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Identification and Role of ETEC by Real-Time PCR in a Sample Collected from Patients with IBD

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Abstract

Inflammatory bowel disease is a common and lifelong debilitating gastrointestinal disease. Several cult-based studies and molecular methods have shown that Escherichia coli bacteria can play a role in IBD. Therefore, the study aimed to investigate the relationship between IBD and bacterial leishmaniosis (ETEC) and their relationship with IBD and non-IBD.

Materials and Methods:

In this cross-sectional study, 81 paraffin blocks including 41 positive IBD samples and 40 control samples (healthy) were examined for ETEC bacteria. Samples were collected from Ghaem Hospital between 2016 and 2018. Initially, the blocks were cut to extract DNA and the DNA was extracted manually. Then, the design of the private premium probe for ETEC identification was done by the Real-Time PCR method. After confirming the primers, Taqman prob Real-time PCR was performed on IBD and control sample.

Results:

DNA extraction was performed and its quality was confirmed by NanoDrop device. Demographic results showed that the mean age of IBD and control patients was 51 and 46, respectively. The results from Real-Time PCR showed that 12 cases (30%) of IBD-positive individuals were positive for ETEC, while no positive cases were observed in control individuals (negative IBD). Statistical study shows that there is a statistically significant difference between the two groups in terms of ETEC bacteria.

Discussion:

Given the conditions of IBD and the results of this study, the presence of the toxin bacterium Escherichia coli is likely to exacerbate the conditions and symptoms of IBD by producing toxin virulence factors.

Keywords: Inflammatory bowel disease (IBD), Enterotoxigenic E. coli (ETEC), Real Time PCR

Introduction

Micro-organisms such as bacteria are causative agents of many Diseases (1, 2). Intestinal epithelium is in constant contact with the contents of the lumen and the intestinal flora. The intestinal barrier is the main defence mechanism for maintaining epithelial integrity and protecting the organism against the environment. The intestinal defence barrier includes the mucosal layer, antimicrobial peptides, secretory IgA, and the epithelial connective tissue complex (1). When epithelial barrier function is impaired, food and bacterial antigens can penetrate beneath the mucous membrane and induce inflammatory responses, which may lead to intestinal abnormalities such as inflammatory bowel disease (2-4). In fact, the intestinal epithelial barrier is protected by a highly viscous microfilm to prevent permanent and unwanted stimulation of the innate immune system. This film prevents close contact between commensal bacteria and intestinal epithelial cells. However, when the contract is formed, enterocytes can send warning messages in the form of chemokines or cytokines to the acquired and innate immune system of the mucous membrane and at the same time, secretes bacterial peptides into the lumen. [5] This complex mechanism is defective in some patients with inflammatory disease; preliminary data suggest that the secretion of antibacterial peptides in a subset of patients with

inflammation is defective or inadequate, in these patients the mucosal layer is thinner and is less effective as an antibacterial filter and is seen. The innate immune response at the level of the intestinal epithelium increases pathologically. The main function of the intestinal microflora is its metabolic activity, which leads to the retention of energy and absorbable nutrients. In inflammatory and infectious diseases, the intestinal microecology balance changes in such a way that the number of bacteria with pathogenic potential increases and the health response between the host and the bacterium is disrupted, as a result, an immune response can be induced by these intestinal bacteria (6). It is widely known that the number of bacteria present in the gastrointestinal tract is about 10 times higher than the number of eukaryotic cells. Also, the bacterial flora of the normal intestine is a complex ecosystem of approximately 500-300 bacteria species (7, 8). It is known that the balance of innate and acquired immunity is vital for the homeostasis of this microenvironment. In this regard, the immune system plays an important role in increasing the immune tolerance and thus avoiding a specific immune response against a large mass of commensal bacteria. The intestinal mucosal immune system is essentially formed by the gut-associated lymphoid tissue (GALT), which is formed by patch (peyer), lymphoid follicles, and peritoneal lymph nodes (9). Lack of regulation of immune responses in the intestinal mucosa along with cellular, environmental and genetic factors is associated with the etiology of inflammatory bowel disease. Changes in autophagy, antigen processing, regulation of cell signaling, and T cell homeostasis usually lead to decreased pathogen clearance and therefore play a role in the onset of inflammatory disorders in susceptible individuals (10, 11).

