Structural shifts of mucosa-associated Lactobacilli and *Clostridium* *leptum* subgroup in patients with ulcerative colitis

Running title: Mucosa-associated bacteria in ulcerative colitis

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Abstract

To understand the role of the mucosa-associated microbiota in pathogenicity of ulcerative colitis (UC), paired biopsies were obtained during colonoscopy from the ulcerated and non-ulcerated gut mucosa of 24 patients with UC. Denaturing gradient gel electrophoresis (DGGE) analysis was employed to profile the composition of the dominant bacteria (16S rRNA gene V3 region) and three important groups Lactobacilli, *Clostridium leptum* subgroup and *Bacteroides* spp.. Pearson coefficient was used to estimate the similarity of the bacterial community between the paired biopsies for each patient. The average similarity values of bacterial composition between the paired samples were $94.8 \pm 3.8\%$ for dominant bacteria, $59.9 \pm 26.1\%$ for Lactobacilli, $79.2 \pm 22.6\%$ for *Clostridium leptum* subgroup and $88.7 \pm 16.4\%$ for *Bacteroides* spp.. The data revealed that Lactobacilli and *Clostridium leptum* subgroup were significantly different between ulcerated and non-ulcerated regions. It also was noted that for Lactobacilli, the composition varied significantly between biopsy sites irrespective of the location of UC in the gut, but the composition of *Clostridium leptum* subgroup showed a significant difference between paired samples from UC in rectum and not in left colon. Localized dysbiosis of the mucosa-associated intestinal microflora, especially for Lactobacilli and *Clostridium leptum* subgroup may be closely related to UC.
Key Words: Ulcerative colitis; Lactobacilli; Clostridium leptum

Introduction

Ulcerative colitis (UC) is an acute and chronic inflammatory disease of the large bowel and is one of the two main forms of inflammatory bowel disease (IBD). The etiology of IBD is unknown, but animal models have shown that resident intestinal bacteria play an important role in the pathogenesis of this disease (27, 39).

Considerable effort has been expended on the study of the intestinal bacteria in patients with inflammatory bowel disease. It has been reported that alteration of the fecal microbiota present in IBD and the dominant bacteria are comprised of unusual bacterial species compared with the healthy subjects (36). Reduced diversity of the bacterial phylum Firmicutes in the faecal microbiota of Crohn's disease patients was observed (19) and furthermore, a decreased number of Lactobacilli, Clostridium leptum and Clostridium coccoides in the fecal microflora while an increased number of Enterobacteria in the patients with inflammatory bowel disease have been observed (3, 32, 36).

Since the mucosa-associated bacteria induce a local immune response (24) and the mucosa-associated bacterial composition is significantly different from that in the feces (43), several more recent studies have focused on the mucosa-associated bacteria in IBD patients. Overall, there
is a greater population of aerobic and facultative-anaerobic bacteria in IBD patients but a decreased number of normal anaerobic bacteria (5, 18, 22).

Since there typically is large person-to-person variation in the mucosa-associated bacteria populating the gut (43), it may be more relevant to compare the intestinal bacteria within the same individual (15). However, no significant difference in the amount of bacteria between inflamed and non-inflamed biopsies was observed in patients with IBD (38); in addition, the dominant microbiota did not differ qualitatively in Crohn’s disease patients (31).

The differences in the intestinal bacterial community adherent to the ulcerated and non-ulcerated mucosa in the same patient with ulcerative colitis have not been explored in detail. In this work, we employed denaturing gradient gel electrophoresis (DGGE) to profile the biodiversity of the dominant bacteria and three important bacteria groups in paired biopsies from patients with UC to search for patterns associated with progression of this important disease.

**Materials and methods**

**Volunteers and colonoscopy biopsies**

Twenty-four ulcerative colitis patients were involved in this study (Table 1). One patient had cecal UC, nine had left colonic UC, one had
recto-sigmoid UC and 13 had rectal UC. Diagnosis of UC was confirmed by histology at the time of colonoscopy and all patients were with active mild to moderate ulcerative colitis (26). Nine of the patients received therapy with mesalazine (5-aminosalicylic acid), three of them received corticoseroides and none of them received antibiotics within the previous four weeks. Colonic cleansing was performed with Polyethylene Glycol-Electrolyte Powder (WanHe Pharmaceutical Co., Ltd, Shenzhen, China). Two biopsies were taken from each patient after the colonic evacuation. One biopsy was collected from macroscopical ulcer or erosion mucosal sites, which was referred as ulcerated mucosa in this study and the other was from adjacent (about 5cm away) mucosa that appeared normal macroscopically, which was referred as non-ulcerated mucosa. In order to minimize contamination, different colonoscopy jaws were used to obtain the ulcerated and the non-ulcerated mucosa biopsies within a given individual. The volunteers underwent routine diagnostic colonoscopy and biopsies were included in this procedure, so this study did not add extra risk to the procedure. Informed consent was obtained from each patient.

