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Investigation of adherent-invasive *E. coli* in patients with Crohn's disease

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Abstract

Background: Crohn's disease and Ulcerative colitis are known as inflammatory bowel disease with high morbidity which are as a result of increasing immune responses to intestinal microbiota in genetically susceptible individuals. The association of adherent invasive Escherichia coli with Crohn's disease in human has been discussed for decades. The principal aim of this study was to assess the relationship between adherent invasive *Escherichia coli* in Iranian patients with Crohn's disease.

Methods: The presence of adherent invasive *Escherichia coli* DNA and viable adherent invasive *Escherichia coli* cells were identified through PCR and conventional culture methods, respectively. All the specimens were subsequently cultured in Hi Chrome Agar medium.

Results: Using molecular assay, the invasive plasmid antigen H and invasion-association locus genes were detected from tissue samples confirming the presence of adherent-invasive Escherichia coli. The invasive plasmid antigen H was detected in 46.7% of CD and 13.3% of healthy peoples. The invasion-association locus gene was found in 36.7% of patients with Crohn's disease and 10% in individuals without IBD.

Conclusion: This study demonstrated an increased frequency of adherent invasive *E. coli* with invasive plasmid antigen H and invasion-association locus genes from patients with CD in comparison to control individuals. Moreover, it was shown that adherent invasive *E. coli* with the invasive plasmid antigen H and invasion-association locus genes can act as a predisposing factor in the development of IBD.

Keywords: Crohn Disease, Inflammatory bowel disease, *Escherichia coli*, PCR

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Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disorder divided into Ulcerative colitis (UC) and Crohn's disease (CD) (1, 2).The rising trend of the disease has observed in Middle Eastern countries as well as in other parts of the world and statistics shows the incidence also is on the rises in North America and Europe as well as other parts of the world. Likewise, a recent study demonstrated that the prevalence of IBD is increasing dramatically in Iran (3).

The incidence and prevalence of ulcerative colitis and Crohn's disease have been increased all over the world, especially among children and adults. It is believed that several factors such as immunology, genetic factors, stress, diet, being an alcoholic or a smoker, as well as bacteria such as the adherent-invasive strains of *Escherichia coli*, *Campylobacter spp.*, *Clostridium dificil*, *Mycobacterium avium* subspecies *Paratuberculosis*, and *Bacteroides fragilis* (4-7) may play a role in the development of IBD. Since gastrointestinal (GI) tract of healthy people is colonized by a wide variety of different microorganisms

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↑What is "already known" in this topic:
Inflammatory bowel disease (IBD) is comprised of Crohn's disease (CD) and Ulcerative colitis (UC). The prevalence and incidence of IBD are continuously rising in Iran. No study has been conducted to investigate the role of adherent invasive *E. coli* in patients with Crohn's disease occurred in the country.

→What this article adds:
Due to the rising trend of IBD prevalence and incidence in Iran, and also the significant role of intestinal microbiota in the development of disease, further investigation into the possible causing factors is vitally imperative.

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hence, dysbiosis of the intestinal microbiota is likely cause of IBD (8).

It is known that intestinal bacteria can cause CEACAM6 expression through inducing inflammatory cytokines. The expression can then leads to production of *E. coli* with AIEC phenotype. The AIEC can attach to epithelial cells by a mechanism involving polymerization of microtubules. In fact, AIEC adheres to GP2 (glycoprotein 2) upon M cells by LPF (long polar fimbriae) and enter to Peyer's patches and exacerbates inflammation in the intestine (9). Moreover, AIEC able to adhere epithelial cells and invade into the cytoplasmic eukaryotic infectious cells with type 1fimbriae (*fimH*), invasive plasmid antigen H (*ipaH*) and invasion-association locus (*ial*) (10).

However, *E. coli* with adherent-invasive phenotype should be regarded as a separate issue of pathogenicity of *E. coli* causing inflammation in the human intestine.

The principal purpose of this study was to investigate of *E. coli* with adherent-invasive phenotype and their relation to Crohn's disease.

Methods

In the present study, sixty subjects including thirty patients with lesions of Crohn's colitis and thirty people without IBD were collected during colonoscopy examinations. Samples with CD were obtained from lesions of colon, or terminal ileum. Moreover, the biopsy of control group was performed on normal areas. DNA was extracted from the tissue samples and PCR assay performed by targeting *ipaH* and *ial* genes. CD diagnosis based on clinical symptoms, laboratory evaluations, and colonoscopy finding was confirmed by histological assessment. The control subjects were taken from people with non-inflammatory IBD (nIBD). None of the individuals presented in this study used antibiotics or probiotics for the 3 months prior to the study. The study was accepted by the ethics committee of Tehran University Medical Sciences.

