

Original Article

Analysis of *clbN* and *clbB* genes in Isolated *Klebsiella pneumoniae* of Biopsies from Patients with Colorectal Cancer

Amir Cheraghi¹ M.Sc., Shahla Mohammad Ganji^{2*} Ph.D., Bita Najmi³ Ph.D.

¹Islamic Azad University, Farahan Branch, Farahan, Iran.

²National Institute Genetic Engineering and Biotechnology, Tehran, Iran.

³Islamic Azad University, Qom Branch, Qom, Iran.

ABSTRACT

Article history

Received 12 Apr 2016

Accepted 2 Sep 2016

Available online 29 Oct 2016

Key words

clbB

clbN

Colorectal cancer

Klebsiella pneumoniae

PCR

Background and Aims: Recently it has been proved that some of Enterobacteriaceae like *Klebsiella pneumoniae*, which carry PKS islands, damage dsDNA by encoding Colibactin genotoxin; and finally they induce some apoptosis in damaged mucosal cells. This study aimed to isolating the *clbN* and *clbB* genes, which are the markers of the PKS genomic island, from PKS cluster in these bacteria

Materials and Methods: In this study, 110 biopsies were obtained from colorectal cancer patients referred to the clinic of Imam Khomeini Hospital. Then, all samples were cultured in LB medium for bacterial isolation. In the next step, *Klebsiella pneumoniae* was detected by biochemical and microbiological tests. Finally, the genomes were extracted by boiling method and then were amplified by PCR with specific *clbN* and *clbB* gene primers.

Results: Our findings have showed 30 *Klebsiella pneumoniae* isolated out of 110 biopsies (27.3%) from patients with colorectal cancer; in which all of biochemical and microbial experiments including Gram-negative staining confirmed this bacteria. Also, 23.23% frequency of *clbN* were positive, 20% frequency of *clbB* were positive genes in the isolated *Klebsiella pneumoniae*, and 13.33% frequency were positive for both *clbB* and *clbN* genes as simultaneous.

Conclusions: It is expected to find a significant correlation between *Klebsiella* bacteria which carries PKS genes and colorectal cancer by increased number of samples. The lower frequencies for *Klebsiella* with *clbB* and *clbN* positive strain in patients with colorectal cancer were shown in European countries based on the results of this study rather than the similar studies.

* **Corresponding Author:** National Institute of Genetic Engineering and Biotechnology (NIGEB), Pajuhesh Blv., 15 Km Tehran-Karaj Highway, Tehran, Iran. **Tel:**+98 2144787466, **Fax:**+98 2144787399, **Email:** shahlamg@yahoo.com

Introduction

Colorectal cancer (CRC), as a multifactorial disease, is a common cancer in the world and Iran. There are some risk factors for this disease such as genetic background and environmental factors, as well as bacterial infections [1]. *Klebsiella pneumonia* is a Gram-negative immobile, encapsulated, lactose-fermenting, facultative anaerobic, and rod shaped bacteria. *Klebsiella pneumonia* is the main factor of about 1% bacterial pneumonia [2, 3, 4]. The studies showed that a certain type of infection of *Enterobacteriaceae* especially *E.coli* and *Klebsiella pneumonia* with PKS has a positive strain. This strain of bacteria can promote colorectal cancer in patients with inflammatory bowel disease (IBD), Crohn's disease, and ulcerative colitis [5, 6, 7].

The bacteria with PKS genes (genome cluster) can produce a kind of exo-toxin colibactin which is able to damage the host genomic cells subjected to compose inflammation all over the colon's wall. Colibactin is a kind of toxin produced from hybrid polyketide and nonribosomal peptide. A perfect knowledge about the mechanism is not in hand. A kind of genomic cluster (54 kbp) has been placed in *asnW* tRNA locus, liable to Colibactin synthesis. This area is consisted of 23 open reading frame (ORF), 3 non-ribosomal peptide megasynthesis (NRPS), 3 polyketide megasynthesis (PKS), 2 hybrid of (PKS-NRPS), and 9 enzymes for rebuilding and slicing [8, 9, 10, 11].