On the other hand, failure to tolerate native antigens in the intestinal mucosa due to injury or genetic background may lead to Crohn's disease (CD) or ulcerative colitis (UC) (12, 13). Intrinsic immune cells, including macrophages and dendritic cells, in line with identifying molecular patterns of microorganisms using pattern receptors, such as Toll-like receptors (TLR) and nucleotide-binding oligomerization domains (NOD) are allocated. In this regard, mutations in the protein gene contain the Caspase Recruitment Domain-Containing protein (CARD-15), which encodes the NOD-2 protein, is associated with inflammatory bowel disease, particularly CD. NOD2 is an intracellular microbial sensor that acts as an important regulator of the immunolamine propria response. Causes severe inflammation in the tissues

Finally, inflammatory bowel disease is a chronic recurrent inflammatory bowel disease (IBD) that is pathologically characterized by inflammation and epithelial damage. The disease includes two main complications: Crohn's disease and ulcerative colitis. These diseases are characterized by abdominal pain, fever, chronic diarrhea, and rectal bleeding due to ulceration of the inner surfaces of the colon and rectum, which can be associated with complications such as fistula, coronary abscess stenosis in the Crohn's and megacolon types in ulcerative colitis (14, 15). Laboratory manifestations of inflammatory bowel disease are non-specific and include anemia, decreased albumin, erythrocyte sedimentation rate (ESR), increased number of defence and white blood cells. In this group of patients, extraintestinal manifestations are seen in different organs of body. For example, skin manifestations of inflammatory bowel disease include inflammation and swelling of the skin, the presence of abscess-like lesions and pus, and multiple and superficial mouth ulcers. In the joints, patients may experience swelling and pain, including the knee, wrist, joint, hand swelling, inflammation, and pain in the pelvic, and lumbar joints. Ocular manifestations of these patients include episcleritis, conjunctivitis, and anterior uveitis, and hepatobiliary manifestations include hepatic steatosis, primary sclerosing cholangitis, bile duct stones, pericholangitis. Other problems in patients with kidney and bladder system problems include urinary stones, ureteral obstruction and urinary fistulas. It should be noted that in general, extraintestinal manifestations of inflammatory bowel disease are more common in Crohn's disease (16).

Research background

In a 2017 study at the Islamic Azad University, Sajedeh Sahari et al. studied the molecular genes of clbS and clbA genes of two genes of the PKS hegemonic island in Escherichia coli bacteria isolated from tumour monolayer tissues. For this purpose, 79 biopsy samples were taken from the dead intestines tissue of 79 patients with colorectal cancer were prepared after obtaining a questionnaire and consent form. Escherichia coli was isolated and identified by microbial and biochemical methods. Then the strains were evaluated for the presence of clbA and clbS genes by PCR. The results of the PCR test

showed that among the 79 strains studied, 66.6% of normal participants, 40.9% of patients with inflammation of the large intestine and 59% of patients with colorectal cancer had the presence of the clbA virulence gene, also in 50% of normal individuals and patients with colitis and 58.62% of patients with colorectal cancer, the presence of the clbS virulence gene was positive. Also, 27.8% of the samples had both genes (clbA / clbS). Finally they concluded that both the clbS and clbA genes were essential components of the PKS gene island. Considering the obtained results and the general importance of bactin and its relationship with colorectal cancer, a more complete study in this field seems necessary (99).

