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Full Length Research Paper

Multiplex polymerase chain reaction (PCR) assay for simultaneous detection of shiga-like toxin (*stx1* and *stx2),* intimin (eae) and invasive plasmid antigen H (i*paH*) genes in diarrheagenic *Escherichia coli*

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Despite the fact that diarrheagenic *Escherichia coli* (DEC) has been identified as a major etiologic agent of diarrhea in children worldwide, few studies have been performed in Iran to evaluate the etiology of these organisms. To evaluate the etiology of shiga toxin-producing E. coli (STEC), enteropathogenic *E. coli* (EPEC) and enteroinvasive *E. coli* (EIEC) in children with diarrhea in Iran a total 300 stool specimens from children with diarrhea were tested for the detection of *E.coli*. Out of 300 samples, 39 were identified as *E. coli* by biochemical tests and were subjected for serogrouping. The most prevalent serogroups among these isolates were serogroup IV, followed by III,I and II respectively. A single multiplex polymerase chain reaction (MPCR) was designed for the detection of target genes of stx1/ stx2, eae and ipaH in DEC. The dominating strain was EPEC (55.6%), followed by STEC (25%) and EIEC (19.4%).

Key words: Multiplex polymerase chain reaction (PCR), diarrheagenic *Escherichia coli*, shiga-like toxin.

INTRODUCTION

Diarrheagenic *Escherichia coli* (DEC) is an important agent of childhood diarrhea which represents a major public health problem in developing countries and is now being recognized as emerging entero-pathogens in the developed countries (Nataro and Kaper, 1998; Soltan Dallal., 2001; Mitchell et al., 2005; Akinjogunla et al., 2009). DEC was usually transmitted through food or water contaminated with human or animal faeces. Person-to-person transmission might also take place, but is probably less common (Wood et al.1983; Harris et al.1985; Nataro et al.1998). Poor sanitation, personal hygiene and environmental conditions are some of the factors that facilitate the transmission of the disease. Thus, DEC is more prevalent in the developing countries (Galane et al., 2001; Campos et al., 2004; Kalantar et al., 2011).

Based on their virulence factors, diarrheagenic*E. coli* have been classified into six groups such as, enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC)

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Abbreviations: DEC, Diarrheagenic *Escherichia coli*; **EPEC,** enteropathogenic *Escherichia coli*; **EHEC,** enterohemorrhagic *Escherichia coli*; **EIEC,** enteroinvasive *Escherichia coli*; **ETEC,** enterotoxigenic *Escherichia coli*; **EAEC,** enteroaggregative *Escherichia coli*; **DAEC,** diffusely adherent *Escherichia coli*; LEE, locus of enterocyte effacement; **A/E,** attaching and effacing; **STEC,** shiga toxin–producing *Escherichia coli*; **NCBI,** National Center for Biotechnology Information; **BLAST,** Basic Local Alignment Search Tool; **MPCR,** multiplex polymerase chain reaction.

Table 1. Isolated DEC types in two hospitals.

DEC types	EPEC		EHEC		EIEC		Others	
	A	B	A	B	A	B	A	B
Isolated	14	6	6	3	5	2	3	-
Total	20		9		7		3	

A, Ali Asghar pediatrics hospital; B, pediatrics department of Imam Khomeini hospital.

