Carriage of CdtB Encoding *Campylobacter* spp., *Salmonella enterica*, and *Yersinia entercolitica* in Patients with Gastroenteritis and Irritable Bowel Syndrome

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Abstract

**Introduction** Cytolethal distending toxin (Cdt) is one of the bacterial toxins that present in a variety of gram-negative human pathogens, such as *E. coli*, *Salmonella* spp., and *Campylobacter* spp. CDT is composed of three subunits encoded by three adjacent genes, including *cdtA*, *cdtB*, and *cdtC*. *cdtB* has been shown to have toxic activity and cause DNA damage in host cells. Despite its presence in different bacterial species, the role of CdtB in acute and chronic infections, such as gastroenteritis and irritable bowel syndrome (IBS), is unclear. To analyze this correlation, we studied the prevalence of *cdtB* among different enteropathogenic bacteria in patients with gastroenteritis and IBS compared with healthy people.

**Materials and Methods** In this cross-sectional descriptive study, 230 stool samples were collected from patients with gastroenteritis, IBS, and healthy people. The presence of CdtB encoding bacteria, including *Escherichia coli*, *Campylobacter* spp., *Yersinia entercolitica*, *Providencia alkalifacience*, and *Salmonella enterica*, was examined by polymerase chain reaction using genus-specific primers.

**Results** Out of 230 stool samples, CdtB encoding *Campylobacter* spp. were found in 34.6% (52/150), 6.25% (5/80), and 4% (2/50) of the patients with gastroenteritis, IBS, and the control group, respectively. Carriage of CdtB encoding *Salmonella enterica* was characterized among 5.3% (8/150) of the patients with gastroenteritis and 17.5% (14/80) of the IBS patients. Although none of the patients carried CdtB encoding *E. coli* and *Providencia* spp., *cdtB* of *Y. enterocolitica* was detected in one of the patients with gastroenteritis (0.6%). Statistical analysis showed significant correlation between infection with CdtB encoding bacteria and other clinical and demographic data.

**Conclusion** Our results confirmed a relatively higher frequency of CdtB encoding bacteria in the intestine of patients with gastroenteritis and those with IBS compared with healthy individuals. Regarding the frequency of CdtB encoding *Salmonella* and *Campylobacter* bacteria, it was proposed that infection with these enteropathogens could be considered a risk factor for the development or progression of IBS among Iranian patients. Further studies are needed to establish this involvement.

**Keywords** Enteric pathogens · Irritable bowel syndrome · Gastroenteritis · Cytolethal distending toxin

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Introduction

Cytolethal distending toxins (CDTs) represent an emerging and unique toxin family. CDT is a heterotrimeric AB2 genotoxin, which consists of cdtA, cdtB, and cdtC. CdtA and CdtC subunits bind to the host cell membrane, whereas CdtB enters the cell nucleolus and causes direct DNA damage due to DNase activity [1, 2]. Although CdtB is the most conserved subunit among all CDT-producing bacterial strains, its overall amino acid sequence showed diversity between 29 to 91% among different bacteria [2–4]. CDT was first described in Escherichia coli by Johnson and Lior in the 1980s. Gammaproteobacteria and Epsilonproteobacteria are among main members of Proteobacteria that carry cdt gene; however, its presence in Firmicutes, e.g., Clostridioides difficile, was also reported [4–7]. Within the Epsilonproteobacteria, CDT was reported in the order of Campylobacteriales, especially among Campylobacter and Helicobacter species [2].

Many gram-negative bacteria are considered clinically important human pathogens that are responsible for gastroenteritis [8]. Involvement of these bacteria in development of chronic bowel disorders, such as irritable bowel syndrome (IBS), was also reported in some studies [9, 10]. Accordingly, 3–30% of people with IBS experience symptoms after an episode of acute gastroenteritis (post-infectious IBS, PI-IBS) [11]. Currently, there is no congruency related to direct or indirect involvement of these pathogens in promotion of the chronic enteric disorders, and no definite virulence factors are characterized to explain their pathogenesis. The gut microbiota dysbiosis could increase the chance for colonization of more virulent bacteria, which promote functional disorders through their interaction with the host [12–14]. This interaction and the functional disorder, including chronic abdominal pain and altered intestinal habits, could occur because of unknown bacterial virulence factors [15, 16]. Campylobacter infection, through elevated antibody titers against one subunit of its genotoxin (CdtB), is among the common infections whose roles in the occurrence of the diarrhea-dominant form of IBS were shown [17, 18]. This involvement may be caused by cross-reaction of the antibodies with vinculin in the host gut [19–21]. Despite wide distribution of this family of toxins in members of the enteric bacteria, little is known about the prevalence of CdtB encoding bacteria and their association with distinct types of IBS (IBS with diarrhea, IBS with constipation, and mixed types), their promotion, or exacerbation. To find any possible correlation, this study was aimed to assess the presence of cdtB gene among different enteric bacteria, including Y. enterocolitica, S. enterica, E. coli, Providencia alcalifaciens, Aggregatibacter actinomycetemcomitans, and Campylobacter spp. in patients with IBS and gastroenteritis compared to healthy people.

