Detection and characterization of verotoxin-producing strains among sorbitol non-fermenting *Escherichia coli*

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اكتشاف وتعيين خصائص الذراري المنتجة للفيروتوكسين بين عصيات الإشريكية القولونية غير المخمرة للسوربيتول

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الخلاصة: تم تقييم وجود جينات الفيروتوكسين 1 و2 من بين مستفرَدات الإشريكية القولونية غير المخمرة للسوربيتول، من حالات الإسهال، باستخدام مقايسة تفاعل سلسلة البوليمراز. وتبيَّن أن 37 مستفرَدة (أي 62%) من 60 مستفردة إيجابية كانت تتوي جينات الفيروتوكسين 1 وأن 23 (أي 38%) منها كانت تتوي جينات الفيروتوكسينَيْن 1 و2. وقد أبدت 48 مستفرَدة (أي 71%) في مقايسة الالتصاق بخلايا HeLa التصاقاً مقاوماً للمانوز بخلايا HeLa. ولوحظت مقاومة 56 (أي 82%) مستفرَدة لأدوية متعددة، وكان أكثرها شيوعاً مقاومة الأمبسلين والكلورامفينيكول، والستربتوميسين، والسلفاميثوكسازول - ثلاثي الميتوبريم، والتتراسيكلين. وتم تحديد 13 نمطاً ظاهرياً كيميائياً حيوياً مشتركاً و22 نمطاً ظاهرياً كيميائياً حيوياً منفرداً. وكانت المستفردات المنتمية إلى الأنماط الظاهرية الكيميائية الحيوية المشتركة تتشابه عادةً من حيث نمط الالتصاق وإنتاج الفيروتوكسين، مع اختلافها إلى حد بعيد من حيث نمط مقاومة المضادات الحيوية، حيث تميزت على هذه المستفردات بارتفاع معدل تحوُّل المقاومة للمضادات الحيوية.

ABSTRACT The presence of genes for verotoxin 1 and 2 (VT1 and 2) among sorbitol non-fermenting *Escherichia coli* isolates from diarrhoeal cases was assessed using polymerase chain reaction assay. Of 60 (88%) positive isolates, 37 (62%) harboured VT1 and 23 (38%) both VT1 and VT2. In HeLa cell adherence assay, 40 (71%) isolates exhibited mannose-resistant adherence to HeLa cells. Multidrug resistance was observed in 56 (82%) isolates, with ampicillin, chloramphenicol, streptomycin, sulfamethoxazole–trimethoprim and tetracycline pattern being the most common. There were 13 common and 22 single biochemical phenotypes identified. Isolates belonging to common biochemical phenotypes normally had a similar pattern of adherence and VT production, but differed greatly in their pattern of antibiotic resistance, pointing to a high rate of antibiotic-resistance transfer among these isolates.

Détection et caractérisation de souches produisant des vérotoxines parmi les *Escherichia coli* ne fermentant pas le sorbitol

RESUME La présence de gènes des vérotoxines 1 et 2 (VT1 et 2) parmi des isolats d'*Escherichia coli* ne fermentant pas le sorbitol et provenant de cas de diarrhée a été évaluée par technique d'amplification génique. Sur les 60 isolats positifs (88 %), 37 (62 %) hébergeaient la VT1 et 23 (38 %) la VT1 et la VT2. Dans la détermination de l'adhérence aux cellules HeLa, 48 (71 %) des isolats ont montré une adhérence aux cellules HeLa résistantes au mannose. Une polypharmacorésistance a été observée sur 56 isolats (82 %). Il y avait 13 phénotypes biochimiques communs et 22 phénotypes biochimiques individuels identifiés. Les isolats appartenant aux phénotypes biochimiques communs avaient normalement un profil d'adhérence et de production de VT similaire, mais différaient grandement dans leur schéma de résistance aux antibiotiques, indiquant un taux élevé de transfert de la résistance aux antibiotiques parmi ces isolats.

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Introduction

Verotoxin-producing *Escherichia coli* (VTEC), to which serotype O157:H7 belongs, is now recognized as an important human pathogen of public health concern [*1*]. Infection with *E. coli* strains, especially O157:H7, which may not be apparent, results in a spectrum of disease ranging from mild, non-bloody diarrhoea to haemolytic-uraemic syndrome [*2*]. Epidemiological studies have shown that serotype O157:H7 accounts for 40%–70% of *E. coli* infections [*3*]. Since this organism does not ferment sorbitol, or fermentation is delayed, sorbitol MacConkey (SMAC) agar is used as a primary screening medium [*4*]. One of the key characteristics of this group of *E. coli* organisms is their ability to produce Shiga-like toxins, also known as verotoxins (VTs), which are cytotoxic to Vero and HeLa cells [*2*]. The development of highly sensitive and specific probes, as well as various polymerase chain reaction (PCR) methods to detect VT gene sequences in *E. coli* has altered the dependence on cell culture assay as the only single method of identification of VTEC strains from clinical and faecal samples [*5*].

