Carriage of Cdt-B Encoding *Campylobacter* spp., *Salmonella* enterica, and *Yersinia enterocolitica* in Patients with Gastroenteritis, Irritable Bowel Syndrome, and Controls

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Keywords: Enteric pathogens, Irritable Bowel Syndrome, Gastroenteritis, Cytolethal distending toxin

Posted Date: February 16th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-196235/v1>

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Abstract

Background: 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 composed of three subunits encoded by three adjacent genes, including *cdtA*, *cdtB* and *cdtC*. It is approved that *cdtB* had toxic activity and caused DNA damage of the host cell. Despite its presence in different bacterial species, role of Cdt 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 Cdt-B encoding bacteria, including *Escherichia coli*, *Campylobacter* spp., *Yersinia enterocolitica*, *Providencia alkalifaciens*, and *Salmonella enterica* was examined by polymerase chain reaction. Demographic data and type of disease was collected through interview and a questionnaire.

Results: Out of 230 stool samples, Cdt-B 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 Cdt-B 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* of *E. coli* and *Providencia* spp., *cdtB* of *Y. enterocolitica* was detected in 1 of the patients with gastroenteritis (0.6%). Statistical analysis showed significant correlation between infection with CdtB-encoding *Campylobacter* spp. and IBS-D subtype. No significant correlation was found between infection with Cdt-B encoding bacteria, and other clinical and demographic data.

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

Introduction:

Cytolethal distending toxins (CDT) represent an emerging and unique toxin family. CDT is a heterotrimeric AB₂ genotoxin, which consist of *cdtA*, *cdtB* and *cdtC*. CdtA and CdtC subunits bind to the host cell membrane, whereas CdtB enters to 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 show diversity between 29 to 91% among different bacteria [3, 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*; however, its presence in

Firmicutes, e.g. *Clostridioides difficile*, was also reported [4–6]. Within the *Epsilonproteobacteria*, CDT was found in the orders *Campylobacterales*, specially *Campylobacter* and *Helicobacter* species [7].

Many of these Gram-negative bacteria are considered as 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 reported in different studies [9, 10]. Accordingly, 3–30% of people with IBS experience their symptoms after an episode of acute gastroenteritis (Post-infectious IBS, PI-IBS) [11].

Currently, no definite virulence factors are characterized in these bacteria in association to PI-IBS. Dysbiosis of the gut microbiota and alteration of microbial population in this organ could accelerate growth 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 by unknown bacterial virulence factors [15, 16]. Increased antibody titers against CdtB of *Campylobacter* as a only proposed virulence factors have been observed in diarrhea-dominant form of IBS [17, 18]. This involvement may cause through 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 Cdt-B encoding bacteria, their association with distinct types of IBS (IBS with diarrhea, IBS with constipation, and mixed-types), and promotion or exacerbation of the disease. This study was aimed to assess the presence of *cdtB* gene among different enteric bacteria, including *Yersinia enterocolitica*, *Salmonella. enterica*, *E. coli*, *Providencia alcalifaciens*, *Aggregatibacter actinomycetemcomitans*, and *Campylobacter* spp. among patients with IBS and gastroenteritis in compare to healthy people.

Materials And Methods:

Sample collection:

This cross-sectional descriptive study was conducted on 230 stool samples that obtained from patients with acute gastroenteritis and IBS. A control group of healthy volunteers (50 samples) was also included in the study. A consent form was filled by all participants. The study was approved by ethical committee of Department of Pathobiology, Tehran university of Medical Sciences, Tehran, Iran (TUMS. Ethics code 92-02-27-22726). Fresh stool samples were collected in clean containers and the samples were immediately transferred to the laboratory under cold chain. Adult patients with functional bowel disorders were interviewed by experienced physicians and fulfilled a questionnaire that was designed according to Rome III criteria for IBS [22]. According to symptoms, they was classified as either diarrhea-predominant (IBS-D), constipation-predominant (IBS-C), or with alternating stool pattern (mixed IBS). Exclusion criteria were 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 systematic inflammatory diseases, defined diet, food allergy, and pregnancy. Healthy controls were selected from people of the same age, who enrolled in routine medical check-ups in the

hospital. These people reported no history of the gastrointestinal disorders and the exclusion criteria described above.

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.enterocolitica*, *S. enterica*, *E. coli*, *Providencia alcalifaciens*, and *Aggregatibacter actinomycetemcomitans* (Table 1). Accordingly, homology of *cdtB* were determined using CLC Sequence Viewer v.6.0, and appropriate regions were selected. Amplification of the *cdtB* were carried out in 25 µl reaction containing 5 µl of DNA template, 0.5 mM of each dATP, dGTP, dCTP and dTTP (gene fanavaran, Iran), PCR (10x) buffer (Gene fanavaran, Iran), 0.3 µl (10 pmol) of each forward and reverse primer, 1x Tag DNA polymerase buffer (Gene fanavaran, Iran), and 0.2 µl (5 U/µl) of Taq DNA polymerase (Fermentase, Germany). Amplified products were visualized in 1.5% agarose gels along with a mixed DNA ladder.

