Characterization of Cytolethal Distending Toxin of *Campylobacter* Species Isolated from Captive Macaque Monkeys†

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An association between certain *Campylobacter* species and enterocolitis in humans and nonhuman primates is well established, but the association between cytolethal distending toxin and disease is incompletely understood. The purpose of the present study was to examine *Campylobacter* species isolated from captive conventionally raised macaque monkeys for the presence of the *cdtB* gene and for cytolethal distending toxin activity. The identity of each isolate was confirmed on the basis of phenotypic and genotypic analyses. The presence of cytolethal distending toxin was confirmed on the basis of characteristic morphological changes in HeLa cells incubated with filter-sterilized whole-cell lysates of reference and monkey *Campylobacter* isolates and examinations by light microscopy, confocal microscopy, and flow cytometry. Although *cdtB* gene sequences were found in both *Campylobacter jejuni* and *Campylobacter coli*, the production of cytolethal distending toxin correlated positively ($P < 0.0001$) only with *C. jejuni*. We concluded that cytolethal distending toxin activity is a characteristic of *C. jejuni*. Our *C. jejuni cdtB* gene-specific PCR assay might be of assistance for differentiating toxigenic *C. jejuni* from *C. coli* in clinical laboratories.

Members of the *Campylobacter* genus are motile, curved, gram-negative, phenotypically heterogeneous bacilli that colonize the mucus on the surface and crypts of the intestine, where certain species can cause epithelial damage and elicit host inflammatory and immune responses (21, 23, 55). *Campylobacter jejuni* is the leading cause of food-borne infectious diarrheal disease in humans in many developed countries, including the United States (47). The primary source of food-borne human *Campylobacter* species infections is contaminated chicken (15).

Campylobacter species account for a large percentage of diarrheal illnesses of colony-raised macaque monkeys (22, 37, 39, 43). Both the duration and the clinical presentation of diarrheal illness in rhesus macaques (*Macaca mulatta*) and pig-tailed macaques (*Macaca nemestrina*) experimentally challenged with *C. jejuni* are similar to those seen in humans (3, 9, 38, 40). The disease in macaques is characterized by diarrhea of variable severity and duration accompanied by prolonged intermittent fecal shedding of *C. jejuni*. Epidemiological studies of infant pig-tailed macaques kept in a nursery facility have further revealed that infection and reinfection with multiple strains of *C. jejuni* and *Campylobacter coli* are common, and as seen in humans (3), recovered monkeys are often asymptomatic (39).

Although several virulence determinants have been identified for pathogenic *Campylobacter* species, the significance of cytotoxins in disease is relatively poorly understood (54). A

newly characterized heat-labile and trypsin-sensitive cytotoxin, known as cytolethal distending toxin (CDT), was first identified from *Shigella*, *Escherichia coli*, and *C. jejuni* isolated from human and nonhuman sources over 15 years ago (18–20). In the last decade, the gene encoding CDT, *cdtB*, has been found in most *Campylobacter* species (8, 27, 35, 56) and in several serotypes of *E. coli* (7, 32, 34, 42, 48). Homologues of *cdtB* and CDT activity have also been found among enterohepatic *Helicobacter* species that associate with the intestinal mucosa (4, 24) and, most recently, in *Salmonella enterica* serovar Typhi (13). Other gram-negative bacteria harboring the *cdtB* gene and producing CDT activity include the periodontopathogenic bacterium *Actinobacillus actinomycetemcomitans* (1, 44, 46, 57) and the venereal pathogen *Haemophilus ducreyi* (1, 5, 36).

With the exception of *S. enterica* serovar Typhi, in which only *cdtB* is found (13), in all other microbes CDT consists of a tripartite polypeptide complex of CdtA (30 kDa), CdtB (29 kDa), and CdtC (21 kDa), encoded by the corresponding genes *cdtA*, *cdtB*, and *cdtC* (26, 33). Although considerable divergence in the predicted amino acid sequences of each CDT subunit exists among different microbes, the *cdtB* genes have conserved structural similarities to the mammalian DNase I catalytic and Mg²⁺-binding sites (6, 25). When it interacts with certain eukaryotic cells, the CDT holotoxin causes progressive cytoplasmic and nuclear distension accompanied by increased DNA contents, leading to growth arrest at the G₂/M transition phase of the cell cycle and, ultimately, cell death (2, 5, 32, 44, 56).

There is some evidence that CDT is a virulence determinant for naturally occurring and experimentally induced enteric diseases associated with *C. jejuni* (11), *E. coli* (30), and *Shigella dysenteriae* (28). Experimental challenge studies support a role for *C. jejuni* as a cause of diarrheal disease in monkeys (9, 38), but *Campylobacter* strains isolated from monkeys have not

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TABLE 1. Classification of macaque species investigated in this study for infection by *Campylobacter* species and for clinical signs

