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Detection of Cytolethal Distending Toxin and Other Virulence Characteristics of Enteropathogenic *Escherichia coli* Isolates from Diarrheal Patients in Republic of Korea

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Cytolethal distending toxins (CDTs) represent an emerging family of newly described bacterial products that are produced by a number of pathogens. The genes encoding these toxins have been identified as a cluster of three adjacent genes, *cdtA*, *cdtB*, and *cdtC*, plus 5 *cdt* genetic variants, designated as *cdt*-I, *cdt*-II, *cdt*-III, *cdt*-IV, and *cdt*-V, have been identified to date. In this study, a general multiplex PCR system designed to detect *Escherichia coli cdt*s was applied to investigate the presence of *cdt* genes among isolates. As a result, among 366 *E. coli* strains, 2.7% were found to carry the *cdtB* gene. In addition, the use of type-specific primers revealed the presence of *cdt*-I, *cdt*IV, and *cdt*-V types of the *cdt* gene, yet no *cdt*-II or *cdt*-III strains. The presence of other virulence genes (*stx1*, *stx2*, *eae*, *bfp*, *espA*, *espB*, and *espD*) was also investigated using a PCR assay. Among the 10 *cdtB* gene-positive strains, 8 were identified as CDT-producing typical enteropathogenic *E. coli* (EPEC) strains (*eae*$^+$, *bfp*$^+$), whereas 2 were identified as CDT-producing atypical EPEC strains (*eae*$^+$, *bfp*$^-$). When comparing the cytotoxic activity of the CDT-producing typical and atypical EPEC strains, the CDT-producing atypical EPEC strains appeared to be less toxic than the CDT-producing typical EPEC strains.

Keywords: Cytolethal distending toxin, atypical EPEC, typical EPEC, bundle-forming pilus

Strains of enteropathogenic *E. coli* (EPEC) are an important cause of infantile diarrhea in developing countries [20]. EPEC can also be identified by their ability to cause the effacement of microvilli and intimate adherence between the bacterium and epithelial cells (A/E lesion) by polymerized actin accumulation and the formation of pedestal-like structures beneath the adherent bacteria [9, 10]. The locus of the enterocyte effacement (LEE) pathogenicity island contains type III secreted effector molecules (*espA*, *espB*, and *espD*) and an *eae* gene that encodes intimin, which in turn allows bacterial attachment to the gut mucosa. Other properties of EPEC include localized adherence (LA; bacteria form characteristic microcolonies on the surface of cells) mediated by the EPEC adherence factor (EAF) plasmid-encoded bundle-forming pili (*bfp*) gene [3, 11]. However, the EAF plasmid has not been detected among many EPEC isolates. EPEC strains can be further classified as typical or atypical and do not carry *stx* (Shiga toxin) genes. Typical and atypical EPEC both carry the LEE region and are distinguished by their phenotypic and genotypic characteristics, virulence properties, and reservoirs. Whereas typical EPEC carry the virulence plasmid EAF and are a leading cause of infantile diarrhea in developing countries, atypical EPEC do not harbor the EAF plasmid and seem to be an important cause of diarrhea in industrialized countries. Furthermore, typical EPEC have rarely been isolated from animals, as humans are the major natural reservoir for these pathogens [31], whereas atypical EPEC strains have been isolated from both animals and humans [1].

Cytolethal distending toxins (CDTs), first detected in 1987 in *Escherichia coli* O128 isolated from the stool of a child who was less than 2 years old and suffering from gastroenteritis [16], are an emerging family of toxins and produced by several pathogenic bacteria, such as *Shigella dysenteriae* [24], *Campylobacter* spp. [16], *Haemophilus ducreyi* [7], and *Actinobacillus actinomycetemcomitans* [17]. The CDTs in the abovementioned species are characterized with a unique structure of three overlapping genes highly homogeneous in all bacteria, yet in widely varying degrees [23, 27–29]. CDTs can be characterized based on their capacity to inhibit cellular proliferation by

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including an irreversible cell cycle block at the G2/M transition [26]. In addition, the CDTs produced by bacteria are composed of three polypeptides, *cdtA*, *cdtB*, and *cdtC*, which have molecular masses of approximately 30, 32, and 20 kDa, respectively. Numerous studies have already revealed the mode of CDT activity [8, 18, 22], where the translocation of the CDT-B catalytic subunit into the host cell rests on the CDT-A and CDT-C subunits. Once in the cytoplasm, CDT-B can reach the nucleus and inflict DNA damage. CDT-B is structurally similar to DNaseI, and its activity depends on the presence of a phosphodiesterase consensus sequence. Accordingly, the aim of this study was to compare typical and atypical CDT-producing strains of EPEC isolated from diarrheal patients to evaluate their pathogenic potential and probable role as a cause of human disease.

