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PCR for Detection of *cdt-III* and the Relative Frequencies of Cytolethal Distending Toxin Variant-Producing *Escherichia coli* Isolates from Humans and Cattle

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A PCR assay that uses primers whose sequences were obtained from the published sequence of the *cdt-III* gene was developed to determine the frequencies of the *cdt-I*, *cdt-II*, and *cdt-III* genes in *Escherichia coli* isolates from humans and animals. *E. coli* isolates producing cytolethal distending toxin (CDT) were infrequently detected. The *cdt-I* gene was preferentially detected in strains with the *cnf1* gene, while the *cdt-III* gene was found in strains carrying the *cnf2* gene. The *cdt-III* genotype was more prevalent in animal isolates, while the *cdt-I* and *cdt-II* genotypes were more evident in human isolates. The presence of further *cdt* gene variants was indicated by the presence of toxin activity in cell culture in the absence of PCR amplification of the *cdt-I*, *cdt-II*, or *cdt-III* gene.

Cytolethal distending toxin (CDT) is a potent bacterial exotoxin that has dramatic effects on target cells in culture (23). Intoxication of eukaryotic cells results in blockage of the cell cycle at the G_2/M transition (2, 22) by a mechanism involving prevention of cdc2 protein kinase dephosphorylation and activation (6). Cellular effects include accumulation of F-actin assemblies resembling stress fibers (2), progressive cell distension, and eventual cell death (14, 15). Epidemiologic studies have not found a statistically significant difference in the incidence of CDT-producing *Escherichia coli* in children with diarrhea and healthy controls (23). However, animal studies with toxigenic *E. coli* strains (18) and *Campylobacter jejuni* CDT-knockout mutants (26) suggest that CDT may be a virulence factor in vivo.

Three genes, *cdtA*, *cdtB*, and *cdtC*, are required for the production of an active toxin in *E. coli* (24, 28), *Shigella dysenteriae* (19), *Campylobacter* spp. (25), *Haemophilus ducreyi* (7), *Helicobacter hepaticus* (32), and *Actinobacillus actinomycetemcomitans* (16). Although these genes appear to be homologous in all bacteria, the degree of relatedness at the genetic level varies widely (23). Three *cdt* genetic variants, designated *cdt-I*, *cdt-II*, and *cdt-III*, have been identified and cloned from *E. coli* (22, 24, 28). However, PCR methods have been developed only for the detection of the *cdt-I* and *cdt-II* variants (19). The distribution of the *cdt-I* and *cdt-II* genes within *E. coli* is not well characterized (23), and little is known about the incidence or prevalence of *cdt-III*. PCR primers were developed for the detection of *cdt-III*. A number of *E. coli* isolates from humans and animals were tested for the presence of all three *cdt* variants.

A group of 46 CDT-producing *E. coli* isolates was collected between 1986 and 1998 from Canadian and international sources. CDT-positive animal isolates collected during this time were also characterized. Enhanced surveillance for *cdt* genes was accomplished by testing 151 non-Shiga toxin-producing non-O157:H7 *E. coli* isolates collected between October 1998 and the end of 2000 together with a randomly chosen subset of 53 *E. coli* O157:H7 isolates. In addition, 72 non-O157 *E. coli* isolates from a series of consecutive blood cultures obtained in the St. Boniface Hospital, Winnipeg, Manitoba, Canada, were examined for CDT.

Culture media for *E. coli*, *Aeromonas* spp., *Campylobacter* spp., *Arcobacter* spp., and *Helicobacter* spp. were optimized for CDT production (14, 15). Bacterial supernatants were obtained by centrifugation and filtration through 0.2-μm-pore-size filters. After a maximum of eight twofold dilutions, the supernatants were added to nonconfluent Chinese hamster ovary (CHO) cells seeded at 2×10^4 cells/ml. Observations for the characteristic effects of CDT were made between 24 and 120 h of incubation. For neutralization studies, rabbit polyclonal antibodies were prepared from partially purified CDT-I. Filtrates were also added to Vero, Y-1 adrenal, and HeLa cells to check for the presence of other toxins (14).