<i>Campylobacter</i> species, animal group, and animal no.	Age (yr)	Sex ^a	Housing ^b	Clinical presentation
<i>C. jejuni</i>				
Symptomatic <i>M. mulatta</i>				
EN69	0.45	F	Indoor cage	Diarrhea with blood for 2 months
EJ92	1.35	F	Outdoor cage	Chronic diarrhea for 1 month
EL84	1.22	F	Outdoor cage	Chronic diarrhea for <2 months, dehydration
EB33	2.31	M	Indoor cage	Chronic diarrhea for 1 month
Mean $\pm$ standard deviation ($n = 4$)	1.33 $\pm$ 0.76			
Asymptomatic <i>M. mulatta</i>				
FI77	4.91	F	Indoor cage	Healthy
FI79	3.05	F	Indoor cage	Healthy
FI83	2.94	F	Indoor cage	Healthy
FI84	3.17	F	Indoor cage	Healthy
Mean $\pm$ standard deviation ($n = 4$)	3.52 $\pm$ 0.93			
Asymptomatic <i>M. nemestrina</i>				
ET03	3.90	F	Outdoor cage	Healthy
ET37	0.82	F	Outdoor cage	Healthy
ET46	1.61	F	Outdoor cage	Healthy
ET51	0.54	M	Outdoor cage	Healthy
Mean $\pm$ standard deviation ($n = 4$)	1.72 $\pm$ 1.52			
<i>C. coli</i>				
Symptomatic <i>M. mulatta</i>				
DC93	2.71	F	Indoor cage	Intermittent diarrhea for >1 year
AE80-7	2.61	M	Indoor cage	Chronic diarrhea found dead
BH12-8	5.32	F	Indoor cage	Diarrhea with blood for <1 month
T818-9	8.31	F	Indoor cage	Diarrhea for >2 months
EE69-12	1.11	M	Indoor cage	Diarrhea <1 month
EH81-48	0.57	F	Indoor cage	Diarrhea <1 month
Mean $\pm$ standard deviation ($n = 6$)	3.44 $\pm$ 2.90			
Asymptomatic <i>M. mulatta</i>				
AE55-3	6.92	M	Indoor cage	Healthy
EE36-13	1.59	F	Outdoor cage	Healthy
L924-46	12.60	F	Outdoor cage	Healthy
Mean $\pm$ standard deviation ($n = 3$)	7.04 $\pm$ 5.51			
Asymptomatic <i>M. nemestrina</i>				
ER94-24	0.43	F	Outdoor cage	Healthy
DA48-27	3.11	F	Indoor cage	Healthy
ET24-29	0.07	F	Outdoor cage	Healthy
ET32-30	4.94	F	Outdoor cage	Healthy
ET34-31	0.63	M	Outdoor cage	Healthy
ET43-34	1.53	M	Outdoor cage	Healthy
Mean $\pm$ standard deviation ($n = 6$)	1.79 $\pm$ 1.89			
<i>C. jejuni</i> and <i>C. coli</i>				
Symptomatic <i>M. mulatta</i>				
EB53	1.46	M	Outdoor cage	Intermittent diarrhea for >6 months

^a M, male; F, female.^b Housing was supplied at biocontainment level 2.

been examined for the presence of the *cdtB* gene and for CDT activity. For this study, we examined *Campylobacter* strains isolated from captive conventionally raised macaques with and without clinical signs of diarrhea for the presence of the *cdtB* gene and CDT activity. We found that both *C. jejuni* and *C. coli* have the *cdtB* gene but that CDT activity was only present in *C. jejuni*.

CASE REPORTS

The clinical presentations and housing of 28 macaques investigated in the present study are outlined in Table 1. The sex distribution of 20 females versus 8 males reflects the natural distribution in the colony. The animals were kept at the Tulane National Primate Research Center in accordance with the standards of the *Guide for the Care and Use of Laboratory Animals* and the Association for Assessment and Accreditation of Laboratory Animal Care. The macaques were housed either

in groups in outdoor breeding cages or individually indoors at biocontainment level 2. Stool specimens were collected either during routine health monitoring of the colony or from macaques with clinical manifestations of diarrheal illnesses of various durations (Table 1). Following an initial sampling in September 2003, rhesus macaque EB53 was treated with the broad-spectrum antibiotic tylosin at a dose of 10 mg/kg of body weight by intramuscular injections twice a day for 10 days and was resampled a month later when diarrhea was still present. The isolation of *Campylobacter* species from stool specimens was the main selection criterion for study assignment.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Campylobacter* strains were isolated from stool specimens by microaerobic incubation at 42°C on *Campylobacter* selective agar (BBL, Cockeysville, Md.) containing cefoperazone, vancomycin, and amphotericin B and then initially identified as previously described (43). The reference human strains *C. jejuni* subsp. *jejuni* VPI H840 (ATCC 29428 [50]) and

LRA 094.06.89 (ATCC 49943), the porcine strains *C. coli* CIP 7080 (ATCC 33559 [45]) and LRA 069.05.89 (ATCC 49941), the human strain *Arcobacter butzleri* LMG 10828 (ATCC 49616 [51]), and the sheep strain *Arcobacter skirrowii* LMG 6621 (ATCC 51132 [51]) were used as controls. Stock cultures were propagated onto Trypticase soy agar with 5% (vol/vol) sheep blood (TSAB; Remel, Lenexa, Kans.) and incubated under microaerobic conditions (Mitsubishi Gas Chemical Co. Inc., New York, N.Y.) at either 24, 37, or 42°C or in Brucella broth (Difco, Detroit, Mich.) supplemented with 5% (vol/vol) sheep serum (Sigma Chemical Co., St. Louis, Mo.) with constant stirring at 37°C in an atmosphere consisting of 5% oxygen, 5% carbon dioxide, and 90% nitrogen. Chemically competent TOP10 *E. coli* cells transformed with the pCR4-TOPO cloning vector (Invitrogen Corp., Carlsbad, Calif.) were grown in Luria-Bertani medium containing ampicillin or kanamycin (Sigma) at 37°C overnight.