In addition to toxin production, adherence of bacteria to the intestinal mucosa is also considered to be a critical initial step in pathogenesis [*6*]. It has been shown that diarrhogenic *E. coli* adhere to various cell lines in characteristic patterns, namely: diffuse, localized and enteroaggregative; the latter two are associated with distinct subgroups of *E. coli* [*7*]. Recently, a new pattern of adherence for enterohaemorrhagic *E. coli* (EHEC) named 'log jam' has been described, which has been observed in both pathogenic and non-pathogenic *E. coli* strains [*8*].

In this study, the occurrence of verotoxin genes and adherence pattern among sorbitol non-fermenting (SNF) *E. coli* strains isolated from children with diarrhoea was investigated. The possible clonal nature of these isolates was also studied, using biochemical fingerprinting, and also the antibiotic-resistance pattern.

Methods

Bacterial strains

On SMAC agar plates, we cultured 1200 *E. coli* strains which did not belong to classical enteropathogenic strains (EPEC) serogroups using poly- and monovalent antisera (Diagnostic Pasteur, France), and which did not produce heat-labile and heat-stable enterotoxin using a reverse passive latex agglutination test (Denka Seiken, Japan) and infant mouse assay respectively. These strains were also tested by the Sereney test for invasiveness [*9*].

Serology

Boiled suspensions of SNF strains were tested by slide agglutination against O157 antiserum (Difco).

PCR assay

DNA was isolated by the method of Kado and Liu [*10*] and amplification was performed according to Cebulla et al. [*11*]. using 35 cycles of 1.5 minutes denaturation at 94 °C, 1.5 minutes annealing at 64 °C, and 1.5 minutes extension at 72 °C with the following primers:

- VT1: 5′-CAGTTAATGTGGTGGCGA AGG 3′

  5′-CACCAGACAATGTAACCGCTG-3′
- VT2: 5′-ATCCTATCCCCGGGAGTT TACG-3′

  5′-GCGTCATCGTATACACAGG AGC-3′

The amplified products (348 and 584 bp for VT1 and VT2 respectively) were visualized by ultraviolet light after electrophoresis on 0.8% agarose stained with ethidium bromide.

Adherence assay

All SNF strains were tested for their adherence to HeLa cells in the presence of 1% D-mannose [*6*]. Adherence patterns were assessed according to Scaletsky et al. [*6*] for localized and diffuse, Nataro et al. [*7*] for enteroaggregative, and Mckee et al. [*8*] for log jam. *E. coli* HB101 was used as negative control.

Antimicrobial susceptibility test

SNF strains were tested for their sensitivity to 10 commonly used antibiotics by a disk diffusion method [*12*]. The following antibiotics were used: ampicillin, nalidixic acid, cephalotin, streptomycin, gentamicin, tetracycline, sulfamethoxazole–trimethoprim, nitrofurantoin, kanamycin, and chloramphenicol. All disks were from bioMérieux, France.

Biochemical fingerprinting

SNF strains were typed with the Phene Plate system specifically developed for typing of *E. coli* strains [*13,14*]. Suspension of bacterial cultures were inoculated into pre-prepared microtitre plates, kept at 4 °C overnight and incubated at 37 °C the following morning. The absorbance at 260 nm of each reaction was measured at different time intervals with a microplate reader and the data were automatically transferred to a computer. After the final reading, the mean value of all readings was calculated to yield a biochemical fingerprint for each isolate. Similarities between the strains were calculated as correlation coefficients and clustered according to the unweighted pair group method with arithmetic averages yielding a dendogram. From the reproducibility of the system, an identity level of 0.975 was chosen. Strains showing similarities to each other higher than this value were regarded as identical and assigned to the same biochemical phenotype. Biochemical phenotypes with more than one isolate were called common (C), and those with only one isolate were called single (S) biochemical phenotypes.

Results

Of the 1200 *E. coli* strains cultured on SMAC agar, 68 (5.7%) did not ferment sorbitol. None of these belonged to serogroup O157. However, of the 68 isolates, 60 (88.2%) were positive with the primers designed for detection of VT1 and VT2, of which 37 (61.7%) possessed VT1 gene alone, and 23 (38.3%) harboured both VT1 and VT2 simultaneously. No isolates carrying VT2 sequences alone were detected, and 8 isolates (13.3%) did not give any PCR product with the primers used (Figure 1).

