Distribution of genes encoding toxins and antibiotic resistance patterns in diarrhoeagenic *Escherichia coli* isolates in Tehran

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توزُّع جينات ترميز الذيفانات، وتحديد أنماط مقاومة المضادات الحيوية في مستفرَدات الإشريكية القولونية، في طهران

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الخلاصة: من الممكن أن تكتنف إمراضية الإشريكية القولونية عدداً كبيراً من عوامل الفَوْعة، التي تُعَدُّ الذيفانات أكثرها وضوحاً. وقد قام الباحثون بتقييم توزُّع جينات ترميز الذيفانات بين مستفرَدات الإشريكية القولونية المأخوذة من عينات الإسهال، باستخدام مسابير الدنا. وتبيَّن أنه من بين 200 مستفرَدة، كانت 92 منها (46٪) تحمل جينات ترمِّز الذيفانات، من بينها 43.5٪ مولدة لذيفانات متعدِّدة. واكتُشِفَ وجود ذيفان معوي متكدِّس معوي صامد للحرارة في 40 مستفرَدة (43.5٪)، ووجود ذيفان فَوْعي في 38 مستفرَدة (41.3٪)، ووجود ذيفان مُوَسِّع قاتل للخلايا في 24 مستفرَدة (26.1٪)، ووجود ذيفان معوي صامد للحرارة في 12 مستفرَدة (13٪)، ووجود ذيفان معوي عَطُوب بالحرارة في 10 مستفرَدات (10.9٪). وتبيَّن كذلك أن 40 ذرية من هذه المستفرَدات (70٪) كانت مقاومة للمضادات الحيوية. واستنتج الباحثون من الدراسة أن توليد الذيفان، ومقاومة المضادات الحيوية هما العاملان الرئيسيان اللذان يحدِّدان الطاقة الفَوْعية لمستفرَدات الإشريكية القولونية.

ABSTRACT Pathogenicity of *Escherichia coli* can involve a large number of virulence factors, toxins being the most obvious. We assessed the distribution of genes encoding toxins among *E. coli* isolates from diarrhoeal cases using DNA probes. From 200 isolates, 92 (46.0%) carried genes encoding for toxins, 43.5% of these being multitoxigenic. Enteroaggregative heat-stable enterotoxin was detected in 40 (43.5%) isolates, verotoxin in 38 (41.3%), cytolethal distending toxin in 24 (26.1%), heat-stable enterotoxin in 12 (13.0%) and heat-labile enterotoxin in 10 (10.9%). Furthermore, 40 strains (70.0%) carried resistance. We conclude that toxigenicity and antibiotic resistance are the main contributing factors leading to the virulence potential of these *E. coli* isolates.

Distribution des gènes codant les toxines et détermination des profils d'antibiorésistance dans des isolats d'*Escherichia coli* diarrhéigène à Téhéran

RÉSUMÉ La pathogénicité d'*Escherichia coli* peut impliquer quantité de facteurs de virulence, les toxines occupant indéniablement la première place. À l'aide de sondes ADN, nous avons évalué la distribution des gènes codant ces toxines dans des isolats d'*E. coli* provenant de cas diarrhéiques. Sur 200 isolats, 92 (46,0 %) étaient porteurs des gènes codant les toxines, 43,5 % d'entre eux s'avérant « multitoxinogènes ». L'entérotoxine thermostable entéro-adhérente a été identifiée dans 40 isolats (43,5 %), la vérotoxine dans 38 (41,3 %), la toxine CDT (pour *cytolethal distending toxin* : toxine provoquant l'arrêt du cycle cellulaire et la distension du cytoplasme des cellules intoxiquées) dans 24 (26,1 %), l'entérotoxine thermostable dans 12 (13,0 %) et l'entérotoxine thermolabile dans 10 (10,9 %). En outre, 40 souches (70,0 %) étaient porteuses de facteurs de résistance. Nous avons conclu que la toxigénicité et l'antibiorésistance sont les principaux facteurs responsables du potentiel de virulence de ces isolats d'*E. coli*.

