Can Colonoscopy Aspirates be a Substitute for Fecal Samples in Analyses of the Intestinal Microbiota?

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There is a growing interest in the study of the human gut microbiota, as correlations between changes in bacterial profiles and diseases are increasingly discovered. Studies in this field generally use fecal samples, but it is often easier to obtain colon content aspirates during colonoscopy. This study used automated ribosomal internal spacer analysis (ARISA) to examine the extent to which the microbiota of colon aspirate samples obtained after bowel cleansing can reflect interindividual differences and serve as a proxy for fecal samples. Pre-bowel preparation fecal samples as well as colonoscopy aspirate samples from the cecum and rectum were obtained from 19 subjects. DNA was extracted from all samples, and comparative analysis was performed, including analysis of similarity (ANOSIM) and nonmetric multidimensional scaling. ANOSIM confirmed that samples from the same individual were well separated from samples from different individuals. Significantly larger differences were found between samples from different individuals than between samples of the same individual (R = 0.7605, p < 0.0001). These findings show that post-bowel preparation aspirates maintain a strong individual signature. Colonoscopy aspirates can therefore serve as a substitute for fecal samples in studies comparing the microbiota of different clinical study groups, especially when fecal samples are unavailable.

Key words: microbiota analysis; ARISA; ITS; colonoscopy; inter-individual variation; intra-individual variation

INTRODUCTION

In recent years, the role of the gut microbiota in health as well as in disease states has become evident. For example, modified microbiotas have been observed in inflammatory bowel disease [1–6], irritable bowel syndrome (IBS) [7–10] and Celiac disease [11, 12].

Fecal samples are most commonly used to study the gut microbiota, but are often difficult to obtain due to low compliance of volunteers who are reluctant to handle them. Availability is further reduced by the need to immediately freeze the samples in one's home freezer, where food is also stored. Furthermore, it is hard to control the duration of the samples' exposure to oxygen, which results in bacterial lysis followed by DNA degradation. In contrast, aspirates of fluid left in the colon after bowel cleansing are easy to obtain during colonoscopy, which is routinely performed in many gastrointestinal disorders and as routine screening for detection and elimination of colonic tumors. The aspirate samples can be immediately frozen, and require no extra effort from the volunteer, resulting in very high compliance. However, it is not clear whether the microbiotas in lavage aspirates are identical to those of fecal samples. Bibiloni and coworkers compared the bacterial composition of fecal and aspirate samples, using temporal temperature-gradient gel electrophoresis (TTGE) of 16S rRNA gene sequences and showed that these samples were not significantly different from one another, having around 80% similarity [13]. However, given that all humans potentially share much similarity in their dominant intestinal microbial species, this similarity could reflect a few highly prevalent core taxa. Thus, the extent to which aspirate samples can represent the fecal microbiota of an individual, should be explicitly examined.

Here we compared the microbial samples of intestinal aspirates obtained during colonoscopy with pre-bowel preparation fecal samples obtained from the same individuals. This was performed using Automated Ribosomal Internal Spacer Analysis (ARISA). This method is based on length polymorphisms in the internal

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transcribed spacer (ITS), located between the 16S and 23S rRNA genes [14], and provides species-level resolution [15, 16].

MATERIALS AND METHODS

Ethics

The study was approved by the ethics committee of Meir Medical Center.

Volunteers and bowel preparation

Nineteen subjects aged 38–74 (mean age 60.4, SD 9.58), who were scheduled for colonoscopy for colon cancer screening at the gastroenterology clinic of Meir Medical Center, were requested over the phone to give a fecal sample at home before bowel cleansing (Table 1). Thereafter, aspirate samples were obtained during colonoscopy from the rectum (first sample) and cecum (second sample). The volunteers varied in terms of medical history (Table 1) but all considered themselves to be generally in good health. Exclusion criteria were use of antibiotics in the 2 weeks prior to the examination and any significant systemic disease, including inflammatory bowel disease (IBD). Of the 19 volunteers, 1 suffered from diarrhea and 2 had irritable bowel syndrome. Three of the patients were taking statins at the time of sampling. Colonoscopy showed that 1 patient had rectal carcinoma, 1 patient had tubulovillous adenoma with high-grade dysplasia and 5 patients had 1–3 rectal polyps.

