Mucosa-associated bacteria in two middle-aged women diagnosed with collagenous colitis

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Abstract

AIM: To characterize the colon microbiota in two women histologically diagnosed with collagenous colitis using a culture-independent method.

METHODS: Biopsies were taken from the ascending colon and the total DNA was extracted. Universal bacterial primers were used to amplify the bacterial 16S rRNA genes. The amplicons were then cloned into competent Escherichia coli cells. The clones were sequenced and identified by comparison to known sequences.

RESULTS: The clones could be divided into 44 different phylotypes. The microbiota was dominated by Firmicutes and Bacteroidetes. Seven phylotypes were found in both patients and constituted 47.5% of the total number of clones. Of these, the most dominating were clones similar to Bacteroides cellulosilyticus, Bacteroides caccae, Bacteroides thetaiotaomicron, Bacteroides uniformis and Bacteroides dorei within Bacteroidetes. Sequences similar to Faecalibacterium prausnitzii and Clostridium citroniae were also found in both patients.

CONCLUSION: A predominance of potentially pathogenic Bacteroides spp., and the presence of clones showing similarity to Clostridium clostridioforme were found but the overall colon microbiota showed similarities to a healthy one. Etiologies for collagenous colitis other than an adverse bacterial flora must also be considered.

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Key words: Microscopic colitis; Collagenous colitis; Lymphocytic colitis; Colonic microbiota; 16S rRNA sequencing

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INTRODUCTION

Collagenous colitis (CC), an idiopathic inflammatory bowel disease, is a subtype of microscopic colitis (MC) together with lymphocytic colitis (LC)\(^1\). It is considered as a common cause of chronic diarrhea. In Sweden the incidence is approx four to five cases per 100 000\(^2\). The incidence for...
both CC and LC in Europe and North America is almost as high as for Crohn’s disease and ulcerative colitis[5].

CC is clinically characterized by chronic non-bloody diarrhea, often combined with abdominal pain and weight loss[2]. The colonic mucosa appears macroscopically normal or near-normal and the diagnosis is made by microscopic examination of mucosal biopsies that reveals diagnostic histopathological changes. CC was first described in 1976 by Lindström[6] in a woman with chronic watery diarrhea in whom histological examination revealed a thick subepithelial collagenous deposition in the rectum. In 1989, Lazenby et al[7] proposed the term lymphocytic colitis in a group of patients with chronic diarrhea and normal colonoscopy with only minor histological changes, where the microscopic evaluation of colonic biopsy specimens revealed modestly increased inflammation in the lamina propria without subepithelial collagen deposition or other mucosal changes.

The peak incidence of MC is in individuals between 55 years and 70 years of age. The female: male ratio is about 7:1 for CC. For LC the female predominance is less pronounced, with a female: male ratio of 2:3:1[8]. However, the disease can occur at all ages, and a few children with CC have been reported[9,10]. Bile acid malabsorption is found in about 27%-44% of patients with CC and 9%-60% in patients with LC[5,8-9]. Treatment with bile acid binding medications is effective in patients without bile-acid malabsorption but can also be effective in patients with bile-acid malabsorption[5,9].

Both etiology and pathogenesis of MC are uncertain. The most widely held hypothesis is that a noxious agent in the lumen, probably originating from the bacterial microflora, may have a major pathogenic role in the chronic intestinal inflammation. This is supported by regression of symptoms and histopathological changes after diversion of the fecal stream, and recurrence after restoration of intestinal continuity[11,12]. Other observations supporting this hypothesis are the sudden onset of diarrhea and that treatment with antibiotics may have positive effects[5,13]. The increased infiltration of lymphocytes in the mucosa also indicates a proinflammatory component in the lumen. There are case reports of linking pathogenic bacteria such as Clostridium difficile, Yersinia enterocolitica, Campylobacter jejuni and Aeromonas hydrophila to MC[2,7,14-16].

The human microbiota in healthy persons as well as in patients with inflammatory bowel disease has been analyzed in several studies using culture-independent methods[15,16]. However, to our knowledge no such studies have been performed on patients diagnosed with CC. The aim of the present study was to characterize the mucosa-associated microflora in the ascending colon in two women histologically diagnosed with CC, by cloning and sequencing of the bacterial 16S rRNA genes.