Phenotypic characterization. Each reference and macaque isolate was examined by wet mounting and gram staining and observed for growth at either 24, 37, or 42°C under microaerobic and aerobic conditions for up to 14 days postinoculation. Catalase and oxidase activities (Remel), the production of urease (Remel), the reduction of nitrate to nitrite (Remel), the hydrolysis of hippurate (Remel), and susceptibilities to nalidixic acid (30 µg) and cephalothin (30 µg) by disk diffusion (Becton Dickinson, Sparks, Md.) were determined by standard laboratory methods (10).

Genotypic identification. *C. jejuni* and *C. coli* were identified on the basis of PCR amplification of the species-specific *ceuE* gene (12). Briefly, 2 µl of bacterial suspension in sterile water was mixed with a total volume of 48 µl containing 1× PCR buffer, 3.5 mM MgCl₂, a 0.2 mM concentration (each) of dATP, dTTP, dGTP, and dCTP, a 1.0 µM concentration (each) of the oligonucleotide primers JEJ1 and JEJ2 or COL11 and COL12, and 0.5 U of *Taq* DNA polymerase (GeneChoice, Inc., Frederick, Md.) in sterile, filtered, autoclaved water. Initial denaturing for 3 min at 94°C was followed by 30 cycles of 30 s at 94°C, 30 s at 57°C, and 1 min at 72°C, with a final extension for 5 min at 72°C, in a thermocycler (GeneAmp PCR System 9600; Perkin-Elmer Cetus, Norwalk, Conn.). The 783-bp *C. jejuni* and the 894-bp *C. coli* products were visualized after electrophoresis in 0.8% agarose gels run at 7.0 V/cm and staining with ethidium bromide. Identification of the *Arcobacter* genus and of *A. butzleri* was accomplished by a multiplex PCR method as previously described (14).

Amplification, cloning, and sequencing of *cdtB* gene. The *cdtB* genes of reference and macaque isolates were amplified by PCRs with the degenerative forward oligonucleotide primer VAT2, extending from base position 67 of *C. jejuni* strain 81-176 (GenBank accession no. U51121; National Center for Biotechnology Information, Bethesda, Md.) with the nucleotide sequence 5'-GTN GCNACBTGGAAYCTNCARGG-3', and the reverse oligonucleotide primer WM11 at position 539, with the nucleotide sequence 5'-RTTRAARTCNCCYA ADATCATCC-3', as previously described (35). Additionally, a *C. jejuni cdtB* gene-specific sequence was amplified with the forward oligonucleotide primer CjcdtBF, extending from position 93 of *C. jejuni* strain 81-176 with the nucleotide sequence 5'-ATCGCAGCCACAGAAAGCAAATG-3', and the reverse oligonucleotide primer CjcdtBR at position 692, with the nucleotide sequence 5'-GCGGTGGAGTATAGGTTTGTGTGTC-3'. Two microliters of bacterial suspension in sterile water was mixed with a total volume of 48 µl containing 1× PCR buffer, 1.5 mM MgCl₂, a 0.2 mM concentration (each) of dATP, dTTP, dGTP, and dCTP, a 0.25 µM concentration of each oligonucleotide primer, and 2.0 U of *Taq* DNA polymerase (GeneChoice) in sterile, filtered, autoclaved water. The PCR parameters for amplification reactions were similar to those for the *Campylobacter* species-specific *ceuE* gene, except that annealing was done for 60 s at 50°C. The 495- and 623-bp amplified products, generated with the degenerative and *C. jejuni cdtB* gene-specific oligonucleotide primers, respectively, were visualized by electrophoresis in 0.8% agarose gels run at 7.0 V/cm after staining with ethidium bromide. The partial nucleotide sequence of the *C. jejuni* EB53-23-01 *cdtB* gene was determined. Briefly, each 495- and 623-bp amplified product was excised, purified (Zymoclean Gel DNA recovery kit; Zymo Research, Orange, Calif.), cloned into pCR4-TOPO, and transformed into chemically competent *E. coli* TOP10 cells according to the manufacturer's instructions. The nucleotide sequences of both strands from five clones of each product were sequenced at the Genomics Core Research Facility, University of Nebraska-Lincoln Center for Biotechnology, by use of an automated dideoxy sequencing method (41) and a commercial kit (CEQ DTCS quick start kit; Beckman Coulter, Fullerton, Calif.) for fluorescence sequencing (CEQ 2000XL/8000 DNA sequencer; Beckman Coulter). A partial 602-bp consensus *cdtB* gene sequence extending from positions 90 to 691 was aligned and edited with the Wisconsin Package, version 9.0 (Genetics Computer Group, Madison, Wis.), and then compared with available sequences from other bacteria in GenBank by use of the BLAST program.

