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Original Research Article

Molecular Investigation of the *Escherichia coli* (*E. coli*) Containing *pks* Region in the Biopsies of Patients with Inflammatory Bowel Diseases

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Abstract

Inflammatory bowel disease (IBD) includes chronic intestinal diseases; two of which are Crohn's disease/and Ulcerative Colitis. *Escherichia coli* (*E.coli*) is among digestive system microbial flora which is composed of the five main phylogenic group including phylogenic groups A, B1, B2, C, and E. Certain group of B2 *E.coli* strains possess a conserved genetic region known as *pks*. This region encodes a genotoxin known as Colibactin. Short term exposure of mammalian epithelial cells to the *E.coli* harboring/*pks* results in the induction of DNA damage. The aim of this study is molecular investigation of the *E.coli* Containing *pks* Region in the Biopsies of Patients with IBD. In this study we isolated *E.coli* pks region from 77 biopsies of the patients with IBD as well as 60 biopsies from the healthy donors. The Duplex PCR for *clbB* and *clbN* genes in *PKS* region was done on DNA extracted of bacteria. The PCR products with 16srRNA primer for all of *E.coli* with *pks* positive were sent for sequencing and phylogetenic tree was drawn. The results showed respectively, 49% and 2% of *E.coli* bacteria isolated from biopsy specimens of the patients with IBD and healthy donors contain *pks* gene. In terms of weight loss a significant differences was observed between the normal control group and patients with IBD (*p*-value= 0.001) as well as patients with UC (*p*-value=0.002). According to the results of this study, it seems most *E.coli* strains isolated to bear much related according to phylogenetic characteristics and their genetic similarity that makes them to compose a family.

Key words: Chron's disease, *E. coli.*, Inflammatory Bowel Disease, *pks*, Ulcerative Colitis

INTRODUCTION

Inflammatory bowel diseases include Ulcerative Colitis and Chron's disease (Baumgart and Carding, 2007). The former disease involves inflammation of the internal wall of large intestine while the later occurs in both outer and inner walls of small and large intestines (Baumgart, 2007). Inflammatory diseases of intestinal tract are more apparent in young individuals of eighteen to twenty nine years of age, nevertheless, disease in the individuals within 2 years of age or elders in their seventh or eighth decades of live have also been reported (Parkin, 2004).

While detailed etiology of the intestinal inflammatory diseases is not well known, several hypothesis have been proposed in this regard which may include: inheritance, altered immune system, stress, and infection (Matricon et al., 2010; Sun et al., 2011). As well, a perturbed immune system of patient is among other features of the disease (Jostins et al., 2012). Perturbed

immune system's function is a common feature of the diseases including IBD, however, the true mechanism of this is not well understood. Many studies have been conducted so far in order to unravel factors involved in the disease etiology, including studies on role of stress and infection (Xavier and Podolsky, 2007). While stress is an important factor in the etiology of inflammatory diseases, however, evidences indicate that stress is not the only cause of the disease, nevertheless, as in case of other diseases stress enhances the signs of the disease and potentiates the requirement**s** for treatment. Ulcerative Colitis is seen in 15 to 40 years old individuals and occurs only in the internal wall of large intestine in addition to rectal region (Dominguez-Bello et al., 2011). The ulcer results in the reduced water absorption which leads to diarrhea. Following disease treatment, relapse is frequent as well (Rickert et al., 1979).

Crohn's disease is a chronic condition which could relapse following earlier recovery (Cummings et al., 2008; Walker et al., 2011). The two causes of the disease are inflammation and ulcer that occur in deeper part of intestine (Jostins et al., 2012). The two most common areas of affection are the lower part of the small intestine (i.e. Il**e**um) and the upper part of the large intestine. Crohn's disease can also affect any part of the upper gastrointestinal tract. Aphthous ulcers in the mouth are common feature**s** of the disease. Ulcers may also develop in the esophagus, stomach and upper small intestine (the duodenum) (Hacker et al., 1997).

Escherichia coli (*E. coli*) is a natural resident of the large intestine in animals and human. The bacteria can also play**a** role as an opportunist in the infections which result in diarrhea as well as contributing to other extra-intestinal infections (Gordon and Cowling, 2003; Yan and Polk, 2010).

