Original Article

Prevalent Phenotypic and Genotypic Profile of Enterotoxigenic *Escherichia coli* among Iranian Children

Shahram Nazarian[1,2], Seyed Latif Mousavi Gargari[1]*, Iraj Rasooli[1],
Masoome Alerasol[1], Samane Bagheri[1], and Shakiba Darvish Alipoor[1]

*[1]Department of Biology, Shahed University Tehran; and
[2]Department of Biology, Imam Hossein University, Tehran, Iran*

SUMMARY: Enterotoxigenic *Escherichia coli* (ETEC) is the most common cause of diarrhea among children. ETEC strains express colonization factors (CFs), which mediate adherence to the small intestinal epithelium and produce entrotoxins that induce diarrhea. Here, we characterized the phenotypes and genotypes of ETEC strains from 261 diarrheal stool samples from Iranian children. The prevalence of ETEC was 8.04%. Most of the isolates were positive for heat-labile and heat-stable toxins. CFA/I, CS3, CS2, and CS5 were detected from some of the clinical isolates. 33.3% of the isolates did not express CFs. The majority of ETEC isolates were identified as O127 and O128 serotypes, and 57% of the strains were resistant to more than 1 antimicrobial agent. Heat-labile enterotoxin activity was confirmed using the Y1 adrenal cell assay, rabbit ileal loop and adenylate cyclase activation tests. Regional phenotypic and genotypic characterization could help to elucidate the ecology and pathogenicity of ETEC to efficiently reduce the burden of illness brought about by ETEC. This study may lead to development of effective prophylactic measures.

INTRODUCTION

Diarrheagenic *Escherichia coli* strains are causative agents of gastrointestinal illness in humans. Five categories of *E. coli*, namely, enteropathogenic, enteroaggregative, enterotoxigenic, enteroinvasive and Shiga toxin-producing are responsible for intestinal and extraintestinal diseases in humans (1,2). Enterotoxigenic *E. coli* (ETEC) is an important causative agent of diarrheal disease (3,4), which is associated with morbidity and mortality in children aged up to 5 years in developing countries (5). It was first recognized as a cause of human diarrheal illness in the 1960s (6,7). ETEC strains have 2 major virulence determinants, the heat-labile toxin (LT) and heat-stable toxin (ST), as well as colonization factors (CFs) (3,8–10). Based on biological and chemical features, the ST can be further differentiated into 2: human ST (STh) and porcine ST (STp) (11,12). Two major LT families, LT-I and LT-II, have been identified to date, of which the latter is rarely found among human-derived ETEC strains. LT-I shows a high similarity to cholera toxin, and both have been intensively studied as virulence factors (13). To cause disease, ETEC must adhere to the epithelium of the small intestine by means of CFs. Secretory diarrhea is then produced owing to the effects of the enterotoxins. Several types of CF antigens (CFAs), coli surface antigens (CSs), and putative CFs (PCFs) have been identified, of which the most common are CFA/I, CS1–CS6, and CS21 (5,8,11,14). More than 23 different human ETEC CSs are known; however, in approximately 30%–50% of strains, none of CS have not been identified, or additional CSs may exist (8,15). ETEC strains produce diversity of toxins or CFs; therefore, it is important to characterize various clinical ETEC isolates with respect to their virulence profiles.

Owing to increasing antibiotic resistance, it is important to consider antimicrobial resistance patterns among ETEC strains in different countries when prescribing antibiotics. The antimicrobial resistance patterns can also serve as a guide for implementation of public health interventions against diarrheal disease (4,14,16,17). The majority of various vaccines rely upon a multivalent approach, including multiple CFs with an LT component for broad coverage. However, the variability of the ETEC phenotype across regions and populations has complicated these efforts over time (8). Gupta et al. highlighted a need for more information on the distribution of ETEC phenotypes (18). The role of ETEC in diarrheal disease in Iran has not been widely studied (19–21). Hence, the present study was designed to characterize the phenotypes and genotypes of ETEC associated with diarrhea among Iranian children aged less than 5 years. The migration of people from surrounding countries to Iran and subsequent spread of ETEC infections through these routes are expected to help in the characterization of the bacteria transmitted from neighboring countries with low human development indices.