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Introduction

Diarrhoea is a leading cause of morbidity and mortality among children in developing countries [*1*]. The bacterial pathogen most commonly associated with endemic forms of childhood diarrhoea is *Escherichia coli* [*2*]. The pathogenicity of *E. coli* isolates is a complex, multi-factorial mechanism involving a large number of virulence factors that vary with pathotype. Some toxins show a strong association with specific pathotypes [*3*]. Verotoxins (VTs) I and II are prominent among the VT-producing pathotype and the enterohemorrhagic subgroup [*4*]. The heat-labile enterotoxins (LTs) are found in enterotoxigenic *E. coli* [*5*]. The heat-stable enterotoxins (STs) are also characteristic of enterotoxigenic *E. coli* pathotypes [*6*]. Another class of heat-stable toxin, enteroaggregative *E. coli* heat-stable enterotoxin (EAST-1) [*7*], is unrelated to STs and is not restricted to a specific pathotype. Cytolethal distending toxin (CDT) is yet another toxin family which was first described in *E. coli* [*8*] isolates, but later on was found in other enteric bacteria such as *Shigella* spp. [*9*], *Campylobacter* spp. [*10*], *Helicobacter* spp. [*11*], *Haemophilus ducreyi* [*12*], and *Actinobacillus actinomycetemcomitans* [*13*].

Another important factor that makes a major contribution to the pathogenic potential of a isolate is antibiotic resistance. Antimicrobial resistance in *E.coli* has been reported worldwide; resistance levels are usually high for broad-spectrum penicillin and trimethoprim and low for third-generation cephalosporins and nitrofurantoin. The emerging resistance to fluoroquinolones and the production of extended-spectrum β-lactamases by multidrug resistant *E.coli* has caused increasing concern recently owing to the limited therapeutic options available if infection with these strains occurs. Pathogen occurrence and susceptibility, however, show substantial geographic variation as well as significant differences in different populations and environments [*14*]. Antimicrobial resistance among clinical isolates is an important public health issue. Studies on the virulence reservoir of the isolates provide additional information on these pathogens. Therefore, in our study, *E. coli* isolates from diarrhoeal cases were analysed using specific DNA probes for genes encoding various toxins using hybridization assay. The distribution of antibiotic resistance in these isolates was also assessed.

Methods

Bacterial strains and plasmids

We identified 200 *E. coli* isolates according to standard procedures [*15*] and used them for this study. The isolates were obtained from children with diarrhoea hospitalized in a medical centre in Tehran during mid-August 2000 to mid-October 2000. Control strains H10407, O157:H7 and pUC18-VT were from our own collection and O42, 17-2 and plasmids CVD427, 403, were obtained from Professor JP Nataro (Center for Vaccine Development, School of Medicine, University of Maryland, USA). Plasmids SS125 and CVD448 were a gift from Dr C. LeBouguenec (Pasteur Institute, Paris, France) and were used as positive controls. We used *E. coli* K12 throughout as negative control for colony dot blot hybridization tests.

DNA extraction

DNA was extracted using the alkaline-lysis method of Birnboim and Doly [*16*] and was used in dot blot hybridization.

DNA probes

The DNA fragments used as probes in this study (Table 1) were obtained after diges-

Table 1 **DNA fragment probes**

Probe	Plasmid	Enzyme	Fragment size (bp)
Heat-stable enterotoxin	pCVD427	*Eco*RI	216
Heat-labile enterotoxin	pCVD403	*Hinc*II	1200
Verotoxin I	pUC18	*Eco*RI & *Kpn*I	1300
Enteroaggregative *E. coli* heat-stable enterotoxin	pSS125	*Kpn*I & *Xmn*I	111
Cytolethal distending toxin	pCVD448	*Acc*I	1375

tion of extracted plasmids with the appropriate enzyme, and were eluted from 1% agarose gel using DEAE membrane (Schleicher & Schuell). The purified fragments were used as probes after labelling with a non-radioactive labelling kit (Boehringer Mannheim).

