Occurrence of genes encoding enterotoxins in uropathogenic *Escherichia coli* isolates

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Abstract

To determine the presence of some toxins of diarrheagenic *Escherichia coli* (DEC) in uropathogenic *E. coli* (UPEC), 138 urinary tract infection (UTI)-causing UPECs were analyzed. The *astA*, *set*, *sen* and *cdtB* genes were detected in 13 (9.4%), 2 (1.3%), 13 (9.4%) and 0 (0%) of UPEC isolates respectively. The results show that some genes encoding toxins can be transferred from DEC pathotypes to UPECs therefore these isolates can transform into potential diarrhea-causing agents.

Key words: uropathogenic *Escherichia coli*, toxins, phylogenetic groups, *set*, *sen*, *astA* and *cdt* genes.

*Escherichia coli* is a commensal of the human intestine. However sometimes it causes extra-intestinal infections such as urinary tract infections (UTIs), in this case they are named uropathogenic *E. coli* (UPEC) (Abe et al., 2008; Oliveira et al., 2011). They differ from commensal and diarrheagenic strains with respect to phylogenetic groups and virulence factors (Sabate et al., 2006). Commensal strains mostly belong to phylogenetic group A and B1 while most extra intestinal pathogenic *E. coli* (ExPEC) strains fall into group B2 or D (Abdallah et al., 2011; Clermont et al., 2000; Johnson and Stell, 2000; Molina-López et al., 2011).

Enteroaggregative heat stable toxin 1 (EAST-1), a 38 amino acid peptide, is encoded by the *astA* gene located on the 60-MDa pAA plasmid common to most enteroaggregative *E. coli* (EAEC) strains (Mendez-Arancibia et al., 2008; Telli et al., 2010; Vila et al., 2000). In addition to the *astA* gene, this plasmid contains genes encoding adherence fimbria (AAFI and AAFII) (Mendez-Arancibia et al., 2008). The *astA* gene is present in commensal, aggregative, and nonaggregative *E. coli* strains (Telli et al., 2010; Vila et al., 2000). The toxin encoded by this gene stimulates the production of high levels of cyclic guanosine monophosphate (cGMP) in the cell such that sodium (Na)/chloride (Cl) ions cotransport system is inhibited and absorption of water and electrolytes from the intestine at villus tips is reduced, resulting in the elevation of secretion of Cl and water in crypt cells (Telli et al., 2010).

*Shigella* enterotoxin 1 (ShET1), a virulence factor in EAEC, was detected for the first time in *Shigella flexneri* 2a. This enterotoxin is encoded by chromosomal *set* genes located on the antisense strand of mucinase gene in *S. flexneri* strains and EAEC (Telli et al., 2010; Vila et al., 2000). The *set* genes encoding this toxin contain 2 contiguous open reading frames (ORFs) of 534 (*setlA*) and 186 (*setlB*) bp (Fasano et al., 1997). These genes are located on the she pathogenicity island (PAI), a 46-kb chromosomal element that carries some genes having potential or established roles in bacterial virulence. The watery phase of diarrhea in shigellosis is caused by this toxin (Thong et al., 2005).

*Shigella* enterotoxin 2 (ShET2), a 62-8 kDa single protein, is encoded by the *sen* gene located on the 140-MDa invasion plasmid (Fasano et al., 1997; Olesen et al., 2012; Telli et al., 2010). This toxin is found in most species of *Shigella* as well as enteroinvasive *E. coli* (EIEC) strains (Farfán et al., 2011; Fasano et al., 1997; Yavzori et al., 2002). Cytotoxic distending toxin (CDT), a complex protein, contains 3 polypeptides CdtA, CdtB, and CdtC. This toxin has DNase I activity and breaks double-strand DNA.
and therefore is called genotoxin or cyclomodulin. Five types of CDTs have been found in \textit{E. coli} strains thus far. Some of these CDTs are encoded by genes located on plasmids; for example, gene encoding CDT-III is carried by pVir, a conjugative plasmid, while others are encoded by genes carried by a lambdoid or P2 phages (Vargas \textit{et al.}, 1999). Because some virulence factors (VFs) of diarrhea-genic \textit{E. coli} (DEC) such as EAST, SHET1, ShET2, and CDT toxins are located on PAIs, plasmids and other mobile genetic elements, this study aimed to investigate the presence of these toxins in UPEC isolates and their relationship with phylogenetic groups in order to understand the genetic diversity of UPEC strains.

One hundred and thirty-eight UPEC clinical isolates were investigated in this study. These bacteria were isolated from urine samples of patients with UTI referred to clinical laboratories of Isfahan, Iran. UPEC was confirmed by a positive urine culture with at least 10^5 cfu of \textit{E. coli} /mL. These isolates were identified by standard laboratory protocols. In addition, 30 \textit{E. coli} isolates were collected from feces of healthy humans and were used as controls. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki (No 63/21/8/90).