Materials and Methods

This cross-sectional descriptive study was conducted on 80 patients with IBS and 150 patients with acute gastroenteritis. A total of 50 healthy subjects with matched gender and age were included in the study as a control group.

Selection and Exclusion Criteria

Adult patients with functional bowel disorders were interviewed by experienced physicians and filled in a questionnaire that was designed according to Rome III criteria. The patients who were diagnosed as having IBS were randomly selected and further subclassified into IBS-C (hard or lumpy stools), IBS-D (loose and watery stools), or IBS-M (a mix of both types) according to their predominant stool composition. Exclusion criteria included intestinal disturbance (celiac disease and lactose intolerance), recent history of hospitalization (> 24 h), antibiotic prescription within the last 3 months, surgery of the gastrointestinal tract, local and systemic inflammatory diseases, defined diet, food allergy, and pregnancy. Healthy controls were selected from people of the same age who enrolled for routine medical check-ups in the hospital. These people reported no history of gastrointestinal disorders and the criteria described above.

Collection of Samples

Fresh stool samples were collected in clean containers, and the samples were immediately transferred to the laboratory under a cold chain. Isolation and characterization of the culturable bacteria were performed using selective and specific culture media as before [14]. Briefly, 100 μl volume of the suspension was cultured on different culture media, including MacConky agar (Merck, Germany) as a selective medium for members of Enterobacteriaceae, Brucella agar supplemented with sheep blood (5%), Campylobacter supplement for detection of Campylobacter spp., and Clostridium selective agar and Mannitol salt agar for isolation of Clostridia and Staphylococci, respectively. The growth of aerobic and anaerobic bacteria was analyzed at 37 °C after 24–48 h incubation under aerobic and anaerobic conditions (Anaeromix: MART Microbiology B.V. 0% O2, 10% H2, 10% CO2, and 80% N2). Suspicious colonies were identified by routine microbiological and biochemical tests [14].
DNA Preparation

Total DNA of the samples was extracted using DNA Stool Kit (Bionner, South Korea) according to the manufacturer’s instructions. The concentration of DNA was measured by Nanodrop (Eppendorf, Germany). All DNA extracts were stored at −20 °C until use.

Identification of cdtB by PCR

In this study specific primers were designed for characterization of cdtB in *Y. entercolitica*, *S. enterica*, *E. coli*, *Providencia alcalifaciens*, and *Aggregatibacter actinomyces-comitans* (Table 1). Accordingly, homology of cdtB was determined using CLC Sequence Viewer v.6.0, and appropriate regions were selected. Amplification of the cdtB was carried out in Mastermix volume of 25 μl containing 5 μl DNA template, 0.5 mM each of dATP, dGTP, dCTP, and dTTP (Gene Fanavaran, Iran), PCR (10 ×) buffer (Gene Fanavaran, Iran), 0.3 μl (10 pmol) of each forward and reverse primer, 1 × Taq DNA polymerase buffer (Gene Fanavaran, Iran), and 0.2 μl (5 U/μl) of Taq DNA polymerase (Fermentase, Germany). Amplified products were visualized in agarose gels (1.5%) in TAE buffer along with a mixed DNA ladder, which was stained with ethidium bromide.

Sequencing of PCR Products

PCR products of each suspected sample were purified using QIA Quick Spin Column (Qiagene, Germany). Then, PCR products were automatically sequenced in Sanger sequencing service. Resulted sequences were aligned and analyzed using Blast, Chromas, and BioEdit software.

Statistical Analysis

Statistical analysis was performed with SPSS software (version 23, Co. Ltd., Tokyo, Japan). Data were expressed as mean ± standard deviation for continuous and frequency percentage for nominal and categorical variables. Comparison of qualitative variables between groups was analyzed by the Pearson chi-square test. The results were considered to be significant if *p* ≤ 0.05.

