Original Article

Comparison of virulence markers and antibiotic resistance in enterotoxigenic *Escherichia coli* isolated ten years apart in Tehran

Nader Shahrokhi, Saeid Bouzari, Anis Jafari

Molecular Biology Unit, Pasteur Institute of Iran, Tehran 13164, Iran

Abstract

Introduction: Enterotoxigenic *Escherichia coli* (ETEC) causes diarrhoea by producing heat-labile (LT) or heat-stable (ST) enterotoxins after colonizing the small intestine by means of colonization factors (CFs). Although detection of the toxins is sufficient for verification of ETEC isolates, toxin-positive strains may be further analyzed for the presence of CFs. Antibiotics may shorten the duration of diarrhoea caused by ETEC, but the rapid emergence of resistant strains limits their usefulness.

Methodology: ETEC isolates collected 10 years apart were compared for the prevalence of toxin types, CFs and antibiotic resistance. DNA/DNA hybridization with digoxigenin (DIG)-labeled probes was used for the detection of toxin types, and CF-typing was performed by DNA hybridization using DIG-labeled probes for *cfaD* and CS6 with slide agglutination. Disk diffusion was used to determine antibiotic resistance. The presence of class 1 integrons was detected by PCR.

Results: ST-positive isolates were the most prevalent among the isolates from 1988, but a significant shift towards LT-gene carriage was observed in the 1998 group. CFA/I and CFA/IV were the most common CF types within both groups. The most prevalent resistance patterns among *these* isolates were ACSTSXT followed by ASTSXT and ASSXT.

Conclusion: Our study of the two groups of isolates showed that the rate of LT and ST gene carriage, as well as antibiotic resistance markers, has changed in the ten years separating the two bacterial populations. These variations show the importance of monitoring pathogenic bacteria to obtain a near realistic picture of the circulating bacterial pathogens.

Key words: Antibiotic resistance; colonization factor antigens; enterotoxigenic Escherichia coli; heat-labile toxin; heat-stable toxin

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Introduction

Enterotoxigenic *Escherichia coli* (ETEC), the most common cause of E. coli-mediated diarrhoea worldwide, is contracted by consumption or use of contaminated food or water [1]. ETEC disease typically manifests as watery diarrhoea which can elicit gastrointestinal symptoms ranging from mild to severe, with or without fever and vomiting, and a relatively high inoculum (~ 10^8 CFU) is required for infection [2].

An important factor in the genesis of diarrhoea is the widespread microbiological contamination of the environment, which, among other factors, is responsible for repeated infections. It has been shown that adequate hygiene practices and good sanitary conditions may significantly lower the prevalent levels of contamination [3]. Therefore, the burden of diarrhoeal diseases may decrease within some countries if their economical conditions improve, but for citizens of low-income nations, diarrhoeal diseases are projected to remain among the ten leading causes of death through to 2030 [4].

The major therapeutic problem of ETEC-associated diarrhoea is loss of fluid and subsequent dehydration. Hence, oral rehydration therapy, and in grave cases, replacement of fluids intravenously, are the cornerstones of treatment. Antibiotics may shorten the duration of diarrhoea caused by ETEC, but the rapid emergence of resistant strains limits their usefulness [5]. Furthermore, the dissemination of antimicrobial resistance genes among bacteria is an increasingly serious problem throughout the world.

The profuse watery diarrhoea caused by ETEC results from elaboration of a heat-labile toxin (LT), a heat-stable toxin (ST), or both (LTST). Pathogenicity is also dependent upon the expression of fimbriae or colonization factors (CFs), which function as adherence factors, allowing the pathogen to colonize the small intestine [2]. To date more than 20 CFs have been characterized with the diversity being due