**DNA extraction and PCR amplification**

Biopsy samples (0.5~1 mg) were suspended in 450 µl 0.05 M potassium phosphate buffer (pH 7.0) in a 2 ml Eppendorf tube. Samples were vortexed for 2 min at max speed using a Mo Bio vortex (model
G-560E). This suspension was incubated at 55°C for 1 hour with 10 μl Proteinase K solution (20 mg/ml) and 50 μl 10% SDS followed by tissue dissociation in 150 μl phenol (pH 7.5) using a mini bead beater (Biospec Products, Bartlesville, Okla.). This step was conducted three times and each time at the max speed for 1 min followed by placing on ice for 1 min.

After dissociation, 150 μl chloroform-isoamyl (vol/vol, 24:1) was added and centrifuged 15,000 × g for 10 minutes. The supernatant was subjected to extraction with an equal volume phenol, followed by phenol/chloroform-isoamyl (chloroform vol/isoamyl vol, 24:1; phenol vol/chloroform-isoamyl vol, 1:1) then chloroform-isoamyl (vol/vol, 24:1). DNA was precipitated with two volumes of ethanol and 1/10 volume of sodium acetate (3 M, pH 5.2) and collected by centrifugation (15,000 × g for 10 min), air-dried and dissolved in 100 μl sterile TE buffer. RNA was digested by adding 3 μl RNase (20 mg/ml) at 37°C for 20 min.

Since the biopsy sample was too small and the DNA extracted from the biopsy containing higher amount of eukaryotic DNA than bacterial DNA, nested-PCR was employed in this study to assure specificity in amplification of 16S rRNA genes. Primers 27F and 1492R (7, 14), which were designed based on the conserved bacterial regions at the 5' and 3' ends of the 16S rRNA gene, were used to amplify the near-full length gene. The 25 μL reaction mixture contained 2.5 μL of 10 × PCR buffer (Mg²⁺ free), 2 μL of
25 mM dNTP mixture, 0.625 U Ex Taq DNA polymerase (Takara, Dalian, China) and 25 pmol of each primer. Amplification was performed by initial denaturation at 94°C for 4 min followed by 20 cycles consisting of 94°C for 45 s, 55°C for 1 min, and 72°C for 1 min; and final extension at 72°C for 6 min. PCR amplification was performed with a thermocycler PCR system (PCR Sprint, Thermo Electron, Corp., UK). The amplification products of 16S rRNA gene were used as the templates in the next amplification.

Dominant bacteria were characterized by amplifying the V3 region of 16S rDNA and group-specific DGGE analysis was used for Lactobacilli, *Clostridium leptum* subgroup, and *Bacteroides* genus. For 16S rDNA V3 region amplification, the 25 µL reaction mixture contained 2.5 µL of 10 × PCR buffer (Mg²⁺ free), 2 µL of 25 mM dNTP mixture, 0.625 U of Ex Taq DNA polymerase, 12.5 pmol of each primer (20). The samples were amplified in a thermocycler PCR system by a touch down PCR protocol (20).

Lactobacilli were detected by specific primers Lac 1 and Lac 2GC that target 16S rDNA gene (13, 40) to amplify a 384 bp product; *Clostridium leptum* subgroup was amplified with group specific primers Clept-F and Clept-GC-R3 producing a 279 bp product (34). *Bacteroides* genus-specific primers Bfr-F and Bfr- GC- R (23), which target the 16S rDNA gene, were
used to amplify a 270 bp product. The PCR mixture (25 µl) contained 0.625 U of Ex Taq DNA polymerase, 10 × PCR buffer (Mg\(^{2+}\) free), 20 pmol of each primer, and 50 mmol each deoxynucleotide triphosphate (dNTP).

Amplification was carried out in a thermocycler PCR system. The amplifications were performed as previously described (23, 40). Five microliters of PCR products were checked by electrophoresis on 1% (wt/vol) agarose gels.