Template DNA was prepared either by alkaline lysis with phenol-chloroform extraction (27) or by boiling two to three single colonies of the target isolate in 100 μl of distilled water for 5 min. PCR primers were designed to amplify the sequence between nucleotides 1066 and 3296 of the *cdt-IIIA* sequence (22). The forward primer (primer *cdt-III*-f) was 5′-AAA CAG GAC GGT AAT AAT GAC TAA TA-3′, and the reverse primer (primer *cdt-III*-r) was 5′-GTG ATC TCC TTC CAT GAA AAT ATA GT-3′. PCR was performed in a mixture that consisted of 1× PCR buffer, 2 mM deoxynucleoside triphosphates, 1.5 mM $MgCl_2$, 20 μM primer oligonucleotides, and

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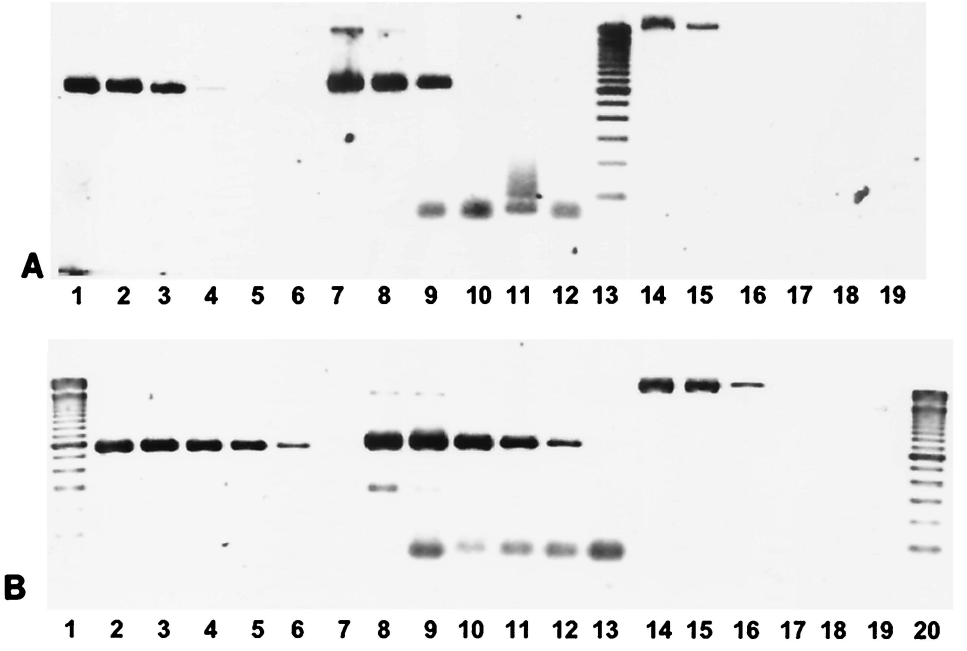

FIG. 1. Sensitivities of *cdt*-specific primers. (A) Different concentrations of boiled cell preparations. The isolates in lanes 1 to 6 were amplified with *cdt-I*-specific primers, the isolates in lanes 7 to 12 were amplified with *cdt-II*-specific primers, and the isolates in lanes 14 to 19 were amplified with *cdt-III*-specific primers. The numbers of boiled bacterial cells serving as templates for each lane were as follows: lanes 1, 7, and 14, 1.25×10^5; lanes 2, 8, and 15, 1.25×10^4; lanes 3, 9, and 16, 1.25×10^3; lanes 4, 10, and 17, 1.25×10^2; lanes 5, 11, and 18, 12.5; lanes 6, 12, and 19, 1.25. Lane 13, 100-bp ladder. (B) Different concentrations of extracted DNA preparations. The isolates in lanes 2 to 7 were amplified with *cdt-I*-specific primers, the isolates in lanes 8 to 13 were amplified with *cdt-II*-specific primers, and the isolates in lanes 14 to 19 were amplified with *cdt-III*-specific primers. The amounts of DNA in each lane were as follows: lanes 2, 8, and 14, 500 ng; lanes 3, 9, and 15, 50 ng; lanes 4, 10, and 16, 5 ng; lanes 5, 11, and 17, 0.5 ng; lanes 6, 12, and 18, 0.05 ng. Lanes 7, 13, and 19, negative controls containing water instead of the DNA template used for each PCR; lanes 1 and 20, 100-bp ladders.

1.25 U of *Taq* polymerase with a Gene Amp PCR System 2400 (Perkin-Elmer Applied Biosystems Ltd., Mississauga, Ontario, Canada). Initial denaturation at 94°C for 2 min was followed by 30 cycles of 94°C for 2 min, 54°C for 2 min, and 72°C for 1 min and 2 cycles of extension at 72°C for 7 min. The amplicon was 2,230 bp long and contained the entire *cdt-III*-coding region. The *cdt-III* PCR product was digested with *Sma*I to give three fragments of 187, 260 and 1,783 bp. PCR for *cdt-I* and *cdt-II* was done by the protocol of Okuda et al. (19).