Most species of *E. coli* could be classified in the five main phylogenic groups: A, B1, B2, D, and E (Escobar-Páramo et al., 2004). These phylogenic groups are different with respect to their phenotypic and genotypic characteristics (Escobar-Páramo et al., 2006).

The extra pathogenic *E. coli* (ExPEC) is able to cause extra intestinal infection (Comito et al., 2014). The pathogenic factors involved in the infection are distributed along the genome (Hacker et al., 1997). For example certain *E. coli* in phylogenic group of B2 carry a genetic region involved in disease called *pks* which encode a polyketide genotoxin in human intestine called Colibactin (Johnson et al., 2002; Homburg et al., 2007). The size of the toxin in *Escherichia coli* strain *Nissele* 1917, has been estimated 55 kb that is located in a region called *GEI* V and in a locus named as *asn* W (Olier et al., 2012; Grabig et al., 2006; Ukena et al., 2007; Rembacken et al., 1999).

This secondary metabolite is a hybrid composed of polyketide (*pks*) and non-ribosomal peptide (NRPS) encoded by *pks* (Putze et al., 2009). For production of bactin, the *pks*, NRPS and 8 other genes are essential. The secreted colibactin induces DNA damage and cell cycle arrest. Irreversible damage to the DNA molecule results in cell death. In addition, colibactin and other virulent factors such as alpha hemolytic *E. coli* strains containing *pks* are also involved in tumor progression (Guerra et al., 2011).

Eukaryotic cells that have been infected with *pks* positive strains of *E. coli*, develop breakages in the DNA double strands. Activation of DNA damage, results in cascade of DNA damage pathway composing ATM-CHK-CDC25-CDK1 and serine139 phosphorylation in histone H2AX which is an indicator of double-stranded DNA damage. Infection with large number of toxin-producing bacteria causes irreversible cell cycle arrest and activation of apoptosis as well as cell death (Cuevas-Ramos et al., 2010).

MATERIALS AND METHODS

Sample collection

Seventy seven biopsy samples from the large intestine of patients with the inflammatory bowel disease and 60 biopsy samples from normal individuals were obtained in sterile condition. Among these samples 26 samples were from patients with Crohn's disease and 51 from ulcerative colitis. Donors of samples were informed of the purpose of the study, their consent obtained, and they participated in filling questionnaires

Bacteriological examination

Samples were kept in phosphate buffered saline (PBS) and subsequently in LB nutrition medium at 37 ˚C incubator. Following to growth and determination of bacterial count in the medium dilutions were prepared and 10^{-5} as well as 10^{-8} of bacterium per ml was added to 1 ml of EMB differential culture medium. Plates of bacterial culture were kept for 24 h in the incubator, stained, and *E. coli* was differentially selected using IMVIC, TSI, and Urea test. Selected bacteria were kept in BHI broth along with 70% glycerol and kept at -20 ˚C.

Molecular examination

Bacterial genome was extracted at logarithmic growth phase using genome extraction kit (Dr. Shayan, MBSA, Iran). Using Duplex PCR, target genes: c1bB and c1bN were amplified applying primers shown in table 1.

Amplification was done in 20 µl according to condition summarized in table 2. Following to preparation of master mix (DreamTaq, Fermentase, Ca.) and distribution in 0.2 ml vials, genomic DNA was added to each tube and PCR amplification was done according to condition in table 2.

Table 1. Sequence of primers sets (5' to 3') used in PCR amplification for c1bB, C1bN, as well as 16s rRNA.

Primer name	Sequences
Primer ClbB F	GAT TTG GAT ACT GGC GAT AAC CG
Primer ClbB R	CCA TTT CCC GTT TGA GCA CAC
Primer ClbN F	GTT TTG CTC GCC AGA TAG TCA TTC
Primer ClbN R	CAG TTC GGG TAT GTG TGG AAG G
27F	AGA GTT TGA TCC TGG CTC AG
1492R	CGG TTA CCT TGT TAC GAC TT

Table 2. The demographic information, in addition to other data, and factors involved in the progress or formation of inflammatory bowel diseases (IBD) in addition to ulcerative colitis (UC) in patients and normal controls. *p*-value < 0.05 was considered as significant. Those cases that are shown with star signify statistical difference.