clbB (5000 bp) and *clbN* (7000 bp) are as molecular markers respectively for spaces of 3"

and 5". These two genomes associated with each other create a pre-reconstruction named N-acyl-D-asparagine which finally produces Colibactin according to the effect of *clbP*. On the other hand, *clbQ* and *clbA* as the final markers are subset to the PKS genomic island [12]. The Colibactin damages to double-stranded DNA and increased level of H2AX histone, lead to the activation of the DNA damage response (DDRs). DDRs are necessary for protection of genetic information and the identification of cell genome against the invasion of environmental factors and other ROS endogenous activities. This mechanism along a molecular cascade causes the stop of cell cycle through G2 process. If the restoration rose in a normally way, the cell proliferation has to become in a natural circle; otherwise, the cell deviates to the apoptosis in which mutation may come to be happened in the genome [13].

So it will lead to the initiation and progression of colorectal cancer by considering this fact that the bacteria with PKS gene disrupt the cell cycle by producing the colibactin as secondary metabolites of bacteria. Therefore the aim of this paper is describing the latest achievements regarding Analysis of *clbN* and *clbB* genes in isolated *Klebsiella pneumonia* of biopsies from patients with colorectal cancer in Iran.

Materials and Method

In this project, as a cross-sectional and experimental study, we collected 110 biopsies from patients with colorectal cancer and IBD who referred to the clinic of colonoscopy in

Imam Khomeini Hospital, and Tumor Bank in Tehran, during two years. This study was approved by Ethics Committee of Islamic Azad university of Farahan branch. All of the participants filled out the questionnaire and consent form and presented them to the ethics committee. All of the specimens had the demographic data and pathology reports which used for future analysis. None of patients hadn't been received any remedy (chemotherapy, drug remedy or radiotherapy) and they hadn't been diagnosed Hereditary non polyposis colorectal cancer (HNPCC) or other cancers. The sterilized tube samples were transported into the National Institute Genetic Engineering and Biotechnology laboratory. All of the biopsies cultured in the LB-broth and then in differential mediums in order to isolation of *Enterobacteriaceae* bacteria including *Klebsiella pneumoniae*. Then the biochemical and microbial tests such as TSI and IMVIC were done to confirm the existence of *Klebsiella pneumoniae*. All of the mediums for microbial experiments were produced in Merk Co. Germany.

The DNA of bacteria were extracted by boiling method. Evaluation of quality and quantity of extracted DNAs was done by electrophoresis on 0.8% agarose gel and spectrophotometry on 260 and 280 nm.

The sequence of designed specific primers for *clbB* and *clbN* genes are *ClbB* F: GAT TTG GAT ACT GGC GAT AAC CG, *ClbB* R: CCA TTT CCC GTT TGA GCA CAC, *ClbN* F: GTT TTG CTC GCC AGA TAG TCA TTC and *ClbN* R: CAG TTC GGG TAT GTG TGG AAG G. Polymerase chain reaction (PCR)

carried out with the suitable reagents as follow: 1 μ l or 100 ng DNA, 6 μ l Master mix (Ampliqon Co. Denmark), 1 μ l of each primers, forward and reverse, with 10 mM concentration (Gene Fanavaran Co.), and 4 μ l deionized water with 12 μ l total volume for each reaction. The PCR was done on the thermocycler, Techno, UK by this program: 94°C for 5 minute denaturation and 30 cycles with 94°C, 58°C and 72°C for 30 second respectively and final elongation temperature was 72°C for 5 minute. All of the PCR products were analyzed by electrophoresis on 1% agarose gel. The expected size of products for *clbB* and *clbN* genes were 500 bp and 700 bp respectively.

Statistical analysis

Statistical analyzes were done by Fisher exact test and Chi-square test by the SPSS software, version 17. and if $\alpha < 0.05$, the *p-value* was considered significant.

Results

In this study, we could isolate 30 *Klebsiella pneumoniae* out of 110 biopsies (27.3%) from patients with colorectal cancer. Fortunately, all of biochemical and microbial experiments, including Gram-negative staining confirmed this bacteria. We found that 4 of the participants in this study (3.63%) had not shown the symptoms of colorectal cancer during sampling, while two years follow up confirmed their infection with colon cancer.