\textit{E. coli} isolates were inoculated in Luria Bertani broth and incubated overnight at 37 °C. Total DNA was obtained by using the boiling method. Bacteria were pelleted from broth, resuspended in sterile distilled water, and boiled at 95 °C for 10 min. Next, the samples were centrifuged at 14,000 rpm for 5 min. The supernatants were collected used as DNA template and stored at -20 °C. For confirming \textit{E. coli} isolates, PCR was performed to amplify a fragment of the gene encoding for the highly specific \textit{E. coli} universal stress protein \textit{A} (\textit{uspA} gene). PCR primers and conditions were described by Chen and Griffiths (1998).

For phylogenetic groups, two genes of \textit{chuA}, \textit{yjaA}, and a DNA fragment TSPE4.C2 were investigated by a triplex PCR method designed by Clermont \textit{et al.} (2000). This reaction was performed in a final volume of 20 μL, containing DNA (2 μL boiling lysate), 3 mM MgCl2, 0.4 mM dNTP, 2.5 U Taq DNA Polymerase (CinaGen, Iran), 1x Taq DNA Polymerase Buffer, and 0.4 μM of each primer. The thermal cycler (Bio-Rad-icycler, America) conditions were as follows: 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s and final extension of 7 min at 72 °C. All primers used are listed in Table 1. Detection of ShET1, ShET2, and EAST-1 enterotoxins encoded by \textit{set}, \textit{sen}, and \textit{astA} genes, respectively, was done by amplifying these genes using primers reported previously (Abe \textit{et al.} 2008). In addition, the \textit{cdtB} gene encoding cytolethal distending toxin was also amplified as suggested by Johnson and Stell (2000). The \textit{astA} gene PCR condition was set up as follows: 94 °C for 3 min followed by 30 cycles of 94 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min and 5 min at 72 °C as final extension. PCR was carried out in a 20 μL volume containing 2 μL of 10x Taq Polymerase buffer, 3 mM MgCl2, 0.8 μM of each primer, 0.4 mM dNTP, 1 U of Taq polymerase (CinaGen, Iran), and 1 μL of template DNA. For \textit{set} and \textit{sen} genes PCR conditions were similar and involved denaturation at 95 °C for 3 min, 30 cycles of denaturation at 95 °C for 30 s, 156

Table 1 - Targets, names, sequences (5’-3’), and product sizes of DNA primers.

| Gene | Primer name | Sequence in 5’ → 3’ direction | Size of product (bp) | Reference |
|------|-------------|-------------------------------|---------------------|-----------|
| \textit{set-1B} | ShET-1B upper | GTGAACCTGCTGCCGATATC | 147 | Fasano \textit{et al.}, 2011 |
| | ShET-1B lower | ATTTGTGGAATAAAAATGACG | | |
| | ShET-2 upper | ATGTGCCTGCTATTATTTAT | 799 | Nataro \textit{et al.}, 1995 |
| | ShET-2 lower | CATATAATAAAGGTCAGC | | Vila \textit{et al.}, 2000 |
| \textit{astA} | East-upper | ATGCCATCAACACAGTATAT | 110 | Johnson and Stell, 2000 |
| | East-lowe | GCCGAGTGCACCGCTTGTAG | | |
| \textit{cdtB} | cdt-a1 | AAATCACCAAGAATACCGTAT | 430 | Chen and Griffiths, 1998 |
| | cdt-a2 | AAATCTCTGCAATACCTCAGTAT | | Clermont \textit{et al.}, 2010 |
| | cdt-s1 | GAAAGTAAATGGAATATAAATGTCCG | | Clermont \textit{et al.}, 2010 |
| | cdt-s2 | GAAAATAAATGGAACACACACATACGTCAG | | Clermont \textit{et al.}, 2010 |
| \textit{uspA} | uspA-up | CCCGCTACCTGCAAATCAT | 883 | Clermont \textit{et al.}, 2010 |
| | uspA-down | ACCGAGCCCTGAGGCGAC | | |
| \textit{ChuA} | ChuA.1 | GACGAAACCAACGTCAGG | 279 | | |
| | ChuA.2 | TGCCGACACATCAAGAAC | | |
| \textit{YjaA} | YjaA.1 | TGAAGTGTGACAGGAGCTG | 211 | | |
| | YjaA.2 | ATGGAGAATGCTTACGTCAC | | |
| \textit{TspE4C2} | TspE4C2.1 | GAGTAAATGTCGCGGCGATT | 152 | Clermont \textit{et al.}, 2010 |
| | TspE4C2.2 | CGCAGAACCAAAGTATTACG | | |
annealing at 55 °C for 90 s, extension at 72 °C for 2 min and one final extension cycle at 72 °C for 7 min. The PCR assays were performed in a final volume of 25 μL, containing DNA (1 μL boiling lysate), 3 mM MgCl₂, 0.4 mM dNTP, 1 U Taq Polymerase (CinnaGene, Iran), 1x PCR Buffer, and 0.4 μM primers. The cdtB gene was also amplified as described previously by Johnson and Stell (2000). The association between different groups and presence of investigated genes was assessed using Pearson Chi-square test or Fisher’s exact test with the SPSS 16.0 software. Results were considered as statistically significant at p < 0.05.

