Original Article

Presence of pathogenicity island related and plasmid encoded virulence genes in cytolethal distending toxin producing *Escherichia coli* isolates from diarrheal cases

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Abstract

Context: Mobile genetic elements such as plasmids, bacteriophages, insertion elements, and genomic islands play a critical role in virulence of bacterial pathogens. These elements transfer horizontally and could play an important role in the evolution and virulence of many pathogens. A broad spectrum of gram-negative bacterial species has been shown to produce a cytolethal distending toxin (CDT). On the other hand, Shiga toxin producing *Escherichia coli* are the one carry virulence genes such as *stx 1* and *stx 2* (Shiga toxin) and these genes can be acquired by horizontal gene transfer. **Aim:** The aim of this study was to investigate the presence of other virulence associated genes among CDT producing *E. coli* strains. **Materials and Methods:** Thirty CDT positive strains isolated from patients with diarrhea were characterized. Thereafter, the association with virulent genetic elements in known pathogenicity islands (PAIs) was assessed by polymerase chain reaction. **Results:** In this study, it was shown that the most CDT producing *E. coli* isolates express Shiga toxin. Moreover, the presence of prophages framing *cdt* genes (like P2 phage) was also identified in each *cdt*-type genomic group. Flanked regions of *cdt-I*, *cdt-IV*, *and cdt-V*-type was similar to plasmid sequences while *cdt-II and cdt-III*-type regions similarity with hypothetical protein (*orf3*) was observed. **Conclusion:** The occurrence of each *cdt*-type groups with specific virulence genes and PAI genetic elements is indicative of horizontal gene transfer by these mobile genetic elements, which could lead to diversity among the isolates.

Key words: Horizontal gene transfer, pathogenic *Escherichia coli*, virulence genes

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Introduction

Intestinal pathogenic *Escherichia coli* are one of the major causes of diarrheal disease. Pathogenic groups of *E. coli* have resulted from the acquisition of virulence genes. Virulence genes are acquired by mobile genetic elements such as prophages and pathogenicity islands (PAIs). Pathogenic *E. coli* are characterized by the production of genes that contribute to virulence. Some virulent genes are located on a large plasmid and encode proteins.[1]

Another virulence factor is PAIs, a subgroup of genomic islands; carry one or more virulence genes and they are present in the genome of a pathogenic bacterium. PAI occupy relatively large genomic regions on chromosomes. The regions are carrying genes for hemolysin production (*hly*) and P-related fimbriae present on PAI I and II of pathogenic

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E. coli 536 chromosome.[2] Cytolethal distending toxin (CDT) production in *E. coli* is dependent on various *cdt* genes (*cdt-I-V*).

The *cdt-I* reported to encode on a lysogenic lambdoid prophage.[3] Moreover, *cdt-I* and *cdt-IV* genes framing with lambdoid prophage genes has been detected in extraintestinal pathogenic *E. coli*.[4] The *cdt-V* flanking regions by P2 phage-related sequences have already been reported.[5]

On the other hand, bacteriophage encoding *cdt-V* was isolated from nonclinical *E. coli* from water samples. P4-like prophage gene location in all *cdt* genes has also been described previously.[6] It has already been found that *cdt-I* genes were also flanked by prophage-related open reading frames (ORFs). The *cdt-I*-flanking ORFs were homologous to flanking ORFs identified in the *cdt-V* loci. Moreover, upstream of the *cdt-I* gene three 28C-related genes, *orf2*, *orf3*, and *orf6* were found.[7-9] A total of 28C strain is the *cdt-IV* prototype with defined orfs.

These putative prophage-related proteins include a lambdoid prophage host specificity protein (*orf1*), a Lom-like protein (*orf2*), a putative tail fiber protein (*orf3*), a putative protease encoded in enterohemorrhagic *E. coli* (EHEC) EDL933 prophage CP-933 (*rorf1*), and a putative OmpT-like outer membrane protease (*rorf2*).[4]

In this study, the presence of prophages framing *cdt* genes was evaluated among our isolates. Virulence-associated genes including *stx 1*, *stx 2*, *hly* were also evaluated by polymerase chain reaction (PCR). In addition, the tRNA insertion site analysis was performed to assess the chromosomal diversity.

Materials and Methods

Bacterial strains

E. coli strains isolated from patients with diarrhea and were used for this study. Ethical approval was granted by the Ethical Committee of Pasteur Institute of Iran.

