Molecular analysis of typical and atypical enteropathogenic *Escherichia coli* (EPEC) isolated from children with diarrhoea

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Diarrhoea continues to be one of the most common causes of morbidity and mortality among infants and children in developing countries. To investigate the incidence, antimicrobial resistance and genetic relationships of enteropathogenic *Escherichia coli* (EPEC) in children with diarrhoea, a total of 612 stool specimens were collected in Tehran, Iran, and cultured to isolate strains of EPEC. The disc diffusion method was used to determine the susceptibility of the isolates according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. The presence of *eae*, *stx* and *bfp-A* genes was determined by PCR. The genetic relationships between EPEC isolates were determined by pulsed-field gel electrophoresis (PFGE). Out of the 412 strains of *E. coli* obtained from 612 diarrhoeal stool specimens, 23 (5.6 %) were identified as EPEC, of which seven (30.4 %) were classified as typical strains of EPEC and 16 (69.6 %) were classified as atypical. Out of the 23 EPEC isolates, 69.5 % were resistant to ampicillin, 39.1 % were resistant to tetracycline and cotrimoxazole, 30.4 % were resistant to cefpodoxime, ceftazidime, ceftriaxone and aztreonam, and 26.1 % were resistant to imipenem. The isolates were classified into 21 pulsotypes by PFGE profiles. The present study shows that typical and atypical EPEC isolates displayed considerable heterogeneity in PFGE profiles and EPEC infections were only sporadic in Tehran. Overall 69 % of isolates were resistant to at least one of the antibiotics tested.

INTRODUCTION

Enteropathogenic *Escherichia coli* (EPEC) is one of the major causes of diarrhoea among children in developing countries (Hernandes et al., 2009; Moura et al., 2009). The pathogenesis of EPEC depends on the locus of enterocyte effacement (LEE), a chromosomal pathogenicity island. The LEE contains a number of different genes, including *eae*, which has an essential role in inducing a characteristic lesion formation in the intestinal epithelium, termed an attaching and effacing (A/E) lesion (Elliott et al., 1998; Vallance & Finlay 2000; Kaper et al., 2004). The *eae* gene encodes intimin, a 94 kDa outer-membrane protein that is responsible for the intimate adherence between bacterial and enterocyte membranes (Vallance & Finlay, 2000; Trabulsi et al., 2002). EPEC strains are classified as typical or atypical, according to the presence or absence of the *E. coli* adherence factor plasmid (EAF) that carries the *bfpA* gene, which encodes the bundle-forming pilin (Hernandes et al., 2009). The most typical EPEC strains belong to the classic O : H serotypes and are *eae-* and *bfpA*-positive (Ochoa et al., 2008). The atypical EPEC isolates are *eae*-positive and *bfpA-* and *stx* (the gene encoding shiga-like toxin)-negative. EPEC are among the most important pathogens infecting children under 2 years of age in the developing world (Ochoa et al., 2008). Recent studies indicate that atypical EPEC is more prevalent than typical...
EPEC in both developed and developing countries and that atypical EPEC is important in both paediatric endemic diarrhoea and diarrhoea outbreaks (Ochoa & Contreras, 2011).

The aim of this study was to investigate the presence of typical and atypical EPEC strains isolated from children with diarrhoea in Tehran. In addition, pulsed-field gel electrophoresis (PFGE) was used to further compare the strains at a molecular level.

