesis (2,10). Shiga toxin is toxic to human colonic, ileal epithelial (11) and endothelial cells (12). Two main groups of Shiga toxins are harbored in STEC (13-15). Shiga toxin 1 (16) is 98% homologous to the Stx produced by Shigella dysenteriae type 1, while Stx2 is about 60% homologous with Stx1 and is antigenically different (9, 17).

E. coli O157:H7 is a common cause of bloody diarrhea in developed countries, but its incidence in developing countries including Iran is not clear. The limited prevalence data in foods and animals in Iran has made the assessment of risks difficult, and also the options for management and control are unclear. In Iran, only a few studies have reported the isolation of STEC (18-20).

**Isolation and identification of E. coli.** All samples were placed in 15 ml of mTSB and incubated overnight at 37°C. The suspension was thoroughly mixed and allowed to stand for a short period before plating. One loop full of enriched broth was streaked onto SMAC-CT. All agar plates were incubated at 37°C for 24 h. Five to six sorbitol negative colonies per sample were collected and streaked on SMAC again. Finally, the bacteria were streaked onto eosin methylene blue (EMB) agar plates and were incubated same as above. The typical E. coli metallic shine on EMB were characterized by biochemical tests, including conventional indol, methyl red, Voges proskauer, citrate and lysine decarboxylase tests. The identity of E. coli O157:H7 was confirmed using an anti-E. coli O157 and H7 antisera agglutination kit (Oxoid DR620).

**Nucleic acid isolation.** One ml of overnight mTSB culture from all the bacterial strains was employed as template for PCR. Cells were pelleted from the cultures at 3,000 rpm for 5 min (Hermle Z23o MA centrifuge) and then continued by DNP™ Sina-gene kit (Cat No.: 8115C). All isolates were examined for verotoxin virulence genes determinants by PCR.

**PCR assay.** DNA samples (1µg nucleic acids) were amplified in a 25µl volumes reaction mixture of the following constitution: 2 mM magnesium chloride, 2.5mM for each of dATP, dCTP, dGTP, and dTTP.

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**Table 1. Specific oligonucleotide primers used for amplification of stx1and stx2 gene.**

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>stx1 F</td>
<td>484</td>
<td>(35)</td>
</tr>
<tr>
<td>stx1 R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>stx2 F</td>
<td>779</td>
<td></td>
</tr>
<tr>
<td>stx2 R</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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2 pM for each of the STX-specific oligonucleotide primers (Oligonucleotide Synthesis Laboratory, Roche, Germany) described in table 1, 1.25 U of Taq polymerase (Fermantas, Sylvius, Lithuania) and the final volume was adjusted with sterile double-distilled water.

The samples were overlaid with 100 µl of mineral oil, denatured at 94°C for 5 min, and subjected to 30 cycles of amplification in a DNA Thermal Cycler (Ependorf Mastercycler Gradient). Parameters for the amplification cycles were: denaturation for 30 s at 94°C, annealing of primers for 30 s at 56°C, and primer extension for 30 s at 72°C with auto-extension. After the last cycle, the PCR tubes were incubated for 10 min at 72°C. Six microliters of the reaction mixture was then analyzed by standard submarine gel electrophoresis (1.5% agarose; 5 V/cm), and the reaction products were visualized by being stained with ethidium bromide (0.5, µg/ml in the running buffer).

Bromocresol broth tubes containing 1% D-sorbitol (Sigma, St. Louis, Mo.) were used for the sorbitol fermentation test. All susceptible strains were separately incubated in 250 µl of phosphate-buffered saline with β-D-glucuronidase tablet (Diatabs, Rosko, Denmark). The incubation time and temperature was 24h at 37°C.

Sequencing: The strains harboring shiga toxin were sequenced both in reverse and forward with the same primers used for stx genes. The obtained sequences were balsted in NCBI databases. The data was analyzed with SPSS (Statistical Package for Social Sciences) for Windows version 11.5 software with assumed confidence level of 95%.

RESULTS

The majorities of the isolated strains were not able to ferment sorbitol within 24 h and had β-D-glucuronidase activity. Only ten strains were able to ferment sorbitol within 24 h, of which only one belonged to the E. coli O157: H serotype. This serotype was also negative for the β-D-glucuronidase test. The data point to the high prevalence of stx in our study both in O157 and non-O157.

All animals came to the Shiraz slaughterhouse from farms located in different regions of the state. No significant difference in STEC isolation rate was observed when the cattle were grouped according to their geographical origin.

Isolation and characterization of STEC in cattle. A total of 146/420 (34.76%, 95% CI) STEC strains were isolated from 51 (12.14%) out of the 420 cattle, that posses stx1 and/or stx2. Fifteen (3.57%) isolates were classified as E. coli O157:H7 and 131(31.19%) non-O157 (Table 2).