E. coli strains were grown on Luria-Bertani (LB) agar or in LB broth at 37°C overnight. A total of 30 CDT positive strains were isolated from patients diagnosed with diarrhea [Table 1]. The *E. coli* strains; 28C, *cdt-IV* AY162217 were provided by Dr. Oswald (Ecole Nationale Veterinaire de Toulouse, France). In this experiment, *cdt* positive strains 163-3 (EF158843), 412 (AF373206), 322 (AF373205) which isolated in our laboratory were also used.[10]

Genotypic characterization

Genomic extraction by phenol-chloroform method was used to provide the target for PCR assays the same as our previous study.[10] Strains were overnight cultured in LB medium and were verified for virulence-associated genes, which were encoded by plasmids and chromosome; including *hly*, *stx 1*, *stx 2*, genes.[1] Primers for phage and flanked regions of *cdt* gene used by Tóth *et al*.[4] and Friedrich *et al*.[5] Putative virulence genes were detected using published PCR protocols.[6] Samples were prepared in a total volume of 25 μl containing: 15.5 μl D.D.W, 2.5 μl PCR buffer, 1 μl dNTP, 1 U of Taq DNA polymerase, 1 μl $MgCl_2$, 1 μl of each primers (forward and reverse), and 2 μl bacterial DNA extraction. The PCR product was visualized in agarose gel electrophoresis. Specific primers were also used to amplify the tRNA loci of *pheV*, *selC*, *leuX* and *pheR*, which are often the site of insertion for virulence genes.[10] The sequences of primers are mentioned in our previous study.[10] *E. coli cdt* producer of five known types of *cdt* alleles (*cdt-I*, *cdt-II*, *cdt-III*, *cdt-IV*, *and cdt-V*) was also assessed by specific primers for *cdt* alleles used by Allué-Guardia *et al*.[6]

Table 1: Genotype characteristics of *cdt* type human isolates

Strain number	Name	*cdt* type	Genotype
1	323	*cdt-I*	*cdt*
2	413	*cdt-I*	*cdt, stx1*
3	440	*cdt-I*	*cdt, stx1*
4	464	*cdt-I*	*cdt, stx1*
5	468	*cdt-I*	*cdt, stx1, stx2*
6	6. 501	*cdt-I*	*cdt, stx1, stx2*
7	2123	*cdt-II*	*cdt, stx1*
8	O157	*cdt-II*	*cdt, stx1*
9	322	*cdt-II*	*cdt, stx1, hly*
10	361	*cdt-II*	*cdt, stx1*
11	363	*cdt-II*	*cdt, stx1*
12	399	*cdt-II*	*cdt, stx1*
13	400	*cdt-II*	*cdt, stx1*
14	376	*cdt-III*	*cdt*
15	378	*cdt-III*	*cdt, hly*
16	378a	*cdt-III*	*cdt, hly*
17	378b	*Cdt-III*	*cdt, stx1*
18	386	*cdt-III*	*cdt, stx1, hly*
19	401	*cdt-III*	*cdt, stx1, stx2, hly*
20	445	*cdt-III*	*cdt, stx1, hly*
21	042	*cdt-IV*	*cdt, stx1*
22	28C	*cdt-IV*	*cdt, stx1, hly*
23	42	*cdt-IV*	*cdt, stx1*
24	63	*cdt-IV*	*cdt, stx1, stx2, hly*
25	356	*cdt-IV*	*cdt, stx1 hly*
26	412	*cdt-IV*	*cdt, stx1*
27	437	*cdt-IV*	*cdt, stx1 hly*
28	40	*cdt-V*	*cdt, stx1*
29	409	*cdt-V*	*cdt, stx1 hly*
30	435	*cdt-V*	*cdt, stx1 hly*

Pulsed field gel electrophoresis

Pulse field gel electrophoresis (PFGE) was performed to determine DNA profiles of *cdt* positive strains. Bacteria were grown on brain heart infusion plates at 37°C for 18 h. Bacterial colonies were suspended in cell suspension buffer (100 mM Tris-HCl, 100 mM EDTA; pH 8.0) and adjusted to an optical density of 1.35–1.45. The cell suspension (250 μl)

was mixed with an equal volume of melted 1% low melting agarose (Pharmacia Biotech Europe). The mixture was carefully dispensed into a sample mold (Bio-Rad). After solidification, the plugs were transferred to a tube containing 1 ml of lysis buffer (50 mM Tris-HCl, 1 mM EDTA, pH 8.0; 1% sarcosyl) and 0.1 mg of proteinase K/ml. Cells were lysed overnight in a water bath at 54°C. Then after lysis, the plugs were washed twice with distilled water and 4 times with TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) for 15 min per wash. Agarose embedded DNA was digested with 40 U of *Xba I* (Fermentas) overnight in a water bath at 37°C. The plugs were placed in agarose gel 1% (Invitrogen). Restricted fragments were separated by electrophoresis in × 0.5 TBE (Tris-borate-EDTA) buffer at 14°C for 18 h using a Chef Mapper (Bio-Rad) with pulse times of 2 to 54 s. The gel was stained with ethidium bromide, and DNA bands were visualized with an ultraviolet transilluminator.

