Differential clustering of bowel biopsy-associated bacterial profiles of specimens collected in Mexico and Canada: what do these profiles represent?

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Bowel commensals appear to be an important source of antigens that drive the chronic immune inflammation characteristic of Crohn's disease and ulcerative colitis [inflammatory bowel diseases (IBD)]. Biopsy-associated bacteria are assumed to be particularly relevant in bacteriological investigations of IBD because they are assumed to be located on the mucosal surface and hence close to immunological cells. This investigation analysed the bacterial collections associated with bowel biopsies, aspirates of residual fluid after bowel cleansing and faeces from IBD patients and non-IBD subjects in Edmonton, Canada, and Mexico City, Mexico. Temporal temperature gradient gel electrophoresis of 16S rRNA gene sequences produced profiles of the bacterial collections and their similarities were compared. Similarity analysis showed that the profiles did not cluster according to disease status, but that Canadian and Mexican profiles could be differentiated by this method. Comparison of biopsy, aspirate and faecal samples obtained from the same subject showed that, on average, the profiles were highly similar. Therefore, biopsy-associated bacteria are likely to represent, at least in part, contaminants from the fluid, which resembles a faecal solution, that pools in the bowel after cleansing prior to endoscopy.

Received 30 March 2007

Accepted 25 September 2007

INTRODUCTION

Crohn's disease and ulcerative colitis are distinct clinical entities but the inflammation in both diseases is considered to be fuelled by antigens associated with the bacterial community that normally inhabits the human bowel. The chronic immune inflammation characteristic of these diseases is likely to be linked to defects in the intestinal epithelial barrier and the immune system of the patients, which are, in part, genetically determined (Podolsky, 2002; Bouma & Strober, 2003). Several studies have been reported concerning the phylogenetic analysis of biopsy-associated bacteria. These studies aimed to determine which commensal bacteria were most likely to be the source of pro-inflammatory molecules (Schultz *et al.*, 1999; Swidsinski *et al.*, 2002; Seksik *et al.*, 2003, 2005; Ott *et al.*, 2004; Prindiville *et al.*, 2004; Lepage *et al.*, 2005;

Bibiloni *et al.*, 2006). It has been reported that these biopsy-associated bacteria are residents of the mucosal surface and that this collection of bacteria is different from that detected in the faeces (Zoetendal *et al.*, 2002). Observations by at least three research groups have shown that the biopsy-associated bacterial collections are similar along the length of the bowel and that they do not differ between inflamed and non-inflamed mucosa (Zoetendal *et al.*, 2002; Lepage *et al.*, 2005; Bibiloni *et al.*, 2006; Sokol *et al.*, 2007).

Biopsies, unlike faeces, provide samples collected from regions of the intestinal tract where inflammation occurs. They are not perfect specimens for bacteriological analysis, however, because they consist of only a few milligrams of tissue and have been collected from subjects that have undergone bowel cleansing prior to endoscopy. Residual bowel cleansing solution pools in the large bowel and can be collected by aspiration. The aspirate, essentially a faecal solution, bathes the mucosal surface of the intestine and in all likelihood contaminates it, as well as contaminating the

Abbreviations: IBD, inflammatory bowel disease; TTGE, temporal temperature gradient gel electrophoresis.

endoscope and its mechanical parts that collect the tissue sample.

According to several reports, in general terms, the bacteriology of biopsies differs among healthy subjects, and Crohn's disease and ulcerative colitis patients (Schultz *et al.*, 1999; Swidsinski *et al.*, 2002; Ott *et al.*, 2004; Prindiville *et al.*, 2004; Lepage *et al.*, 2005; Bibiloni *et al.*, 2006). Unfortunately, there is little agreement between the results obtained by different laboratories located in different countries as to specific differences in the composition of the bacterial collections. This may reflect differing methodological approaches used to analyse the biopsy-associated collections of bacteria. Alternatively, it may reflect variation in the composition of the bacterial communities resident in the intestines of humans who, being of different nationalities, had different diets and lifestyles (Lay *et al.*, 2005).