Bowel cleansing was generally performed using polyethylene glycol electrolyte lavage solution (Meroken™, Taro, Israel), composed of 315 g polyethylene glycol, 8.424 g sodium chloride, 4.2840 g sodium bicarbonate and 1.1175 g potassium chloride, dissolved in 3 L of water. Five patients received a preparation composed of 9.53% w/v Disodium Phosphate Anhydrous + 41.75% w/v Monosodium Phosphate Anhydrous (Soffodex, Dexon, Israel) (see Table 1).

Sample collection

All stool samples were obtained fresh prior to the use of any bowel preparation. Stools were frozen immediately after defecation at –20°C on the day before colonoscopy. The samples were transferred to the hospital on ice, kept frozen at –20°C for up to 4 weeks and then stored at –70°C until analyzed. Colonic aspirates, the intestinal content obtained from the cecum and rectum using the colonoscope during the colonoscopy procedure, were also frozen at –20°C and then stored at –70°C until analyzed together with the fecal samples.

DNA extraction

DNA was extracted from feces using a PowerSoil DNA kit (Mo Bio Laboratories Inc) in a biosafety cabinet (HB 2448, Holten LaminAir, Denmark) to avoid sample contamination by exogenous DNA. Fecal samples were kept on ice and handled using all safety precautions advised when handling samples of human origin.

Bacterial DNA fingerprinting

PCR reactions for ARISA (25 µl) were performed in duplicate and contained 1.25 U of Taq DNA polymerase (BIOTAQ™, BIOLINE), 3 mM of MgCl2, 2.5 µl 10xPCR buffer, 0.1 mM of each dNTP, ultrapure water (Biological Industries, Israel) and 1 pmol of each primer: 16S-1392F, 5'-GYACACACCGCCCGT-3', and a 5' TET labeled 23S-125R, 5'-GGTTBCCCCATTCRG-3'. Reactions were performed as follows: 3 min of preliminary denaturation, at 94°C; 32 cycles of 1 min of denaturation at 94°C, 1 min of annealing at 52°C, 1.5 min of elongation at 72°C; and then 20 min final elongation at 72°C.

PCR products were analyzed using an ABI PRISM 3100 Genetic Analyzer. The labeled fragments were separated on the capillary sequencer along with a custom-made ROX-labeled 250-1150 bp size standard (BioVentures) for ARISA and a GeneScan™ 600 LIZ® (Applied Biosystems) size standard for LH-PCR. Raw data generated by the ABI PRISM 3100 Genetic Analyzer were initially analyzed using GeneMarker™ (SoftGenetics).

Length heterogeneity determination

After performing accurate size calling using the program, all data were exported to Microsoft Excel for further analysis. In Excel, operational taxonomic units (OTUs) were determined by applying a cutoff from the total intensity of the sample of 0.1% in ARISA and 0.25% in LH-PCR. Fragment length data were binned according to the precision confidence levels, in order to compensate for uncertainty in size-calling when comparing different samples [17]. Thus, all OTUs were assigned to bins of 3 bp (±1 bp) for fragments up to 700 bp in length, bins of 5 base pairs for fragments between 700–1000 bp in length and bins of 10 base pairs for fragments above 1000 bp in length. Duplicates were compared with each other, and OTUs that appeared in only one of the duplicates were excluded. New relative intensities were calculated, and duplicates were united by averaging their corresponding values of relative intensity. Finally, the averaged values for each sample

Table 1. Research subjects (for additional details see materials and methods)

