The biodiversity and composition of the dominant fecal microbiota in patients with inflammatory bowel disease ☆

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ABSTRACT

Clinical and experimental observations in animal models indicate that intestinal commensal bacteria are involved in the initiation and amplification of inflammatory bowel disease (IBD). As the majority of colonic bacteria cannot be identified by culture techniques, the aim of this study was to use sequence-based methods to investigate and characterize the composition of the dominant fecal microbiota in both patients with inflammatory bowel disease and healthy subjects. Fecal microbiota was isolated and quantified using real-time quantitative polymerase chain reaction. Denaturing gradient gel electrophoresis (DGGE) of 16S rDNA was used to evaluate the diversity of the dominant species. Analysis of individual bacterial groups showed a greater change in the fecal microbiota of patients with IBD, especially in those with active ulcerative colitis and active Crohn's disease. DGGE demonstrated the diversity of microbial flora in ulcerative colitis and Crohn's disease was less than in healthy subjects. Our results provide a better understanding of changes in fecal microbiota among patients with inflammatory bowel disease.

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1. Introduction

Observations of inflammatory bowel disease (IBD) have led to the theory that the pathogenesis of IBD is the result of an aberrant immune response to normal commensal bacteria in genetically susceptible individuals (Khor et al., 2011; Xavier and Podolsky, 2005). In humans, the intestinal bacterial community (microbiota) that inhabits in the gut appears to be an important source of antigens, which drive the chronic immunologic processes that are hallmarks of both Crohn's disease (CD) and ulcerative colitis (UC). Although several bacterial and viral pathogens have been suggested as causes of IBD, none has gained general acceptance. Current views assert that the pathology of IBD is a result of members in gut microbiota. These commensal bacteria are believed to be the source of antigens to which the dysfunctional immune system reacts. The distal gut of humans is one of the most densely populated microbial ecosystems on Earth. The endogenous gastrointestinal microbiota fulfills a fundamental and important role in human health and disease (Hooper and Gordon, 2001), including metabolic activities involved in salvaging energy and absorbing nutrients, trophic effects on the intestinal epithelia, promotion of gut maturation and integrity, maintenance of intestinal immune homeostasis, and defense against pathogenic bacteria (Conte et al., 2006). Most species in microbiota still cannot be identified using culture-based techniques. First pioneered in the 1990s, novel, culture-independent methods now allow for comprehensive comparisons of the intestinal microbiota in health and disease. Molecular techniques such as real-time quantitative polymerase chain reaction (Q-PCR) circumvent biases traditionally imposed by enrichment culturing; they allow for the direct detection and amplification of genes in DNA isolated from the environment. It has a great potential for analyzing fecal microbiota (Malinen et al., 2003). Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA genes is now routinely used to assess the diversity of microbial communities. This technique generates a DNA fingerprint for each sample, which permits the subsequent identification of components of the micro-biota by sequence analysis to monitor their dynamics (Wu et al., 2010). This process can also provide a rapid and comprehensive comparison of the majority of fecal microbiota's composition, as it can separate nucleotide sequences that differ by as little as 1 nucleotide (Stebbings et al., 2002).

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Table 1
Demographics of patients with IBD and HS.

	UC	CD	HS
Number of patients	26	10	14
Male/female	11/15	8/2	9/5
Age (years)	21–65	21–52	22–51
Active/remission	11/15	10/0	0
5-ASA	26	10	0
Corticosteroids	9	6	0
Purine analogues	1	1	0
Antibiotic	0	0	0

IBD = Inflammatory bowel disease; UC = ulcerative colitis; CD = Crohn's disease; 5-ASA = 5-aminosalicylic acid
There was no statistically significant difference in the age or sex ratio between the IBD and HS groups.

The aim of this study was thus to compare the bacterial composition in the fecal microbiota of patients with IBD and their matched controls and to identify the underlying differences. Nucleic acid–based methods of analysis were used. DNA extractions from samples were subjected to 2 different methods of microbiome analysis. Specific bacterial groups were quantified by real-time Q-PCR, and the bacterial diversity in the same DNA extracts was assessed by PCR-DGGE.

2. Materials and methods

2.1. Patients

Fecal samples were collected from 26 patients with UC (11 active UC [A-UC], 15 UC in remission [R-UC]) and 10 patients with active CD (A-CD). Diagnoses were confirmed by endoscopy and histology performed by pathologists at the Xijng Hospital of Digestive Diseases, Fourth Military Medical University (Xi'an, China). A-UC and A-CD were defined by a clinical activity index of UC, also called Mayo Index greater than 3 (Lewis et al., 2008; Truelove and Witts, 1955) and a CD activity index greater than 150 (Best et al., 1979), respectively. Fecal samples were also obtained from 14 matched healthy subjects (HS). None of them had taken antibiotics or undergone colonic cleansing for at least 4 weeks prior to sample collection. The sampling process was performed with the approval of the local ethics committee, and informed consent was obtained from patients.

