MICROBIAL ECOLOGY

Mucosa-associated bacterial flora of the human colon

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Biopsy samples of mucosa were taken during colonoscopy from the proximal colon and rectum of 12 patients, six with ulcerative colitis (UC) and six with non-inflammatory conditions. After anaerobic transport to the laboratory, biopsy specimens were examined by quantitative bacteriological culture on selective and non-selective media for total aerobic count, total anaerobic count, *Bacteroides* spp., lactobacilli, bifidobacteria and asaccharolytic, lactic acid producers. Isolates of the genus *Bacteroides* were identified to species level. Counts from proximal colonic and rectal biopsy samples in the same patient were not significantly different. Viable aerobic counts (aerobes and facultative organisms) ranged from 2.4×10^3 to 1.3×10^6 cfu/sample biopsy (5.6 mg) and total anaerobic counts were 10 – 10^2 times higher at (1.4×10^5) – (3×10^7) cfu/sample. *Bacteroides* spp. predominated at both sites (range 8.6×10^4 to 1.4×10^7 cfu/sample), comprising 66% of total counts from proximal colon (range in individual patients 31–80%) and 68.5% from rectum (range 38–91%). Lactobacilli were isolated from eight biopsy samples from five patients, counts ranging from 3.6×10^2 to 1×10^5 cfu/sample; bifidobacteria were isolated from both sites from 10 of the 12 patients, counts ranging from 50 to 1.8×10^6 cfu/sample. From the 24 biopsy samples, 235 isolates representing 11 species of *Bacteroides* were identified. For any individual patient, only a few species (2–7; mean 4.4) of *Bacteroides* were found, with just one or two species predominating. *B. vulgatus* was cultured from both samples of seven patients (where it was the major isolate in four) and from single samples of two others; *B. fragilis* was cultured from both sites in six patients, being the major isolate in one patient and second commonest in three, but was not detected in the other six; the majority of other isolates were *B. merdae/distasonis*, *B. ovatus*, *B. thetaiotaomicron* and *B. uniformis*. *B. thetaiotaomicron* was isolated from both biopsy samples in all three UC patients with active inflammation (16 of the 60 isolates from these patients) but from only four of the other 18 samples from non-inflamed colonic mucosa (nine of 175 isolates).

Introduction

In faeces, strictly anaerobic bacteria outnumber facultative species by a factor of 10^2 – 10^3 , with concentrations of anaerobes reaching levels of 10^{10} – 10^{12} cfu/g [1]. The predominant cultivable species in faeces belong to the genus *Bacteroides*, and the majority of these species are well characterised, belonging to the former '*B. fragilis* group'. Since the recent changes in classification of the gram-negative anaerobic rods, the intestinal bacteroides represent the entire genus *Bacteroides sensu stricto*. *B. vulgatus* is usually considered

one of the most common faecal bacteroides, with *B. fragilis* being one of the less common [2].

Bacteria of the commensal intestinal flora possess a range of important nutritional, immunological, metabolic and pathophysiological properties, and it seems likely that those in the immediate vicinity of the mucosal surface will be particularly relevant to some of these functions. In contrast to an enormous amount of literature on the bacteria of faeces, there are few comprehensive published data on the quantitative bacteriology of the mucosa-associated bacteria of the human colon. Most quantitative studies, e.g., those of Peach and Tabaqchali [3], Hudson *et al.* [4], Hudson [5] and Hartley *et al.* [6] did not identify bacteria to species level. For colonic mucosa-associated counts, the reported difference between strict anaerobes and

facultative organisms is not of the same order as in faeces, the ratio being nearer unity, with some studies showing anaerobes outnumbering facultative organisms by a factor of between 5 (biopsy samples) and 40 (resected samples) [5]. There is a single report by Namavar *et al.* [2], in which the colonic luminal and mucosa-adherent *Bacteroides* species in 10 patients with colon cancer were assessed quantitatively in faeces, gut lavage fluid and biopsy samples from operative resection specimens. *B. fragilis* was found with a low frequency in the faeces (only 4% of the total *Bacteroides* spp.), but had a more intimate association with the gut mucosa than other *Bacteroides* spp. representing 42% of the total bacteroides. The authors suggested that this might explain why *B. fragilis* is commonly isolated from patients with gut-associated abdominal sepsis.