to differences in the primary amino acid sequences of the fimbrial structural subunits [6]. These proteins have been grouped into families or as distinct fimbriae on the basis of the underlying genetic diversity and are named colonization factor antigen I (CFA/I) and coli surface antigen 1 (CS1) to coli surface antigen 22 (CS22) [7,8]. All of the genetically related class 5 family of ETEC fimbriae which includes CFA/I, CS1, CS2, CS4, CS14, CS17, CS19 and putative CF O71 (PCFO71) as well as CS3 and CS20 are regulated by the transcriptional activator Rns or its functional homolog CfaD [7,9,10]. CfaD, also known as CfaR, shares 97% identity with Rns [10]. CS3 is expressed either alone or in combination with CS1 or CS2, and in the CFA/IV group, which contains CS4, CS5, and CS6 fimbriae, CS6 is carried alone or in combination with CS4 or CS5 [6]. Of all the known CFs, the most frequently encountered are CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS14, CS17, and CS21, of which to our knowledge only CS6, CS7 and CS21 are not regulated by the CfaD/Rns activators. Moreover, despite the important role that CFs play in the pathogenesis of ETEC, in approximately 30-50% of the isolates worldwide, no CF is detected [11]. ETEC fimbriae have generally been identified by agglutination or immunoblotting with poly or monoclonal antibodies, but these reagents are not widely available, leading to the publication of various genotypic methods, including mono- and multiplex PCRs for the identification of CFs [8,12-16].

In this study, a group of ETEC isolates that had been collected in 1988 were compared with isolates obtained in 1998 from children under the age of 5 years with diarrhoea. Antibiotic resistance profiling, toxin and CF typing were performed to identify changes that might have occurred in the interim period. Initial screening for CF types was performed by DNA hybridization using a probe for *cfa*D, which is the regulator for nearly half of all the known CFs and *css*A, the structural subunit of CS6, but immunoblotting with monoclonal antibodies was subsequently used for CF typing.

Methodology

Bacterial isolates

Two collections of isolates obtained from children under 5 years of age in Tehran with diarrhoea in 1988 and 1998, and previously indicated as ETEC without CF determination, were studied. LT and ST production in the 1988 isolates (179 isolates) had been determined by VET-RPLA and E. COLIST-EIA kits (Denka Seiken, Tokyo, Japan) and in 1998 (84 isolates) by DNA/DNA hybridization. The isolates had been kept as stab cultures at 4°C. Prior to use, isolates were examined for purity and identity confirmation was performed by standard microbiological methods and streaked out onto CFA agar. Subsequently a colony was cultured in LB broth and used for phenotypic and genotypic tests.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the disk diffusion method [17] for ampicillin (A 10 µg), cefalothin (30 µg), chloramphenicol (30 µg), gentamycin (G 10 µg), kanamycin (K 30 µg), nalidixic acid (30 µg), streptomycin (10 µg), tetracycline (T 30 µg), and trimethoprim/sulfamethoxasole (12.5/23.7µg). For data analysis, intermediate isolates were counted as resistant. All the disks used in this study were locally produced (PadtanTeb, Tehran, Iran).

DNA extraction, dot-blot hybridization and PCR

DNA was obtained by boiling the pellet from 1.5 ml of overnight bacterial culture in 500 µl of double distilled water for 10 minutes. After centrifugation at 12,000 rpm for 5 minutes, the supernatant was collected and used for dot-blot hybridization and PCR.

Probes for ST and LT were obtained from digestion of plasmids CVD427 and CVD403 (a gift from Professor J.P. Nataro, University of Maryland, USA) with EcoRI and *Hinc*II (Fermentas, Lithuania) to produce fragments of 216 and 1,200 bp respectively. The fragments were subsequently labeled by digoxigenin following the manufacturer's instructions (Roche, Mannheim, Germany). The DNA-coated (2 µl) and cross-linked nylon membranes (Schleicher and Schuell, Dassel, Germany) were hybridized with 25 ng of the labeled probes according to the manufacturer's instructions (Roche, Mannheim, Germany). *E. coli* K12, distilled water, and hybridization buffer were used for negative controls and plasmids containing the probe fragments were used as positive controls. Each isolate was tested at least twice to confirm the reproducibility of the results.