In 2016, Atieh Silagheh *et al.* investigated the frequency of vat1 and fimC genes in tissue Escherichia coli isolates in patients with colorectal cancer and inflammatory bowel disease in Iran population. Thirty eight biopsy samples were taken from intestinal tissue and isolated and identified by microbial and biochemical methods of bacteria. After extracting the genome of strains from the presence of virulence vat1 and fimC genes they were evaluated by polymer chain reaction (PCR) method. A molecular study showed that there was a significant difference between the studied groups in terms of genes, while there is no significant difference between groups in terms of fimC gene. The results of this study confirmed the relationship between the virulence genes studied and the induction of inflammation and the induction of the mutation rate (100).

In 2012, Zhoubin *et al*. studied the E. coli NC101 strain isolated from the studied intestine and the relationship between PKS island and IBD and CRC diseases and they found that out of 35 IBD samples and 21 CRC samples and 42 healthy samples in terms of inflammatory bowel disease and colorectal cancer, 20.8% of control samples, 40% of IBD patients and 33.0% of CRC samples were positive in terms of PKS genomic islands (71).

Research Method

First, all paraffin blocks related to IBD endoscopic specimens were collected from Ghaem and Razavi hospitals. The IBD blocks of this bank are available to these centres from 2016 to 2018. The criteria for entering the study in this study were biopsy samples of patients with IBD that were removed after endoscopic surgery and were fixed and maintained in the form of paraffin blocks. Samples taken from people with various malignancies and patients with acute gastrointestinal infections were excluded from the study.

Patients were selected after examining 80 patients' records (40 positive IBD patients and 40 negative IBD patients) paraffin blocks were cut and their DNA was extracted.

DNA extraction

1. 100 microliters of xylene were placed on a microchip containing a sample of two supplements for 15 minutes on a shaker.
2. The samples were then centrifuged at 16000 RPM for 3 minutes and the top solution was drained. This step was repeated 5 times. Washing with xylene is done to remove paraffin.
3. 800 µl of 100% ethanol was microtubed and Vertexed. The sample was then centrifuged at 14,000 rpm for 3 min.
4. The top solution was carefully removed and discarded, and 800 µl of 70% ethanol was sampled and Vertex. The sample was 14,000 centrifuged for 3 precision RPM headers.
5. The top solution was carefully removed and discarded, and 800 ml of 50% ethanol was sampled and Vertex was taken. The sample was centrifuged at 3,000 rpm for 3 minutes.
6. The top solution was carefully removed and discarded and the sample was left at room temperature for 1 minute to dry the alcohol.
7. 800 µ l of lysis buffer and 10 l of protinase k were added to the microtube containing the sample and the sample was placed in a 56 ° C laboratory water bath overnight and then the sample was transferred to a 90 ° C laboratory water bath for 10 minutes.
8. The sample was then centrifuged for 15 minutes at 12000 g at 4 ° C and finally the top solution containing the extracted DNA was transferred to the appropriate microtube.

SPSS statistical software version 20 was used to analyse the data. Also, quantitative variables were analysed by student t-test relationships and quantitative variables were analysed by Chi-square relationship.

Results

DNA extraction and adjustment of PCR reaction conditions

DNA extraction

After collecting the samples, DNA extraction from each of the samples was done manually and the quality of the extracted DNA after reading with the NanoDrop device with a wavelength of 280/260 (1.8) nanometers was confirmed.