In the present study, endoscopic biopsy samples from the proximal colon and rectum of 12 patients (six with ulcerative colitis) were examined by quantitative aerobic and anaerobic culture and the individual *Bacteroides* species in each patient were identified.

Materials and methods

Patients and specimens

Endoscopic biopsy samples from 12 patients (seven men and five women; age range 21–67 years, mean 48) in whom colonoscopy was being done in the course of routine clinical care were examined. Six had chronic ulcerative colitis (UC); this was clinically, endoscopically and histologically inactive in three cases, and three patients had active disease with mucosal inflammation. Two UC patients, both with inactive disease, were taking maintenance sulphasalazine (containing sulphapyridine). Of the remaining six patients, three had previous colonic neoplasms, two had irritable bowel syndrome and one had diverticulosis. Because, with one exception discussed below, there were no differences in the bacteriological findings in UC and other patients, data for all 12 patients are presented together.

Preparation for colonoscopy was as follows: clear fluids only by mouth for 48 h before the procedure; co-danthrusate 50/60, two capsules 60 h before and two capsules 36 h before the procedure; and Picolax (Ferring Pharmaceuticals, Feltham, Middlesex) one sachet 24 h and one sachet 16 h before the procedure. Colonoscopy was performed with midazolam and pethidine sedation; instruments and biopsy forceps were cleansed with glutaraldehyde (Cidex: Johnson and Johnson, Skipton, Yorkshire). Forceps were rinsed in sterile saline before sampling. In each patient, one biopsy sample was taken from the proximal colon (ascending colon in 10, transverse colon in two) and one from the rectum. The mucosal samples were immediately (within 30 s) transferred with a sterile

needle from the forceps into the transport system (see below). Previously, a separate evaluation had shown that the weight of 21 biopsy samples taken with the standard forceps was reasonably consistent (5.6 mg, SD 1.6 mg).

This use of colonic mucosal biopsies was approved by the Lothians Medicine and Medical Oncology Ethics Committee.

Processing of biopsy material and quantitative bacteriology

Biopsy samples were transported to the laboratory within 45–60 min after collection in the Anatube type II anaerobic transport system [7] containing 0.2 ml of Stuart's transport medium (Unipath) supplemented with yeast extract (Unipath) 0.5%. In the laboratory, the whole transport system was placed inside an anaerobic cabinet (N₂ 80%, H₂ 10%, CO₂ 10%) and the biopsy sample and the 0.2 ml of transport medium were transferred into a sterile 'Stomacher' bag, together with 1.8 ml of pre-reduced anaerobe investigation medium (AIM) [8]. The sample was broken up by squeezing it manually through the bag, and then the suspension was vigorously aspirated into and out of a Pasteur pipette to ensure complete homogenisation. For anaerobic incubation, 10-fold dilutions were made in pre-reduced AIM, 20- μ l volumes were seeded on to the culture plates and the inoculum was spread with a sterile glass spreader. Portions of the relevant dilutions were transferred out of the cabinet to inoculate plates for aerobic incubation. After incubation at 37°C, plates were examined and colonies were counted after 2–3 days and again after 7 days. The total anaerobic counts (as indicated in Table 1) included strict anaerobes and facultative organisms.

The following media were used: Columbia Agar (Unipath) supplemented with horse blood 5% for total counts of aerobic and facultative organisms (BA); Fastidious Anaerobe Agar (Lab M) supplemented with horse blood 4% and freeze-thaw lysed horse blood 1% for total anaerobic counts (FAA); FAA supplemented with kanamycin 75 mg/L and vancomycin 2.5 mg/L for *Bacteroides* spp. (FAAKV); Rogosa Agar (Unipath) for *Lactobacillus* spp. (RA); Reinforced Clostridial Cotton Blue Agar [9] for *Bifidobacterium* spp. (RCCBA).