To investigate these topics further, we compared the DNA profiles of bacteria associated with biopsies collected from inflammatory bowel disease (IBD) patients and non-IBD subjects examined in Edmonton, Canada, and Mexico City, Mexico. For the latter subjects, we compared the profiles generated from biopsy-associated bacteria with those of aspirates and faeces collected from the same individuals.

METHODS

Human subjects. Intestinal biopsies (rectal and transverse colon) were obtained from patients recruited to the study at two medical centres: the Instituto Nacional de Ciencias Medicas y Nutricion Salvador Zubrian (INCMNSZ) and the University of Alberta. The approval of the ethics committees at the institutions involved in collecting samples was obtained (University of Alberta Faculty of Medicine Research Ethics Board, permit 4833; INCMNSZ Medical Ethics Committee, permit 1250) and laboratory manipulations were approved by the Faculty of Agriculture, Forestry and Home Economics Research Ethics Board (permit 0302) at the University of Alberta. Written consent was given by all participants prior to collection of samples. Biopsies were collected during endoscopy, after standard bowel cleansing of subjects (NuLYTELY or GoLYTELY) and were stored at -70°C until examination. Diagnosis of IBD was made according to established criteria by a pathologist at each medical centre before shipment of the specimens on dry ice to the analytical laboratory at the University of Alberta. Biopsy and faecal samples and aspirates were obtained from 15 of the Mexican subjects; a complete set of samples was not obtained from the remaining subjects. Aspirates were predominantly from the caecal region of the large bowel. Faecal samples were collected prior to bowel cleansing. Patients with conditions that indicated colonoscopy but who were found to be free of intestinal inflammation were included as non-IBD subjects. Patients were excluded if they had consumed probiotic products or yogurt (more than three servings per week). The number of patients recruited and their diagnoses and treatments are shown in Table 1.

Biopsies. Immediately after collection, biopsy samples were agitated in a buffered solution [5 mM Tris/HCl (pH 8.0), 4 mM Sigma 7-9, 150 mM NaCl]. The buffer was then removed and replaced with fresh solution, and the process was repeated three times. The tissue, in buffer, was then stored at -70°C . DNA was extracted from the biopsies, faeces and aspirate as described previously (Tannock *et al.*, 2004; Bibiloni *et al.*, 2006).

Table 1. Human subjects

Characteristic	Mexico (n=38)	Canada (n=64)
Mean age (range) in years	46 (22–78)	46 (24–75)
Gender (male/female)	14/24	34/30
Diagnosis		
Crohn's disease	2	24
Ulcerative colitis	17	20
Non-IBD	19	20
Treatment		
Antibiotics	0	5
Sulfasalazine	3	3
Mesalazine	13	19
Steroids	2	13
Smokers	5	15

Generation of temporal temperature gradient gel electrophoretic (TTGE) profiles and identification of bacterial origins of DNA.

