Detection of typical and atypical enteropathogenic *Escherichia coli* (EPEC) in Iranian children with and without diarrhoea

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The present study was performed to investigate the contribution of typical and atypical enteropathogenic *Escherichia coli* (EPEC) as a cause of infectious diarrhoea among children less than 10 years old in Iran. During the summer months, 247 specimens from children with diarrhoea and 1108 from asymptomatic children were analysed for the presence of EPEC and other bacterial pathogens. Potential enteric pathogens were identified in 140 cases of children with diarrhoea (56.7%). EPEC was the most frequently identified agent (111 cases), followed by Shiga toxin-producing *E. coli* (13), *Shigella* (9), *Salmonella* (6) and *Aeromonas* sp. (1). EPEC isolates were examined for the presence of *eaeA*, *bfpA* and *stx* genes by PCR. EPEC isolates were classified as typical (*eaeA*⁺ *bfpA*⁺) or atypical (*eaeA*⁺ *bfpA*⁻). Typical EPEC was diagnosed in 35 cases (11.8%), compared with 8 (0.4%) in the asymptomatic group ($P < 0.05$). Atypical EPEC strains were isolated from 23 cases (9.3%), compared with 13 (1.2%) of the healthy control group ($P < 0.05$). In conclusion, the data suggest that typical and atypical EPEC are an important cause of diarrhoea in Iranian children.

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INTRODUCTION

Infectious diarrhoea is one of the world's leading causes of morbidity and mortality, resulting in about two million deaths per year (<http://www.who.int>). The majority of cases of serious diarrhoea occur among children in developing countries (World Health Organization, 1995). Enteropathogenic *Escherichia coli* (EPEC) is a major cause of infantile diarrhoea among children in developing countries (Levine, 1987; Donnenberg & Kaper, 1992). The central mechanism of EPEC pathogenesis is a lesion called 'attaching and effacing' (A/E), which is characterized by intimate adherence of bacteria to the intestinal epithelium (Vallance & Finlay, 2000). The *eaeA* gene, which is located in the 'locus of enterocyte effacement' pathogenicity island, and the *bfpA* gene, located on a plasmid called the EPEC adherence factor (EAF), have both been used for identification of EPEC and for subdivision of this group of bacteria into typical and atypical strains (Nataro & Kaper, 1998). *E. coli* strains with the A/E genotype (*eaeA*⁺) that harbour the EAF plasmid (*bfpA*⁺) are classified as 'typical EPEC' and strains with the A/E genotype that do not possess the EAF

plasmid (*bfpA*⁻) are classified as 'atypical EPEC' (Kaper, 1996). *E. coli* strains with and without the *eaeA* genotype that harbour Shiga toxin genes (*stx*₁ and/or *stx*₂) are classified as Shiga toxin-producing *E. coli* (STEC) (Nataro & Kaper, 1998). Typical EPEC is well recognized as a cause of gastroenteritis in infants (Nataro & Kaper, 1998), whereas the role of atypical EPEC in childhood diarrhoea is still controversial. Most case-control studies have not been able to demonstrate any significant association between atypical EPEC and diarrhoea (Knutton *et al.*, 2001; Scaletsky *et al.*, 2002; Nunes *et al.*, 2003; Afset *et al.*, 2003, 2004; Regua-Mangia *et al.*, 2004). However, the majority of these studies have shown a higher prevalence of atypical EPEC among the group of subjects with diarrhoea than among the healthy controls, and it has been argued that atypical EPEC are probably pathogenic, as such strains are capable of colonizing the intestinal mucosa and produce A/E lesions (Knutton *et al.*, 2001). There have also been reports of atypical EPEC being significantly associated with endemic diarrhoea (Vieira *et al.*, 2001; Dulguer *et al.*, 2003) or outbreaks (Yatsuyanagi *et al.*, 2002; Jenkins *et al.*, 2003). The aim of the present study was to investigate the relative contribution of typical and atypical EPEC among a randomly selected group of Iranian children with and without diarrhoea.

