

Molecular detection of lactose fermenting enteroinvasive *Escherichia coli* from patients with diarrhea in Tehran-Iran

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ABSTRACT

Background and Objectives: Enteroinvasive *Escherichia coli* (EIEC) is one the cause of acute diarrhea and bacillary dysentery in developing countries. Routine diagnostic microbiology tests are not capable to distinguish EIEC from other pathogenic or non-pathogenic *E. coli*. PCR, targeting *ipaH*, *virF*, *virB* and other virulence genes, is a diagnostic method for detecting *E. coli* pathotypes. Using PCR, we identified EIEC by PCR targeting *ipaH* and *virF* genes among *E. coli* isolates from patients with diarrhea at the selected hospitals in Tehran.

Materials and Methods: Isolates of *E. coli* were cultured from 140 specimens of patients with diarrhea using culture and IMViC test. DNA was extracted using commercial kits and tested for *uidA*, *ipaH* and *virF* genes by PCR.

Results: Totally, 140 *E. coli* isolates were confirmed by IMViC tests and PCR targeting *uidA* gene. Of 140 *E. coli* isolates, 5 (3.6%) were positive for the *ipaH* gene, 4 (2.9%) contained *virF* gene and 4 (2.9%) were positive for both *ipaH* and *virF* genes.

Conclusion: These results indicated that EIEC is a considerable acute diarrheagenic pathogen in adults and infants. Moreover, *virF* gene is suggested for evaluation of invasiveness of EIEC.

Keywords: Enteroinvasive *Escherichia coli*, diarrhea, *ipaH*, *virF*

INTRODUCTION

Enteroinvasive *Escherichia coli* (EIEC) is one of the intestinal pathotypes of *E. coli*, that cause diarrhea, very similar to *Shigellosis* (1, 2). This organism is capable to invade and penetrate to the epithelium of the colon leading to dysentery that is an important cause of morbidity and mortality among children in developing countries (3, 4). Dysentery can be characterized with abdominal pain, fever and diarrhea, sometimes vomiting, and the stool contains blood

and leukocyte. In addition EIEC might cause an invasive inflammatory colitis, small bowel syndrome and watery diarrhea that is not distinguishable from infections by other *E. coli* pathotypes (5).

Pathogenesis and especially the ability to invade the epithelium of the colon is dependant on a 220kb plasmid, called *pInv*, which carries the genes for a type III secretion system that is used as the virulence factor (6, 7).

There are no biochemical tests for distinguishing of EIEC from other *E. coli* pathotypes and routine laboratory tests can not discriminate *E. coli* pathotypes from other non-infectious *E. coli* and very few biochemical tests can distinguish EIEC from *Shigella*. Therefore, in many cases EIEC pathotype is misreported as *Shigella* (8). Regarding to remarkable EIEC acute diarrhea and poor isolation tests,

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molecular methods like PCR are more applicable for detection of this pathotype. Since detection of EIEC leads to appropriate therapeutic strategies.

The PCR of virulence genes has been used for detection of EIEC and *Shigella*, which all are located in *pInv* plasmid. The invasion associated pathogen antigen or *ipaH* is present both on the chromosome and the invasion plasmid of *Shigella* and EIEC strains (9, 10). The *virF* is a member of the AraC family gene, which is a transcription activator and their action is necessary for virulence regulon (11). The *uidA* gene is a housekeeping gene that encode beta D-glucuronidase in *E. coli*. This gene can be used for confirmation of *E. coli* isolates and differentiation of *E. coli* from non-*E. coli* isolates by PCR (12).

Considering the high prevalence of gastrointestinal disorders, including diarrhea in populations of developing countries, appropriate detection and treatment of diarrhea has high value of importance. However, there is a very limited study concerning *E. coli* pathotypes in these regions. The aim of this study was to study the EIEC distribution in patients with diarrhea referring to the selected governmental hospitals in Tehran during 2013 to 2014 using molecular techniques.

MATERIALS AND METHODS

Studied population and sampling. In this descriptive study a total of 140 patients with diarrhea were investigated by culture. Diarrhea was defined as three or more watery, loose or mucoidal stool in one day in the current study. Prior to sampling, the medical information was collected from each patient. The stool samples were collected from all age groups between April 2013 to September 2013 from selected hospitals of Tehran considering standard protocols for microbial analysis of stool (13). Diarrhea samples were evaluated for PMN, RBC and microscopic situation.