Of the 68 SNF isolates, 57 (83.8%) were resistant to more than two antibiotics, and a total of 25 different antibiotic resistance patterns were observed among the isolates. There were 3 strains sensitive to all antibiotics tested. Resistance to five antibiotics was the most prevalent pattern observed in 21 strains (31%), with the ampicillin, chloramphenicol, streptomycin, sulfamethoxazole–trimethoprim, tetracycline pattern being the most common in 14 isolates (21%). Ampicillin resistance marker alone or in combination with others was carried by 95% of the isolates.

There were 48 isolates (71%) that exhibited mannose-resistant adherence to HeLa cells in four different characteristic patterns (Figure 2). Localized adherence

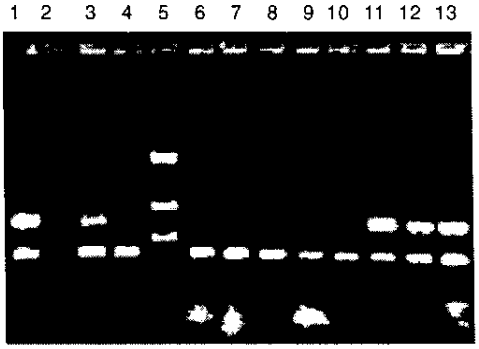

Figure 1 **Polymerase chain reaction products from isolates harbouring VT1 and VT2. Lane 1: 0157, lane 2: HB101, lane 5: MW marker (1444, 736, 476 bp). The remaining lanes show wild isolates.**

was seen in 22 strains (46%), log jam in 11 (23%), enteroaggregative in 10 (21%) and diffuse in 5 (10%). There were 20 isolates (29%) that were non-adherent.

Based on biochemical phenotypes, 35 patterns comprising 35 common and 22 single biochemical phenotypes were identified. The most common biochemical phenotypes (i.e. C6 and C8) contained 8 and 6 isolates respectively. The relation between biochemical phenotype and other virulence markers tested among these strains is shown in Figure 3. Generally, isolates belonging to common biochemical phenotypes had a similar pattern of adherence and VT production, although the correlation was less pronounced with antibiotic resistance.

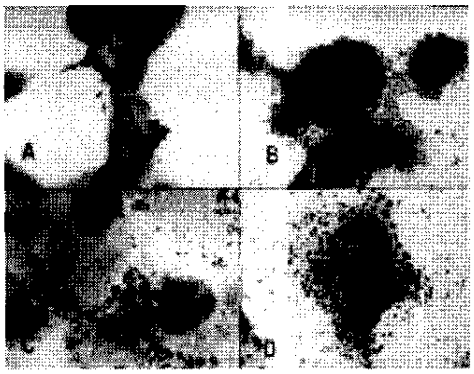

Figure 2 **Cultured Hela cells incubated with strains of *Escherichia coli* showing: a) localized, b) enterohaemorrhagic, c) log jam and d) diffuse**

Discussion

SMAC agar has been used in many laboratories for presumptive identification of O157:H7 strains followed by agglutination with specific antisera for serotype confirmation [*1*]. In our study, no SNF isolates were agglutinated with O157 antisera — indicating that this particular serotype is not a common cause of diarrhoea in the Islamic Republic of Iran. This result accords with reports from Asia, Serbia and Spain, and also from western Islamic Republic of Iran [*15–18*]. This finding also confirms the observation made by Ojeda et al. [*4*] that sorbitol negativity, although a common phenotype among the EHEC group, is not serotype specific.

Based on the primary screening with SMAC, there was a prevalence rate of 5% for isolates harbouring the gene for VTs, whereas in a report by Aslani et al. [*18*], in which diarrhoeal isolates were screened indiscriminately, a rate of 9.6% for VT-producing isolates was found. The discrepancy might be due either to variation in geographical distribution of these strains, or SMAC screening may have resulted in an underscoring of VTEC strains. Recently, the results of an evaluation comparing Vero cell assay, PCR and a sandwich ELISA "RIDASCREEN Verotoxin" showed