Isolates giving a positive reaction in the DNA dot blot hybridization were randomly subjected to PCR for *ltB*. PCR was performed in 25 µl containing 1 X PCR buffer, 1.5 mM MgCl2, 200 mM dNTPs, 200 mM of each primer, 0.5 unit of Taq DNA polymerase

Table 1. List of primers used in this study.

Virulence Factor	Primer sequence (annealing site)	Size (bp)	GenBank No.	Refs
*cfa*D-F	5'-ttgccttttgtaggtataagatgga-3' (539-563)	799	M55609	This
*cfa*D-R	5'-gtaaaaaactgctttggtgtaacac-3' (1337-1337)			study
*cfa*B-F	5'-catagaaaaagagcaagggctaataca-3' (1521-1548)	696	M55661	This
*cfa*B-R	5'-gcaactgaaatcgctacgactaatct-3' (2219-2190)			study
*css*A-F	5'-tcattcggcagccatgccagaa-3' (685-706)	505	U04844	This
*css*A-R	5'-atttaccactccggaagttcctg-3 (1190-1168)			study
ltb-F	5'-atgaataaagtaaaattttatg-3' (17-38)	396	M17874	This
ltb-R	5'-ctagttttccatactga-3' (391-375)			study
*int*I F	5'-ggcatccaagcagcaagc-3'	variable		[28]
*int*I B	5'-aagcagacttgacctgat-3'			
*qacEΔ*IF	5'-atc gcaatagttggcgaa gt-3'	~800		[28]
*sul*I B	5'-gca aggcggaaa ccc gcg cc-3'			

(Fermentas, Vilnius, Lithuania) and 5 µl of boiled bacterial DNA as a template. The PCR program for all the genes amplified in this study was as follows; [(95 °C, 5 min) 1 cycle; (95°C, 45 sec., 65°C, 30 sec., 72°C, 30 sec) 30 cycles; 72°C, 5 min]. This program was used for all amplifications, but the annealing temperature for *int*I and *qacEΔ*I F-*sul*I B primers was 58°C. PCR products were visualized after staining with ethidium bromide (Sigma, St. Louis, USA). CFA/I-positive isolates were also tested by PCR and an amplicon sequenced. The primers (Metabion, Martinsried, Germany) are listed in Table 1.

Production of LT and ST was detected in randomly selected probe-positive isolates by GM1-ELISA and inhibition GM1-ELISA respectively, the materials for which were a gift from Professor Ann-Mari Svennerholm, University of Göteborg, Sweden, with instructions as described by Sjöling *et al*. [15].

The probes for CfaD and CS6 were generated by PCR, sequenced commercially (MWG, Ebersberg, Germany), and labeled with digoxigenin using a DIG-labeling kit (Roche, Mannheim, Germany). Initially all the isolates were hybridized with *cfa*D and those which did not produce any signal were screened by the CS6-labeled probe. *cfa*D probe-positive isolates were subjected to immunodot blot with monoclonal antibodies (MAbs) for CFA/I, CS3, CS4, and CS6 probe-positives with CS5 which were kindly provided by Professor Ann-Mari Svennerholm, University of Göteborg, Sweden, with instructions as described by Sjöling *et al*. [15]. CS4 positive isolates were tested by PCR using *css*A primers for the presence of CS6 structural subunit gene and the amplicons were hybridized with the labeled probe for CS6.

Statistical analysis

A Z-test was used to determine the statistical significance of the differences between the percentages obtained.

