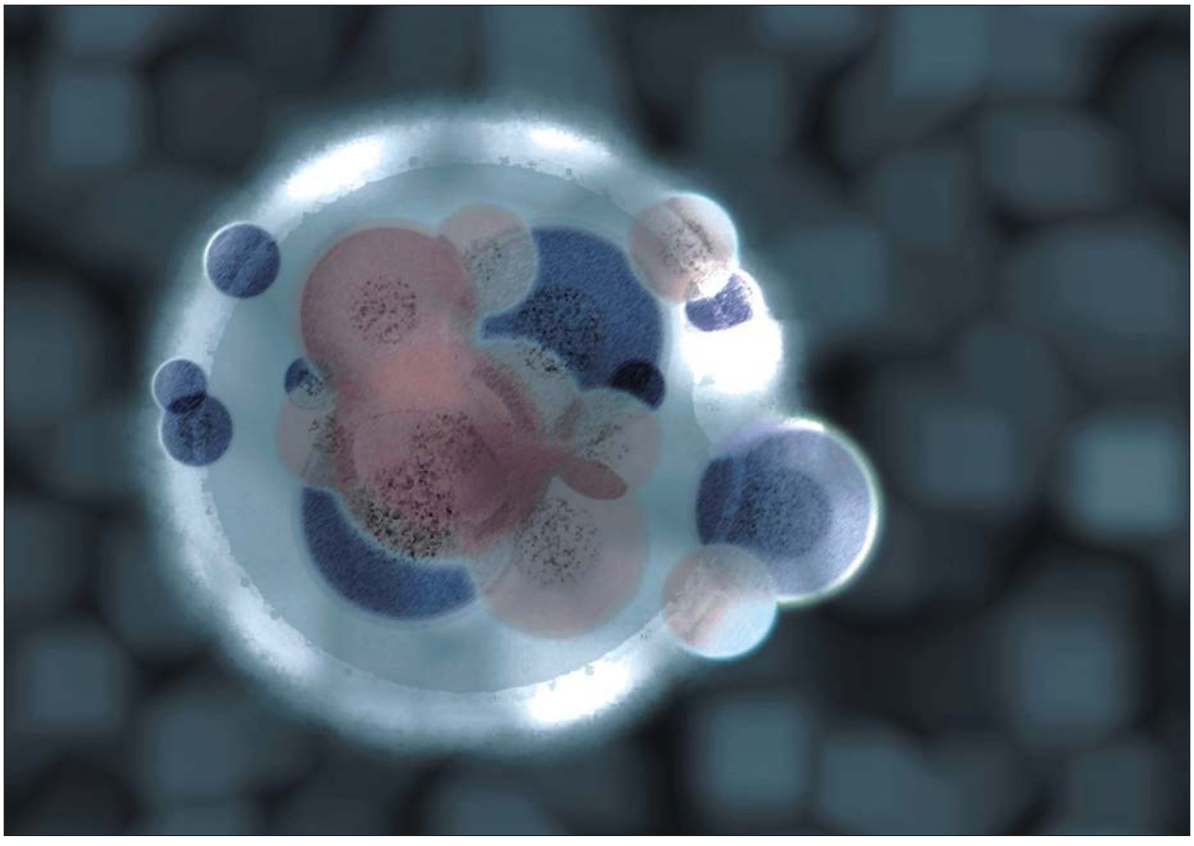

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Detection of Shiga Toxin Producing *E. coli* Strains Isolated from Stool Samples of Patients with Diarrhea in Abadan Hospitals, Iran

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Abstract: The aim of present study is to apply the PCR method for detection of *stx1* and *stx2* genes in *E. coli* strains isolated from stool samples. Shiga Toxin-producing *Escherichia coli* (STEC) have emerged as pathogens that can cause food-borne infections and severe and potentially fatal illnesses in humans, such as Haemorrhagic Colitis (HC) and Haemolytic Uraemic Syndrome (HUS). Of the numerous serotypes of *E. coli* that have been shown to produce stx, *E. coli* O157:H7 are frequently implicated in human disease. So, due to serious infections associated with STEC in human, early recognition of STEC is critical for effective treatment of patients. In total 130 isolates of *E. coli* were recovered from patients by using standard identification procedures. The isolates were subjected to PCR technique for investigation of presence *stx1* and *stx2* genes. Results revealed the presence of *stx1* in 20 isolates (35.5%), *stx2* in 28 isolates (49.1%) and *stx1* and *stx2* both in 9 isolates (15.7%). The present study shows the relatively high prevalence of STEC producing *stx1* and *stx2* in the region of study. The higher detection rate of *stx2* represented the important role of this gene in diarrheal diseases.