Preparation of bacterial cell lysates. Confluent cultures of reference and macaque bacterial isolates grown on TSAB for 2 to 3 days under microaerobic conditions at 42°C were harvested by washing the surface growth with phosphate-buffered saline (PBS; pH 7.2) and then centrifuging it at 800 × *g* for 20 min at 4°C. The pellet was suspended in 1.0 ml of ice-cold PBS and lysed by sonication as previously described (4). Unlysed bacteria and cell debris were removed by centrifugation at 6,000 × *g* for 20 min at 4°C, and the supernatant was filtered through 0.22-µm-pore-size filters and stored at -20°C until needed. The concentration of protein present in each filter-sterilized lysate was determined by use of a commercially available kit (BCA protein assay kit; Pierce, Rockford, Ill.).

HeLa cell culture and CDT bioassay. A human epithelioid cervical carcinoma HeLa cell line (ATCC CCL-2) was maintained in Eagle's minimum essential medium (MEM; Sigma) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, Ga.) and gentamicin (50 µg/ml; Sigma) at 37°C in a humidified atmosphere of 5% carbon dioxide in air. The CDT bioassay was performed as previously described by Johnson and Lior (20), with some modifications. Approximately 2 × 10⁴ cells in 10% FBS-MEM were placed in each well of a chamber slide (Lab-Tek II chamber slide system; Nalge Nunc International, Naperville, Ill.) and incubated for 3 h at 37°C in 5% carbon dioxide in air. Either 100 µl of PBS (negative control), 20, 40, or 80 µM (final concentration) apigenin (positive control; apigenin is a compound known to arrest the cell cycle at the G₂/M phase [53]), or 10.0, 20.0, or 40.0 µl of filter-sterilized lysate obtained from a reference or macaque *Campylobacter* isolate or a reference *Arcobacter* strain was then added to individual wells and incubated for an additional 72 to 96 h. At the end of the incubation, each culture was washed with PBS, fixed with 10% neutral buffered formalin (VWR International, West Chester, Pa.), stained with hematoxylin and eosin, and examined under a light microscope. The mean diameter and standard deviation of nuclei from 10 HeLa cells in cultures incubated with either PBS or 10 µl of filter-sterilized lysate obtained from *C. coli* EB53-02-12 or *C. jejuni* EB53-23-01 were determined. The nuclear morphology of control and HeLa cells incubated with bacterial lysates was examined further by use of a confocal laser scanning microscope (model FV500; Olympus Optical Co., Tokyo, Japan) after staining with propidium iodide (PI; Sigma) as previously described (2). Lysates from reference and macaque *Campylobacter* isolates were examined in at least two separate experiments, and positive and negative controls were included in each assay.

Cell cycle analysis by FACS. The G₂/M-phase cell cycle arrest of HeLa cells incubated with lysates obtained from reference and macaque *Campylobacter* isolates and reference *Arcobacter* strains was determined quantitatively by fluorescence-activated cell sorter (FACS) analysis and compared with that of either untreated cells or HeLa cells incubated with PBS (negative control) or apigenin (positive control). Confluent monolayers of HeLa cells were treated with 0.05% (wt/vol) trypsin-EDTA (Invitrogen), washed with PBS, suspended in 10% FBS-MEM to obtain 3 × 10⁵ cells in 5.0 ml, and placed into 25-cm² flasks (Costar, Cambridge, Mass.). After 3 h, either 100 µl of PBS, 20, 40, or 80 µM (final concentration) apigenin, or 1.0, 10.0, 100.0, or 200.0 µl of filter-sterilized bacterial cell lysate was added to the culture medium, and the cells were incubated for an additional 72 h. HeLa cells were prepared for FACS analysis as previously described (5), with some modifications. After being washed with PBS, the cells were treated with trypsin and centrifuged at 1,000 × *g* for 2 min. The cell pellet was then suspended in PBS, transferred to 70% ethanol, and held at -20°C for 5 min. The cells were harvested by centrifugation and rehydrated with 1.0 ml of PBS at room temperature for 15 min. Following centrifugation, the cells were suspended in PI staining buffer (3 µg of PI/ml, 0.02 mg of RNase A/ml, 100 mM Tris [pH 7.2], 150 mM NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 0.1% Triton X-100) and incubated at room temperature in the dark for 15 min. For DNA content analysis, approximately 1.0 × 10⁴ cells were examined by FACS analysis, with the excitation set at 488 nm and the emission set at 630 nm (FACScan flow cytometer; Becton Dickinson) and with the data analyzed by use of Cell Quest and ModFit LT software (Becton Dickinson). Lysates from reference and macaque *Campylobacter* isolates and reference *Arcobacter* strains were examined in at least two separate experiments, and positive and negative controls were included in each assay.

Data analysis. FACS analysis data for HeLa cells in G₂/M phase were expressed as mean percentages of HeLa cells with 4N DNA contents ± standard deviations. An association between the presence of CDT and the species of *Campylobacter* was determined by using the Student *t* test. The significance of association was tested at an α value of 0.05.

Nucleotide sequence accession number. The 602-bp partial nucleotide sequence of the *C. jejuni* EB53-23-01 *cdtB* gene has been deposited in GenBank under accession number AY652840.