*Corresponding author: Mailing address: Department of Biology, Shahed University, Tehran-Qom Express Way, Opposite Imam Khomeini's shrine, Tehran-3319118651, Iran. Tel: +98(21)51212600, Fax: +98(21)51212601, E-mail: slmousavi@shahed.ac.ir

MATERIALS AND METHODS

Study design and population: All ETEC specimens were obtained from children aged less than 5 years. The study was conducted in Iranian communities between

April 2010 and September 2011 and from May to October 2012. After initial screening, 261 stool samples from children residing in central, southern, and northern regions of Iran (i.e., Tehran, Kerman, and Golestan provinces, respectively) were analyzed for the presence of ETEC. The inclusion criteria included the passing of unformed stools 2 or more times during a 24-h period with at least 1 abdominal symptom of enteric infection such as vomiting. The children with diarrhea attended different health care centers or hospitals. The hospitals and care centers were selected on the basis of the fact that they had the largest number of diarrhea cases. The personnel of the participating hospitals and care centers were instructed to obtain samples on the day of admission before antibiotics were administered and to obtain written consent from the patients for the use of their stool samples for both treatment and research purposes. Clinical patient data, including age, sex, type of diarrhea, fever, vomiting, duration of diarrhea, etc., were obtained through questionnaires.

Microbiological studies: For ETEC detection, fresh stool specimens were diluted, plated on MacConkey agar, and incubated at 37°C for 18 h. Five lactose-fermenting individual colonies morphologically resembling *E. coli* were selected and submitted for biochemical testing. Stock samples were prepared in Luria–Bertani (LB) broth containing 20% glycerol and preserved at −80°C for further studies.

Primer design: Nucleotide sequences of genes encoding for enterotoxins and CFs were retrieved from the GenBank database at the National Center for Bioinformatics (http://www.ncbi.nlm.nih.gov/genbank). Specific primers were designed for detection of CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS21, LT, STh, and STp (Table 1). The specificities of the primers were confirmed using the Basic Local Alignment Search Tool (http://www.ncbi.nlm.nih.gov/BLAST).

DNA template preparation: A loopful of a freshly prepared culture from each of the cultured *E. coli* isolates was suspended in 200 μl of sterile deionized water, heated at 100°C for 10 min, and transferred into ice bath for 2 min. Cell debris was pelleted by centrifugation at 12,000 g for 2 min, and the supernatant was transferred to a new microfuge tube and stored at −20°C (22).

Multiplex polymerase chain reaction (PCR): ETEC

strains were identified using primers designed for LT, STp and STh to amplify the toxin-encoding genes (Table 1). PCR amplification was performed in a total reaction mixture of 25 μl containing 1 μl of cell lysate; 0.2 mM dNTPs; 3 mM MgCl$_2$; 10 mM Tris/HCl, pH 8.3; 50 mM KCl; 10 pM of each primer; and 1 U of *Taq* DNA polymerase (26). Cyclic conditions were initiated at 95°C for 5 min followed by 30 cycles of 94°C for 1 min, 55°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 10 min. Once strains were confirmed as ETEC by PCR, they were sequentially tested to detect CFA-encoding genes using the primers listed in Table 1. Conditions for the multiplex PCR were the same as those described above, except for the annealing temperature, which was set at 54°C for 1 min. The amplicons were analyzed by agarose gel electrophoresis.

Dot blot assays: Dot blot tests to detect CFs were performed by introduction of 2 μl of the bacterial culture on to a nitrocellulose membrane, which was dried and blocked with 3% BSA in PBST (PBS [pH 7.4], 0.05% [v/v] Tween-20) at 37°C for 1 h. The membrane was incubated with 1:200 dilutions of specific CF sera in PBST at room temperature for 1 h. After washing, the membrane was incubated for 2 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G. Detection was performed by the addition of 3,3′-diaminobenzimidine (Sigma-Aldrich, St. Louis, Mo., USA) and H$_2$O$_2$ (23). The antibodies used in this study were developed in-house.

Reverse transcriptase (RT)-PCR analysis of mRNA expression: RT-PCR was used to analyze CF mRNA expression under culture conditions at 37°C. Total RNA was isolated from 10^9 bacterial cells in exponential phase cultures grown in LB broth using an RNA purification kit (Fermentas Inc., Hanover, Md., USA). The cDNA amplification reaction was performed using specific primers (Table 1) and purified RNA. RT-PCR was conducted using gene-specific first strand cDNA.

Serotyping: The O serotypes of *E. coli* strains were determined by agglutination analysis using a commercially available serotype kit (Bahar-afshan, Tehran, Iran) according to the manufacturer's instructions.

