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Original Article

Colibactin Genes Isolated from *Escherichia Coli* Related to Patients with Colorectal Cancer in Qom, Iran

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Abstract

Introduction: Colorectal cancer is one of the four most common cancers in the world. The results of various studies have shown that various factors, including pathogenic bacteria, play a role in the development of colorectal cancer. This study was performed to detect colibactin N (*clb*N) and colibactin B (*clb*B) genes in *Escherichia coli* (*E. coli*) isolated from patients with colorectal cancer.

Methods: In this case-control study, 200 (136 colorectal cancer and 64 healthy colon tissues) specimens were collected from patients referring to health centers in Qom province, Iran (April 2018-May 2019). The biopsy culture, microbiological, and biochemical tests were applied to detect *E. coli*. Polymerase chain reaction (PCR) was performed to determine *clb*P, *clb*N, *and clb*B genes. Data were analyzed using statistical package for social science (SPSS)-22.

Results: *E. coli* was detected in 58.1% of colorectal cancer and 17.2% of non-cancer specimens. The most frequent was related to *clb*B in colorectal cancer specimens (12.5%). *clb*N (14.28%) and *clb*B (12.28%) showed the highest frequency in female and male specimens. There was no significant difference between frequencies of colibactin genes in colorectal cancer and non-colorectal cancer specimens, and also gender (P-value>0.05).

Conclusion: Most *E. coli* strains were isolated from cancer-related samples. Also, most types of genes identified were related to *clb*B and *clb*N. Although no significant relationship was found between colibactin genes and colorectal cancer, infection control and identification of bacterial pathogenic genes in cancer patients are necessary.

Keywords: Colibactin, *Escherichia coli*, Colorectal cancer

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Introduction

Colorectal cancer develops from polyps in the lining of the large intestine. Symptoms include abdominal pain or blood in the stool. Colon cancer screening tests are needed to detect cancer early and treat it early.[1,2] Studies of the last two decades have shown that different mechanisms are involved in the initiation and progression of colon cancer at the tissue level.[3,4] Causes of cancer are divided into three main groups: physical or radiation, chemical, and infectious.[5,6] Although the role of microbiota in the development of colorectal cancer is still unclear, microbes appear to be important factors in the host immune system and genetics.[7,8] Pili, fimbria, and toxins such as colibactin are among the virulence factors that affect the pathogenicity of bacteria.[9,10] *Escherichia coli (E.coli)* can cause digestive infections, urinary tract infections (frequently in female patients due to anatomical conditions and poor hygiene), pneumonia, septicemia, and suprainfection of skin lesions.[11,12] Colibactin is one of the major toxins of *E.coli* that is involved in colon cancer.[13] The enzymes required for the overall synthesis of the colibactin are encoded and expressed by a 54 kb gene range.[14] The total colibactin synthesized genomic range ranged from 3 to non-ribosomal peptide synthases (NRPS) and 3 to polyketide synthases (PKS), and 2 NRPS / PKS hybrids and 9 to the editing and cleavage helper enzymes, including colibactin I, J, N, and O is formed.[15,16] The overall chemical properties of the bacterium are due to the presence of three *clb*N, *clb*B, and *clb*P enzymes that are involved in the synthesis and elongation, and failure of the overall folding of the colibactin.[16] Colibactin is secreted by the bacterial outer membrane and has a cytotoxic effect on host enterocyte cells. Colibactin damages DNA by activating the signaling pathway.[17,18] A study in Iran showed that 43580 new colorectal cancer cases were identified during 2014 and 2017.[19] The annual

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incidence rate of colorectal cancer in the Iranian National Cancer Registry was 8.46 per 100,000 population.[20] One study showed that *E. coli* was more abundant in colorectal cancer biopsies than in healthy mucosa. Furthermore, most mucosa-associated *E. coli* isolated from colorectal cancer harbored the pks genomic island (pks + E. coli), which is responsible for the synthesis of colibactin, a genotoxic compound.[21] In another study, genes encoding *clb*B were each identified in 5 isolates (11.4%) of 44 *E. coli* strains from patients with colorectal polyps.[22] As mentioned, pks + *E. coli* strains can damage the host cells of the gut lining, and these bacteria are commonly found in the colon of infected patients and are not usually found in the intestines of healthy individuals.[21,23] The prevalence and causes of cancer vary geographically, and risk factors associated with cancer can change over time, so this study was performed in Qom province, Iran. Also, the role of infectious agents, particularly bacteria, in the disease must also be considered. This study aimed to detect *clb*P, *clb*N, and *clb*B genes in *E. coli* isolated from colon tissue samples in patients with suspected cancer.