Key words: Shiga toxin-producing, *E. coli*, PCR, *stx1*, *stx2*

INTRODUCTION

Diarrheal diseases caused by microorganisms and their toxins are a major cause of mortality and morbidity throughout the world (Kaur and Ganguly, 2003). There are over 2 million deaths occurring each year, particularly among infants younger than 5 years (Clarke *et al*., 2003). It is now recognized that there is a very broad spectrum of human disease associated with Shiga toxin (Stx)-producing organisms. Stx-producing *Escherichia coli* (STEC) are an important cause of haemorrhagic colitis and the diarrhea-associated form of the Haemolytic Uraemic Syndrome (HUS). Of the numerous serotypes of *E. coli* that have been shown to produce stx, *E. coli* O157:H7 and *E. coli* O157:NM (non-motile) are most frequently implicated in human disease (De Boer and Heuvelink, 2000). The STEC-related disease may involve either sporadic cases or large outbreaks involving a common contaminated food source (Dontorou *et al*., 2003). However, all the STEC strains are not pathogenic, so the pathogenic STEC are divided as a subgroup of enterohaemorrhagic *E. coli* (EHEC). These can cause disease with a low infectious dose of about 1-100 bacteria (Jaeger and Acheson, 2000).

Stx are classified into main types of *stx1* and *stx2*, which are encoded by *stx1* and *stx2* genes, respectively. Studies in the early 1980s established that *stx1* and *stx2* were encoded on a variety of bacteriophages. The pathogenic strains of STEC could produce *stx1* or *stx2* or both (Franz *et al*., 2007). Stx are able to inhibit protein synthesis leading to cell death by inactivating the 60s ribosomal subunit in host cells. These cytotoxins can cause diarrhea or bloody diarrhea by destroying the intestinal epithelium (Montville and Matthews, 2005).

More than 200 different serotypes of STEC have been described until 2003 (Brett *et al*., 2003). Stx1 and 2, elaborated by serotypes of *E. coli* O157:H7 (ECO157), are well established as the major cause of hemorrhagic Colitis and complications such as HUS and thrombotic thrombocytopenic purpura, typically affecting children, the elderly and immunocompromised patients (Park *et al*., 2002; Rivas *et al*., 2006). Apart from this serotype, a subset of STEC strains including serogroups O26, O103, O111 and O113, often referred to as EHEC, are also commonly associated with such serious afflictions (Schmidt *et al*., 2001).

Cattle have long been regarded as the principal reservoir of STEC strains. However, epidemiological

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surveys have revealed that STEC strains are also prevalent in the gastrointestinal tracts of other domestic animals, including sheep, pigs, goats, dogs and cats with or without symptoms (Bonardi *et al*., 1999; Nielsen and Scheutz, 2002). Although, contaminated vegetables and unpasteurized dairy products could serve as a source of STEC food infections (Coia *et al*., 2001; Dontorou *et al*., 2003), however the minced meat is the most prevalent source of such infections (Conedera *et al*., 2004; Mora *et al*., 2007).