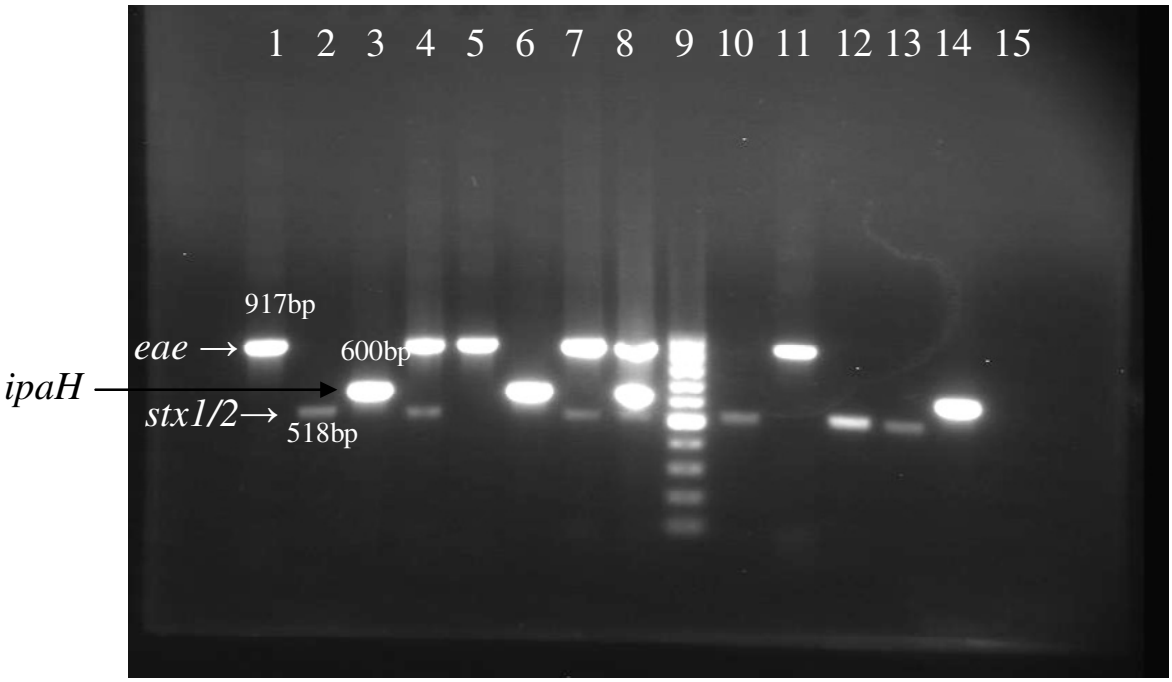


Figure 1. Gel electrophoresis of virulence genes of detected DEC types. Lines 1,5 and 11**,** EPEC (***eae*** gene); Lines 2, 10, 12 and 13, EHEC(*Stx1/2* gene); Lines 3,6 and 14, EIEC (*ipaH* gene); Lines 4 and 7, EHEC *(eae* and *stx1/2* genes); Line 8, positive control; Line 15, negative control.

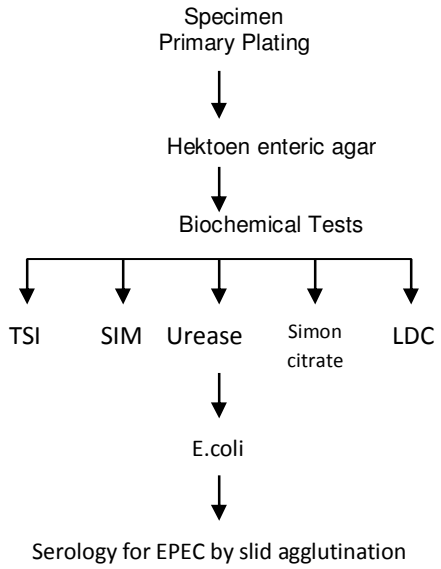
and diffusely adherent *E. coli* (DAEC).

Commonly isolated diarrheagenic *E. coli* in children are EPEC strains, which contained pathogenicity island (locus of enterocyte effacement or LEE containing *eae* gene).The *eae* gene is responsible for encoding proteins involved in the formation of attaching and effacing (A/E) lesions on host intestinal cells. EHEC or shiga toxin-producing *E. coli* (STEC) is the cause of hemolytic uremic syndrome, which may contain the locus of enterocyte effacement and by definition either or both of the shiga toxins (*stx1 and stx2*). Some of EHEC strains harbors the chromosomal gene of *eae* which is responsible for the encoding of the outer membrane protein intimin, same as EPEC strains. The *ipaH* gene in EIEC strains is similar to shigella species, which causes shigella-like dysenteric enteritis in human (Katia et al., 2007; Maricel et al., 2005; Sunabe and Honma, 1998; Stacy-Phipps et al., 1995; Rappelli et al., 2001). Multiplex polymerase chain reaction (PCR) systems have been used to reduce the number of tests needed for diagnosis of diarrheagenic *E. coli* (Osek, 2001; Pass et al., 2000; Paton and Paton, 2002; Rappelli et al., 2001; Ratchtrachenchai et al.,1997; Rich et al., 2001). The potential of diarrheagenic *E. coli* to cause diarrhea in children in other developing countries have been reported previously (Mitchell et al., 2005; Maricel et al., 2005). Therefore, the aim of this study is to use multiplex PCR to simultaneously detect diarrhea-agenic *E. coli* such as EPEC, EHEC and EIEC in fecal samples of children less than 5 years with diarrhea.