Bacterial culture and identification. Diarrhea samples initially were cultured on MacConkey and Hekton enteric agar (Merck, Germany) and incubated for 24 hours at 37 °C. The lactose fermented colonies were tested by biochemical routine tests including citrate, Methyl Red, Voges Proskauer, indole production, and lysine decarboxylation for iden-

tification of *E. coli*. All *E. coli* isolates were saved at -20 in Luria Bertani broth (Merck, Germany) with 20% glycerol for molecular procedures.

Nucleic acid extraction. DNA was extracted with a commercial genomic DNA extraction kit (Bioneer, South Korea) according to the manufacturer's recommendations. Total DNA was extracted from 0.5 ml of Luria Bertani broth culture grown overnight for all the bacterial isolates. The concentration of DNA samples was measured as micrograms per milliliter based on A260 on A280 values by the nanodrop system (Thermo, USA).

PCR assays. All *E. coli* isolates were evaluated for the presence of the *uidA*, *ipaH* and *virF* genes by PCR assay using specific primers (Table 1).

PCR amplification was performed in a 25µL reaction mixture containing 200 ng of DNA template (1µL), 12µL ready to use Mastermix (Fermentas, Germany), 10 µL of distilled water and 1µL of 20 pmols forward and reverse primers. DNA amplification was carried out with a thermal cycler (Eppendorf, Germany) with the following thermal cycling program: initial denaturation at 95°C for 5 min followed by 30 cycles of amplification (denaturation at 95°C for 30 sec, annealing for 30 sec at 67°C for *uidA*, 58°C for *virF* and 61°C for *ipaH* and extension at 72°C for 30sec) ending with a final extension at 72°C for 5 min. The *E. coli* K12 was used as positive control for *uidA* and *Shigella flexneri* ATCC 12122 was used as positive PCR control for *ipaH* and *virF*. PCR products were visualized after electrophoresis on 1.5% agarose gel stained with ethidium bromide and the product size estimated using a 50bp DNA ladder (Fermentas, Germany).

Data analysis. Chi squared and Fisher's exact tests were used for analysis of categorical data and ANOVA was used for containing data. Analyses were done using Sigma Stat for Windows V2.03 (SPSS, Chicago, IL, USA). A P value less than 0.05 was accepted as statistically significant.

RESULTS

A total of 140 (100%) *E. coli* isolates were detected from 140 patients with diarrhea throughout three gen-

Table1. The sequences of primers and sizes of PCR products of the oligonucleotide primers to amplify specific, fragments of the *uidA*, *ipaH* and *virF* genes.

Primer	Sequence (5'-3')	Product size bp	Reference
<i>uidA</i>	5-GCGTCTGTTGACTGGCAGGTGGTGG-3 5- GTTGCCCCGCTTCGAAACCAATGCCT-3	503 bp	(14)
<i>ipaH</i>	5-CTCGGCACGTTTTAATAGTCTGG-3 5-GTGGAGAGCTGAAGTTTCTCTGC-3	933 bp	(14)
<i>virF</i>	5-AGCTCAGGCAATGAAACTTTGAC-3 5-TGGGCTTGATATCCGATAAGTC-3	618 bp	(14)

eral hospitals. The patients age range was between 0 to 66 years and mean age of the patients was 32 years. Among 140 patients, 31(22.1%) had abdominal pain and 13 (12.1%) had a fever.

Among diarrhea samples, PMNs and RBCs were seen in 38 (27.1%) and 14 (10%) of samples, respectively (Table 2).

As we expected, all *E.coli* isolates were positive for

uidA. Of 140 *E. coli* isolates, 5 (3.6%) were positive for *ipaH*, while *virF* was detected in 4 *ipaH* positive isolates. On the other hand, 4 (2.9%) isolates were positive for both of *ipaH* and *virF* genes.

All patients infected with *ipaH*⁺/*virF*⁺ *E. coli* isolates, had abdominal pain and fever while one patient infected with *ipaH*⁺/*virF*⁻ showed no symptoms (Table 3).

Table 2. Microscopic situations of diarrheal samples and clinical symptoms in patients infected with EIEC and non-EIEC isolates.

	Sex		Age	WBC	RBC	Abdominal pain	Fever
	Female	Male					
EIEC n=5	2(40%)	3(60%)	44±22	23±18	28± 20	4(80%)	4(80%)
Non- EIEC <i>E. coli</i> n=135	51(38%)	84(62%)	32±34	4±1	6±2	27(20%)	9(6.6%)
P value	0.581		0.544	0.021	0.003	0.009	0.001

Table 3. Microscopic situations of diarrheal samples and clinical symptoms in patients infected with *virF*⁺ and *virF*⁻ EIEC isolates.