**MATERIALS AND METHODS**

**Subjects and samples**

Two female patients, 51 years and 60 years old (A and B) with a known diagnosis of MC, took part in the study. Patient A, otherwise healthy, started to experience watery, non-bloody diarrhea after an antibiotic treatment for gastroenteritis 10 years earlier. Colonoscopy was performed and she was diagnosed with LC. She was treated with Loperamid® (Merck NM AB, Stockholm, Sweden). Two years later she had a relapse of watery, non-bloody diarrhea and a second colonoscopy was performed, still indicating LC. This time she improved spontaneously. At the time of the present study, after a period of stress and a viral gastroenteritis, she started to lose weight and had frequent, watery, non-bloody diarrhea. The present colonoscopy showed a slightly swollen mucosa and increased vascular pattern. The histological examination revealed a thickened subepithelial collagen layer as well as inflammation in the lamina propria and a damaged surface epithelial layer. Patient B had a history of chronic thyroaiditis but was otherwise healthy. She was diagnosed with CC as well as with bile acid malabsorption 4 years before the study. At that time she improved spontaneously but had a recurrence after a period of major stress. Previously, she was treated with non-steroidal anti-inflammatory drugs due to muscular stiffness and actually experienced an improvement of her bowel function by this treatment. At the time of the present colonoscopy her symptoms had improved due to dietary fat reduction. Colonoscopy showed an increased vascular pattern in the right colon but was otherwise normal. Histological examination could verify a collagenous colitis.

Neither patient had any medication at the time of the colonoscopy. Celiac disease had been excluded in both women. They were both non-smokers.

The patients were asked to avoid fiber-rich foods such as fruits, vegetables, grains and seeds some days before the colonoscopy. The day before the examination they ate a plain breakfast, and no solid food was allowed after noon. Intestinal cleansing was carried out with Phosphoral® (Clean Chemical Sweden AB), a salt preparation with osmotic effects. Colonoscopy was performed and serial biopsies throughout the colon as well as two extra biopsies from the right colon were collected. The histological examination followed routine procedures. The latter were placed in tubes with TE-buffer [10 mmol Tris-HCl, 1 mmol ethylenediaminetetraacetic acid (EDTA), pH 8.0], frozen immediately in liquid nitrogen and stored at -80°C. The study was approved by the Ethics Committee at Lund University. The women gave written, informed consent before entering the study.

**DNA extraction and amplification**

Frozen tissue samples were thawed on ice and a single biopsy was transferred to a 1.5 mL tube with 190 µL Buffer G2 (DNA Tissue Kit; Qia-gen, Gmbh, Hilden, Germany) and 10 µL of Proteinase K (Qiagen). Eight to ten sterile glass (2 mm) beads were added and the cells were lysed at 56°C for 3-4 h in a shaking water bath. Tubes were cooled on ice and shaken for 30 min on an Eppendorf Mixer 5432 (Eppendorf, Hamburg, Germany) at 4°C.
to disintegrate all bacteria. After centrifugation at 300 × g for one minute, the solution was transferred to a Qia-gent sample tube, and total DNA was extracted by using Bior sor EZ1 (Qiagen) according to the manufacturer’s instructions. DNA was eluted in 200 µL.

