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 STEE 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
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 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 stx2 gene in the infecting strain was previously reported to correlate with severe disease in humans (14), and the administration of purified Stx2, but not of Stx1, 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 stx1, stx2 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 37°C. 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 (stx1, stx2, and eaeA) simultaneously in a single reaction (Table 1). EPEC and STEC were distinguished by the presence of the Shiga toxin-encoding (stx) genes (stx1 and/or stx2), being present only in STEC.

**Single PCR assays.** Each PCR assay was performed with a final reaction volume of 25 µl containing 2 µl
of the template DNA, 200 mM deoxynucleoside triphosphates, 4 mM MgCl₂, 1.5 unit U Taq DNA polymerase (Sinagen, Iran), 0.2 mM of each primer. Cycling parameter was used as follow: 95°C for 5 min to initially denature the DNA, then 35 cycles of 1 min at 94°C, 1 min at 58°C to, 1 min at 72°C, and finally single prolonged extension at 72°C 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 (stx₁, stx₂, 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°C for 5 min followed by 45 cycles of 95°C for 30 s and 58°C for 40 s. Finally, melt curve analysis was performed from 70-99°C with ramping rate of 2.5°C/s and analysis of fluorescence at each 2°C 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

| Pathotype | Gene       | Primer sequence (5’-3’) | Amplicon size (bp) | Reference |
|-----------|------------|-------------------------|--------------------|-----------|
| STEC      | stx₁, stx₂ | F: CTGGATTTAATGTCGCATAGTG  
R: AGAACGCCCACTGAGATCATC | 150                  | 23         |
| STEC      | stx₁, stx₂ | F: GGCACGTGCCTGAAACCTGCTCC  
R: TCGCCAGTTATCTGACATTG | 255                  | 23         |
| EPEC/STEC | eaeA       | F: ATGCTTAGTGCTGGTTTAGG  
R: GCCTTCATCATTTCGCTTT | 248                  | 24         |

**Fig. 1.** Agarose gel of amplicons from the Single PCR. The molecular weight ladder is shown in lane M (100bp); STEC strain (stx₁, stx₂, 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 (stx₁, and stx₂), and eaeA virulence genes.

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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. 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 stx1+, 8 isolates stx2+, 3 isolates were stx1+, stx2+ and 2 isolates were stx1+, stx2+, 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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