Antimicrobial resistance phenotyping: Antimicrobial resistance phenotyping was performed using commercial grade antimicrobial disks (ampicillin, nalidixic acid, chloramphenicol, tetracycline, ciprofloxacin, gentami-

Table 1. Primers sequences used for amplification of toxin and colonization factor of ETEC

Virulence factor	Target gene	Primer sequence		Product (bp)	GenBank accession no.
		Forward	Reverse		
LT	*eltBI*	TCTTTATGATCACGCGAGAGGA	TCTTCTCTCCATAGCTTGGTGATC	412	S60731.1
STh	*estA2-4*	TGCTAAACCAGTAGAGTCTTCAAAAG	TAATAGCACCCGGTACAAGCAG	157	M29255.1
STp	*estA1*	AGGTAACATGAAAAAGCTAATGTTG	AGGCAGGATTACAACAAAGTTCA	209	M58746.1
CFA/I	*cfaB*	ACAATGTTTGTAGCAGTGAGTGC	AGATACTACTCCTGAATAGTTTCCTG	450	M55661.1
CS1	*csoA*	ACTGTTGACCTTCTGCAATCTGATG	ACGTTGACTGAGTCAGGATAATTG	403	AY513487.1
CS2	*cotA*	ACTATCGATCTGATGCAATCTGATG	TACAATATTAGTTTGCTGGGTGC	355	Z47800.1
CS3	*cstA*	TCCTCAGGATAATTTAACATTATCC	ACCTTCAGTGGTAATAAACTTAACTG	304	X16944.1
CS4	*csaB*	TAGTAGTTTACCTACTGCTGTAGAATTAA	GACGTTCCAAATTTAATTCTGC	252	AF296132.1
CS5	*csfA*	TCCTTCCGCTCCCGTTA	TAGCTGACGTGTCACGCGT	206	AJ224079.2
CS6	*cssA*	TGATGATTCCCAATCAATAATCTAC	AGGTATTTCTTTATCTCCGCATG	142	U04844.1
CS21	*lngA*	TGCTGGAAGTTATCATTGTTCTTG	ACTTAGAATTTTGTCTGCAGTCAAG	569	AF004308.1

cin, cefoxitin, cefalothin, and trimethoprim/sulfame-thoxazole), with *E. coli* ATCC 25922 as a control. *E. coli* strains positive for ETEC genes were subjected to antimicrobial susceptibility analysis using the disk diffusion method (24), according to breakpoints established by the National Committee for Clinical Laboratory Standards (25).

Phenotypic detection of LT and ST: For detection of the ST, *E. coli* strains cultivated from single colonies in 30 ml of casamino acids-yeast extract (CAYE) broth (2% casamino acids, 0.6% yeast extract, 43 mM NaCl, 38 mM K_2HPO_4, and 0.1% trace salt solution consisting of 203 mM $MgSO_4$, 25 mM $MnCl_2$, and 18 mM $FeCl_3$) were incubated at 37°C overnight with continuous vigorous shaking. Cultures were centrifuged at 900 g for 30 min at 4°C, and the supernatants were used as test samples. ST production was determined using the E. COLIST-EIA kits (Denka Seiken, Tokyo, Japan).

The GM1 ganglioside-enzyme-linked immunosorbent assay (GM1-ELISA) was performed for detection of the LT antigen. Aliquots of 100 μl from each of the *E. coli* strains in lincomycin-supplemented LB broth were added to microtiter wells pre-coated with GM1 (0.5 μg/ml in PBS) and incubated overnight at 37°C. The released LT bound to GM1 was detected with rabbit anti-cholera toxin antiserum (Sigma-Aldrich) and HRP-conjugated goat anti-rabbit immunoglobulin (Dako, Roskilde, Denmark), using H_2O_2 and o-phenylenediamine dihydrochloride as substrates (26).

Rabbit ileal loop test: *E. coli* strains cultivated from single colonies in 30 ml of CAYE broth were incubated overnight at 37°C. The rabbit ileal loop test was performed according to the methods described by De and Chatterje (27). New Zealand white rabbits (1.5 kg) were starved for 48 h before bacterial inoculation. Under general anesthesia, 6 loops, each approximately 10 cm in length, were constructed using the mid portion of the ileum and 500-μl aliquots containing 10^7 cfu/ml were injected into each. The animals were sacrificed 18 h later and the length of each loop (cm) and amount of fluid accumulation in each (g) were measured. The ratio of fluid accumulation against loop length (g/cm) was calculated as an index of enterotoxigenicity. Experimental infection of the rabbits was performed by the Faculty of Veterinary Medicine, University of Tehran. All protocols were performed in compliance with the Animal Welfare Act and regulations related to animal experimentation.

