Molecular detection of *Campylobacter jejuni* in patients with Crohn's disease in Iran

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Abstract

Background: Crohn's disease is one of the most significant intestinal disorders and is known as inflammatory bowel disease; *Campylobacter* spp. are one of the leading causes of bacterial gastroenteritis in humans.

Methods: In this study, 60 tissue samples, including 30 cases with Crohn's disease and 30 cases with no inflammatory bowel disease, were collected. Patients were referred to Taleghani hospital and Behboud clinic between March 2015 and May 2016. Biopsies were used for DNA extraction and assessment of *Campylobacter jejuni* in patients with Crohn's disease and controls using polymerase chain reaction and quantitative real-time polymerase chain reaction. All positive amplified fragments were sequenced. The gene encoding 16S rRNA, specific to *Campylobacter* genus, was amplified.

Results: The results were positive for *Campylobacter* genus in patients with Crohn's disease compared to healthy individuals. The quantitative real-time PCR showed a significantly higher prevalence of *Campylobacter jejuni*, particularly in hippurate hydrolase in tissue specimens of patients with Crohn's disease compared to control group. The correlation between *Campylobacter jejuni* and diarrhea symptoms in patients with Crohn's disease and controls was investigated. One positive case of *Campylobacter jejuni* found in patients without diarrhea was compared with 13 patients with diarrhea.

Conclusion: The present study demonstrated the alarmingly high rate of *Campylobacter jejuni* prevalence in Crohn's disease patients with diarrhea symptoms. However, further investigation is needed to determine the possible causing factors of this disease.

Keywords: Crohn disease, *Campylobacter* genus, *Campylobacter jejuni*, Real-time PCR, Inflammatory bowel disease

Introduction

Crohn's disease (CD) is a chronic inflammatory disorder of the gastrointestinal tract, which is considered as inflammatory bowel disease (IBD). The cause of IBD is still unknown.

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↑What is "already known" in this topic:
The prevalence of inflammatory bowel disease is increasing in developing countries in Asia. However, the etiology of inflammatory bowel disease is not known yet. Several studies have indicated *Campylobacter* spp. may play a significant role in the development of inflammatory bowel disease.

→What this article adds:
The present research finding demonstrates that *C. jejuni* may pose a predisposing factor in the development of inflammatory bowel disease. Humans could be exposed to *C. jejuni* through poultry products, contaminated water, livestock, and unpasteurized dairy products. However, more research is needed to detect the cause of inflammatory bowel disease and to identify the correlation of *C. jejuni* with Crohn's disease in Iran.

A variety of factors are implicated in this disorder, including intestinal microbiota, genetic, immunological and environmental factors (1). In addition, a bacterial component, such as *Campylobacter* spp., in humans and animals is responsible for the pathogenesis of IBD (2). Moreover, the most common isolated *Campylobacter* species from colonic biopsies and human diarrheal feces samples are composed of *C. jejuni* and *C. jejuni* (3).

Chickens, farmed animals, such as cattle, pigs, and contaminated food or water are reservoirs of this organism (4). *C. jejuni* is the leading cause of severe diarrhea and gastrointestinal distress worldwide. The symptoms of infected humans are mild to acute diarrhea (5).

Cytotoxic effects and/or host-cell invasion of *C. jejuni* may be responsible for cellular damage (6). The cell cycle is usually arrested by producing cytolethal distending toxin (CDT) from *C. jejuni.* Consequently, this toxin can lead to apoptotic and nonapoptotic death of lymphocytes, monocytes, and endothelial cells, respectively (7). In terms of the ability of invasion of *C. jejuni,* it seems that these bacteria can cause intestinal damage through invasion in the mucosa.