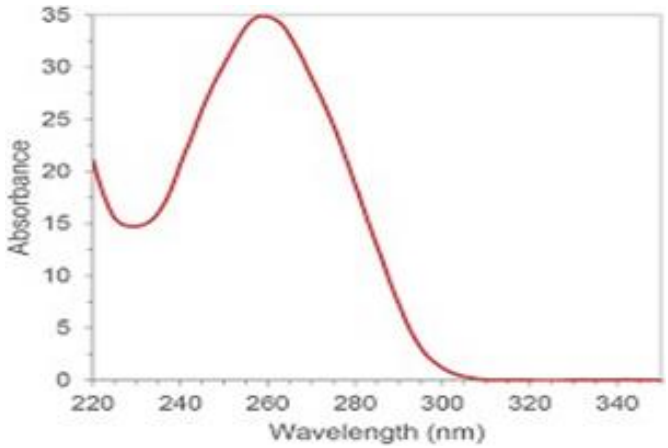

Figure 4-1 Absorption diagram of the NanoDrop device for the extracted sample

Real-Time PCR Results

After the approval of the primers for the toxigenic Escherichia coli and the approval of the bp 100 product, the Real-Time PCR reaction for the samples was set using the primers and the projector with the Roche device.

After real-time PCR for 81 samples (41 positive IBD patients and 40 negative IBD patients) among forty negative IBD patients, none of the samples had a specific reproduction, after 40 cycles of Real-Time PCR reaction no positive chart was obtained and analysis of melt graphs also confirmed that the samples were negative. Twelve (94%) of the 40 IBD patients were seen and had a positive response chart. To confirm the reaction in the Real-Time PCR, the reaction products were electrophoresed on an agarose gel and indicated that only bp 100 was multiplied. In addition, the housekeeping gene GAPDH (internal control) was reproduced.

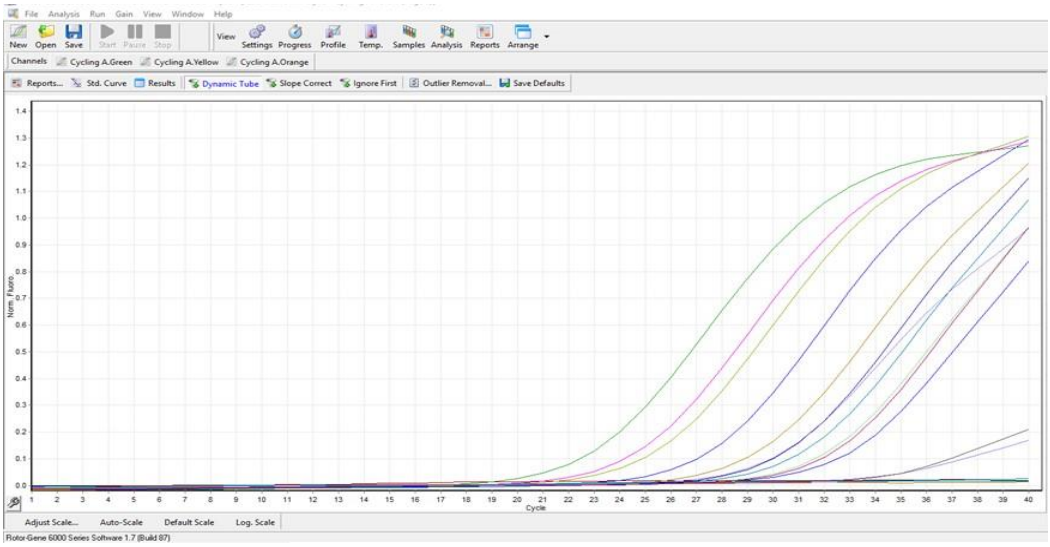

Figure 4-2 Real-time PCR reaction for toxigenic escherichia coli in positive samples

Based on the results of Real-time PCR, it can be said that there is a significant relationship between IBD and Enterotoxigenic Escherichia coli bacteria.

Discussion

Inflammatory bowel disease is a common and lifelong debilitating gastrointestinal disease. The disease is highly prevalent in developing countries. Crohn's disease and ulcerative colitis differ in their cytokine profile and histologic profile. Inflammatory diseases of the intestine are due to a complex interrelationship between monologue, environmental, and genetic factors. The presence of one or more genetic defects leads to an extreme reaction of the host mucosal immune system with the natural dissociation of the intestinal mucosal microflora (16).