Detection of *E. coli* 0157:H7 on routine enteric media is time consuming and expensive. Unlike most *E. coli* strains, serotype 0157:H7 does not ferment sorbitol and testing for sorbitol fermentation, therefore, has been suggested as a simple means to screen for this organism (Shelton *et al*., 2008). All stool specimens submitted for bacterial culture are inoculated onto sorbitol MacConkey agar (SMAC) and in fact most laboratories are using only SMAC as the primary plate medium. However, the sensitivity of this medium is relatively low for the detection of ST-O157, reported by investigators ranges from 50 to 82.5% (Park *et al*., 2002) and by disease progression, the detection rate even falls to 33% (Kehl, 2002). Besides, despite that *E. coli* O157:H7 is easily differentiated from other *E. coli* by its inability to rapidly ferment sorbitol, but non-O157:H7 STEC are phenotypically similar to commensal nonpathogenic *E. coli* and are not detected with SMAC agar (Fey *et al*., 2000).

The profound sensitivity of Vero cells to Stx and cytotoxicity for this cell line remains the gold standard for confirmation of putative Stx-producing isolates (Cermelli *et al*., 2002). But the technique is labour extensive and time consuming. Serological tests such as ELISA, have limited value in detection, due to variety of stx-producing serotypes (Feng *et al*., 1998).

Considering the clinical significance of the ST-O157, rapid, specific and sensitive detection methods are required to identify toxin-producing isolates. The PCR amplification technique is a sensitive and specific method for detecting shiga toxins and other virulence factors. The PCR technology will allow large-scale screening of many colonies from clinical material or contaminated foods (Bielaszewska and Karch, 2000).

Due to serious infections associated with STEC in human, early recognition of STEC is critical for effective treatment of patients. Furthermore, rapid microbiological diagnosis of individual patients enables the prompt notification of outbreaks and implementation of control measures to prevent more cases. In this study, we applied PCR method for detection of *stx1* and *stx2* genes in *E. coli* strains isolated from stool samples.

MATERIALS AND METHODS

Sampling: Three hundred and eighty six samples from patients referred to Hospitals in Abadan, Iran, Southwest, during a six month period from March to August, 2008 were screened. Among them, 291 samples (75.3%) were obtained from patients with simple diarrhea and 95 samples were belonged to patients with bloody diarrhea.

The colonies of isolated *E. coli* strains, were inoculated in Trypticase Soy Broth (TSB) containing 20% glycerol and were kept at -70°C until use.

DNA extraction: The TSB suspension were then subcultured on McConcky agar and DNA was extracted from colonies using simple boiling method (Jothikumar and Griffiths, 2002). In brief, a few colonies were suspended in 0.5 mL deionized distilled water and was heated in a 100°C water bath for 10 min with subsequent centrifugation at 12000 rpm for 5 min. The supernatant was then used for amplification.

RCR: The composition of PCR mixture was 50 mM KCl, 10 mM HCl (pH 8.3), 4.4 mM $MgCl_2$, 200 mM dNTPs, 0.5 μM of each primer, 2 U of Taq polymerase and 5 μL of DNA template in a final volume of 25 μL. All the reagents were purchased from Cinnagen Company, Tehran, Iran, and the PCR amplification was performed according to the kit instructions. The used primers are shown in Table 1. The *stx1* and *stx2* primers used previously by other investigators, can amplify 130 and 478 bp regions of the target gene, respectively (Keskimäki *et al*., 1998). The control positives *E. coli* ATCC 43890 (*stx1*) and ATCC 43899 (*stx2*) and control negative (*E. coli* non-O157) were included in each set of reaction.

The PCR conditions for *stx1* were initial denaturation of 95°C for 5 min followed by 33 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1 min and a final extension at 72°C for 7 min using a thermocycler (Eppendorf, UK). The conditions for *Stx2* was slightly different as: initial denaturation of 95°C for 4.5 min followed by 35 cycles of 95°C for 1 min, 62°C for 1 min and 72°C for 1 min and a final extension at 72°C for 5 min. The PCR products were analyzed on 1.5% agarose gel. The gels were then stained with 0.5 mg mL^{-1} ethidium bromide and photographed. A 100 bp DNA size marker was used for band interpretation.