Polymerase chain reaction amplification and cloning

The bacterial 16S rRNA genes were amplified by the universal primers ENV1 and ENV2 annealing to positions 8-27 and 1492-1511, respectively, according to Escherichia coli (E. coli) numbering [10]. The reaction mixture contained 5 µL of 10X polymerase chain reaction (PCR) buffer (100 mmol Tris-HCl, 15 mmol MgCl₂, 500 mmol KCl, pH 8.3), each deoxyribonucleotide phosphate at a concentration of 200 µmol, 2.5 U of Tag DNA Polymerase (Roche Diagnostics, GmbH, Mannheim, Germany) and 10 pmol of each primer. To each tube, 5 µL of extracted sample DNA was added and sterile water was added to 50 µL. As negative controls, water was added to the reaction mixture instead of DNA. Amplification was performed on an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany). Initially, the reaction was heated to 94 ºC for 3 min, followed by 25 cycles of denaturing at 94 ºC for 1 min, annealing at 50 ºC for 45 s and elongation at 72 ºC for 2 min. Finally, the reaction was held at 72 ºC for 7 min before cooling down to 4 ºC. Six PCR tubes were prepared from each sample and then pooled. Forty-two µL of the pooled reaction mixture from one sample was separated on a 1.5% (w/v) agarose gel (Agarose Type I, Sigma Aldrich, St Louis, Mo., United States) in TBE-buffer (89 mmol Tris, 89 mmol boric acid and 2.5 mmol EDTA, pH 8.3). The agarose gel was stained with ethidium bromide (0.5 mg/L) and the band was cut out from the gel. DNA was purified by using Wizard® SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI, United States). For cloning Promega pGEM®-T Vector System and E. coli JM109 (Promega Corp.) competent cells were used as described previously [20]. Colonies were selected randomly and recultivated on LB-agar containing ampicillin, and then harvested and stored in freezing buffer at -80 ºC.

Sequencing

Selected clones were single-strand sequenced by MWG Biotech (Ebersberg, Germany). ENV1 primer was used as sequencing primer. Sequences were edited using Bioedit Sequence Alignment editor 7.0.5.3 [21]. Sequences were identified by comparing them to sequences using the “simmatch” option available at the Ribosomal Database Project [25]. Sequences were checked for chimeric artifacts by using the Bellerophon server 2 and by creating phylogenetic trees of both 5’- and 3’-ends of the sequences. DNAseq calculations were performed using the Phylip DNAdist program using the “similarity table” option (available at: http://mobyle.pasteur.fr/cgi-bin/portal.py?form=dnadist) [24]. Sequences representing the different phylotypes have been submitted to Genbank and the accession numbers are HQ992999- HQ993602.

Diversity calculations

Shannon and Simpson’s indices were used for diversity calculations. The Shannon index is based on the proportional abundance of species and accounts for both evenness and species richness. Simpson’s index is the dominance measure where the abundance of commonest species is considered more than species richness [23]. The Simpson’s index was expressed as 1/D.

RESULTS

Two clone libraries were constructed, one for patient A with 87 clones and one for patient B with 90 clones. Five clones were suspected chimeras and were removed from the dataset before analysis. The lengths of the sequenced fragments were approximately 750 bp. Sequences showing > 98% similarity to each other were assigned to a single phylotype and a total of 44 phylotypes were identified (Table 1). Sequences could be grouped into 22 phylotypes in patient A and 29 phylotypes in patient B. Shannon’s and Simpsons diversity indices were calculated and both the patients showed similar values. The Shannon index was 2.61 for patient A and 2.78 for patient B, and the Simpson index was 8.13 for A and 9.29 for patient B. Firmicutes and Bacteroidetes were the dominating phyla with 50.6% and 47.2% in patient A and 57.8% and 42.2% in patient B, respectively (Figure 1).

In patient A Porphyromonadaceae constituted 1.2% of the clones and in patient B, Porphyromonadaceae and Rikenellaceae constituted 11.1% of the clones. Only two clones (2.3%) similar to Enterobacteriaceae were found in patient A.

The most common phylotypes were sequences similar to Bacteroides thetaiotaomicron (23 clones) and Faecalibacterium prausnitzii (13 clones) and Clostridium citroniae (9 clones) within Firmicutes, and Bacteroides dorei (29 clones), Bacteroides caccae (16 clones) and Bacteroides cellulosolvens (9 clones) within Bacteroidetes (Table 1). These phylotypes showed > 97% similarity to the closest type strain except for C. citroniae. Out of the 44 phylotypes identified, the two patients had 7 in common and 5 of these were assigned to Bacteroidetes and two to the Firmicutes. The phylotypes in common were 84 clones (47.5%) of the total number of clones. Sequences similar to F. prausnitzii and C. citroniae were found in both patients (Table 1). Within Bacteroidetes the shared phylotypes were most similar to, Bacteroides thetaiotaomicron, Bacteroides uniformis, B. cellulosolvens, B. caccae and B. dorei.