Factors	Normal Subjects n=60(%)	IBD Patients n=26(%)	UC Patients n=51(%)	*p*-value	
				For IBD	For UC
Mean (Years± SD*)	47.24±15.8	50.2±18.1	51.5±17		
Age (years)					
>50	25(50%)	12(48)	20(39.2)	1	0.37
<50	25(50%)	13(52)	31(60.8)		
Mean Weight(kg± SD)	67±19.6	70.6±12.2	71.9±21.5		
Mean Losing weight(Kg± SD)	4.6±1.2	9.3±2.3	6.6±1.5		
Losing weight (Kg± SD)					
Yes	13(23.6)	16(61.5)	22(55)	0.001*	
No	42(76.4)	10(38.5)	18(45)		0.002*
Gender					
Male	28(46.6)	13(50)	26(51)	0.8	
Female	32(53.4)	13(50)	25(49)		0.7
Ethnicity			15(38.5)		
Persian	40(85.1)	10 (38.5)	9(23)	0.02*	
Azari	5(10.6)	6(23)	12(30.8)		0.000*
Gilaki and Kord	0	6(23)	3(7.7)		
Others	2(4.3)	4(15.5)			
Material status			6(14.6)		
Single	3(5.2)	3(11.5)	35(85.4)	0.3	
Married	55(94.8)	23(88.5)			0.15
Smoking			6(14.3)		
Yes	5(9)	5(19)	36(85.7)	0.27	
No	50(91)	21(81)			0.5
using meat per week					
Yes					
No	39(70.9)	18(85.7)	29(70.7)	0.1	
	16(29.1)	3(14.3)	12(29.3)		1
using vegetable/per week					
Yes				0.03	
No	54(96.4)	15(78.9)	35(72.9)		0.0007*
using multi vitamin	2(3.6)	4(21.05)	13(27.1)		
Yes					
NO			6(14.6)	0.06	
using Aspirin	15(55.5)	4(23.5)	35(85.4)		0.0005*
YES	12(44.5)	13(76.5)			
NO			5(12.2)		
family history of cancer	7(28)	4(15.3)	36(87.8)	0.32	
YES	18(72)	22(84.6)			0.18
NO				0.02*	
		7(30.4)	19(46)		0.0002*
E. coli isolated	2(6.4)	16(69.6)	22(54)		
Pks gene	29(93.6)				
Positive				0.0006*	
Negative					0.005*
	47/60(78.3%)	9/26(34.6%)	24/51(47%)		
	2(4)	5(55.5)	7/24(29)		
	45(96)	4(44.5)	17/24(71)		

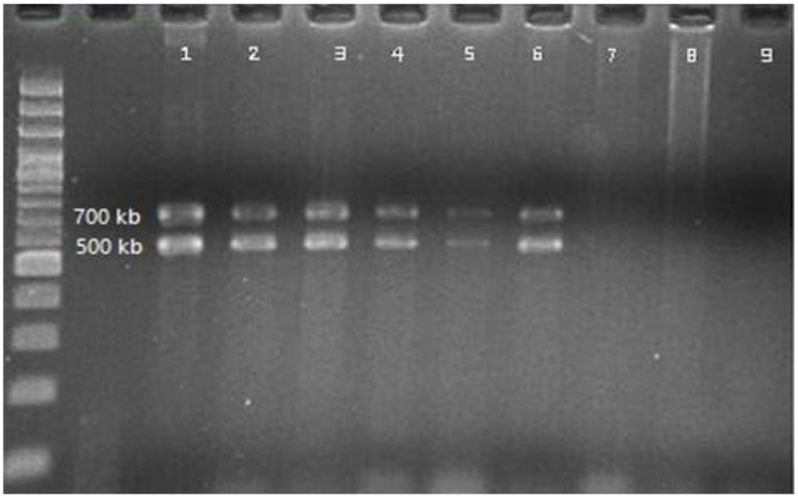

Figure 1. Duplex PCR amplification of the isolated *pks* region of *E. coli* DNA and running on 2% agarose. From the left: wells 1 to 3 compose ladder, negative control, and positive control. Wells 4 to 6 include amplification product of *E. coli* DNA samples obtained from patients containing *pks* region. Wells 7 to 9 include amplification of bacterial DNA of the patients whose genome was negative for *pks* region.