METHODS

Specimens and bacterial isolates. Fresh stool specimens were collected during 2009 and 2010 from children <10 years of age with diarrhoea immediately after admission to the children’s hospital, a referral tertiary care centre of the Tehran University of Medical Sciences. For E. coli isolation, all stool specimens were cultured on MacConkey agar and incubated at 37°C for 24 h. The identification process was performed based on the standard biochemical tests. Oxidase-negative Gram-negative bacilli were identified as E. coli based upon the following test reactions; acid on acid reaction with gas production and no hydrogen sulfide production on Kligler iron agar, catalase-positive, indole-positive, citrate-negative, methyl red positive, urease-negative and Voges–Proskauer-negative (Mahon et al., 2007). Strains biochemically identified as E. coli were differentiated as typical or atypical EPEC strains by a PCR-based method. The eae, stx and bfp-A genes were amplified using the primers eae-F (5′-CTGAAACCCGGTTACGGCA-3′) and eae-R (5′-CCAGACGGATACCTACG-3′), stx-F (5′-GAGCCGAAATTATTTATATGTG-3′) and stx-R (5′-TGATGATGGCAATTCAGTAT-3′), and bfpA-F (5′-AATG-CTG-GGCGGGCGGTATCCCTG-3′) and bfpA-R (5′-GCCGTTTATCATCAACCTTGTA-3′) (Toma et al., 2003; Aranda et al., 2007). The current study was approved by the Ethics Committee of the Tehran University of Medical Sciences.

Susceptibility testing. Antimicrobial susceptibility testing was performed by using the disc agar diffusion method, according to Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2011). These tests were performed twice at separate times. The antibiotics tested were as follows (μg per disc): ampicillin (10), aztreonam (30), gentamicin (10), ciprofloxacin (2), cotrimoxazole (25), oxolinic acid (5), tetracycline (30), cefpodoxime (10), ceftazidime (30), cefotaxime (30), tobramycin (10) and imipenem (10). The antimicrobial discs were purchased from the Mast Group. E. coli ATCC 25922 was used as the control strain and all the susceptibility test results were assessed after 20 h of incubation at 35°C.

Pulsed-field gel electrophoresis. Entire genomic DNA was prepared as described by Aligholi et al. (2011). After digestion with XbaI endonuclease, DNA was separated by using a CHEF electrophoresis system (AP-Zoha) for 24 h at 14°C with an electric field of 6 V cm−1 in 0.5× TBE buffer. The pulse time was increased from 1 to 30 s (16 h) and 1 to 3 s (8 h). The gels were stained with ethidium bromide (1 μg ml−1) and visualized by UV illumination. Assessment and interpretation of PFGE patterns were performed according to the criteria of Tenover et al. (1995) using TotalLab software (BioSystematica).

Statistical analysis. The correlation between patient gender, age and antimicrobial resistance pattern and the distributions of typical and atypical EPEC isolates was determined with a 95% confidence interval and compared by using Fisher’s exact test.

RESULTS

Out of the 612 stool specimens from children with diarrhoea, 412 were positive for E. coli, using biochemical methods. Among the 412 E. coli isolates, 23 (5.6%) were identified as EPEC. All the EPEC strains tested positive for the eae gene and none was positive for the stx gene by PCR. The presence of the bfpA gene was detected in only seven (30.4%) strains classified as typical EPEC (eae- and bfpA-positive). The remaining 16 (69.6%) strains (eae-positive and bfpA-negative) were classified as atypical EPEC strains.

Antibiotic susceptibility testing data are shown in Table 1. Obvious zones of growth inhibition of the 23 EPEC isolates using the antibiotics tested were used as criteria for the identification of resistant or susceptible categories; no intermediate category was found. Of the 23 EPEC isolates, 69.5% were resistant to ampicillin, 39.1% were resistant to tetracycline and cotrimoxazole, 30.4% were resistant to cefpodoxime, ceftazidime, ceftriaxone and aztreonam, 26.1% were resistant to imipenem, 8.6% were resistant to gentamicin and tobramycin, and 4.3% were resistant to ciprofloxacin and ofloxacin. Detailed analysis showed that 16 (69%) EPEC isolates were resistant to at least one of the antibiotics tested.

Macro-restriction and PFGE analysis of the 23 EPEC isolates showed 21 unique pulsed types (Fig. 1). These pulsed types were grouped into 21 PFGE types. Based on differences in the number of restriction fragments, the isolates showed seven or more band differences.

There was no statistically significant correlation regarding the frequency of typical or atypical EPEC isolates in relation to patient age, gender or antimicrobial resistance pattern.