The results showed that the frequency of *Klebsiella pneumoniae* with *clbN* positive strains was 23.23%. It means that 7 samples out of 30 were positive for *clbN* (Fig 1).

Moreover, the frequencies of *Klebsiella pneumoniae* with *clbB* positive strains was 20% or in the other words 6 samples out of 30 were positive for *clbB* gene (Fig 2). The analysis also showed that 4 samples with 13.33% frequency

were positive for both *clbB* and *clbN* genes as simultaneous; so there were no significant differences between *clbB*, *clbN* and colorectal cancer ($P>0.05$).



Fig 1. Analysis of *clbB* gene on 1% agarose gel. From left to right; lane1, ladder 100bp, Fermentase Co. lane 2: Positive control, gifted from France, University of Toulouse; lane 3: Negative control; lanes 4-7, PCR products of a few samples with *clbB* gene. The size of PCR product for *clbB* gene is 500 bp.

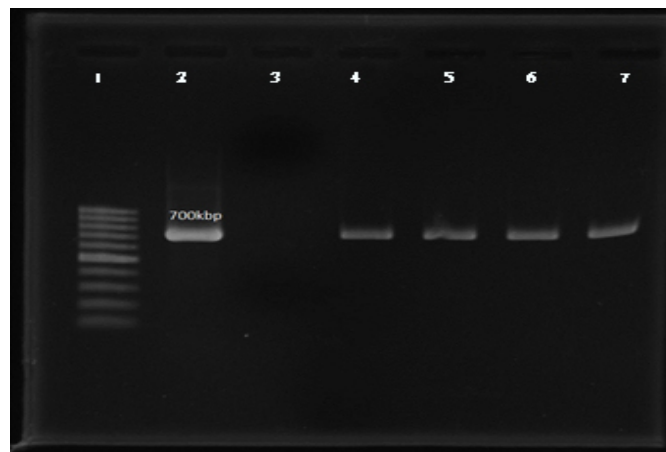


Fig 2. Analysis of *clbN* gene on 1% agarose gel. From left to right; lane1, ladder 100bp, Fermentase Co. lane 2: Positive control, gifted from France, University of Toulouse; lane 3: Negative control; lanes 4-7, PCR products of a few samples with *clbN* gene. The size of PCR product for *clbN* gene is 700 bp.

Discussion

Nowadays, a study has recently been confirmed that there is a specific relation among bacteria with colorectal cancer and the other long intestinal infections; so that the IBD

can be good examples for this claim. The first level of intestinal protective mechanism (epithelium) starts by campaign for anti-genes and harmful bacteria. The epithelium has a

covering of secretive cells owning the quality of mucosal secretion named goblet, which sticks to epithelium cells and its function is preventing direct contacts to touch harmful bacteria. Thus, each one of these protective covers do their task perfectly whether the gets damaged or not, so colonization and bacteremia infections shall be happen [14]. In 2004, Martin, *et al* attended in a work on mucosal glycosylation changes around the colon cancer and the other cases of inflammatory colon infection by focusing on the role of intestinal adhesion with mucosal bacterial. The experts had attended to set up clinical testing in *E. coli*. in especial. The result has confirmed that adhesion mucosal bacterial has a strong effect in colon injuring or in other meaning colorectal cancer [2]. Oswald, *et al* acknowledged *PKS* genome region and chromosomal abnormalities which are effective on the colibactin toxin over a line of CHO cells. The results obtained from the data showed that this toxin causes tetraploidy, aneuploidy, and makes anaphase bridges [15]. The strains carrying this genomic clusters, have a land of genome split in double-stranded eukaryote genome, cells in a state of both two conditions of in vivo and in vitro simultaneously. So it has the ability of forcing inflammatory. Now the majority of the characteristics of this mechanism is unknown; so further studies are mostly around the genetic structure and the function of colibactin for this remark. The present report states the role of *clbB*, *clbN* in the beginning of colibactin biosynthesis. *clbB* and *clbN* make a pre-drug (N-acyl-D Asparagine), so finally the

