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Production of Cytolethal Distending Toxins by Pathogenic *Escherichia coli* Strains Isolated from Human and Animal Sources: Establishment of the Existence of a New *cdt* Variant (Type IV)

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Three types of cytolethal distending toxin (CDT), namely, CDT-I, CDT-II, and CDT-III, have been described in *Escherichia coli*. Using primers designed for the detection of sequences common to the *cdtB* genes, we analyzed by PCR a set of 21 CDT-producing *E. coli* strains of intestinal and extraintestinal origins isolated from human and different animal species in several European countries and in the United States. On the basis of the existing differences in the *cdtB* genes, *cdt*-I-, *cdt*-II-, and *cdt*-III-specific primer pairs were designed and used for *cdt* typing. These new primers successfully differentiated all of the previously described *cdt* genes. Six strains proved to be *cdt*-I; eight strains proved to be *cdt*-III. However, none of the type I-, II-, and III-specific primers generated amplicons from six CDT$^+$ strains, suggesting the existence of a new *cdt* variant. Sequence analysis of the amplicons from two untypeable genes confirmed the existence of a new *cdt* variant that we called *cdt*-IV. Using the new specific primers, *cdt*-IV was detected in human, porcine, and poultry strains of intestinal and extraintestinal origins. To validate all sets of *cdt* specific primers, a group of 353 human *E. coli* strains isolated in Hungary was then investigated for the presence of *cdt* genes. This included 190 strains isolated from patients with urinary tract infections (UTI), 51 strains isolated from other (nonurinary) extraintestinal infections, and 112 intestinal strains isolated from healthy individuals. Of 190 UTI strains, 15 (7.9%) had *cdt* genes. Of 51 non-UTI extraintestinal strains 3 (5.9%) contained the *cdt* gene, and 1 (0.9%) of 112 healthy intestinal strains was PCR positive. Five strains proved to be *cdt*-I, and fourteen strains proved to be *cdt*-IV. The CDT-producing extraintestinal strains belonged to a wide variety of serogroups, including O2, O6, O75, and O170. In conclusion, we have developed a new PCR typing system for CDT able to detect a new CDT variant present in pathogenic *E. coli* strains obtained from animals and humans.

Cytolethal distending toxins (CDT) represent an emerging and unique toxin family (12, 33). CDT was first described in *Escherichia coli* by Johnson and Lior (22). The culture filtrates of CDT-producing *E. coli* strains induced characteristic morphological changes, and the distended cells died in 3 to 4 days (23). CDT production was reported from various serotypes of enteropathogenic *E. coli* (EPEC) (2, 6, 17) and from several bacterial species of medical importance (10, 26, 32, 37). The CDTs induce the formation of giant mononucleated cells of sensitive eukaryotic cell lines and block the cell cycle in G_2 phase (3, 30). The block is due to the maintenance of mitosis promoting factor in an inactive form (9, 30).

Molecular genetic studies of CDT-producing *E. coli* strains were conducted in three different laboratories. Scott and Kaper (34) used an EPEC strain of O86:H34 serotype (15). Pickett et al. (31) used an EPEC O128:H$^-$ serotype strain (23), and Pérès et al. (30) used a cytotoxic necrotizing factor type 2 (CNF2)-producing *E. coli* strain of serogroup O78 isolated from a septicemic calf (28). In all of these cases, it was established that CDT was encoded by three adjacent or slightly overlapping genes, *cdtA*, *cdtB*, and *cdtC*, all of which were required for toxin production (30, 31, 34). The *cdtABC* genes in EPEC strains were located on the chromosome (31, 34), and in the O78 calf strain the *cdtABC* genes were located on a transferable large virulence plasmid called pVir that also coded for CNF2 toxin and F17 fimbrial adhesin (30). The function of the proteins encoded by the *cdt* genes is not fully elucidated, but recent studies have shown that CdtB protein contains an enzymatic motif with homology to several mammalian and bacterial phosphodiesterares, such as the human DNase I and the sphingomyelinase from *Bacillus cereus* (12, 15, 24). The *cdt* operon of the O86:H34 EPEC strain E6468/62 was referred to as *cdt* type I. The operon of the EPEC O128:H$^-$ strains 91422-88 and the operon of the O78 calf strain 1404 were referred to as *cdt* types II and III, respectively (33).