Table 1
Primers that were used in this study.

Bacteria	Sequence (5'-3')	PCR product (bp)	Reference
<i>Campylobacter jejuni</i>	GTTGGCACTTGGAATTTGCAAGGC	470	[23]
	RTTRAARTCNCCYAADATCATCC		[24]
<i>Yersinia enterocolitica</i>	TAGCAATAGCAAATGGATAG ATCTGCTCTAATTCTTGA	376	This study
<i>Salmonella enterica</i>	TTCTGACCATGATCATCTG AGATTCCAGGTGTATTCATC	283	This study
<i>Escherichia coli</i>	AGGCCATTAAGTGGATGATT TTTCCWRCTACHGCATAATC	178	This study
<i>Providencia alkalifaciens</i>	GTAGCGAGCTTGGAATTTGCAAGGC TTTRAATTTAGAAAGATCATCC	680	This study

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 $\pm$ standard deviation for continuous and frequency percentage for nominal and categorical variables. The comparison of qualitative variables between groups analyzed by the Pearson Chi-square test. The results were considered to be significant if p -values were ≤ 0.05 .

Results:

Clinical information of patients and controls.

Out of 80 patients with IBS, 18 had IBS-D (mean age, 37.55 ± 3.7 y), 29 had IBS-C (mean age, 37.26 ± 2.8 y), and 33 had IBS-M (mean age, 42.68 ± 2.1 y). In addition, 150 patients with gastroenteritis (mean age, 41.3 ± 2.1 y), and 50 healthy controls (mean age, 37.9 ± 2.1 y) were recruited into the study.

The results showed that the prevalence of IBS was significantly higher in women than in men (56/24) ($p < 0.05$). The commonest subtype of IBS in female patients was IBS-C (86.2%); while, IBS-M was the most frequent type among males (33.33%). Diarrhea-predominant IBC was the same in men and women. Anxiety was significantly higher among women in compare to men ($P < 0.05$). Similarly, several other symptoms, including abdominal bloating, cramp, and stress were reported more common in females, which was not statistically significant (Table 2). The most common symptoms associated with constipation in subjects with IBS-C was abdominal bloating (89.65 %), followed by bellyache (75 %), while subjects with IBS-D type felt a higher degree of abdominal cramp ($P = 0.01$). Additionally, there was a significant correlation between IBS-C and IBS-M with anxiety. Anxiety degree was significantly associated with bloating ($P = 0.02$) and abdominal pain in IBS patients ($P < 0.01$). No statistically significant difference was measured between age and IBS disease subtypes.

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

Type of symptoms	IBS-C N (%)	IBS-D N (%)	IBS-M N (%)
Total number	29/80 (14.5%)	18/80 (22.5%)	33/80 (15.4%)
Anxiety	21 (72.41 %)	8 (44.44%)	17(51.51%)
Yes	8 (27.58 %)	10 (55.55%)	16 (48.48%)
No			
Abdominal pain	21 (72.41 %)	14 (77.77%)	27 (81.81%)
Yes	8 (27.58 %)	4 (22.22%)	6 (18.18%)
No			
Abdominal Cramp	9 (31.03%)	15(83.33%)	17 (51.51 %)
Yes	20 (68.96%)	3 (16.66%)	16 (48.48%)
No			
Abdominal bloating	26 (89.65%)	3 (16.66%)	30 (90.9%)
Yes	3(10.34%)	15(83.33 %)	3(9.09%)
No			
Stress	22 (75.86%)	12(66.66%)	20 (60.6%)
Yes	7 (24.13%)	6 (33.33%)	13 (39.39%)
No			
Infection with CdtB encoding bacteria	4 (13.79%)	2 (11.11%)	8 (24.24%)
<i>Salmonella</i>	1 (3.44%)	3 (16.66%)	1 (3.03%)
<i>Campylobacter</i>			
Others			

Diversity of *cdtB* gene variants in the fecal DNA extracts

The frequency of *cdtB* varied between patients with IBS, gastroenteritis, and healthy people. *cdtB* of *Campylobacter* showed high- frequency in stool samples of patients with gastroenteritis (34.6%, 52/150); which was higher than those characterized in patients with IBS and healthy people (6.25%, 5/80 and 4%, 2/50, respectively). The difference was statistically significant ($p < 0.05$). Significant association 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* didn't show a correlation with IBS types (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, gastroenteritis, and in the control group. The results also indicated that only 0.6% of the patients with gastroenteritis carried *cdtB* of *Y. enterocolitica* (Table 3). Statistical analyses showed no significant correlation between Cdt-B encoding bacteria and gender or other demographic data among the studied patients with IBS.

Table 3
Presence of *cdtB* in enteric pathogenic bacteria among patients with Irritable bowel syndrome, gastroenteritis, and controls.

	<i>P. alkalifaciens</i>	<i>Y. enterocolitica</i>	<i>E. coli</i>	<i>S. enterica</i>	<i>Campylobacter</i> 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%)

Sequence accession number

Nucleotide sequence analysis of related amplified PCR products showed 100% identity to *cdtB* of *Campylobacter* spp., *S. enterica* and *Y. enterocolitica*. The accession numbers of the sequences of the PCR products submitted to GenBank were as follows: KT008107.1, KR778819.1, KT008106.1 (<https://www.ncbi.nlm.nih.gov>).

