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ORIGINAL ARTICLE

A Molecular Analysis of Fecal and Mucosal Bacterial Communities in Irritable Bowel Syndrome

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Abstract
Purpose The objectives of this study were, firstly, to determine the diversity of the host's gut microbiota in irritable bowel syndrome (IBS) using a culture-independent method (DGGE of the 16S rRNA gene) and, secondly, to examine mucosal biopsies of IBS patients and compare them to their own fecal microbiota.
Methods The diversity of the dominant microbiota in the fecal material of IBS patients was compared to a healthy control group. In addition, we compared the mucosal and fecal microbiota of IBS patients.
Results Statistical analysis of the mean similarity data for these groups indicated a significant difference ($P < 0.001$) between IBS ($n = 47$) and healthy controls ($n = 33$) with significantly more variation in the gut microbiota of healthy volunteers than that of IBS patients. The average intra-individual similarity between the mucosa and luminal microbiota was 84%, which indicates that different communities were present at the two sites. This difference, however, is similar to that previously described between these two niches in control subjects. The average inter-individual similarity of the bacterial communities on the mucosa and in the lumen of IBS was not significantly different ($P > 0.05$).
Conclusions IBS impacts equally on both bacterial communities in the IBS host and a significant difference in the gut microbiota exists between fecal samples from IBS patients and healthy controls. The reason for this difference is unclear and various possible explanations are available, but much more work is required to determine the underlying reason for this observation.

Keywords Irritable bowel syndrome · Colonic microbiota · Gut flora · Fecal flora · DGGE · Mucosal microbiota

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Introduction

Irritable bowel syndrome (IBS) is a common and potentially disabling disorder [1, 25] that afflicts between 10 and 15% of the population in Western Europe and North America, but is also common in other parts of the world [7]. The cause of the disease is thought to be multifactorial and may include dysmotility, abnormal gut sensation, genetic and dietary factors, and low-grade inflammation. Microbial factors have also been suggested to be involved in the disease with some investigators identifying small intestinal bacterial overgrowth (SIBO) as a contributory factor [4, 21–23] and others describing qualitative changes in the colonic microbiota or altered bacterial metabolism in the colon. However, no specific microbe has been

implicated [12]. Since an altered microbial composition has been implicated in several other gastro-intestinal diseases, such as colitis [30], several groups have also investigated whether a dysbiosis exists in IBS. The community diversity of dominant bacterial groups in fecal material of IBS patients has been investigated using culture-independent and dependent methods [2, 3, 13, 16, 17, 26, 31]. However, the results are conflicting and are probably explained by the variations in experimental design. Si et al. [31] found significant changes in the number of viable cells of *Bifidobacteria*, *Clostridium perfringens*, and *Enterobacteriaceae*, while Mättö et al. [16] did not, but reported changes in total aerobes and coliforms. This inherent bias of culturing led Mättö et al. to analyze the diversity of the dominant fecal bacteria using a culture-independent approach. They concluded that IBS patients had a greater instability in their microbiota, i.e., over time the microbiota was changing more than in a healthy group. In a more recent investigation [17] of the clostridial groups in fecal samples of IBS subjects, instability in the community structure was also reported. The members of this phylogenetic group are documented producers of H_2, which is a primary energy source for many other bacteria in the gut [18] and any changes in these functionally relevant bacteria may imply that they are involved in IBS pathophysiology [9]. In the most recent investigation of the bacteria diversity in IBS Kassinen et al. [8] fractionated the genomic DNA from clinically defined IBS subjects by %GC content and showed both qualitative and quantitative differences compared to a control group. Thus the case for a dysbiosis in the microbiota of IBS patients is growing. However, it is unclear whether these changes are a primary event or a mere epiphenomena, secondary to the disease process. In the present study we investigated the diversity of the dominant microbiota in the fecal material of IBS patients and compared them to a healthy control group. In addition we examined mucosal biopsies of IBS patients and compared them to their own fecal microbiota.

Materials and Methods

Sample Collection

Forty-seven female patients diagnosed with irritable bowel syndrome, according to Rome II criteria [33], were selected (mean age 43.6, median 44, range 24–66). In addition, fecal samples were also taken from 33 healthy female volunteers (mean age 27.1, median 27, range 21–38). None of the subjects had taken antibiotics or any other medication prior to (within 3 months), or during, the sampling period. Fecal samples were taken from each patient and colonic biopsy samples taken from nine patients and stored at −80°C until DNA extraction was performed. All samples were used for culture-independent analysis of the gut microbiota. Biopsies were obtained by flexible sigmoidoscopy following Fleet enema and were placed into sterile PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) for transport to the lab and immediately frozen at −80°C. All biopsies were obtained from the sigmoid colon.