Subject no.	Age	Gender	Bowel cleansing	Colonoscopy findings	Other medical information
1	56	M	PEG	Rectal carcinoma	None
2	67	F	PEG	Hemorrhoids	Obesity
3	55	F	PEG	None	Past: inguinal hernioplasty, nephrectomy
4	51	M	Sodium phosphate	Polyp- rectum	Past: Duodenal ulcer
5	62	M	Sodium phosphate	Hemorrhoids	Past: Duodenal ulcer
6	58	M	PEG	Polyp- sigma	Prostate cancer 2 years ago, flatulence, diarrhea
7	54	F	PEG	Polyp x2- sigma	None
8	50	M	Sodium phosphate	Polyp x2- sigma	Hyperlipidemia, diabetes, hypertension
9	60	F	PEG	None	None
10	68	M	PEG	Polyp- sigma	Hyperlipidemia, diabetes, irritable bowel syndrome
11	68	F	PEG	None	None
12	74	F	PEG	None	None
13	38	F	Sodium phosphate	None	Anemia (Gastric polyp)
14	68	M	PEG	Polyp x2- sigma, polyp- ascending	Benign tumor of kidney, ischemic heart disease, prostatectomy
15	50	F	Sodium phosphate	None	Anemia
16	67	F	PEG	None	Irritable bowel syndrome
17	61	F	PEG	None	Hypertension, reflux esophageal
18	67	F	PEG	None	Obesity, hyperlipidemia, diabetes, osteoporosis
19	74	M	PEG	None	None

were normalized to reflect relative intensity values, by dividing the intensity of each binned peak by the total intensity of all peaks in the sample. All data were then exported to PAST, a statistical data analysis package [18], where similarity index (Bray-Curtis) was computed and clustering was performed.

One-way analysis of similarities (ANOSIM) test

ANOSIM (analysis of similarity) nonparametric tests were performed using the statistical software PAST (Paleontological Statistics, v.1.79). ANOSIM tests the null hypothesis that within-individuals similarity in microbial composition equals between-individual similarity [19] and provides a test statistic R, with values close to 1 meaning high dissimilarity among groups and high similarity within groups, i.e., high group specificity.

Nonmetric multidimensional scaling (NMDS)

We performed NMDS of the samples, as implemented in PAST, using the Bray-Curtis similarity index.

RESULTS

Intestinal samples obtained from the same individual tend to have a more similar bacterial composition

For colonoscopy aspirates to qualify as a proxy for fecal samples, in terms of bacterial composition, they should not only be similar to fecal samples from the same individual but also individual specific, at least to some extent. Thus, we applied ARISA to test the person specificity of aspirate samples. NMDS of the Bray-Curtis similarities derived from the ARISA, showed that bacterial profiles from the same individual tended to be relatively closer compared with other samples (Fig. 1), although in several cases, fecal samples were somewhat distant from the aspirate samples. ARISA-based Analysis of Similarity (ANOSIM) of all samples (including feces and aspirates obtained from the cecum and rectum), grouped by individual, was performed. ANOSIM confirmed that differences were larger (R = 0.7605, p < 0.0001) between samples from different individuals than within the same individual. Removing the rectum samples from the analysis and grouping the cecum and fecal samples yielded similar results (R=0.7338, P < 0.0001). When removing the fecal samples from the analysis, the person specificity between the cecum and rectum aspirates was even higher (R=0.9765, p < 0.0001), indicating that aspirates from these two sites are highly similar. Notably, groups that produce ANOSIM R values greater than 0.75 are considered to be well separated [20]. Conversely, ANOSIM conducted with grouping samples by type (i.e., fecal, cecum aspirate or rectum aspirate) showed no type-specific signal (R= −0.0125, p=0.6854). Furthermore, overall there were no specific peaks (taxa) present only in fecal samples and not in aspirate samples

Fig. 1.   Nonmetric multidimensional scaling of ARISA-based Bray-Curtis similarity values of bacterial profiles from cecum (C) and rectum (R) aspirates and feces (F) from 19 individuals. Subjects are denoted by their individual numbers, which correspond to those in Table 1.

and vice versa. Thus, it appears that aspirate samples do not have a strong taxonomic bias either for or against specific taxa when compared with fecal samples.

