

Full Length Research Paper

Fecal microbial flora in colorectal cancer

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The human bowel contains a large and dynamic bacterial population (more than 500 species of bacteria). Some intestinal bacteria such as *Streptococcus bovis*, *Enterococcus* spp., *Bacteroides fragilis* and *Clostridium perfringens* have previously been suggested to be implicated in the promotion of colon carcinogenesis probably through the conversion of mutagen metabolites. In this study 30 patients with colorectal cancer confirmed by colonoscopy examination and pathological methods were evaluated. In control group, 30 healthy people matched by sex and age with the patient group were identified. Fecal samples were collected from patient and control groups and were cultured in specific and non-specific culture media (aerobic and anaerobic conditions) organisms were isolated by microbial and biochemical methods. *S. bovis* was the predominant fecal microbial flora in 9 of the patients (39.1%) and 6 of the control group (26.1%) P=0.657. *Enterococcus* spp. was the predominant fecal microbial flora in 6 of the patients (26.1%) and 2 of the control group (8.7%) P=0.657. *Bacteroides fragilis* was the predominant fecal microbial flora in 8 of the patients (21.6%) and 2 of the control group (5.4%) P= 0.062. *C. perfringens* was the predominant fecal microbial flora in 11 of the patients (29.7%) and 16 of the control group (43.2%) P=0.062. Based on our sample size in this study, the results, when analyzed using Fisher statistical analysis, showed that the incidences of fecal *S. bovis*, *Enterococcus*, *B. fragilis* and *C.perfringens* in colorectal cancer were not significantly higher than healthy people.

Key words: Colorectal cancer, fecal microbial flora, *Streptococcus bovis*, *Enterococcus* spp., *Bacteroides fragilis*, *Clostridium perfringens*.

INTRODUCTION

The human bowel contains a large and dynamic bacterial population that is not only essential for intestinal health, but also critical for the development of diseases such as cancer (Tjalsma et al., 2006). *Bacteroides fragilis* and *Clostridium perfringens* are obligate anaerobes which are found in the colon microbial flora. Possible roles of *B. fragilis* enterotoxin in the etiology of colorectal cancer were investigated in stool specimens from 73 patients with colorectal cancer and from 59 control patients. The enterotoxin gene (bft) was detected by PCR in 38% of the *B. fragilis* isolated from colorectal cancer patients, compared with 12% of isolates from control group (p=0.009) (Potter et al., 1998; Topark et al., 2006). *C.*

perfringens has been regarded as one of the intestinal bacteria increasing colon cancer risk by producing α -toxin and enterotoxin, resulting in gas gangrene and sitotoxim. Furthermore, they have been shown to contain γ -dehydroxylase, the enzyme converting bile acids to tumor-promoting secondary bile acids and also shown to have β -glucuronidase which reactivates detoxified glucuronized mutagens and carcinogens (Arimochi et al., 2006).

Streptococcus bovis has been associated with colon cancer for many years. Moreover, infectious endocarditis from *S. bovis* associated with colonic carcinoma has been reported (Gold et al., 2004; Waisberg et al., 2002; Wentling et al., 2006).

Enterococcus is a group of commensally micro-organisms of the human intestinal tract that produces substantial extracellular superoxide, and derivative reactive oxygen species such as H₂O₂ and hydroxyl

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radical, through autoxidation of membrane-associated demethylmenaquinone. These oxidants may be important as a cause of chromosomal instability (CIN) associated with sporadic adenomatous polyps and colorectal cancer (Rafter et al., 2007; Huycke et al., 2002).

The aim of the present study was carried out in order to isolate *B. fragilis*, *C. perfringens*, *S. bovis* and *Enterococcus* spp. from the fecal microbial flora of patients with colorectal cancer and control subjects. *B. fragilis* and *C. perfringens* are obligated anaerobic bacteria while *S. bovis* and *Enterococcus* spp. are facultative aerobic bacteria.

MATERIALS AND METHODS

Patients who referred to cancer institute for confirmatory diagnosis of colorectal cancer were selected for this study. Further investigations such as colonoscopy; biopsy and pathologic methods were performed in order to confirm the colorectal cancer in patients. Healthy individuals of the same age and sex were selected as members of the patients group.

In this study, 30 patients with confirmed diagnosis of colorectal cancer from different region of Iran as indicated above were selected. They did not take any treatment such as surgery, radiotherapy or antibiotic treatments before. A general patient information questionnaire was completed and a stool sample was collected from both patient and control group subjects. Stool sampling in patients group was done before any antibiotics treatments. In first stage, stool samples cultured on two non specific media, brain heart infusion agar for anaerobic condition (isolation *B. fragilis* and *C. perfringens*) and blood agar for aerobic condition (isolation *S. bovis* and *Enterococcus* spp.). The predominant colonies in each of the plates were identified. Anaerobic condition, were achieved through the use of commercial gas packs and for preparing culture media for isolating *B. fragilis* and *C. perfringens*, Gentamycin (100 µg/ml) was added to brain heart Infusion agar to inhibit growth of other anaerobic bacteria.