DISCUSSION

In the present study the microbiota of the ascending col on in the two female patients with CC showed similarities to a normal colon microbiota with Firmicutes and
Bacteroidetes as dominating phyla, making up 97.7% and 100.0% of the clones in patient A and B, respectively. Only two clones close to Enterobacteriaceae were found in patient A. In several studies, the microbiota of healthy persons have been analyzed by sequencing of the 16S rRNA genes using either fecal samples or tissue samples from the intestinal mucosa\textsuperscript{[17,18,26,25]}. All these studies showed a predominance of Firmicutes and Bacteroidetes while Verrucomicrobia, Actinobacteria and gamma proteobacteria were detected at lower frequency.

The proportion of clones belonging to Bacteroides was 47.0% in patient A and 31.1% in patient B. These were higher figures than Wang et al\textsuperscript{[8]}, using a similar methodology, found in biopsies taken from the ascending colon from a healthy, 54-year old woman where Bacteroides constituted 24.4% of the clones. Hayashi et al\textsuperscript{[3]} analyzed fecal samples of 3 healthy men aged 27, 34 and 54 years, and the proportion of Bacteroides was 4.2%, 3.4% and 14.9%, respectively. In another study of fecal samples from a healthy 40-year old man, Bacteroides constituted 14.4% of the total number of clones\textsuperscript{[9]}. Delgado et al\textsuperscript{[3]} analyzed clones from the descending colon from

