

Prevalence and distribution of the *stx*₁, *stx*₂ genes in Shiga toxin producing *E. coli* (STEC) isolates from cattle

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ABSTRACT

Background and Objectives: Shiga toxin-producing *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 *stx*₁ and *stx*₂ gene in *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 *stx*₁ and *stx*₂ gene using multiplex-PCR every 1 week over a 1-year period (2007-2008).

Results: A total of 146 strains carrying the *stx*₁ and *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 *stx*₂ and *stx*₁ genes were detected in 51 (34.93%) STEC isolates. Genotypes *stx*₁ and *stx*₂ were detected in 15 (10.27%) and 78 (53.42%) respectively. Seasonal distribution of *stx* genes revealed high percentage of positive animals in warm seasons. The gene sequence similarity ranged from 94 to 100%.

Conclusion: Frequency of *stx*₁ and *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 *stx*₂ gene than *stx*₁ in strain populations isolated from cattle indicates a risk alert of *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, *stx*₁, *stx*₂, cattle, Iran.

INTRODUCTION

The broad group of *E. coli* are known as Enterohemorrhagic *Escherichia coli* (EHEC), including *E. coli* O157:H7 and non-O157 (1). EHEC refers to a subset of Shiga toxin-producing *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

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Table 1. Specific oligonucleotide primers used for amplification of *stx1* and *stx2* gene.

Oligonucleotides	Size	Reference
Stx1 F CTT CGG TAT CCT ATT CCC GG Stx1 R GGA TGC ATC TCT GGT CAT TG	484	(35)
Stx2 F CCA TGA CAA CGG ACA GCA GTT Stx2 R CCT GTC AAC TGA GCA GCA CTT TG	779	

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 and characterization of STEC in humans (18-20). *E. coli* O157:H7/ H⁻ was sporadic reported to be present in up to 11.5% of cattle in Iran (21). Unfortunately, there is no conclusive data from Middle-East particularly from Iran. Therefore, the objective of this study was to determine the frequency of bacterial virulence profiles of *stx* in STEC O157 and non-157 in cattle in a one-year period.

MATERIALS AND METHODS

Sample collection. In one year, from September 2007 to August 2008, 420 samples consisting of recto-anal mucosal swabs from cattle were collected in Shiraz-Iran. All samples were collected aseptically, placed in coolers, transported to the laboratory, and kept at 4°C. The samples were immediately processed upon arrival to the laboratory. A questionnaire was carried out for risk factor of *E. coli* O157:H7 determination that included season of the year. The prevalence ratio (PR) was determined using Win Episcope 2.0 at 95.0% level of confidence for association between risk factors.

Seasonal variations in the presence of the *stx* genes in bacterial populations from rectum were studied by sampling weekly over a 1-year period. For groups

of cattle derived from one herd, a random selection of 10% of the total number of cattle was sampled, with a maximum of 10 cattle being sampled. The enumeration of *stx* gene-carrying bacteria was performed by the MPN-PCR method (Most Probable Number).

Bacterial strains and media. *E. coli* O157:H7 strain EDL933 (kindly from Prof. David Gally, University of Edinburgh, UK), which produces Stx1 and Stx2, was used as positive control. *E. coli* O157:H7 strain SHT87 (isolated from cattle in Shiraz-Iran, unpublished) which do not possess the genes *stx*₁ and *stx*₂, was used as negative control. The bacteria were grown in modified tryptic soy broth and on sorbitol MacConkey agar (supplementation with cefixime and potassium tellorite) (mTSB, SMAC-CT) (Difco, Le Pont de Claix, France) and incubated at 37°C overnight.

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 Z230 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 (1 µg nucleic acids) were amplified in a 25 µl volumes reaction mixture of the following constitution: 2 mM magnesium chloride, 2.5mM for each of dATP, dCTP, dGTP, and dTTP,

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

Strains (No.)	Stx1	Stx2	Stx1+stx2	-stx	Total %
O157: H7	6	8	6	6	15(3.57)
Non- O157	60	121	45	15	131(31.19)
Total	66	129	51	21	146(34.76)

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-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*₂ 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 *stx*₁ and/or *stx*₂. 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 3. Sequence comparison of *stx2* genes in different strains.

Strains (Stx1)	Identity to <i>stx1,2</i> gene of EDL933 (AE005174.2) %		Strains (Stx2)
	Identity to <i>stx1</i>	Identity to <i>stx2</i>	
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 slt-II
AB8SF, ECLR2	91	99.1	O157:H7 str. Sakai

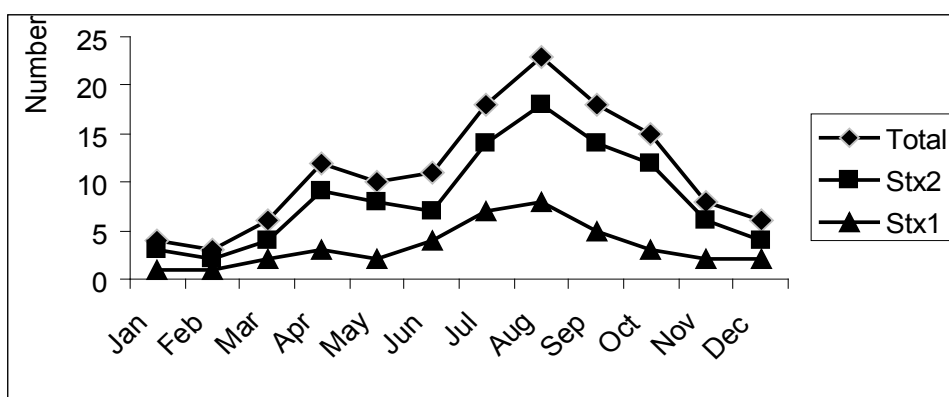


Fig. 1. Seasonality STXs from STEC isolates from cattle Shiraz-Iran, 2007-8

harboring at least one shiga toxin gene was considered positive for STEC. Both stx_2 and stx_1 genes were detected in 51 (34.93%) isolates, but stx_1 was detected in 15 (10.27%) and stx_2 was detected in 78 (53.42%). One or more cattle from each Shiraz farm was positive for stx . The ratio of stx_2 to stx_1 gene-carrying bacteria was 5.2:1. Except for the six strains that apparently lost the genes, the presence of the stx 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 stx_2 and/or stx_1 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) amplicons 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 stx_2 gene-carrying bacteria in fecal samples from different cattle. STEC harboring stx_2 was isolated significantly more (53.42%) than STEC stx_1 (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). Zahraee 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 stx_1 in 35.5 and stx_2 in 49.1% of human isolates (19). This is in contrast with Askari *et al.* finding with a report of stx_1 and stx_2 , among 5% and 1.9% of calves respectively (23). 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, stx_1 gene was not detected in any strains tested while 93.1% of the isolates were found to carry the stx_2 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 *stx*₁ and *stx*₂ 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 *Stx*₂ and *Stx*₁ in animal and people in close contact to HUS patients in Iran. The greater observation of the *stx*₂ gene relative to the *stx*₁ gene in strain populations indicates a risk alert of this gene between these populations (32). Some studies have revealed that strains possessing only *stx*₂ are potentially more virulent than strains harboring *stx*₁ or even strains carrying both *stx*₁ and *stx*₂ (17, 33). It is of note that most HUS-associated clinically relevant STEC isolates produce *Stx*₂, but at least in Europe, *Stx*₁ is rarely highly relevant (32). *Stx*₂ has been found to be approximately 400 times more toxic (as quantified by LD₅₀ in mice) than *Stx*₁ (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.

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