Dot blot hybridization

Hybridization was carried out by a standard method according to the manufacturer's instructions after blotting the extracted DNA onto positively charged nylon membranes (Schleicher & Schuell). We used 25 ng of the DNA probes labelled with non-radioactive DIG-labelling kit for hybridization. Isolates were screened for presence of genes encoding LT and ST enterotoxins, VT, EAST-1 and CDT. An example of the results of the dot blot test with the EAST-1 probe is shown in Figure 1.

Antibiotic sensitivity test

The antibiotic resistance profile of the strains was assessed using a standard disk diffusion test [*17*] for the following antibiotics: chloramphenicol 30 µg, ceftizoxim 3 µg, ciprofloxacin 5 µg, cephalothin 30 µg, gentamicin 10 µg, kanamycin 30 µg, nalidixic acid 30 µg, sulfamethoxazole-trimethoprim 23.75 µg + 1.25 µg, and tetracycline 30 µg, all of which were obtained from bioMérieux, France.

Results

The hybridization assay using dot blot analysis showed that 92 (46.0%) of the 200 isolates examined were toxigenic. Of these, the most prevalent gene encoding for a toxin was EAST-1, encountered in 40 (43.5%) toxigenic isolates, followed by VT in 38 (41.3%) and CDT in 24 (26.1%). We found LT in 10 (10.9%) isolates and ST in 12 (13.0%).

The presence of a gene encoding only a single toxin occurred in 52 (56.5%) of the toxigenic bacteria. In the remaining isolates, 26 (28.3%) had genes encoding 2 toxins, 12 (13.0%) isolates carried genes encoding 3 different toxins and 2 (2.2%) isolates were positive with all 4 DNA probes.

The antibiotic susceptibility test showed that 140 (70.0%) isolates carried resistance markers. Of these, resistance to 1 antibiotic was observed in 36 (25.7%) of the isolates; 42 (30.0%) carried markers for 2 antibiotics, 41 (29.3%) for 3 antibiotics, followed by 13 (9.3%) for 4 antibiotics and 4 (2.9%) for 6 antibiotics. Sulfamethoxazole-trimethoprim, tetracycline and chloramphenicol were the least effective antibiotics since 112 (80.0%), 90 (64.3%) and 78 (55.7%) of the isolates were resistant to these antibiotics respectively. Ciprofloxacin, ceftizoxim and cephalothin were the most effective since all isolates (100%)

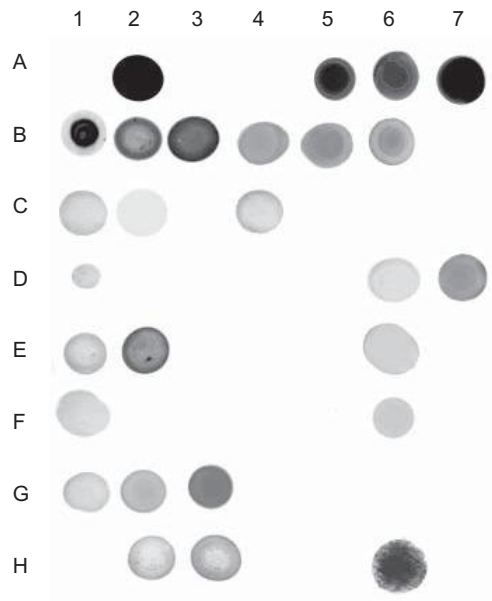

Figure 1 **Dot blot hybridization with EAST-1 probe (A1: blank, A2: plasmid SS125 [positive control], A3: K12 [negative control], A4: distilled water; A5–H5: clinical isolates, H6: strain 17-2)**

were sensitive to ciprofloxacin and only 10 (7.1%) were resistant to ceftizoxim and 6 (4.3%) resistant to cephalothin.