In molecular epidemiological studies, different DNA probes were used for detecting *cdt* genes in *E. coli*. By colony hybridization, *cdt* genes were detected in 3.1% of *E. coli* strains isolated from Bangladeshi children with diarrhea, while 0.9% of the control strains had *cdt* genes (2). By using *cdtI*- and *cdtII*-specific DNA probes, *cdt* genes were detected in 1.6% of diarrheagenic *E. coli* strains isolated in Nigeria (25). It can be assumed that more *cdt*-bearing strains could have been detected if a PCR system detecting all of the known types of CDT had been available. However, at the beginning of the present study we did not have such a system.

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TABLE 1. *E. coli* strains used in this study

Strain	Serogroup and/or serotype	CDT producer[a]	Origin	Country	Source or reference
H173	O2	+	Human, UTI	Hungary	This study
H52	O2	+	Human, UTI	Hungary	This study
34	O2	+	Human, septicemia	Northern Ireland	This study
66LS	O2	+	Human, septicemia	Northern Ireland	This study
33KH89	O2	+	Bovine, septicemia	Belgium	This study
AII-40	O6	+	Porcine, diarrhea	Austria	38
S5	O15:K?:H21	+	Lamb septicemia	England	35
B20a	O15	+	Bovine, diarrhea	Spain	This study
67LS	O18	+	Human, septicemia	Northern Ireland	This study
KS-159	O23	+	Food, poultry origin	Hungary	This study
H78	O75	+	Human, UTI	Hungary	This study
1404	O78	+	Bovine, septicemia	France	28
B26a	O78:K80	+	Bovine, diarrhea	Spain	This study
E6468/62	O86:H34	+	Human, EPEC	United States	34
OS (Outbreak Strain)	O86:K61	+	Finch, EPEC	Scotland	16
BM2-10	O88	+	Bovine, diarrhea	France	This study
E253	O115	+	Poultry, septicemia	Hungary	This study
89-201-2/3	O123	+	Bovine, diarrhea	France	This study
8	O123	+	Human, diarrhea	Northern Ireland	This study
B28b	O123:K$^-$	+	Bovine, diarrhea	Spain	This study
28C	O75:K95	+	Porcine septicemia	Spain	13
E2348/69	O127	−	Human, EPEC	England	14
HB101(p2123)	K-12	+	Cloned *cdt*-II operon	United States	31
J96	O4:K6	−	Human, UTI	Germany	5
DH5α	K-12	−	Laboratory strain		36

[a] +, producer; −, nonproducer.

In the present study we designed PCR primers suitable for detecting any type of sequenced *cdt* genes in *E. coli*. An additional goal was to use type-specific primers suitable for the detection and identification of known *cdt* types I, II, and III. In addition, the present study leads to the identification of a novel type (IV) of *cdt* gene.

MATERIALS AND METHODS

Bacterial strains. The origins of CDT-producing *E. coli* strains used in the present study are given in Table 1. The CDT-I-producing prototype strain E6468/62 (O86:H34) was kindly provided by J. B. Kaper, and strain HB101(p2123) containing the cloned *cdt*-II operon was kindly provided by C. Pickett. A further 353 *E. coli* strains were isolated and identified by standard bacteriological procedures in three different Hungarian hospitals and in two public health laboratories between 1998 and 2001. A total of 190 *E. coli* strains were isolated from urine specimens of patients with urinary tract infections (UTI), 51 strains were isolated from patients with different extraintestinal infections, and 112 *E. coli* strains were isolated from stool samples of healthy individuals.

Phenotypic tests. O-typing was carried out as described by Orskov and Orskov (27). Hemolysin production was examined on Luria-Bertani agar containing 5% sheep red blood cells.

Sequence analysis. DNA sequences *cdt* operons sequenced thus far were retrieved from GenBank and included human EPEC strains E6468/62 and 9142-88 and the bovine septicemic strain 1404. Comparison of DNA sequences was made by using the database at the National Center for Biotechnology Information (National Institutes of Health, Bethesda, Md.) with the basic local alignment search tool (BLAST) search algorithm and GCG alignment software and with CLUSTAL W multiple sequence alignment software.

Primers. On the basis of previously published sequences, two pairs of general PCR primers were designed for the consensus region of all *cdtB* genes. Furthermore, *cdt* type-specific primers were designed on the basis of unique *cdtB* sequences of the different types of *cdt* genes. All primers used are given in Table 2.