All the 168 UPEC and commensal isolates were confirmed as E. coli by standard laboratory protocols. In addition the uspA gene was detected in all the UPEC isolates. Of the 138 UPEC isolates, 16 (12%), 76 (55%), 29 (21%), and 17 (12%) strains belonged to phylogenetic groups B1, B2, D, and A respectively. Concerning to the 30 commensal E. coli isolates, 9 (30%), 12 (40%), and 9 (30%) bacteria were allocated into B2, D, and A groups, respectively but none of the isolates tested clustered in B1 group. A comparison of UPEC strains in different phylogenetic groups showed that B2 group isolates were significantly higher than those belonging to other phylogenetic groups (p ≤ 0.001). For the commensal isolates, D group isolates were statistically more significant than those belonging to other groups (p ≤ 0.001). These details are shown in Table 2. Presence of 4 genes of set, sen, astA, cdtB was investigated in UPEC and commensal E. coli isolates. The astA gene was detected in 13 (9.4%) UPEC and 5 (16.6%) E. coli isolates collected from feces. The set gene was detected in only 2 (1.3%) UPEC isolate and was not amplified for any of the commensal E. coli isolates tested. The sen gene was detected in 13 (9.4%) UPEC isolates and in 2 (6.6%) commensal isolates. The cdtB gene was not found among the UPEC and E. coli commensal strains analyzed. None of these differences were statistically significant (p > 0.05). Subsequently, the association between phylogenetic groups and these toxins was investigated. No statistically significant correlation was seen between phylogenetic groups and these factors (p > 0.05). Of the 13 astA-positive UPEC isolates, 9 (69.2%), 2 (15.4%), 1 (7.7%), and 1 (7.7%) belonged to B2, B1, D, and A phylogenetic groups, respectively. Both the set-positive isolates belonged to the B2 phylogenetic group. Six (46%) UPEC isolates carrying the sen gene belonged to the D group, 4 (31%) to the B2 group, 2 (15%) to the B1 group and 1 (8%) to the A group. Although more enterotoxins were observed in the B2 phylogenetic group than in other groups, the different was not statistically significant. Three UPEC isolates had both set and sen genes and belonged to the B2 phylogenetic group.

In the present study, we investigated the prevalence of different toxins and phylogenetic groups in UPEC isolates. Our data showed few differences and similarities with other studies performed on UPECs. The high prevalence of the B2 phylogenetic group observed in our study is in agreement with that observed in previous studies by other researchers who found a high prevalence of the B2 phylogenetic group in ExPEC pathogenic strains (Abdallah et al., 2006; Clermont et al., 2000; Molina-López et al., 2011). The structural astA gene, identified first in EAEC, encodes a low-molecular weight enterotoxin EAST-1 (Yatsuyanagi et al., 2003). In addition to EAEC, this gene has been found in enterohemorrhagic E. coli (EHEC), enteropathogenic E. coli (EPEC), atypical enteropathogenic E. coli (A-EPEC), Enterotoxigenic E. coli (ETEC), and Shiga Toxin-Producing E. coli (STEC), (Contreras et al., 2011; Paiva de Sousa et al., 2001; Yatsuyanagi et al., 2003) and other members of Enterobacteriaceae such as Salmonella (Paiva de Sousa et al., 2001). Abe et al. (2008) found that some UPEC isolates carried the gene sequences aggR, aggC, aap, and astA, which are located in the conserved and large plasmid pAA. In their study, 7.1% out of 225 UPEC isolates were found to be astA positive. Soto et al. (2009) detected the astA gene in 8% of 170 UPEC clinical isolates; in the present study, this gene was detected in 9.4% UPEC isolates. Furthermore, the astA gene was found in commensal isolates. This result is in accordance with that reported by Vila et al. (2000), who found the astA gene in a significant proportion of E. coli intestinal isolates that did not cause diarrhea, suggesting that this toxin is insufficient to cause diarrhea without presence of other virulence fac-

