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Full Length Research Paper

Characterization of virulence genes in typical and atypical enteropathogenic *Escherichia coli*

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Enteropathogenic *Escherichia coli* (EPEC) is a major cause of diarrhea in infants and children in developing countries. The aim of this study was to investigate the frequency of EPEC in children with diarrhea by polymerase chain reaction (PCR) method targeting the eae gene. The presence of virulence gene including bfpA was also investigated by PCR, for the differentiation of typical and atypical EPEC strains. We also sought to determine the presence of virulence gene including efa-1, fimH and lpfDo113 of the isolated strains. Stool samples were collected from 313 less than 5 years old children with diarrhea at Shahid Beheshti Hospital, Kashan, Iran, from November 2009 to May 2010. Specimens were examined by PCR test to determine the eae gene to detect of EPEC pathotype and also the frequency of genes coding virulence factors of EPEC such as bfpA, efa-1, fimH and lpfDo113 were analyzed. Results showed that 51 cases (28.6%) out of 178 *E. coli* isolated were EPEC. The percentage of isolated EPECs carrying virulence genes were bfpA (tEPEC) (11.8%), efa1 (2%), fimH (98%) and lpfDo113 (13.7%). Variable frequency of EPEC virulence factors isolated from children with diarrhea needs further studies for creating epidemiological map of diarrheal infections caused by EPEC.

Key words: Enteropathogenic *Escherichia coli*, virulence genes, diarrhea, children, polymerase chain reaction.

INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) is an important diarrheal pathogen of young children living in developing countries (Scaletsky et al., 2002). EPEC through eae chromosomal gene which codes intimin causes a histopathological lesion known as attaching and effacing (A/E) (Nataro and Kaper, 1998). The genes necessary for A/E lesion formation are located on a pathogenicity island on the *E. coli* chromosome called locus of enterocyte effacement (LEE). EPEC strains are classified as typical if they possess the *E. coli* adherence plasmid (EAF) with bfp genes encoding bundle-forming pili. A/E *E. coli* strains which do not possess the EAF plasmid or bfp genes are classified as atypical EPEC (Hernandes et al., 2009). Whereas typical EPEC is well recognized as a leading cause of severe pediatric diarrhea in developing countries (Trabulsi et al., 2002), the role of atypical EPEC in childhood diarrhea has been controversial (Nataro and Kaper, 1998). Atypical EPEC has been shown to be prevalent among children in both developing (Dulguer et al., 2003; Valentiner-Branth et al., 2003; Gomes et al., 2004) and developed countries (Knutton et al., 2001; Beutin et al., 2003; Cohen et al., 2005). Long polar fimbriae (LPF) have been shown to facilitate attachment of the bacteria to murine Peyer's patches (Doughty et al., 2002). Type 1 fimbriae of *E. coli* are surface organelles which mediate binding to D-mannose-containing structures. Furthermore, FimH protein is located laterally in the structure of the type 1 fimbriae (Krogfelt et al., 1990). Molecular characterization has shown a great diversity of virulence factors among different strains of atypical EPEC (Vieira et al., 2001; Dulguer et al., 2003; Beutin et al., 2005).

In this study we have examined the contribution of a large gene, efa1, fimH and lpfDo113 which is present in A/E pathogens, to the adherence phenotype of EPEC.

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Table 1. PCR primers and conditions used in this study and sizes of PCR amplicons.

Target	Forward primer	Reverse primer	Amplicon (bp)
Eae	CCCGAATTCGGCACAAGCATAAGC	CCCGGATCCGTCTCGCCAGTATTC	863
bfpA	ATTGAATCTGCAATGGTGC	ATAGCAGTCGATTTAGCAGCC	461
efa1	AAGGTGTTACAGAGATTA	TGAGGCGGCAGGATAGTT	268
fimH	TGCAGAACGGATAAGCCGTGG	GCAGTCACCTGCCCTCCGGTA	508
lpfDo113	GAACTGTAGATGGGTAC	AGCAGGCATAACGCAAG	798

Table 2. Frequency of virulence factors in enteropathogenic *E. coli* isolated from hospitalized children in Kashan in 2009-2010 based on sex, age (years) and hospitalization (day).