Y-1 adrenal tumor cell assay: Y-1 adrenal tumor cells (Pasteur Institute of Iran) were grown in Dulbecco's modified Eagle media (Gibco, Eggenstein, Germany) supplemented with 10% heat-inactivated fetal bovine serum (Gibco). Approximately 10^4 cells were seeded in 96-well cell culture plates and allowed to adhere to the plate by incubating under 5% CO_2 at 37°C. Next, the cells were inoculated with 100 μl of bacteria-free CAYE culture supernatant and incubated at 37°C under 5% CO_2 for 12 h. The cells were then examined for typical cell rounding (28).

Adenylate cyclase activation: The isolated ETEC strains were analyzed for their ability to produce and release LT after growing at 37°C for 18 h in CAYE medium. The ability of the toxin to stimulate adenylate cyclase was determined by incubating CHO cells with the toxin and measuring the amount of cyclic AMP (cAMP) production. CHO cells were seeded in 24-well sterile culture plates at a density of 5 × 10^4 cells per well and grown overnight to approximately 80% confluence. Bacteria-free CAYE culture supernatant was added and the plates were incubated in a CO_2 incubator. After washing with PBS, the cells were lysed with 0.1 M HCl and the lysate was centrifuged (29,30). The supernatant was collected and tested for intracellular cAMP levels using a cAMP immunoassay kit (Abnova, Taipei City, Taiwan).

ETEC binding assay: Caco-2 cells were grown to confluence in a culture flask, trypsinized, and distributed on sterile cover slips placed on the bottom of each well of a 6-well cell culture plate. The ETEC cells were adjusted to an optical density of 0.5 at 600 nm. An aliquot of the cell suspension (200 μl) was added to Caco-2 cells prewashed with PBS and incubated for 1 h at room temperature. The Caco-2 cells on the cover slips were fixed in methanol, stained with Giemsa staining solution, destained with PBS, and examined under a light microscope. The number of ETEC cells adhering to each Caco-2 cell was counted, and the adhesion index was determined by examining 100 Caco-2 cells on each cover slip (31,32).

Statistical analyses: Statistical analyses were performed using the Student's t-test. A probability (P) value > 0.05 was considered statistically significant. Binding of the bacteria to the cells was expressed as the mean percentage of cells with bound bacteria ± standard deviation. Results were obtained from 3 different experiments.

RESULTS

Clinical data: A total of 322 fecal samples were collected and screened for the presence of *E. coli* strains, of which 261 (69.9%) were positive for *E. coli* and 21 (8.04%) were positive for ETEC (12 females and 9 males; 57.14% vs. 42.85%). The mean age of ETEC-infected patients was 28.23 month (range, from <1 to 5 years), with 42.85% ($n = 9$) under 24 months old (Table 2). The most common symptoms of diarrheagenic children with ETEC infection were watery stools (80.95%), vomiting (52.3%), and abdominal pain (80.95%) (Table 2).

Genotypic characterization of ETEC isolates: Of the 21 ETEC-positive fecal samples, multiplex PCR analysis of enterotoxin genes identified 1 LT strain, 4 STh strains, 15 LT/STh strains, and 1 LT/STp strain, with no non-specific products (Fig. 1). Agarose gel electrophoresis of the PCR products showed that the CF genes were of the expected sizes (Fig. 2). Sequencing analysis of the PCR products confirmed their identity.

CFA/I was the most commonly detected, followed by CS3, CS2, and CS5. It was interesting that none of the Iranian samples were of type CS21. Some of the ETEC strains (33.3%) had no detectable CFs (CFA/I, CS1-CS6) but expressed both the LT and ST (Table 3). Further, RT-PCR analysis of CFs showed mRNA production from CF-encoding genes.

