Real Time PCR for Characterization of Enteroinvasive *E. coli* (EIEC) in Children with Diarrhea in Shiraz

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**Background:** Diarrheal disease is still a health problem, especially in developing countries, where it is considered one of the foremost causes of death in children, accounting for approximately two million deaths annually worldwide. Enteroinvasive *Escherichia coli* (EIEC) is known to cause shigellosis-like symptoms in both adults and children.

**Patients 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. We used Real time PCR and PCR to detect the presence of ipaH gene in EIEC. Susceptibility testing to 18 antimicrobial agents was determined by diffusion methods according to clinical and laboratory standards institute (CLSI, 2011) guidelines.

**Results:** In total, 285 stool samples were tested in which 49 (17%) were contaminated with *E. coli* by biochemical tests. In the present study, EIEC was detected in seven (14.3%) children with diarrhea. Of seven patients, five children were younger than 12 months and two were 13-24 months old. In total, four EIEC strains were isolated from watery diarrhea and three EIEC strains from bloody diarrhea. In the present study, EIEC strain exhibited high frequency of drug resistance to penicillin (100%), trimethoprim/sulfamethoxazole (71%) and tetracycline (71%).

**Conclusions:** We reported the first study performed in Shiraz to identify EIEC intestinal pathogens in children with diarrhea. This type of pathogen should be considered when designing preventive strategies for children in Iran.

**Keywords:** Enteroinvasive *Escherichia coli*; Real-Time PCR; Children; ipaH Gene; Shiraz

1. **Background**

Diarrheal diseases continue to be a health problem worldwide, especially in developing countries, where they are estimated to be responsible for 2.5 million infant deaths annually, with an annual mortality rate of 4.9 per 1000 children and an incidence of 3.2 episodes per child annually in children younger than five years (1). Among bacterial causes of diarrhea, diarrheagenic *Escherichia coli* (DEC) is the most important etiologic agent of childhood diarrhea and represents a major public health problem in developing countries (2). DEC is one of the predominant species of facultative anaerobes in the human gut and usually harmless to the host; however, a group of pathogenic *E. coli* has been emerged responsible for diarrheal disease in humans (3). *E. coli* strains associated with diarrhea have been classified into six groups based on clinical, epidemiological and molecular criteria (4, 5) namely, enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteraggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (EIEC) (6-8). Most *E. coli* isolated bacteria from stool are nonpathogenic, but DEC pathotypes represent a leading bacterial cause of pediatric diarrhea in developing regions, with some responsible for traveler’s diarrhea and an emerging cause of diarrhea in industrialized countries (9-11).

Enteroinvasive *Escherichia coli* (EIEC) was first found in 1971 to cause diarrheal disease in otherwise healthy volunteers. It is known to cause shigellosis-like symptoms in both adults and children (12). Like Shigella, EIEC invades the colonic epithelium, allowing cell-to-cell spread of the bacteria. Invasion is mediated by the genes located in virulence plasmid pINV coding, e.g. Ipa proteins and their transcription regulator invE (13). EIEC might cause an invasive inflammatory colitis, and occasionally dysentery, but in most cases, EIEC elicits watery diarrhea indistinguishable from that caused by infection by other *E. coli* pathogens. Conventional identification of pathogens from fecal specimens by selective culturing and biochemical assays is complex and time consuming. An invasion-associated plasmid antigen ipaH, has been described as a
possible target for diagnostic tools, as the gene is multiply present both on the invasion plasmid and EIEC chromosome (14). This genetic marker is currently used to detect EIEC in feces of patients with diarrhea.

2. Objectives

The aim of the present study was to estimate the incidence of EIEC infection and to investigate its epidemiology and pathogenesis from fecal specimens in infants with diarrhea. In this study, a molecular methodology was described to screen children samples regarding the presence of EIEC by detection of a target designed on the invasion plasmid antigen H (ipaH) gene, which represents a genetic marker characteristic for this group of pathogenic *E. coli*.

3. Patients and Methods

A total of 285 stool samples were collected from patients with diarrhea in Shiraz, in 2012. Clinical symptoms from children with diarrhea such as fever, vomiting, nausea and stool properties were recorded. Fecal samples from children were transported to the laboratory in Phosphate buffered saline (PBS) transport mediums on ice packs. Single *E. coli* isolate was obtained from each stool sample by culturing stool on MacConkey agar and subculturing on Eosin methylene blue agar for isolation of pure strains. Presumptive *E. coli* strains capable of fermenting lactose, beta-glucuronidase, positive indole, negative citrate and positive motility were confirmed as *E. coli*.