Identification of isolates

This was done initially by examination of colonial morphology on primary isolation plates and cell morphology in gram-stained films. Isolates from RA and RCCBA that were gram-positive rods were subcultured to AIM broth containing glucose 1% and the resultant culture was analysed by gas chromatography for short-chain fatty acids [8]. Isolates that produced only lactic acid in major amounts were

Table 1. Summary of quantitative bacteriology of 24 biopsy samples from 12 patients

Diagnosis	Biopsy site	Viable counts (cfu/sample)					Asaccharolytic lactic acid producers
		Total aerobic count	Total anaerobic count	<i>Bacteroides</i> spp.	Lactobacilli	Bifidobacteria	
UC active	AC	1.4×10^5	9.4×10^5	7.4×10^5	—	1.4×10^3	—
	R	2.4×10^5	$c.9.0 \times 10^6$	$c.8.6 \times 10^6$	$c.1.0 \times 10^5$	$c.6.4 \times 10^4$	—
UC active	AC	3.8×10^4	2.4×10^6	1.5×10^6	1.1×10^3	$c.4.2 \times 10^4$	—
	R	2.4×10^5	9.2×10^6	5.8×10^6	2.2×10^3	$c.1.2 \times 10^5$	—
UC active	PTC	4.8×10^4	8.6×10^5	7.0×10^5	—	1.0×10^2	—
	R	3.0×10^3	1.4×10^5	8.6×10^4	—	5.0×10^1	—
UC inactive	AC	4.0×10^4	3.2×10^6	2.2×10^6	—	—	—
	R	6.0×10^4	9.0×10^6	6.2×10^6	—	—	—
UC inactive	AC	3.0×10^4	7.8×10^6	2.4×10^6	—	1.9×10^6	—
	R	2.2×10^4	3.8×10^6	1.4×10^6	—	9.8×10^3	—
UC inactive	AC	1.8×10^5	2.4×10^6	1.6×10^6	—	6.0×10^2	—
	R	1.3×10^5	1.1×10^6	5.4×10^5	—	6.0×10^2	—
DD	AC	$c.1.3 \times 10^6$	$c.4.0 \times 10^6$	$c.2.0 \times 10^6$	—	5.0×10^4	—
	R	1.4×10^5	5.6×10^5	2.8×10^5	—	9.8×10^3	—
IBS	AC	1.6×10^5	1.1×10^6	8.6×10^5	—	1.5×10^5	—
	R	4.8×10^4	2.2×10^5	1.6×10^5	—	4.0×10^4	—
IBS	AC	8.8×10^3	1.5×10^6	1.2×10^6	—	4.6×10^4	—
	R	5.4×10^3	1.5×10^6	8.4×10^5	$3.8 \times 10^4*$	9.0×10^3	—
Previous Ca AC	PTC	$c.7.6 \times 10^5$	5.2×10^6	3.4×10^6	1.0×10^4	2.8×10^3	2.4×10^3
	R	6.2×10^5	6.0×10^6	2.6×10^6	2.2×10^4	5.6×10^3	$c.6.0 \times 10^4$
Previous Ca AC	AC	3.6×10^5	3.0×10^7	1.4×10^7	1.1×10^3	1.9×10^3	—
	R	1.1×10^5	9.4×10^6	8.2×10^6	3.6×10^2	3.0×10^2	—
Adenoma Fup	AC	2.8×10^3	1.4×10^6	1.1×10^6	—	—	$c.7.0 \times 10^3$
	R	2.4×10^3	1.6×10^6	1.3×10^6	—	—	$c.3.2 \times 10^3$

UC, ulcerative colitis; DD, diverticulitis disease; IBS, irritable bowel syndrome; Ca AC, cancer, ascending colon; Fup, follow up; AC, ascending colon; R, rectum, PTC, proximal transverse colon; —, not isolated, <50 cfu/sample.

*May be bifidobacteria.

considered to be *Lactobacillus* spp. and those that produced major amounts of acetic and lactic acids in a ratio of >1:1 were considered to be *Bifidobacterium* spp. Colonies that were *Bacteroides*-like (0.5–4 mm, round and entire, convex, smooth or mucoid, shiny, translucent or opaque, grey, haemolytic or non-haemolytic) from FAAKV and were gram-negative bacilli or cocco-bacilli were subcultured to AIM-cooked meat broth and identified [8]. Ten such colonies were selected from the dilution that gave 100–200 colonies on the FAAKV plate for identification to species level.