Microbial identification

Samples were immediately transferred into sterile vials containing either thioglycolate broth or saline (Sigma-Aldrich, Hi Media) and stored at -20˚C. The tissue biopsy were homogenized and inoculated into Hi Chrome *E. coli* agar (Sigma-Aldrich, Hi Media) and incubated for 18-24h at 35±2°C. The bacteria were stored in TSB broth containing 30% glycerol at a temperature of -70°C for further analysis.

DNA Extraction

Tissue biopsies were crushed and DNA extracted through RTP® Mycobacteria kit (Berlin, Germany).

PCR assay

All samples were tested for the presence of *ipaH* and *ial* genes using molecular PCR. The nucleotide sequence of primers (Macrogen, Pishgam) and production size (base pairs) for amplification of the *ipaH* and *ial* genes are shown in Table 1 (11, 12). PCR was performed in 12.5µL comprising 5µL master mix (Amplicon, Pishgam), 0.5 µL of each primer, 2 µL of the DNA template (50 ng) and 4.5µL of ddH_2O. Subsequently, the thermal cycling status was used: 300 seconds at 94°C and 25 cycles of amplification consisting of 60 seconds at 95°C, 55 seconds at 58°C, and 60 seconds at 72°C, with 300 seconds at 72°C for the final extension. PCR products were investigated 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_2EDTA (pH 8.0)] (13).

Statistical Analysis

Data were analyzed using Pearson Chi-Square test, and *P* value below 0.05 considered as statistically significant.

Results

Clinical information of patients and controls

The average age of patients with IBD and controls were 38.8 and 52.86 years old respectively. The participants included 14 males and 16 female patients with CD, and 18 males and 12 females without IBD. In addition, the age range for patients with CD and without IBD was 18-64 and 25-76 years old respectively.

Detection of adherent-invasive E. coli with ipaH and ial genes

Of 60 tissue samples collected, *E. coli* were isolated from 20 (66.6%) patients with CD and 22 (73.3%) from healthy people (Table 2). All isolated bacteria were confirmed as AIEC by biochemical tests and PCR assay. The

Table 1. The primers used for PCR reaction

Genes	Nucleotide Sequences(5´-3´)	Size of product, bp
ipaH	F-GTTCCTTGACCGCCTTTCCGATACCGTC R-GCCGGTCAGCCACCCTCTGAGAGTAC	619
ial	F-CTGGATGGTATGGTGAGG R-GGAGGCCAACAATTATTTCC	320

Table 2. *E. coli* Bacterial Culture in various condition

Group	Culture of		Total
	Positive	Negative	
Crohn's disease			
Count	10	20	30
CD (%)	33.4	66.6	100.00
nIBD			
Count	8	22	
nIBD (%)	26.7	73.3	100.00
Total			
Count	18	42	60
Total (%)	30	70	100.00

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Fig. 1. Amplification of the ipaH gene. Lanes: 1, DNA molecular size marker (100–bp ladder); 2-7, Crohn's disease samples; 8, negative control (Water).

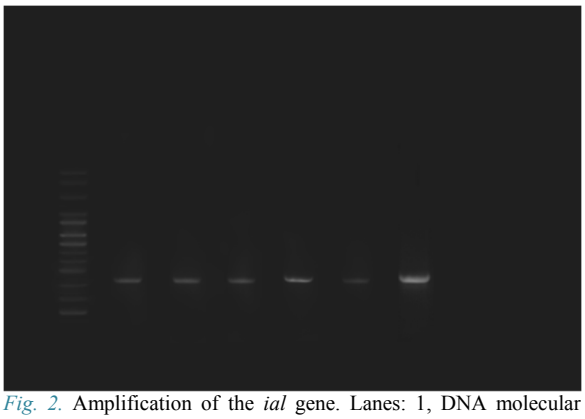

Fig. 2. Amplification of the *ial* gene. Lanes: 1, DNA molecular size marker (100–bp ladder); 2-7, Crohn's disease samples; 8, negative control (Water).

ipaH, *ial* genes were amplified by utilizing particular primers and become visible as a band of approximately 619 and 320 kb on agarose gel respectively (Figs. 1 and 2). The difference between patients with Crohn's disease and controls was not significant (p=0.59). Of 30 patients with CD, the positive and negative rate of *ipaH* gene was 46.7% (n=14) and 53.3% (n=16) respectively.