The cells were evaluated by PCR for the presence of the following other virulence factors: Shiga toxin genes stx_1 and stx_2 (17, 21); the *eae* gene (11, 21); the *elt* gene, which encodes the *E. coli* heat-labile toxin (10, 20); the *est* gene, which encodes the *E. coli* heat-stable toxin (20, 30); the invasion-associated locus (*ial*) of the enteroinvasive *E. coli* virulence plasmid or invasion genes (20, 29); cytotoxic necrotizing factor genes *cnf1* and *cnf2* (4, 20); and a gene associated with the enteroaggregative phenotype (20).

The *cdt-III* PCR product was subjected to automated fluorescent cycle sequencing with a Wizard Miniprep kit (Promega; Fisher Scientific Ltd., Ottawa, Ontario, Canada) and an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (version 2.0; Applied Biosystems) on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Primers *cdt-III*-f and *cdt-III*-r were used for the initial sequencing reactions. The internal primer sets used to complete the sequencing were *cdt-III* seq (5′-GGC TCC ACA TTG TTA GAA AGC-3′), *cdtIII* seq-int3 (5′-GTC TAC AGG TTT ATG CAT CAG CAG AGC-3′), *cdtIII* seq-int4′ (5′-CCA TTG CTT GGC ATA CGG ATT GGC-3′), *cdtIII* seq-3′ (5′-CAT TCC GCA ATC TCC AAA GTG GGC-3′), *cdt-III* seq-f (nucleotide 1724; 5′-GAA ACT TCG AAT TTA TGT GG-3′), and *cdtIII* seq-r (nucleotide 2629; 5′-AAG ACA TCT GAG TCC TTC TG-3′). LaserGene-DNAStar software was used for sequence editing and comparisons.

Evaluation of the PCR method for detection of the *cdt-III* gene indicated that the primer sets were specific. Of 315 *E. coli* isolates examined, 24 possessed the *cdt-I* gene alone, 5 carried the *cdt-II* gene alone, and 21 carried the *cdt-III* gene alone. No isolates carried combinations of *cdt* genes. Isolates of *Campylobacter* (11 isolates), *Helicobacter* (8 isolates), *Aeromonas* (31 isolates), *Salmonella* (4 isolates), and *Arcobacter* (4 isolates) did not amplify a product by use of primers specific for *cdt-III* even when they were positive for CDT by the CHO cell assay. Detection of the *cdt-III* gene required 1.25×10^4 bacterial cells, while detection of the *cdt-I* and *cdt-II* genes required 1.25×10^2 and 1.25×10^3 bacterial cells, respectively (Fig. 1A). Both the *cdt-I*-specific and *cdt-II*-specific primers had high degrees of sensitivity when pure DNA was used, detecting 0.05 ng of DNA, while with the *cdt-III*-specific primers a minimum of 5 ng of DNA was required for detection (Fig. 1B). The sequence of the *cdt-III* PCR product obtained from three representative isolates had 100% homology with the published sequence (data not shown). Digestion of the amplified prod-

TABLE 1. Association of other *E. coli* virulence genes with *cdt* genes

cdt variant (no. of isolates)	No. of isolates with each combination of genes[a]							
	A *cdt* gene alone	*eae*	stx_1 + stx_2	stx_2	*cnf1*	*cnf2*	*cnf1* + *eae*	*cnf2* + stx_1
cdt-I (24)	10	1	1	0	12	0	0	0
cdt-II (5)	4	0	0	0	0	0	1	0
cdt-III (21)	1	0	0	1	0	10	0	9

[a] Fifty of 322 *E. coli* strains were positive for one or more variants of *cdt*.

ucts with *Sma*I gave the 187-, 260-, and 1,783-bp DNA fragments expected, confirming the high degree of sequence homology.

The distribution of the *cdt* genes among the isolates and their association with other virulence factors are shown in Table 1. *cdt-III* was strongly associated with *cnf2*, and *cdt-I* was strongly associated with *cnfI*. All *cdt-III*-positive, *cnf2*-positive strains were isolated from cattle. Several of these strains also carried the stx_1 gene. All *cdt-I*-positive and *cnf1*-positive strains were derived from humans, as were all except one of the other strains carrying *cdt-I*; the strain that was the exception was isolated from a pig. Similarly, all *cdt-II*-positive *E. coli* were from human patients. A minority (2 of 50) of the CDT-producing *E. coli* isolates were found to carry the *eae* gene. While some *cdt*-positive *E. coli* that also express the heat-stable toxin have been found (5), none were found in this study.