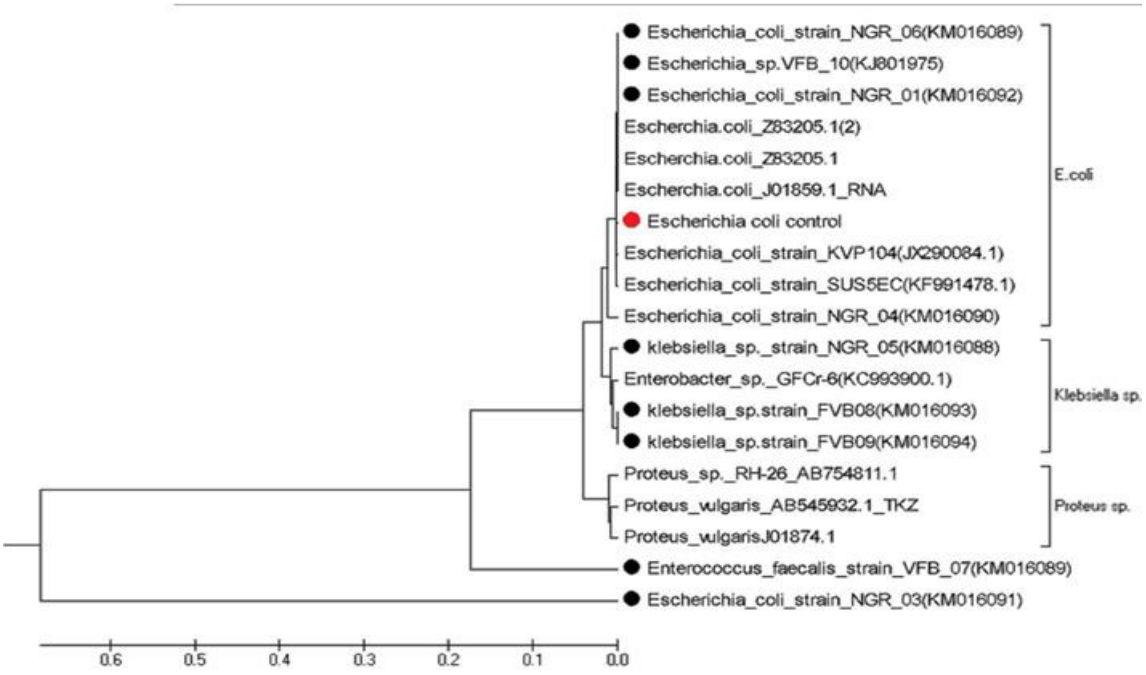

Figure 2. Plotted phylogenic tree, and placement of the isolated *E. coli* in the tree. Bacterium shown with the red color indicates the positive control bacterium that was used in the present report.

PCR products were run in 2% agarose a sample of which is depicted in figure 1. Positive control was *E. coli* gifted to us by Prof. Jean-Philippe Nougayre from Toulouse university.

Samples containing *pks* region were sequenced. Furthermore, in order to identify *E. coli* strain, amplification of 16s ribosomal RNA was carried out. The primer sets for 16s RNA are shown in table 1. The sequence of bacteria was obtained and deposited in NCBI: http://www.ncbi.nlm.nih.gov. As well, the phylogenic tree of the bacteria was plotted and location of the identified bacteria was assigned in the phylogenic tree (figure 2).

RESULTS

Bacteria which contain *pks* genomic region are capable of producing a genotoxin called colibactin which causes genomic instability and double strand DNA breaks. Such factors can stimulate progress of the inflammatory bowel disease toward colon cancer. Reports have shown that bacteria whose genome is devoid of *pks* region are not able to produce the toxin (Putze et al., Provide year). Using Duplex PCR we found that 49% of *E. coli* bacteria isolated from biopsy specimens of the patients with IBD contain *pks* gene and 2% of *E. coli* bacteria isolated from healthy individuals carry *pks* gene.

Results obtained in this study in addition to demographic information as well as important factors involved in the progress of inflammatory bowel disease and ulcerative colitis and normal individuals were evaluated using different statistical tests including: Fisher exact test, t-test, and Z-test. Results were evaluated and considered significant if *p*-value were < 0.05. Also, in cerain statistical calculations there might have not been total factor of 60 for normal participants and 77 for patient respectively.

