Characterization of Shiga-toxin producing *E.coli* (STEC) and enteropathogenic *E.coli* (EPEC) using multiplex Real-Time PCR assays for stx_1, stx_2, *eaeA*

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ABSTRACT

Background and Objective: Diarrheal disease is still a major health problem, especially in developing countries, where it is considered as one of the leading causes of morbidity and mortality especially in children. Studies showed that Diarrheagenic *E.coli* (DEC) such as STES and EPEC strains are among the most prevalent causative agents in acute diarrhea, particularly in children. Aim of the present study was to investigate the presence and the frequency of STEC and EPEC as etiologic agent of diarrhea in children less than 2 years of age with diarrhea in Shiraz.

Materials and Methods: A total of 285 stool samples were collected from patients with diarrhea in Shiraz, in 2012. Diarrheagenic *E.coli* (DEC) strains were isolated by standard biochemical analysis. In this study, we used multiplex Real time PCR and single PCR to detect the presence of indicator genes stx_1, stx_2 and *eaeA* for STEC and EPEC strains, respectively.

Results: A total of 285 stool samples were tested in which 49 (17%) were identified as contaminated with *E.coli* by biochemical tests. Out of total samples, 15 STEC (31%) and 13 EPEC (27%) were identified using multiplex Real-Time PCR assay. Among STEC isolates, 2 strains were $stx_1{}^+$, 8 isolates $stx_2{}^+$, 3 isolates were $stx_1{}^+$, $stx_2{}^+$ and 2 isolates were $stx_1{}^+$, $stx_2{}^+$, *eaeA*$^+$.

Conclusion: In this study, we found rather high occurrence of STEC and EPEC virulence genes in children with diarrhea. The results of this study showed that, real time PCR can be used as a replacement for conventional PCR assay in the detecting virulence genes of STEC and EPEC strains. Real-time PCR offers the advantage of being a faster, more robust assay, because it does not require post-PCR procedures to detect amplification products.

Keywords: *Multiplex Real-Time PCR, STEC, EPEC, Children, Shiraz*

INTRODACTION

Diarrheal disease is still a major health problem, especially in developing countries, where it is considered as one of the leading causes of morbidity and mortality especially in children. Among the bacterial pathogens, diarrheagenic *Escherichia coli* (DEC) is one of the important etiological agents of diarrhea (1, 2, 3). Commensal *Escherichia coli*, which was discovered in 1885, is the predominant facultative anaerobe of the human gut microbiota (4). Pathogenic strains are divided into intestinal pathogens causing diarrhea and extraintestinal *E.coli* (ExPEC) causing

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a variety of infections in both humans and animals, including urinary tract infections (UTI), meningitis and septicemia (5, 6).

Diarrheagenic *E.coli* (DEC) strains can be divided into six main categories on the basis of distinct epidemiological and clinical features, and specific virulence determinants (7): Enteropathogenic *E.coli* (EPEC), Enterotoxigenic *E.coli* (ETEC), Enteroinvasive *E.coli* (EIEC), Enterohaemorrhagic *E.coli* (EHEC) or Shiga-toxin producing *E.coli* (STEC), Enteroaggregative *E.coli* (EAEC), and diffusely adherent *E.coli* (DAEC) (8).

Shiga toxin-producing *E.coli* is a heterogeneous group of organisms (9, 10) and the clinical spectrum caused by gastrointestinal infection with STEC varies widely. Symptomatic clinical manifestations of STEC infection are including hemorrhagic colitis, hemolytic uremic syndrome (HUS), and a life-threatening thrombotic microangiopathy leading to acute renal dysfunction. (11). STEC is characterized by the production of two potent cytotoxins denominated Shiga-like toxins 1 and 2 (Stx1 and Stx2) (12) and in some strains the presence of the LEE locus related to the attaching and effacement lesion (13). Intimate attachment of bacteria to the host cell is mediated by the binding of intimin, the product of the *eae* gene, to the translocated intimin receptor. The virulence of STEC for humans may also be related to the Stx type which is produced by the bacteria. The presence of the stx_2 gene in the infecting strain was previously reported to correlate with severe disease in humans (14), and the administration of purified Stx_2, but not of Stx_1, was shown to cause HUS in experimentally treated primates (15).