The percentage of *ipaH* among 30 individuals without IBD was 13.3% (n=4) but 86.6% (n=26) was not detected (Table 3). Furthermore, the positivity of *ial* gene were 36.7% (n=11) and 10% (n=3) in patients with CD and healthy people respectively (Table 4). Moreover, the presence of *ipaH* and *ial* genes in adherent-invasive *E. coli* in patients with CD was more than control populations.

Due to its high sensitivity, PCR assay was more reliable than cultivation. The difference between the correlation of adherent-invasive *E. coli* with *ipaH* and *ial* in CD patients with control populations was statistically significant ($p<0.05$). All positive amplified fragments were sequenced and high percentage of adherent-invasive *E. coli* was identified.

Furthermore, a chi square test was performed and the correlation between adherent-invasive *E. coli* with *ipaH* and *ial* genes in patients with CD found to be (N=30) =7.937, p= .005 and (N=30) =5.963, p=.015 respectively. Nonetheless all positive amplified fragments were sequenced and high percentage of adherent-invasive *E. coli* was detected.

Discussion

Crohn's disease is considered as a chronic inflammatory of GI disorder.

Although the etiology of inflammatory bowel disease is not still known (14), but numerous studies demonstrated that the mortality rate of this disease is high (15). Furthermore, it is demonstrated that several factors may play role in induction of the disease. In a research by Sartor RB et al, the intestinal microbiota effect on epithelial cells, and inflammation of the intestinal mucosa have shown to trigger the pathogenesis of both CD and UC (16). Moreover, CD patients demonstrated an altered intestinal microbial community, with different kind of bacteria in colonic and ileal areas (17).

Previous studies showed that *Enterobacteriaceae* especially *E. coli* could contributed the onset of IBD. Nevertheless other bacteria can trigger the pathogenesis of in-

Table 3. PCR Analysis of the ipaH gene for CD and nIBD samples

Group	ipaH	
	Positive	Negative
Crohn's disease		
Count	14	16
CD (%)	46.7	53.3
P value	.005*	
nIBD		
Count	4	26
nIBD (%)	13.3	86.7
P value	0.24	

*Significant at alpha=0.05

Table 4. PCR Analysis of the ial gene from CD and nIBD samples

Group	ial	
	Positive	Negative
Crohn's disease		
Count	11	19
CD (%)	36.7	63.3
P value	.015*	
nIBD		
Count	3	27
nIBD (%)	10.0	90.0
P value	0.34	

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flammatory bowel disease including Bacteroidales, Clostridiales, Pasteurellaceae (*Haemophilus* sp.), Veillonellaceae, Neisseriaceae, and Fusobacteriaceae (18). Significant information exists regarding the role of adherent invasive *E. coli* in the CD. Numerous studies reported that adherent invasive *E. coli* could be a candidate for the development of CD (19).

An emerging evidence indicated an association between adherent invasive *E. coli* with IBD and colorectal cancer (20, 21 and 22). The present study shows no difference in the incidence of *E. coli* positive culture between CD and control patients (Table 2). The lack of *E. coli* in some tissue sample was probably due to consumption of antibiotics which in turn can cause dysbiosis in the human gastrointestinal tract (23). In some studies, using the culture-based method demonstrated that *E. coli* bacteria were found in the intestinal mucosa as well as in ulcers of both CD and UC patients compared to controls (24). In comparison with nIBD, our finding shows that the abundance of adherent invasive *E. coli* harboring *ipaH* and *ial* genes was higher in CD individuals. Additionally, we found that 46.7% and 36.7% of CD specimens harbored *ipaH* and *ial*, an increased proportion of AIEC, compared to control individuals. In spite of interesting debate, Dogan et al conducted a similar prevalence of AIEC in the ileum of patients with CD (25). Several studies showed a high proportion of AIEC harboring *fimH*, *fimA* and *Cas* genes in CD (26, 27). According to the present study, in comparison with nIBD, PCR assay displayed a high abundance of *ipaH* and *ial* genes in CD patients with statistically significant difference. These data proposed that AIEC most likely harbored *ipaH* and *ial* genes which in turn could be effective in the initiation of the CD. Nonetheless, since other virulence factors for AIEC were not analyzed, so it may be taken into consideration in the future study.

Conclusion

Despite the role of several factors in the progression of IBD, the present study displays a high prevalence of AIEC with *ipaH* and *ial* from patients with CD. According to these findings, *E. coli* with AIEC phenotype could be a predisposing factor in the development of inflammatory bowel disease. In addition to AIEC, host immune response, genetics of patients and environmental factors play an important role in the initiation of IBD. Therefore the present study suggests that appropriate treatment against AIEC could be practically effective in patients with inflammatory bowel disease.

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Conflict of Interests

The authors declare that they have no competing interests.

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