Phenotypic detection of CFs: Dot blot analysis was used, which was based on antibodies for CFs. Comparisons of strains for CFs by dot blot analysis and PCR

Table 2. Clinical characteristics of ETEC affected diarrheal patients

| Patient ID	Age (months)	Sex	Stool feature	Vomiting	Duration of diarrhea (days)	Abdominal pain	Hospital care	Fever (°C)	No. of stools per 24 h
19	29	F	Watery	+	4–8	+	+	≥38	≤4
25	28	F	Watery	−	1–3	+	−	<38	≤4
51	60	F	Watery	+	4–8	+	+	≥38	≥5
56	28	M	Watery	−	1–3	+	−	<38	≤4
71	22	F	Watery	+	1–3	−	−	<38	≤4
85	30	M	Watery	+	4–8	+	+	≥38	≤4
89	24	M	Watery	−	1–3	+	−	<38	≤4
105	20	F	Mucosal	−	1–3	+	−	<38	≥5
130	28	M	Watery	+	1–3	+	−	<38	≤4
141	60	F	Mucosal	+	4–8	−	+	≥38	≥5
152	20	M	Watery	+	1–3	+	−	<38	≥5
198	34	F	Watery	−	1–3	−	−	<38	≥5
210	28	M	Watery	−	4–8	+	+	<38	≤4
212	24	F	Blood	+	1–3	+	−	<38	≤4
320	18	F	Watery	+	1–3	−	+	<38	≤4
354	20	M	Watery	−	1–3	+	−	<38	≥5
362	26	M	Mucosal	−	1–3	+	−	<38	≤4
385	30	F	Watery	−	1–3	+	−	<38	≤4
401	28	F	Watery	−	1–3	+	−	≥38	≥5
403	24	F	Watery	+	1–3	−	−	<38	≤4
420	12	M	Watery	+	4–8	+	+	≥38	≥5
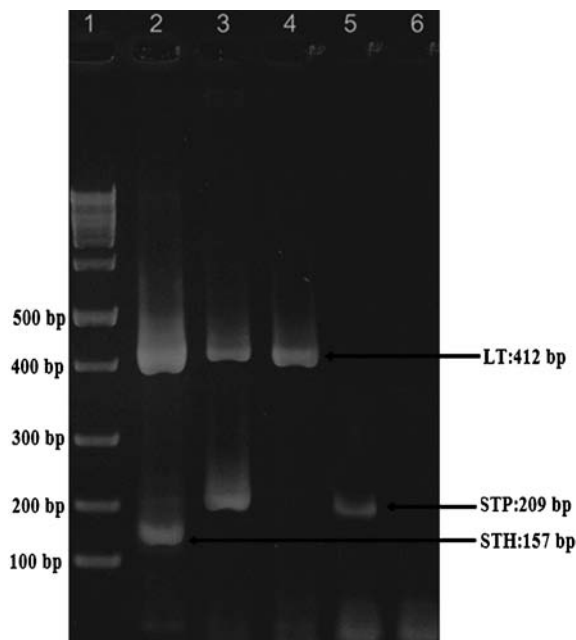
Fig. 1. Gel electrophoresis of PCR products obtained by using multiplex PCR for ETEC toxin genes in clinical isolates of ETEC strains. Lane 1, 100-bp DNA ladder marker; lane 2, ETEC strain with LT/STh genes (*eltBI*: 412 bp, *estA2–4*: 157 bp); lane 3, ETEC strain with LT/STp genes (*eltBI*: 412 bp, *estA1*: 209 bp); lane 4, ETEC strain with LT gene (*eltBI*: 412 bp); lane 5, ETEC strain isolated with STp gene (*estA1*: 209 bp); lane 6, negative control.
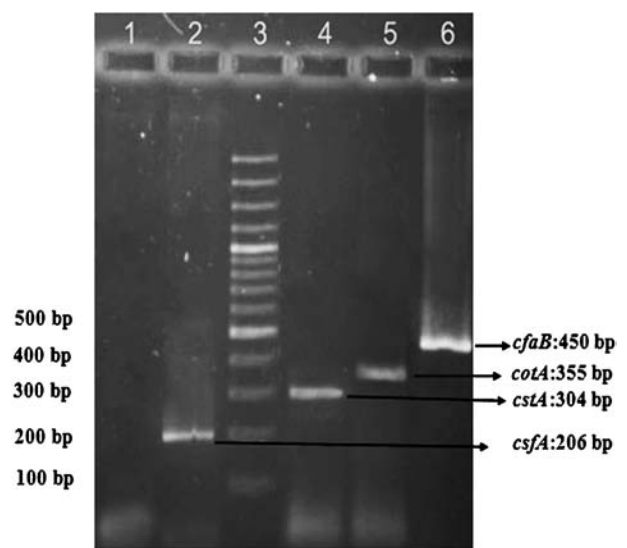