MATERIALS AND METHODS

A total of 300 stool samples were collected from children with diarrhea in Ali Asghar pediatrics hospital and pediatrics department of Imam Khomeini hospital in Tehran, from April to Jul 2008. The samples were cultured on hekteon enteric agar (MERCK) and incubated at 37°C for 24 h.

The following schema outlines the cultivation, biochemical



Lysine decarboxylase	v	E. coli O Antisera slide
Triple sugar iron	A/AG	agglutination with heated
Simmon citrate	-	culture
Motility	+	Serotyping with the
Urease	-	following poly valent antisera:
Indole	+	
H2S		
G - gas	-	poly I (O26,O55, O111)
Phenylalanine deaminase		- Poly II (O86, O127)
Interpretation:		Poly III (O44, O125, O126, O128)
v - variable		Poly IV
A - acid		(O20, O114)

and serological methods employed in the isolation and identification of Lactose-fermenting colonies were confirmed as *E. coli* on the basis of morphological and standard biochemical tests (Nataro and Kaper , 1998; Maricel et al., 2005). After identification , the *E. coli* isolates suspension were heated and suspended in saline and mixed on a slide with polyvalent EPEC serogrouping antisera.

DNA was extracted by bolling 5 colonies of overnight bacterial culture in 200 µl sterile distilled water for 5 min, followed by centrifugation at 12000 rpm for 10 min. 2 µl of supernatant was used as a template in PCR. The primers were selected to specifically amplify theree different virulence genes eae, stx1/ stx2 and *ipaH* in the one reaction (Katia et al., 2007). The primer pairs were used in this study was designed in table 1. The primer sequences were further analyzed by evaluation of the sequences against those in the National Center for Biotechnology information (NCBI) database with the Basic Local Alignment Search Tool (BLAST) program. To develop the multiplex polymerase chain reaction (MPCR), the progressive incorporation of primers corresponding to the different genes and several combinations of melting temperatures and primer concentrations were tested.

The control strains were *E. coli* ATCC 35218 (for eae, stx1, and stx2 genes). *E. coli* EATCC 7852 (for eae gene) and shigella sonnei ATCC 9290 (for *ipaH* gene). The isolated DEC strains were subjected to MPCR for detection of eae, stx1 / stx2 and *ipaH* genes. The optimized PCR protocol was carried out with a 50 µl mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.2). 1.5 mMMgCl2, a 2 mM concentration of each deoxynucleoside triphosphate, 1.5 U of Taq DNA polymerase, 2 µl of the DNA template and the primers. The optimal concentration of primer pairs in the mixture was determined empirically, that each primer pair concentration independently was varies. DNA mixture of the three reference *E. coli* strains was used as the PCR template in each of the reaction. The mixtures were subjected to the following cycling: 94°C (5 min, 1 cycle); 35 cycles consisted of denaturation at 95°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min, with a final extension stage at 72°C for 7 min. In all the experiments, the DNA mixture from the reference strains served as the positive control and distilled water as negative control. 5 µl of PCR products were separated by electrophoresis in 1% agarose gels containing ethidium bromide stain, in Tris-borate-EDTA buffer and visualized with UV illumination and imaged with GelDoc 1000 fluorescent imaging system (Bio-Rad). The amplicons were identified based on the size of the amplified product with DNA ladder markers.