Materials and Methods

Bacterial Strains

A total of 366 *E. coli* strains were isolated from diarrheal patients in 2005, and the presence of the *eae* gene was investigated in all the strains using a PCR.

PCR Analysis

DNA from 100-μl aliquots of overnight cultures was extracted using a MegaExtract genomic kit (Toyobo, Japan) according to the manufacturer's instructions. The primers and PCR conditions are summarized in Table 1. The PCR (30 cycles) was performed in a total volume of 20 μl containing 25 pM concentrations of each primer, 200 μM concentrations of each dNTP, 1.5 mM $MgCl_2$, 1 U *Taq* polymerase (TaKaRa), 2 μl of a 10× polymerase buffer, and 1 μl of the DNA preparation. The PCR products were visualized by ethidium bromide staining after electrophoresis on agarose gels (1.8%). The amplicon produced using this method was 466 bp. To detect the *cdt*-I, II, III, and IV (*cdt*B) genes, the PCR conditions were as previously described by Toth *et al.* [30] using the primers shown in Table 1. The PCR conditions described by Bielaszewska *et al.* [4] were used to amplify the *cdt*-V (*cdtB*) gene. Additional PCR tests using the primer sequences and PCR conditions described in Table 1 were conducted to determine the presence of the *stx1*, *stx2*, *eae*, *bfp*, *espA*, *espB*, and *espD* genes.

Serotyping

The determination of O antigens was carried out using the method described by Guinee *et al.* [12] employing all available O (O1-O185) antisera. The O antisera were produced by LREC (Lugo, Spain).

Table 1. Primers used in this study.

Gene	Orientation	Primer sequence (5' - 3')	Product size (bp)	Condition	Reference
*esp*A	F	5'-GTT TTT CAG GCT GCG ATT CT	187		This study
	R	5'-AGT TTG GCT TTC GCA TTC TT			
*esp*B	F	5'-GCC GTT TTT GAG AGC CAG AA	106	94°C, 25 sec	This study
	R	5'-AAA GAA CCT AAG ATC CCC A		55°C, 25 sec	
*esp*D	F	5'-AAA AAG CAG CTC GAA GAA CA	145	72°C, 25 sec	This study
	R	5'-CCA ATG GCA ACA ACA GCC CA			
eae	F	5'-ATG CTG GCA TTT GGT CAG GTC GG	233		This study
	R	5'-TGA CTC ATG CCA GCC GCT CAT GCG			
bfp	F	5'-AAT GGT GCT TGC GCT TGC TGC	320	94°C, 25 sec	[13]
	R	5'-GCC GCT TTA TCC AAC CTG GTA		65°C, 25 sec	
				72°C, 25 sec	
*cdt*B	F1	5'-GAA AGT AAA TGG AAT ATA AAT GTC CG	466	94°C, 1 min	
	R1	5'-AAA TCA CCA AGA ATC ATC CAG TTA		55°C, 1 min	
	F2	5'-GAA AAT AAA TGG AAC ACA CAT GTC CG		72°C, 1 min	
	R2	5'-AAA TCT CCT GCA ATC ATC CAG TTA			
cdt type I	F	5'-CAA TAG TCG CCC ACA GGA	411		[30]
	R	5'-ATA ATC AAG AAC ACC ACC AC			
cdt type II	F	5'-GAA AGT AAA TGG AAT ATA AAT GTC CG	556		
	R	5'-TTT GTG TTG CCG CCG CTG GTG AAA		94°C, 1 min	
cdt type III	F	5'-GAA AGT AAA TGG AAT ATA AAT GTC CG	555	54°C, 1 min	
	R	5'-TTT GTG TCG GTG CAG CAG GGA AAA		72°C, 1 min	
cdt type IV	F	5'-CCT GAT GGT TCA GGA GGC TGG TTC	350		
	R	5'-TTG CTC CAG AAT CTA TAC CT			
cdt type V	F	5'-AGC ACC CGC AGT ATC TTT GA	1363		[15]
	R	5'-AGC CTC TTT TAT CGT CTG GA			
*cnf*1	F	5'-GGC GAC AAA TGC AGT ATT GCT TGG	533	94°C, 1 min	
	R	5'-GAC GTT GGT TGC GGT AAT TTT GGG		62°C, 1 min	[5]
*cnf*2	F	5'-GTG AGG CTC AAC GAG ATT ATG CAC TG	552	72°C, 1 min	
	R	5'-CCA CGC TTC TTC TTC AGT TGT TCC TC			