Abbreviations: A/E, attaching and effacing; EAF, EPEC adherence factor; EPEC, enteropathogenic *Escherichia coli*; STEC, Shiga toxin-producing *E. coli*.

METHODS

Subjects and strains. The strains examined in this study were isolated during the summer months in three Iranian provinces, Tehran, Ilam and Mazandaran. Random sampling was employed in two stages. In the first stage, the total population of the provinces was divided into 400–500 blocks, of which 50 were selected randomly. In the second stage, 8–15 families in each block were selected and a total of 1355 children under 10 years of age were chosen randomly for investigation for the presence of EPEC or other bacterial pathogens in their faeces.

Culture and identification methods. Samples were cultured for enteric pathogens by using standard methods (Murray *et al.*, 2002). In brief, stool samples were cultured on Salmonella–Shigella agar and in Selenite F broth for *Salmonella* and *Shigella* spp., on two MacConkey agar plates for *Yersinia* spp. and on one Thiosulfate Citrate Bile Sucrose agar plate for *Vibrio* and *Aeromonas* spp. All plates were incubated at 37 °C for 24–48 h, except one MacConkey plate that was incubated at 25 °C. Presumptive colony identification was carried out according to Edwards and Ewing (Ewing, 1986). For both children with diarrhoea and healthy children, five colonies from the MacConkey plate were characterized for the detection of *E. coli*. Identification of *E. coli* was carried out by using standard biochemical methods (Forbes *et al.*, 2002).

EPEC serogrouping. Strains biochemically identified as *E. coli* were selected and both lactose-positive and lactose-negative *E. coli* strains were serogrouped by using slide agglutination tests into the different EPEC serogroups (O26, O55, O86, O111, O114, O119, O124, O125, O126, O127, O128, O142) using O-specific antisera, according to the manufacturer's instructions (Bio-Rad). Strains that agglutinated with 4 % saline were defined as rough.

Detection of virulence genes by PCR. EPEC isolates were examined by using PCR with the primers listed in Table 1 for the following virulence genes: *eaeA* (Beaudry *et al.*, 1996), *bfpA* (Gunzburg *et al.*, 1995) and *stx* (Lin *et al.*, 1993). PCR was performed in a 25 µl reaction mixture containing 100 µM (each) dNTP, 1.5 mM MgCl₂, 0.5 µM (each) primer, 2.5 µl 10-fold-concentrated polymerase reaction buffer (Fermentas), 1 U *Taq* polymerase (Fermentas) and 2 µl template DNA. Primers and cycling conditions are listed in Table 1. Amplification reactions were performed in a thermal cycler (Eppendorf Mastercycler) and, for all amplification reactions, the mixture was heated at 94 °C for 5 min prior to thermocycling. The mixture was held at 72 °C for 7 min after the final cycle before cooling at 4 °C. Amplified products were analysed by using 2 % agarose gel electrophoresis and visualized by staining with ethidium bromide. A 100 bp DNA ladder (Fermentas) was used as a molecular

size marker in all gels. Positive controls for PCR were *E. coli* strains 2348/69 (*eaeA*⁺ *bfpA*⁺) and EDL933 (*stx*⁺). *E. coli* HB101 was used as a negative control.

Statistical analysis. χ^2 test and Fisher's exact test were used for statistical analysis. Means $\pm$ SD are given.

RESULTS AND DISCUSSION

Subjects

The 1355 stool specimens studied for the presence of EPEC were obtained from 247 children with diarrhoea (0–10 years old; mean age, 5.5 years) and 1108 children without any gastrointestinal symptoms (0–10 years old; mean age, 6.8 years).

