

## Prevalence of Virulence Genes and Cytolethal Distending Toxin Production in *Campylobacter jejuni* Isolates from Diarrheal Patients in Bangladesh<sup>∇</sup>

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**From 300 stool samples, 58 *Campylobacter* strains were isolated by standard microbiological and biochemical methods. Of these, 40 strains were identified as *Campylobacter jejuni* and 5 as *Campylobacter coli*. The presence of *flaA* (100%), *cadF* (100%), *racR* (100%), *dnaJ* (100%), *pldA* (100%), *ciaB* (95%), *virB11* (0%), *ceuE* (82.5%), *cdtA* (97.5%), *cdtB* (97.5%), *cdtC* (97.5%), and *wlaN* (7.5%) genes was detected in *C. jejuni* by PCR. All *C. jejuni* strains but one produced cytolethal distending toxin in a HeLa cell assay.**

*Campylobacter* is a major cause of human bacterial gastroenteritis, and may be responsible for as many as 400 to 500 million cases worldwide each year (9). Although the genus *Campylobacter* is composed of 17 described species, human illness is associated primarily with *Campylobacter jejuni* and *Campylobacter coli* (17). Previous studies in Bangladesh conducted in the 1990s showed that *C. jejuni* is typically associated with predominantly watery diarrhea and isolated in frequencies varying from 17 to 26% (2, 12). Specific properties involved in adhesion, colonization, invasion, and toxin production appear necessary in the process of infection (15). In this study, *flaA*, *cadF*, *racR*, and *dnaJ* were selected as pathogenic genes responsible for the expression of adherence and colonization; *virB11*, *ciaB*, and *pldA* were selected as pathogenic genes responsible for the expression of invasion; *cdtA*, *cdtB*, and *cdtC* were selected as pathogenic genes responsible for the expression of toxin production; and *wlaN* was selected as a gene that is presumably involved in the expression of ganglioside mimics in Guillian-Barré syndrome (8). Another putative virulence gene, *ceuE*, encoding a lipoprotein (a component of a protein-binding-dependent transport system for the siderophore enterochelin) of *C. jejuni* was also selected in this study (23). In this study, the prevalence of *Campylobacter* species and their biochemical properties were determined. The prevalence of different pathogenic genes among *C. jejuni* strains was detected by PCR, and cytolethal distending toxin (CDT) activity was tested on HeLa cells.

Three hundred stool specimens collected from 2% of patients attending the ICDDR,B diarrhea treatment center under the systematic surveillance system (26) between January and December 2002 were examined. From these samples, 58 strains were isolated and presumptively identified as *Campy-*

*lobacter* species by standard microbiological and biochemical methods (1, 7). Species identifications of *C. jejuni* and *C. coli* strains were confirmed by hippurate hydrolysis test and PCR (19).

Template DNAs for PCR were extracted by the conventional boiling method (8). Four sets of primers for *cadF* (14), *cdtA* (11), *ceuE* (10), and *wlaN* (18) were described earlier. The remaining eight sets of primers were designed by Datta et al. (8). All primers were purchased from Integrated DNA Technologies, Inc., Coralville, IA. All PCR assays were performed according to the procedure described earlier (27), but the cycling was as follows: denaturation at 94°C for 1 min, annealing at a temperature specific for the primer pairs (listed in Table 1) for 1 min, and extension at 72°C for 1 min.

Bacterial cell lysates were prepared and assayed for CDT activity according to the method described earlier (13, 24, 25), with minor modifications. Briefly, assays were performed with HeLa cells grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and an antibiotic supplement (Gibco, Invitrogen Corporation) containing penicillin, streptomycin, and glutamine. Ninety-six-well microtiter plates were seeded with  $1 \times 10^4$  to  $2 \times 10^4$  HeLa cells per well. After 18 h of incubation, 20  $\mu$ l of the toxin preparation in 200  $\mu$ l DMEM was applied to these wells. The plates were incubated at 37°C in a humidified 5% CO<sub>2</sub> incubator. Morphological changes of HeLa cells were studied at every 24 h for 4 days. Toxin titers were determined by performing twofold serial dilution of the sonic lysate in HeLa cell culture medium. Toxin titers were expressed as the reciprocal of the highest dilution that caused over 50% of the cells to be distended in a well. After 3 days, cellular distension and morphological changes were assessed by staining actin filaments with Alexa Fluor 488-conjugated phalloidin (Molecular Probes, Invitrogen) according to the method described earlier (5). The average cell size was determined by using the NIH Image J package (W. Rasband, NIH, Bethesda, MD; <http://rsb.info.nih.gov/ij/download.html>).