For diagnosis of *B. fragilis* the following procedures were performed in 2 steps:

1. Second passage was performed from the colony in Brain Heart Infusion agar (BHI) agar and 2 antibiotic disks of Vancomycin (5 µg) and Kanamycin (1mg) were used over the culture under anaerobic condition. *B. fragilis* is resistant to both of antibiotics.
2. Another passage was performed from *B. fragilis* colonies isolated in first step on bile esculin agar under anaerobic condition. As such, *B. fragilis* can hydrolyse bile esculin.

C. perfringens produces double zone around colonies on egg yolk agar. For preparation of blood agar media containing egg yolk, the following procedures were performed:

After making base blood agar media and autoclaving process, for each 250 ml of the media, 2 eggs were used. When the culture temperature prepared was 45, the yolks of eggs were added to the medium, mixed well and plated (Onderdonk and Hannele, 1999).

For confirmation of diagnosis of *C. perfringens*, Vancomycin and Kanamycin disks were used on cultures in BHI media. After 48 h in anaerobic condition, the inhibitory zone around the disks was evaluated. *C. perfringens* is sensitive to Kanamycin but resistant to Vancomycin.

Colonies on blood agar cultured were investigated after overnight incubation on aerobic condition. For diagnosis of *S. bovis* and *Enterococcus* spp., bile esculin agar media and 6.5% salt broth media were used. Catalase negative colonies and Gram-positive

cocci were cultured in bile esculin agar and also some colonies were suspended in 6.5% salt broth media. After 24 h of incubation, *S. bovis* and *Enterococcus* spp. were examined for hydrolysis of bile esculin and growth in 6.5% salt broth media (Ruoff, 1999; Verneuil et al., 2005). *S. bovis* and *Enterococcus* spp. hydrolysis bile esculin. *Enterococcus* spp. grows in 6.5% salt broth media but *S. bovis* do not grow in 6.5% salt broth media.

RESULTS

In the present study 30 patients (23 male and 7 female) and 30 controls (20 male and 10 female) were evaluated. There was no significant difference about sex between patients and control group ($p < 0.05$).

Age range was 18 to 79 years in patient group and 16 to 78 years in control group. Average age in patient group was 55.97 years (SD = 17.14) and in control group was 50.73 years (SD = 16.71) there was no significant difference about age between patients and control group. ($p < 0.05$). Tables 1 and 2 show the fecal aerobic and anaerobic bacteria isolated from patient and control groups.

B. fragilis was the dominant bacteria isolated from 8 patients and 2 of control group. *C. perfringens* was the dominant bacteria isolated from 11 patients and 16 control group. Although *C. perfringens* and *B. fragilis* were more isolated in colorectal cancer patients versus healthy individuals, statistical analysis by Fisher test showed that there was no significant difference between patients and control groups ($P = 0.062$).

S. bovis was the dominant bacteria isolated from 9 stool samples of patients and 6 samples of control group. *Enterococcus* spp. was the dominant bacteria isolated from 6 patients and 2 control group. Statistical analysis by Fisher test did not show any significant difference between patients and control groups ($P = 0.657$). *B. fragilis* and *Enterococcus* spp. was isolated from 3.3% of patient group and 3.3% of control group. *B. fragilis* and *S. bovis* was isolated from 3.3% of patient group but it was not found in control group.

C. perfringens and *Enterococcus* spp. was isolated from 3.3% of patient group but it was not found in control group. *C. perfringens* and *S. bovis* was isolated from 6.6% of patient group and 10% of control group.

Both of *B. fragilis* and *C. perfringens* (anaerobic bacteria) were not isolated from patient and control groups. Both of *S. bovis* and *Enterococcus* spp. (aerobic bacteria) were not isolated from patient and control groups.

DISCUSSION

Colorectal cancer is among one of the common types of cancer in people age 50 and older in the United States and is considered the second cause of death in cancer patients. Based on the data of the Cancer Institute of Tehran University, the prevalence of colorectal cancer in

Table 1. Comparison of fecal *C. perfringens* and *B. fragilis* isolated from patients and control groups.