Potential enteropathogens other than EPEC

A potential or established microbial pathogen was detected in 140 (56.7 %) and 92 (8.3 %) children with diarrhoea and asymptomatic controls, respectively. No mixed infections were observed. *Shigella* spp. were isolated from ten children, nine with diarrhoea and one without. Six *Salmonella* spp. were detected, all from diarrhoeal cases. Of the four *Aeromonas* spp. isolated, one was from a diarrhoeal case and the others were from asymptomatic children. STEC were isolated from 21 children, 13 with diarrhoea and 8 without. *Vibrio cholerae* and *Yersinia* spp. were not isolated in this study. No bacterial pathogens were isolated from the remaining 107 children with diarrhoea.

Typical and atypical EPEC

EPEC serogroups were isolated as the sole pathogen from 44.9 % (111 of 247) of children with diarrhoea, compared with 7.2 % (80 of 1108) of those without diarrhoea ($P < 0.05$). EPEC strains isolated in this study belonged to 11 EPEC serogroups (O26, O55, O86, O111, O114, O119, O125, O126, O127, O128 and O142). In children with diarrhoea, O111 serogroup EPEC (8.9 %) was the most prevalent, followed by O142 (8.5 %) and O127 (8 %). This is consistent with the findings of previous studies from

Table 1. Primer sequences, annealing temperatures and size of amplified products from selected genes of EPEC and STEC

bfpA, Bundle-forming pilus gene; *eaeA*, attaching and effacing-associated gene; STEC, Shiga toxin-producing *E. coli*; *stx*, Shiga toxin gene.

Category	Target	Sequence (5'–3')	Annealing temp. (°C)	No. of cycles	Fragment size (bp)	Reference
EPEC	<i>eaeA</i>	F, CATTATGGAACGGCAGAGGT R, ATCTTCTGCGTACTGCGTTCA	55 (1 min)	35	790	Beaudry <i>et al.</i> (1996)
EPEC	<i>bfpA</i>	F, AATGGTGCTTGCGCTTGCTGC R, GCCGCTTTATCCAACCTGGTA	60 (1 min)	30	326	Gunzburg <i>et al.</i> (1995)
STEC	<i>stx</i>	F, GAACGAAATAATTTATATGT R, TTTGATTGTTACAGTCAT	43 (1.30 min)	39	900	Lin <i>et al.</i> (1993)

Bangladesh (Albert *et al.*, 1995), Saudi Arabia (El-Sheikh & El-Assouli, 2001) and Uruguay (Torres *et al.*, 2001). In children with diarrhoea, eight (3.2 %) cases were colonized with two different 'O' serogroups of EPEC, whereas no healthy children were colonized with more than one 'O' serogroup of EPEC. In accordance with many studies in developing countries (Albert *et al.*, 1995), a strong association of EPEC with diarrhoea was found. In other studies, children colonized with EPEC were rarely infected with other enteric pathogens (Echeverria *et al.*, 1991; Gomes *et al.*, 1991). A similar trend was found in the present study for the children infected with the EPEC serogroups. Surprisingly, EPEC was the most frequently identified potential pathogen in this study, identified in 44.9 % of patients. This is a high prevalence compared with most other reports (Tompkins *et al.*, 1999; Svenungsson *et al.*, 2000; Keskimäki *et al.*, 2001; Knutton *et al.*, 2001; Vieira *et al.*, 2001).

PCR was positive for the *eaeA* gene in 45 of 111 (40.5 %) strains isolated from patients, compared with 16 of 80 (20 %) from children without diarrhoea (Table 2). The presence of the *eaeA* gene was also significantly more frequent (46 %) among strains belonging to serogroups O55 (9 of 14), O86 (12 of 23), O127 (11 of 33) and O114 (1 of 3) than among strains belonging to other serogroups. Interestingly, these serogroups are included among those that were isolated frequently from diarrhoeal cases. Case-control studies have found *eaeA*⁺ *E. coli* strains more frequently in children with diarrhoea than in healthy controls in developing countries (Nataro & Kaper, 1998; Rosa *et al.*, 1998). Only 35 of 111 (31.5 %) strains isolated from children with diarrhoea and 8 of 80 (10 %) strains isolated from healthy controls gave positive results for the *bfpA* gene (Table 2). In total, 45 of 247 (18 %) strains isolated from children with diarrhoea were negative for the two virulence markers (*eaeA* and *bfpA*) (non-EPEC). In this study, 29 of 111 (26.1 %) and 5 of 80 (6.2 %)