Fig. 2. Agarose gel electrophoresis of PCR-amplified products for detection of colonization factor genes in clinically ETEC strains. Specific CFAs based on the amplicon size on the agarose gel are shown. Lane 1, negative control; lane 2, ETEC strain with CS5 (*csfA*: 206 bp); lane 3, 100-bp DNA ladder marker; lane 4, ETEC strain with CS3 (*cstA*: 304 bp); lane 5, ETEC isolate with CS2 (*cotA*: 355 bp); lane 6, ETEC strain with CFA/I (*cfaB*: 450 bp).

showed a high level of agreement. Only 1 PCR positive strain for CFA/I was not detected by the dot blot assay.

Analysis of Caco-2 cell monolayers revealed that ETEC H10407 cells and CFA/I-, CS2-, CS3-, and CS5-positive clinical strains were distributed among the Caco-2 cells. Almost all of the Caco-2 cells were found

to bind 1 or more bacteria with an adhesion index of 14.5 bacteria per cell for H10407 and 15.5 ± 0.8, 14.6 ± 0.9, 12.7 ± 0.8, and 12 ± 0.6 bacteria per cell for CFA/I-, CS2-, CS3-, and CS5-positive clinical strains, respectively.

Phenotypic detection of enterotoxins: Results of ST and LT expression analysis using the ST detection kit and GM1-ELISA are given in Table 3 and Fig. 3. LT/ST-producing ETEC strains were the most fre-

Table 3. Characterized ETEC strains collected from Iranian children with diarrhea

Patient ID	Toxin	CF	O serotype
19	LT/STh	CFA/I	O127
25	LT	CFA/I	NT
51	LT/STh	CS2	O6
56	LT/STh	CS3	O86
71	LT/STh	—	O26
85	LT/STh	CS3	O86
89	LT/STh	CFA/I	NT
105	LT/STh	—	O55
130	LT/STh	CS3	O86
141	STh	—	O127
152	LT/STh	CFA/I	O127
198	LT/STh	—	O111
210	STh	CFA/I	NT
212	LT/STh	CFA/I	O78
320	LT/STp	—	O114
354	STh	CFA/I	NT
362	STh	CFA/I	O127
385	LT/STh	CFA/I	O127
401	LT/STh	CS5	O128
403	LT/STh	—	NT
420	LT/STh	—	O20

quently detected, followed by those producing ST and LT only. One ETEC strain was found to be *eltB*-positive by multiplex PCR but LT-negative by GM1-ELISA.

Stimulation of the cAMP levels in cell culture after addition of bacterial culture supernatant indicated LT
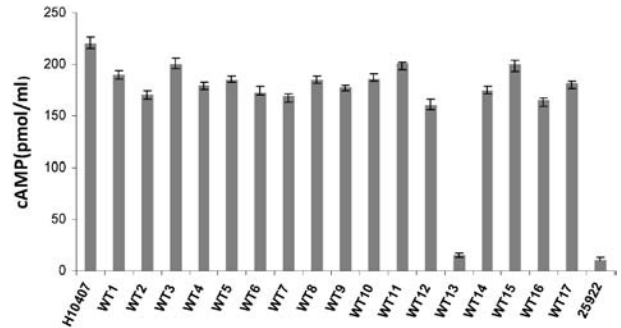

Fig. 4. Toxicity detected in cAMP ELISA. The biological activities of LT were assessed by measurement of intracellular cAMP levels following exposure of the cells to supernatants of overnight-grown cultures of ETEC strains. Results carried out with ETEC H10407 and *E. coli* 25922 strains as positive and negative controls. WT1–17, ETEC strain containing LT gene; WT13, ETEC strain detected *eltB* positive by multiplex PCR and LT negative by GM1-ELISA. Error bars represent standard deviations from 3 independent replicates.