All 58 strains showed morphological properties and biochemical reactions typical of *Campylobacter* species. These strains were oxidase and catalase positive and urease negative,

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TABLE 1. PCR primers for virulence gene detection

| Target gene                      | Primers                                  | Sequence (5'→3')  | Annealing temp (°C) | Product size (bp) | Reference |
|----------------------------------|--|---|---------------------|-------------------|-----------|
| <i>flaA</i>                      | <i>flaA</i> 664<br><i>flaA</i> 1494      | AATAAAAAATGCTGATAAAACAGGTG<br>TACCGAACCAATGTCTGCTCTGATT | 53                  | 855               | 8         |
| <i>cadF</i>                      | <i>cadF</i> -F2B<br><i>cadF</i> -R1B     | TTGAAGGTAATTTAGATATG<br>CTAATACCTAAAGTTGAAAC            | 45                  | 400               | 14        |
| <i>racR</i>                      | <i>racR</i> -25<br><i>racR</i> -593      | GATGATCCTGACTTTG<br>TCTCCTATTTTTACCC                    | 45                  | 584               | 8         |
| <i>dnaJ</i>                      | <i>dnaJ</i> -299<br><i>dnaJ</i> -1003    | AAGGCTTTGGCTCATC<br>CTTTTTGTTTCATCGTT                   | 46                  | 720               | 8         |
| <i>virB11</i>                    | <i>virB</i> -232<br><i>virB</i> -701     | TCTTGTGAGTTGCCTTACCCCTTTT<br>CCTGCGTGTCTGTGTTATTTACCC   | 53                  | 494               | 8         |
| <i>ciaB</i>                      | <i>ciaB</i> -403<br><i>ciaB</i> -1373    | TTTTTATCAGTCCTTA<br>TTTCGGTATCATTAGC                    | 42                  | 986               | 8         |
| <i>pldA</i>                      | <i>pldA</i> -84<br><i>pldA</i> -981      | AAGCTTATGCGTTTTT<br>TATAAGGCTTTCTCCA                    | 45                  | 913               | 8         |
| <i>cdtA</i>                      | DS-18<br>DS-15                           | CCTTGTGATGCAAGCAATC<br>ACACTCCATTTGCTTCTG               | 49                  | 370               | 11        |
| <i>cdtB</i>                      | <i>cdtB</i> -113<br><i>cdtB</i> -713     | CAGAAAGCAAATGGAGTGTT<br>AGCTAAAAGCGGTGGAGTAT            | 51                  | 620               | 8         |
| <i>cdtC</i>                      | <i>cdtC</i> -192<br><i>cdtC</i> -351     | CGATGAGTTAAAAACAAAAAGATA<br>TTGGCATTATAGAAAATACAGTT     | 47                  | 182               | 8         |
| <i>wlaN</i>                      | <i>wlaN</i> -DL 39<br><i>wlaN</i> -DL 41 | TTAAGAGCAAGATATGAAGGTG<br>CCATTTGAATTGATATTTTTG         | 46                  | 672               | 18        |
| <i>ceuE</i> ( <i>C. jejuni</i> ) | JEJ1<br>JEJ2                             | CCTGCTACGGTGAAAGTTTTGC<br>GATCTTTTTGTTTTGTGCTGC         | 57                  | 793               | 10        |

did not ferment glucose, and were resistant to cephalothin. Of 58 isolates, 40 strains were identified as *C. jejuni* and 5 as *C. coli* by hippurate hydrolysis test and species-specific PCR for the presence of *hipO* and *ask* genes. The remaining 13 strains

were classified as *Campylobacter* species. The amplicons of five pathogenic genes (*flaA*, *cadF*, *racR*, *dnaJ*, and *pldA*) were detected in all of the *C. jejuni* isolates tested. All *C. jejuni* strains except one (KC-1375) were positive for *cdtA*, *cdtB*, and *cdtC*