The invasive action of *C. jejuni* in epithelial cells is correlated with increased permeability of human intestinal cells (8). In addition to *C. jejuni,* a leading cause of gastroenteritis, other members of *Campylobacter* genus consisting of *C. concisus, C. coli, C. gracilis, C. upsaliensis,* and *C. lari,* may also play a role in intestinal disease (9). The ability of hydrolase hippurate encoded by *hipO* gene in *C. jejuni* can distinguish this bacterium from other *Campylobacter* spp. (10). In addition to IBD, irritable bowel syndrome (IBS) is another type of chronic disease of the intestine that was recently shown to be associated with post infectious events.

Campylobacteriosis has been identified as one of the most usual risk factors for IBS. The induction of intestinal pathology occurs by pathological mechanism, such as damage to the epithelial cells, interruption of adherents junctions, and host cell death (11).

The aim of this study was to determine the prevalence of *C. jejuni* with *hipO* gene in intestinal biopsy samples, collected from patients with CD and control participants using molecular assay.

Methods
Study participants and biopsy collection
In this study, 60 samples, including 30 samples of patients with CD and 30 without IBD, were collected from ileum, caecum, descending colon, ascending colon and rectum during colonoscopy. Patients were referred to Taleghani hospital and Behboud clinic between March 2015 and May 2016. The study was approved by the ethics committee of Tehran University of Medical Sciences. All the CD biopsy samples were taken from lesions of colon, terminal ileum. Moreover, the control biopsy samples were collected from healthy areas. CD diagnosis was based on clinical symptoms, laboratory evaluations, and colonoscopy finding which was confirmed by histological assessment. The control samples were taken from people with noninflammatory IBD (nIBD). None of the cohorts in

this study used any antibiotics or probiotics for at last 3 months. All the samples were transferred in sterile plastic containing thioglycollate medium (Merck, Germany).

DNA extraction from biopsies
DNA was extracted from active ulcer and normal mucosa of individuals with CD and control participants. Biopsies were crushed and DNA was extracted by RTP® Mycobacteria kit (Berlin, Germany) and stored at −20 °C for PCR and real-time PCR assays.

Statistical analysis
Data were analyzed using chi-squared tests and Kruskal-Wallis test for the presence of *Campylobacter jejuni*. P value <0.05 was considered statistically significant. The positive samples from the experimental infection were evaluated in parallel with the real-time PCR assays. All data analyses were performed with Microsoft Excel.

Polymerase chain reaction (PCR)
All samples were tested for the presence of *Campylobacter* genus using PCR. PCR was performed in 12.5μL comprising of 5μL master mix (Amplicon, Pishgam), 0.5 μL of each primer, 2 μL of the DNA template (50 ng) and 4.5μL of ddH$_2$O. Subsequently, the PCR was performed using the following thermal cycling conditions: 300 second at 94°C and 25 cycles of amplification consisting of 60 seconds at 95°C, 55 seconds at 58°C, followed by 60 seconds at 72°C, and 300 second at 72°C for the final extension. PCR products were examined by electrophoresis on a 1% agarose gel in 1X TBE buffer [10.8 g Tris and 5.5 g Boric acid, 0.5 M Na$_2$EDTA (pH 8.0)] (12).

Real-time PCR assay
The presence of *hipO* gene was evaluated using real-time PCR assay. Therefore, quantitative real-time PCR was performed on a Line GeneK bioerr biosystem using Taq Man Probe. At the 5'end, the *hipO* gene probe was linked to the fluorophore FAM. For the real-time PCR, 12.5 μl Taq Man Master Mixes, 0.5 μL of each primer and probe, 10 μL ddH$_2$O and 2 μL of the DNA template was added to a final volume of 25 μL. The thermocycler condition for *hipO* gene was 95°C for 10 minutes for the initial duration, followed by 40 cycles of a 2-stage temperature profile of 95°C for 10 seconds and 62°C for 1 minute. Cycle threshold (Ct) was assessed to provide standard curves for the quantification of *hipO* gene. PCR amplification efficiency (E) was estimated by the slope of the standard curve using the following formula E = $10^{(-1/\ slope)}$-1. A reaction with 100% efficiency will generate a slope of -3.32.