Gene factors		*pfpA*	efa_1	*fimH*	lpfDo113	P eulav
Sex	Male	3 (14.3%)	0 (0%)	20 (95.2%)	4 (19%)	N.S*
	Female	3 (10%)	1 (3%)	30 (100%)	3 (10%)	
	Total	6 (11.8%)	1 (2%)	50 (98%)	33 (13.7%)	
egA (raey)	<1	4 (16.7%)	0 (0%)	24 (100%)	4 (16.7%)	N.S*
	1-2	1 (11.1%)	0 (0%)	8 (88.9%)	1 (11.1%)	
	>2	1 (5.6%)	1 (5.6%)	18 (100%)	2 (11.1%)	
	Total	6 (11.8%)	1 (2%)	50 (98%)	7 (13.7%)	
noitazilatipsoh fo noitaruD (yad)	≤1	2 (8.3%)	0 (0%)	24 (100%)	3 (12.5%)	N.S*
	2-3	1 (7.1%)	1 (7.1%)	14 (100%)	4 (28.6%)	
	4-6	3 (23.1%)	0 (0%)	12 (92.3%)	0 (0%)	
	Total	6 (11.8%)	1 (2%)	50 (98%)	7(13.7%)	

*Not significant.

MATERIALS AND METHODS

From November 2009 to May 2010, a total of 313 less than five years old children with diarrhea hospitalized in Kashan Shahid Beheshti Hospital were studied. Stool samples collected in Cary-Blair transport medium (Merck, Germany) were cultured on the surface of MacConkey agar (Merck, Germany) for the selection of *E. coli* isolates. Verification of *E. coli* was carried out by using standard biochemical methods (Bueris et al., 2007). EPEC identification was based on PCR amplification of the eae and bfpA genes. Detection of virulence genes including efa-1, fimH and lpfDo113 was carried out by using PCR. A boiling method was used for DNA extraction (Aranda et al., 2004). DNA was extracted from bacteria by re-suspending one bacterial colony in 100 µl of deionized water, boiling the suspension for 10 min and centrifuging it at 14,000 × g for 5 min. The supernatant was then used as the DNA template for PCR. PCR for each of the genes was performed separately in a 25 µl reaction mixture containing 2.5 µl 10X PCR buffer, 1 mM MgCl2, 1 mM concentration of each deoxynucleoside triphosphate, 0.5 U of Taq DNA polymerase (Fermentas), 5 µl of the DNA template and 1 µl of primers 1 or 2 containing 10 ng primer/µl (Table 1). Amplified products were analysed by using 1.5% agarose gel electrophoresis and visualized by staining with ethidium bromide. A 100 bp DNA ladder (Fermentas, Germany) was used as a molecular size marker in all gels. The positive PCR control was EPEC strain E2348/69 (eae+ bfpA+).

The primer sequences and cycling conditions used in the PCR analysis are listed in Table 1. All data were entered into SPSS software and comparative statistics were evaluated by chi-square test and Fisher's exact test. P≤0.05 was considered to be significant.

RESULTS

The diarrhea surveillance study for the presence of virulence genes was conducted from November 2009 to May 2010. The mean ± SD age of the children studied was 16.6 ± 12.4 months. 178 *E. coli* was isolated from 313 stool specimens (57.8%), then 51 (28.6%) EPEC pathotypes were isolated from 178 *E. coli* cases. Frequency of virulence factors in enteropathogenic *E. coli* isolated from hospitalized children based on sex, age (years) and hospitalization (day) is shown in Table 2. In children with diarrhea, 21 were male and 30 were female (Table 2). The frequency of EPEC was higher in children under 1 year old. The results of PCR on 51 EPEC showed that 6 cases (11 .8%) of these samples with bfpA gene were considered as typical EPEC and 45 specimens (88.2%) without bfpA gene were considered as atypical EPEC. The results of this study showed that the frequency of genes fimH, lpfDo113, and efa-1 was 98, 13.7 and 2%, respectively. Pictures are shown in Figures 1 to 5. Among the studied genes, fimH was observed in 50 EPEC pathotypes. The results of the presence of virulence genes based on age showed that children less than one year old had the highest frequency of genes fimH, lpfDo113 and bfpA, respectively. Fisher's