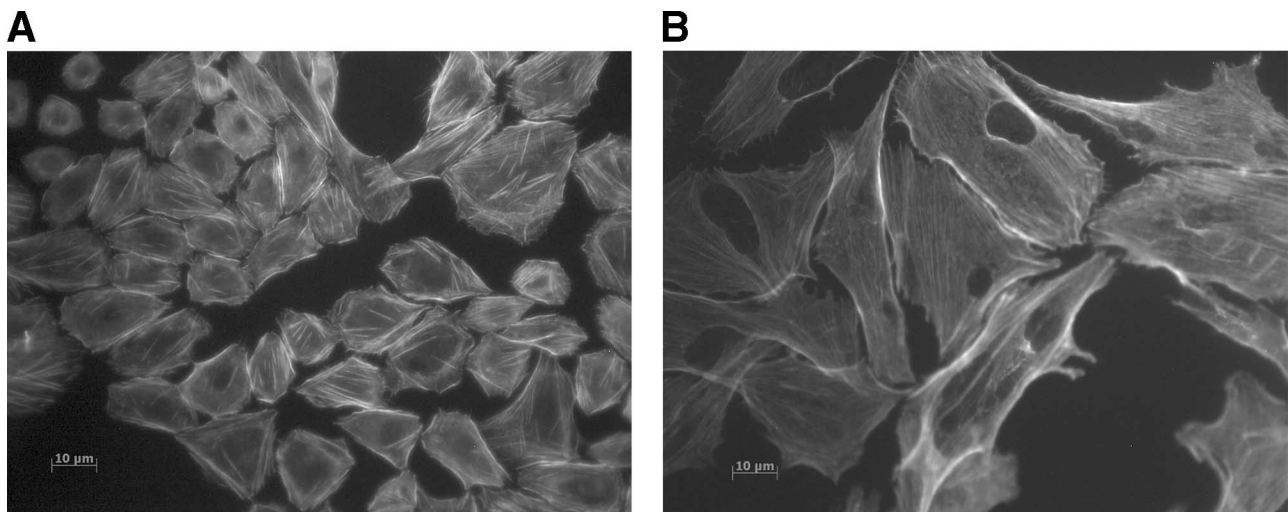


FIG. 1. Immunocytochemistry of HeLa cells exposed to CDT for 72 h. Actin was stained with Alexa Fluor 488-labeled phalloidin. (A) Phosphate-buffered saline-treated control cells. (B) Cells treated with filtered *C. jejuni* cell sonicate. All photographs were taken at a magnification of  $\times 40$ ; the scale bar in the microphotographs represents 10  $\mu\text{m}$ .

genes. The prevalences of the *ciaB* and *ceuE* genes were 95% and 82.5%, respectively. The *virB11* gene, which is found on the pVir plasmid (6), was not detected in any of the strains. A similar observation was obtained by Louwen et al. and Muller et al. (20, 21). This result is more likely as the majority of the strains contained no plasmid (K. Talukder, M. Aslam, Z. Islam, I. J. Azmi, M. A. Islam, D. K. Dutta, A. S. G. Faruque, Y. Kabir, A. Cravioto, and H. P. Endtz, unpublished data). The *wlaN* gene was detected in three (7.5%) strains only.

*C. jejuni* strains isolated in Bangladesh from patients with gastroenteritis caused morphological changes in cells of a tissue culture monolayer. In the HeLa cell assay, all but one (KC-1375) of the *C. jejuni* (97.5%) isolates produced CDT. When HeLa cells were treated with the sonic lysates, the cells did not show any characteristic changes in morphology within 24 h. However, after 48 h, the cells were enlarged, whereas control cells treated with phosphate-buffered saline continued to divide and grow to confluence. Cellular distension and morphological changes were more evident after staining with Alexa Fluor 488-conjugated phalloidin (Fig. 1). Measurement of cell size of 100 cells from each group was determined. The average diameter of the control cells was  $475 \pm 39 \mu\text{m}^2$ , whereas the average diameter of CDT-treated cells was  $1,504 \pm 119 \mu\text{m}^2$ . The CDT titer of the toxin positive strains ranged from 10 to 40.

CDT activity requires the function of three genes: *cdtA*, *cdtB*, and *cdtC*. CdtA, CdtB, and CdtC are all necessary for toxin production and form an active tripartite holotoxin that exhibits full cellular toxicity (16). Despite numerous reports on the toxic effects of CDT on different cultured mammalian cells, its role in pathogenesis is still unclear. Recently, Purdy et al. (25) have shown that *C. jejuni* isogenic *cdt* mutants have reduced invasiveness in a mouse model of enteric infection. An epidemiological study in Bangladesh showed a trend toward increased numbers of CDT-positive *Escherichia coli* cells in diarrheal patients compared to asymptomatic controls, but the difference did not reach statistical significance (3). In another report, a CDT-producing *E. coli* strain (O55:K59:H4) was isolated from the stool of a child suffering from gastroenteritis and encephalopathy (4). No other known toxins or virulence factors were detected from this strain, and no other viral or bacterial pathogens were isolated. Recent evidence from the use of a suckling mouse model suggests that the toxin causes secretory diarrhea and necrosis of colonic epithelium (22). CDT-mediated intestinal secretion in the mouse model was rapid, with diarrhea being evident within 4 h of toxin administration (22). In our study, all samples examined were obtained from diarrheal patients. Our observations suggest that CDT is a putative virulence factor in diarrhea caused by *Campylobacter* in Bangladesh and is responsible for a toxin-induced increase in intestinal fluid secretion.

Further studies in developing countries are necessary to further clarify the pathogenesis of the campylobacteriosis and the role of CDT.

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