| Phylotype No. | Closest type strain | Acc. No.\textsuperscript{1} | Similarity (%)\textsuperscript{2} | No. of clones\textsuperscript{3} | Distribution of clones\textsuperscript{4} | Assignment of clones |
|---------------|---------------------|-----------------|------------------|----------------|-----------------|----------------|
| 1             | Faecalibacterium prausnitzii | AJ413954        | 98.4-98.5        | 9              | 8 (A); 1 (B)    | Ruminococcaceae |
| 2             | Faecalibacterium prausnitzii | AJ413954        | 98.4-99.1        | 4              | 4 (B)           | Ruminococcaceae |
| 3             | Subdoligranulum variabile   | AJ58869         | 97.0             | 1              | 1 (B)           | Ruminococcaceae |
| 4             | Anaerostipes caccae        | AJ270487        | 99.2             | 2              | 2 (A)           | Lachnospiraceae |
| 5             | Ruminococcus lactaris      | AJ312384        | 94.9             | 3              | 3 (A)           | Lachnospiraceae |
| 6             | Subdoligranulum variabile   | AJ58869         | 97.0             | 1              | 1 (B)           | Ruminococcaceae |
| 7             | Ruminococcus lactaris      | AJ312384        | 100.0            | 3              | 3 (B)           | Lachnospiraceae |
| 8             | Clostridium jejuni         | AJ320509        | 96.9             | 2              | 2 (B)           | Lachnospiraceae |
| 9             | Subdoligranulum variabile   | AJ58869         | 97.1             | 1              | 1 (B)           | Lachnospiraceae |
| 10            | Roseburia intestinalis      | AJ312385        | 94.1             | 1              | 1 (B)           | Lachnospiraceae |
| 11            | Anaerostipes caccae        | AJ270487        | 95.8             | 1              | 1 (B)           | Lachnospiraceae |
| 12            | Roseburia intestinalis      | AJ312385        | 100.0            | 3              | 3 (B)           | Lachnospiraceae |
| 13            | Roseburia faccis           | AJ320509        | 96.9             | 2              | 2 (B)           | Lachnospiraceae |
| 14            | Roseburia intestinalis      | AJ312385        | 97.1             | 1              | 1 (B)           | Lachnospiraceae |
| 15            | Pseudobutyrivibrio ruminis | X95985          | 94.9-94.3        | 2              | 2 (A)           | Lachnospiraceae |
| 16            | Dorea longicatena           | AJ312382        | 94.9-95.2        | 3              | 3 (A)           | Lachnospiraceae |
| 17            | Dorea longicatena           | AJ312382        | 96.4-97.0        | 5              | 5 (A)           | Lachnospiraceae |
| 18            | Dorea longicatena           | AJ312382        | 100.0            | 3              | 3 (B)           | Lachnospiraceae |
| 19            | Dialister pneumosintes     | X82500          | 99.6             | 1              | 1 (A)           | Veillonellaceae |
| 20            | Escherichia coli            | AJ312382        | 96.4-97.0        | 5              | 5 (A)           | Lachnospiraceae |
| 21            | Streptococcus thermophilus  | X73440          | 99.9             | 23             | 23 (B)          | Lachnospiraceae |
| 22            | Faecalibacterium prausnitzii | AJ320509    | 95.1             | 9              | 9 (A); 7 (B)    | lachnospiraceae |
| 23            | Clostridium citroniae       | DQ279737        | 95.1             | 9              | 9 (A); 7 (B)    | Lachnospiraceae |
| 24            | Clostridium citroniae       | M95089          | 95.0             | 5              | 5 (A)           | Lachnospiraceae |
| 25            | Clostridium citroniae       | DQ279737        | 95.0             | 5              | 5 (A)           | Lachnospiraceae |
| 26            | Clostridium citroniae       | M95089          | 94.9-99.7        | 3              | 3 (A)           | Lachnospiraceae |
| 27            | Clostridium citroniae       | DQ279737        | 99.1             | 1              | 1 (A)           | Lachnospiraceae |
| 28            | Clostridium citroniae       | M95089          | 95.1             | 5              | 5 (B)           | Lachnospiraceae |
| 29            | Clostridium citroniae       | M95089          | 95.1-95.9        | 5              | 5 (B)           | Lachnospiraceae |
| 30            | Clostridium ramorum         | X73440          | 100.0            | 2              | 2 (A)           | Lachnospiraceae |
| 31            | Clostridium ramorum         | X73440          | 100.0            | 2              | 2 (A)           | Lachnospiraceae |
| 32            | Bacteroides uniformis       | AB267809        | 97.1             | 1              | 1 (B)           | Bacteroidaceae |
| 33            | Bacteroides uniformis       | AB267809        | 92.1             | 1              | 1 (B)           | Bacteroidaceae |
| 34            | Barnesiella viscericola     | AB267809        | 93.0             | 3              | 3 (A)           | Bacteroidaceae |
| 35            | Parabacteroides distasonis | AB230992        | 99.4-100.0       | 4              | 4 (B)           | Bacteroidaceae |
| 36            | Bacteroides cellulosoliticus| AJ383243        | 97.6-98.9        | 9              | 9 (A); 5 (B)    | Bacteroidaceae |
| 37            | Bacteroides asaccae        | X83951          | 99.4-99.9        | 16             | 16 (A); 14 (B)  | Bacteroidaceae |
| 38            | Bacteroides xylanivorans    | AM230650        | 97.7             | 1              | 1 (A)           | Bacteroidaceae |
| 39            | Bacteroides thaeotaomicron | AE015930        | 99.9             | 6              | 4 (A); 2 (B)    | Bacteroidaceae |
| 40            | Bacteroides thaeotaomicron | AE015930        | 99.9             | 6              | 4 (A); 2 (B)    | Bacteroidaceae |
| 41            | Bacteroides uniformis       | AB105110        | 99.7-100.0       | 3              | 3 (A); 3 (B)    | Bacteroidaceae |
| 42            | Bacteroides dorei           | AB242142        | 97.3-98.7        | 29             | 29 (A); 26 (B)  | Bacteroidaceae |
| 43            | Alistipes putredinis        | L16497          | 92.4-92.7        | 2              | 2 (B)           | Rikenellaceae |
| 44            | Alistipes oneidokkii        | L7602           | 95.1             | 9              | 9 (A); 7 (B)    | Lachnospiraceae |