Epidemiological relationships between strains were assessed by studying the PFGE patterns of genomic DNA after restriction by *Xba* I. PFGE patterns were compared using Gel-Compar software (Applied Maths http://www.applied-maths.com/).

Sequence analysis

The framing genes and associated CDT alleles were cloned, and the resulting ampilicons were sequenced. The sequences were analyzed by EMBL-GENBank database (http://www.ncbi.nlm.nih.gov/BLAST).

Nucleotide sequences

The partial sequence of flanked regions for some strains were sequenced and deposited in EMBL-GenBank (Accession no. KC769190-KC769195).

Results

Virulence genes in chromosome and plasmids

In this study, the presence of PAI related genes, plasmid encoded virulence genes; tRNA insertion site and framing location in different *cdt* allelic types were assessed. CDT-producing *E. coli* strains that were already established in our previous study were considered for the present investigation.[10] Genotype characteristics of 30 human CDT producer strains along with *Stx* and *hly* were also assessed [Table 1]. It was shown that six strains carried *cdt-I* genes (20%), seven strains carried *cdt-II*, *cdt-III*, *and cdt-IV* genes (each 23.3%), and 3 strains (10%) carried *cdt-V* genes [Table 2]. The *stx 1* and *hly* plasmid genes existence were shown in Table 1. Regarding different *cdt*-types, the most prominent gene in our isolates is a *stx 1* gene. The prevalence of *stx 1* gene was 83.3% in *cdt-I*-type while in *cdt-II*, *cdt-IV*, and *cdt-V*-type, expression of the gene was observed in 100% of isolates. The most prevalence of *stx 2* gene expression was observed in 33.3% of *cdt-I*-type. The most *hly* gene expression was detected in 71.42% of *cdt-III*-type. Furthermore, the frequency of *stx 1* gene expression was 57.14% in *cdt-III*-type [Table 2].

Whole genomic profiles

Two strains of each *cdt*-type group were applied for PFGE assay. Phylogenic tree from DNA profiles of these strains was compared. It was shown that there is genomic diversity in different types of CDT positive *E. coli* strains. On the other hand, DNA profile of *cdt* groups was shown in similarity between *cdt-I* and *cdt-II* group then between *cdt-III* group, with maximum 60% genomic diversity. Moreover, the similarity was also shown in *cdt-IV* and *cdt-V* group in different strains with maximum 40% genomic diversity.

tRNA genes

The insertion of PAI in tRNA sites results in PCR negative amplicon indicating the presence of PAI inserted into tRNA gene. The *pheV* and *leuX* tRNA insertion sites were mostly detected among *cdt* groups [Table 3]. Since, *pheR* tRNA was rarely detected, and *selC* tRNA insertion was not observed at all. *pheR* tRNA insertion was not detected in *cdt-I* type groups, and no tRNA insertion was observed for *cdt-V* type groups in our isolates. The most prevalence of tRNA insertion was observed in *cdt-III* type group [Table 3]. The *pheV* and *leuX* tRNA prevalence in *cdt-III* type group was 57.1% as the most prevalence of *cdt*-type tRNA insertion. Even *pheR* tRNA detection in *cdt-III* type was 71.4%, as the most frequent insertion in *pheR* tRNA group [Table 3].

Flanked genes

The flanking regions of *cdt* genes were also assessed by PCR [Table 4]. The prophage genes upstream of the *cdt* genes may encode several proteins. In this regard, we cloned

Table 2: The percentage of target genes in different *cdt*-type isolates

cdt type	Target gene (%)		
	stx1	*stx2* (%)	*hly* (%)
cdt type-*I* (20%)	83.33	33.33	0
cdt type-*II* (23.3%)	100	0	14.28
cdt type-*III* (23.3%)	57.14	14.28	71.42
cdt type-*IV* (23.3%)	100	14.28	57.14
cdt type-*V* (10%)	100	0%	66.66