material would be *clbP* which is a kind of protease owning an effect on it [12]. The biology of colibactin living is placed in its early occasion yet, because it is detected in modern time and cannot be achieved artificially in any laboratory. But there are some unanswered questions awaiting now. 1) How does this toxin can damages DNA, 2) how does it get stick to the nucleus cell and get into it 3) Does it contribute to long-term bacterial colonization in vivo [10]. Colibactin genes were found earlier in *E. coli* by having marks of ability to transmit genes horizontally. Genes horizontally transmitted among the bacteremia are one of the most significant ways of genetic elements transferring. In respect to these remarks, chromosomal insertion into *asn* WtRNA locus, presence of a P4like-integrase, the presence of flanking 16 bp direct repeats, and elevated G + C content are related to the *E. coli* core genome [9]. Therefore, this problem can itself be a logical reason for investigation over these genome islands. Experimental activities around cancer and bacteria especially on genotoxin issue are complicated in relation to bacteria and host cell interaction. However, it is possible to verify this matter in the area of medical sciences by developing animal models and arising the quality of tests [10]. Johnson (2008), stated that *clbB* and *clbN* have a significant effect on bacteremia cycling. So that he isolated *clbB* and *clbN* positive bacteria in 58% blood and 32% fecal strains. The relationship between these genes and other virulence factors is investigated. However, they can be a therapeutic target or prevention

of bacteremia [16]. In contrast to his research, the bacteria in this study were isolated from colorectal biopsies and a significant relationship between *clbN*, *clbB* (p -value >0.05) and colorectal cancer was investigated, but there was no significant relationship. Putze, et al (2009) have done their investigation over genetic structure of *PKS* and genetic distribution between other *Enterobacteriaceae* bacteria, and reported that there are other members of *Enterobacteriaceae* such as *Enterobacter aerogenes*, *Citrobacter koseri*, and *Klebsiella pneumonia* in addition to *E. coli*. The results of this study have indicated that among 1565 isolated samples, %9.5 was related to *E. coli*, 3.5% with *Klebsiella pneumonia*, 27.3% with *Enterobacter aerogenes* and 100% *Citrobacter koseri*, in which all of them were *clbB* and *clbN* positive. So this result showed that the frequency of these genes in *E. coli* are more than other isolated *Enterobacteriaceae* [16]. In present research, for the first time in Iran, it was attempted to investigate dispersion and participating of *clbB*, *clbN*, as two important genes in the *PKS* Island. In the other *Enterobacteriaceae* strains, except *E. coli*, *Klebsiella pneumonia* was isolated from mucosal tissues infected in colorectal cancer. The positive control strain that used in this testing was *E. coli* strain of Nissel, which is the name of its detector that had been gifted here by Toulouse university department in France. The 3.5% dispersion of *clbB* and *clbN* in 104 samples had been reported in Putze's studying about the *Klebsiella pneumonia*. It is considered this study confirmed their data since we found

13.33% frequency for both *clbB* and *clbN* positive genes as simultaneous in 30 isolated *Klebsiella pneumonia*. The reason of disparity in statistical ending may return to nutrition, colon normal Flora, and different routine infections among two population memberships. Although sampling was the most important imitating factor in this study, so we suggest a similar study with increased sample size to find significant results. Totally, the lower frequencies for *Klebsiella* with *clbB* and *clbN* positive strain in patients with CRC were shown based on the results of this study rather than similar studies in European countries.

Conclusion

The aim of current research is analyzing the presence of the *PKS* Island within some strains of *Enterobacteriaceae*, except *E. coli* over the isolated bacteria from biopsies of colorectal cancer patients in which the positive marks were selected. It also statistically indicated that positive and volunteer's samples were being in quantity more than people infected in cancer. On the other hand, there was not any sign of a meaningful relationship between *clbB* and *clbN* clusters. The main reason of not having a kind of meaningful relation should come back to the difficulty of handling more infected samples and small statistical population indeed. Collecting demographic factors, frequency analysis of the *PKS* Island in other members of *Enterobacteriaceae*, and their sources are the next aims of this study. At the end, the PCR products of some positive strains will be sent to sequencing and phylogenetic studies.