Among the isolates carrying genes encoding various toxins, 56 (60.9%) were resistant and 36 (39.1%) were sensitive to ≥ 1 of the antibiotics tested. Among isolates not harbouring genes encoding toxins, 84 (77.8%) were resistant and 24 (22.2%) were sensitive to ≥ 1 of the antibiotics used in this study.

Discussion

Owing to the vast knowledge of the genetic basis of *E. coli* virulence, genetic methods are more and more frequently applied in the detection and typing of *E. coli* [*3*]. In our study, *E. coli* isolates were analysed for the presence of genes encoding various toxins using a hybridization assay. We found that 46.0% of the isolates carried gene(s) for various toxins, although it has to be emphasized that reports on studies of this type are very rare in the Islamic Republic of Iran and other countries of this region. Although the importance of diarrhoeagenic *E. coli* in the Region has been indicated by other investigators [*18*], the real importance and the epidemiological value of these findings could be corroborated if more data were available.

The EAST-1 gene was the most prevalent, carried by 43.5% of the isolates. In a study conducted in Spain, *E. coli* strains producing EAST-1 were found to be significantly associated with diarrhoea, whereas enteroaggregative strains of *E. coli* (EAEC) (a group of diarrhoeagenic strains) were not [*19*]. Savarino et al. reported that 41% of the EAEC strains tested carried the EAST-1 gene (encoding the toxin), similar

to the findings of our study; not all EAST-1 positive isolates, however, belonged to the EAEC group [*7*]. Recent studies have indicated the importance of diarrhoeagenic *E. coli* that possess the EAST-1 gene [*20,21*].

E. coli isolates producing VT are considered potential pathogens, but a suitable marker to predict the virulence potential of this group of organisms is not yet known [*22*]. In our study, just over 40% of the isolates showed the presence of VT genes. In some recent studies, the virulence potential of VT-producing *E. coli* isolates has been proven [*6,23*].

E. coli isolates producing CDT are a recently recognized group, whose potential virulence is not yet fully defined. In our study, 12.0% of the isolates (26.1% of the toxigenic isolates) carried the genes encoding CDT. This is the first report of the presence of CDT genes among *E. coli* isolates in the Islamic Republic of Iran. Consequently, to establish the role of this group, further epidemiological studies are required. Reports from other parts of the world have, however, indicated the production of this toxin in *E. coli* isolates from cases of diarrhoea [*6,24,25*]. Although in our study the strains carrying LT and ST genes were not found to be widespread, differences in the prevalence of these toxins among *E. coli* isolates has been reported in epidemiological studies from different geographical regions [*26,27*]. The low prevalence of isolates carrying LT and ST genes in our study could be related to the time of sample collection, since the prevalence of these toxins has been shown to be dependent on season [*25*].

In brief, toxigenicity was found to be prevalent among *E. coli* isolates in our setting. Although the occurrence of EAST-1 and VT was high, in order to establish the epidemiological importance of these toxins, further detailed studies are warranted.

Resistance to various antibiotics was another prevalent feature of our *E. coli* isolates, and a widely distributed characteristic. Reports from other countries [*14,26–28*] also indicate that resistance to > 1 antibiotic is prevalent among clinical isolates. In our study, a high-level of resistance to sulfamethoxazol-trimethoprim, tetracycline and chloramphenicol was observed. Similar findings were reported by investigators from other regions [*14,28*]. The increasing prevalence of resistance to various antibiotics is one of the most important public health concerns, and is mostly a result of environmental conditions and high consumption of antimicrobial agents [*28*]. Consequently, updating of guidelines for the appropriate use of antibiotics in developing countries and constant monitoring to safeguard against inappropriate use of these substances is necessary.

In conclusion, it seems toxigenicity and resistance to antibiotics are the main contributors to the virulence potential of the *E. coli* isolates in our setting.

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