PCR analysis. All PCRs contained a 0.2 mM mix of each deoxynucleoside triphosphate, 1× *Taq* DNA polymerase buffer, 0.25 μM concentrations of each primer, and 2.5 U of *Taq* polymerase. The amplification protocol was as follows: denaturation at 94°C for 5 min, followed by 30 cycles of denaturation (94°C, 1 min), annealing (55°C, 1 min), and extension (72°C, 1 min). A final extension was done at 72°C for 10 min. When the amplicons were produced for sequencing, *Pfu* DNA polymerase (Promega) was used, and sequence analysis was done by Genome Express, Grenoble, France. The presence of the *eae* genes was also determined by PCR as described previously (7, 29).

Detection of cytotoxic activity of bacterial lysates and supernatants. Experiments and the preparation of bacterial lysates were conducted as described previously (11, 30). Briefly, *E. coli* strains were grown at 37°C in Tryptic soy broth medium with vigorous (200 rpm) shaking for 2 days. Supernatants of bacterial cultures were saved, and bacterial cells were sonicated. Supernatants and sonic lysates were sterile filtered separately by using 0.22-μm-pore-size filters. The amounts of proteins in each extract and supernatant was determined by using a protein assay kit (Bio-Rad). Nonconfluent HeLa cell monolayers were infected in 96-well plates with the culture supernatants and sonic lysates used in twofold dilution, diluted in tissue culture medium. Plates were incubated at 37°C in 5% CO_2 atmosphere for 4 days. After 4 days of interaction, the infecting materials were removed by several washings of the HeLa cell monolayers, morphological changes characteristic to CDT were determined, and the cytotoxic activity (expressed as the highest twofold dilution yielding 50% transformed cells after 96 h of incubation [CD_{50}]) values were calculated as the means of the highest twofold dilution of toxic material yielding 50% transformed HeLa cells after 96 h of coincubation. As a negative control, DH5α (36) and the *cnf cdt* mutant enteropathogenic prototype strain E2348/69 (19) was used. In addition, a CNF1-producing uropathogenic strain (J96 of serotype O4:K6) was used (5). All experiments were conducted in triplicate.

Nucleotide sequence accesion number. The newly determined *cdt*-IV sequence reported in the present study has been deposited in the GenBank database under accesion number AY162217.

RESULTS

Detection of *cdt* genes by PCR with *cdt*-specific universal primers. Two pairs of *cdtB*-specific primers were designed on the basis of available sequences in GenBank (Table 2). A *cdt* multiplex PCR system containing these primers was used to detect *E. coli* strains producing CDT in a set of 25 *E. coli* strains isolated from humans and different animals. Among these strains, 21 have been tested as CDT producer-positive by

TABLE 2. Primers used in this study

PCR	Primer	Sequence	Orientation[a]	GenBank accession no.	Position	PCR product size (bp)
Multiplex *cdt*	CDT-s1	5′-GAAAGTAAATGGAATATAAATGTCCG	F	U04208	1292–1317	466
				U89305	1966–1991	
	CDT-s2	5′-GAAAATAAATGGAACACACATGTCCG	F	U03293	1019–1044	
	CDT-as1	5′-AAATCACCAAGAATCATCCAGTTA	R	U04208	1735–1758	
				U89305	2409–2432	
	CDT-as2	5′-AAATCTCCTGCAATCATCCAGTTA	R	U03293	1462–1485	
Multiplex *cnf*	CNF-s	5′-TTATATAGTCGTCAAGATGGA	F	U42629	1638–1658	633
				X70670	1636–1656	
				U01097	892–912	
	CNF-as	5′-CACTAAGCTTTACAATATTGA	R	U42629	2251–2271	
				X70670	2249–2269	
				U01097	1505–1525	
cdt-I	CDT-Is	5′-CAATAGTCGCCCACAGGA	F	U03293	1186–1203	411
	CDT-IIas	5′-ATAATCAAGAACACCACCAC	R	U03293	1587–1597	
cdt-II	CDT-IIs	5′-GAAAGTAAATGGAATATAAATGTCCG	F	U04208	1291–1317	556
	CDT-IIas	5′-TTTGTGTTGCCGCCGCTGGTGAAA	R	U04208	1824–1847	
cdt-III	CDT-IIIs	5′-GAAAGTAAATGGAATATAAATGTCCG	F	U89305	1966–1991	555
	CDT-IIIas	5′-TTTGTGTCGGTGCAGCAGGGAAAA	R	U89305	2498–2521	
cdt-IV	CDT-IVs	5′-CCTGATGGTTCAGGAGGCTGGTTC	F	U03293	1078–1095	350
	CDT-IVas	5′-TTGCTCCAGAATCTATACCT	R	AY162217	340–360	
cnf-1	CNF-1s	5′-GGGGGAAGTACAGAAGAATTA	F	U42629	2700–2720	1111
				X70670	2698–2719	
	CNF-1as	5′-TTGCCGTCCACTCTCACCAGT	R	U42629	3791–3811	
				X70670	3789–3809	
cnf-2	CNF-2s	5′-TATCATACGGCAGGAGGAAGCACC	F	U01097	1942–1965	1240
	CNF-2as	5′-GTCACAATAGACAATAATTTTCCG	R	U01097	3159–3182	