EIEC	PMN	RBC	Abdominal pain		Fever	
			Yes	No	Yes	No
<i>ipaH</i> ⁺ , <i>virF</i> ⁺ n=4	22±16	28±18	4	0	4	0
<i>ipaH</i> ⁺ , <i>virF</i> ⁻ n=1	4±1	2±1	0	1	0	1
P value	0.007	0.001	0.002		0.001	

DISCUSSION

EIEC strains are known as one of clinically important *E. coli* pathotypes that cause watery diarrhea to dysentery (2, 3). EIEC are closely related to *Shigella* spp in terms of pathogenicity (1, 3). Given the importance of diarrhea caused by the EIEC, and the inability of conventional cultivation methods in the distinguishing pathogenic *E. coli* from non-pathogenics, the EIEC is usually ignored and most of the time it is misreported as *Shigella* spp. Thereby molecular methods seem to be an ideal option for this purpose. Due *ipaH* wide distribution in EIEC chromosome and plasmid, often was used for detecting of this pathotype (4).

A few studies have been done on EIEC in Iran. In the present study, *ipaH* gene was used for targeting EIEC in *E. coli* isolates from diarrheal samples. Our results showed 3.6% of isolated *E. coli* from diarrheal samples were EIEC.

Recently Alizadeh et al (15) in a study in the south of Iran showed EIEC in 1.4% of total *E. coli* isolates from <5 years children with diarrhea by PCR on *ipaH*. Akbari et al (201) in a similar study was evaluated EIEC in under 5 years, infants with acute diarrhea in Tehran (16). They were reported EIEC in 2.7% of samples by amplifying *ipaH* gene.

Mostafa et al (17) studied diarrheagenic *E. coli* pathotypes in Libyan children with diarrhea in 2012 using *ipaH* gene but did not detected EIEC in any sample. Hein et al (2009) were found only 0.8% of isolates as EIEC <5 years Vietnamese children (18).

Hegde et al (19) detected EIEC from diarrhea samples among Indian children <5 years by amplifying *iaL* gene. They indicate EIEC were present in 3.5% of total *E. coli* isolates.

ipaH gene was also used for detection of EIEC for the same purpose in Ecuador. EIEC were found in 3.2% of isolates and diarrhea samples among <5 years children in that country which is comparable to the results of our study.

Considering our result and data extracted from literature, the frequency of EIEC varies from 0 to 3.5% (15, 20). This prevalence shows that EIEC in the last years and in different geographic areas are generally considered to be one of the causes of acute diarrhea. Because of differences in time, place and the study conditions it seems to be reasonable for different reported results in reviewing studies.

In spite of majority of studies carried out on children under 5 years, we included patients without any

age limitation (age 32±34). However, there were no significant differences between current study results with others. Our study indicates that EIEC is a considerable acute diarrhea producing pathogen in adults as well as infants.

The invasive EIEC illness is characterized by abdominal cramps, diarrhea, vomiting, fever, chills, and a generalized malaise (5). In the current study, we studied clinical symptoms of patients as well as microscopic parameters. In the 80% of EIEC isolates from diarrheal samples PMNs and RBCs were present and patients, which were infected with this isolates had fever and abdominal pain that is evidence of invasive intestinal infection.

The EIEC virulence genes are carried by *pInv*. Two *pInv*-encoded activators, the VirF and VirB, are involved in the transcriptional control of the invasion genes. The VirF activates transcription of the secondary regulator *virB* gene, which activates the operons that are associated with EIEC invasion (21). In addition to *ipaH* gene, the *virF* gene was used as a gene involved in the EIEC invasion in our study. Among 5 EIEC isolates 4 (2.8%) isolates were *virF*⁺ and one isolate was *virF*⁻. The *virF* negative EIEC was isolated from a sample which has any PMNs and RBCs and associated patient had no fever and abdominal pain. Also the presence of RBCs in samples and abdominal pain and fever in patients was associated with *virF*⁺ isolates. This can confirm the role of *virF* in EIEC invasion.

The present study showed that EIEC can be cause of acute diarrhea in all age groups. Also applying the molecular diagnostic methods for detecting EIEC and other diarrheagenic *E. coli* pathotypes and subsequent appropriate treatment can be helpful.

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