Methods

Sample collection: In this case-control study, a total of 200 biopsy specimens (paraffin-embedded tissue) of colorectal cancer suspected collected randomly from April 2018 to May 2019 from people who were referred to medical centers in Qom province, Iran. The criterion for admission was a biopsy of Iranian patients with colorectal cancer after colonoscopy. After scoring the research questionnaire forms and the consent form, it was completed and signed by the patients. According to Cochran's formula, with 95% confidence and an expected prevalence of 25%, those referring to cancer treatment centers in Qom province were collected. The prevalence of colorectal cancer in the world and in different populations varies. According to studies in Iran, their frequency is 25%, and according to the accuracy of the study (d = 0.08), using the relationship below, the sample size was calculated. A significant level in this study (alpha) was considered equal to 5%. Z1-α/2 with a 95% confidence interval is 1.96. According to the formula used, the minimum sample size is 112 specimens, of which at least 61 healthy specimens and 61 patient specimens should be selected, but in this study, 64 healthy specimens (case) and 136 patient specimens (control) were examined (n = 200), which is more than the minimum number of samples. Overall, 56 females and 144 males were sampled, of whom 38 females and 98 males (case = 136) had colorectal cancer, and 18 females and 46 males (control = 64) were without colorectal cancer.[22]

$$n = \frac{1.96^2 \quad p(1-P)}{d^2}$$

The specimens that were pathologically healthy after examination by the pathologist were considered control samples. Samples (Patients' colon tissue) were then sent to the laboratory for examination.[24]

DNA extraction and polymerase chain reaction (PCR): All biopsies were cultured in brain heart infusion (BHI) broth (Himedia, India) and then in differential culture media. Microbiological and biochemical tests were used for identification. DNA extraction was prepared according to the Kit (Beyonce, Korea) instructions. The PCR was performed with the primers (The primers in this study were designed by the researchers) (Table 1).[25]

PCR buffer 10 X, deoxynucleoside triphosphates (dNTPs) Mix (10 millimolar (mM)), MgCl2 (50 mM), Taq DNA polymerase, and DNA extracted from bacterial strains. The following PCR conditions: initial denaturation at 95 °C for 5 min, 34cycles of 95°C for 1min, 50-52°C (for each gene) for 30 s, and 72°C for the 30s, and final extension of 72°C for 5 min. Five µl of PCR products were subsequently loaded on an agarose gel with 1.5%, 1X Tris acetate (ethylene-diamine-tetraacetic acid (EDTA) (TAE), and electrophoresed.

Statistical analysis: Matching was performed to control for confounding variables (For each person in the experimental group, a similar person was selected in the control group). Data were analyzed using SPSS version 22.0, Microsoft Excel, Chi-square, Fisher, and t-test ($P < 0.05$).

Results

One hundred and thirty-six (136/200: 68%) specimens were related to colorectal cancer patients, and 64 (64/200: 32%) were related to healthy colon tissue. Based on PCR results, *E. coli* was detected in 79/136 (58.1%) colorectal cancer and 11/136 (17.2%) non-cancer specimens. Frequencies of *clb*P, *clb*N, and *clb*B in colorectal cancer specimens were 8.1%, 11.8%, and 12.5%, respectively. In non-cancer specimens, frequencies of *clb*P, *clb*N, and *clb*B were; 1.6%, 4.6%, and 6.2%, respectively. There was no significant difference between frequencies of colibactin genes in colorectal cancer and non-colorectal cancer specimens (*P*-value > 0.05) (Table 2, Figure 1).

The highest frequency of known genes in females was related to *clb*N (14.28%) and in males to *clb*B (12.28%). There was no significant difference between the frequency of colibactin genes and gender (Table 3 and Figure 2).

Discussion

This study aimed to detect colibactin genes isolated from *E. coli*. *E. coli* strains were also isolated from patients with colorectal cancer. In our study, *E. coli* was isolated from 58.1% of colorectal cancer and 17.2% non-cancer specimens. An increase in the prevalence of *E.coli* in inflammatory bowel disease and colorectal cancer has recently been reported. In spite of the biological

Table 1. Sequences of primers used to detect *clbP, clbN, and clbB* genes

Genes	Sequences of primers	Size (bp)
*clb*P	Forward: 5'-GCCGCAGACAATCGTATCC-3' Reverse: 3'-CCTGGAGATAGTATACCCGGTG-5'	461
*clb*N	Forward: 5'-TTCAGTTCGGGTATGTGTGG-3' Reverse: 3'-AGCTGCCGTAAAGACTCGAC-5'	562
*clb*B	Forward: 5′-TTAGTCGAGGGCGGGATAC-3′ Reverse:5′-AGCACCATAGCGATAGTAATAAGTC -3′	525