Table 2. Serotype, *cdt* types, and virulence gene profiles of *E. coli* strains possessing *cdtB*.

Strain	Serotype	PCR result of virulence genes[c]								*cdt* type	Reciprocal titer of *cdt* in CHO cell assay
		eae	*bfp*	*espA*	*espB*	*espD*	*stx 1*	*stx 2*	*cdtB*		
05-137	O167	+	+	+	+	+	–	–	+	IV	128
05-204	O6	+	+	–	–	–	–	–	+	IV	128
05-654	OUT[a]	+	+	+	+	+	–	–	+	I	32
05-1070	OUT[a]	+	+	+	+	+	–	–	+	I	128
05-1072	OUT[a]	+	–	–	–	–	–	–	+	I	8
05-1394	OUT[a]	+	+	–	+	+	–	–	+	IV	128
05-1569	O179	+	+	–	+	+	–	–	+	IV	64
05-1633	O142	+	+	–	+	+	–	–	+	I	64
05-1745	O88	+	+	+	+	+	–	–	+	V	ND[b]
05-2119	O21	+	–	–	–	–	–	–	+	I	16

[a]OUT, not typeable.

[b]ND, Not determined.

[c]Virulence genes: *eae*, intimin; *bfp*, bundle-forming pilus; *espA*, EPEC-secreted protein A; *espB*, EPEC-secreted protein B; *espD*, EPEC-secreted protein D; *stx* 1, Shiga toxin 1; *stx* 2, Shiga toxin 2; *cdtB*, cytolethal distending toxin B.

CHO Cell Assay

The CDT assays were conducted using Chinese hamster ovary (CHO) cells. Briefly, supernatants taken from cultures of *E. coli* grown overnight at 37°C in a DMEM with 5% fetal calf serum were filter-sterilized, and then 1 ml of the supernatants or 2-fold dilutions were added in duplicate to 10^3 freshly seeded CHO cells in 1.5 ml of the DMEM in 6-well tissue culture plates (Falcon 3502). The assay mixtures were incubated for 5 days at 37°C under 5% CO_2, and examined daily for typical distending effects [15]. The CDT titer was defined as the highest filtrate dilution that caused distension in 50% of the CHO cells.

RESULTS

PCR Analysis

The 366 strains isolated from diarrheal patients were initially screened using primers designed for the detection of EPEC *cdtB*. The results indicated that 10 (2.7%) strains yielded an amplicon with the predicted size (466 bp). Nine of these strains were found to be CDT production-positive when a conventional cytotoxic assay was used (Table 2). Additional analyses were then conducted using primers derived from the *cdtB* region, which is very highly conserved in all *cdtB*s, to detect *cdt* sequences in several different genera (Table 1). Four primers designed to detect the presence of the *bfp*, *espA*, *espB*, and *espD* genes were used in the PCR assay. In the PCR, amplicons with the expected sizes of 326 bp, 187 bp, 106 bp, and 145 bp were generated from 8 (*bfp*), 4 (*espA*), 7 (*espB*), and 7 (*espD*) of the tested isolates, respectively. In addition, 8 of the EPEC strains were found to contain the *bfp* gene encoding a bundle, which is the structural subunit of the bundle-forming pilus expressed by typical EPEC (eae^+ and bfp^-) strains. However, two of the EPEC strains did not possess the *bfp* gene expressed by atypical EPEC (eae^+ and bfp^-) strains (Table 2).