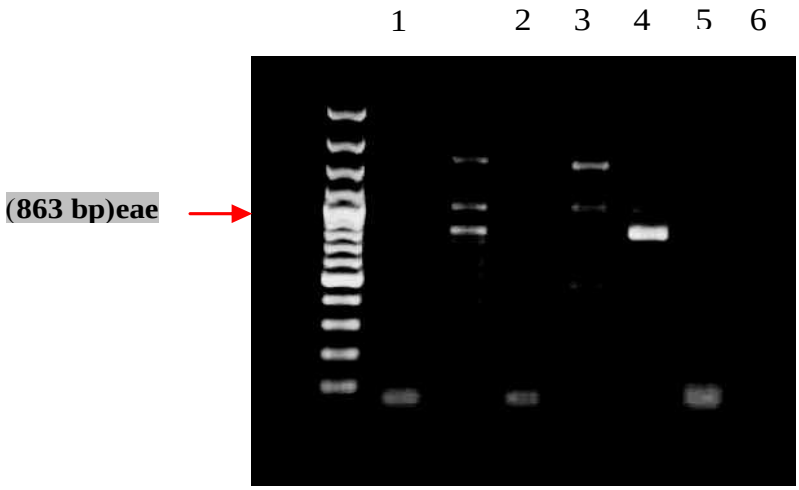

Figure 1. Agarose gel electrophoresis of products from PCR associated gene eae. *E. coli* in some diarrhea samples. Lane 1, DNA molecular size markers (100-bp ladder) (Fermentas,Germany); lane 2, patient sample (eae); lane 3, patient sample (without eae gene); lane 4, patient sample (without eae gene); lane 5, EPEC E2348/69 (positive control) and lane 6, patient sample (without eae gene).

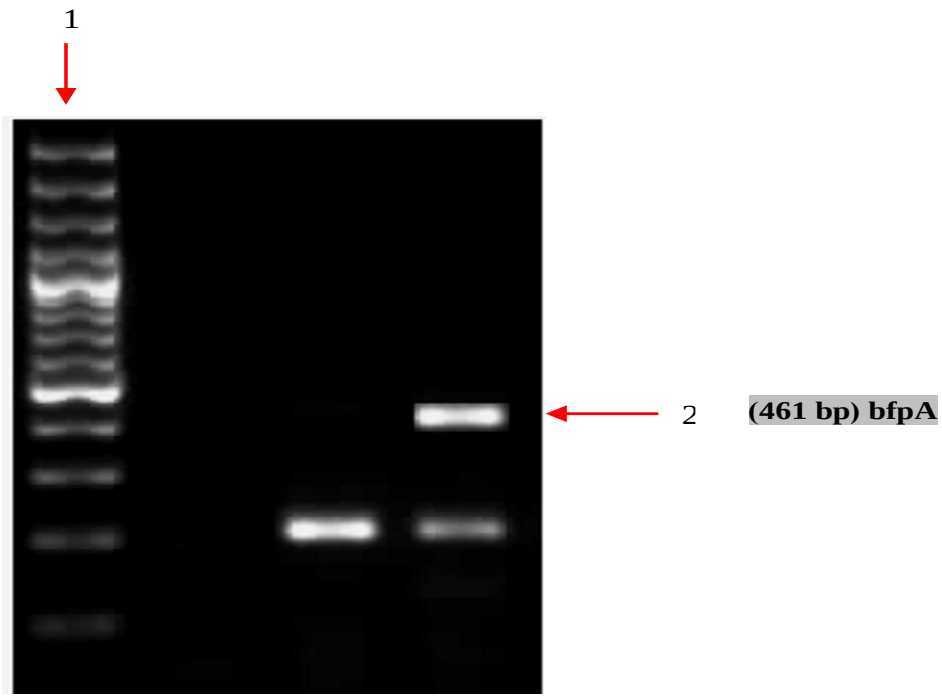

Figure 2. Agarose gel electrophoresis of products from PCR associated gene bfpA *E. coli* in some diarrhea samples. Lane 1, DNA molecular size markers (100-bp ladder) (Fermentas,Germany) and lane 2, patient sample (bfpA).

exact test showed that there is no statistically significant difference in male and female and also in the all age groups with the virulence genes (P>0.05).