Results

Of the total of 179 isolates obtained in 1988, LT-only was detected in 56 (31.3%), ST-only in 108 (60.3%), and LTST in 15 (8.4%). The toxin profile for the 84 ETEC isolates obtained in 1998 was 31 LT-only (36.9%), 30 ST-only (35.7%) and 23 LTST (27.4%). No loss of toxin genes was observed in the 1988 isolates. ST-only was the most frequent toxin-type among the 1988 isolates followed by LT only, with the least frequent being the LTST type, whereas among the 1998 strains no significant difference in the prevalence of these toxin types was observed. The GM1-ELISA performed on randomly selected isolates confirmed the hybridization results. Combining the number of LT- or ST-positive only isolates with those producing both toxins (LTST) in each year showed that among the 1988 isolates, 71 (39.6%) and 113 (63.1%) carried the gene for LT or ST either alone or in combination respectively. In the 1998 isolates, 54 (64.2%) and 53 (63%) carried the LT or ST genes alone or concomitantly with the other toxin. These results showed that the rate of isolation of LT gene-carriage had significantly increased (Table 2). In this study, the available MAbs identified

Table 2. Distribution of toxin types among ETEC isolates in 1988 and 1998.

	No. (%) of toxigenic types			
Year	LT only	ST only	LTST	Total
1988	56 (31.3)	108 (60.3)	15 (8.4)	179
1998	31 (36.9)	30 (35.7)	23 (27.4)	84

Table 3. Prevalence of CF types in the two ETEC populations under study.

Toxin types	CF types (%)					Year
	CFA/I	CS3	CS4+ CS6	CS5+ CS6	CS6	
LT (n=56)	2 (3.6)	3 (5.3)	2 (3.6)	1 (1.8)	3 (5.3)	1988
ST (n=108)	25 (23.1)	10 (9.2)	2 (1.8)	2 (1.8)	15 (13.9)	
LTST (n=15)	2 (13.3)	1 (6.7)	1 (6.7)	1 (6.7)	2 (13.3)	
LT (n=31)	3 (9.7)	1 (3.2)	1 (3.2)	1 (3.2)	6 (6.4)	1998
ST (n=30)	*9 (30)*	*1 (3.3)*	*1 (3.3)*	*1 (3.3)*	*6 (20)*	
LTST (n=23)	*5 (21.8)*	*1 (4.3)*	*1 (4.3)*	*1 (4.3)*	*1 (4.3)*	

CFA/I, CS3, CS4, and CS5 with 16.2, 7.8, 6.7 and 5.6% respectively among the 1988 group; the rate of detection of these CFs for the 1998 population was 20.2, 3.6, 7.1 and 9.5%, making CFA/I and CFA/IV , which is composed of CS4, CS5, and CS6, the most prevalent CF types in both groups of isolates.

Hybridization of the isolates with the probe for the cfaD gene produced positive results in 45.8% (82/179) of the 1988 isolates and 46.4% (39/84) of the 1998 isolates respectively. The CS6 probe hybridized with 13.4% (24/179) and 14.3% (12/84) of the 1988 and 1998 isolates. Among the *cfa*D probe-positive isolates of both years, nearly 64.5% (78/121) were identified with the available MAbs. A total of 73 (40.8%) of the 1988 and 33 (39.3%) of the 1998 isolates did not react with either probe.

Among the bacterial populations from both years, CFA/I and CS6 were detected with almost equal frequency, whereas CS3 had a significantly lower rate of detection. Immunodot blot results with the available MAbs, as well as the data for CS6 probe, are given in Table 3.

Sensitivity to all the antibiotics used was observed in 25% (21/84) of the 1998 isolates compared to approximately 9% (16/179) of the 1988 isolates (Z value = 3.302). The percentage of resistant bacteria to various antibiotics showed some variations between the two study years, but apart from resistance to kanamycin, which showed a significant reduction (23.9% to 12.7%), the differences among other antibiotics were not statistically significant. High resistance to ampicillin (91.4% - 82.5%), trimethoprim /sulfamethoxazole (86.5% - 71.4%), streptomycin (54.6% - 50.8 %), chloramphenicol (47.8 - 41.3%) and tetracycline (42.9 - 34.9%) was observed. The percentage of resistance to cefalothin and nalidixic acid increased among the 1998 isolates and, although the differences were not statistically significant, the increasing trend was of note (Table 4).