Table 3: tRNA genes detection in different *cdt* allelic type

cdt type	Target gene (%)			
	pheV	*pheR*	*selC*	*leuX*
cdt type-*I*	50	100	100	50
cdt type-*II*	71.4	85.7	100	57.2
cdt type-*III*	57.1	71.4	100	57.1
cdt type-*IV*	85.7	85.7	100	85.7
cdt type-*V*	100	100	100	100

and sequenced some PCR products obtained from our isolates [Table 5]. Hence, the results of sequencing and homology search of their blast search yield the following; The primer designed for the amplification of *rorf1* yield the amplicon, which its sequence on blast search showed similarity with hypothetical protein in PAI II, strain 536 in *cdt-III* type strain. While using primers designed for bacteriophage P2 the sequence similarity with enterotoxigenic *E. coli* (ETEC) H10407 p52 plasmid was shown in *cdt-I* strain. However, in *cdt-II* strain, the similarity with *E. coli* ETEC H10407 hypothetical protein was observed. Meanwhile, in *cdt-III* strain the amplicon shows similarity with chromosomal putative type 1 fimbrial protein. Although, in *cdt-IV* strain, the similarity with *E. coli* ETEC 1392/75 plasmid p746 and *E. coli* O104:H4 strain 2011C-3493 and pESBL-EA11 plasmid similarity was observed [Table 5].

Table 4: *cdt* related genes and flanking regions in *cdt*-type isolates with different genotype

Strain	*cdt* type	*orf5*	*orf6*	*orf5/cdtA*	*rorf1*	*cdt IV/rorf1*
1	*cdt-I*	+	–	–	–	–
2	*cdt-I*	–	–	–	–	–
3	*cdt-I*	–	–	–	–	–
4	*cdt-I*	–	–	–	+	–
5	*cdt-I*	–	–	–	–	–
6	*cdt-I*	–	–	–	+	–
7	*cdt-II*	–	–	–	+	–
8	*cdt-II*	–	–	–	+	–
9	*cdt-II*	–	–	–	+	–
10	*cdt-II*	–	–	–	+	–
11	*cdt-II*		–	–	+	–
12	*cdt-II*	+	–	–	+	–
13	*cdt-II*	+	–	–	+	–
14	*cdt-III*	–	–	–	+	–
15	*cdt-III*	–	–	–	+	–
16	*cdt-III*	+	–	–	+	–
17	*cdt-III*	+	–	–	-	–
18	*cdt-III*	–	–	–	+	–
19	*cdt-III*	+	–	–	–	–
20	*cdt-III*	+	–	–	+	–
21	*cdt-IV*	+	–	–	+	+
22	*cdt-IV*	+	+	+	+	+
23	*cdt-IV*	+	+	+	+	+
24	*cdt-IV*	-	–	–	+	+
25	*cdt-IV*	+	–	–	+	+
26	*cdt-IV*	–	–	–	+	+
27	*cdt-IV*	–	–	–	+	+
28	*cdt-V*	–	–	–	+	–
29	*cdt-V*	+	–	–	+	–
30	*cdt-V*	+	–	–	+	–

(+) indicate positive PCR product and the existence of mentioned gene while (–) indicate negative PCR product. PCR: Polymerase chain reaction

In our isolates, *orf5* and *rorf1* were mostly found but their location and association with *cdt* were hardly detected as already mentioned by Tóth *et al*. [Table 4]. The same size of insertion (in *orf5* and *rorf1*) that already observed was only detected in *cdt-IV* and *cdt-II* isolates [Table 4]. This indicates that the integrity of PAI related genes is not maintained in our isolates, although they were all CDT producer.

Discussion

Five different CDTs have been reported for *E. coli*, so far. CDT-I[11] and CDT-II[12] were identified in enteropathogenic *E. coli* serotype O86:H34 and O128:NM strains, respectively.[12] CDT-III was cloned from *E. coli* serotype O15:H21 strain from the calf.[13] CDT-III is encoded by pVir, a conjugative plasmid, which codes for some other genes.[14] CDT-IV was isolated from *E. coli* strains of intestinal and extraintestinal origin.[15] CDT-V was identified in Shiga toxin producing *E. coli* strain serotype O157:NM and non-O157 strains.[16] However, the presence of *stx* genes in all types of *cdt I-V* was detected in most of our isolates. It was already shown that *cdt-I* and *cdt-IV* genes were flanked by lambdoid prophage genes.[4] Although CDTs are produced by other diverse pathogenic bacterial species, the mechanism associated with the possible horizontal transfer of