Real-time PCR primers and probes
The nucleotide sequence of primers for *Campylobacter* genus-specific 16SrRNA was designed manually and checked with Primer 3 plus software (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi); also, the primer and probe for *C. jejuni* with *hipO* gene were used as described by Toplak et al (13). Oligoanalyzer (http://eu.idtdna.com/home/home.aspx) and

Table 1. The primers and probe used for quantitative real-time PCR reaction

Gene name	Primer	(5´- 3´)	Probe	Product size
16S rRNA	AATCTAATGGCTTAACCATTA GTAACTAGTTTAGTATTCCGG		-	850
hipO	AATGCACAAATTTGCCTTATAAAAGC TNCCATTAAAATTCTGACTTGCTAAATA		FAM-ACATACTACTTCTTTATTGCTTG-BHQ1	123

VNTI database were used to check the specificity of primer target sites. Primers and probes are presented in Table 1.

Results

Clinical information of patients and controls

Thirty samples were collected from CD patients (15 males and 15 females; mean age: 36.5 years, range: 18-64) and 30 samples were taken from nIBD (15 males and 15 females; mean age: 56.5 years, range: 25-76).

Detecting Campylobacter genus-specific 16S rRNA in tissue samples of cases with CD and controls using PCR assay

In this study, a *Campylobacter* genus-specific 16S rRNA PCR assay was used to examine the presence of *Campylobacter* genus in the current populations with CD and controls (Fig. 1). Of 30 control samples examined, 16.6% were positive and 83.4% were negative, and among 30 samples of patients with CD, 63.3% were positive and 36.7% were negative (Table 2).

Molecular detection of C. jejuni in biopsies using real-time PCR

The specificity of each primer-probe for the identification of *hipO* gene in *C. jejuni* was tested. No signal was observed for any of the other *Campylobacter* species that could be present in biopsy samples. A serial dilution of bacterial DNA of *C. jejuni* and biopsy DNA samples was tested with the specific primers and FAM-labeled specific probes; and a strong linear correlation (R^2 values were all equal to 0.98) was observed. The threshold cycle (C_t) values were defined in quantitative real-time PCR. Figure 2 displays the standard curves of the template DNA for *C. jejuni* with FAM-labeled specific probes and specific primers. Among 30 patients with CD and 30 individuals without IBD, 70% (n=21) and 6.6% (n=2) were positive for *C. jejuni* gene, respectively. Therefore, of the 60 tissues examined, 23 (38.33%) were positive and 37 (61.6%) were negative for *hipO,* and the difference between patients with CD and control participants were statistically significant (p<0.05) (Table 2).
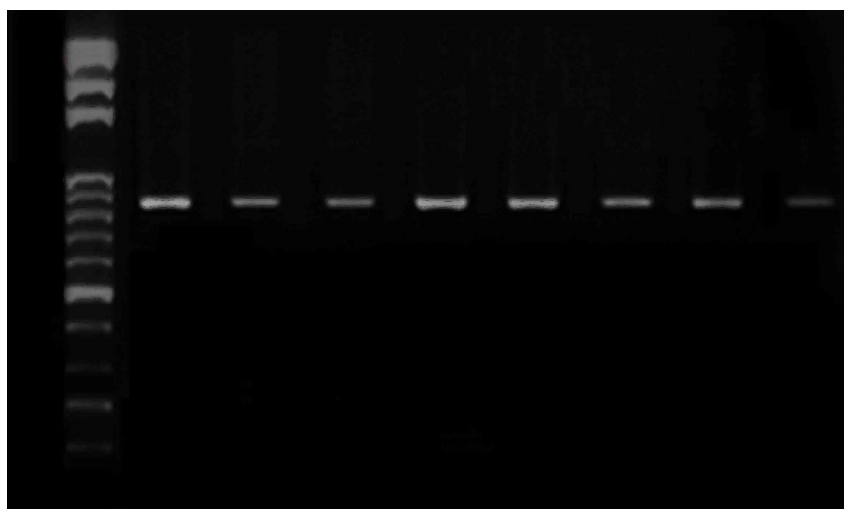

Fig. 1. Agarose gel electrophoresis of PCR amplification of *Campylobacter* genus-specific (16S rRNA). Lanes: 1, 100-bp DNA Ladder; 2-8, Crohn's disease samples; 9, Water (negative control).

