Original Article

Iran J Pediatr
Dec 2013; Vol 23 (No 6), Pp: 637-642

Frequency of Shiga Toxin-Producing Genes of Escherichia Coli Isolated from Diarrheic Stools of Iranian Children by PCR

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Received: *Jan 20, 2013*; Accepted: *Aug 14, 2013;* First Online Available: *Nov 08, 2013*

Abstract

Objective: Shiga toxin-producing *E. coli* (STEC) is a pathogenic *E. coli* that may cause hemolytic uremic syndrome (HUS) after diarrheal disease through Shiga toxins. Management of the patients with STEC infection is different from that of other diarrheal diseases due to increase in frequency of HUS after antibiotic administration. Few studies were conducted in Iran and epidemiology of STEC remains obscure; this necessitates examination of stools especially in young children for this bacterium.

Methods: We determined the frequency of STEC in 947 *E. coli* strains isolated from diarrheal stools of children less than 14 years in Tehran with conventional culture methods and multiplex-PCR via determining the *STX1* and *STX2* genes, between October 2008 and September 2009. We also evaluated the association between stool exam findings and presence of STEC.

Findings: Twenty seven (2.8%) of *E. coli* isolates were positive for *STX1* or *STX2* genes, most of which occurred in spring ($P<0.05$). There was no significant association between STEC positivity and stool exam findings. Eighteen out of 27 (66.7%) Shiga toxin positive samples were isolated from males and the rest from females. The most common *STX*-positive diarrheal samples showed loose consistency ($P<0.017$).

Conclusion: Although the low frequency of STEC in our population indicates that it is not a major problem in our population, STEC should be regarded as an important infection because of its severe consequences. Further studies with greater sample size are needed to confirm our results.

Iranian Journal of Pediatrics, Volume 23 (Number 6), December 2013, Pages: 637-642

Key Words: Shiga Toxin; E. coli; Hemolytic Uremic Syndrome; Polymerase Chain Reaction

Introduction

Most of Escherichia coli isolated from stool are nonpathogenic. During the last decade, many researchers reported isolation of pathogenic strains as causes of large food–borne outbreaks in developed counties. From 5 main pathogenic E. coli strains, Shiga toxin-producing *E. coli* (STEC) that produces Shiga-like toxins received more attention. Although *E. coli* O157:H7 is the most common serotype that produces this toxin[1,2], non O157:H7 has also been reported to cause infection in children with no significant differences in severity of illness. Cattle, in which the bacterium inhabits intestinal flora, is the natural reservoir of STEC[3,4]. The infection is mostly acquired by

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human beings through the ingestion of contaminated water and under-cooked food although human to human transmission has been described[2-5]. Mortality and morbidity of diarheal disease in *STEC* infection is an important health care problem especially in young children. Meanwhile, hemolytic uremic syndrome (HUS), acute renal failure and end stage renal disease are other complications of this infection[2,6].

STX1 and *STX2*, enterotoxins which are produced by *STEC*, are structurally related to the toxin produced by *Shigella dysenteriae*. Shiga-toxins have A and B subunits, the latter binds to the surface of enterocytes, polymerizes in plasma membrane, and supplying a route for the other subunit to enter the cytoplasm. Being proteolytically activated in cytoplasm, the A subunit, irreversibly inhibits protein synthesis by inactivating the ribosome, and kills the cell. Carried to kidneys by blood stream, Shiga-toxins cause endothelial damage in glomerular capillaries, which accompanied by platelet aggregation and fibrin deposition, finally results in renal failure[5,7].

There are several studies that determined the frequency of infection by *STEC* in diarrheal patients and evidence show that *STEC* can be a major problem in developed and developing countries, but there are only few published data from Iran[1,4,8-13]. *STEC* can be diagnosed by different methods; stool culture on Sorbitol-MacConkey agar (SMAC) mostly used for detection of *E. coli* O157. However, this medium is not suitable for detection of non-O157 STEC. Enzyme immunoassay for Shiga toxins 1 and 2 and molecular assay for *STX* genes are other methods[4,12,14]. Here we report the frequency of *STEC* infection with PCR in patients with diarrhea referred to Children's Medical Center in Tehran.

Subjects and Methods

Bacterial isolation:

All patients with diarrhea or dysentery admitted to Children's Medical Center from October 1, 2008, to September 1, 2009, were enrolled in this cross sectional study. The study was approved by ethical committee of Tehran University of Medical Sciences. The patients were not charged. Stool samples were collected and cultured on EMB (Eosin-methylene blue) agar at 37˚C. After 24 hours, recognizable colonies with metallic shine examined biochemically for isolating *E. coli* strains. Indole (+), Methyl red (+), Simon citrate (-), Urea hydrolase (-), Lysine decarboxylase (+), ONPG (+) and TSI [Alk/A, H_2S(-), Gas(-)] colonies were inoculated into two Cary-Blair transport media and preserved there at 27˚C for 24 hours. One of the two Cary-Blair media was stored at -20 ºC for further study, and the second one was inseminated to Muller agar and incubated at 37˚C for 24 hours before extraction.

