Real-time polymerase chain reaction quantification of specific butyrate-producing bacteria, \textit{Desulfovibrio} and \textit{Enterococcus faecalis} in the feces of patients with colorectal cancer

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Abstract

Background and Aim: Bacterial metabolites produced in the bowel are potentially related to the genesis of colorectal cancer. Butyrate is protective against cancer, whereas hydrogen sulfide and oxygen free radicals can be toxic to the epithelium. The present study was designed to quantitate \textit{Eubacterium rectale}, \textit{Faecalibacterium prausnitzii} (both butyrate-producing bacteria), \textit{Desulfovibrio} (sulfate-reducing bacteria), and \textit{Enterococcus faecalis} (that produces extracellular superoxide) in the feces of patients with colorectal cancer.

Methods: DNA was extracted from feces of 20 patients with colorectal cancer, nine patients with upper gastrointestinal cancer and 17 healthy volunteers. Real-time polymerase chain reaction using primers aimed at 16S rDNA was used to quantitate the above bacterial species or genus, and this was expressed relative to amplification of universal sequences conserved among all bacteria.

Results: Levels of \textit{E. rectale} and \textit{F. prausnitzii} were decreased approximately fourfold ($P = 0.0088$ and 0.0028, respectively) in colorectal cancer patients compared to healthy control volunteers. Levels of \textit{Desulfovibrio} were not significantly different between the three groups. \textit{E. faecalis} populations were significantly higher in colorectal cancer patients compared to healthy volunteers ($P = 0.0294$).

Conclusions: Butyrate producers were decreased and \textit{E. faecalis} increased in the feces of colon cancer patients. These shifts in the colonic bacterial population could potentially lead to epithelial cell damage and increased turnover and may be a factor leading to colon cancer.

Key words
butyrate-producing bacteria, colorectal cancer, \textit{Enterococcus faecalis}, India, quantitative polymerase chain reaction, sulfate-reducing bacteria.

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hydrogen sulfide (H₂S) is generated from sulfate during anaerobic respiration by SRB.¹²

Enterococci are the predominant Gram-positive cocci in human stools, and Enterococcus faecalis is the most common Enterococcus species found. These bacteria possess the unique ability among commensals to produce extracellular superoxide by non-enzymatic reduction of oxygen by membrane-associated demethylmenaquinone.¹³ The extracellular superoxide can cause DNA damage in mammalian cells, which may result in chromosomal instability and eventually lead to colorectal cancer.¹³,¹⁴

Molecular methods targeted at the 16S rDNA of bacteria allow the anaerobic microbial flora of the intestine and colon to be examined with greater ease than in earlier times.¹⁵ The present study was designed to quantitate E. rectale, E. faecalis, Desulfovibrio and E. faecalis in the feces of colorectal cancer patients and to compare them with the fecal flora in appropriate control subjects.

**Methods**

**Subjects**

Twenty consecutive patients with colorectal cancer presenting to the Department of Gastrointestinal Sciences, nine patients diagnosed during the same period to have upper gastrointestinal (esophageal or gastric) cancer, and 17 healthy volunteers from in and around Vellore town, were included in the study. The diagnosis of colorectal cancer was established by colonoscopy and biopsy. Participants with any concurrent medical illness and any who had used antibiotics within the past month were excluded. Informed consent was obtained from all participants. The study protocol was approved by the institutional review board. Patients and healthy volunteers provided fresh stool specimens, which were transported to the laboratory where they were stored in duplicate aliquots at −80°C until DNA extraction.

**DNA extraction**

Stool specimens were processed in batches. Fecal DNA was extracted from 200–250 mg (wet weight) feces using a QIAamp DNA stool extraction mini kit (QIAGEN, Hilden, Germany), with subsequent isolation of bacterial DNA according to the instructions of the manufacturer and the final eluate of 200 μL was stored at −20°C.

**Primers and polymerase chain reaction**

Oligonucleotide primers were targeted at the 16S rDNA sequences and designed to amplify a conserved sequence specific for two butyrate-producing bacteria (E. rectale, F. prausnitzii), the sulfate-reducing bacteria of the Desulfovibrio genus and E. faecalis (Table 1).¹⁶–¹⁸ Primers were also used to amplify a conserved sequence present in all bacteria (universal primer set),¹⁹ the amplification of which served as the denominator against which amplification of other bacterial nucleic acid was compared. To check for specificity, the selected primers were compared to all available 16S rDNA sequences in the Ribosomal Database Project-II²⁰ and the BLAST database search program (http://www.ncbi.nlm.nih.gov/BLAST). Primers were synthesized by Sigma Genosys (Bangalore, India). Gradient polymerase chain reaction (PCR) was carried out initially to standardize the PCR conditions. The final conditions were: initial denaturation at 95°C for 10 min followed by 44 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. PCR products were analyzed by agarose gel electrophoresis for specific band of the amplified product (Fig. 1). The specificity of primers was checked by performing PCR using target and a variety of non-target test bacteria, to add to the results from in-silico PCR and sequence searches mentioned above. The above standardized conditions were used to carry out real-time PCR. Quantification of bacterial DNA was performed on a Chromo4 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA) using SYBR Green Master Mix from Eurogentec (Liege, Belgium) (Fig. 2). All simplex PCR reactions were performed in duplicate in a volume of 20 μL, using high-profile tubes and ultra clear sealing caps (Bio-Rad). Melting curve analyses were carried out from 40 to 95°C with a plate read step after every 1°C, which was held at a particular temperature for 10 s, in order to check the specificity of the product formed (Fig. 2).