Typing of *cdt* Genes and Serotype

The CDT-producing EPEC strains were tested for their *cdt* types (Fig. 1). However, *cdt*-II and *cdt*-III were not identified in any of these strains (Table 2). As such, the CDT-producing *E. coli* isolates belonged to serotypes O21, O142, and not typeable (*cdt*-I-positive strains), serotypes O6, O167, O179, and not typeable (*cdt*-IV-positive strains), and serotype O88 (*cdt*-V-positive strain).

CHO Cell Assay

The CDT activity was evaluated by microscopic observation of Chinese hamster ovary cells 5 days after exposure to the CDT-producing *E. coli* cultured supernatants. Whereas the supernatant from *E. coli* DH5α did not alter the morphology of the Chinese hamster ovary cells, the Chinese hamster

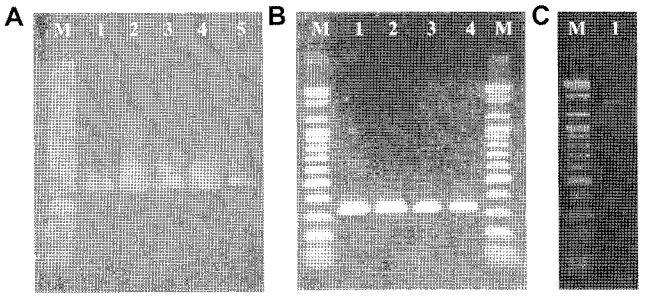

Fig. 1. PCR products produced by *cdt*-I, *cdt*-IV, and *cdt*-V CDT-producing EPEC strains.

A. PCR products (411 bp) amplified from indicated EPEC using *cdt*-I-specific primers described by Toth *et al.* [29]. Lanes: 1, 05-654; 2, 05-1070; 3, 05-1072; 4, 05-1633; 5, 05-2119; M, Molecular weight marker (100-bp ladder). **B**. PCR products (350 bp) amplified from indicated EPEC using *cdt*-IV-specific primers described by Toth *et al.* [29]. Lanes: 1, 05-137; 2, 05-204; 3, 05-1394; 4, 05-1569. **C**. PCR products (1,363 bp) amplified from indicated EPEC using *cdt*-V-specific primers described by Bielaszewska *et al.* [4]. Lane: 1, 05-1745.