Table 5: Sequence similarity of flanked genes in *cdt*-types

Strain/*cdt* type	Primer feature	Length (bp)	GenBank accession number
378/ III	*Rorf* 1		
	Hypothetical protein	357	KC769190
	Integrative element		
	Predicted protein *orf3*		
	Pathogenicity island II, strain 536		
440/I	Bacteriophage P2/*cdt*		
	E. coli ETEC H10407 p52 plasmid	348	KC769191
361/II	*E. coli* ETEC H10407, complete genome		
	Hypothetical protein	784	KC769192
401/III	*E. coli* O111:H-, complete genome		
	Putative type 1 fimbrial protein precursor	588	KC769193
437/IV	*E. coli* ETEC 1392/75 plasmid p746	627	KC769194
356/IV	*E. coli* O104:H4 strain 2011C-3493 plasmid pESBL-EA11		
	E. coli O104:H4 strain 2011C-3493 plasmid pESBL-EA11	589	KC769195

E. coli: Escherichia coli; ETEC: Enterotoxigenic *Escherichia coli*

the various *cdt* genes resulting in the wide distribution of *cdt* genes among pathogenic bacteria is not completely known.[17] However, bacteriophages are supposed the major vehicles for the transfer of genes including virulence genes between bacteria.[17] Furthermore, the *stx* genes location in the genomes of heterogeneous, lambdoid genes has been confirmed.[7,9] It has been shown that the *cdt-I* gene cluster is transferred by CDT-1Φ (CDT-I converting phage) to a recipient strain, which then produces biologically active CDT-1 toxin.[3] The *cdt-I* and *cdt-IV* genes acquisition by phage transduction from a common ancestor resulting to evolution of the CDT-encoding phages in different bacterial hosts, might generate differences in the *cdt* genes and their flanking DNA contents, the finding similar to our results.[4] Genomic diversity in our isolates was also shown by PFGE. Comparison of the *cdt-I* and *cdt-IV* genes and their flanking regions have revealed that *cdt* upstream flanking regions are similar, containing similar prophage genes.[18]

Previously, *cdt V* operon framing by P2 prophage sequences demonstrated in EHEC O157:NM strain 493/89.[18,19] However, the *cdt-IV* and *cdt-I* flanking genes relation to P2 phage genes has not been shown. *cdt-IV* operon framing by two prophages and PAI-associated DNA sequences, including integrase and tRNA genes detected.[8] It is shown that, the presence of *cdt* genes in different bacterial species and its flanking regions suggest that this gene has been acquired from heterologous species by horizontal gene transfer or through a phage.[12,13,18,20] In uropathogenic *E. coli*, serotype 536 insertions at *leuX* observed in PAI II and in (ETEC) insertion at *pheV* also reported.[2]

In this study, *leuX* and *pheV* insertion sites were detected in *cdt* positive strains. It was shown that CDT producer strains mostly containing Stx which, in combination with phages could drives for the dissemination of different type of *cdt* genes among bacterial strains. The relationship between CDT production and *stx* gene expression in O157 and non-O157 strains and involvement of phages was also mentioned. In our study, it was also demonstrated that CDT family of different *cdt* genes within the chromosome or plasmid could also frame with phages and their association with other virulence genes would help to the diversity among them. It could be resulted that all *cdt* positive strains containing *cdt-I* to *cdt-V* genes are along with phages or converting bacteriophages. The *rorf1* and *orf5* gene existence in most *cdt* types were detected in this study. The result of *rorf1* genes sequencing showed the existence of hypothetical proteins located in a PAI.

On the other hand, in our study *rorf1* existence among our isolates was similar to ECs1662 from *E. coli* O157:H7 that confirm the association of *cdt-I-V* genes with the Stx producer strain.

Collectively, our study is supporting the hypothesis that *cdt-I* to *cdt-V* could evolve by phage transduction in bacterial progeny. Moreover, an association of CDT and Stx producing strains with mobile genetic elements could result to the evolution of more pathogenic bacterial strains. The presence of bacteriophage P2 sequences flanking the *cdt-V* cluster in each of the 30 strains was also investigated in this study. The association of *cdt* with several particular phage types had been reflected the ability of such strains to be transduced by the *cdt*-containing phage, which, in turn, may depend on the phage integration sites within the chromosomes of these strains.

Conclusion

The *cdt* positive isolates association with *stx 1*, and *hly* plasmid genes could lead us to the idea that these virulence genes are associated with mobile genetic elements, which help them to spread among a wide spectrum of bacteria. The existence of prophages in almost all types of CDT producer strains could be the evidence of recombination events between bacterial chromosome, plasmids, and phages that bring the existing diversity among the strains.

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Conflicts of interest
There are no conflicts of interest.

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