Ann Colorectal Res. 2015 January; 2(3): e22721. DOI: 10.17795/acr-22721

Published online 2014 December 9. **Research Article**

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

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Received: August 14, 2014; **Revised:** October 5, 2014; **Accepted:** November 11, 2014

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.
Objectives: The aim of this study was to identify EIEC in children with diarrhea using Real-time PCR assays in Shiraz, Iran.
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), enteroaggregative *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.

Table 1. Primers for PCR Assays of Enterotoxigenic *E. coli* (EIEC) Gene

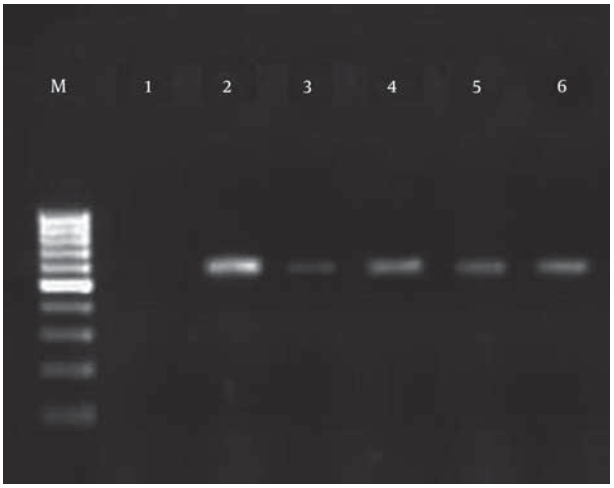
Pathotype	Gene	Primer Sequence (5´-3´)	Amplicon Size	Reference
EIEC	ipaH	F: GTTCCTTGACCGCCTTTCCGATACCGTC	619	(15)
		R: GCCGGTCAGCCACCCTCTGAGAGTAC		

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 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 extention 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 electrophoresisin 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



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

Ax2 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. We reported the first study performed in Shiraz to identify EIEC in children with diarrhea. Our finding was approximately similar to that reported by Javadzadeh et al., in which the rate of EIEC was 13% (16).

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,

Table 2. Distribution *E. coli* and EIEC Strains According to Seasons, Age, Gender and Clinical Symptoms

Clinical and Other Characterization	*E. coli*, (n = 49)	EIEC, (n = 7)
Season		
Spring	17	2
Summer	21	5
Fall	8	not seen
Winter	3	not seen
Age, month		
0-2	8	1
3-5	6	1
6-11	14	3
12-24	21	2
Gender		
Male	29	4
Female	20	3
Clinical symptoms		
Diarrhea bloody	13	3
Diarrhea watery	38	4
Fever	33	2
Vomiting	21	3

Table 3. Susceptibility of EIEC Isolated From Stools of Patients With Diarrhea in Shiraz [a]

Antimicrobial	EIEC (n = 7)		
	Susceptible	Intermediate	Resistant
Cefotaxime	71	0	29
Ceftriaxone	71	0	29
Cefixime	57	14	29
Imipenem	86	0	14
Levofloxacin	86	0	14
Ciprofloxacin	71	0	29
Nalidixic Acid	43	0	57
Chloramphenicol	57	29	14
Tetracycline	14	14	71
Trimethoprim/Sulfamethoxazole	0	29	71
Streptomycin	57	29	14
Gentamicin	86	0	14
Amikacin	57	29	14
Nitrofurantoin	71	14	14
Ampicillin	43	14	43
Penicillin	0	0	100
Azithromycin	57	29	14
Clarithromycin	43	0	57
Erythromycin	29	14	57

[a] Data are presented as %.

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 cefotaxime 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.

Acknowledgements

The authors acknowledge the Biotechnology Research Center of Islamic Azad University of Shahrekord Branch in Iran for their sincere support.