Results

There were no significant differences between the counts from proximal colonic and rectal biopsy samples from the same patient. Aerobic viable counts ranged from 2.4×10^3 to 1.3×10^6 cfu/sample, and total anaerobic counts (essentially, total counts) 10 – 10^2 -fold higher at 1.4×10^5 to 3.0×10^7 cfu/sample (Table 1). Facultative anaerobes (essentially *Escherichia coli* and other coliforms) were included in both counts. In most cases, where the difference in counts was ≥ 10 -fold, the total aerobic count was equivalent to the *E. coli* count and the total anaerobic count represents strict anaerobes. However, where the counts are of a similar order of magnitude, a significant proportion of the anaerobic count will be made up of facultative organisms.

Bacteroides spp. predominated in the flora at both sampling sites (range 8.6×10^4 to 1.4×10^7 cfu/sample), comprising 66% of the total anaerobic counts from proximal colon (range in individual patients 31–80%) and 68.5% of total anaerobic counts (range 38–91%) from rectum. Lactobacilli were isolated from eight biopsy samples from five patients, with counts in the range (3.6×10^2) – (1×10^5) cfu/sample. Bifidobacteria were isolated from both samples taken from 10 of the 12 patients, with counts in the range 50 – (1.9×10^6) cfu/sample. Asaccharolytic lactic acid producers were isolated in low numbers – (2.4×10^3) – (6×10^4) cfu/sample – from only two patients, neither of whom had inflammatory conditions.

From the 24 biopsy samples, 235 isolates representing 11 species of *Bacteroides* were identified (Table 2). Generally, there was concordance between the proximal colon and rectum, as to the presence or absence of a particular species. For any individual patient, only a few species (2–7: mean 4.4) of *Bacteroides* were found, with just one or two species predominating. *B. vulgatus* was cultured from both samples of seven patients (where it was the major isolate in four) and from single biopsy samples of two others. *B. fragilis* was cultured from both sites in six of the 12 patients, being the major isolate in one patient and the second most frequent in three, but was not detected in the other six. The majority of other isolates were *B. merdae/distasonis*, *B. ovatus*, *B. thetaiotaomicron* or *B. uniformis*.

Table 2. Details of *Bacteroides* species and other gram-negative anaerobic bacilli isolated from biopsy samples

Diagnosis	Biopsy site	Total	Indole-negative <i>Bacteroides</i>							Indole-positive <i>Bacteroides</i>					Other GNAB
			<i>B. fragilis</i>	<i>B. vulgatus</i>	<i>B. m/d</i>	<i>B. caccae</i>	<i>B. sp.</i>	<i>B. ovatus</i>	<i>B. tho</i>	<i>B. uniformis</i>	<i>B. v/s</i>	<i>B. splanchnicus</i>			
UC active	AC	10	0	3	0	0	0	2	3	2	0	0	0	0	
	R	10	0	5	0	0	0	2	2	1	0	0	0	0	
UC active	AC	11	6	0	2	0	0	1	2	0	0	0	0	0	
	R	9	1	0	3	0	0	0	5	0	0	0	0	0	
UC active	PTC	10	0	4	1	0	0	1	2	1	0	0	0	0	
	R	10	0	5	1	0	0	0	2	0	0	0	0	0	
UC inactive	AC	10	3	0	3	0	0	2	1	1	0	0	0	0	
	R	10	1	2	4	0	0	2	0	0	0	0	1	0	
UC inactive	AC	10	0	0	6	0	0	0	0	0	0	0	0	0	
	R	10	0	0	4	0	0	4	0	0	0	0	0	0	
UC inactive	AC	10	1	6	2	0	0	0	0	1	0	0	0	0	
	R	10	2	5	2	0	0	0	0	0	1	0	0	0	
DD	AC	9	2	3	0	0	0	0	0	0	0	0	0	0	
	R	8	2	0	2	1	0	4	0	0	0	0	0	0	
IBS	AC	9	7	2	0	0	0	0	0	0	0	0	0	0	
	R	9	8	1	0	0	0	0	0	0	0	0	0	0	
IBS	AC	10	0	8	0	1	0	0	0	0	0	0	0	1	
	R	10	0	6	2	0	0	0	0	2	0	0	0	0	
Previous Ca Ac	PTC	10	0	8	0	0	0	2	0	0	0	0	0	0	
	R	10	0	4	0	0	0	6	0	0	0	0	0	0	
Previous Ca Ac	AC	10	1	0	4	0	0	0	3	1	1	0	0	0	
	R	10	2	0	2	0	0	0	3	1	1	0	0	0	
Previous adenoma	AC	10	0	4	1	0	0	0	2	2	0	0	1	0	
	R	10	0	1	0	0	0	1	0	7	0	0	1	0	
Total isolates	...	235	36	67	39	2	4	35	25	19	4	3	1	1	