Table 1: Primers used in present study

Genes	Primers' sequences (5'-3')	Amplicon size (bp)
stx1F	GAAGAGTCCGTGGGATTACG	130
stx1R	AGCGATGCAGCTATTAATAA	
stx2 F	CTTCGGTATCCTATTCCCGG	478
stx2 R	GTTACTGGTCTCTACGTAGC	

RESULTS

From total 386 samples screened in this study, 137 samples were positive for bacteria with 130 *E. coli* strains isolated. Nineteen (14.6%) out of *E. coli* strains, were isolated from bloody diarrhea and 111 (85.3%) were from simple diarrhea. Seventy seven samples (59.2%), belonged to male and 23 samples (40.8%) were obtained from females. The majority of positive *E. coli* isolates were from infants and young children; age range 1-5 years (30%) (Fig. 1).

The 130 *E. coli* strains were further screened for detection of *E. coli* O157:H7 by presence of *stx1* or *stx2* genes. Fifty seven strains were identified as *E. coli* O157:H7 (43.8%) and contained *stx1* and/or *stx2*. For *stx1* gene, the detection rate was six (25%) in simple diarrheal cases and 18 (75%) in bloody diarrhea, however for *stx2* gene the detection rates were 23 (69.6%) and 10 (30.4%) from simple and bloody diarrheas, respectively. Among the positive isolates of *E. coli* O157:H7, nine cases were carried both *stx1* and *stx2* genes (Table 2). The nested PCR amplification is shown in Fig. 2.

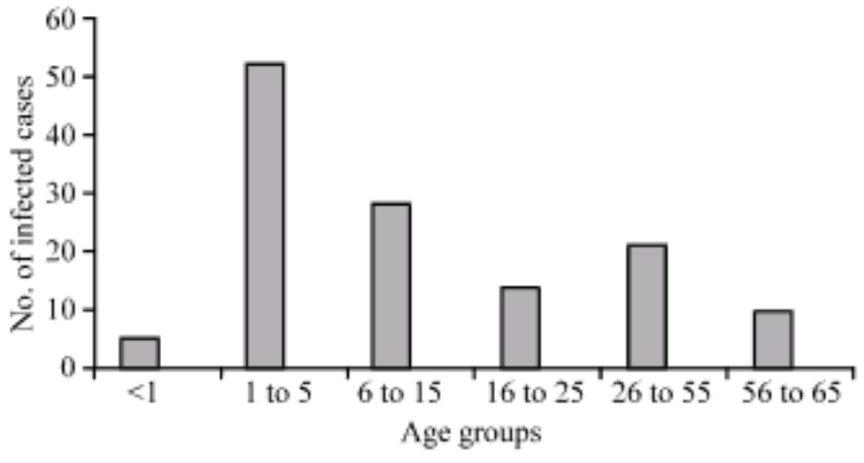

Fig. 1: Age distribution among patients with positive *E. coli* O157:H7

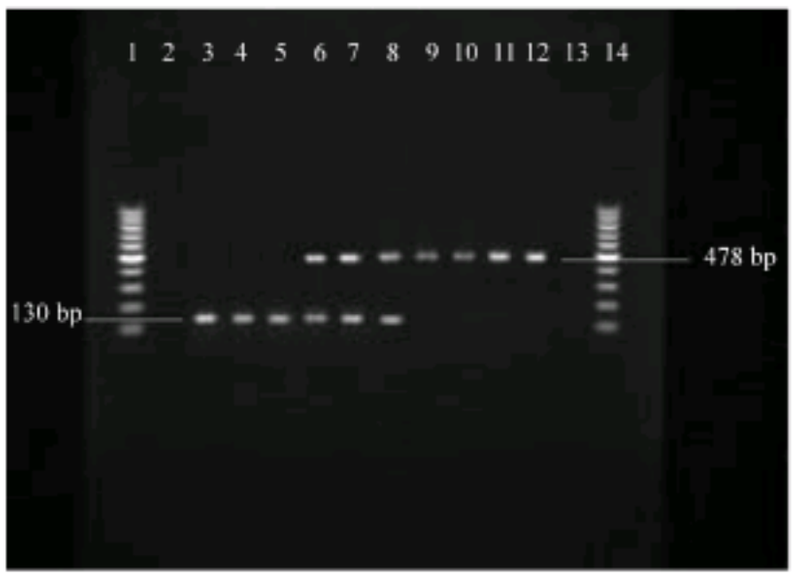

Fig. 2: Gel electrophoresis of the PCR amplification using STEC specific primers. Lanes: 1 and 14, molecular size marker; 2 and 13, negative control; 3, positive control for *stx1*; 4-5 positive isolates for *stx1* gene; 6-8, positive isolates for *stx1* and *stx2* genes; 9-11 positive isolates for *stx2*; 12, positive control for *stx2*