Acknowledgments

The authors should thank the colonoscopy department, Imam Khomeini Hospital, Iran Tumor Bank, for their help in collecting samples; and National Institute of Engineering and Biotechnology in Tehran for their facility to do this

research. Also the authors appreciate Mr. Masood Cheraghi for his help in writing this paper.

Conflict of Interest

The authors confirm that this article content has no conflict of interest

References

- [1]. Boleij A, Tjalsma H, Gut bacteria in health and disease: a survey on the interface between intestinal microbiology and colorectal cancer. *Biol Rev Camb Philos Soc.* 2012;87(3): 701-30.
- [2]. Martin HM, Campbell BJ, Hart CA, Mpofo C, Nayar M, Singh R, et al. Enhanced *Escherichia coli* adherence and invasion in Crohn's disease and colon cancer. *Gastroenterology* 2004;127(1): 80-93.
- [3]. Mohandas Km. Colorectal cancer in India: controversies, enigmas and primary prevention *Indian Journal of Gastroenterology.*2011; 30(1): 3-6.
- [4]. Parkin DM. International variation. *Oncogene* 2004;23(38): 6329-340.
- [5]. Kefalas CH. Gastrointestinal Crohn's disease. *Proc (Bayl Univ Med Cent).*2003;16(2): 147-51.
- [6]. Parry FQ, Wani M, Bijli AH, Thakur N, Irshad I, Nayeem-ul-Hassan. Crohn's disease: a surgeon's perspective. *Saudi J Gastroenterol.* 2011; 17(1): 6-15.
- [7]. Hawker PC, Gyde SN, Thompson H, Allan RN. Adenocarcinoma of the small intestine complicating Crohn's disease. *Gut.* 1982;23(3): 188-93.
- [8]. Jawetz, Jawetz, Melnick & Adelbergs's medical microbiology, ed. 26th ed 2013.
- [9]. Putze J, Hennequin C, Nougayrède JP, Zhang W, Homburg S, Karch H, et al. Genetic structure and distribution of the colibactin genomic island among members of the family Enterobacteriaceae. *Infect Immun.* 2009;77(11): 4696-703.
- [10]. Guerra L, Guid R, Frisan T. Do bacterial genotoxins contribute to chronic inflammation, genomic instability and tumor progression? *FEBS J.* 2012;278(23): 4577-588.
- [11]. Homburg S, Oswald E, Hacker J, Dobrindt U. Expression analysis of the colibactin gene cluster coding for a novel polyketide in *Escherichia coli*. *FEMS Microbiol Lett.*2007;. 275(2): 255-62.
- [12]. Brotherton CA, Balskus EP. A produg resistance mechanism is involved in colibactin biosynthesis and cytotoxicity. *J Am Chem Soc.* 2013;135(9): 3359-362
- [13]. Kurokawa K, Itoh T, Kuwahara T, Oshima K, Toh H, Toyoda A, et al.. Comparative metagenomics revealed commonly enriched gene sets in human gut microbiomes. *DNA Res.* 2007;14(4): 169-81.
- [14]. Chichlowski M, Hale LP. Bacterial-mucosal interaction in inflammatory bowel disease: an alliance gone bad. *Am J Physiol Gastrointest Liver Physiol.* 2008;295(6): G1139-149.
- [15]. Cuevas-Ramos G, Petit CR, Marcq I, Boury M, Oswald E, Nougayrède JP. *Escherichia coli* induces DNA damage in vivo and triggers genomic instability in mammalian cells. *Proc Natl Acad Sci U S A.*2010;107(25):11537-1542
- [16]. Johnson JR, Johnston B, Kuskowski MA, Nougayrede JP, Oswald E. Molecular epidemiology and phylogenetic distribution of the *Escherichia coli* pks genomic island. *J Clin Microbiol.* 2008;46(12): 3906-911.