DISCUSSION

According to findings, 51 (28.6%) of 178 *E. coli*

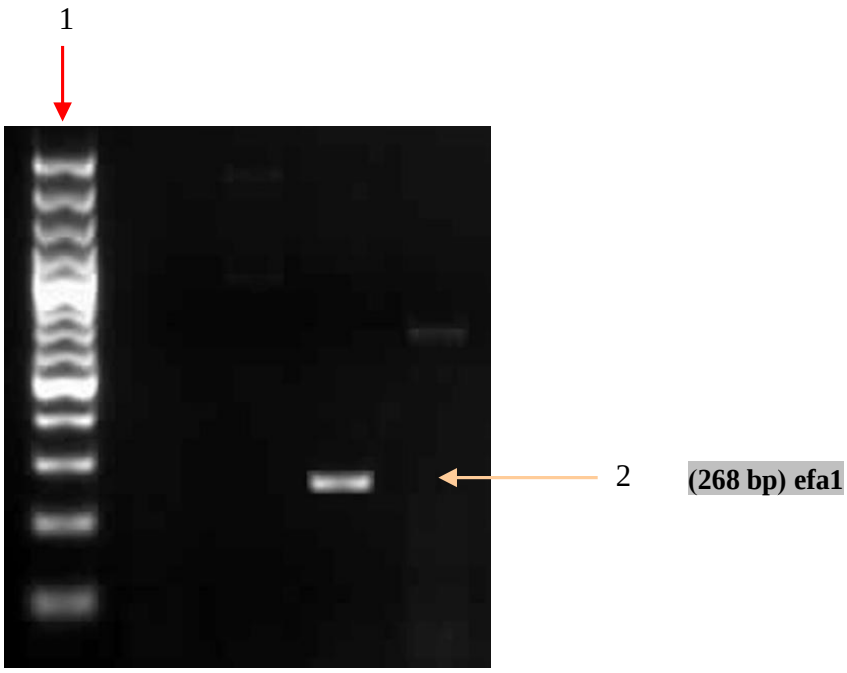

Figure 3. Agarose gel electrophoresis of products from PCR associated gene efa1 *E. coli* in some diarrhea samples. Lane 1, DNA molecular size markers (100-bp ladder) (Fermentas, Germany) and lane 2, patient sample (efa1).

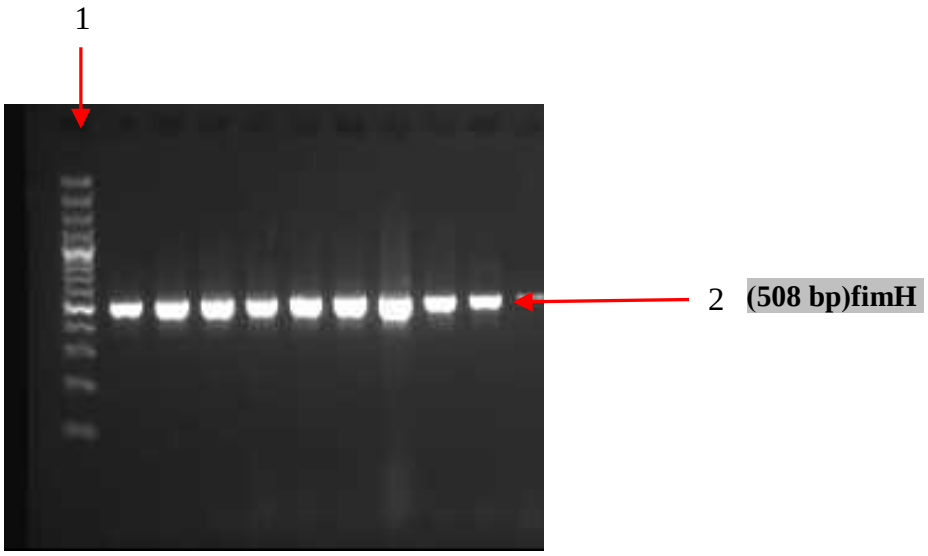

Figure 4. Agarose gel electrophoresis of products from PCR associated gene fimH *E. coli* in some diarrhea samples. Lane 1, DNA molecular size markers (100-bp ladder) (Fermentas,Germany) and lane 2, patient sample (fimH).

were positive for EPEC pathotype. The frequency of EPEC was higher in children less than 1 year old. The results showed that EPEC was one of the important causes of diarrhea in children less than five years old referred to shahid Beheshti Hospital in kashan. Cravioto and et al have reported that the rate of diarrhea caused by EPEC in children younger than one year was 51.3% (Cravioto et al., 1996). Different studies on the prevalence of diarrheal infections in children have indicated that this prevalence was a wide range. These