[a] F, forward; R, reverse.

using the conventional cytotoxic assay (Table 1). The two pairs of *cdtB*-specific primers generated a product with the expected size (466 bp) of DNA from all of the wild-type strains producing CDT and from the recombinant strains containing the cloned *cdt*-II operon in a multicopy vector plasmid. On the other hand, no product was detected with templates obtained from strains that did not produce CDT: the uropathogenic *E. coli* (UPEC) strain J96, the EPEC strain E2348/69, and the laboratory strain DH5α. Further, another set of 50 wild-type *E. coli* strains that tested as CDT negative by the conventional cytotoxic assay turned out to also be PCR negative (data not shown).

Typing and sequence analysis of the *cdt* genes by PCR with specific primers. On the basis of existing sequence differences in the *cdtB* genes of CDT-I, CDT-II, and CDT-III, type-specific primers were designed and used for typing the *cdt* genes (Table 2). Seven strains proved to be *cdt* type I, and eight strains proved to be *cdt* type III. Our collection did not contain any CDT-II-producing wild-type strains, but the *cdt*-II operon containing the HB101(p2123) recombinant strain reacted specifically with the type II-specific primers. None of these primers generated amplicons from six CDT^+ strains, suggesting the existence of a new *cdt* variant. To confirm this hypothesis, all of the amplicons were sequenced. The seven *cdt*-I-specific amplicons had identical sequences. The eight type III-specific sequences were also identical to each other. The sequences of the six untypeable amplicons were identical to each other but were different from the sequences of types I, II, and III, indicating the existence of a new variant of *cdt*, referred to as type IV, for which *E. coli* strain 28C was selected as the prototype strain. The alignment of the internal sequences of *cdt* types I, II, III, and IV is given in Fig. 1. Sequence comparisons revealed that *cdt*-IV is closely related to *cdt*-I, with a homology of 84%. The *cdt*-IV sequence is less related to *cdt*-II and *cdt*-III (Table 3). This result suggests the presence of two CDT families represented by types II and III and types I and IV, respectively.

On the basis of the new sequence, type IV-specific primers were designed. By using these primers, a 326-bp product was amplified from all six previously *cdt* untypeable strains. These six strains were referred to as *cdt* type IV strains (Table 4). PCR based typing is shown in Fig. 2.

CDT effect of *E. coli* strains. Nonconfluent HeLa monolayers were incubated with the supernatants and the sonic lysates of the study strains, and the CD_{50} values were determined as described in Materials and Methods. All of the 21 CDT-producing strains had cell-associated toxicity, and 8 of them secreted the CDT to a high titer. All but one of them was of the CDT-I type (Table 4). Sonicates and culture supernatants of CDT-I strains showed equally strong toxicity. The sonicates of CDT-III- and CDT-IV-producing strains were less toxic than those of CDT-I-producing strains, and the supernatants of