2.2. Collection of stool sample and DNA extraction

Samples were placed in a freezer at −70 °C immediately after arrival, which was at most 30 min after sample collection, and they were stored until analysis. Frozen fecal samples were thawed at room temperature, and an aliquot of approximately 200 mg (wet weight) of each sample was added to a 2-mL sterile tube containing 1.4 mL of ASL buffer from the TIANamp stool DNA kit (Tiangen, Beijing, China). DNA was isolated according to the manufacturer's instructions with an initial bead-beating step of 30 s at 5000 rpm. Extracts were then stored at −20 °C.

2.3. PCR Amplification assays

All of the primer sequences targeting the 16S rRNA gene (rDNA) of the bacterial species or groups were derived from the previously published studies mentioned in Table 2. The PCR amplification and optimal annealing temperatures of the PCR primers were initially optimized with a gradient PCR in a Chromo 4 system (Bio-Rad, Hercules, CA, USA), and these conditions were used to quantify bacterial levels from fecal samples.

2.4. Purification and cloning of PCR products

PCR reaction products were purified and then used for cloning. Subsequently, they were ligated into the pMD18-T Vector, as specified by Takara (Dalian, China). Competent *Escherichia coli* DH5α cells were transformed with ligation products by heat shock at 42 °C. LB medium supplemented with ampicillin (100 mg/mL) was used for cloning and subculture procedures. Cells containing the correct plasmid insert were confirmed.

2.5. Calculation of starting 16S rRNA gene copy numbers and standard curves

Plasmid DNA was purified using the TIANprep Mini Plasmid Kit (Tiangen). DNA concentrations were then converted into 16S rRNA gene copy numbers as previously described (Fite et al., 2004). The plasmids were used for sequencing (Takara) simultaneously. To generate standard curves, plasmid standards for Q-PCR assays were

Table 2
Primers based on 16S rDNA sequences.

Target bacteria	Primer	Sequence (5′ to 3′)	Product size (bp)	Reference
Bacteroides–Porphyromonas–Prevotella	Bac708F	CACGAAGAACTCCGATTG	385	Hopkins et al., 2005
	Bac1080R	CACTTAAGCCGACACCT		
Bifidobacterium[a]		TCGCGTC(C/T)GGTGTGAAAG	243	Rinttila et al., 2004
		CCACATCCAGC(A/G)TCCAC		
Helicobacter	658f	TGGGAGAGGTAGGTGGAAT	374	Zhang et al., 2005
	1067R	GCCGTGCAGCACCTGTTTTCA		
Lactobacillus[b]	Lac1	AGCAGTAGGGAATCTTCCA	380	Walter et al., 2001
	Lac2	ATTYCACCGCTACACATG		
Enterococcus	Ent.1017F	CCTTTGACCACTCTAGAG	300	Vanhoutte et al., 2004
	Ent.1263R	CTTAGCCTCGCGACT		
Escherichia coli	uidAF	GCGAAAACTGTGGAATTGGG	252	Cebula et al., 1995
	uidAR	TGATGCTCCATAACTTCCTG		
Bacteroides fragilis subgroup[c]	g-Bfra-F	ATAGCCTTTCGAAAGRAAGAT	501	Matsuki et al., 2002
	g-Bfra-R	CCAGTATCAACTGCAATTTTA		
Clostridium phylogenetic clusters XI and XIVa[d]	Erec 688F	GCGTAGATATTAGGAGGAAC	211	Vanhoutte et al., 2006
	Erec 841R	TGCGTTWGCKRCGGCACCG		

  [a] Target species: *Bifidobacterium longum, B. minimum, B. angulatum, B. catenulatum, B. pseudocatenulatum, B. dentium, B. ruminantium, B. thermophilum, B. subtile, B. bifidum, B. boum, B. lactis, B. animalis, B.choerinum, B. gallicum, B. pseudolongum* subsp. *globosum, B. pseudolongum* subsp. *pseudolongum, B. magnum, B. infantis, B. indicum, B. gallinarum, B. pullorum, B. saeculare,* and *B. suis.*
  [b] The *Lactobacillus* group comprising the genera *Lactobacillus, Leuconostoc, Pediococcus, Weissella,* and *Aerococcus* (the last-named genus was originally not described as a target of the Lac1/2 primers).
  [c] The *Bacteroides fragilis* subgroup comprising *B. fragilis, B. acidifaciens, B. caccae, B. eggerthii, B. ovatus, B. stercoris, B. thetaiotaomicron, B. uniformis,* and *B. vulgatus.*
  [d] *Clostridium* phylogenetic cluster XI represents the *Clostridium lituseburense* group, whereas *Clostridium* phylogenetic cluster XIVa represents the *Clostridium coccoides–Eubacterium rectale* group.