B. m/d = *B. merdae/distasonis*; *B. sp.* = *Bacterioides* species; *B. tio* = *B. thetaiotaomicron*; *B. v/s* = *B. variabilis/stercoris*.
See footnote to Table 1 for diagnosis and biopsy site.

B. thetaiotaomicron was isolated from both biopsy samples from all three UC patients with active inflammation (16 of the 60 isolates from these patients) but from only four of the other 18 biopsy samples from non-inflamed colonic mucosa (nine of the 175 isolates).

Discussion

It is likely that mucosal adherence of colonic bacteria is influenced by factors within the lumen, properties of the organisms themselves and the local immune response. Many of the bacteria in the gastrointestinal tract, especially the gram-negative rods, are opportunist pathogens and are capable of eliciting injurious, pro-inflammatory responses if viable bacteria or soluble cell-surface components interact with cells of the systemic immune system. Therefore, it is important to know which species are in the most intimate association with the mucosa, and how the immune response to these species is mounted and controlled.

There are probably several reasons why there are few published data on this important topic. Faeces, and the contents of the intestinal lumen, are readily available for study whereas there are logistic and ethical difficulties in obtaining fresh biopsy tissue to allow investigation of the mucosal flora. In the past, tissue has become available mainly as operative specimens [2, 5]. In these, the microbial flora may have been influenced by pre-operative preparation which would normally include starvation, bowel cleansing and often antibiotic treatment pre-operatively; intra-operatively, oxygenation and viability of the colon will have been influenced by the operative technique and, in particular, the amount of time between interruption of the arterial supply and removal of the specimen from the body to allow samples to be collected for study.

The size of the clinical specimen, resected material or biopsy, varies greatly, and in most studies, the number of bacteria is related to weight (of sample) as the unit of measurement, even though surface area is much more appropriate. In previous studies, specimens have

varied from *c.* 3 cm² of visually normal colon dissected from resected specimens [2], to *c.* 5-mg biopsy samples [3]. By weight, mean total counts obtained were up to 6×10^7 cfu/g of tissue [2], but most studies have reported counts of around 10^6 cfu/g [3, 6]. If the results of the present study were expressed per gram of tissue, the counts would be up to 5×10^9 cfu/g. Even acknowledging the possible errors of using counts/unit weight, the techniques employed in the present study appear to have achieved much higher isolation rates than those of other studies. Nevertheless the ideal denominator is probably mucosal surface area rather than biopsy weight. The surface area of the biopsy samples in this study was estimated as 20 mm².

The proportions of different *Bacteroides* spp. identified in this study were different from those reported in the only other study where *Bacteroides* spp. were identified, especially in regard to *B. fragilis*. This comparison is summarised in Table 3, but it should be noted that the study by Namavar *et al.* was performed on colonic mucosal specimens obtained at operation.

Preparation for colonoscopy has the potential to influence the colonic flora. Patients in this study were given stimulant laxative treatment for 3 days and a fluid diet for 2 days before colonoscopy. Nevertheless the observation that the mucosa-associated flora of the rectum and the proximal colon are similar indicates that valuable information may be obtained from rectal or sigmoid biopsy samples which are readily collected from an unprepared colon. Although two of the UC patients in the present study were receiving sulphasalazine, a recent study on the use of this drug in the treatment of UC concluded that it had little effect on the bacteria colonising the colorectal mucosa [6].