**DGGE profiling and data analysis**

Parallel DGGE was performed with a Dcode System apparatus (Bio-Rad) as described by the manufacturer. Amplification products were separated on 8% (wt/vol) polyacrylamide gels. Electrophoresis was performed in 1 × Tris-acetate-EDTA (TAE) buffer at a constant voltage of 200 V and a temperature of 60°C for 200 minutes. The DNA bands were stained with SYBR green I (Amresco) and photographed using a UVI gel documentation system (UVItec, Cambridge, United Kingdom).

For 16S rDNA V3 region analysis, the DGGE gel contained a linear 35% to 55% denaturing gradient (100% denaturant corresponds to 7 M urea and 40% deionized formamide). For Lactobacilli and *Clostridium leptum* subgroup analysis the DGGE gel contained a linear 28% to 48% denaturing
gradient, and for *Bacteroides* genus-specific analysis, the DGGE gel contained a linear 22.5% to 45% denaturing gradient.

Gelcompare II software (Applied Maths, Kortrijk, Belgium) was used for gel analysis and the similarity score was calculated using the Pearson correlation. The methodological bias caused by the DNA extraction, PCR amplification and DGGE analysis was estimated by performing these whole procedures on four samples twice (15), and the average similarity value of these four pairs of replicated samples was 93.8 ± 4.2%. In this study, the DGGE patterns with similarity values higher than 93.8% were not significantly different.

The bacterial diversity in the ulcerated site and non-ulcerated site was evaluated as bands number of DGGE profiles (32) and as weighted diversity according to Shannon and Weaver (22, 33).

All data sets were expressed as the mean ± SD. Student’s *t* test was applied for the comparison of variables with normal distribution and Wilcoxon’s test for non-normal distributions.
Results

Biopsy samples and PCR amplification

Forty-eight bacterial DNA samples were extracted from ulcerated and non-ulcerated mucosa of 24 patients diagnosed with ulcerative colitis. Since amplification of some DNA samples did not yield a positive result, 19 subjects were involved in Lactobacilli and *Clostridium leptum* subgroup analysis. Seventeen subjects were involved in the *Bacteroides* genus-specific DGGE analysis.

Comparisons of bacterial community structure within a given individual

The DGGE patterns of three representative individuals are shown in Fig. 1. The DGGE patterns indicated that the bacterial composition varied from person to person. When the microbiota biodiversity within a given individual was considered, the dominant bacterial composition within each patient showed a high similarity. The similarity value ranged from 79.9% to 99.1%. The mean similarity percentage was 94.8±3.8% and this value was higher than 93.8%, which indicated that the dominant bacterial composition adhered was similar between these two mucosal sites.

For Lactobacilli analysis, the similarity value ranged from 13.6% to
96.6% and the mean value was 59.9\(\pm\)26.1%. The mean similarity value for *Clostridium leptum* subgroup analysis was 79.2\(\pm\)22.6% and the similarity percentage ranged from 27.6% to 98.8%. The similarity value of *Bacteroides* genus-specific DGGE patterns ranged from 37.2% to 99.3% and the mean value was 88.7\(\pm\)16.4%.

When the results of all patients were taken into account, the similarity value of Lactobacilli and *Clostridium leptum* subgroup showed significant reduction compared with the dominant bacteria (P<0.01), while the similarity value of *Bacteroides* did not show a significant difference (Table 2). These results indicated that the dominant bacteria were more similar between two adjacent biopsies within the same intestinal region while for other groups, especially for Lactobacilli and *Clostridium leptum* group, the bacterial composition varied significantly.

The bacterial diversity in ulcerated and non-ulcerated sites was evaluated as bands number and as weighted diversity. No significant difference of the bacterial diversity between the two mucosal sites was observed when we focused on the dominant bacteria and these three groups (data not shown).

Comparisons of bacterial composition from separate compartments of the lower intestine
Four intestinal compartments, cecum, left colon, recto-sigmoid and rectum, were involved in this study and most patients had ulcerative colitis localized to the left colon and rectum. The similarity percentage for the dominant bacteria and three groups were compared in these patients with left colonic UC and rectal UC. The results indicated that in left colonic UC, the average similarity index of only Lactobacilli between the two biopsies (60.4 ± 26.1%) was decreased significantly than that of the dominant bacteria (95.8 ± 0.8%, P<0.05). In rectal UC, the average similarity index of both Lactobacilli (64.1 ± 26.3%) and Clostridium leptum subgroup (74.2 ± 26.8%) was reduced significantly compared with that of dominant bacteria (95.2 ± 2.7%, P<0.01) (Table 2). These results suggested that different diseased regions might have differences in the type and extent of the bacterial community alteration.