DNA Extraction:

DNA from biochemically confirmed isolates was extracted with method described by Dhanashree and Mallya[15] with minor modifications. Briefly, a loopful of bacterial colonies, from Muller agar, admixed with 200 µL sterile distilled water and then placed in a water bath at 97˚C for 15 minutes. The samples were centrifuged for 5 minutes at 8000g, and then the supernatant was transferred to a fresh tube and stored at –20°C for Polymerase Chain Reaction.

Polymerase Chain Reaction:

For each reaction, master mix contained 10x PCR buffer (BioFlux, Tokyo, Japan), 2.5 millimolar of $MgCl_2$, 1 millimolar of dNTP (BioFlux, Tokyo, Japan), 2 IU of taq polymerase (BioFlux, Tokyo, Japan) and 200 nanomolar of each primer (CinnaGen, Tehran, Iran). PCR amplification was performed by using a Mastercycler Gradient PCR machine (Eppendorf, Netheler-Hintz GmbH, Hamburg, Germany). The duplex PCR conditions for *STX1/STX2* involved denaturation of template DNA for 4 minutes at 95°C followed by 35 cycles of 30 seconds at 95°C, 60 seconds at 55°C and 60 seconds at 72°C, final extension period was 7 minutes at 72°C. After PCR completion, gel electrophoresis was preformed and the lengths of amplicons were compared with predetermined ladder (Fig. 1). Positive and negative controls were included in each run. The primer sequences[17] are shown in Table 1.

Amplicon detection:

The PCR products were detected by electrophoresis on 1% agarose gel at 100 volts for

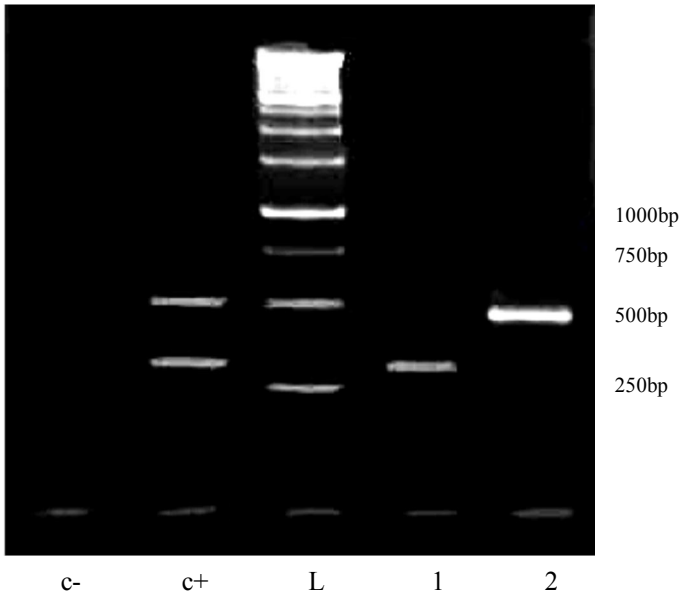

Fig. 1: Gel electrophoresis of *E. coli* isolates. c-:negative control, c+:positive control, L: Ladder, 1 and 2 (two samples, one of them *STX1* and second *STX2* positive)

60 minutes. In each run of electrophoresis, a DNA ladder (Fermentas Gene Ruler 1 KB, CinnaGen, Tehran, Iran) was placed in one of the wells. Gel was stained with ethidium bromide and then visualized on UV transilluminator, PCR products with 302 bp and 516 bp length were considered positive for *STX1* and *STX2* respectively (Fig. 1).

Statistical analysis

Quantitative data were expressed as frequency and percent. For statistical comparisons Chi square test was used for proportions. SPSS Version15.0 was used for statistic analysis. A *P*-value of less than 0.05 was supposed to be significant.

Findings

From all 6500 samples received, 967 E. coli strains were isolated and tested by PCR method. E. coli was Isolated from 524 males and 443 females. Twenty seven (2.8%) cases were positive for *STX1* or *STX2* genes (Fig. 1). Positive samples were from 18 males and 9 females. Most common season for *STX*-positive E. coli was spring (17 out of 27=63%, *P*<0.05) (Table 2). The most common *STX*-positive diarrheal samples had loose consistency (*P*<.017). Most of *STX*-positive samples had few WBC or RBC and none was bloody. There was no significant association between *STEC* positivity and stool exam findings. (All *P*-value>0.05). The details of results are summarized in Table 3.