Isolation and characterization of stx gene-carrying STEC bacteria. Any E. coli isolated

<table>
<thead>
<tr>
<th>Strains (No.)</th>
<th>Stx1</th>
<th>Stx2</th>
<th>Stx1+stx2</th>
<th>-stx</th>
<th>Total %</th>
</tr>
</thead>
<tbody>
<tr>
<td>O157: H7</td>
<td>6</td>
<td>8</td>
<td>6</td>
<td>6</td>
<td>15(3.57)</td>
</tr>
<tr>
<td>Non- O157</td>
<td>60</td>
<td>121</td>
<td>45</td>
<td>15</td>
<td>131(31.19)</td>
</tr>
<tr>
<td>Total</td>
<td>66</td>
<td>129</td>
<td>51</td>
<td>21</td>
<td>146(34.76)</td>
</tr>
</tbody>
</table>

Table 2. Distribution of E. coli O157, Non-O157 stx1, stx2 Genes in Cattle. Shiraz-2007-8.

| Identity to stx1,2 gene of EDL933 (AE005174.2) % |
|---------------------------------|--------|--------|
| Strains (Stx1)                  | Identity to stx1 | Identity to stx2 | Strains (Stx2) |
| DEC10J, EC108                   | 100    | 98     | c466-01B |
| EC127, EC120                    | 99     | 98.2   | EC130   |
| BCN26                          | 94     | 98.1   | EC176, EC125 |
| EC152                          | 99     | 98.6   | EC169, EC131 |
| EC176                          | 99     | 97.9   | H2687 serotype O157:NM |
| O157:H7 Str. Sakai             | 99     | 99     | I8257, 933W srl-II |
| AB8SF, ECLR2                   | 91     | 99.1   | O157:H7 str. Sakai |

Table 3. Sequence comparison of stx2 genes in different strains.
harboring at least one shiga toxin gene was considered positive for STEC. Both stx2 and stx1 genes were detected in 51 (34.93%) isolates, but stx1 was detected in 15 (10.27%) and stx2 was detected in 78 (53.42%). One or more cattle from each Shiraz farm was positive for stx. The ratio of stx2 to stx1 gene-carrying bacteria was 5.2:1. Except for the six strains that apparently lost the genes, the presence of the stx1 gene was confirmed by specific PCR for all of these isolates.

Seasonal distribution of the stx genes. The proportion of each of these bacterial populations that carried stx2 and/or stx1 were not similar (Fig. 1). There was a significance seasonal difference for any of the measured parameters, as indicated by an analysis of variance test (P < 0.05). The percentage of positive animals range from 24.28 to 40.9% in warm seasons of May to August (in Iran) compared to winter seasons with the average frequency dropping (8.96 to 11.11%).

Sequencing. Some of the 484 and 779 bp (Table 1) amplimers were sequenced. These phages were isolated from strains summarized in Table 3.

DISCUSSION

This is the first study which describes the detection and frequency of major virulence genes of STEC isolated from cattle in Shiraz, Iran. Our data revealed high levels of stx2 gene-carrying bacteria in fecal samples from different cattle. STEC harboring stx2 was isolated significantly more (53.42%) than STEC stx1 (10.27%) (P < 0.01). Most human epidemiological studies in Iran have revealed that the prevalence of STEC infection ranges between 0.7 to 15%, but none of them belonged to the O157:H7 serotype (18, 20). Isolation of STEC from bovine reservoirs from other parts of the country has already been documented (8, 21-24). Zahrae Salehi and his colleagues identified STEC O157 among 7 isolates (11.5%), from cattle, whereas non-O157 strains that are frequently associated with sporadic cases of HUS (25, 26), were isolated from 4 (6%) of animals. They showed 5 (8.2%) isolates carried stx genes (21). This finding was in parallel with presence of stx1 in 35.5 and stx2 in 49.1% of human isolates (19). This is in contrast with Askari et al. finding with a report of stx1 and stx2, among 5% and 1.9% of calves respectively (22). Recently Ludwig Kerstin et al. reported that 71% of children with HUS were due to Stx2-producing E. coli strains (16). In a study in the USA, stx1 gene was not detected in any strains tested while 93.1% of the isolates were found to carry the stx2 gene (27).

The gene belonging to strains detected from animals showed more expression of protein toxin than human samples (28), hence the strain within animal origin maintain the characteristic and are more cytotoxic than the gene from human origin (16). This supports the suggestion that cattle may have been the source of the organism for the HUS patients.

The seasonal shedding of STEC and distribution of stx ranged from 8.69 to 40.9%. There are significant seasonal differences in the levels of shedding of stx gene-carrying bacteria. The results revealed decrease in the number of stx gene carrying bacteria during the winter. These results are in agreement with those studies indicating that STEC shedding has seasonal variations by cattle in different countries (5). High isolation rate was observed in late spring and summer in the UK (29), Sweden, Washington State in the USA.
(30) and Italy (5). In this study, we observed a marked decrease in the prevalence of STEC from October, which in Iran is the beginning of fall, to the end of spring (June). This trend seems to parallel what is usually observed in the summer for the occurrence of human STEC, with very few episodes notified during the winter season (31).

Sequence variations in stx1 and stx2 genes of fifteen E. coli O157 isolates in this study were investigated. The similarity ranged from 94 to 100%. As demonstrated in the present investigation, the genetic diversity of organisms causing disease is considerable. (GenBank accession number DQ235775).

In conclusion, there is no data available about the frequency of Stx2 and Stx1 in animal and people in close contact to HUS patients in Iran. The greater observation of the stx1 gene relative to the stx2 gene in strain populations indicates a risk alert of this gene between these populations (32). Some studies have revealed that strains possessing only stx1 are potentially more virulent than strains harboring stx1 or even strains carrying both stx1 and stx2 (17, 33). It is of note that most HUS-associated clinically relevant STEC isolates produce Stx2, but at least in Europe, Stx1 is rarely highly relevant (32). Stx2 has been found to be approximately 400 times more toxic (as quantified by LD50 in mice) than Stx1(4).

These findings are important for public health and preventive veterinary medicine. Therefore, emergency cautions are necessary to decrease the incidence of STEC infections in animals and people. In order to achieve this, good hygienic practice and HACCP systems are necessary from the farm to the family table especially in the abattoirs to prevent contamination of meat and abattoir environment with intestinal content.

ACKNOWLEDGMENTS

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