Table 2. The prevalence of Campylobacter genus-specific 16S rRNA and *Campylobacter jejuni hipO* gene in CD and control subjects by PCR and real-time PCR

	PCR		Real time PCR	
Group	16S rRNA		hipO	
	Positive	Negative	Positive	Negative
Crohn's disease				
Count	19	11	21	9
CD (%)	63.3	36.7	70	30
nIBD				
Count	5	25	2	28
nIBD (%)	16.6	83.3	6.6	93.3
p	0.043*		0.007*	

* Significantly higher as compared with the controls (p<0.05)

In this study, there were 30 nIBD controls, of whom 26.6% (8/30) had diarrhea. One (1/8) was *C. jejuni* positive and 73.3% (22/30) did not have diarrhea, and none of them (0/22) was *C. jejuni* positive. Therefore, the differences between the percentages of *C. jejuni* positive in non-IBD controls with diarrhea, compared to those without diarrhea, was not statistically significant (p= 0.53).

In the CD samples, 60% (18/30) had diarrhea, of which 72/2% (13/18) was *C. jejuni* positive. In the CD group, 40% (12/30) did not have diarrhea, of whom 25% were *C. jejuni* positive. Differences between the percentages of *C. jejuni* positive in the CD group with diarrhea, compared to those without diarrhea, was statistically significant (p=0.011) (Table 3). All positive amplified fragments were sequenced and confirmed for *C. jejuni*.

Quantification of C. jejuni

The results of PCR confirmed the higher rate of *C. jejuni* with *hipO* gene in CD patients compared to the controls, which clearly indicated that the average number and std. The error of the mean (SEM) of *C. jejuni* was significantly higher in patients with CD (6.80E01± 2.90E01 bacteria per gram of tissue samples, p=0.004) compared to control participants (median 3.00E-02) (Table 4).

Discussion

CD is the most common form of IBD. There is limited information about the etiology of this disease in Iran (14). However, it was reported that the prevalence of IBD is increasing in Asia and developing countries (15). In the
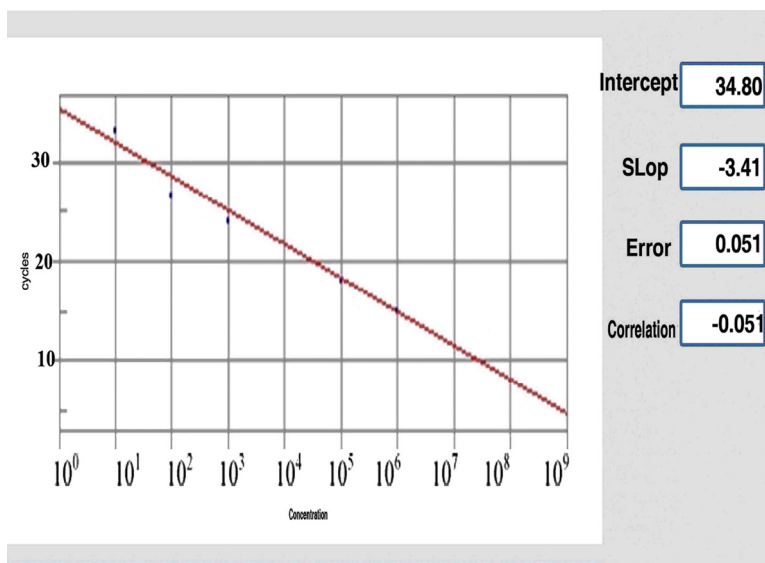

Fig. 2. Standard curve of DNA extracted from *C. jejuni* by quantitative real-time PCR assay. In standard curve, X and Y showed the concentration of *C. jejuni* and number of cycles respectively for control positive sample.