Extraction of Bacterial DNA from Fecal Samples

Fecal material (200 mg) was suspended in 500-µl lysozyme (20 mg/ml in 20 mM Tris-HCl pH 8, 2 mM EDTA, 1.2% w/v Triton X-100) and incubated at 37°C for 2 h. Bacterial DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's instructions. DNA was visualized by agarose gel electrophoresis and quantified using a Nanodrop spectrophotometer.

Extraction of Bacterial DNA from Biopsy Samples

Biopsy material (approximately 20 mg) was suspended in 500-µl lysozyme (as above) and incubated at 37°C for 2 h. Proteinase K was added (400 µg/ml final concentration) and the samples incubated at 55°C for 1 h. Bacterial DNA was extracted using a FastDNA SPIN kit for soil (Qbiogene) according to the manufacturer's instructions.

Comparison of IBS and Healthy Fecal Samples by DGGE of the V1–V3 Region of the 16S rRNA Gene

DNA was amplified in an MJ Research PTC-200 Thermal Cycler using the primers 27F plus GC clamp and 519R (Table 1) under the following conditions: 94°C for 2 min, 20 cycles of 94°C for 45 s, 70°C for 45 s (decreasing by 1°C every cycle), 75°C for 45 s, 20 cycles of 94°C for 45 s, 50°C for 45 s, 75°C for 45 s and a final extension at 75°C for 5 min. The reaction mixture (50 µl) contained dNTPs (0.25 mM each), 1× reaction buffer (20 mM Tris pH 8.4, 50 mM KCl), $MgCl_2$ (3 mM), primers (0.4 pM each), *Taq* DNA polymerase (1.25 units) and 1 ng template DNA (all PCR reagents were purchased from Invitrogen). DGGE was performed using a CBS DGGE system run at 60°C, 85 V for 16 h, in 0.5× Tris–acetic acid–EDTA buffer (TAE; 0.02 M Tris, 0.5 mM EDTA, 0.01 M acetic acid, pH 8.2-8.4). Polyacrylamide gels (8% v/v) were prepared using acrylamide/bis acrylamide 37.5:1 in 0.5× TAE with a denaturant concentration gradient of 50–65% (a 100% denaturant solution contained 7 M urea and 40% v/v formamide). A DNA ladder (Invitrogen 100-bp ladder) was used at a concentration of 500 ng per lane. Gels were stained with 1× SYBR gold in 1× TAE (0.04 M Tris, 1 mM EDTA, 0.02 M acetic acid, pH 8.2–8.4) and

visualized by illumination with UV light (300 nm). Images were captured using an Alpha Innotech Fluorchem system and analyzed using Gelcompar II software (Applied Maths). Statistical analysis of the results was carried out using the program SPSS (Chicago, USA) and Sigmaplot. All the pairwise comparisons were plotted using a boxplot to identify outliers, which were removed from the final analysis.

Comparison of IBS Fecal and Biopsy Samples by DGGE of 16S rRNA Gene Products

Partial 16S rRNA gene products were amplified from DNA extracted from nine fecal and biopsy samples (fecal and biopsy samples were from the same nine patients) using primers that targeted the V6–V8 region of the 16S rRNA gene. PCR primers 968F plus GC clamp and 1401R were used to amplify the V6–V8 region of the 16S rRNA gene. To amplify the V6–V8 region of the 16S rRNA gene, the following PCR conditions were used: 95°C for 5 min, 30 cycles of 95°C for 30 s, 56°C for 40 s, 72°C for 1 min, final extension at 72°C for 5 min. The PCR conditions and recipe were the same as described for the amplification of partial 16S rRNA gene products from fecal samples. DGGE was performed in a DCODE universal mutation detection system (Bio Rad) run in 0.5× TAE buffer at 60°C, 85 V for 16 h, using an 8% v/v polyacrylamide gel with a denaturant concentration gradient of 30–55%. All other conditions were the same as described above.