Discussion

STEC is one of the six groups of diarrheagenic Ecoli. Its diagnosis from non pathogenic Ecoli is important because of its ability to produce

Table 1: Primer sequences and length of amplification products

gene	Primer Sequence (5′ – 3′)	Product size (bp)	Reference
STX1	CGCTGAATGTCATTCGCTCTGC CGTGGTATAGCTACTGTCACC	302	Blanco et al (2003)[19]
STX2	CTTCGGTATCCTATTCCCGG CTGCTGTGACAGTGACAAAACGC	516	Blanco et al (2003)[19]

Table2: Seasonal prevalence in diarheal specimens with *STX*-positive *E.coli* isolates and all *E.coli* isolates

Variable	Season			
	Spring	Summer	Autumn	winter
STX-Positive *E.coli* isolates	17 (63%)	8 (29.6%)	2 (7.4%)	0 (0%)
All *E. coli* isolates	181 (18.7%)	531 (54.9%)	242 (25%)	13 (1.4%)

hemolytic uremic syndrome, a life-threatening disease in 15% of patients with 5% mortality rate[2,7]. Treatment of infection with antibiotics is controversial because of increasing risk of HUS[16,17,18]. Thus knowing the frequency, seasonal and epidemiologic pattern of *STEC* in each population can guide clinicians in test ordering and management of diarrheic patients. Also, it can help health care providers in controlling outbreaks.

Although E. coli O157:H7 is the most common serotype of *STEC*[1,2], non O157:H7 has also been reported to cause infection in children. Culture on specific medium (SMAC) and serological assay were used mostly for diagnosis. Since non -O157 STEC usually cannot be recognized on SMAC agar, evaluation of stool samples for toxins (proteins or gens) has been highly recommended[4]. PCR methods are rapid and more accurate methods in this regard. So we decided to use a PCR method to determine the frequency of Shiga toxin producing E. coli in diarrhea samples in our population and evaluate the relationship between stool exam findings and Shiga toxin positivity.

In our study 967 E. coli strains were isolated from diarrheal stool samples of children younger than 14 years from October 2008 to September 2009 and amplification of *STX1* and *STX2* genes using PCR method revealed that 2.8% of them were STEC .This frequency was significantly less than previous study conducted in Iran. Salmanzadeh-Ahrabi, S et al in 2005 reported that STEC was isolated in 15.5% of patients under 5 years of age with diarrhea[9]. In another study in Iran, Alikhani et al in 2007 found that 8.7% of the children aged less than 10 years with diarrhea were infected with STEC[10]. These differences may be from differences in methods and sample sizes. Frequency infection with STEC is different in other populations. In a study conducted in Australia the rate of EHEC was 1.7% in children with diarrhea[11]. Another study in Netherlands revealed the prevalence of STEC in 3.8% of samples with macroscopic blood and 1.4% in diarrheal stool samples[8]. In Bennett-Wood VR study the frequency of STEC was 2.5 times more in samples that contained blood[11].

We examined all specimens to find any association between stool exam findings and STEC positivity. In this regard we classified our data based on gross and microscopy such as stool color, pus, mucus, WBC and RBC. There was no significant association between these findings and STEC; this indicates that all diarrheal stools should be examined for Shiga toxin-producing E. coli if no STEC is to be missed. In contrast to our results Appleman et al showed that 83% of the children with STEC had blood in their stool samples[20].

Table 3: Stool analysis findings of diarheal specimens with *STX*-positive *E.coli* isolates and all *E.coli* isolates

Variable		STEC	All E. coli
White Blood Cells	<=3	22(81.5%)	697 (71.6%)
	>3	5 (18.5%)	275 (28.4%)
Red Blood Cells	<=2	20 (74.1%)	646 (66.8%)
	>2	7 (25.9%)	326 (33.2%)
Stool consistency and form	Loose	17 (63%)	492 (50.9%)
	Soft	8(29.6%)	442 (45.7%)
	Watery	1 (3.7%)	25 (2.6%)
	Mucoid	1 (3.7%)	6 (0.6%)
	Bloody	0 (0%)	27 (2.8%)

STEC: Shiga toxin-producing *E. coli*

We found that the rate of STEC positive diarrhea was increased in the spring. In a study in Germany Ducker C. et al reported an outbreak of Shiga-toxin-producing Escherichia coli (EHEC) infections occurred from May to June and the great number of patients had HUS[21].

There was no significant sex differences in the frequency of STEC positive isolates in our study, although some studies found it higher in younger and female patients[20].

Our study had some limitations. We did not determine the serotype of the isolates and could not compare our results with other studies to find if E. coli O157:H7 is the most prevalent STEC in Iran or not. Also, if we could have used SMAC agar for isolation, we could have differentiated E. coli O157:H7 from other E. coli strains.

Conclusion

The low frequency of STEC in our population indicates that the STEC and HUS is not a major problem in our population but, using rapid and accurate methods for detection of it, is important because STEC infection may lead to serious complications like HUS. Further studies with greater sample size are needed for determining STEC frequency and also frequency of Shiga-toxin-associated HUS.

Acknowledgment

We thank all staff of Laboratory of Children's Medical Center (Department of Microbiology & Genetics) for their help in data collection and performing the STEC PCR Specially Leila Kashi and Dr. Saeeid Sepehri. This work was financially supported by the Tehran University of Medical Sciences.

Conflict of Interest: None

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