Amplification of 16S rRNA gene sequences from bacterial DNA was achieved using the universal bacterial primers HDA1-GC and HDA-2 and a previously described PCR program (Tannock *et al.*, 2000). PCR products (200 bp) were checked by electrophoresis in a 2 % agarose gel and then subjected to TTGE analysis using a DCode universal mutation detection system apparatus (Bio-Rad) on a 6 % polyacrylamide gel prepared in $1.25 \times \text{TAE}$ [50 mM Tris/HCl (pH 8.3), 25 mM acetic acid, 1.25 mM EDTA] and containing 7 M urea (Merck). TEMED (Sigma) (40 μl) and 10 % ammonium persulfate (Bio-Rad) (400 μl) were added to 40 ml polyacrylamide/urea solution prior to pouring the gels. Samples of PCR product (35 μl) were loaded in each well of the electrophoretic gel, which was run at a fixed voltage of 40 V for 16 h with an initial temperature of 63°C and a final temperature of 70°C (ramp rate of $0.4^{\circ}\text{C h}^{-1}$). For better resolution, voltage was fixed at 20 V for 15 min at the beginning of electrophoresis (Seksik *et al.*, 2003). A reference sample with bands distributed throughout the whole gel was included in all runs to normalize profiles. After the runs, gels were stained with ethidium bromide solution ($5 \mu\text{g ml}^{-1}$) for 20 min, washed with deionized water for 20 min and viewed by UV transillumination. TTGE profiles were compared using Dice's similarity coefficients with the BioNumerics software package (version 4.01; Applied Maths) at a sensitivity of 2–3 %. Graphics generated to show only the positions of fragments in gels showed that even faintly staining DNA fragments were included in the analysis. DNA fragments of interest were selected on the basis of the common presence of DNA with similar electrophoretic migration in Mexican samples (Fig. 1, mid- or lower-gel fragments 3–8 and 17) or Canadian samples (Fig. 1, upper-, mid- or lower-gel fragments 1, 2 and 9–16). The fragments were excised from the polyacrylamide gels using sterile scalpel blades, placed in 100 μl diffusion buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1 % SDS, pH 8.0) and stored overnight at 4°C to allow elution of the DNA. DNA was recovered and treated with S1 nuclease (Roche) as described previously (Tannock *et al.*, 2004). S1-treated DNA fragments were used as templates in PCR with the HDA primers prior to ligation in a cloning vector. The amplicons were cloned in the pCR2.1 TOPO plasmid vector (Invitrogen), and One Shot Top10 (Invitrogen) competent *Escherichia coli* cells were chemically transformed as described by the supplier. Recombinant cells were cultured on Luria-Bertani (LB; Becton Dickinson) agar plates containing 100 μg ampicillin ml^{-1} (Sigma) and 40 μg X-Gal ml^{-1} (Sigma). Four white colonies from each transformation reaction were transferred to a second LB plate containing ampicillin/X-Gal to confirm colour selection. Plasmids were isolated from colonies using a Wizard *Plus* SV minipreps DNA purification system (Promega) according to the manufacturer's

Fig. 1. Dendrogram showing relatedness of biopsy-associated bacterial profiles. The majority of Mexican profiles were greater than 74% similar (shown clustered within a box). Numbers correspond to excised fragments used to obtain identification in Table 2. CD, Crohn's disease; E, Edmonton; H, non-IBD; M, Mexico; UC, ulcerative colitis.

instructions. Prior to sequencing, the plasmid DNA and the original template DNA, from which the band was excised, were amplified using the HDA1-GC and HDA-2 primers and were checked by TTGE. Only products that migrated as a single band and to the same position with respect to the original sample were used for sequencing. Cloned sequences were amplified using plasmid-targeted primers (M13 forward 5'-GTAAAACGACGGCCAG-3' and M13 reverse 5'-CAGGAAACAGCTATGAC-3') and the following PCR program: 94 °C for 4 min and 25 cycles of 94 °C for 1 min, 56 °C for 1 min, 72 °C for 2 min, followed by 72 °C for 7 min. Amplified DNA (4 µl) was used as the template for sequencing. The dideoxy chain terminator reaction was conducted using the M13 reverse primer, a CEQ DTCS kit (Beckman Coulter) and a CEQ8000 genetic analyser (Beckman Coulter) following the manufacturer's instructions. The sequences retrieved were compared with the GenBank database using the BLASTN algorithm (Altschul *et al.*, 1990). Although of short length, the sequences gave high-resolution gels and provide sufficient taxonomic power to provide useful bacterial identifications (Bibiloni *et al.*, 2005; Snart *et al.*, 2006).

RESULTS

Comparison of TTGE profiles of biopsy-associated bacteria

A dendrogram comparing the similarity among profiles of Crohn's disease patients, ulcerative colitis patients and non-IBD subjects from Edmonton and Mexico City is

shown in Fig. 1. There were 10–20 16S rRNA gene fragments per profile. Strikingly, 30/36 biopsy profiles collected from Mexican subjects clustered at a node with 74.0% similarity and 58/62 Canadian profiles clustered at the 68.9% level. Two Canadian and two Mexican samples did not produce profiles of acceptable quality for analysis. IBD patients could not be differentiated from non-IBD subjects on the basis of TTGE profiles generated from biopsy-associated bacteria. Thus, the profiles clustered according to national (geographical) origin of the subjects rather than disease status.