Results

Comparison of IBS and Healthy Fecal Samples Using DGGE Profiles of the V1–V3 of the 16S rRNA Gene

Bacterial DNA was successfully extracted from all samples and used for PCR-DGGE analysis. Similarity values for each group of samples and between patients within the groups were generated and analyzed. To compare groups, the mean percentage similarity of the DGGE profiles was calculated from pairwise comparisons of each patient's DGGE profile compared to every other patient in the same sample group. For the test group of 47 individuals this results in 1,081 pairwise comparisons from which the average % similarity was calculated, likewise for the control group there were 528 pairwise comparisons. The boxplot for the data is shown in Fig. 1; any outliers were removed from the analysis of whether or not there was a statistical significant difference between the two data sets. From the analysis of the DGGE profiles for both IBS and healthy subjects we can show that the gut microbiota was highly variable. The IBS cohort showed a mean similarity value of 48.3 ± 10.4% and the healthy group 31.7 ± 10.3%. Statistical analysis of the mean similarity data for these groups indicated a significant difference ($P < 0.001$) between IBS and healthy controls with the controls exhibiting greater variability. The values for the IBS cohort were much lower than those obtained for the same IBS fecal samples using the primers for V6–V8 (87.7 ± 8.3%). Cluster analysis of the DGGE profiles is shown in Fig. 2. No significant clustering of the two test groups was observed.

Table 1 PCR primers used in this study

Primer	Primer sequence 5′-3′	Reference
27F[a]	AGA GTT TGA TC(AC) TGG CTC AG	[10]
519R	GTA TTA CCG CGG CTG GCT G	[28]
968F[a]	AAC GCG AAG AAC CTT AC	[19]
1401R	CGG TGT GTA CAA GAC CC	[28]

[a] GC clamp (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG-3′) on forward primers

Comparison of Fecal and Biopsy Samples Using the V6–V8 Region of the 16S rRNA Gene

The second aim of this study was to compare the fecal and colonic mucosal microbiota in IBS using culture-independent analysis. The amplification of the V1–V3 region of 16S rRNA gene from the biopsy DNA proved to be problematic as it did not consistently provide an amplicon from the biopsy DNA samples or produced DGGE profiles, which were artifacts of the PCR, in this case they were >97% similar and contained amplicons of human origin

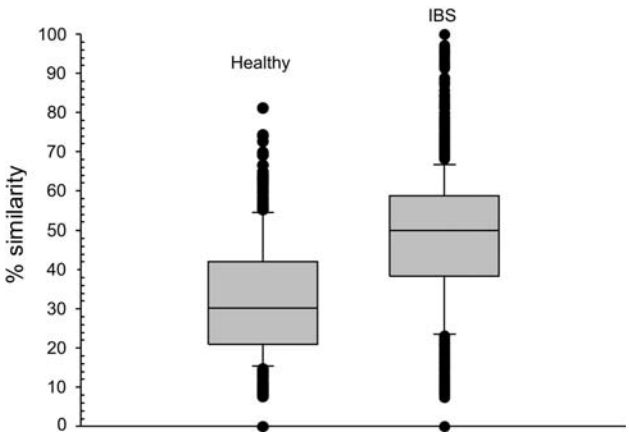

Fig. 1 Boxplot of the pairwise comparisons for the DGGE profiles of IBS versus healthy luminal community

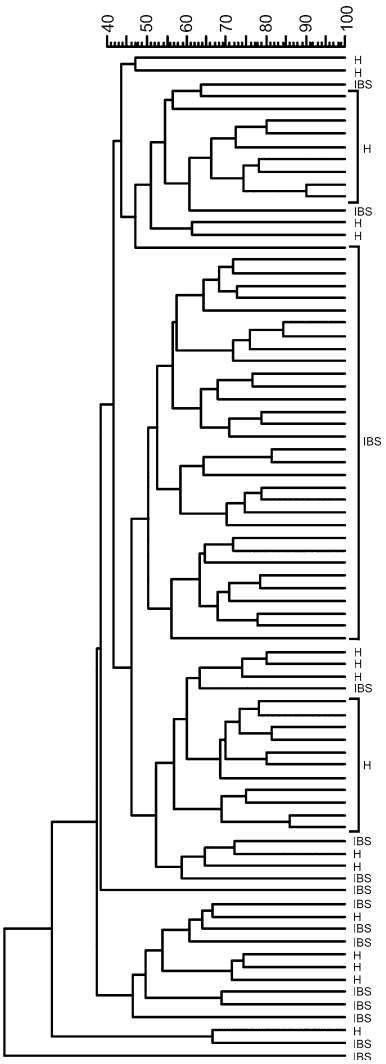

Fig. 2 Dendrogram of IBS and healthy fecal (H) samples from pairwise comparisons of the DGGE profiles from the V1–V3 region of the 16S rRNA gene. *Scale bar* indicates similarity (%)