Resistance to more than one antibiotic was observed in 90.8% (148/163) of the 1988 and 81% (51/63) of the 1998 isolates respectively. The difference between the prevalence of multidrug resistant isolates in the two study years was not statistically significant (Table 4). The most prevalent of the five antibiotic resistance patterns between both groups of isolates was ampicillin (A), chloramphenicol (C), streptomycin (S), tetracycline (T), and trimethoprim/sulfamethoxazole (SXT) (ACSTSXT) carried by 12.83% (29/226) of these bacteria. The most frequent pattern for resistance to four antibiotics was ASTSXT, which was observed in 7.96% (18/226) of the isolates and for resistance to three antibiotics, the ASSXT pattern was the most prevalent (11%).

Screening of the resistant isolates with *qacEΔ*I-*sulI* primers gave positive results with an amplicon size of 800 bp in 77.4% (175/226) of the isolates, of which 82.9% (145/175) gave amplicons of 700 bp (54 isolates), 1000 bp (13 isolates), and 1600 bp (78 isolates) with the *int*I primers. The most frequent variable region length among the isolates was 1600 bp and contained *dfrA1* and *aadA1* cassettes.

Discussion

Diarrhoea caused by ETEC is the most common cause of travellers' diarrhoea and can have fatal consequences for children under five years of age

Table 4. Antimicrobial resistance among the isolates studied.

	Percentage of resistance (no/subtotal)		
	1988	1998	Both
ampicillin	91.4 (149/163)	82.5 (52/63)	88.93 (201/226)
cefalothin	0.61 (1/149)	6.34 (4/63)	2.21 (5/226)
chloramphenicol	47.85 (78/163)	41.26 (26/63)	46.01 (104/226)
gentamycin	3.68 (6/163)	3.17 (2/63)	3.53 (8/226)
kanamycin	23.92 (39/163)	12.69 (8/63)	20.79 (47/226)
nalidixic acid	0.61 (1/163)	7.93 (5/63)	2.65 (6/226)
streptomycin	54.6 (89/163)	50.79 (32/63)	53.53 (121/226)
tetracycline	42.94 (70/163)	34.92 (22/63)	40.70 (92/226)
trimethoprim/ sulfamethoxasole	86.50 (141/163)	71.42 (45/63)	82.3 (186/226)
Multidrug resistance			
5-7 resistant markers	37.42 (61/163)	41.26 (26/63)	38.49 (87/226)
2-4 resistant markers	53.37 (87/163)	61.90 (39/63)	55.75 (126/226)

18]. Variation in the prevalence of toxin types as well as CFs may occur from year to year and among different geographic areas. In Bangladesh, a study conducted in 1980 reported a 4% prevalence for LT-only, 34% for ST-only and 62% for LT and ST isolates; these ratios, however, for 1992 were 58%, 38% and 4% respectively, a significant change in the ratio of LT-producers [11]. Katouli *et al*. showed that ST-only with a 78% rate was the most frequent toxin type followed by LT-only (14%) and LTST (8%) [19]. Dominance of the ST-expressing ETEC has been documented in Egypt, Bangladesh, and Tunisia [11,20,21]. However, a recent study conducted in Bangladesh concluded that ETEC strains have shifted from ST-producers to significantly more LT-producing strains similar to those observed in Latin American countries, such as Mexico, Peru, and Argentina, which have shown a high prevalence of LT-producing ETEC [22,11,23]. The results of this study also showed the predominance of the ST-producing strains in the 1988 isolates, but a significant increase in the detection rate of the LT-producers was observed among the 1998 ETEC isolates, and those previously reported [19]. Comparison of phenotypic and genotypic methods of toxin and CF detection has shown a good general agreement between the results obtained, although discrepancies in a few strains were reported [14,15]. We randomly compared only the results obtained for LT by DNA/DNA hybridization and PCR, and no discrepancy between these methods was observed.