Table 2. Genotypic characterization of typical, atypical and non-EPEC serogroups

Serogroup	No. of strains	Clinical sign		PCR result	Virulence category
		Diarrhoea (<i>n</i> =247)	Asymptomatic (<i>n</i> =1108)		
O111	5	0	5	<i>eaeA</i> ⁺ <i>bfpA</i> ⁺ <i>stx</i> ⁻	Typical EPEC
O111	12	5	7	<i>eaeA</i> ⁺ <i>bfpA</i> ⁻ <i>stx</i> ⁻	Atypical EPEC
O111	1	0	1	<i>eaeA</i> ⁻ <i>bfpA</i> ⁺ <i>stx</i> ⁻	Atypical EPEC
O111	2	1	1	<i>eaeA</i> ⁻ <i>bfpA</i> ⁻ <i>stx</i> ⁺	STEC
O111	34	16	18	<i>eaeA</i> ⁻ <i>bfpA</i> ⁻ <i>stx</i> ⁻	Non-EPEC
O127	4	4	0	<i>eaeA</i> ⁺ <i>bfpA</i> ⁺ <i>stx</i> ⁻	Typical EPEC
O127	7	5	2	<i>eaeA</i> ⁺ <i>bfpA</i> ⁻ <i>stx</i> ⁻	Atypical EPEC
O127	1	1	0	<i>eaeA</i> ⁻ <i>bfpA</i> ⁺ <i>stx</i> ⁻	Atypical EPEC
O127	1	1	0	<i>eaeA</i> ⁻ <i>bfpA</i> ⁻ <i>stx</i> ⁺	STEC
O127	20	9	11	<i>eaeA</i> ⁻ <i>bfpA</i> ⁻ <i>stx</i> ⁻	Non-EPEC
O142	4	4	0	<i>eaeA</i> ⁺ <i>bfpA</i> ⁺ <i>stx</i> ⁻	Typical EPEC
O142	5	4	1	<i>eaeA</i> ⁺ <i>bfpA</i> ⁻ <i>stx</i> ⁻	Atypical EPEC
O142	6	5	1	<i>eaeA</i> ⁻ <i>bfpA</i> ⁺ <i>stx</i> ⁻	Atypical EPEC
O142	16	7	9	<i>eaeA</i> ⁻ <i>bfpA</i> ⁻ <i>stx</i> ⁻	Non-EPEC
O86	10	10	0	<i>eaeA</i> ⁺ <i>bfpA</i> ⁺ <i>stx</i> ⁻	Typical EPEC
O86	2	2	0	<i>eaeA</i> ⁺ <i>bfpA</i> ⁻ <i>stx</i> ⁻	Atypical EPEC
O86	1	1	0	<i>eaeA</i> ⁻ <i>bfpA</i> ⁺ <i>stx</i> ⁻	Atypical EPEC
O86	10	4	6	<i>eaeA</i> ⁻ <i>bfpA</i> ⁻ <i>stx</i> ⁻	Non-EPEC
O26	1	1	0	<i>eaeA</i> ⁺ <i>bfpA</i> ⁺ <i>stx</i> ⁻	Typical EPEC
O26	1	1	0	<i>eaeA</i> ⁻ <i>bfpA</i> ⁻ <i>stx</i> ⁺	STEC
O26	4	2	2	<i>eaeA</i> ⁻ <i>bfpA</i> ⁻ <i>stx</i> ⁻	Non-EPEC
O55	9	9	0	<i>eaeA</i> ⁺ <i>bfpA</i> ⁺ <i>stx</i> ⁻	Typical EPEC
O55	5	1	4	<i>eaeA</i> ⁻ <i>bfpA</i> ⁺ <i>stx</i> ⁻	Non-EPEC
O119	1	1	0	<i>eaeA</i> ⁺ <i>bfpA</i> ⁺ <i>stx</i> ⁻	Typical EPEC
O119	7	3	4	<i>eaeA</i> ⁻ <i>bfpA</i> ⁻ <i>stx</i> ⁻	Non-EPEC
O126	8	5	3	<i>eaeA</i> ⁻ <i>bfpA</i> ⁻ <i>stx</i> ⁺	STEC
O126	2	2	0	<i>eaeA</i> ⁻ <i>bfpA</i> ⁻ <i>stx</i> ⁻	Non-EPEC
O114	1	0	1	<i>eaeA</i> ⁺ <i>bfpA</i> ⁻ <i>stx</i> ⁻	Atypical EPEC
O114	2	1	1	<i>eaeA</i> ⁻ <i>bfpA</i> ⁻ <i>stx</i> ⁻	Non-EPEC
O128	6	3	3	<i>eaeA</i> ⁻ <i>bfpA</i> ⁻ <i>stx</i> ⁺	STEC
O125	3	2	1	<i>eaeA</i> ⁻ <i>bfpA</i> ⁻ <i>stx</i> ⁺	STEC
Total	191	111	80		