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**Table 2 - Distribution of different genes and relationship with phylogenetic groups in clinical and commensal isolates of E. coli.**

| Gene | Number of cases | UPEC isolates | Phylogenetic groups of UPEC isolates | Commensal E. coli isolates | Phylogenetic groups of commensal E. coli isolates |
|------|----------------|--------------|-------------------------------------|---------------------------|--------------------------------------------------|
|      |                |              | A | B1 | B2 | D | A | B1 | B2 | D |
| set  | 138 (%)        | 17 (%)       | 16 (%) | 76 (%) | 29 (%) | 30 (%) | 9 (%) | 0 (%) | 9 (%) | 12 (%) |
| sen  | 13 (9.4)       | 1 (5.8)      | 2 (12.5) | 4 (5.2) | 6 (20.7) | 2 (6.6) | 0 (%) | 0 (%) | 0 (%) | 0 (%) |
| astA | 13 (9.4)       | 1 (5.8)      | 2 (12.5) | 9 (11.8) | 1 (3.4) | 5 (16.6) | 0 (%) | 0 (%) | 4 (44) | 1 (8) |
| cdtB | 0 (0)          | 0 (0)        | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (%) | 0 (%) | 0 (%) | 0 (%) |
| astA+sen | 3 (2.2) | 0 (0) | 0 (0) | 3 (3.9) | 0 (0) | 0 (0) | 0 (%) | 0 (%) | 0 (%) | 0 (%) |

UPEC, uropathogenic E. coli.
tors. ShET1 is a 55-kDa protein encoded by the set gene located on the antisense strand of a mucinase gene in S. flexneri and EAEC (Roy et al., 2006; Telli et al., 2010). This PAI has been found in other bacteria such as Yersinia enterocolitica, Salmonella typhimurium, pathogenic E. coli isolates but not in any diarrhea-causing bacteria (Telli et al., 2010, Vila et al., 2000). ShET2, encoded by the sen gene located in the large invasion plasmid, has been reported in different Shigella species as well as in EIEC, EAEC, ETEC-ST, and among E. coli isolates not associated to diarrhea (Farfán et al., 2011; Nataro et al., 1995; Roy et al., 2006; Vila et al., 2000). In a study on E. coli isolates causing bacteremia, 21 and 8 out of 100 UTI cases were positive for set and sen genes, respectively. In this study UTI-causing agents were not distinguished, and bacteria were isolated from blood not urine (Telli et al., 2010). Another study performed by Soto et al. (2009) analyzing the presence of set and sen genes in 170 UPEC isolates showed that 16% of the isolates had the set gene; however, the sen was not detected in any of the isolates. In contrast, in our study, the set gene was detected in 1.3% isolates, which is lesser than that detected by Soto et al. (2009) while the sen gene was detected in 13 (9.4%) UPEC isolates. We could not find any report on the presence of the sen gene in UPEC isolates. The cdt gene was detected for the first time in DEC and subsequently among other gram-negative bacteria such as Campylobacter spp., Shigella spp., Helicobacter spp., Aggregatibacter actinomycetemcomitans, Escherichia albertii, Haemophilus ducreyi, and Providencia alcalifaciens (Asakura et al., 2007; Okuda et al., 1995; Scott et al., 1994; Vargas et al., 1999). In C. coli and C. jejuni, cdt genes are not associated with any mobile genetic element, whereas in E. coli CDT encoding-genes are present on a plasmid or bacteriophage (Vargas et al., 1999). In the study by Johnson and Stell (2000) on urosepsis isolates of E. coli, 8% of the isolates were cdtB positive, suggesting that cdt gene should also be investigated as possible extraintestinal VF even though they have been primarily regarded as an enteric VF. In contrast to the study by Johnson and Stell (2000), cdt genes were not found in any case or control isolates. Although the presence of enterotoxins in the B2 phylogenetic group was more frequently detected than that of other phylogenetic groups, there seems to be no relationship between the presence of enterotoxins and the B2 phylogenetic group. Our results, along with those by Soto et al. (2009) and Abe et al. (2008), raise the probability that E. coli strains acquire these toxins to become potential diarrhoea-causing agents. However it should be remarked that not all isolates carrying these genes express these toxins (Vila et al., 2000). The presence of EAST, ShET-1, and ShET-2 in UPEC strains shows that horizontal transfer of virulence factors present on plasmids, PAIs, and other mobile genetic elements in bacteria belonging to different or similar species can take place. A study on DEC pathotypes in Brazil showed that 45% and 22% EAEC and EPEC strains, respectively, carried at least one of the urovirulence sequences (Regua-Mangia et al., 2010). Although this and other studies report the presence of some enterotoxin genes in Shigella and DEC pathotypes of UPEC strains, we do not know whether these genes are expressed in vivo and play any role in bacterial pathogenesis. These questions remain to be answered (Abe et al., 2008).

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