Similar to the prevalence of enterotoxins, variation in the detection rate of CFs has also been reported, ranging from 30-60%, and the inability to detect any known CFs in approximately 30-50% of ETEC strains worldwide has been attributed to the absence of CFs, their loss, or lack of specific tools for their detection [11]. The most frequently encountered CFs are CFA/I, CFA/II and CFA/IV [1], although the rate for each group may vary from year to year and region to region [11]. In Tunisia, CFA/I (44.6%) was reported as the most common CF, followed by CS6 (11%), and in 44.6% of the isolates no CF was detected [21]. However, in Bangladesh, the rate for CFA/I varied between 23.5% and 27%, the rate for CFA/II was 21-22%, and the rate for CFA/IV was 31% to 29% in different years, and 44-45% of the isolates were CF negative in the studies reported [11]. In Egypt, the rate for CFA/I was reported as 9.7% in 2004, increasing to 15.6 % in 2009; for CFA/II, the rate was 7.3%, changing to 1.8%, and the CFA/IV rate increased from 12.6% in 2004 to 17.2% in 2009. More than half (59% and 53%) of the ETEC isolates in this region were not associated with any known CFs [20,2]. In this study, the prevalence of the CF types also showed variation between the two groups isolated 10 years apart, with CFA/I, CS4, CS5 and CS6 (CFA/IV)being the most common MAb-identified CFs (Table 3).

Classification of the CFs by antibody typing is time-consuming and requires reagents that are not readily available except in reference laboratories; additionally, the phenotypic expression is easily

affected by environmental factors such as culture conditions as well as genetic variations, changing the target epitope [8,12-16]. Although the PCR method is easier and faster to perform, it is intolerant of sequence variation, whereas the DNA-based hybridization is largely tolerant of minor base pair differences between alleles of the same genes. Furthermore, usually 30% to 50% of the ETEC isolates are without a detectable CF; therefore, a means of addressing this group of isolates at the start, followed by confirmatory tests for the positive strains, would be ideal. CfaD/Rns seems to be a suitable candidate and with a DNA probe there is always the possibility that as yet unknown CFs with similar relationships might be identified. DIG-labeling the PCR products using labeled primers has made this phase much easier and faster than labeling the PCR product by random hexamer or even PCR labeling kits, and DIG-labeled probes can be stored at -20°C for at least a year [12].

High resistance to antibiotics, especially ampicillin, trimethoprim/sulfamethoxazole (SXT), streptomycin, chloramphenicol and tetracycline, has been reported in recent studies dealing with both human and avian *E. coli* isolates in Iran [24,25], which follows the global trend [20]. This study showed that, by 1998, resistance to quinolones as well as cefalothin was emerging and, by 2008, resistance to cefalothin was reported in 37% of diarrhoeagenic *E. coli* [24].

Class 1 integrons are found extensively in clinical isolates and these elements confer resistance to all known β-lactams, all aminoglycosides, chloramphenicol, trimethoprim, and antiseptics of the quaternary-ammonium-compound family [26]. Integrons were detected in 64% and *sul*1-*qacEΔ*1 in 77.4% of the isolates. These results are in complete contrast to the 6.2% rate of class 1 integrons in a collection of multidrug resistant-uropathogenic *E. coli* in Shiraz, Iran [27]. The *sul1* gene is frequently located on class 1 integrons, which could be confirmed by the fact that, of the 175 *sul1*-positive isolates, 145 (82.8%) harboured integrons, but the inability to detect an integron in the remaining *sul1*-positive isolates might have been due to the suboptimal PCR conditions for longer products.

In conclusion, changes in the prevalence of virulence markers of the bacterial pathogens as are shown here are recognized to exist, emphasizing the need for constant monitoring of pathogenic microorganisms to determine the prevalence of virulence markers in bacterial populations.

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Corresponding author
Anis Jafari
Molecular Biology Unit
Pasteur Institute of Iran
Pasteur Avenue, Tehran 13164, Iran
Email: anisjafari@yahoo.com

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