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Short Communication

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 enteroaggrigative *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).Cytolethal distending toxin (CDT), a complex protein, contains 3 polypeptides CdtA, CdtB, and CdtC. This toxin has DNase I activity and breaks double-strand DNA

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and therefore is called genotoxin or cyclomodulin. Five types of CDTs have been found in *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 *et al.*, 1999). Because some virulence factors (VFs) of diarrheagenic *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 *E. coli* /mL. These isolates were identified by standard laboratory protocols. In addition, 30 *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). *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 *E. coli* isolates, PCR was performed to amplify a fragment of the gene encoding for the highly specific *E. coli* universal stress protein A (*uspA* gene). PCR primers and conditions were described by Chen and Griffiths (1998).

For phylogenetic groups, two genes of *chuA*, *yjaA,* and a DNA fragment TSPE4.C2 were investigated by a triplex PCR method designed by Clermont *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 *set*, *sen*, and *astA* genes, respectively, was done by amplifying these genes using primers reported previously (Abe *et al*. 2008). In addition, the *cdtB* gene encoding cytolethal distending toxin was also amplified as suggested by Johnson and Stell (2000). The *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 *set* and *sen* genes PCR conditions were similar and involved denaturation at 95 °C for 3 min, 30 cycles of denaturation at 95 °C for 50 s,

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
set-1B	ShET-1B upper	GTGAACCTGCTGCCGATATC	147	Fasano *et al.*, 2011
	ShET-1B lower	ATTTGTGGATAAAAATGACG		
Sen	ShET-2 upper	ATGTGCCTGCTATTATTTAT	799	Nataro *et al*., 1995
	ShET-2 lower	CATAATAATAAGCGGTCAGC		Vila *et al*., 2000
astA	East-upper	ATGCCATCAACACAGTATAT	110	Johnson and Stell, 2000
	East-lowe	GCGAGTGACGGCTTTGTAGT		
cdtB	cdt-a1	AAATCACCAAGAATCATCCAGTTA	430	
	cdt-a2	AAATCTCCTGCAATCATCCAGTTTA		Chen and Griffiths, 1998
	cdt-s1	GAAAGTAAATGGAATATAAATGTCCG		Clermont *et al*., 2010
	cdt-s2	GAAAATAAATGGAACACACATGTCCG		Clermont *et al*., 2010
uspA	uspA-up	CCGATACGCTGCCAATCAGT	883	Clermont *et al*., 2010
	uspA-down	ACGCAGACCGTAGGCCAGAT		
ChuA	ChuA.1	GACGAACCAACGGTCAGGA	279	
	ChuA.2	TGCCGCCAGTACCAAAGACA		
YjaA	YjaA.1	TGAAGTGTCAGGAGACGCTG	211	
	YjaA.2	ATGGAGAATGCGTTCCTCAAC		
TspE4C2	TspE4C2.1	GAGTAATGTCGGGGCATTCA	152	
	TspE4C2.2	CGCGCCAACAAAGTATTACG		

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 $MgCl_2$, 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 ($p \leq 0.001$). For the commensal isolates, D group isolates were statistically more significant than those belonging to other groups ($p \leq 0.001$). 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 enterohemorragic 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-

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
		138 (%)	17 (%)	16 (%)	76 (%)	29 (%)	30 (%)	9 (%)	0 (%)	9 (%)	12 (%)
set		2 (1.3)	0 (0)	0 (0)	2 (2.6)	0 (0)	0(0)	0 (0)	0 (0)	0 (0)	0 (0)
sen		13 (9.4)	1 (5.8)	2 (12.5)	4 (5.2)	6 (20.7)	2(6.6)	0 (0)	0 (0)	2 (22)	0 (0)
astA		13 (9.4)	1 (5.8)	2 (12.5)	9 (11.8)	1 (3.4)	5 (16.6)	0 (0)	0 (0)	4 (44)	1 (8)
cdtB		0 (0)	0 (0)	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)	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 *Providenica 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 diarrhea-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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