DISCUSSION

In the current study, the prevalence rate of EPEC in the E. coli isolates was 5.6%. This is higher than prevalence rates mentioned in similar reports from Thailand (3.2%) and Tanzania (4.6%) and lower than those mentioned in reports from Vietnam (6.6%) and Kuwait (8.4%) (Ratchtrachenchai et al., 2004; Nguyen et al., 2005; Moyo et al., 2007; Albert et al., 2009). However, EPEC rates vary greatly among different countries (Robins-Browne et al., 2004; Al-Gallas et al., 2007), which may reflect geographical specificities and other factors.

EPEC strains are classified into two types: the typical EPEC isolates, which are positive for both the eae gene and the bfp gene and mostly belong to the classical EPEC serotypes, and atypical EPEC isolates, which are only positive for the eae gene and belong to non-classical serotypes (Trabulsi et al., 2002; Albert et al., 2009). In the current study, among the 23 EPEC isolates tested, approximately 70% were classified as atypical EPEC strains. Based on these data and the results of several recent studies, the prevalence of
atypical EPEC seems to be on the rise (Afset et al., 2003; Nguyen et al., 2006; Albert et al., 2009). The role of atypical EPEC in childhood diarrhoea, however, still remains controversial (Moyo et al., 2007).

Although most EPEC infections resolve without antimicrobial therapy, antimicrobials should be administered in persistent infections to reduce the duration of illness and the choice of effective antimicrobials may be critical for patient healing and even survival (Diniz-Santos et al., 2006; Scaletsky et al., 2010). In the current study, as in many reports, the resistance rates of ampicillin, cotrimoxazole (trimethoprim–sulfamethoxazole) and tetracycline antibiotics were high and more than 39 % of the isolates showed resistance to these antibiotics (Putnam et al., 2004; Estrada-Garcı́a et al., 2005; Nguyen et al., 2005). Resistance rates of EPEC to different antibiotics, however, differ noticeably between countries (Putnam et al., 2004; Estrada-Garcı́a et al., 2005; Diniz-Santos et al., 2006; Nguyen et al., 2006; Pérez et al., 2010), which may reflect differences in the effectiveness of antimicrobial stewardship programs.

EPEC infection is primarily a disease of infants younger than 2 years of age. Nguyen et al. (2006) found that atypical EPEC generally occurred in children <2 years of age. Afset et al. (2003) found that EPEC was significantly more common among children who were 12–23 months old than in those who were 12 months of age. Nweze (2010) showed that the distribution of EPEC strains was significantly different with respect to age-group and was mostly confined to the age-group of 0–4 years. However, in our study, there were no statistically significant differences between the frequency of typical and atypical EPEC isolates in relation to age, gender or antimicrobial resistance pattern.

In recent years, typing methods have been used as a means to characterize and distinguish isolates based on their genotypic characteristics. These methods may be used to establish genotypic relationships between strains and to trace the geographical dissemination of bacterial isolates (Beutin et al., 2005; Blanco et al., 2006; Emaneini et al., 2011). DNA macro-restriction analysis by PFGE has been successfully used for the typing of EPEC in epidemiological

### Table 1. Resistance patterns, presence of eae, bfpA, and stx genes and PFGE profiles of enteropathogenic Escherichia coli (EPEC) isolates tested in this study.

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*t, Typical; a, atypical.
studies, even though no international database for comparisons of patterns has been established (Beutin et al., 2005; Blanco et al., 2006). In the current study, PFGE analysis of 23 EPEC isolates produced 21 distinct pulsotypes. This genetic heterogeneity may demonstrate that there are several distinct EPEC strains present in children with diarrhoea in Tehran and shows that EPEC infection is sporadic in this area and there is no common source of infection. This finding has also been seen in another study where each of 36 typable strains displayed a unique genotypic pattern (Afset et al., 2003).

In conclusion, the present study shows that typical and atypical EPEC were present among specimens of children in Tehran and that they belonged to 21 different pulsotypes. There was considerable heterogeneity in PFGE profiles and 69% of the isolates were resistant to at least one class of the tested antibiotics.

ACKNOWLEDGEMENTS

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REFERENCES


**Fig. 1.** Dendrogram of clinical isolates of EPEC based on PFGE patterns. The tree was reconstructed using the unweighted pair-group method with arithmetic mean (UPGMA). Percentage similarity is indicated above.