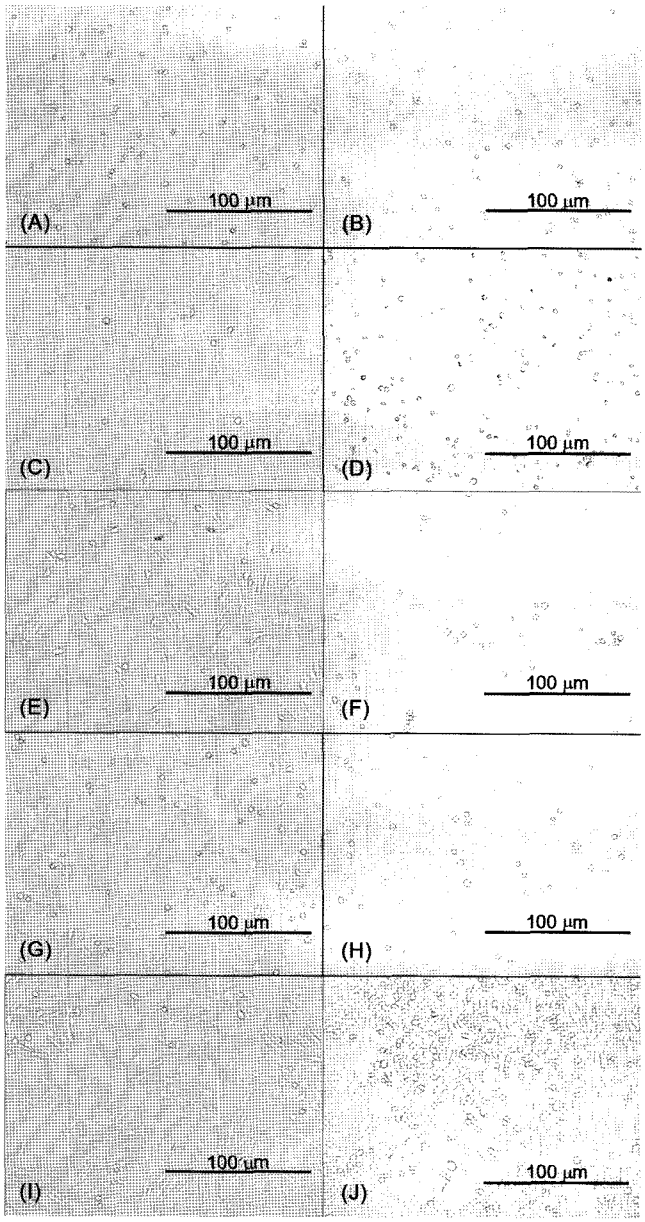

Fig. 2. Appearance of Chinese hamster ovary cells after 5 days of exposure to culture supernatant of CDT-producing EPEC strains.
(A) CDT-producing typical EPEC (05-137) culture supernatant at 1/8 dilution; (B) Typical EPEC (05-204); (C) Typical EPEC (05-654); (D) Typical EPEC (05-1070); (E) Atypical EPEC (05-1072); (F) Typical EPEC (05-1394); (G) Typical EPEC (05-1569); (H) Typical EPEC (05-1633); (I) Atypical EPEC (05-2119); (J) Negative control.

ovary cells treated with supernatants from cultures of the CDT-producing *E. coli* strains exhibited the characteristic alterations in shape caused by CDT (Fig. 2). Moreover, the distending effects of increasing the concentration titers of the total cell supernatants from the various CDT-producing *E. coli* isolates on the Chinese hamster ovary cells are shown in Table 2.

Discussion

Since CDT was first identified in *E. coli* by Johnson and Lior in 1987 [16], several studies have reported that *cdt* can be produced by intestinal and extraintestinal pathogenic bacteria [17, 21, 24]. In addition, other studies have revealed differences in the sequences of *E. coli cdt*, allowing it to be divided into different types [18, 25, 27, 30]. Nonetheless, when the *cdt* operons obtained from *Campylobacter coli* and *C. jejuni* were sequenced, they were shown to be much more homologous than the *E. coli cdt* genes [28]. Furthermore, Albert *et al.* [2] found no significant association between CDT-positive *E. coli* strains and diarrhea; however, the toxin was recently shown to have diarrheagenic properties in an animal model [23].

This study was conducted to detect and identify different *cdt* genes among EPEC strains isolated from diarrheal patients in Korea. A general PCR system [30] designed to detect *E. coli cdt*s was employed to investigate the presence of *cdt* genes among the isolates, and among 366 *E. coli* strains, 2.7% were found to carry the *cdtB* gene. CDT production has been associated with EPEC serogroups [5]. For example, the frequency of the *cdt* gene in EPEC strains has been reported as 2.8% and 6.4% in studies conducted in Brazil [14] and India [6], respectively, similar to the frequency observed in this study. Using type-specific primers, it was demonstrated that *cdt*-I, *cdt*-IV, and *cdt*-V types of the *cdt* gene were present in the isolates evaluated in this study, although the present collection did not contain any *cdt*-II and *cdt*-III strains. The distribution of virulence genes is presented in Table 2. All the CDT-producing EPEC strains were positive for *eae* (intimin). Eight out of 10 strains were *bfp* (EAF plasmid-encoded bundle-forming pili)-positive. CDT production is associated with EPEC [2, 6], and it has also been reported to be associated with the *eae* gene [4, 14, 18]. When comparing the cytotoxic activity of culture supernatants of the CDT-producing atypical EPEC (eae^+, bfp^-) and typical EPEC (eae^+, bfp^+) strains, the CDT-producing atypical EPEC strains appeared to be less toxic than the CDT-producing typical EPEC strains. Although the underlying mechanisms to explain the associations observed in the present study are not known, the results imply that bundle-forming pili (*bfp*) were related to the CDT distending effect.

In conclusion, this study would appear to be the first to report the possible association of CDT-producing EPEC with diarrheal patients in the Republic of Korea. Thus, the present results would seem to suggest that CDT-producing *E. coli* represent a new category of diarrheagenic *E. coli* attributed to diarrhea. Further studies are required to investigate a correlation among CDT-producing *E. coli*, virulence genes, and crucial diarrhea.

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