Evaluation of 151 non-O157:H7 *stx*-negative *E. coli* strains from humans, animals, and the environment for the presence of the *cdt-I*, *cdt-II*, and *cdt-III* genes showed that 5 (3.3%) of the strains carried a *cdt* gene variant. Carriage of *cdt* genes was more frequent among the non-O157 blood isolates (5 of 72; 6.9%). None of 53 randomly selected *E. coli* O157:H7 isolates were found to carry any of the *cdt* variant genes. The results of CHO cell assays for CDT correlated well with those of PCR for the detection of *cdt* gene variants. However, among 46 CDT-producing human isolates collected between 1986 and 1998 and used to estimate the frequency of carriage of *cdt* gene variants, 3 isolates (7%) carried the *cdt-I* gene but did not produce detectable toxin. In addition, seven strains (15%) were CDT positive only by the cell culture assay. Toxin identity was confirmed in these seven strains by neutralization with specific anti-CDT antiserum.

CDT production has been associated with enteropathogenic *E. coli* (EPEC) serogroups (4), and an association between the *cdt* gene and the *eae* gene has been demonstrated (1, 5, 12). The frequency of *cdt* gene carriage by EPEC strains was found to be 2.8% in Brazil (12) and 6.4% in India (5). Similar frequencies of carriage were found among the non-O157 *E. coli* isolates in the present study. However, carriage of *cdt* genes is not strongly correlated with the presence of the *eae* gene alone. Both the low prevalence of *cdt* genes and their association with other virulence genes suggest that the *cdt* genes are acquired independently in a number of *E. coli* lineages, possibly as the result of horizontal gene transfer. Among blood culture isolates, *cdt* was detected in only a minority of strains within the *E. coli* B2 virulence group (13). Furthermore, analysis of the sequence surrounding the *A. actinomycetemcomitans cdt* genes and of naturally occurring *cdt* deletions suggested that the *cdt* genes are contained within a virulence-associated region resembling a pathogenicity island (16). Whether this is also true for *cdt*-positive *E. coli* strains is not known.

In some strains, the genes encoding *cdt-III* and *cnf2* are carried on the pVir plasmid (22), suggesting they may be readily transferred among *E. coli* strains. These observations support the hypothesis that *cdt* genes may have been acquired recently by a small proportion of *E. coli* strains. The strains were not assessed for the presence of the pVir plasmid in the present study; however, there was a strong association (19 of 21 isolates) between the presence of *cdt-III* and the presence of *cnf2*. In contrast to a previous study (13), there was a strong association between carriage of the *cdt-I* variant and carriage of *cnf1*. The origins of the *E. coli* strains in this study and the previous study (13) differ, in that many strains in the present study were obtained from stools of patients with diarrhea. Further investigations into the association of these genes in different *E. coli* subpopulations are required to resolve this issue.

Previous studies indicated a strong association of *cnf1*-positive strains with humans and *cnf2*-positive strains with animals (3). The association of *cdt-III* with cattle was therefore not surprising given that *cdt-III* is carried on the same plasmid as *cnf2.* The presence of the *cdt-III* gene was associated with the stx_1 genotype. *E. coli* strains carrying only stx_1 are also prevalent in cattle (8, 9, 31). In contrast, carriage of the *cdt-I* gene was not strongly correlated with the presence of *stx* genes. Although the underlying mechanisms responsible for the associations seen in the present study are not known, the results imply that cattle and humans provide ecological niches for *E. coli* strains that impose different selective pressures on the organism, resulting in the carriage of different virulence gene sets. In this case selection may be for genes other than *cdt-III* on the pVir virulence plasmid.

The *cdt-III*-specific PCR described here was effective in detecting the *cdt-III* gene from isolated DNA and should prove useful for studies aimed at characterizing the distribution of *cdt* genes in *E. coli* populations. However, CDT was detected by cell culture assays in strains that were negative by PCR with primers specific for the *cdt-I*, *cdt-II*, and *cdt-III* gene variants. This supports the hypothesis of Pérès and colleagues (22) that there are additional *cdt* gene variants that cannot be found by the available PCR methods and suggests that both PCR and cell culture assays are necessary for enumeration of CDT-positive *E. coli* strains. Characterization of these additional variant genes will aid in the development of improved PCR-based assays for the detection of *cdt*.

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