TABLE 2. Biochemical and PCR characterization of *Campylobacter* species isolated from macaques^a

Species and strain	Date of isolation	Growth pattern	Hippurate hydrolysis	Drug sensitivity		PCR amplification of <i>ceuE</i> gene		PCR amplification of <i>cdtB</i> gene with indicated primers	
				Nalidixic acid	Cephalothin	<i>C. jejuni</i>	<i>C. coli</i>	VAT2 and WMI1	CjcdtBF and CjcdtBR
<i>C. jejuni</i> (n = 16)									
EN69-49-09	10/08/03	Colonies	—	—	—	+	—	+	+
EJ92	08/16/04	Colonies	+	+	—	+	—	+	+
EL84	08/17/04	Colonies	+	+	—	+	—	+	+
EB33	08/16/04	Colonies	+	+	—	+	—	+	+
FI77	08/24/04	Colonies	+	—	—	+	—	+	+
FI79	08/24/04	Colonies	+	—	—	+	—	+	+
FI83	08/24/04	Colonies	+	—	—	+	—	+	+
FI84	08/24/04	Colonies	+	—	—	+	—	+	+
ET03-26-14	10/02/03	Spreading haze	+	+	—	+	—	+	+
ET03-26-15	10/02/03	Colonies	—	+	—	+	—	+	+
ET37-32-17	10/03/03	Colonies	+	+	—	+	—	+	+
ET46-36-20	10/03/03	Spreading haze	+	+	—	+	—	+	+
ET46-36-21	10/03/03	Colonies	+	+	—	+	—	+	+
ET51-37-18	10/03/03	Spreading haze	+	+	—	+	—	+	+
ET51-37-19	10/03/03	Colonies	+	+	—	+	—	+	+
EB53-23-01	10/01/03	Colonies	+	+	—	+	—	+	+
<i>C. coli</i> (n = 16)									
DC93-53-05	10/14/03	Colonies	—	+	—	—	+	+	—
AE80-7	02/02/99	Colonies	—	—	—	—	+	+	—
BH12-8	09/11/03	Colonies	—	+	—	—	+	+	—
T818-9	09/17/03	Colonies	—	+	—	—	+	+	—
EE69-12	09/24/03	Colonies	—	+	—	—	+	+	—
EH81-48	10/08/03	Colonies	—	+	—	—	+	+	—
AE55-3	09/04/03	Colonies	—	+	—	—	+	+	—
EE36-13	09/25/03	Colonies	—	+	—	—	+	+	—
L924-46	10/08/03	Colonies	—	+	—	—	+	+	—
ER94-24	10/01/03	Colonies	—	+	—	—	+	+	—
DA48-27	10/02/03	Colonies	—	+	—	—	+	+	—
ET24-29	10/03/03	Colonies	—	+	—	—	+	+	—
ET32-30	10/03/03	Colonies	—	+	—	—	+	+	—
ET34-31	10/03/03	Colonies	—	+	—	—	+	+	—
ET43-34	10/03/03	Colonies	—	+	—	—	+	+	—
EB53-02-12	09/04/03	Colonies	—	+	—	—	+	+	—

^a +, present; —, absent.

RESULTS

Clinical findings. Although *C. jejuni* and *C. coli* were isolated from rhesus and pig-tailed macaques, diarrheal illness was present only in rhesus macaques. Approximately equal numbers of symptomatic and asymptomatic rhesus macaques were infected with *C. jejuni* or *C. coli*. At the initial clinical presentation in September 2003, a stool specimen taken from rhesus macaque EB53 contained *C. coli* (Table 2, strain EB53-02-12), while clinically this monkey showed weakness and dehydration. After antibiotic therapy, the clinical status of the monkey improved and diarrhea ceased, but recurrent episodes of diarrhea continued through April 2004, and a culture of a stool specimen taken in October 2003 yielded *C. jejuni* (Table 2, strain EB53-23-01).

Phenotypic and genotypic characterizations. The macaque *Campylobacter* isolates formed distinct gray colonies, but cultures of ET03, ET46, and ET51 also formed a spreading haze that was maintained after subculture (Table 2). All of the isolates grew under microaerobic conditions at 37 and 42°C, but not at 25°C or under aerobic conditions. They were gram negative with a curved-rod morphology and had darting motility. The biochemical and genotypic characteristics of each isolate are presented in Table 2. Additionally, the isolates produced catalase and oxidase but did not produce urease or reduce nitrate to nitrite. With the exception of isolates EN69-49-09

FIG. 1. Ethidium bromide-stained 0.8% agarose gel with PCR-amplified 495-bp products obtained with degenerative oligonucleotide primers VAT2 and WMI1 (A) and 623-bp products obtained with *C. jejuni cdtB* gene-specific oligonucleotide primers CjcdtBF and CjcdtBR (B) from reference and monkey *Campylobacter* isolates. Lanes: 1, molecular size standard in base pairs (HyperLadder I; Bioline, Randolph, Mass.); 2, *C. jejuni* ATCC 49943; 3, EB53-23-01; 4, ET03-26-14; 5, ET46-36-20; 6, EN69-49-09; 7, ET37-32-17; 8, ET51-37-18; 9, *C. coli* ATCC 49941; 10, EB53-02-12; 11, DC93-53-05. All of the isolates contained the *cdtB* gene, as determined with the degenerate primers, whereas only the *C. jejuni* isolates had the *C. jejuni cdtB* gene-specific product.