Subsequently, a sweep of three colonies were inoculated in Luria-Bertani (LB) broth (1%) tryptone, 0.5% yeast extract, 0.5% NaCl and incubated overnight at 37°C with shaking. All isolated *E. coli* strains were grown on Luria-Bertani agar (Sigma, St. Louis, MO) over night at 37°C. *E. coli* genomic DNA was extracted using DNA extraction kit (QIAGEN Ltd., Crawley, UK) according to manufacturer’s instructors. These primer and amplification fragment length are shown in Table 1.

| Pathotype | Gene | Primer Sequence (5’-3’) | Ampli- | Reference |
|-----------|------|-------------------------|--------|-----------|
| EIEC      | ipaH | F: GTTCCCTTAC-CGGCTTTCTCGATAC-CGT C | 619    | (15)      |
|           |      | R: GCCTGTCAGC-CACCCCTCGAG-TAC |        |           |

3.1. 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 MgCl$_2$, 1.5 U Taq DNA polymerase (Cinagen, Iran) and 0.2 mM of each primer. Cycling protocol was performed as follows: 95°C for 5 minutes to initially denature DNA, then 35 cycles consisting of one minute at 94°C, one minute at 58°C, one minute at 72°C and finally single prolonged extension at 72°C for 5 minutes.

A negative control lacking the DNA template was included in each experiment to exclude the possibility of reagent contamination. Shigella strain used as positive control in the PCR test was *Shigella sonnei* ATCC: 9290 (ipaH). The amplified product was separated by gel electrophoresis 1.5% agarose gel containing ethidium bromide for 45 minutes at 100 V and then visualized under UV light (Figure 1).

![Figure 1. Agarose Gel of Amplicons From the PCR](image)

The molecular weight ladder is shown in lane M; nonpathogenic *E. coli* is shown in lane 1; *Shigella sonnei* ATCC: 9290 is shown in lane 2; strain identification and amplicons are shown in lanes 3 to 6.

3.2. Real Time PCR Assays

Real-time PCR assay for detection of EIEC strains was conducted in a final volume of 25 µL, the same as PCR plus 1 µL of CYBR Green I (Invitrogen, USA). Reactions were performed on Rotor-Gene 6000 (Corbett Research, Australia) by cycling condition of 95°C for 5 minutes followed by 45 cycles of 95°C for 30 seconds and 58°C for 40 seconds. Finally, Melt curve analysis was performed from 70-99°C with ramping rate of 2.5°C/second and analysis of fluorescence at each 2°C for 5 seconds. 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 Corbett Research, Australia.

3.3. Antibiotic Susceptibility Testing

Susceptibility testing to 19 antimicrobial agents (MAST Co., UK) including imipenem, ciprofloxacin, levofloxacin, trimethoprim-sulfamethoxazole, amikacin, gentamicin, streptomycin, ceftriaxone, cefixime, cefotaxime, nalidix-
ic acid, tetracycline, azithromycin, ampicillin, chloramphenicol, clarithromycin, penicillin, erythromycin and nitrofurantoin was determined by diffusion methods according clinical and laboratory standards institute (CLSI, 2011) guidelines.

3.4. Data Analysis

Aχ² test or Fisher’s exact test was used to determine data statistical significance. P value below 0.05 was considered statistically significant.

4. Results

In this study, 285 children with diarrhea aged 1 to 24 months were evaluated from two different hospitals in Shiraz, Iran. In total, 285 stool samples were tested in which 49 (17%) were contaminated with E. coli by biochemical tests. The present analysis revealed that 29 cases (59%) were males (P = 0.199). Children were categorized into four different groups according to their age as: (0-2), (3-5), (6-11) and (12-24) months (Table 2). Infant diarrhea was more frequent in the summer than other seasons (P = 0.028). Macroscopic analysis of stool samples showed that 38 of 49 cases (77%) were diarrhea watery and 33% bloody diarrhea.

In this study, we used Real time PCR and PCR to detect the presence of ipaH gene in EIEC. In the present study, EIEC was detected in seven (14.3%) children with diarrhea. Seven EIEC strains were observed in dry season samples. Four EIEC strains were isolated from watery diarrhea and three EIEC strains from bloody diarrhea. Among the total samples, five children were younger than 12 months and two were 13-24 months old.