Discussion

In this work, DGGE patterns with a similarity value higher than 93.8% was thought of as having no difference and a similar percentage also was regarded as the positive cutoff of the similarity value by previous reports (15, 31). In this study, the average similarity value of the dominant bacteria between two adjacent biopsies was higher than the positive cutoff of our study, and this meant that the dominant bacterial composition at the two
biopsy sites was similar. Due to the host-specificity of the intestinal bacteria and the limited sample size and bias produced during the sample collection and processing, the positive cutoff for the three groups was not titrated with replicate samples, instead, the similarity for the three groups between the paired samples in each patient was statistically analyzed against the similarity value of the dominant bacteria within the same host.

In order to avoid amplification of eukaryotic DNA from the biopsy samples, nested-PCR was performed. In the first step, a nearly complete 16S rRNA gene fragment was amplified using the universal primer pair 27F/1492R. The product obtained was used as a template for a second amplification with dominant bacteria and three group-specific primers. Nested-PCR can improve the specificity and make it possible to characterize the diversity of bacteria in low numbers from mixed microbial communities.

The mucosa-associated bacterial composition is host-specific and significantly different from the fecal bacteria (43) and they are also stable in a period of time (22). This suggested that the mucosa-associated bacterial composition alteration found in this study was not due to transient factors.

The dominant bacteria are relatively stable when non-ulcerated tissues were collected along the distal intestine (15) and the dominant bacteria in
ulcerated and non-ulcerated biopsies in Crohn’s disease are similar (31). In this study, the universal primer-based DGGE analysis indicated that the dominant bacterial structure between the ulcerated and non-ulcerated tissue showed high similarity. Due to the complexity of the intestinal bacteria, the resolution of the universal primer-based analysis was not enough to reflect the bacterial composition in detail.

More recently, group-specific PCR-DGGE has been developed for important groups such as *Bacteriodes* and *Clostridium leptum* subgroup to increase resolution and sensitivity of this technology for evaluating gut ecology (23, 34). This method allows the visualization of the bacterial group of interest in a highly diverse background (2, 35). In our study, group-specific DGGE analysis indicated that the bacterial composition in ulcerated and non-ulcerated biopsies within the same intestinal segment showed lower similarity than the dominant bacteria. Furthermore, great intra-individual variations existed. Among the bacteria we investigated, the composition of *Lactobacilli* and *Clostridium leptum* subgroup varied significantly between the ulcerated and the non-ulcerated areas within the same individual.

*Lactobacilli* are thought of as beneficial to the host and many studies have demonstrated that some strains of *Lactobacilli* can reduce the severity and maintain the remission of this disease (1). A significant decrease in the
number of Lactobacilli is found in the mucosa of patients with inflammatory bowel disease (9, 22) and it has been suggested that the changing condition in the intestine may influence the Lactobacilli composition (43). We found the composition of Lactobacilli adhered to the ulcerated and non-ulcerated tissue from within the same patient varied greatly and may be caused by differences in the physiological status of the mucosa.

*C. leptum* subgroup (cluster IV) (4) is one of the most predominant populations of human fecal microflora (12, 37), which contains a large number of butyrate-producing bacteria (25, 28). Their metabolic activities have a significant effect on the health of the human colon. It has been reported that decreased fecal population level of *Clostridium leptum* group is observed in patients with inflammatory bowel disease (36). When we compared the bacteria adhered to the ulcerated mucosa and the non-ulcerated mucosa, we found that the composition of *Clostridium leptum* group differed significantly in the rectal UC but not in left colonic UC. It has been demonstrated that protein can be degraded into branched-chain fatty acids by many *Clostridia* (8) and the breakdown of proteins by the microbiota becomes more important in the distal bowel than in the proximal region (17). Therefore, the results from those studies suggest that the change of the mucosal physiology in the rectum may have a greater influence on the composition of *Clostridium leptum* group than in the left colon.
*Bacteroides* is the most dominant part of the normal indigenous flora in the human gut. It makes up more than 25% of bacteria in human fecal flora (10, 37, 41, 42). These bacteria are significant contributors to the metabolism, nutrition and health of man and animals; some *Bacteroides* species frequently are found in clinical infections and are thought to be opportunistic microorganisms (6, 21, 30). The role of *Bacteroides* spp. in inflammatory bowel disease has been explored in many investigations. Several studies reported an increase of *Bacteroides* spp. population level in IBD using both culture-dependent and culture-independent methods (11, 16, 29, 38). However, Conte *et al* found a decreased population level of anaerobic, especially *Bacteroides vulgatus* (5). The age difference between the individuals involved in these studies may cause these discrepant results. Moreover, these studies focused on the population level of *Bacteroides* spp., while the present study assessed the biodiversity of this genus. Our results indicated that *Bacteroides* spp. composition did not differ markedly between the two biopsy sites within the same individual.