In this study, 81 paraffin blocks consisting of 41 positive IBD samples and 40 control block samples were considered in the presence of Real-Time PCR with the Taqman Moore de Berber C probe. A collection of samples from Ghaem Hospital (AS) has been done. These samples are kept between the years of 2016 to 2018 in the archives and patient blocks of Ghaem Hospital. Out of 41 positive samples, 12 samples were positive in terms of Toxigenic Escherichia coli. Statistical analysis of healthy IBD group showed that there is a significant difference in the presence of this bacterium in these two groups In other words, this bacterium is more likely to be isolated in the IBD group, given that It produces a wide range of virulence and toxins factors, it can exacerbate inflammatory responses in these people and can exacerbate the symptoms of IBD. In 2001, James *et al.* worked on the distribution of E. coli bacterial evolutionary breeding outside the gut. In this study, investigations were performed on E.coli strains of groups A1, B1, B2, D in which the traits related to each of the pathogenesis cases, including the determination of some genes involved in the production of sidrophore of E. coli strains outside the intestine (18). Due to the prevalence of inflammatory bowel disease (IBD), many studies have been performed to isolate and identify the genomes of isolated bacteria, and most of these bacteria have the PKS genomic range. Their role in the disease is similar to that of Helicobacter pylori in gastric cancer (19). A study by Arlette Darfeuille-Michaud (2004) showed On Crohn's disease showed that in this disease the number of E. coli bacteria in the mucosa of the colon and ilium has increased. E. coli attaches to the ilium and attacks epithelial cells in vivo. In patients with ulcerative colitis or Crohn's disease, the risk of developing colorectal cancer increases fivefold (20). Inflammation of the large intestine causes intestinal pathogens such as *E. coli*. If the epithelium does not function properly as the first line of defence against intestinal antigens and bacteria, the risk of bacterial infection and intestinal inflammation increases in patients with intestinal inflammation. Studies have shown that in some diseases, such as IBD, the relationship between host coexistence and microbial flora fails. History of intestinal diseases such as IBD, ulcerative colitis, and Crohn's disease increases the risk of colorectal cancer because in these diseases, the large intestine is inflamed for a long time. In 2009, Putze investigated the presence of the bacterial toxin in the Enterobacteriaceae family and concluded that, in addition to *E. coli* strains, the gene was present in much smaller numbers in the Klebsiella pneumoniae, citrobacter, and Enterobacter (21). Rolhion *et al.* (2007) reported an increase in mucus-related Escherichia coli bacteria in inflammatory bowel disease and also examined their relationship in response to the immune system (110). One of the members of the human gastrointestinal microbiota is Escherichia coli, which forms a colony in the intestine a few days after birth and is present in the intestine throughout the life of the host. To induce bacterial inflammation, it may directly damage DNA by producing oxygenase genes, and this has been well observed in Enterobacteriaceae and bacteroid strains. Research shows that some strains of Escherichia coli can induce mutation rates. Infection with Escherichia coli is caused by virulence factors. Two fimC, vat1, virulence genes play an important role in bacterial pathogenicity in this organism. Escherichia coli, and in particular the invasive pathotype (AIEC), is increasingly involved in the pathogenesis of Crohn's disease (CD). Escherichia coli strains with pathogenic properties similar to AIEC are associated with other gastrointestinal diseases such as colic blistering, Colorectal, and BA-celiac disease. There is disagreement about the prevalence of Escherichia coli in the mucosa of UC patients. Several studies have reported no increase in the number of healthy individuals (22-23), while other studies have reported an increase in the prevalence of Escherichia coli in patients with UC (24, 25). These differences may be due to differences in the severity of the disease. Using, Escherichia coli FISH was detected in active UC epithelial samples more frequently than inactivated or controlled UC and Real Time PCR showed an increase in the frequency

of Escherichia coli in active UC patients compared to inactive UC patients as well as in the inflammatory form of non-inflammatory UC ratio (26).

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