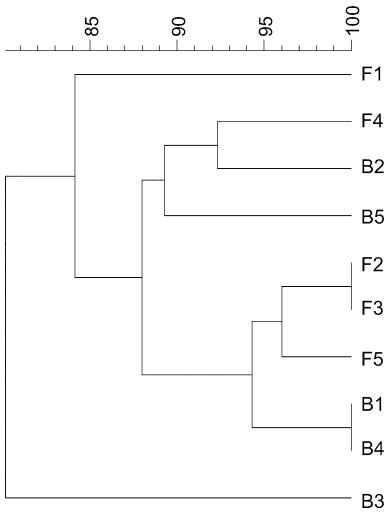

Fig. 3 Dendrogram of IBS fecal and biopsy samples from pairwise comparisons of the DGGE profiles from the V6–V8 region of the 16S rRNA gene. *Scale bar* indicates similarity (%)

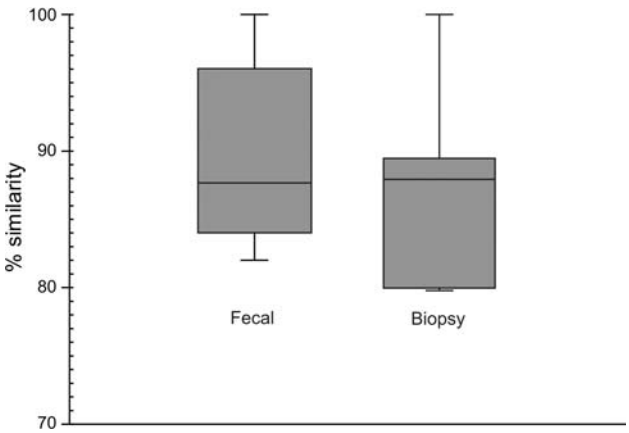

Fig. 4 Boxplot of the pairwise comparisons for the DGGE profiles of IBS fecal versus biopsy bacterial community

(Fig. S1). In order to perform the comparison between luminal and biopsy microbiota, we turned to amplifying the V6–V8 region of the 16S rRNA gene for DGGE analysis. Initially we compared the community structure of the biopsies to each other and the luminal communities to each other, i.e., inter-individual pairwise comparisons. The key result from this data was that no significant difference in the mean inter-individual similarity of fecal samples (87.7%) compared to biopsy samples (87.9%) was observed, when using the V6–V8 region of the 16S rRNA gene. Furthermore, the two sample types do not form separate clusters when their profiles were analyzed nor are the data significantly different (Figs. 3, 4). When we measured the intra-individual similarity between the bacterial community in the fecal sample and that attached to the mucosa for the IBS cohort, the average value was $84 \pm 5.4\%$.

Discussion

The microbiota of the gut plays an important role in the maintenance and function of this ecosystem. Dysbiosis has been associated with the development of inflammatory bowel disorders such as Crohn's disease [15, 28] and ulcerative colitis [36]. More recently, evidence has accumulated to suggest an inflammatory basis for irritable bowel syndrome (IBS), hence the search for a microbial dysbiosis in irritable bowel syndrome is highly germane. Since the majority of microbes in the gut are refractory to traditional culturing approaches [27], culture-independent methods have been used to explore the large diversity in this habitat. In this study we explored the diversity of the microbiota from the distal gut and compared the luminal microbiota of IBS and healthy volunteers. The mean percentage similarity between individuals in both cohorts was low, but in

agreement with previously published studies in the gut [6, 13, 14, 29, 35]. The reason for this low similarity was due to the primers we used, which covered the V1–V3 regions of the 16S rRNA gene and encompassed the three most variable regions of the gene [34], hence they were expected to give the greatest variability from DGGE. When compared to the mean similarity for the same sample fecal samples using the PCR primers covering the V6–V8 regions of the 16S rRNA gene, we see that this prediction was true. Analysis of the dendrogram derived from these DGGE profiles led us to conclude that healthy samples clustered together into two main groups and the level of variation between samples fell in the range of 50–80%, from which we also concluded that the samples do not cluster very strongly (Fig. 2).