In anaerobic infections outside the gastrointestinal tract it is well established that certain species of *Bacteroides* are much more likely to be isolated than others, and this has been related to the relative virulence, or pathogenic potential of the species. *B. fragilis* is the *Bacteroides* species most commonly isolated from clinical specimens, ranging from 78% in

Table 3. Comparison of results with those of Namavar *et al.* [2]

<i>Bacteroides</i> species	Percentage of <i>Bacteroides</i> isolates		
	Namavar <i>et al.</i> [2]		This study (mucosa)
	Faeces	Mucosa	
<i>B. fragilis</i>	4.4	42.8	15.6
<i>B. vulgatus</i>	45	25.6	28.1
<i>B. distasonis</i>	9.3	9.8	16.9
<i>B. uniformis</i>	22.2	10.3	7.4
<i>B. thetaiotaomicron</i>	15.1	8	10.8
<i>B. ovatus</i>	3.6	3.6	15.2
Others	...	...	6.1

blood cultures and 69% in wounds in a survey in two military hospitals [10] to 54% in bacteroides infections of children [11]. *B. thetaiotaomicron* is usually the second most common species at 10–20% followed by *B. vulgatus*, *B. distasonis* and *B. ovatus* at <10%. The relative virulence of different *Bacteroides* spp. is not clearly understood, but has been ascribed to possession of a capsule [12], and resistance to serum complement [13–15] and to phagocytosis and intracellular killing [16, 17]. The complexity of unravelling these pathogenic mechanisms has been reviewed recently for *B. fragilis* [18], and the importance of the chemistry of the capsular polysaccharide in the mechanism of abscess formation by *B. fragilis* has been demonstrated [19, 20]. The lipopolysaccharides (LPS) of *Bacteroides* spp. vary in their biological activity (endotoxicity), with the LPS of *B. fragilis* being the most active in a range of models [21–23]. Most authors agree that *B. fragilis* is the most virulent species of the genus, and this was supported in the investigations cited above. Moreover, as the results of the present study did not confirm the previous suggestion that *B. fragilis* is particularly mucosa-associated, this indicates that the biological properties of this particular species of *Bacteroides*, rather than the fact that it is mucosa-adherent, are relevant to its pathogenic potential.

The mechanisms of virulence of the lesser pathogens of the genus are largely unexplored. The indication from this study that *B. thetaiotaomicron* is a possible inflammatory agent in UC does not correlate with the production of an LPS of high endotoxicity; the single strain of *B. thetaiotaomicron* investigated produced one of the least endotoxic of the LPS preparations in the model systems used [22], which suggests that other mechanism(s) may be operating.

Observations from parallel studies on the systemic [24] and local [25] antibody responses to *Bacteroides* LPS in health and disease are relevant to this study. Analysis of the local IgA response to *B. fragilis* and *B. vulgatus* in the colon, by measuring antibodies in a whole gut lavage fluid, has demonstrated that there is a major response to a common epitope within the LPS molecule of both species. Speculatively this may be a protective mechanism, neutralising potentially harmful endotoxin (LPS) within the healthy gut – the production of a cross-reactive antibody being much more efficient than multiple antibodies to all of the different serotype determinants [25]. Within the colon, the gut-associated lymphoid tissue may be multiply exposed to LPS from both the adherent and luminal (faecal) bacterial populations. Therefore, a response to common determinants might be more likely. However, a different mechanism appears to operate in the systemic immune system, where there is more evidence of the production of species-specific rather than cross-reactive anti-LPS IgG antibodies [24]. It is possible that individuals respond systemically more to

the mucosa-associated populations than to the faecal organisms, because of proximity and ease of translocation.

These studies have shown the variation of the bacterial populations of the colonic mucosa and the antibody responses to them both locally and systemically within an individual patient. Some common themes are becoming apparent, but much more work is required to attempt to relate the observations to the mechanisms that operate to maintain homeostasis, and to further knowledge of the complex relationship between the normal bacterial flora and the local and systemic immune responses in the gastrointestinal tract in health and disease.

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