Results

Clinical Information of Patients and Controls

This study included 80 patients with IBS (mean age, 38.71 ± 1.33 years), 150 patients with acute gastroenteritis (mean age, 41.3 ± 2.1 years), and 50 healthy controls (mean age, 37.9 ± 2.1 years) with a statistically insignificant difference between patients and control regarding age and sex (*p* = 0.7). Patients were classified according to Rome III criteria into 18 patients with IBS-D (mean age, 37.55 ± 3.7 years), 29 patients with IBS-C (mean age, 37.26 ± 2.8 years), and 33 patients with IBS-M (mean age, 42.68 ± 2.1 years). The IBS-M subtype was the most prevalent (41.25%), followed by IBS-C (36.25%) and IBS-D (22.5%). The results showed that the prevalence of IBS was significantly higher in women than in men (56/24) (*p* < 0.05). The most common subtype of IBS in female patients was IBS-C (86.2%), while IBS-M was the most frequent type among men (33.33%). Diarrhea-predominant IBS was the same in men and women. Anxiety was significantly higher among women compared with men (*p* < 0.05). However, several symptoms including abdominal bloating, cramps, and stress were more common in women than men. However, this difference was not significant (Table 2).

### Table 1 Primers that were used in this study

| Bacteria                  | Sequence 5'-3'                              | PCR product (bp) | Reference |
|---------------------------|----------------------------------------------|------------------|-----------|
| *Campylobacter jejuni*    | GTTGGCACCTTGGGAATTGCAAGGC                   | 470              | [7]       |
|                           | RTRRAARTCNCCYADATCATCC                      |                  | [4]       |
| *Yersinia entercolitica*  | TAGCAAATAGCAATGATAG                         | 376              | This study|
|                           | ATCTGCTCTTAATTCTTTGA                       |                  |           |
| *Salmonella enterica*     | TCTTGACCATGACATCTCG                        | 283              | This study|
|                           | AGATTCCAGGTGTATCCATC                      |                  |           |
| *Escherichia coli*        | AGGCCATTAACCTGGATGATT                      | 178              | This study|
|                           | TTCCWRCTACHGCAATAATC                     |                  |           |
| *Providencia alkalifaciens* | GTAGCGACTTGGGAATTGCAAGGC | 680              | This study|
|                           | TTATTTTCAGAAAGATCCATCC                  |                  |           |
The most common symptom associated with constipation in subjects with IBS-C was abdominal bloating (89.65%) followed by bellyache (75%). IBS-D subjects felt a higher degree of abdominal cramps in their daily lives than those in the two other subtypes ($p = 0.01$). Additionally, there was a significant correlation between IBS-C and IBS-M with anxiety. Interestingly, the anxiety degree was significantly associated with bloating ($p = 0.02$) and abdominal pain ($p < 0.01$). Regarding IBS patient subgroups, there was no statistically significant difference between age and subtype of IBS.

The frequency of $cdtB$ gene varied between patients with IBS, gastroenteritis, and healthy people. $cdtB$ of Campylobacter showed higher frequency in stool samples of gastroenteritis patients (34.6%, 52/150) than those characterized in patients with IBS and healthy people (6.25%, 5/80, and 4%, 2/50, respectively). This difference was statistically significant ($p < 0.05$). Significant associations also were seen between $cdtB$ of Campylobacter spp. and IBS-D patients. $cdtB$ of $S$. enterica was detected in 8 (5.3%) and 14 (17.5%) of the patients with gastroenteritis and IBS, respectively. There was a significant difference between the presence of $cdtB$ of $S$. enterica in patients with IBS and gastroenteritis compared with healthy subjects ($p < 0.05$). However, the presence of $cdtB$ of $S$. enterica did not show a correlation with IBS subtypes (24.24%, 13.79%, and 11.11% in IBS-M, IBS-C, and IBS-D patients, respectively). $cdtB$ of $E$. coli and $P$. alkalifaciens was not detected in any of the patients with IBS and gastroenteritis and in the control group. The results also indicated that only 0.6% of the patients with gastroenteritis carried $cdtB$ of $Y$. entercolitica (Table 3). Statistical analyses showed no significant correlation between $cdtB$ encoding bacteria and gender or other demographic data among the studied patients with IBS.

### Discussion

In this study, diversity and frequency of $cdtB$ gene among different enteropathogenic bacteria were investigated in patients with IBS and gastroenteritis. Among the studied pathogens, $cdtB$ encoding Campylobacter spp. were