EPEC was the first pathotype of *E. coli* to be described. A characteristic intestinal histopathology; known as 'attaching and effacing' (A/E), is associated with EPEC infections. The bacterium initially attaches to intestinal of host and cause changes in cytoskeletal of epithelial cells, including accumulation of polymerized F-actin directly beneath the adherent bacteria (16, 17).

Many types of STEC and EPEC have the *eae* chromosomal gene, encoding the outer membrane protein intimin, and both strains elicit attaching and effacing lesions on the intestinal mucosa. EPEC and STEC are distinguished by the presence of the Shiga toxin–encoding gene, being present only in STEC (18, 4).

Although EPEC are among the most important pathogens infecting children less than 2 years of age in the developing world, but the prevalence may vary depending on differences in study population, age group, diagnostic criteria and diagnostic tools used (5). The gastrointestinal pathogens EPEC and STEC continue to pose a threat to human health worldwide. While EPEC remains a significant cause of diarrhea in low-income countries, EHEC is more common as a food or water-borne pathogen in industrialized countries (19).

DEC is the most important etiologic agent of children's diarrhea in the Iran (20). Also these studies showed that DEC such as STES and EPEC strains are among the most prevalent causative agents in acute diarrhea, particularly in children (1, 21, 22). Therefore, the aim of the present study was detection of STEC and EPEC as etiologic agent of diarrhea in children less than 2 years of age with diarrhea in Shiraz. In this study we used Multiplex Real time PCR and single PCR to detect the presence of stx_1, stx_2 and *eaeA* genes.

MATERIALS AND METHODS

A total of 285 stool samples were collected from children with diarrhea in Shiraz, in 2012. Fecal samples, from patients were transported to the laboratory in PBS transport mediums on ice packs. A loop full of diarrheal sample was streaked on MacConkey agar and incubated for 24 h at 37ºC. Pink colonies then subcultured on Eosin Methylene Blue (EMB) agar. Colonies that exhibit green metallic sheen color were isolated for a further confirmation set of biochemical tests.

Subsequently, a sweep of three colonies were inoculated in Luria-Bartani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) and incubated overnight at 37°C with shaking. All the isolated *E.coli* strains were grown on Luria-Bertani agar (Sigma, St. Louis, MO) overnight at 37°C. *E .coli* genomic DNA was extracted using DNA extraction kit (QIAGEN Ltd., Crawley, UK) according to manufacturer's instructors. The primers were selected to detect three different virulence genes (stx_1, stx_2 and *eaeA*) simultaneously in a single reaction (Table 1). EPEC and STEC were distinguished by the presence of the Shiga toxin-encoding (*stx*) genes (stx_1 and/or stx_2), being present only in STEC.

Single PCR assays. Each PCR assay was performed with a final reaction volume of 25 µl containing 2 µl

Table 1. Primers for PCR assays for STEC and EPEC genes.

Pathotype	Gene	Primer sequence (5'-3')	Amplicon size (bp)	Reference
STEC	stx_1	F: CTGGATTTAATGTCGCATAGTG R: AGAACGCCCACTGAGATCATC	150	23
STEC	stx_2	F: GGCACTGTCTGAAACTGCTCC R: TCGCCAGTTATCTGACATTCTG	255	23
EPEC/STEC	$eaeA$	F: ATGCTTAGTGCTGGTTTAGG R: GCCTTCATCATTTCGCTTTC	248	24

of the template DNA, 200 mM deoxynucleoside triphosphates, 4 mM $MgCl_2$, 1.5 unit U Taq DNA polymerase (Sinagen, Iran), 0.2 mM of each primer. Cycling parameter was used as follow: 95ºC for 5 min to initially denature the DNA, then 35 cycles of 1 min at 94ºC, 1 min at 58ºC to, 1 min at 72ºC, and finally single prolonged extension at 72ºC for 5 min. A negative control lacking the DNA template was included in each experiment to exclude the possibility of the reagent contamination. The *E.coli* strain used as control in the PCR test included *E.coli* ATCC 43894 (stx_1, stx_2, *eaeA*) and *E.coli* ATCC 7852 (*eaeA*). The amplified product was visualized by gel electrophoresis in 1.5% agarose gel containing ethidium bromide for 45 min at 100 V and then visualized under UV light (Fig. 1).