The type of bowel preparation influences the similarity between aspirates and feces

When separately analyzing samples of subjects who used a phosphate-based prep and samples of subjects who used the PEG-based formulation, an interesting trend was observed. The ANOSIM statistic R values, denoting separation of the cecum, rectum and feces samples of the same individual from those of others, was much higher in the PEG group compared with the phosphate group (R=0.8275, p<0.0001, vs. R=0.4607, p=0.0002, respectively). Similarly, ANOSIM R values between the cecum and rectum contents from the same individual were also more similar in samples in the PEG group (R=0.9874, p<0.0001, and R=0.88, p=0.0026,

respectively). With the exception of subject 8, all other samples from the phosphate-based prep were relatively distant from one another in the NMDS (Fig. 1). Correspondingly, Bray-Curtis similarity values of ARISA profiles from samples of the same individual were also more similar on average to one another in the PEG samples than the phosphate-based prep samples (mean of 0.41 vs. 0.32, SE of 0.04 and 0.06, for PEG and phosphate, respectively). This may indicate that different bowel prep formulations may influence colonic bacteria and the extent to which colonic aspirates can represent stool. For instance, phosphate-based formulations may damage the adherence of bacteria to the intestinal lumen and could thus affect the diversity represented in the colonoscopy aspirates, and aspirates obtained from milder, PEG-based bowel preps, may be more suitable as a substitute for stool samples for microbiome analysis.

DISCUSSION

The data presented here show that in general the similarity in microbial composition between intestinal and fecal samples taken from the same individual is higher than that observed across individuals. Nonetheless, several fecal profiles were relatively distant from their corresponding aspirate samples. Since the fecal samples were obtained a day before obtaining the cecal and rectal aspirate samples, there could be intraindividual temporal changes in microbiota. A study that compared fecal samples of healthy volunteers over a period of 3 months, using denaturing gradient gel electrophoresis, found a similarity of about 85% between samples [21]. Although day-to-day heterogeneity should be smaller than monthly variation, the issue of daily temporal variation has not been adequately investigated in the past and requires further study.

It has been shown, in both humans and rodents, that the composition of tissue-associated microbiota is different than that of the fecal microbiota [22, 23]. The degree to which aspirates resemble the fecal or tissue-adherent microbiota is likely to depend on the bowel preparation type. In our study, the effect of the bowel cleansing was greater when a phosphate-based formulation was used, and aspirate samples taken from subjects who underwent this preparation were less similar to feces. Thus, when considering the suitability of aspirate samples as a substitute for fecal samples, the type of bowel cleansing undertaken has great importance, and different formulations could bias the sample towards particular microbial taxa. Since phosphate-based formulations have been recently withdrawn due to their potential nephrotoxic effects, this is of lesser practical concern at present.

Despite the fact that PCR-based fingerprinting techniques do not identify the taxa in the samples, these rapid and inexpensive methods are known to accurately assess similarity levels between samples/communities [24]. Furthermore, fingerprinting results have been shown to be in good agreement with 16S rRNA gene sequencing [25]. Nevertheless, with sequencing costs ever on the decrease, it will be interesting to perform a similar analysis in the future using high-throughput 16S rRNA or ITS sequencing.

Microbial profiles from aspirate samples can differ substantially from fecal profiles from the same individual, and that difference is highly dependent on the bowel prep formulation. Thus, one cannot currently envision practical clinical applications that rely on aspirates as a proxy for feces. Nevertheless, since the post-bowel preparation aspirates still maintain much similarity in their microbial composition to the pre-prep stool samples from the same individuals, these samples (rectal aspirates in particular) could be used to compare the microbiota of different study groups in clinical research, when fecal samples are unavailable.

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