The type strain showing the highest similarity to the sequence is shown. Assignment of the clones to bacterial family level was done using the “sequence match” option in the Ribosomal data base\textsuperscript{[20]}. \textsuperscript{1}Accession number for the type strain; \textsuperscript{2}Similarity to the closest type strain; \textsuperscript{3}The total number of clones assigned to the phylotype; \textsuperscript{4}Number of clones found in patient A and B, respectively.
a healthy 45-year old man and found one clone out of 20 (5%) belonging to Bacteroides. Of the 44 phylotypes found here, the two patients had only 7 in common. However, these shared phylotypes constituted 47.5% of the total number of clones. Within Bacteroides five phylotypes were common to both patients. Of these the most dominating were clones similar to B. caccae and B. dorei making up 25.4% of the total number of clones (Table 1). Both species belong to the Bacteroides fragilis group that are opportunistic pathogens isolated from a variety of anaerobic infections and cause about 50% of all anaerobic bacteremias.

A subgroup of B. fragilis, enterotoxigenic B. fragilis (ETBF), that can secrete a proinflammatory enterotoxin, has been found to be implicated in traveller’s diarrhea. In a study by Zhang et al, significantly more ETBF were found in patients with watery diarrhea (26.8%) than in the control group (12.4%). ETBF was also found at a higher frequency in patients over 30 years of age compared to the control group. Additionally, it was shown that 27.0% of patients over the age of 60 carried ETBF compared to 3.7% for the control group. It has been suggested that Bacteroides fragilis toxin can bind to receptors on the epithelial cells, leading to a signal cascade and cleavage of cadherines promoting an increased intestinal permeability. An increased intestinal permeability was shown in one patient with CC, using an Ussing chamber. Permeability was measured on biopsies taken on the colonic mucin layer and the intestinal permeability, leading to an immune response.

The clones resembling Clostridium clostridiiforme, Clostridium cietroniae, Clostridium asparagiforme and Clostridium aldenense were distributed into 7 phylotypes showing 95%-99.7% similarity to the different type strains. Four clones from patient A showed high similarity (99.3%-99.9%) to the type strain B. thetaiotaomicron NCTC 10582 was shown to express glycosidases and glycosulphatase and could degrade pig gastric mucin. In the present study 4 clones from patient A and 3 clones from patient B showed high similarity (99.3%-99.9%) to the type strain B. thetaiotaomicron NCTC 10582 (Table 1). Clones belonging to Akkermansia muciniphila were not found. However, it has been shown that this species represents only about one percent of the microbiota in healthy children and adults. One might speculate that specific components present within the microbiota of the CC patients, i.e., Bacteroides spp., that has an impact both on the colonic mucin layer and the intestinal permeability, leading to an immune response.

Clones identified as F. prausnitzii of the Ruminococcaceae family were found in both patients and constitute about 7% of the total number of clones. These bacteria together with Eubacterium rectale and Roseburia spp, are known as butyrate producers and usually make up about 5%-10% of the human microbiota and can be re-
garded as commensals. No clones resembling *Lactobacillus* nor Actinobacteria or Verrucomicrobia were found. This can probably be explained by the fact that too few clones were sequenced and that they usually constitute a minor part of the microbiota. Previously published case reports have suggested *Clostridium difficile*, *Yersinia enterocolitica*, *Campylobacter jejuni* and *Aeromonas hydrophila* to CC as possible pathogens. This could not be confirmed in the present study. As different pathogens are described, and the fact that the colonic microbiota was similar to a healthy one, the etiology to CC may not primarily depend on abnormal microbiota, and antibiotics may not be the treatment of choice in this entity, as it is sometimes considered.

This study has some limitations. Only two patients were examined and the method applied here only detects the dominant bacteria. Future research needs to examine the presence of common pathogens in the bowel, but also etiologies of CC other than bacteria must be considered. To the best of our knowledge, this is the first study of the intestinal microbiota in patients with a histologically diagnosed CC, by a culture-independent method. The overall composition of the colonic microbiota was similar to a healthy one with dominance of Firmicutes and Bacteroidetes. Due to the fact that only two patients were analyzed it is difficult to draw any conclusions, but in both patients a high proportion of potentially pathogenic species of *Bacteroides* and clones related to *C. drosidioforme* were found.

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