The key observation was that there was significantly more variation in the gut microbiota of healthy volunteers than that of IBS patients. While the patients were not age matched, statistical analysis of the data did not show any impact of this variable on the clustering pattern ($P > 0.05$, data not shown). We concluded that, in IBS, the gut microbiota within the colon is being influenced by the disease. One could hypothesize that small intestinal bacterial overgrowth (SIBO) may "dampen down" the variation in the large intestine. However, the evidence for a role of SIBO in IBS is weak [24] and is, therefore most probably not the cause of this observation. Alternatively, reports of immune activation, or even a low-grade inflammatory process, in IBS [20] raise the possibility that these processes may be responsible for the greater similarity in the gut microbiota in this disorder. However, the mechanism of this effect on the microbiota needs clarification. In contrast to our findings, Mättö et al. [16] have reported instability in the microbiota of IBS, but failed to control for antibiotic usage. In a follow-up study, Maukonen et al. [17] targeted the clostridial groups in IBS and reported a similar instability in this functionally important group of bacteria. If an unstable bacterial community did exist we would expect to see much more inter-individual between IBS subjects, but this was not the case and we observed the opposite. We concede, however, that to verify these findings, further observations at additional time points are needed. Alternatively, our observation may be a consequence of our inclusion of a larger study group than in prior studies and of controlling for antibiotic use. Analysis of the microbial community in this cohort using more robust culture-independent methods such as deep-sequencing of 16S rRNA gene libraries, as used by Eckburg et al. [5], could provide further insights into the role of bacteria in this disease.

The second aim of the project was to compare luminal and mucosa-associated microbiota from the same subject and between subjects, i.e., intra- and inter-individual comparisons. Since the PCR primers for the V1–V3 region were not sufficiently robust for this purpose (Fig. S1), we changed to using the V6–V8 region of the 16S rRNA gene for community structure analysis. The lack of significant differences between the two niches, either intra- or inter-individual, led us to conclude that the presence of IBS had no influence on the bacterial composition when comparing either niche, but, that it was influencing the composition of the luminal microbiota, when compared to a healthy control group. While we did not study the mucosally associated microbiota of control subjects in this study, it is interesting to note that when Swidsinski et al. [32] compared mucosal biopsies from IBS with a healthy control group they found that they were similar. The average values for intra-individual similarity between the fecal and mucosal communities were very similar to values that have been reported for similar studies of the microbiota in both IBD patients and healthy volunteers in the two niches. Lepage et al. [11] investigated the mucosa-associated bacteria and fecal microbiota in CD and UC and the average intra-individual similarity was reported to be 87.0 and 88.2% for the left colon and rectum, which are the closest areas to those we sampled, hence not statistically different to our values. Furthermore, studies on healthy volunteers showed a very similar difference between luminal and mucosal communities [5, 37]. These results lead us to conclude that IBS exerted the same influence on the composition of the bacterial community found on either the mucosa or in the lumen in an IBS subject. In order for us to understand better what is occurring in these two separate bacterial communities, a more robust quantitative and qualitative analysis needs to be undertaken. These types of analyses would reveal the types and numbers of bacteria in the respective sites and whether they were similar to healthy subjects or those with inflamed mucosa, e.g., IBD.

We acknowledge the limitations of the study. Thus, we did not segregate IBS subjects according to bowel habit sub-type or severity but chose instead to study sequential subjects as they presented to a busy gastroenterology clinic. We concede that further studies will be required to determine whether bowel habit, per se, has a significant effect on the microbiota in IBS. Similarly, we did not stratify our IBS subjects according to severity or impact on quality for life; these issues will also need to be addressed in subsequent studies.

These shortcomings notwithstanding we believe that this study provides further evidence for the presence of a significant difference in the distal gut microbiota between IBS patients and healthy controls. The reason for this difference is unclear and various possible explanations are available, but much more work is required to determine the underlying reason for this observation. Furthermore, IBS was not seen to play a role in preferentially influencing the mucosal or luminal bacterial communities in an IBS subject and we conclude that both communities are being impacted equally by the disease.

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