Table 2: Results from PCR amplification for presence of *stx1* and *stx2* genes among tested *E. coli*

Isolates No.	Percentage	Identified genes
24	42.2	*stx1*
33	57.8	*stx2*
57	100.0	

DISCUSSION

The STEC is an emerging pathogen which is recognized as an important cause of human sporadic and epidemic diarrhea, hemorrhagic colitis and HUS (Gavin and Thomson, 2004). The development of molecular techniques capable of identifying both toxin and serogroup-specific genetic determinants holds promise for a more comprehensive characterization of stool samples and isolation of STEC strains (Gilmour *et al*., 2009). One of the convenient methods suggested for detection of STEC is PCR, which is successfully applied by other investigators in human samples (Nielsen and Scheutz, 2002; Khan *et al*., 2002; Gilmour *et al*., 2009) and animal origin samples (Bonardi *et al*., 1999; Leotta *et al*., 2008).

In present study, *stx1* and/or *stx 2* genes were detected in 57 detected cases of *E. coli* O157:H7 (43.8%) among 130 *E. coli* isolates, with *stx2* as the most frequent gene (57.9%). The technique was able to detect successfully all the strains containing *stx1* and *stx2* genes. No primer-specific amplification was detected when the DNA template was isolated from stx-negative *E. coli* or from other strains of enteric pathogens tested.

In an investigation from Europe, the highest rate of isolation of *E. coli* O157:H7 in England and Scotland was reported (Smith *et al*., 1998). While in later study, the significant prevalence of non-O157 including O111, O103, O26 and O145 from Europe was also reported (Tozzi and Caprioli, 2001). These investigators were also reported a high prevalence of STEC infection (diarrheal form and asymptomatic) in Thailand and Iran. However, none of their isolates was belonged to *E. coli* O157:H7.

In another survey, among 66 isolates of *E. coli* O157:H7, the most frequent *stx* genotypes were *stx2* (91%) in Argentina, *stx2* (89%) in New Zealand and *stx1*/*stx2* (30%) in Australia. No *stx1*-positive strains were identified in Argentina or New Zealand (Leotta *et al*., 2008). In similar report from Argentina, *stx1* and *stx2* were identified in 9.7 and 90.3% of *E. coli* O157:H7 strains, respectively (Rivas *et al*., 2006). The detection of *sxt2* was higher compared to our work in both mentioned studies. In an investigation from Tunisia, 11 isolates out of 127 *E. coli* isolates were carrying *stx1* and *stx2* genes, which was lower compared to our work (Gallas *et al*., 2006). Persent experience in the region of study with very hot climate, strongly confirms a seasonal variation and the higher

prevalence of diarrheal diseases in spring and summer time. Due to this, present study design was sampling during the period of the year from March to August. This may explain the higher detection rate of *E. coli* in comparison with other surveys.

In present study, all the patients with positive STEC, who referred to Abadan hospitals, were from rural area and in direct contact with domestic animals and their feces as important natural reservoirs of STEC. Other sources of acquiring infection may include consumption of contaminated foods or contaminated drinking water.

The results of this study indicate that when extremely sensitive methods such as PCR, are applied to the analysis of primary cultures, STEC-related genes can be detected in a high proportion of children. The rate of STEC serotypes identified, indicates the need for further studies in this region.

In conclusion, the present study shows the relatively high prevalence of STEC producing *stx1* and *stx2* in the region of study and could be implicated as a major causal agent of diarrhea, but because many patients infected with the organism do not develop bloody diarrhea, we recommend that clinical laboratories screen all stools submitted for culture for *E. coli* O157: H7. The higher detection rate of *stx2* represented the important role of this gene in diarrheal diseases.

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