The results of antimicrobial drug susceptibility tests are shown in Table 3 indicating resistance, intermediate and susceptibility (Table 3).

Penicillin, Tetracycline and Trimethoprim/Sulfamethoxazole were the highest resistant antibiotics, while levofloxacin, Imipenem and Gentamicin were the highest susceptible antibiotics in patients with diarrhea.

5. Discussion

Diarrheal disease is still a health problem, especially in developing countries, where it is considered one of the foremost causes of death in children, accounting for approximately two million deaths annually worldwide. Features of diarrhea vary from place to place depending on local meteorology, geography and socioeconomic variables (10). In the present study, EIEC strains were detected in seven (14.3%) children with diarrhea. Seven EIEC strains were observed in dry season samples. Four EIEC strains were isolated from watery diarrhea and three EIEC strains from bloody diarrhea. Among the total samples, five children were younger than 12 months and two were 13-24 months old.

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Between August 2003 and July 2005, high prevalence rates of EIEC (3.2 cases/100) infection in diarrhea were reported in Ecuador (17). Between May 2009 and May 2010, 2011, high prevalence rates of EIEC (3.2 cases/100) infection in diarrhea were reported in Ecuador (17). Between May 2009 and May 2010, and 2011.
one (0.29%) EIEC sample was identified from 466 stool samples in patients and 349 stool samples from controls were obtained in Colombia (8). In 1989, 15 EIEC samples were identified in 221 cases of childhood diarrhea in a Beijing hospital (18); in 1985, 17 cases of EIEC were found in 410 children with diarrhea in a Bangkok hospital (19); and in 1982-1986, 17 cases of EIEC were detected in 912 infants with diarrhea in Chile (20). In the mid-1990s, EIEC was identified in 87 of 1579 stool samples from patients with travel-associated diarrhea (17). Features of this pathogen as causative agents of diarrhea vary from place to place depending on local meteorology, geography and socioeconomic variables. This variation is also seen between and within countries in the same geographical area (5).

Our study showed the importance of EIEC in clinical practice and surveillance of incidence and complications. The present research suggests that EIEC may be an important and unrecognized cause of diarrhea in infancy, not only in developing countries but also in developed areas. A primary important modality to reduce distribution of these pathogenic agents is improvements in sanitary measures (10). EIEC is the causative agent of invasive, dysenteric form of diarrhea in humans due to the ability to invade the colonic mucosa. The results of this study showed that three of seven strains were isolated from watery diarrhea and four from bloody diarrhea. Our finding is similar to Javadzadeh et al. in Zahedan and Akbari et al. studies in Tehran (16, 20).

PCR amplified DNA sequences encoding IpaH, a multiple copy sequence located on the chromosome and the invasion plasmid. Traditional PCR methods require amplification in a thermocycler followed by product separation by gel electrophoresis, time-consuming and laborious processes. However, as shown here, PCR products can also be practically detected using a DNA binding dye, such as SYBR green in a format (21, 22). Advantages of real-time PCR over traditional PCR include its closed tube system requiring no post-PCR processing. Therefore, real-time PCR offers the advantage of a faster and more robust assay, because it does not require post-PCR procedures to detect amplification products (15, 23). Real-time PCR has higher precision, increased sensitivity (down to 1 copy), increased dynamic range (greater than 8 logs) and high resolution (less than two fold differences) (15).

In this present study, EIEC strain exhibited higher frequencies of resistance to penicillin (100%), trimethoprim/sulfamethoxazole (71%) and tetracycline (71%). In Tehran, Alikhani et al. reported higher percentage of resistance to tetracycline, trimethoprim/sulfamethoxazole and cephotaxime in *E. coli* (23). Another report in Tehran reported higher frequencies of resistance to tetracycline, trimethoprim/sulfamethoxazole and ampicillin in *E. coli* (24). In Kenya, the highest prevalence of antimicrobial resistance was against ampicillin followed by trimethoprim/sulfamethoxazole and tetracycline (24). In this study, our finding was approximately similar to studies performed in Iran and Kenya (23-25).

The present study was performed to identify the incidence of EIEC as a potential etiologic agent of diarrheal disease in children in Iran. This type of pathogen should be considered when designing preventive strategies for people living in Iran. Molecular analysis using Real-time PCR is a universal method to identify EIEC and using this assay is suggested for evaluation and genotyping this bacterium in all regions of the country.

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