Multiplex real time PCR assays. Real-time PCR assay for detection of STEC and EPEC strains was conducted in a final volume of 25 µl as same as
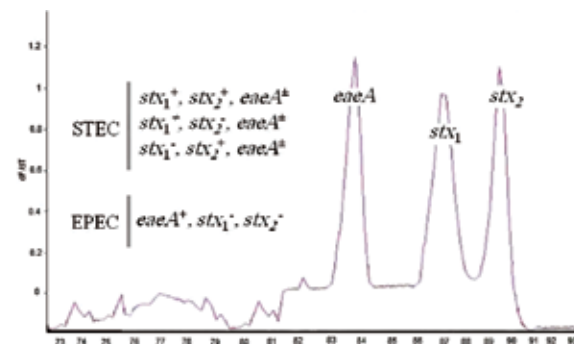
PCR plus 1 µl of SYBR Green I (Invitrogen, USA). Reactions were performed on Rotor-Gene 6000 (Corbett Research, Australia) by cycling conditions of 95 ºC for 5 min followed by 45 cycles of 95ºC for 30 s and 58ºC for 40 s. Finally, melt curve analysis was performed from 70-99ºC with ramping rate of 2.5ºC/s and analysis of fluorescence at each 2ºC for 5s. All reactions were repeated in triplicates and positive and negative control samples were used in each run. All data were analyzed by rotor-gene 6000 software version 1.7. The results of Multiplex Real time PCR assays showed in Fig. 2.

RESULTS

In this study, a total of 285 stool samples were collected from children (1 to 24 months old) with diarrhea in Namazi hospital and Shahid Dastghaib Hospital in Shiraz, Iran. A total of 285 stool samples were tested in which 49 (17%) were identified as contaminated with *E.coli* by biochemical tests. The present analysis revealed that 29 contaminated cases (59%) were males ($P = 0.199$). The children were categorized into four different groups according to

Fig. 1. Agarose gel of amplicons from the Single PCR. The molecular weight ladder is shown in lane M (100bp); STEC strain (stx_1, stx_2, *eaeA*) is shown in lanes 1 to 3, EPEC strain (*eaeA*) is shown lane 4 and nonpathogenic *E. coli* is shown in lane 5.

Fig .2. Identification of EPEC and STEC strains by Multiplex Real-time PCR on *stx* (stx_1 and stx_2), and *eaeA* virulence genes.

Table 2. Distribution of STEC and EPEC strains in accordance to seasons, age, sex and clinical symptoms.

Clinical and other characterization		STEC. No	EPEC.No
Season	Spring	5	5
	Summer	7	6
	Fall	3	1
	Winter	Not Seen	1
Age	(0-2)	0	2
	(3-5)	2	1
	(6-11)	5	4
	(12-24)	8	6
Sex ratio (M/F)	Male	11	8
	Female	4	5
Clinical symptoms	Diarrhea Bloody	5	2
	Diarrhea Watery	10	11
	Fever	12	9
	Vomiting	8	8

their age: (0-2), (3-5), (6-11) and (12-24) (Table 2). Infant diarrhea was more frequent in the summer than in other season ($P = 0.028$). The macroscopic analysis of the stool samples showed that 38 of the 49 cases (77%) were diarrhea watery and 33% bloody diarrhea.

All EPEC strains lack genes encoding shiga toxin (*stx*). Presence of *stx* gene can distinguish STEC strains from EPEC strains. In this study, we used Real time PCR and PCR to detect the presence of stx_1, stx_2 and *eaeA* genes. *E.coli* strains that carried *stx* (stx_1 and/or stx_2) genes were interpreted as STEC and strains that were negative for *stx* (stx_1 and/or stx_2) genes were considered as EPEC. The strains positive for stx_1, stx_2 genes and were positive or negative for *eaeA* were interpreted as STEC. Out of total samples, 15 STEC (31%) and 13 EPEC (27%) were identified using multiplex Real-Time PCR assay. Among STEC isolates 2 strains were stx_1^+, 8 isolates stx_2^+, 3 isolates were stx_1^+, stx_2^+ and 2 isolates were stx_1^+, stx_2^+, $eaeA^+$.