RESULTS

300 stool samples were tested in which 39 were identified as *E. coli* by biochemical tests (Table2). Out of 39 E.coli isolated which were subjected for serogrouping were classified as EPEC and most of them were isolated from among children under one year age. The serogroup IV was the dominant serogroup, followed by III,I and II respectively as it is shown in table 3. Out of these 39 identi-fied, 36 were confirmed as DEC by PCR method. Out of 36 DEC , 20 (55.6%) possessed *eae*, 7 (19.4%) *ipaH* and 9 (25%) both stx1/2 and *eae* genes, which are designa-ting as EPEC, EIEC and EHEC respectively. The three stains which were identified as *E. coli* by biochemical tests do not have *eae*, stx1/2 or *ipaH* genes. Out of 39 isolates which were identifies as *E. coli* , 20 have *eae*, 9 stx1/2 and 7 *ipaH* genes, which are classified as EPEC, EHEC and EIEC, respectively (Figure 1).

DISCUSSION

It is widely accepted that, the characteristics of several specific virulence genes are sufficient for the identification of six categories of DEC strains (Nataro and Kaper, 1998; Katia et al., 2007).

Historically, EPEC was defined as a category of *E. coli* belonging to certain serogroups that had been associated with outbreaks of infantile gastroenteritis. Several studies (Smith et al.,1990; Knutton et al.,1991; Scotland et al.,1991; Robins-Browne et al.,1993; Law et al.,1994; Morelli et al.,1994) have recently demonstrated that this group of organisms is actually quite heterogeneous in the possession of putative virulence properties. EPEC strains associated with outbreaks (Moyenuddin et al.,1989; Robins-Browne et al.,1993) and it is of significant value in the detection of EPEC in developing countries. The serogroup IV was the dominant serogroup. Some of these serotypes could be originally avirulent and so they have contributed to the larger number of avirulent strains

Table 2. isolated DEC types in two hospitals. A, Ali Asghar pediatrics hospital; B, pediatrics department of Imam Khomeini hospital.

DEC types	EPEC		EHEC		EIEC		Others	
	A	B	A	B	A	B	A	B
Isolated	14	6	6	3	5	2	3	-
Total	20		9		7		3	

Table 3. serological results of EPEC serogroups

percentage	frequency	EPEC srogroups
10.5	2	Poly I
5.3	1	Poly II
21.1	4	Poly III
63.1	12	Poly IV
100	19	Total

in these serogroups. (Soltan Dallal et al.,2006; Galane and Le Roux 2001;Savulescu et al.,2007).

In this study, we used multiplex PCR to detect the presence of target genes of stx1/stx2 , *eae* and *ipaH* in EPEC, EHEC and EIEC. In this study, *eae* gee was dp,omating in isolates while EPEC was the dominating strain to cause diarrhea in children. Results of a similar study in Iran (Soltan Dallal., 2001; Alikhani et al., 2006; Kalantar et al.,2011), also showed that, EPEPC is dominating DEC type causing diarrhea in children which is correlated with our results. The high percentage of stx1/2 genes (231%) was obtained in this study compared to other studies (Estelle et al., 2006; Sahilah et al., 2010) and this might be due to geographical differences. High percentage of eae gene (75%) was found among the samples of children less than 1 year old and the percentage of stx1/2 genes in girls was more than that of boys. In total, 3 out of 39 tested *E. coli* did not have *eae*, stx1/2 and *ipaH* genes, perhaps these strains belong to ETEC, EAEC or DAEC that were not included in this study. Also, 3 out of 39 isolates possessed simultaneously both *eae* and stx1/2 genes, this might be due to virulence factors of EHEC and other DEC strains which are mobile within bacterial populations (Agin et al., 1996; Estelle et al., 2006; Boerlin, 1999).

This study also showed that, multiplex amplification of nucleic acid can be used as a replacement for conventional method in detection of DEC strains in Iranian children and for epidemiological study of these pathogens, particularly the emerging strains such as EHEC and EIEC in Iran.

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