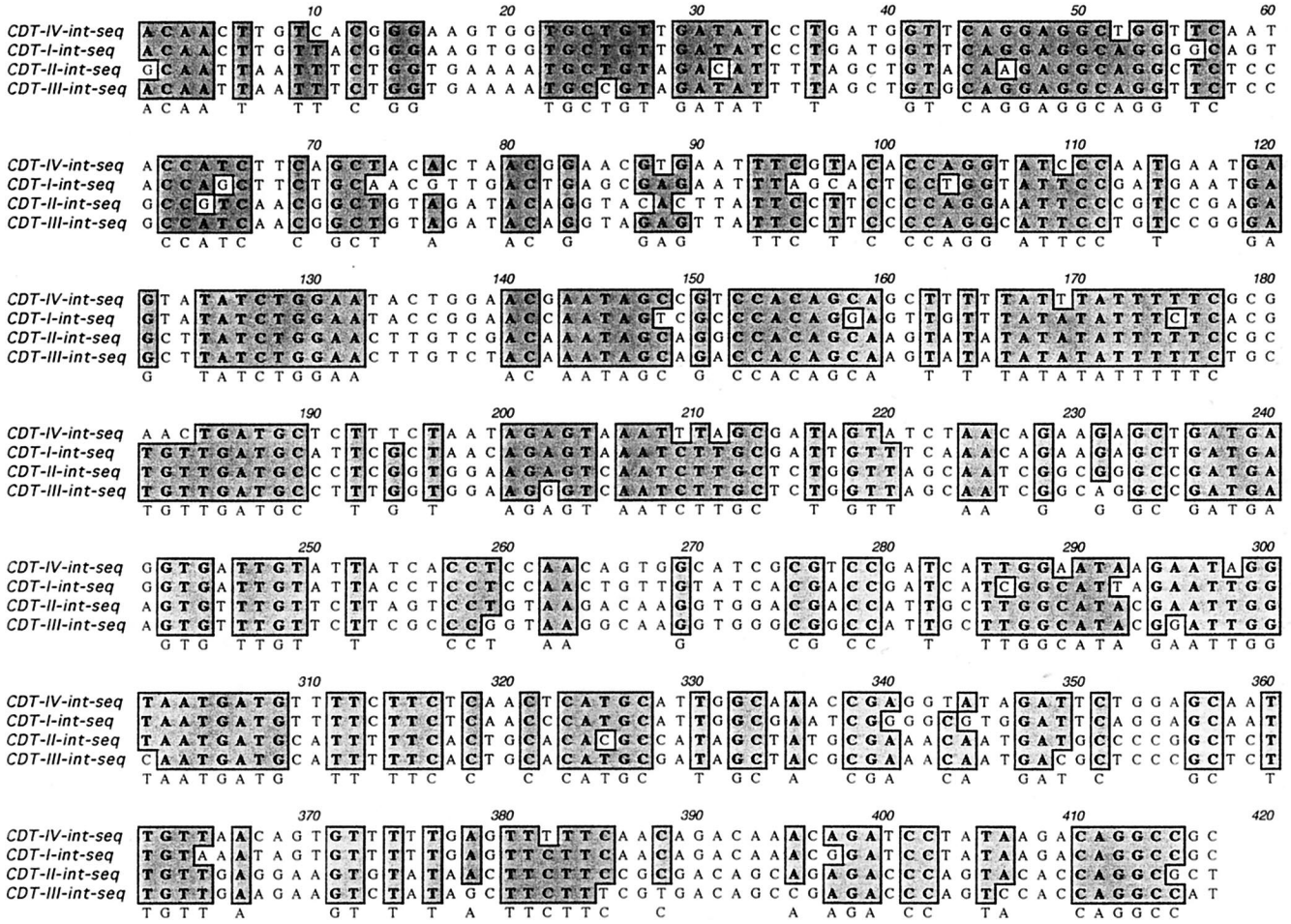

FIG. 1. CLUSTAL W-formatted alignments of *cdtB* type I, II, III, and IV genes. The positions of *cdt*-IV-specific primers are indicated by arrows starting at positions 34 and 359, respectively.

CDT-III and CDT-IV strains were negative, except for one CDT-III strain (Table 4). Eight CDT-producing strains also had the *cnf-2* gene, and five had *cnf-1*. Four strains were hemolytic, and two had the *eae* gene. Among the CDT- and CNF-producing strains a common tendency was observed. At lower dilutions the morphological transformation typical for CDT could be observed: almost all of the HeLa cells became enlarged and mononucleated. However, at higher dilutions, the CNF-specific polynucleation appeared as well and almost all of the transformed HeLa cells appeared as large polynucleated cells. The cytotoxic activity ranged between 100 and 12,500 CD_{50}/mg of protein. The results of these experiments are shown in Table 4.