FIG. 2. Light photomicrographs of HeLa cells incubated with macaque *Campylobacter* lysates for 72 h at 37°C. HeLa cells incubated with either PBS (A) or a lysate obtained from macaque *C. coli* EB53-02-12 (B) had normally sized cytoplasmic boundaries and nuclei. HeLa cells incubated with a lysate from macaque *C. jejuni* EB53-23-01 (C) had a mean size of 2.0- to 2.5-fold that of control cells and displayed marked nuclear enlargement and occasional multinucleated giant cells (arrow). The images shown were stained with hematoxylin and eosin.

and ET03-26-15, all of the *C. jejuni* isolates hydrolyzed hippurate. All of the isolates were resistant to cephalothin, and while the *C. jejuni* and *C. coli* isolates were generally susceptible to nalidixic acid, five *C. jejuni* isolates and one *C. coli* isolate were resistant. None of the isolates yielded products in multiplex PCR assays for the *Arcobacter* 16S rRNA and *A. butzleri* 23S rRNA genes.

Detection and characterization of *cdtB* gene. A putative 495-bp *cdtB* gene product was obtained by PCR amplification of all of the *Campylobacter* isolates by use of the degenerative oligonucleotide primers VAT2 and WMI1 (Table 2 and Fig. 1A). Conversely, only the *C. jejuni* isolates generated the expected 623-bp product by PCR amplification with *C. jejuni cdtB* gene-specific oligonucleotide primers (Table 2 and Fig. 1B). With the exception of a single silent nucleotide substitution (C instead of T at position 126), the consensus sequence of the cloned products obtained by PCR amplification of EB53-23-01 with the *cdtB* gene-specific oligonucleotide primers was identical to the *cdtB* gene sequence of the reference human strains *C. jejuni* subsp. *jejuni* NCTC 11168 (AL139074) (31), *C. jejuni* 81-176 (U51121) (35), and *C. jejuni* CH5 (AF038283) (16).

Morphological changes in HeLa cells incubated with bacterial lysates. Significant changes were not observed in HeLa cells incubated with PBS (Fig. 2A) or in lysates obtained from either the reference and macaque *C. coli* isolates (Fig. 2B), the

C. jejuni isolate EJ92, or the reference *Arcobacter* isolates at any of the times and concentrations examined. HeLa cells incubated with either apigenin or the reference and remaining macaque *C. jejuni* isolates displayed morphological changes that were characteristic of CDT in a time- and dose-dependent manner. HeLa cells incubated with 20 and 40 µM apigenin had larger cytoplasmic boundaries than control cells by 48 h, whereas cellular enlargement was apparent by 24 h with the 80 µM concentration. Between 48 and 72 h of incubation, the cellular enlargement became progressively more prominent. A marked enlargement of the cytoplasmic boundaries and the nucleus was first seen by 48 h for HeLa cells incubated with lysates obtained from the reference and macaque *C. jejuni* isolates. Between 72 and 96 h, the mean size of distended cells was approximately 2.0- to 2.5-fold that of control cells (Fig. 2C). By 96 h, nuclear fragmentation and cytoplasmic vacuolation were also seen. A dose effect similar to that for apigenin was seen with the *C. jejuni* lysates. Ten microliters of *C. jejuni* lysate was sufficient to exert a cytotoxic effect, whereas larger volumes caused more rapid and prominent morphological changes. Although the magnitude of the cellular changes over time varied among the *C. jejuni* isolates and with the protein concentration of the lysate, only the reference and macaque *C. jejuni* isolates produced detectable cellular and nuclear enlargement. In addition to the characteristic cellular enlargement, HeLa cells incubated with either apigenin or nearly all *C. jejuni* lysates, but not with the *C. coli* or *Arcobacter* isolates, contained between 1 and 2% multinucleated giant cells, depending on the isolate (Fig. 2C). When HeLa cells were incubated with either PBS, lysates from reference and macaque *C. coli* isolates, the *C. jejuni* isolate EJ92, or reference *Arcobacter* strains, stained with PI, and examined under a confocal laser scanning microscope, they had small nuclei with widely dispersed small nucleoli (Fig. 3A and B). In contrast, HeLa

FIG. 3. Confocal laser scanning photomicrographs (A, C, and E) and corresponding phase-contrast images (B, D, and F) of HeLa cells incubated with a macaque *C. jejuni* lysate for 72 h at 37°C. HeLa cells incubated with PBS (A and B) had normally sized nuclei with widely dispersed small nucleoli and normally sized cytoplasmic boundaries. HeLa cells incubated with a lysate from macaque *C. jejuni* EB33 had an increased nuclear size, prominent nucleoli (C and D), and occasional multinucleated giant cells (E and F). The images shown were stained with propidium iodide. Bar = 50 μ m.

cells incubated with lysates from reference strains and the remaining macaque *C. jejuni* isolates had a mean nuclear size of approximately 1.5- to 2.0-fold that of control cells, contained fewer and more prominent nucleoli (Fig. 3C and D), and had occasional multinucleated giant cells (Fig. 3E and F) by 48 h.