Bacterial origin of selected 16S rRNA gene fragments

BLASTN alignments of sequences obtained from DNA eluted from TTGE gels with those of the GenBank showed that commonly occurring (based on common migration distances), intensely stained fragments in the upper part of the gels were likely to be of *Bacteroides* origin, and in the lower regions of gels were likely to be from *Bifidobacterium* or *Collinsella* (Fig. 1, Table 2). Intensely stained fragments in the middle of gels were likely to have originated from *Enterobacteriaceae* in the case of Canadian samples, or unknown or known anaerobic bacteria in the case of samples from Mexico (Fig. 1, Table 2).

Table 2. Bacterial origins determined by sequencing cloned 16S rRNA gene fragments (200 bp)

DNA fragment no.	Identity (%)	Closest relative	GenBank accession no.
1	98.9	Uncultured bacterium	AY343263
	98.3	<i>Collinsella aerofaciens</i>	AJ245920
2	96.6	Uncultured bacterium	AY668499
3	99.4	' <i>Roseburia faecalis</i> '	AY804150
4	99.0	Uncultured bacterium	AF128638
5	99.4	Uncultured bacterium	AY916300
6	100	<i>Bifidobacterium adolescentis</i>	AB125906
	100	<i>Bifidobacterium ruminantium</i>	D86197
	100	<i>Bifidobacterium thermacidophilum</i>	AY166524
		<i>Bifidobacterium pseudocatenulatum</i>	AY166523
7		<i>Bifidobacterium catenulatum</i>	AF432082
	100	Uncultured bacterium	AF132279
8	98.5	<i>Bacteroides vulgatus</i>	AB050111
9	97.4	<i>Bacteroides acidifaciens</i>	AB021157
10	97.4	<i>Ruminococcus gnavus</i>	X94967
11	98.8	<i>Clostridium polysaccharolyticum</i>	X77839
12	100	<i>Shigella boydii</i>	AY696681
	100	<i>Escherichia coli</i>	AF527825
	100	<i>Hafnia alvei</i>	Z83203
	100	<i>Serratia marcescens</i>	AF076038
13	100	Uncultured <i>Bacteroides</i> sp.	AY494687
14	100	<i>Shigella boydii</i>	AY696681
	100	<i>Escherichia coli</i>	AF527825
	100	<i>Hafnia alvei</i>	Z83203
	100	<i>Serratia marcescens</i>	AF076038
15	99.4	<i>Ruminococcus gnavus</i>	X94967
16	96.6	Uncultured bacterium	AY668449
17			

Comparison of profiles generated from biopsies, aspirates and faeces

Pairwise calculations showed that there was a high degree of similarity between the bacterial collections associated with biopsies, aspirates and faeces [biopsy/aspirate similarity (mean $\pm$ SD) $83.8 \pm 7.9\%$; aspirate/faeces $80.2 \pm 10.6\%$; biopsy/faeces $79.2 \pm 9.9\%$; $n=15$]. Thus, the profiles generated from the three types of specimen did not differ significantly ($P>0.05$ by t -test using SigmaStat; Systat Software). Examples of TTGE profiles comparing biopsy, aspirate and faecal samples are shown in Fig. 2.