EPEC strains isolated from cases and healthy controls, respectively, were typical EPEC (*eaeA*⁺ *bfpA*⁺). Typical EPEC was much more prevalent in cases with diarrhoea (11.8%) than in healthy children (0.4%) ($P < 0.05$). This shows that the typical EPEC strains continue to be an important cause of diarrhoea in children. These strains were isolated from patients with acute or persistent diarrhoea (Scaletsky *et al.*, 1999). In this report, typical EPEC strains were mostly encountered among the strains belonging to serogroups O55 and O86 (Table 2), which differs from the findings of a previous study in Iran (Bouzari *et al.*, 2000).

In 23 of 111 (20.7%) and 13 of 80 (16.2%) EPEC strains isolated from cases and healthy controls, respectively, only one of these determinants (*eaeA*, *bfpA*) was found (atypical EPEC). Most atypical EPEC isolates (72.2%) constituted the *eaeA*-positive isolates. This finding is in accordance with recent reports from the UK (Knutton *et al.*, 2001) and Brazil (Dulguer *et al.*, 2003). Atypical EPEC strains were mostly encountered among strains belonging to serogroups O114 and O142 (Table 2), which is different from another report (Gonçalves *et al.*, 1997). Atypical EPEC strains were isolated from cases with diarrhoea (9.3%) and healthy controls (1.2%) ($P < 0.05$). Atypical EPEC has been implicated as the causative agent in some outbreaks (Scotland *et al.*, 1993; Viljanen *et al.*, 1990), whereas in other studies atypical EPEC has not been recovered more frequently from diarrhoeal cases than from controls (Echeverria *et al.*, 1991). In some studies, the rate of isolation of atypical EPEC from children without diarrhoea was twice that of cases with diarrhoea (Gomes *et al.*, 2002; Dulguer *et al.*, 2003). There have also been reports of atypical EPEC associated with both endemic diarrhoea (Bokete *et al.*, 1997; Vieira *et al.*, 2001) and outbreaks (Hedberg *et al.*, 1997; Yatsuyanagi *et al.*, 2002). Nevertheless, the role of atypical EPEC in diarrhoea has not been established conclusively. Although in our study the association of atypical EPEC with diarrhoea was statistically significant, to elucidate the role of atypical EPEC in diarrhoeal disease among Iranian children, more epidemiological studies and further investigation of the virulence properties of these strains are warranted.

Conclusions

We conclude that EPEC strains that possess the *eaeA* gene are a common cause of diarrhoea in Iranian children. Typical EPEC was more prevalent in children with diarrhoea (11.8%) than in healthy cases (0.4%). Atypical EPEC is prevalent among children with diarrhoea in Iran and was diagnosed in 9.3% of patients in this study. Detection of the *eaeA* and *bfpA* genes is a useful method for the identification of and differentiation between typical and atypical EPEC strains.

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