Validation of all of the sets of PCR primers. To validate all sets of the PCR primers, a collection of human wild-type pathogenic extraintestinal and nonpathogenic intestinal strains was tested. Altogether, 353 *E. coli* strains isolated from humans were examined: 190 strains were isolated from individuals with UTI, 51 strains originated from different extraintestinal infections, and 112 strains were isolated from feces of healthy individuals. All of these strains were tested for both CDT activity on HeLa cells by using the conventional cytotoxic assay and for the presence of the *cdtB* gene by PCR. The testing of 353 *E. coli* strains by our PCR protocol (19 CDT-positive strains and 334 CDT-negative strains in HeLa cell culture assay) yielded no false-negative and no false-positive results. The presence of *cdt* genes was further verified by colony dot blot hybridization (data not shown) (4, 36). As high as 7.9% (15 of 190) of the human UPEC strains and 5.9% (3 of 51) of the other extraintestinal strains possessed *cdt* genes, and only one (0.9%) strain from a healthy control had a *cdt* gene. Twelve UPEC strains and two other extraintestinal strains had the *cdt*-IV gene alone, and three UPEC strains and one extraintestinal strain had the *cdt*-I gene alone. The *cdt*-bearing strain isolated from the healthy individual carried the *cdt*-I

TABLE 3. Percentage of nucleotide identities in the *cdt* internal sequences

cdt type	% Nucleotide identity with *cdt* type:			
	I	II	III	IV
I	100	55	54	84
II		100	89	55
III			100	54
IV				100

TABLE 4. Genotypes and phenotypes of *E. coli* strains[a]

Strains	Sero-group	Genotype					Phenotype	
		cdt	*cdt* type	*cnf*	*eae*	Hly	Lysate	Super-natant
E6468/62	O86	+	I	−	+	−	+++	+++
34	O2	+	I	1	−	−	+++	+++
66LS	O2	+	I	−	−	−	+++	+++
H52	O2	+	I	1	−	+	+++	++
67LS	O18	+	I	−	−	−	+++	+++
KS159	O23	+	I	−	−	−	++	++
OS (outbreak strain)	O86:K61	+	I	−	+	−	+++	+++
HB101(p21213)	K-12	+	II	−	−	−	ND	ND
1404	O78	+	III	2	−	−	++	−
33KH89	O2	+	III	2	−	+	++	++
B20a	O15	+	III	2	−	−	++	−
B26a	O78	+	III	2	−	−	++	−
BM2-10	O88	+	III	2	−	−	+	−
89-201-2/3	O123	+	III	2	−	−	+	−
B28b	O123	+	III	2	−	−	+	−
S5	O15	+	III	2	−	−	++	−
28c	O75	+	IV	1	−	+	++	−
H173	O2	+	IV	−	−	−	+	−
AII-40	O6	+	IV	1	−	+	++	−
H78	O75	+	IV	1	−	+	++	−
E253	O115	+	IV	−	−	−	++	−
8	O123	+	IV	−	−	−	+	−
J96	O4	−	−	1	−	+	*	−
DH5α	K-12	−	−	−	−	−	−	−
E2348/49	O127	−	−	−	−	−	−	−

[a] The cytotoxic activity in the sonic lysate and the culture supernatant was titrated as previously described (30) by using as the endpoint the CD_{50} value. The amounts of proteins in each extract were determined by using a bicinchoninic acid assay (Pierce), and the cytotoxic activity was expressed as the number of CD_{50} per milligram of protein (+++, ≥1,000; ++, 1,000 to 100; +, <100; −, no specific CDT or CNF effect). If a strain produced CNF and CDT, both cytopathic effects were observed. At the endpoint dilution the transformed HeLa cells were enlarged and polynucleated (CNF effect), and at lower dilutions only the CDT-specific enlargement effect was observed (large mononucleated cells). ND, not done; *, CNF cytopathic effect only.

