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

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 (**orf1**), and a putative OmpT-like outer membrane protease (**orf2**).[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** AT162217 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 MgCl₂, 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**, **seIC**, **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.[4]

**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)

### 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, stx 1 |
| 3             | 440  | cdt-I        | cdt, stx 1 |
| 4             | 464  | cdt-I        | cdt, stx 1 |
| 5             | 468  | cdt-I        | cdt, stx 1, stx 2 |
| 6             | 6.501| cdt-I        | cdt, stx 1, stx 2 |
| 7             | 2123 | cdt-II       | cdt, stx 1, stx 2 |
| 8             | 0157 | cdt-II       | cdt, stx 1, stx 1, stx 2 |
| 9             | 322  | cdt-II       | cdt, stx 1, hly |
| 10            | 361  | cdt-II       | cdt, stx 1 |
| 11            | 363  | cdt-II       | cdt, stx 1 |
| 12            | 399  | cdt-II       | cdt, stx 1 |
| 13            | 400  | cdt-II       | cdt, stx 1 |
| 14            | 376  | cdt-III      | cdt |
| 15            | 378  | cdt-III      | cdt, hly |
| 16            | 378a | cdt-III      | cdt, hly |
| 17            | 378b | Cdt-III      | cdt, stx 1 |
| 18            | 386  | cdt-III      | cdt, stx 1, hly |
| 19            | 401  | cdt-III      | cdt, stx 1, stx 2, hly |
| 20            | 445  | cdt-III      | cdt, stx 1, hly |
| 21            | 402  | cdt-IV       | cdt, stx 1 |
| 22            | 28C  | cdt-IV       | cdt, stx 1, hly |
| 23            | 42   | cdt-IV       | cdt, stx 1 |
| 24            | 63   | cdt-IV       | cdt, stx 1, stx 2, hly |
| 25            | 356  | cdt-IV       | cdt, stx 1, hly |
| 26            | 412  | cdt-IV       | cdt, stx 1 |
| 27            | 437  | cdt-IV       | cdt, stx 1, hly |
| 28            | 40   | cdt-V        | cdt, stx 1 |
| 29            | 409  | cdt-V        | cdt, stx 1, hly |
| 30            | 435  | cdt-V        | cdt, stx 1, hly |
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 flanking 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-Ill-type. Furthermore, the frequency of stx 1 gene expression was 57.14% in cdt-IIll-type [Table 2].

Whole genomic profiles
Two strains of each cdt-type group were applied for PFGE assay. Phylogenetic 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-IIll type group [Table 3]. The pheV and leuX tRNA prevalence in cdt-IIll type group was 57.1% as the most prevalence of cdt-type tRNA insertion. Even pheR tRNA detection in cdt-IIll 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         | 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         | 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 I 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].

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. [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.[13] 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.[1] Although CDTs are produced by other diverse pathogenic bacterial species, the mechanism associated with the possible horizontal transfer of

### 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/orf1 |
|------------|----------|------|------|-----------|-------|-------------|
| 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

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

| Strain/cdt type | Primer feature | Length (bp) | GenBank accession number |
|-----------------|----------------|-------------|--------------------------|
| 378/III         | Rorf1          | Hypothetical protein | 357 | KC769190 |
|                 |                | Integrative element |              |          |
|                 |                | Predicted protein orf3 |        |          |
|                 |                | Pathogenicity island II, strain 536 | 589 | KC769195 |
| 440/I           | E. coli ETEC H10407 p52 plasmid | 348 | KC769191 |
| 361/II          | E. coli ETEC H10407, complete genome | 784 | KC769192 |
| 401/III         | E. coli O111:H-, complete genome | 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 | 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.\cite{17} However, bacteriophages are supposed the major vehicles for the transfer of genes including virulence genes between bacteria.\cite{17} Furthermore, the stx genes location in the genomes of heterogeneous, lambdoid genes has been confirmed.\cite{7,13} It has been shown that the cdt-I gene cluster is transferred by CDT-I-Φ (CDT-I converting phage) to a recipient strain, which then produces biologically active CDT-I toxin.\cite{9} 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.\cite{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.\cite{18}

Previously, cdt V operon framing by P2 prophage sequences demonstrated in EHEC O157:NM strain 493/89.\cite{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.\cite{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.\cite{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.\cite{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 drive 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 I, 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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