We used Opticon 3.1 software (Bio-Rad) to plot the rate of change of the relative fluorescence units (RFU) with reference to time (T) (d(RFU)/dT) on the Y-axis versus the temperature on the X-axis, with the curve peaking at the melting temperature (Tm), and melting curve analysis was always done to check the specificity of the amplification. Quantification was based on the fluorescence intensity obtained from the intercalated SYBR Green dye. The cycle number at which the signal was first detected, the threshold cycle (Ct), correlated with the original concentration of the DNA template. DNA copy was not expressed as absolute

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**Table 1** Primers used in the present study

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Sequence 5’ to 3’</th>
<th>Amplicon (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eubacterium rectale</td>
<td>5′-AAGGAAAGCAAGCTGTGAA-3′</td>
<td>200</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>5′-CGGTATGTCAGCTGCTC-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Faecalibacterium prausnitzii (Fp)</td>
<td>5′-GGGAGAATGACCCCTCATGT-3′</td>
<td>203</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>5′-CTGGTCCCGAAGAAACCAT-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desulfovibrio sp.</td>
<td>5′-CCGATATTCTGGAGGAACATCAG-3′</td>
<td>136</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>5′-ACATCTAGCATCCATTTACAGC-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>5′-CCCTATTGTATGCTGCACTCAT-3′</td>
<td>144</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>5′-ACTCGTTGATACCTCCCATT-3′</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Universal (Univ)</td>
<td>5′-TCTCAGGAGGACGACATT-3′</td>
<td>466</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>5′-GGACTACCCAGGTATACCTCGTT-3′</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 1  Agarose gel showing electrophoretic separation of polymerase chain reaction (PCR) products of the different bacterial rDNA targets that were amplified. Panel on the left shows amplification of the universal sequence (lane 1), *E. rectale* (lane 2), *F. prausnitzii* (lanes 3,4) and *Desulfovibrio* (lane 5). Panel on the right shows the universal primer amplicon (lanes 1,4) and *Enterococcus faecalis* (lanes 2,3). Lane M, DNA ladder.

Figure 2  Representative graphs of (top) relative quantification curve of universal primer set, butyrate-producing bacteria and *Desulfovibrio* and (bottom) melting curves of the same amplicons.
number, but was expressed by the relative cycle threshold at which DNA for each target was detected relative to the cycle threshold at which ‘universal bacterial’ DNA was detected after amplification. This relative quantification was done automatically by the Opticon 3.1 software using the formula $2^{-\Delta Ct}$ and expressed as relative fold difference compared to the reference (universal) amplicon. The relative difference of the target bacterial population is thus the ratio of the target bacterial rDNA compared to universal rDNA, providing a quantitative comparison between different samples. The ratio calculated for each of the duplicate samples always correlated well with the other, with an adjusted $R^2$ in the region of 0.80.

**Statistics**

All values for bacteria are presented as mean (SEM). Significance of differences between groups was assessed by the Mann–Whitney U-test and a two-tailed $P$-value $< 0.05$ was considered to be significant.

**Results**

Patients with colorectal cancer (15 male, five female) ranged in age from 18 to 77 years (median 51.5 years). The tumors were located in the rectum (seven), cecum or ascending colon (six), transverse colon (four), sigmoid (two) and one patient with synchronous tumors in the rectum and ascending colon. The tumors were moderately differentiated or poorly differentiated adenocarcinoma or signet ring carcinoma. Patients with upper gastrointestinal cancer (six male, three female) ranged in age from 40 to 67 years (median age 54 years). They included five patients with carcinoma of the esophagus, three with gastric carcinoma and one with periampullary carcinoma. Healthy volunteers (seven female, 10 male) ranged in age from 18 to 60 years (median 39 years).