### Table 2 Frequency of symptoms in patients with IBS and gastroenteritis

| Type of symptoms | IBS-C | IBS-D | IBS-M |
|------------------|-------|-------|-------|
| Anxiety          | N (%) | N (%) | N (%) |
| Yes              | 29/80 (36.25%) | 18/80 (22.5%) | 33/80 (41.25%) |
| No               | 8 (27.58%) | 10 (55.55%) | 16 (48.48%) |
| Abdominal pain   | N (%) | N (%) | N (%) |
| Yes              | 21 (72.41%) | 14 (77.77%) | 27 (81.81%) |
| No               | 8 (27.58%) | 4 (22.22%) | 6 (18.18%) |
| Abdominal cramps | N (%) | N (%) | N (%) |
| Yes              | 9 (31.03%) | 15 (83.33%) | 17 (51.51%) |
| No               | 20 (68.96%) | 3 (16.66%) | 16 (48.48%) |
| Abdominal bloat- | N (%) | N (%) | N (%) |
| ing              | Yes | 26 (89.65%) | 3 (16.66%) | 30 (90.99%) |
| No               | 3 (10.34%) | 15 (83.33%) | 9 (9.09%) |
| Stress           | N (%) | N (%) | N (%) |
| Yes              | 22 (75.86%) | 12 (66.66%) | 20 (60.66%) |
| No               | 7 (24.13%) | 6 (33.33%) | 13 (39.39%) |
| Infection with   | N (%) | N (%) | N (%) |
| CdtB encoding    | Salmonella | 4 (13.79%) | 2 (11.11%) | 8 (24.24%) |
| bacteria         | Campylobacter | 1 (3.44%) | 3 (16.66%) | 1 (3.03%) |
| Others           | P. alkalifaciens | 0 | 0 | 14 (17.5%) |
| Y. enterocolitica | 0 | 1(0.6%) | 8 (5.3%) |
| E. coli          | 0 | 0 | 52 (34.6%) |
| S. enterica      | 0 | 0 | 3 (3.75%) |
| Campylobacter spp. | 0 | 0 | 5 (6.25%) |

### Table 3 Presence of $cdtB$ in enteric pathogenic bacteria among patients with irritable bowel syndrome, gastroenteritis, and controls

|                | P. alkalifaciens | Y. enterocolitica | E. coli | S. enterica | Campylobacter spp. |
|----------------|------------------|-------------------|--------|-------------|-------------------|
| IBS ($n = 80$) | 0                | 0                 | 0      | 14 (17.5%)  | 5 (6.25%)         |
| Gastroenteritis ($n = 150$) | 0 | 1(0.6%) | 0 | 8 (5.3%) | 52 (34.6%) |
| Control ($n = 50$) | 0 | 0 | 0 | 0 | 3 (3.75%) |
detected in higher frequency among patients with gastroenteritis (34.6%) and IBS (6.25%), compared with healthy ones (3.75%). In a study by Peters et al., infection with Campylobacter isolates was shown in 22.8% of the patients with gastroenteritis, where 67% of the isolates were CdtB positive [22]. In a study by Burliava et al., CdtB encoding Campylobacter strains was detected in 5% of patients with IBS [23], which was relatively similar to our results. In our study, the frequency of CdtB encoding Campylobacter spp. was significantly higher among IBS-D patients (16.66%). The higher frequency of CdtB of Campylobacter among patients with the diarrhea subtype disease could be explained through the function of Campylobacter CdtB in the intestine [24]. Burliava et al. showed that wild-type CdtB encoding C. jejuni strain is able to induce chronic altered bowel patterns and mild chronic rectal inflammation [23]. Although new developing commercial diagnostic kits for detection of anti-CdtB antibodies, as specific biomarker for diarrhea-predominant irritable bowel syndrome, are currently in use in some countries [17, 18], further studies are needed to establish whether CdtB-positive Campylobacter strains are associated with diarrhea in patients with irritable bowel syndrome. It seems that CDT has the ability to attack the cells of intestinal villi and causes the disease [24, 25]. While the direct effect of the toxin on the enterocytes seems to be the cause of acute gastroenteritis, evidence from animal and human studies indicates a different mechanism for the induction of symptoms in IBS-D patients [17, 19, 21, 26]. According to the mechanism proposed by Pimentel et al., production of antibodies against CdtB following last infection with CdtB-producing bacteria and anti-vinculin antibodies, which may negatively interact with the cells of Cajal, is responsible for inducing the chronic symptoms in these patients [19]. Previous studies in animal models showed that CDT is important for bacterial pathogenicity and inflammation. It seems that CDT has the ability to attack the cells of intestinal villi to induce cell death leading to epithelial barrier permeabilization [24, 25]. Moreover, CDT appears to mainly target the immune system, especially lymphocytes, which are extremely sensitive to CDT-mediated cell cycle arrest and death. Cell death of enterocytes will increase intestinal permeability and barrier dysfunction, which may help the bacterium to better colonize itself while hijacking the immune system [27]. The involvement of CdtB encoding Campylobacter and Salmonella strains in induction of inflammatory response could also describe the pathophysiology of IBS, where they can promote inflammation in the intestinal mucosa and nervous system through the gut-brain axis [28, 29].