In terms of age, patients were classified in two groups: above 50 years of age and below 50 years for which *p*-value= 1 was obtained. This *p*-value means that inflammatory bowel disease (IBD) is common among people older than 50 years that, is in accordance with the worldwide published reports. In terms of weight loss a significant differences was observed between the normal control group and patients with IBD (*p*-value= 0.001) as well as patients with UC (*p*-value= 0.002).

With respect to history of the disease, we found a significant association as well. In fact 30.4% of the patients had at least one individual with the IBD (7 out of 23 patients) in their first cousin and second cousins. In ulcerative colitis patients, 22 patients (54%) were negative with regard to any correlation with the family history, but, 19 patients (46%) were positive with regard to cancer in their first, second, or third cousins. The type of observed cancers in family history were either related to gastrointestinal tract, as could be expected with respect to IBD such as: esophagus, stomach, intestine, colon, rectum or unrelated and less related to the IBD such as hematopoietic, mammary, lung, and laryngeal cancer.

Analysis of samples has shown that 78.3% of the normal individuals (47 out of 60) were positive for bacterial (*E. coli*) contamination. For Crohn's individuals this amount was 34.6% (9 out of 26) and in ulcerative colitis 47% (24 patents among 51 patients) were positive for *E. coli*.

We found a significant correlation between consumption of vegetables in the week (p <0.0007) as well as consumption of multi-vitamins (p< 0.0005) in UC patients, but, no correlation was seen in case of IBD patients (Table 2). Bacteria containing *pks* region were sequenced and their sequence were deposited in http://www.ncbi.nlm.nih.gov. As well, their phylogenic tree was plotted (figure 2).

DISCUSSION

Applying Duplex PCR, in present study we were able to isolate 49% of *E. coli* positive for *pks* from IBD patients and 2% from normal individuals. Among these bacteria 28% have shown both 500 kb (c1Bb) and 700 kb (c1bN) bands and 17.2% of bacteria were containing a single 700 kb band.

Our results show that among various ethnic groups whose samples underwent the present evaluation, Fars is more susceptible than the other two ethnic groups (Azery and Gilac) to both Crohn's disease and ulcerative colitis. Compared to Crohn's disease for which the growth of bacteria was not seen to have a significant correlation with the consumption of vegetable and multivitamins, there was a positive correlation between consumption of vegetables and multivitamin with UC. Thus consumption of these two dietary factors has positive impact on UC prevention. As mentioned earlier, for both types of diseases, a history of cancer in their family has positive impact on susceptibility to the diseases. Regarding cigarette smoking, we didn't find a direct correlation between cigarette smoking and affliction with the diseases.

Plotting the phylogenic tree, among the three main groups of bacteria including: *E. coli*, *Proteus*, and *Klebsiella*, the bacteria isolated and studied in the present report: NGR01, NGR04, and NGR06 belong to the *E. coli* main group. Also the positive control bacteria belong to this group. Our isolated bacteria were much similar to positive control as results of sequencing suggests this notion. Among these bacteria, the *E. coli* strain NGR03 is far phylogenetically related to *E. coli* which indicates a far genetic relationship with the other bacteria in *E. coli* main group (Putze et al., 2009). have worked on genetic structure and distribution of colibactin among members of Enterobacteriacea. They found that this region is present in other Enterobacteriacea such as *Entrobacter aerogenes*, *Klebsiella pneumonia*, and *Citrobacter koseri*, in addition to *E. coli* (Putze et al., 2009).

E. coli strains in B2 group are more involved in the extra intestinal infections such as urinary tract. This group contains different adhesive polypeptide factors which are involved in pathogenesis of urinary tract…….**,** through adhesion to this tract and possibly in the large intestine cancer (Rhodes, 1996).

Our results indicate that most *E. coli* strains isolated in our study belong to B2 family. These bacteria seem to be much related according to phylogenetic characteristics and their genetic similarity that make them to compose a family. Among these group of bacteria, the *E. coli* strain

NGR03 is more distant to other members, thus, it could be assumed to belong to another group of bacteria.

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