Fig. 3. LT detection by GM1 ELISA in ETEC clinical isolates. Samples were either identified as LT positive (■) or LT negative (□). ETEC H10407 and *E. coli* 25922 were used as positive and negative controls.
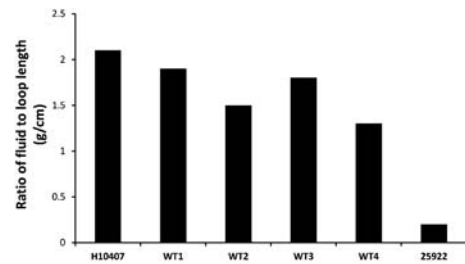

Fig. 5. Fluid accumulation in rabbit ileal loops inoculated with ETEC strains. Exponentially growing ETEC cells harvested from cultures were inoculated into ileal loops. After 18 h, animals were sacrificed and the volume of liquid in each ileal loop was measured. Results carried out with ETEC H10407 and *E. coli* 25922 strains as positive and negative controls. WT1, 2, 3, and 4, ETEC strain containing LT/STh, LT, LT/STp, and STh, respectively.
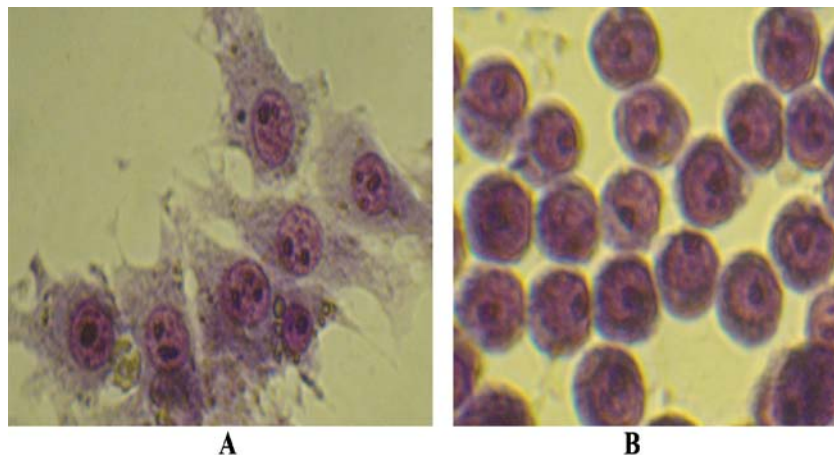

Fig. 6. (Color online) Photomicrographs of monolayer cultures of Y1 cells. (A) Untreated cells. (B) Y1 cells after addition of culture filtrate of LT positive ETEC strain.

release and toxic activity (Fig. 4). Fluid accumulation in the ligated ileal loops further confirmed the in vivo enterotoxic activities of the isolated strains (Fig. 5). As indicated in Fig. 5, the ratios of fluid accumulation (g) against loop length (cm) were 1.31–1.89. The cell culture cAMP levels as well as the fluid accumulation in the loops from the clinical strains did not show any significant differences with that from the ETEC strain H10407. The morphological changes induced in the Y1 cells by bacteria-free culture supernatant (Fig. 6) were identical to those induced by ETEC strain H10407. The supernatants from the negative control strain did not cause cell rounding.

Serotyping: Serotyping results of ETEC isolates for detection of somatic antigens are presented in Table 3. A wide array of O-type antigens was observed among 16 (76.19%) of the ETEC isolates. Serogroups O20, O128, O6, and O8, which are typically associated with ETEC, were also detected. The majority of ETEC isolates were serotyped as O127 and O86.

Antimicrobial drug susceptibility: Antimicrobial drug susceptibility testing of the ETEC isolates showed that 38% were resistant to tetracycline, 71.4% to ampicillin, 19% to chloramphenicol, 57.1% to sulfamethoxazole-trimethoprim, 19% to cephalothin, 47.6% to amoxicillin–clavulanate, and 9.5% to ciprofloxacin. Most of the tested strains were not resistant to ciprofloxacin, but all were susceptible to nalidixic acid and gentamicin. In our study, 57% of the isolates were resistant to more than 1 antimicrobial agent, and 14.28% were resistant to more than 4 antibiotics. The most common multidrug resistance phenotype was ampicillin-trimethoprim/sulfamethoxazole-chloramphenicol resistance, and 85.71% of the most common CF-positive strains harbored antimicrobial resistance.

DISCUSSION

ETEC is one of the most common bacteria responsible for diarrhea in different parts of the world (1,3,5,7,33,34). Here, the prevalence of ETEC among pediatric Iranian patients with diarrhea referred to hospitals and medical centers in central, southern and northern Iran was studied. The proportion of Iranian children hospitalized with acute diarrhea owing to ETEC infections were reportedly 21.9% (20), 7.3% (19), and 5.1% (35), whereas ETEC was confirmed in 8.04% of the patients in this study. Multiplex PCR findings were supported by toxin ELISA results for all ST- and LT-ETEC strains tested. However, 1 ETEC strain was found to be *eltB*-positive by multiplex PCR but LT-negative by GM1-ELISA. Therefore, it is likely that this strain contained silent genes or an enterotoxin level inadequate for detection by ELISA.