Quantitative fluorescent cell cycle analysis of HeLa cells incubated with bacterial lysates. Approximately $10.6\% \pm 0.2\%$ of the cells in untreated HeLa cell cultures and cultures incubated with PBS were in the G₂/M phase (Fig. 4A). Conversely, HeLa cell cultures incubated in the presence of apigenin had an increase in the percentage of cells that were arrested at the G₂/M phase in a dose-dependent manner: $<10\%$ of the cells were in the G₂/M phase with 20 μ M apigenin, whereas $57.0\% \pm 18.0\%$ and $78.5\% \pm 16.2\%$ of the cells were in the G₂/M phase with 40 and 80 μ M apigenin, respectively. For HeLa cells incubated with any concentration of lysate obtained from either the reference or macaque *C. coli* isolates, $<7.6\% \pm$

1.8% of the cells were in the G₂/M phase (Fig. 4B). Similarly, for HeLa cells incubated with lysates obtained from the reference *Arcobacter* strains, $<9.8\% \pm 0.3\%$ of the cells were in the G₂/M phase. In contrast, $81.1\% \pm 4.8\%$ of HeLa cells were arrested in the G₂/M phase when they were incubated with a lysate obtained from the reference strain *C. jejuni* ATCC 49943. The percentage of HeLa cells arrested in G₂/M phase ranged between $35.6\% \pm 12.0\%$ and $98.6\% \pm 0.2\%$ after incubation with lysates obtained from the macaque-derived *C. jejuni* isolates (Fig. 4C). Compared with those from other isolates, the lysate obtained from the macaque *C. jejuni* isolate EJ92 did not cause a significant increase in the percentage of HeLa cells arrested in the G₂/M phase of the cell cycle, with $17.1\% \pm 3.0\%$ of the cells being in this phase. In all of the assays, the progressive increase in the percentage of cells in G₂/M phase (4N DNA content) was accompanied by a corresponding decline in the number of cells in G₁ phase (2N DNA content). The variable percentages of HeLa cells arrested in the G₂/M phase with different *C. jejuni* lysates could not be attributed to the concentration of protein, but rather depended on the absolute amount of CDT produced by individual isolates. Thus, 1 μ l of EB53-23-01 lysate containing approximately 3 μ g of protein caused $>96\%$ of HeLa cells to arrest in the G₂/M phase, whereas 20 μ l of ET03-26-15 lysate containing approximately 40 μ g of protein was required to achieve a similar level of arrest. In addition to HeLa cells with 4N DNA contents, cells with 8N DNA contents were seen after incubation with apigenin and lysates from reference and macaque *C. jejuni* isolates, but not with lysates from either reference and macaque *C. coli* or reference *Arcobacter* strains. The production of CDT was positively correlated with lysates obtained from *C. jejuni* ($P < 0.0001$) but not with those obtained from *C. coli*.

DISCUSSION

Phenotypic and genotypic characterizations of *Campylobacter* strains isolated from captive conventionally raised macaques revealed that *cdtB* gene sequences were present in both *C. jejuni* and *C. coli* but that CDT activity was only present in the 2 reference human *C. jejuni* isolates and 15 of the 16 *C. jejuni* isolates from macaques. This is consistent with previous reports indicating that although the *cdtB* gene is present in both *C. jejuni* and *C. coli* (8), the production of CDT is a characteristic of *C. jejuni* (16, 35). A previous study found CDT activity in *C. jejuni* strains isolated from 13 of 14 humans, 3 of 4 cows, a goat, and a lamb, whereas *C. coli* strains isolated from 10 humans, a cow, and a sheep had no significant CDT activity (35). Our data extend these previous observations to include *Campylobacter* strains isolated from nonhuman primates. Because the membrane fraction of *C. jejuni*, but not *C. coli*, can elicit the release of interleukin-8 by human intestinal epithelial cells (16), the absence of CDT activity in macaque *C. coli* isolates and the *C. jejuni* isolate EJ92 might be attributable to either an inability to produce CDT or the production of abnormal or inactive CDT in these isolates. Alternatively, as recently suggested for *A. actinomycetemcomitans* (57), polymorphisms in the *cdtABC* flanking regions might account for the absence of CDT activity in some strains. The absence of the *cdtB* gene and of CDT activity in the reference *Arcobacter*

FIG. 4. Quantitative fluorescent cell cycle analysis of HeLa cells incubated with macaque *Campylobacter* lysates for 72 h at 37°C. Less than 12% of HeLa cells that were incubated with either PBS (A) or a lysate obtained from macaque *C. coli* DC93-53-05 (B) had 4N DNA contents, whereas >96% of HeLa cells incubated with a lysate obtained from macaque *C. jejuni* EB53-23-01 had 4N DNA contents and were arrested in the G₂/M phase of the cell cycle, a characteristic of cytotoxic distending toxin activity. The y axes indicate cell numbers, and the x axes indicate DNA contents. The first and second arrowheads below the x axes indicate 2N and 4N DNA contents, respectively. The cells were stained with propidium iodide stain, and data are for 1.0×10^4 cells.

strains was consistent with a previous report indicating that these strains do not have this virulence determinant (17).