In summary, the composition of Lactobacilli and *Clostridium leptum* was altered between the ulcerated and the non-ulcerated biopsy sites and the bacterial structure alteration was host-specific. These results suggest these bacteria may be closely related to UC and that this alteration may be caused by the differential physiology of the intestinal mucosa. The potential role
of Lactobacilli and *Clostridium leptum* in the etiology of ulcerative colitis should be more closely evaluated in future research.
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Figure legend:

FIG. 1. DGGE patterns of the mucosa-associated microbiota in three patients, A, B, C, three individuals. Pearson coefficient was calculated between the two biopsy sites in each individual. The left lane of each individual reflected the bacteria from the ulcerated mucosa and the right lane from the non-ulcerated mucosa.
TABLE 1. Information about the patients

<table>
<thead>
<tr>
<th>Sampling Position</th>
<th>Cecum</th>
<th>Left colon</th>
<th>Recto-Sigmoid</th>
<th>Rectum</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>1</td>
<td>9</td>
<td>1</td>
<td>13</td>
<td>24</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>1/0</td>
<td>4/5</td>
<td>0/1</td>
<td>4/9</td>
<td>9/15</td>
</tr>
<tr>
<td>Mean age (yr) (range)</td>
<td>16 (16)</td>
<td>47 (34-72)</td>
<td>38 (38)</td>
<td>46 (19-70)</td>
<td>40 (16-72)</td>
</tr>
</tbody>
</table>

<table>
<thead>
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<th>Treatment</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosteroids</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Mesalazine</td>
<td>1</td>
<td>3</td>
<td>0</td>
<td>5</td>
<td>9</td>
</tr>
</tbody>
</table>
TABLE 2. Average similarity coefficients (% ± SD) between the mucosa-associated bacteria in ulcerated and non-ulcerated position of 24 ulcerative colitis patients

<table>
<thead>
<tr>
<th>Position</th>
<th>Dominant bacteria</th>
<th>Lactobacilli n=19</th>
<th>Clostridium leptum n=19</th>
<th>Bacteroides spp. n=17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cecum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=1</td>
<td>96.4</td>
<td>18.3</td>
<td>92.1</td>
<td>87.3</td>
</tr>
<tr>
<td>Left Colon</td>
<td>95.8 ± 0.8</td>
<td>60.4* ± 26.1</td>
<td>81.5 ± 20.2</td>
<td>88.2 ± 15.4</td>
</tr>
<tr>
<td>n=9</td>
<td></td>
<td>(n=7)</td>
<td>(n=8)</td>
<td>(n=6)</td>
</tr>
<tr>
<td>Recto-Sigmoid</td>
<td>79.9</td>
<td>55.7</td>
<td>93.0</td>
<td>94.1</td>
</tr>
<tr>
<td>n=1</td>
<td></td>
<td>(n=1)</td>
<td>(n=1)</td>
<td>(n=1)</td>
</tr>
<tr>
<td>Rectum</td>
<td>95.2 ± 2.7</td>
<td>64.1** ± 26.3</td>
<td>74.2 * ± 26.8</td>
<td>88.5 ± 19.6</td>
</tr>
<tr>
<td>n=13</td>
<td></td>
<td>(n=10)</td>
<td>(n=9)</td>
<td>(n=9)</td>
</tr>
<tr>
<td>Total</td>
<td>94.8 ± 3.8</td>
<td>59.9** ± 26.1</td>
<td>79.2 ** ± 22.6</td>
<td>88.7 ± 16.4</td>
</tr>
<tr>
<td>(n=24)</td>
<td></td>
<td>(n=19)</td>
<td>(n=19)</td>
<td>(n=17)</td>
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* P<0.05 compared with dominant bacteria.

**P<0.01 compared with dominant bacteria.