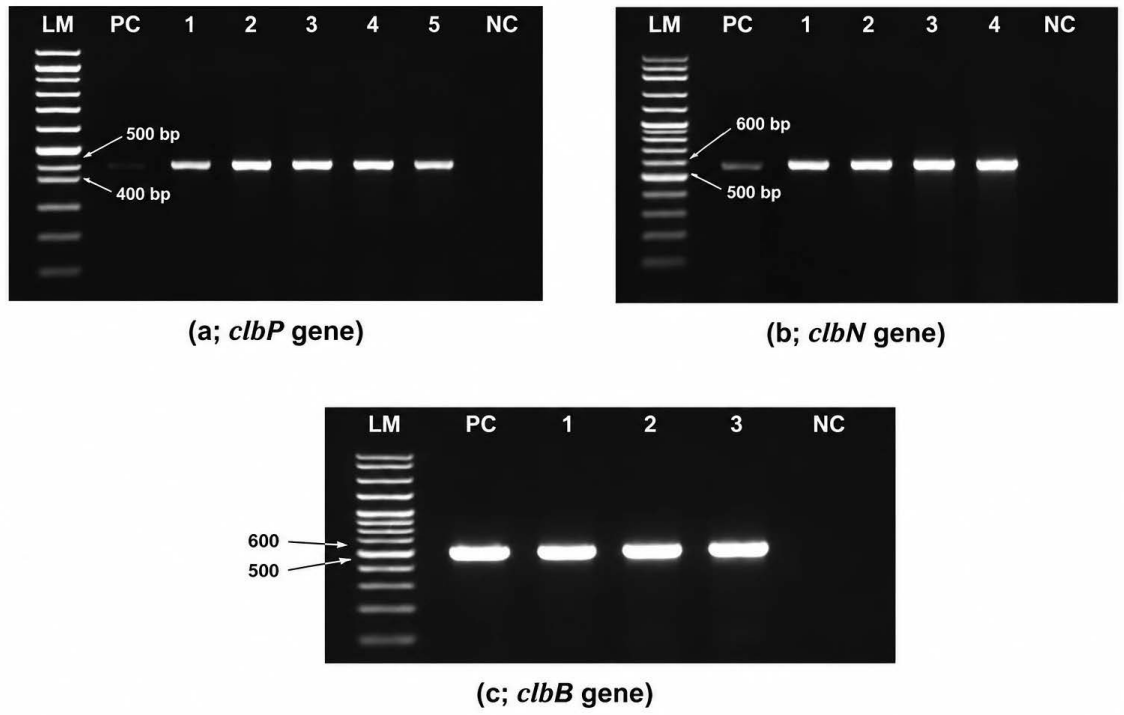


Figure 1. Agarose gel electrophoresis, a) *clb*P gene amplification [LM: DNA Ladder (100 bp), PC: positive control (1-5: positive sample (461 bp)), NC: negative control], b) *clb*N gene amplification [LM: DNA Ladder (100 bp), PC: positive control (1-4: positive sample (562 bp)), NC: negative control], c) *clb*B gene amplification LM: DNA Ladder (100 bp), PC: positive control (1-3: positive sample (525 bp)), NC: negative control]

Table 2. Frequencies of colibactin genes in colorectal cancer and non-colorectal cancer specimens

Colibactin genes	Colorectal cancer (*E. coli*; 79 = n; %)	Non-colorectal cancer (*E. coli*; 11 = n; %)	*P*-value
*clb*P	11 (8.1)	1 (1.6)	0.061
*clb*N	16 (11.8)	3 (4.6)	0.10
*clb*B	17 (12.5)	4 (6.2)	0.22
*clb*N-B	7 (8.86)	4 (36.36)	0.420
*clb*P-N-B	4 (5.06)	2 (18.18)	0.502

Table 3. Frequencies of colibactin genes in female and male specimens

Colibactin genes	Females (n = 56, %)	Males (n = 114, %)	*P*-value
*clb*P	3 (5.35)	9 (7.89)	0.055
*clb*N	8 (14.28)	13 (11.40)	0.112
*clb*B	7 (12.5)	14 (12.28)	0.069
*clb*N-B	5 (8.92)	6 (4.38)	0.220
*clb*P-N-B	3 (5.35)	3 (2.63)	0.101

similarities between diffuse inflammatory bowel disease and colorectal cancer, it seems that the intermediates that are produced during the inflammatory process can inhibit the apoptosis pathway.[9] The lymphoid tissue of the mucosa of the gastrointestinal tract is the first line of defense against pathogenic germs.[1-3] *clb*P, *clb*N, and *clb*B were detected in 8.1%, 11.8%, and 12.5% of colorectal cancer specimens, respectively. Olier et al. demonstrated that inactivation of the *clb*A gene in *pks* islands induces DNA harm and chromosomal irregularities in intestinal epithelial cells in rats.[8] Bonnet et al. evaluated the carcinogenic properties of *E. coli* in intestinal neoplasia mice. Multiple intestinal neoplasias in mice infected with