Anaerobic bacteria Groups	Culture positive for <i>C. perfringens</i>		Culture positive for <i>B. fragillis</i>		Total culture positive for <i>C. perfringens</i> and <i>B. fragillis</i>	
	Number	Percentage	Number	percentage	Number	percentage
Patients	1	29.7	8	21.6	19	51.4
Controls	16	43.2	2	5.4	18	48.6

Table 2. Comparison of fecal *S. bovis* and *Enterococcus* spp. isolated from patients and control groups.

Anaerobic bacteria Groups	Culture positive for <i>S. bovis</i>		Culture positive for <i>Enterococcus</i> spp.		Total culture positive for <i>S. bovis</i> and <i>Enterococcus</i> spp.	
	Number	Percentage	Number	percentage	Number	percentage
Patients	9	39.1	6	26.1	15	65.2
Controls	6	26.1	2	8.7	8	34.8

2003 was about 6,000 cases. 3,700 cases were discovered in year 2003 and 2,300 cases were patients with a history of hospitalization. Ninety patients had confirmed colorectal cancer and they were under chemotherapy or surgery.

Several studies have been performed to investigate the association between colorectal cancer and certain bacteria in stool (Wentling et al., 2006). Bacteria that have been isolated among colorectal cancer patients include: *S. bovis*, *Enterococcus*, *B. fragillis* and *C. perfringens* (Potter et al., 1998; Topark et al., 2006). Toprak et al. (2006) investigated about the relationship between *B. fragillis* enterotoxin and colorectal cancer. In their study, stool specimens were collected from 73 patients with colorectal cancer and also from 59 control group. The enterotoxin gene of *B. fragillis* was detected in 38% of the isolates from colorectal cancer patients, compared with 12% of the isolates from the control group ($P = 0.009$).

Various theories about the cause of some carcinogenic bacteria in colorectal cancer has provided. Based on Klein's research, *Streptococcus bovis* were isolated from fecal cultures of 11 of 105 controls and 35 of 63 patients with carcinoma of colon ($P < 0.01$). (Klein et al., 1977) Production of carcinogenic metabolites from these bacteria in some stages of their growth can cause cancer or can facilitate the growth and dissemination of cancer (Sangelo and Kurzrock, 2007; Rafter et al., 2007; Winters et al., 1998).

Different hypothesis about the high prevalence of these bacteria in gastrointestinal tract of colorectal patients have been presented. Production of carcinogenic metabolites from those bacteria in some stages of their growth can cause cancer or can facilitate the growth and dissemination of cancer (Sangelo and Kurzrock, 2007; Rafter et al., 2007; Winters et al., 1998). *C. perfringens* and *B. fragillis* are both anaerobic and, with their effect on fats

and bile, they can increase the production of fatty acids and secondary bile acids in colon. These chemicals damage the colon mucosa and cause uncontrolled proliferation, which can lead to cancer in colon and rectal area (Arimochi et al., 2006).

In this study, 30 patients with confirmed diagnosis of colorectal cancer survived. In addition, 30 healthy individuals as control group were selected. Some factors that might have had an impact on the results of this study include their diets and habits, and environmental exposure. For reducing these Interfering factors, patients and control groups included in this study were selected from different region of Iran who has the same nutritional habits. For examples, they had eaten 2 or 3 meat meals in a week and about patients, they did not take any treatment before been included in this study. Then stool samples were cultured as described in material and methods. *B. fragillis* was the dominant bacteria isolated from 8 patients and 2 of the control group. *C. perfringens* was the dominant bacteria isolated from 11 patients and 16 of the control group. Although *C. perfringens* and *B. fragillis* were dominant in colorectal cancer patients versus healthy individuals, statistical analysis by Fisher test were not significant ($P = 0.062$).

S. bovis was the dominant bacteria isolated from 9 stool samples of patients and 6 samples of the control group. *Enterococcus* spp. was the dominant bacteria isolated from 6 patients and 2 control group. Statistical analysis by Fisher test did not show any significant difference between the patients and the control groups ($P = 0.657$).

Results obtained were based on colorectal cancer patients in Iran. All patients and controls studied had normal diet and they were on average twice a week meat meal. Thus, these results may arise from other countries where meat consumption may be higher and a little bit different.

Conclusion

Clostridium perfringenes, *Bacteroides fragilis*, *Streptococcus bovis* and *Enterococcus* spp. isolated in patients fecal samples more than control group (specialy *Clostridium perfringenes*), but based on statistical analysis, there were no significant difference between patients and control groups, Although carcinogen metabolites produced by each of these bacteria were proved and isolation of these bacteria from cases of colorectal cancer were higher than normal individuals but based on statistical results obtained from patient and control groups in the present study, there were not significant differences between isolation of these bacteria in patients and controls which may require further studies.

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