DISCUSSION

Our study has shown that the TTGE profiles generated from bacteria associated with biopsies are influenced by geographical origin. TTGE profiles generated by universal bacterial PCR primers represent at least 90% of the inhabitants of the bacterial community (Zoetendal *et al.*, 1998). Although there are inherent biases in analytical methods requiring PCR, our study was strictly comparative

and all DNA samples used in electrophoresis were obtained using identical extraction and PCR amplification procedures. The influence of geographical origin is a particularly important observation for at least two reasons. Firstly, Canada has the highest incidence and prevalence of Crohn's disease so far reported worldwide, whereas this disease is rare in Mexico (Pérez Torres *et al.*, 1992; Bernstein *et al.*, 2006). Thus, the bowel microbiota of Canadians may contain bacterial species critical to fuelling chronic immune inflammation in hosts with dysfunctional immune systems, whereas most Mexicans may lack them. It was noteworthy that TTGE profiles of Canadians commonly contained DNA fragments originating from members of the *Bacteroidetes*, a bacterial group that has been associated with biopsies of Crohn's disease patients in a previous study (Bibiloni *et al.*, 2006), as well as with an experimental animal model of colitis (Rath *et al.*, 1999; Bibiloni *et al.*, 2005). Secondly, although it has been assumed that lifestyle, including different dietary components, could impact the composition of commensal communities inhabiting the bowel, supporting evidence has been difficult to obtain. Analytical methods used to compare community composition in the faeces of humans of different geographical origins have been conducted at a broad phylogenetic level (Lay *et al.*, 2005) and may have lacked the necessary sensitivity. There is evidence that dietary supplementation with oligosaccharides can increase the metabolic activity of specific bacteria in the bowel (Tannock *et al.*, 2004), but the genetic constitution of the human host is likely to be the most important factor that dictates, through physiological idiosyncrasies, the composition of the bacterial community (Rawls *et al.*, 2006). The lack of concurrence between the results of studies of Crohn's disease and ulcerative colitis patients conducted in disparate nations inhabited by populations of differing ethnicities is perhaps, therefore, not anomalous.

Our observations also revealed that, despite washing of the biopsy samples immediately after collection, the TTGE profiles of biopsy-, aspirate- and faeces-associated bacteria were highly similar. This result supports the view that bacteria detected in association with biopsies are mostly contaminants from a faecal solution (aspirate) that pools in the bowel and bathes the mucosal surface after bowel cleansing. As reported in other studies, biopsy-associated bacterial profiles do not differ between small and large bowel sites (Lepage *et al.*, 2005; Bibiloni *et al.*, 2006), a surprising observation when the differing compositions of the microbiota in the two sites are considered (Hayashi *et al.*, 2005). This suggests mucosal contamination from the faecal fluid, rather than seeding of the fluid by mucosa-associated bacteria. The endoscope may be contaminated with faecal fluid during insertion to the site of biopsy collection, thus leading to contamination of the sample. Our results contradict the conclusions of Zoetendal *et al.* (2002) who reported biopsy-associated profiles and faecal profiles of ten Dutch subjects to be different. The selected profiles reproduced in their paper appeared visually

Fig. 2. Examples of TTGE profiles obtained from biopsy, aspirate and faecal samples. Similarities (%) derived by BioNumerics software analysis are shown for each biopsy/aspirate comparison.

similar, although mean similarity between faecal and biopsy profiles was reported to be about 50 %, but with a large SD. These and other investigators have not compared aspirate and biopsy bacterial profiles, nor have they commented on the potential contamination of the mucosa and instrumentation by bowel fluids.

Evidence that nationality/ethnicity influences the composition of the biopsy-associated bacterial profiles, and that these bacteria are likely to be contaminants from the residual bowel cleansing fluid, further confounds the already difficult task of investigating the role of gut commensals in the pathogenesis of Crohn's disease and ulcerative colitis. Inter-individual variation in the composition of bowel communities (Zoetendal *et al.*, 1998; Lay *et al.*, 2005), even at the level of bacterial strains (McCartney *et al.*, 1996), and a 16S rRNA gene database that has been polluted with unreliable sequences (Ashelford *et al.*, 2005), together with our novel observations, all indicate that realistic comparisons of human bowel communities are difficult to achieve. Certainly, our results show that the same information can be obtained from the bacteriological examination of aspirated bowel fluid as from biopsies, thus removing the need for biopsy collection and hence lowering the risk to patients.

ACKNOWLEDGEMENTS

The support of the Crohn's and Colitis Foundation of Canada is gratefully acknowledged. G. W. T. was supported by the Alberta Value Added Corporation.

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