Table 3. C. jejuni in patients with and without diarrhea

Group	C. jejuni	
	Positive	Negative
CD		
Diarrhea	13	5
No diarrhea	3	9
Total	30	
p	0.011	
nIBD		
Diarrhea	1	8
No diarrhea	0	22
Total	30	
p	0.53*	

* Significantly higher as compared with the controls (p<0.05).

Table 4. Quantitative analysis of *C. jejuni* from CD and nIBD biopsy samples

Group	C. jejuni
Crohn's disease	
Mean	68
N	30
SEM	29
nIBD	
Mean	0.03
N	30
SEM	0.03
p	0.01*

*The mean difference is significant at the 0.05 level.
SEM: Standard error of mean

past decades, IBD was diagnosed more than ever in various parts of Iran (16).

In Europe and North America, more information has been reported about the epidemiology, clinical symptoms and etiology of IBD, as a consequence of the high prevalence of the disease in these areas (17). However, it seems that combinations of genetic and environmental factors that can affect the immune responses are implicated in causing the disease. There is numerous information on the transfer of *Campylobacter* spp., particularly *C. jejuni*. Previous studies demonstrated that *C. jejuni* is one of the causing factors of human infections (18) and could be transmitted through poultry products, contaminated water (19), livestock, such as cattle (20), and unpasteurized dairy products (21).

A study investigated whether chicken (38% to 77%) and cattle (16% and 54%) are the first and second most common reservoir of *Campylobacter* infection, respectively (22).

Rahimi et al reported that *C. jejuni* (88.5%) was the most prevalent of *Campylobacter* species isolated from row duck and goose meat in Iran (23). In another study, Rahimi et al demonstrated that the high percentage of *Campylobacter* spp. (41.7%) was found in poultry meat in Esfahan, Iran (24).

Nevertheless, a higher prevalence of *C. concisus* was reported both in colonic biopsy samples from patients with ulcerative colitis (31%; 4/13) in the samples from CD patients (53%; 8/15) than in nIBD (18%; 6/33 individuals) (P<0.05) (25).

A recent study by our group addressed this issue by investigating the prevalence of *C. jejuni* with *hipO* gene in patients with CD in Tehran, Iran. The high positive rate of *Campylobacter* genus-PCR and intestinal prevalence of *C. jejuni* in patients with CD observed in this study are consistent with previous findings in CD patients (26). In the present study, *C. jejuni* was detected in 62.5% of CD patients with diarrhea and in 28.5% without diarrhea symptoms.

In a later study, Man SM *et al* detected a significantly higher prevalence of *C. concisus* in stool samples of children with CD (27).

Another finding of this study was the increased prevalence of *C. jejuni* in the patients with CD compared to the control participants. Some previous studies detected a high prevalence of *C. jejuni* in patients with gastroenteritis and *C. concisus* (28).

In a follow-up study, Zhang et al reported a high presence of *C. concisus* (75%) in oral samples with healthy individuals and 95% in intestinal specimens through PCR (29). In early 2015, it was reported that several factors and different gut microbiota may be associated with IBD (30). Moreover, our evidence revealed that *C. jejuni* may be an important predisposing factor for the initiation and development of CD.

In this study, *C. jejuni* was detected in a significantly larger proportion of CD patients compared to control populations.

Conclusion

In summary, in this study, a significantly higher correlation of *C. jejuni* was detected in colonic biopsies from patients with CD as compared to control participants. However, the absence of *C. jejuni* in CD patients suggests that different bacterial genera may be associated with IBD. The present study proposed that infection with *C. jejuni* can be one of the significant reasons in the development of IBD.

Conflict of Interests
The authors declare that they have no competing interests.

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