Methods to differentiate STp from STh may help to identify differences in the epidemiology of ST subtypes (18). In this study, we used multiplex PCR to differentiate STh and STp. The results showed that LT/ST-ETEC was the most commonly detected ETEC enterotoxin subgroup. A total of 50 studies assessing the relative frequency of the 3 ETEC enterotoxin subgroups indicated that ST-ETEC was the most common enterotoxin subgroup (18). The referenced studies indicated a higher frequency of ST-ETEC in Latin America/the Caribbean, the Middle East/North Africa, and South Asia. In Iran, Katouli et al. showed that ST was the most frequent toxin type (60%) followed by LT/ST and LT (20). Samples collected in 1998 showed a prevalence of the LT type (36). In fact, previous studies on ETEC isolates revealed a change in the prevalence of enterotoxin types from 1992 until now.

Although detection of toxins can be used to confirm the presence of ETEC, positive strains can be further analyzed by dot blot or PCR analysis for the presence of CFs (26). The PCR method for detection of CFs is highly sensitive and affordable; however, the occurrence of mutations in the primer binding site can cause errors in PCR amplification. In phenotypic detection of CFs, phenotypic silencing may occur owing to storage or subculture techniques of the ETEC isolates and may have a genetic explanation, such as loss of regulatory genes (26). Among the ETEC strains isolated in the present study, CFA/I and CS3 were the most common CFs combinations detected by the multiplex PCR. Although most studies found CS3 either alone or in conjunction with CS1 or CS2 ETEC strains (14,17,34), the co-expression of CS3 and CS1 or CS2 component antigens was not identified in our study. A similar observation was reported in a review by Isidean et al., in which CS3 alone was the predominant phenotype among CFA/II-expressing ETEC strains (8). CFA/I-expressing strains are reportedly common in different parts of the world (8). Shahrokhi et al. showed that CFA/I and CFA/IV were the most common CF types within ETEC isolates (36). Although CS6-producing ETEC is the most prevalent strain of ETEC in various developing countries (18,34,37), none of the ETEC isolates in our study displayed the CS6 antigen. Despite the usual co-expression of CS5 with CS6, 1 ETEC CS5 strain was detected in our study. Thus, variations in the prevalence of CF antigens may be related to location. CFs were also detected by dot blot, indicating that the genes were translated and displayed on the bacterial surface. In this study, 33.3% of the detected ETEC isolates did not express the most prevalent CFs (CFA/I, CS1–CS6, and CS21). Our findings are in agreement with those of others who reported no detectable CFs in 30%–50% of ETEC strains worldwide (3,14,23,34). We did not include other CFs in this investigation because they have been reported to escape detection by their absence or owing to the loss of genes encoding CFs during culture storage or in subculture (3,26). The local and regional distributions of enterotoxin type are related to the estimation of vaccine coverage against ETEC strains. Using a multivalent vaccine containing the LT toxoid and the most common CFs as a model along with the data from this study, the estimated vaccine coverage rate among Iranian children is expected to be 80.95% against the LT and 66.6% against CFs. High antibiotic resistance in *E. coli* has been previously reported in Iranian isolates (19,36). The pattern of multidrug resistance in our isolates was similar to those reported by Aslani et al. (19). Our results indicated wide-spread antimicrobial properties of ETEC strains, suggesting that antimicrobial treatment should be limited against ETEC diarrhea other than for more severe or persistent cases, which may hamper further development of antimicrobial resistance. Our results showed a significant correlation

between expression of the most common CFs and the presence of antimicrobial resistance.

There was a wide diversity of O antigens represented among the ETEC isolates from children in this study, of which serotypes O6, O8, O78, O128, and O153 were the most common antigens (38). Twenty percent of our isolates expressed the aforementioned common O-antigens. This result further confirms that ETEC from different geographical areas belonged to a relatively large number of serotypes, and ETEC serotypes may change over time (38–40).

In conclusion, elucidation of the ecology and pathogenicity of ETEC may help to reduce the burden of illness, and effective prophylactic measures can be developed by phenotypic and genotypic characterization of regional ETEC outbreaks.

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