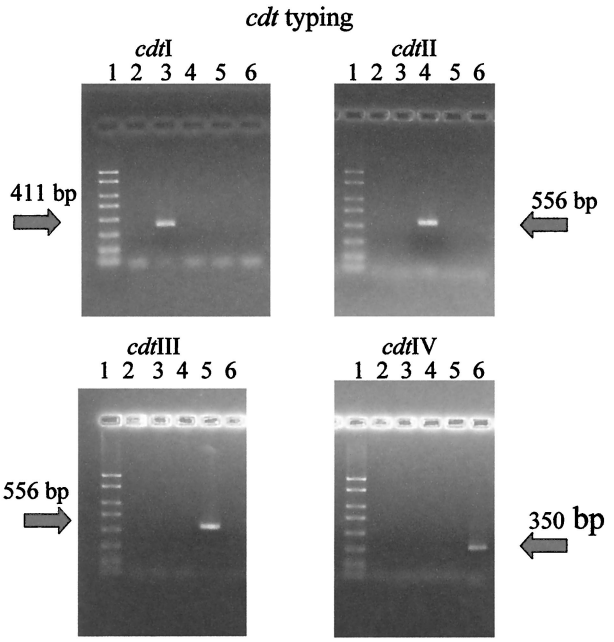

FIG. 2. *cdt* gene typing. In each panel the *cdt* type specific amplicons were loaded in the same order. Lanes: 1, PCR marker (Sigma) containing eight defined double-stranded DNA markers from 50 to 2,000 bp; 2, *E. coli* DH5α; 3, KS159 (*cdt*-I); 4, HB101(p2123) (*cdt*-II); 5, 1404 (*cdt*-III); 6, AII-40 (*cdt*-IV).

gene. No strain had combinations of different types of *cdt* genes. The CDT-producing strains belonged to a wide variety of serogroups, including O2, O6, O75, O170, and O rough, and most of them also had the *cnf-1* gene and were hemolytic (Table 5).

DISCUSSION

Since CDT was first identified by Johnson and Lior from *E. coli* in 1987 (22), several studies reported that CDT can be produced by strains belonging to other intestinal (26) and extraintestinal (10, 37) pathogenic bacteria. Molecular genetic studies have revealed that the *E. coli cdt* operons sequenced thus far are different (30, 31, 34). Interestingly, the sequenced *cdt* operons of *Campylobacter coli* and *C. jejuni* are much more homologous than the *E. coli cdt* genes (32, 33). In the present study, we developed PCR-based techniques and successfully applied them for a general and specific identification of the known *cdt* types in *E. coli*. Since the *cdtB* gene is the most conserved gene, we have designed primers for the consensus region of the *cdtB* genes and developed a general multiplex PCR system that can identify all of the known types of *E. coli cdt.* Further, we have designed primers specific for *cdt* types I, II, and III, and we have demonstrated that these type-specific primers can be used for typing *cdt* genes. The use of these primers suggested the existence of a novel *cdt* variant. Sequencing results validated all of the PCR typing results, including the existence of a new *cdt* gene, termed *cdt*-IV. The *cdt*-IV gene has 84% homology to *cdt*-I and less homology to *cdt*-II and *cdt*-III.

Recently, Clark et al. (8) successfully used PCR to detect and identify *cdt*-I, *cdt*-II, and *cdt*-III genes among CDT-producing *E. coli* strains from humans and animals. Interestingly,

TABLE 5. Characteristics of *cdt*-bearing *E. coli* human strains isolated in Hungary[a]

Strain	Source	City	O group	Genotype		
				cdt	*cnf*	Hly
H52	Urine	Debrecen	O2	I	1	+
H149	Urine	Budapest	O rough	I	1	−
H154	Urine	Budapest	O rough	I	1	−
H260	Bile	Budapest	ND	I	−	−
H173	Urine	Budapest	O2	IV	−	−
H4	Urine	Debrecen	O6	IV	1	+
H29	Urine	Debrecen	O6	IV	1	+
H161	Urine	Budapest	O6	IV	1	+
H58	Urine	Debrecen	O75	IV	1	+
H61	Urine	Debrecen	O75	IV	1	+
H62	Urine	Debrecen	O75	IV	1	+
H78	Urine	Debrecen	O75	IV	1	+
H83	Urine	Debrecen	O75	IV	1	+
H104	Urine	Debrecen	O rough	IV	1	+
H155	Urine	Budapest	O rough	IV	−	−
H193	Urine	Pécs	O170	IV	−	+
H327	Wound	Budapest	ND	IV	−	−
H329	Wound	Budapest	ND	IV	−	−
H462*	Feces	Budapest	ND	I	−	−

[a] ND, not determined; *, only *cdt*-bearing strain isolated from a healthy person.

that report indicated the existence of a further *cdt* gene variant, but these authors did not identify a novel *cdt*.