Highest STEC and EPEC strains were observed in dry season samples. 10 STEC strains were isolated in watery diarrhea and 5 STEC strains were isolated in bloody diarrhea. Highest EPEC strains were isolated in watery diarrhea. Among the total samples of STEC, 11 children were younger than 12 months and 4 children were between 13–24 months. Also, 10 EPEC strains were isolated from children younger than one year old and 5 EPEC strains were isolated from children from 13 to 24 months (Table 2).

DISCUSSION

Diarrhea is one of the important illnesses with high morbidity and mortality in children, resulting in about 1.6-2.5 million deaths annually (2). Among the bacterial cause of diarrhea, DCE is the most important etiologic agent of childhood diarrhea and represents a major public health problem in developing countries. Studies in Iran showed that DEC such as EPEC and STEC strains are among the most prevalent causative agents in acute diarrhea, particularly in children (21, 22, 1). In Shiraz, the status of STEC and EPEC prevalence and contribution to disease is uncertain. In this study, with appropriate scale, which covered diarrhea season of one year was to conducted to determine the prevalence of STEC and EPEC strains associated with children less than 2 years of age in Shiraz, Iran. In this study, STEC and EPEC strains were identified by a combination standard biochemical tests and molecular diagnostic methods for children with diarrhea. STEC infections continue to be a significant health burden in the United States. There are typically 15–20 outbreaks of STEC in the United States per year (25) that result in 265,000 illnesses, 3,600 hospitalizations, and 30 deaths annually (26, 27)

Pourakbari *et al.*, and Jafari *et al.*, reported that prevalence of STEC strains in children with diarrhea in Tehran, Iran, was 17% and 18.9% respectively (20, 21). In another report, STEC strains were isolated in 15.5% of children with diarrhea in Tehran (28).

Our finding is approximately similar to that reported from Tehran and the results of studies from Tunisia, Nigeria, America, Swaziland, Central African Republic and the Cameroon (29, 19, 30). The results of this study showed that among 15 STEC isolates 2 strains were stx_1^+, 8 isolates stx_2^+, 3 isolates were stx_1^+, stx_2^+ and 2 isolates were $stx_1^+, stx_2^+, eaeA^+$. Our finding is similar to the results of the study of Jafari *et al.* and Sharifi Yazdi *et al.* (21, 31).

Epidemiologically, despite large outbreaks of infant diarrhea due to EPEC in industrialized countries in the past (32), EPEC strains still remain a major cause of mortality in infants in developing countries (7, 33, 32). In 2007, the prevalence levels of EPEC (4.6%) infection in diarrheal in Tanzania (2). In 1991, prevalence of EPEC strains in children with diarrhea in Thailand and Brazil was 4.6% and 5.5% respectively (34, 35). In 2003, EPEC strains were isolated in 16% of children with diarrhea in Switzerland (36) in the present study, EPEC were detected in 13 (%) children with diarrhea. We report the first study performed in Shiraz to identify EPEC intestinal pathogens with children's diarrhea. Our results showed that the prevalence of STEC strains was higher than EPEC strains which similar to results reported by Jafari *et al.*, in Tehran, but a study by Asadi Karam *et al.*, showed predominance of EPEC strains (1, 21). In this study, highest STEC and EPEC strains were observed in dry season samples. No difference proportion was found between the dry and rainy season samples (21, 20).

In this study, we found rather high occurrence of STEC and EPEC virulence genes in children with diarrhea. Thus, more research is required, about the source model transition and risk factor of 2 pathotypes of DEC in children less 1 year in Shiraz, Iran. The results of this study showed that, real time PCR can be used as a replacement for conventional PCR assay in the detection of genes virulence STEC and EPEC strains.

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