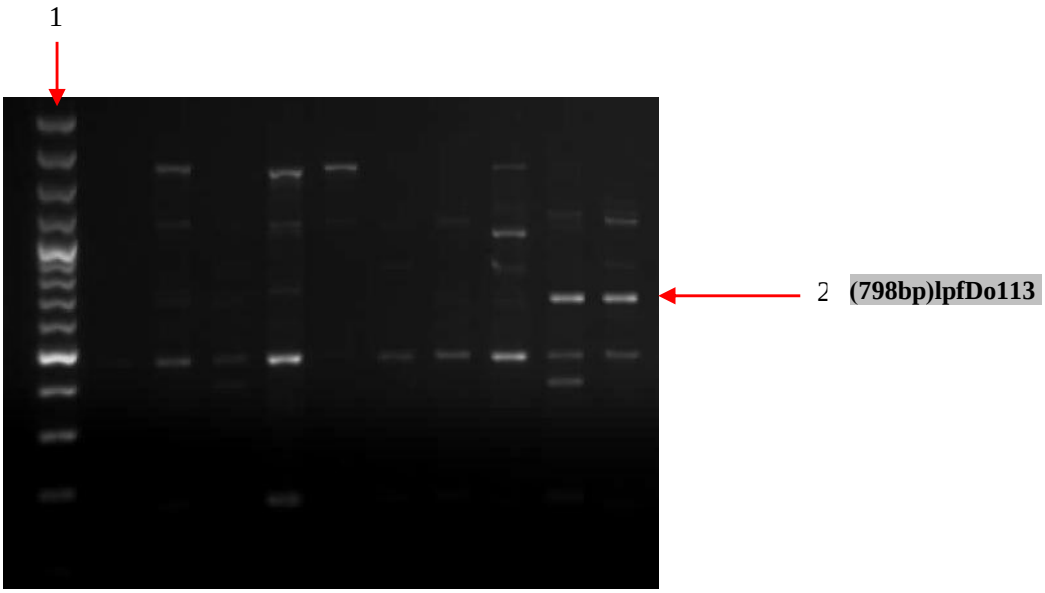

Figure 5. Agarose gel electrophoresis of products from PCR associated gene lpfDo113 *E. coli* in some diarrhea samples. Lane 1, DNA molecular size markers (100-bp ladder) (Fermentas, Germany) and lane 2, patient sample (lpfDo113).

values in Southeast Asia studies were of different from 2.7% in Singapore to 12.6% in Thailand (Sunthadvanich et al., 1990; Lim et al., 1992). Three studies in Brazil have reported that the prevalence of EPEC isolates were from 10.1 to 32.7% (Rosa et al., 1998; Franzolin et al., 2005). The results showed that the rates of diarrhea caused by EPEC in female and male were 30 (58.8%) and 21 (41.2%), respectively. Similarly, in some studies the high prevalence of EPEC was reported in female children along with diarrhea (Afset et al., 2004). Thus, all studies indicate the role of EPEC as a main factor in the creation of diarrheal infections, especially in children less than five years old, but it seems that the prevalence is highly variable. These results need further studies for creating epidemiological map of diarrheal infections caused by EPEC. The results of this study showed that 45 cases (88.2%) out of 51 EPEC pathotype were atypical and 6 cases (11.8%) were typical EPEC. The study was conducted by Contreras et al. (2010), the frequency of atypical and typical EPEC isolates was reported as 73 and 27%, respectively. In a study conducted by Vanessa et al. (2007), the frequency of atypical and typical EPEC was reported as 99.1 and 0.8%, respectively. The comparison of these results with the findings of our study revealed that the frequency of atypical EPEC strains is higher than typical strains. And similarly, many previous studies show that the prevalence of atypical EPEC is higher than typical EPEC in infants with diarrhea. Until the 1990s, typical EPEC strains was more than atypical EPEC strains, while recent studies have indicated that the prevalence of typical EPEC strains decreased and they are gradually replaced by atypical EPEC (Trabulsi et al., 2002).

Vieira et al. (2001) analyzed the virulence potential of atypical EPEC and concluded that a lack of expression of the LEE genes was related to virulence. Results about the frequency of virulence genes were efa1 (2%), lpfDo113 (13.7%) and fimH (98%). An examination of the distribution of lpfDo113 among other pathogenic *E. coli* strains and enteric pathogens revealed that lpfO113 is closely associated with EHEC strains. It seems that lpfDo113 gene is effective in EPEC attaching to epithelial cells and cause diarrhea. However, in addition to lpfDo113, other genes involved in EPEC are attaching to epithelial cell. Due to the high percentage of the fimH in isolated EPEC, fimH is an ideal candidate protein for vaccine development to prevent devastating squeal of enteric infections in Kashan region.

Conclusion

This study has showed that the presence of these virulence factors vary according to geographic region. Also EPEC attaching mechanisms to epithelial cell are different.

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