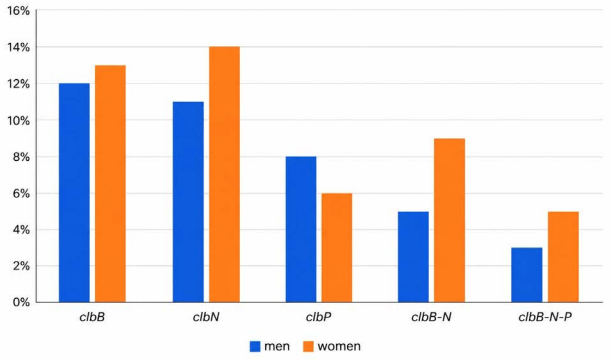


Figure 2. Frequencies of *clb*P, *clb*N, and *clb*B in female and male specimens

E. coli strain 11G5 showed an increase in the number of polyps compared to the control. Our results also showed that *E. coli* was detected from 58.1% of colorectal cancer specimens. These results support the idea that the pathogenic *E. coli* could be a cofactor in colorectal cancer pathogenesis.[24] Dastjani Farahani et al. showed that the frequency of *E. coli* with *pks*-positive strains in colorectal cancer patients and healthy individuals was 12.2% and 4%, respectively. In our study, *E. coli* was detected in 58.1% colorectal cancer specimens and 17.2% non-cancer specimens.[9] In our study, *clb*N and *clb*B were detected in 11.8% and 12.5% of colorectal cancer specimens, respectively. Falah Al-Fatlawi et al. investigated the genes of the *clb*A and *clb*P of *E.coli* in 50 patients with colorectal polyps and 50 healthy controls. The frequency of *clb*A and *clb*P genes in patients with colorectal polyps was 14.28 and 0%, respectively, and in healthy individuals was 2

and 0%, respectively. Frequencies of *clb*P, *clb*N, and *clb*B according to our results were; 1.6%, 4.6%, and 6.2%, in non-colorectal cancer, respectively. The results showed that the presence of total colibactin genes in patients with colorectal polyps could make them more susceptible to the development of colorectal cancer. Also, they can be found in non-colorectal cancer specimens.[1] Biosynthesis of hybrid nonribosomal peptide-polyketide(s) gives bacteria the ability to induce DNA double-strand breaks in eukaryotic cells. Expression of the *clb* gene can enhance the ability of *E. coli* to induce DNA damage and chromosomal aberrations.[8] The bacterial genotoxin colibactin is produced by the *pks* genomic island in *E. coli* strains. Mammalian cells exposed to live pks+bacteria exhibit DNA double-strand breaks and undergo cell cycle arrest and death. Colibactin induces DNA double-strand breaks and increases senescence-associated β-galactosidase (SA-β-Gal) activity in cells. Senescent cells promote tumor cell growth. Consequently, the *E. coli* genotoxin colibactin induces cellular senescence and genotoxic effects.[13] Studies in different places show that the frequency of genes is different. The reason for this can be attributed to the difference in the natural flora of the colon in different places. The incidence and risk factors associated with infection of this type of cancer depend on various factors. Genetic and environmental components, age over 50, screening and early diagnosis, polyp formation, diet, and hereditary factors can be the cause of this disease, and based on these factors, its prevalence also varies in different societies.[1,5,8,9,24] The strengths of this study include laboratory estimation in a specific context. The results of this study showed that control of bacterial infections in affected patients and early treatment are essential. One of the limitations of this project is the small number of samples prepared. If the number of samples increased, comprehensive and complete results would be obtained. Single geographic location and lack of sequencing confirmation are other limitations. Examining more geographical locations gives more credibility to the information and results. Molecular sequences were also recommended to examine gene mutations and obtain molecular information about the *E. coli* strains.

Conclusion

Colorectal cancer and non-cancer specimens were infected with *E. coli*. *clb*P, *clb*N, and *clb*B were detected in Colorectal cancer and non-cancer specimens. The highest frequency genes detected in females and males were related to *clb*N and *clb*B. no significant difference between the frequency of *clb*P, *clb*N, and *clb*B in colorectal cancer and non-colorectal cancer specimens, and by gender.

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Writing–Review & Editing: Azam Ghorbannia-Delaver

Competing Interests

The authors declare that they have no conflict of interest.

Ethical Approval

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