All of the *cdt*-bearing strains had only one type of *cdt* gene; these genes proved to be *cdt*-I, *cdt*-III, or *cdt*-IV types, but our collection did not contain any *cdt*-II strains. The *cdt*-I and *cdt*-IV genes were detected in human, porcine, and poultry strains, and the *cnf-1* gene was detected among *cdt*-I (4 of 11) and *cdt*-IV (11 of 18) type strains, but none of these strains had *cnf-2.* In correlation with previous observations, all eight CDT-III-producing strains were isolated from bovine sources, and all of them also had the *cnf-2* gene.

We wanted to further characterize the CDT-producing strains and have compared the cytotoxic activity of bacterial lysates and supernatants of the CDT-producing strains from different *cdt* gene types. Interestingly, the CDT-IV-producing strains seem less toxic than the strains producing CDT-I, whereas the toxic activities of the CDT-IV- and CDT-III-producing strains were almost the same. None of the CDT-IV-producing strains seemed to secrete toxins into the supernatant. However, CDT-I was secreted by CDT-I-positive strains. These in vitro results indicate that CDT-I could be the most potent type of CDT. In our study, all six tested CDT-IV-producing strains had cell-associated CDT activity, and none of these strains' supernatants were toxic in HeLa cell cultures. Clark et al. (8) recently reported that the some CDT-producing strains' supernatants showed a CDT effect in cell culture in the absence of PCR amplification of the *cdt*-I, *cdt*-II, or *cdt*-III genes. Based on these two observations, it is possible that there are other *cdt* types among pathogenic *E. coli*, or that in some strains CDT-IV toxin is also secreted, or that there are additional *cdt* types yet to be described.

As reported earlier, the EPEC strain E6468/62 (34) and the finch outbreak strain OS (16) had the *eae* gene encoding for intimin, but none of the other study strains had *eae*. These results clearly show that CDT production is not limited to EPEC strains, as some epidemiological studies have suggested (1, 2, 6, 17).

Since there were several strains of extraintestinal origin among the CDT-producing strains, a collection of extraintestinal *E. coli* strains was screened for *cdt*, and the *cdt* genes were typed. As expected, the *cdt* gene was found, and the incidence in extraintestinal strains was even higher than that reported earlier for EPEC strains in India (6) and in Brazil (17). Interestingly, most of the of *cdt* genes proved to be *cdt*-IV. On the basis of the existence of *cdt*-IV, we propose that in the former epidemiological reports the incidence of CDT-producing strains was underestimated. In an earlier study (21), *cdt* genes were detected in urosepsis *E. coli* isolates when the strains were tested for as many as 29 genes. These genes were virulence factor genes and potential virulence factor genes characteristic for UPEC. The study did not include strains isolated from healthy individuals. Recently, Johnson et al. (20) reported that among *E. coli* strains isolated from infants with neonatal bacterial meningitis (NBM) the *cdtB* gene was more prevalent than other well-established NBM-associated virulence genes. We have found that these strains produced CDT-I or CDT-IV.

Our results also highlight that a new CDT type, *cdt*-IV, could be widely disseminated. We found CDT-IV production among intestinal and extraintestinal strains of human, poultry, and porcine origin. It has been first reported that *cdt* was encountered among extraintestinal isolates in only two clonal groups: *E. coli* O2:K5/K7:H1 (21) and *E. coli* O6:K53:H1 (18, 19). Additional clonal groups (O83:K1 and O18:K1) have been identified among NBM-associated *cdt*-bearing strains (20). In the present study, the CDT-I-producing strains belonged to the O2, O18, O23, and O28 serogroups; the CDT-III-producing strains to belonged O2, O15, O78, O88, and O123 serogroups; and the CDT-IV-producing strains belonged to several serogroups, including serogroups O2, O6, O75, O115, O123, and O170.

In summary, we describe here a new PCR system that specifically detects all three known types of CDT, leading to the identification of a new member of the CDT family (CDT-IV). Although there are great quantitative differences in toxicity between the CDT-I-, CDT-III-, and CDT-IV-producing strains in vitro, it seems that CDT could be an important virulence factor of intestinal and extraintestinal *E. coli* in animals and humans.

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