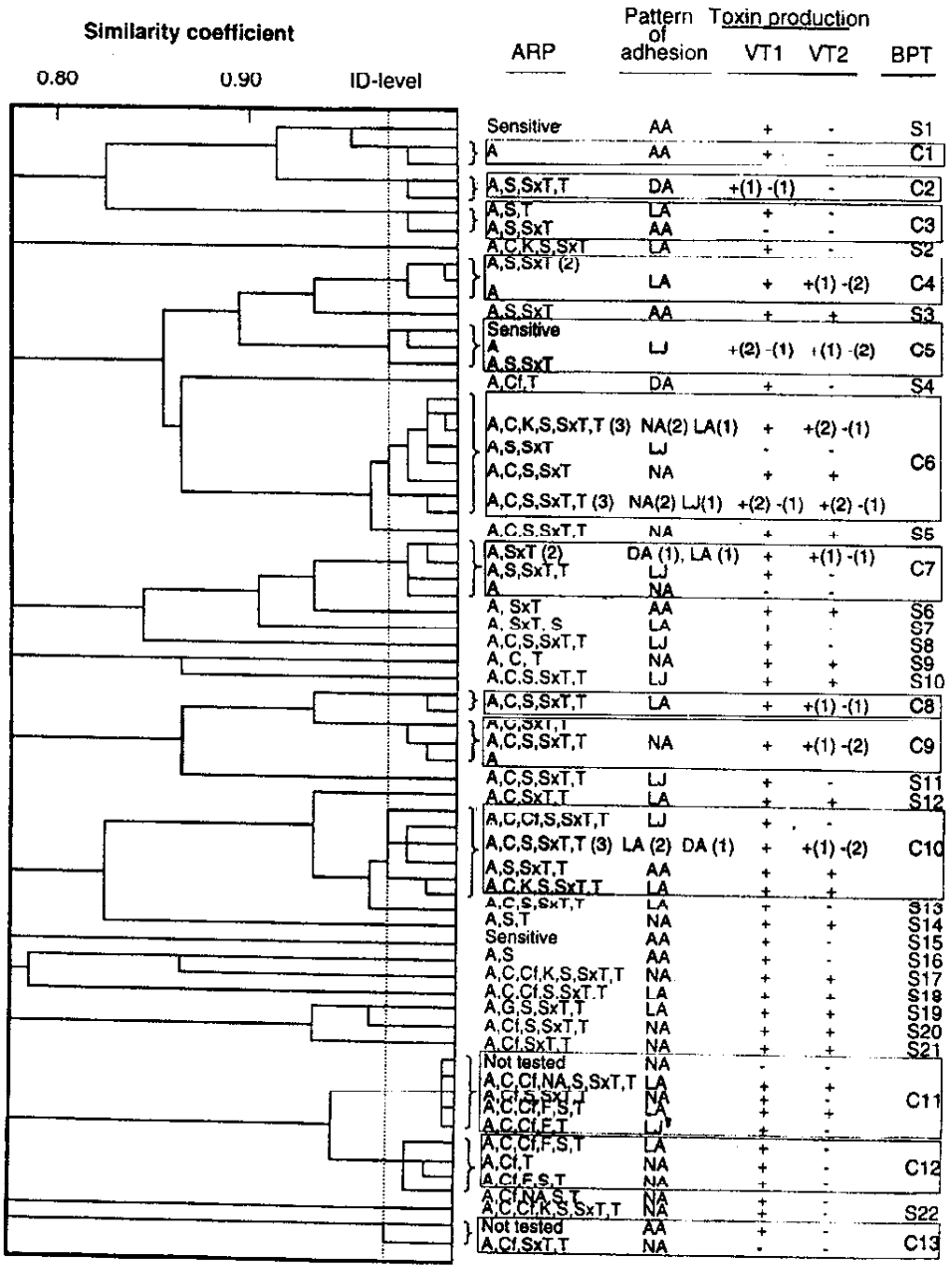

Figure 3 **Dendogram showing similarity coefficient of sorbitol non-fermenting isolates in association with adhesion pattern, toxin production and antibiotic resistance**

that the commercial ELISA system was as sensitive and specific as PCR, with the added advantage of rapidity and ease of performance for identification of VTEC O157:H7 [*19*].

In addition to the production of verotoxin, adhesion of VTEC strains to epithelial cells is a critical primary step in bacterial colonization of the host [*20*]. We found that 71% of the isolates were adherent, with the localized pattern being dominant. Localized adherence in association with attaching and effacing lesions has been considered the hallmark of EPEC strains [*21*]. Although no strains in our study belonged to the classical EPEC serotypes, a homologous chromosomal *eae* gene essential for attaching and effacing lesion has also been identified in some VT-producing strains [*22*]. In our investigation, 23% of the isolates adhered to HeLa cells, in a pattern termed log jam by Mckee et al. [*8*], a previously unrecognized pattern. Association of this pattern with virulence has not been established.

Further characterization of the SNF strains with biochemical fingerprinting revealed that strains belonging to the same biochemical phenotypes generally had similar virulence properties. However, this was not consistent with the antibiotic resistant pattern since in most cases, different antibiotic resistant patterns were observed in strains belonging to a common biochemical phenotype. This is probably due to the fact that antibiotic resistance is transferable to other strains. Nevertheless, the presence of several different biochemical phenotypes among SNF strains in our study points to the biochemical heterogeneity of these isolates, emphasizing the endemic status of VT-producing *E. coli* strains in the Islamic Republic of Iran.

Conclusion

The data obtained in this study suggest that while O157:H7 strains are not a common cause of childhood non-bloody diarrhoea in Teheran, non-O157:H7 SNF VTEC strains may play an important role in the pathogenesis of *E. coli* diarrhoea in this part of country.

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