Although CDT has been suggested as a virulence determinant, four of eight rhesus macaques and four pig-tailed macaques with toxigenic *C. jejuni* did not exhibit clinical signs of enteric disorders. This may be attributable to either (i) sam-

pling prior to clinical disease, (ii) sampling after recovery from infection while the animals were subclinically infected, or (iii) a requirement for either additional or other virulence determinants for clinical diarrhea associated with *C. jejuni* infection. On the basis of experimental challenge studies, the possibility that macaques are not susceptible to *C. jejuni* infection is unlikely (9, 38, 40). However, because *C. jejuni*-specific immunity can prevent reinfection of recovered hosts, including macaques (3, 9, 38), and because the four asymptomatic rhesus macaques were older than those with clinical diarrhea (mean age $\pm$ standard deviation, 3.52 ± 0.93 versus 1.33 ± 0.76), prior exposure and protective immunity might explain the absence of clinical signs in these monkeys. Conversely, two of the pig-tailed macaques were less than a year old, suggesting that maternal immunity might have prevented clinical disease in these monkeys. It is likely that virulence determinants other than CDT play a role in diarrheal disease caused by *C. jejuni*. Because the toxigenic *C. jejuni* isolates were not examined for virulence determinants other than the presence of the *ceuE* gene (12), it is unknown whether or not these isolates lack other determinants required for disease expression in their natural host. Nevertheless, CDT alone was recently shown to

play an essential role in disease by use of an NF- κ B-deficient mouse model of campylobacteriosis (11).

Stool cultures taken from six rhesus macaques with diarrhea had *C. coli* only. Although *C. coli* can cause diarrhea in susceptible hosts, including monkeys, the possibility that *C. jejuni* or some other enteropathogen was responsible for chronic diarrhea cannot be ruled out completely. As seen with rhesus macaque EB53, from which repeated stool cultures yielded *C. coli* at the first sampling and toxigenic *C. jejuni* a month later, a single stool culture might not be sufficient in order to assess the role of *Campylobacter* species in diarrhea of colony-raised macaques. As previously shown, continuous exposure to pathogenic *Campylobacter* and reinfection with different strains over time are common among conventionally raised macaques kept in close contact in primate colonies (39, 43).

Although most of the macaque *C. jejuni* and *C. coli* isolates conformed to their respective phenotypic characteristics, three macaques had *C. jejuni* isolates that either grew as a spreading haze or formed individual colonies, two others had isolates that did not hydrolyze hippurate, and six had *C. jejuni* or *C. coli* isolates that were resistant to nalidixic acid. Isolates of *C. jejuni* with a spreading haze phenotype might represent motility variants (3). The ability of *C. jejuni* to hydrolyze hippurate into benzoic acid and glycine is commonly used for rapid laboratory differentiation between *C. jejuni* and *C. coli* or other, less common *Campylobacter* species (10, 29). The isolation of a macaque *C. jejuni* strain that did not hydrolyze hippurate was consistent with previous reports indicating that certain *C. jejuni* strains isolated from humans and other hosts, including macaques, do not hydrolyze hippurate (39, 49, 52). In spite of these various phenotypes, CDT activity was found exclusively among *C. jejuni* isolates. As suggested previously (8, 35), the *cdtB* genes of *C. jejuni* and *C. coli* appear to be sufficiently divergent to allow the differentiation of each species by use of a *C. jejuni cdtB* gene-specific PCR assay. In contrast with the variability of phenotypes for the hydrolysis of hippurate, the *C. jejuni cdtB* gene-specific PCR assay might be useful for differentiating toxigenic *C. jejuni* from *C. coli* in clinical laboratories.

In addition to the progressive cytoplasmic and nuclear enlargement and G₂/M-phase cell cycle arrest characteristic of CDT, HeLa cell cultures incubated with either apigenin or lysates obtained from reference and macaque *C. jejuni* strains contained cells with 8N DNA according to FACS analysis. These cells with four times the normal amount of DNA likely corresponded to the 1 to 2% of multinucleated giant cells seen by direct microscopic examination. The presence of multinucleated giant cells in HeLa cell cultures treated with CDT was originally reported by Johnson and Lior (20) as a feature of *Campylobacter fetus* subsp. *fetus*. Similar multinucleated giant cells have also been found in HeLa cell cultures treated with lysates from certain enterohepatic *Helicobacter* species (4, 24) and *E. coli* (2, 48). An interruption of cytokinesis while nuclear division continues has been suggested as a possible mechanism for the formation of multinucleated cells in the presence of CDT (2). In addition to nuclear enlargement, HeLa cells incubated with *C. jejuni* lysates and examined by confocal laser scanning microscopy had nuclei with few but expanded nucleoli, suggesting that there was enhanced ribosome biogenesis.

In conclusion, we found the *cdtB* gene and CDT activity in *C. jejuni* strains isolated from healthy macaques and from

macaques with clinical signs of diarrhea. Although the *cdtB* gene was also present in *C. coli* strains isolated from symptomatic and asymptomatic macaques, only lysates obtained from the *C. jejuni* isolates caused cytoplasmic and nuclear enlargement together with the cell cycle arrest of HeLa cells in the G₂/M phase that is characteristic of CDT activity.

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