Discussion:

The overall objectives of the current study were to determine diversity and frequency of *cdtB* gene among different enteropathogenic bacteria in subjects with IBS and gastroenteritis and their possible roles in the occurrence of these diseases.

According to our results, higher frequency of *cdtB* of *Campylobacter* spp. was detected in patients with IBS (6.25%) and in patients with gastroenteritis (34.6%), compared with healthy people (3.75%). Similar to our results, high prevalence of *cdtB* was detected among *Campylobacter* isolates in patients with gastroenteritis [25]. Peter KE *et al* characterized *cdtB* gene among 67% of *C. jejuni* and 19% of *C. coli* isolates in patients with gastroenteritis. In a study by Burliaeva *et al*, *cdtB* encoding *Campylobacter* strains was detected in 5% of patients with IBS [26], which was relatively similar to our results. In our study, the frequency of Cdt-B encoding *Campylobacter* spp. was significantly higher among IBS-D patients (16.66%). The higher frequency of Cdt-B of *Campylobacter* among patients with the diarrhea

type disease could be explained through function of *Campylobacter* CdtB with the intestine. Burliaeva *et al* showed that wild-type Cdt-B encoding *C. jejuni* strain is able to induce chronic altered bowel patterns and mild chronic rectal inflammation [25, 26]. Although, new developing commercial diagnostic kits for detection of anti-CdtB antibodies as specific biomarker for diarrhea-predominant irritable bowel syndrome is currently in use by 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 cause disease [27, 28]. In this study, no significant association was seen between the *Campylobacter* encoding *cdtB* and other clinical symptoms, including abdominal bloating. No significant correlation also was found between Cdt-B encoding *Campylobacter* spp. and gender among IBS patients, while most of IBS patients were female.

cdtB of *S. enterica* was detected in 14% of patients with IBS and 5.3% of patients with gastroenteritis in current study, while it was not detectable in the control group. Few studies have been done on *Salmonella* spp.; however, the presence of *cdtB* in *Salmonella* is reported both for typhoidal and non-typhoidal *S. enterica* (NTS) serovars [5, 29–32]. In a study by Mezal *et al.*, the carriage of *cdtB* gene was detected among all isolates of *Salmonella enterica* serovar *Javiana* isolated from food, environmental, and clinical samples [5]. An increased level of CDT-mediated invasiveness for these isolates was shown in HeLa cell culture, which could support possible involvement of this toxin in different diseases of the gastrointestinal tract. .

Recent evidence demonstrated that *Y. enterocolitica* is linked with chronic gastrointestinal diseases, including IBS, dyspepsia, constipation, and Inflammatory bowel disease (IBD) [33]. However, this involvement was not confirmed in our study, since only one Cdt-B encoding *Y. enterocolitica* sample was characterized. Similarly, none of the samples of patients with IBS and gastroenteritis, and healthy people were positive for *cdtB* of *E. coli* and *P. alcalifaciens*. In a study by Meza-Segura *et al*, out of 1306 young children with acute diarrhea, *cdt* encoding *E. coli* strains detected in 1% of the patients [34]. In another study, *cdtB* gene were found in 1.4% of 366 *E. coli* strains isolated from stool specimens of patients with acute diarrhea in Calcutta, India [35].

In our study, we didn't find *cdtB* of *P. alcalifaciens* among our samples. However, Shiam *et al.* detected *cdtB* harboring *P. alcalifaciens* among 9.7% of patients with diarrhea [36]. According to our knowledge, no report for this bacterium and CDT exists in patients with IBS, which is consistent to our finding.

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 proposed 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. No significant correlation was found between CdtB encoding *Campylobacter* spp. and *S. enterica* and gender among the IBS patients. Further studies are needed to establish this correlation.

Declarations:

Acknowledgment

Many thanks go to Prof. Saeid Bouzari (Pasture Institute of Iran, *E. coli* Reference Laboratory, Iran) for providing DNA of *CdtB* encoding *E. coli*.

Ethical approval and consent to participate.

The study was approved by ethical committee of Department of Pathobiology, Tehran university of Medical Sciences, Tehran, Iran (TUMS. Ethics code 92-02-27-22726).

Consent for publication.

Not declared

Availability of data and materials.

All data generated or analyzed during this study are included in this published article.

Competing interests.

None declared.

Funding:

This study was supported by Department of Pathobiology, Tehran university of Medical Sciences, Tehran, Iran (92-02-27-22726), Tehran, Iran.

Authors' Contributions.

Leila Ganji provide the samples and do laboratory tests. Write the manuscript. Parisa Eslami, Dr. Mohammad Rahbar, and Dr. Nase Ebrahim Daryani provide the clinical samples. Dr. Mohammad Hassan Shirazi advised the project and provide technical helps. Dr. Masoud Alebouyeh designed and supervised the study.

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