$E. \text{rectale}$ was significantly lower in patients with colorectal cancer (0.0181 ± 0.0100) compared to healthy volunteers (0.0850 ± 0.0508) or patients with upper gastrointestinal cancer (0.0967 ± 0.0358) ($P = 0.0088$ and $P = 0.0117$, respectively) (Fig. 3). $F. \text{prausnitzii}$ was also significantly lower in patients with colorectal cancer (0.0013 ± 0.0006) compared to healthy volunteers (0.0052 ± 0.0018) ($P = 0.0028$). Patients with upper gastrointestinal cancer had intermediate levels (0.0024 ± 0.0009) of this bacterium that were not significantly different from either the colorectal cancer group or from healthy volunteers ($P = \text{NS}$). Levels of $\text{Desulfovibrio}$ were not significantly different between colorectal cancer patients (0.0242 ± 0.0213), healthy volunteers (0.0024 ± 0.0011) and patients with upper gastrointestinal cancer (0.0428 ± 0.0229). Levels of $E. \text{faecalis}$ were increased in patients with colorectal cancer (0.0500 ± 0.0426) compared to healthy volunteers (0.0106 ± 0.0033) ($P = 0.0294$). The levels were not significantly different between the healthy volunteers and patients with upper gastrointestinal cancer (0.0213 ± 0.0086) (Fig. 3).

**Discussion**

Commensal bacteria have been linked to the genesis of colorectal cancer as the colonic region coincides with the greatest luminal bacterial concentrations.21 Gut bacteria are capable of chemical transformation, metabolic activation of procarcinogens, disrupting the intracellular redox homeostasis and damaging epithelial cell DNA. The present study points to specific alterations in fecal bacterial populations in patients with colorectal cancer that may be of pathogenetic significance in these patients.

$F. \text{prausnitzii}$ and $E. \text{rectale}$ represent two major butyrate-producing bacteria in the colon.8,22 Short chain fatty acids are produced by fermentation of unabsorbed carbohydrate in the colon. Among the short chain fatty acids, acetate is quantitatively most predominant, with lesser amounts of propionate and butyrate.
being formed. Butyrate has the most noticeable effects on colonic epithelial physiology and function, and on colorectal carcinogenesis. Of specific interest to colon cancer is that butyrate influences gene expression through histone hyperacetylation and the ability to induce apoptosis in a variety of cancer cell lines. It may inhibit the genotoxic effect of nitrosamides and hydrogen peroxide in human colon cells. A combination of these effects is likely to be responsible for the protection afforded by butyrate against early tumorigenic events. There is no simple way to selectively isolate butyrate-producing bacteria and the majority of these bacteria are also highly oxygen sensitive. However, current developments in molecular analysis have overcome many of the restrictions and provided a means to identify and quantify particular bacterial species in complex microbial communities. The PCR primers used here specifically detected and quantified two important butyrate-producing bacteria. Reduction in these two species in patients with colorectal cancer, compared with healthy volunteers or with disease controls, could conceivably reduce luminal butyrate availability and predispose to cancer. It is likely that this is of pathogenetic significance. In mice, giving a butyrate-producing bacterium has been shown to decrease the formation of aberrant crypt foci, an early stage of carcinogenesis, in the colon and rectum.  

Sulfate-reducing bacteria of the genus *Desulfovibrio* are present in the feces of healthy persons. *Desulfovibrio* are the predominant sulfate-reducing bacteria in the human large intestine and are involved in a number of important processes in the bowel, including hydrogen disposal and other nutrient cross-feeding reactions. Sulfate-reducing bacteria in the large bowel reduce sulfate to hydrogen sulfide, which is toxic to colonic epithelial cells, interfering with butyrate oxidation in these cells. Hydrogen sulfide, when incubated with colonic biopsies, significantly increases the proliferation of cells in the upper crypt region. Sulfate-reducing bacteria bear an inverse relationship with the presence of methanogens in the large bowel. Predominant methane excreters (with presumably low levels of sulfate-reducing bacteria) were significantly less common in populations (European and North American) with a high incidence of colorectal cancer compared to a population (rural black Africans) with a low incidence of colorectal cancer, providing indirect evidence for an association of sulfate-reducing bacteria with colorectal cancer. The present study showed that *Desulfovibrio* were not significantly different between colorectal cancer patients and healthy volunteers. It is possible that *Desulfovibrio* may be relevant to the genesis of colitis-associated colorectal cancer than to sporadic colorectal cancer.  

*E. faecalis* was isolated from the feces of healthy individuals in concentrations of 10^5–10^7/g. It produces extracellular superoxide, hydrogen peroxide and hydroxyl radicals, all of which are oxygen free radicals which can directly, or through intermediates, damage DNA leading to single- and double-strand breaks. Oxidative damage produced by endogenous redox sources is a potentially important mechanism for somatic mutations that give rise to cancer. *E. faecalis* has been shown to produce severe distal colitis in interleukin-10 knockout mice, progressing to dysplasia and adenocarcinoma. The finding of an expanded population of this organism in the feces of colorectal cancer patients, compared to healthy volunteers or to disease controls, suggests the possibility of a causal association.  

Shifts in the large bowel luminal population of several beneficial and harmful bacteria in patients with colorectal cancer suggest that the luminal bacteria should remain an important consideration in the genesis and prevention of colorectal cancer.

**Acknowledgments**

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**References**