It is known that acute gastroenteritis induced by Campylobacter spp. is a major risk factor for PI-IBS [30]. In a study by Scuron, CdtB encoding Campylobacter was detected in 5% of IBS patients, which was relatively similar to our results [25]. In studies that were done using an adult Sprague-Dawley rat model, symptoms consistent with IBS-D, including bacterial overgrowth, development of chronic altered bowel patterns, mild chronic rectal inflammation, and reduction of interstitial cells of Cajal, were induced by C. jejuni expressing CDT [31]. As was shown using C. jejuni-infected rats, level of circulating antibodies cross-reacting with vinculin, a cytoplasmic protein in interstitial of Cajal cells and myenteric ganglia required for gut motility, was correlated with levels of exposure to CdtB encoding C. jejuni [19]. This observation was later confirmed through a comparative study on differentiation of IBS-D from IBD, celiac diseases, and healthy controls, where increased levels of anti-CdtB and anti-vinculin antibodies were detected in IBS-D patients compared with non-IBS subjects [17]. In our study, no significant association was seen between the fecal carriage of CdtB encoding Campylobacter and clinical symptoms associated with IBS. No significant correlation also was found between CdtB encoding Campylobacter spp. and gender among IBS patients, while most IBS patients were female.

In our study, fecal carriage of cdtB encoding S. enterica was shown in 14% of the patients with IBS and 5.3% of those with gastroenteritis, while it was absent in the control group’s samples. Among the IBS patients who had CdtB-positive S. enterica, 13.75%, 11.25%, and 6.25% had bloating, abdominal pain, and abdominal cramps, respectively. The presence of cdtB gene in typhoidal and non-typhoidal S. enterica serovars was reported previously [5, 32–35]. Mezal et al. showed carriage of cdtB among all isolates of S. enterica serovar Javiana [5]. An increased level of invasiveness for these isolates was shown in HeLa cell cultures, mediated by CDT. Possible involvement of CDT of Salmonella in the development or progression of IBS could be also supported by previous results that described association of salmonellosis and IBS. This association was described in a retrospective study 16 years after an outbreak, where Salmonella gastroenteritis during childhood increased the risk for IBS in adulthood [36].

In our study, infection with CdtB encoding Y. enterocolitica was not shown in the stool samples of IBS patients, while it was shown in only one patient with gastroenteritis. In a study by Porter et al. that investigated pathogen-specific risk for chronic gastrointestinal complications, Y. enterocolitica showed the strongest association with IBS by the greatest relative risk [37]. Low frequency of CdtB encoding E. coli in patients with gastroenteritis, as shown in our study, was similarly recorded among children (1% and 1.4%, respectively) by Meza-Segura et al. and Pandey et al.; however, data about its frequency in adults are scanty [38, 39]. These findings suggest that CDT-producing E. coli strains are infrequent and probably do not play an important role in acute and chronic diarrheal illness. CdtB encoding P. alcalfaciens was reported in just one isolate, as reported by Shiam et al.,
which is consistent with our findings [40]. Although there are some other reports presenting the link between microbial agents and development of post-infectious IBS following gastroenteritis, further investigations are needed to establish the possible role of these pathogens, their virulence factors, mainly CdtB or its homologous proteins in other pathogens, and the antibodies induced in this interplay.

Besides the small number of samples, there are some other limitations in this study. Although conventional culture and molecular methods were used for isolation and characterization of enteric bacteria in this study, expression of CdtB and its translocation into enterocytes and levels of the induced anti-CdtB and anti-vinculin antibodies were not analyzed in the studied patients. Moreover, no long-term follow-up was done for patients with gastroenteritis to investigate possible outcomes, mainly development of IBS in the cases infected with CdtB encoding bacteria. The number of patients with chronic diarrhea (IBS-D) was lower than in other IBS subtypes, which was one of the limitations of this research. Future studies on a higher number of patients experiencing diarrhea-predominant IBS are required to investigate direct or indirect roles of CdtB-producing bacteria in the development of chronic diarrhea.

**Conclusion**

Our data showed high distribution of cdtB gene among *Campylobacter* spp. and *Salmonella* spp. in stool samples of patients with IBS and gastroenteritis. These findings indicated that infection with CdtB encoding bacteria could be a risk factor for development of IBS. In addition, significant association was seen between CdtB encoding *Campylobacter* spp. and diarrhea-predominant irritable bowel syndrome.

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**Declarations**

**Conflict of interest** The authors declare that there are no competing or potential conflicts of interest.

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