References

1. Estrada-Garcia T, Lopez-Saucedo C, Thompson-Bonilla R, Abonce M, Lopez-Hernandez D, Santos JI, et al. Association of diarrheagenic Escherichia coli Pathotypes with infection and diarrhea among Mexican children and association of atypical Enteropathogenic E. coli with acute diarrhea. *J Clin Microbiol.* 2009;**47**(1):93–8.
2. Heydari H, Shokrollahi MR, Movahedi Z. Frequency of type 1 fimbriae among E. coli subtypes isolated from patients with urinary and gastrointestinal tract infection. *Life Sci J.* 2013;**10**(7).
3. Kagambega A, Martikainen O, Siitonen A, Traore AS, Barro N, Haukka K. Prevalence of diarrheagenic Escherichia coli virulence genes in the feces of slaughtered cattle, chickens, and pigs in Burkina Faso. *Microbiologyopen.* 2012;**1**(3):276–84.
4. Kalantar E, Alikhani MY, Naseri MH, Torabi V. Antibiotic resistance patterns of STEC and ETEC strains: a study on frozen foods of animal origin and children with acute diarrhea. *J Microbiol Infect Dis.* 2013;**3**(1):31–5.
5. Hegde A, Ballal M, Shenoy S. Detection of diarrheagenic Escherichia coli by multiplex PCR. *Indian J Med Microbiol.* 2012;**30**(3):279–84.
6. Guion CE, Ochoa TJ, Walker CM, Barletta F, Cleary TG. Detection of diarrheagenic Escherichia coli by use of melting-curve analysis and real-time multiplex PCR. *J Clin Microbiol.* 2008;**46**(5):1752–7.
7. Kuwayama M, Shigemoto N, Oohara S, Tanizawa Y, Yamada H, Takeda Y, et al. Simultaneous detection of virulence factors from a colony in diarrheagenic Escherichia coli by a multiplex PCR assay with Alexa Fluor-labeled primers. *J Microbiol Methods.* 2011;**86**(1):119–20.
8. Gomez-Duarte OG, Romero-Herazo YC, Paez-Canro CZ, Eslava-Schmalbach JH, Arzuza O. Enterotoxigenic Escherichia coli associated with childhood diarrhoea in Colombia, South America. *J Infect Dev Ctries.* 2013;**7**(5):372–81.
9. Shams S, Haghi-Ashtiani MT, Nasrollahi L, Shahsiah R, Monajemzadeh M, Tahbaz-Lahafi B, et al. Frequency of shiga toxin-producing genes of Escherichia coli isolated from diarrheic stools of Iranian children by PCR. *Iran J Pediatr.* 2013;**23**(6):637–42.
10. Amisano G, Fornasero S, Migliaretti G, Caramello S, Tarasco V, Savino F. Diarrheagenic Escherichia coli in acute gastroenteritis in infants in North-West Italy. *New Microbiol.* 2011;**34**(1):45–51.
11. Nataro JP, Kaper JB. Diarrheagenic Escherichia coli. *Clin Microbiol Rev.* 1998;**11**(1):142–201.
12. Pereira AL, Giugliano LG. Adhesion of Diarrheagenic Escherichia coli and Inhibition by Glycocompounds Engaged in the Mucosal Innate Immunity. *Biology (Basel).* 2013;**2**(2):810–31.
13. Fujioka M, Kasai K, Miura T, Sato T, Otomo Y. Rapid diagnostic method for the detection of diarrheagenic Escherichia coli by multiplex PCR. *Jpn J Infect Dis.* 2009;**62**(6):476–80.
14. Song T, Toma C, Nakasone N, Iwanaga M. Sensitive and rapid detection of Shigella and enteroinvasive Escherichia coli by a loop-mediated isothermal amplification method. *FEMS Microbiol Lett.* 2005;**243**(1):259–63.

15. Smith CJ, Osborn AM. Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS Microbiol Ecol.* 2009;**67**(1):6–20.
16. Javadzadeh M, Dabiri S, Zangiabadi M. Role of Shigella, Enteroinvasive Escherichia coli (EIEC) and Entamoeba Histolytica in causing dysentry in children and antibiotic sensitivity testing. *J Mazandaran Univ Med Sci.* 2003;**39**(13):29–35.
17. Vieira N, Bates SJ, Solberg OD, Ponce K, Howsmon R, Cevallos W, et al. High prevalence of enteroinvasive Escherichia coli isolated in a remote region of northern coastal Ecuador. *Am J Trop Med Hyg.* 2007;**76**(3):528–33.
18. Hidalgo M, Realpe ME, Munoz N, Sicard D, Silva E, Agudelo CI, et al. [Acute diarrhea outbreak caused by Shigella flexneri at a school in Madrid, Cundinamarca: phenotypic and genotypic characterization of the isolates]. *Biomedica.* 2002;**22**(3):272–9.
19. Munoz N, Realpe ME, Castaneda E, Agudelo CI. [Characterization by pulsed-field gel electrophoresis of Salmonella typhimurium isolates recovered in the acute diarrheal disease surveillance program in Colombia, 1997-2004]. *Biomedica.* 2006;**26**(3):397–407.
20. Akbari A, Pourmand MR, Sharifi Yazdi MK, Soltan Dallal MM. Isolation of Enteroinvasive E. coli (EIEC) Strains In Children With Diarrhea By polymerase chain reaction (PCR) assay. *J Fac Tehran Univ Med Sci.* 2009;**3**(3,4):25–3.
21. Kagkli DM, Folloni S, Barbau-Piednoir E, Van den Eede G, Van den Bulcke M. Towards a pathogenic Escherichia coli detection platform using multiplex SYBR(R)Green Real-time PCR methods and high resolution melting analysis. *PLoS One.* 2012;**7**(6).
22. Souza TB, Lozer DM, Kitagawa SM, Spano LC, Silva NP, Scaletsky IC. Real-time multiplex PCR assay and melting curve analysis for identifying diarrheagenic Escherichia coli. *J Clin Microbiol.* 2013;**51**(3):1031–3.
23. Alikhani MY, Hashemi SH, Aslani MM, Farajnia S. Prevalence and antibiotic resistance patterns of diarrheagenic Escherichia coli isolated from adolescents and adults in Hamedan, Western Iran. *Iran J Microbiol.* 2013;**5**(1):42–7.
24. Sang WK, Oundo V, Schnabel D. Prevalence and antibiotic resistance of bacterial pathogens isolated from childhood diarrhoea in four provinces of Kenya. *J Infect Dev Ctries.* 2012;**6**(7):572–8.
25. Jafari F, Hamidian M, Rezadehbashi M, Doyle M, Salmanzadeh-Ahrabi S, Derakhshan F, et al. Prevalence and antimicrobial resistance of diarrheagenic Escherichia coli and Shigella species associated with acute diarrhea in Tehran, Iran. *Can J Infect Dis Med Microbiol.* 2009;**20**(3):e56–62.