INFLAMMATORY BOWEL DISEASE

High prevalence of *Escherichia coli* belonging to the B2+D phylogenetic group in inflammatory bowel disease

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Background: It is not clear which species of bacteria may be involved in inflammatory bowel disease (IBD). One way of determining which bacteria might be likely candidates is to use culture-independent methods to identify microorganisms that are present in diseased tissues but not in controls.

Aims: (1) To assess the diversity of microbial communities of biopsy tissue using culture-independent methods; (2) to culture the bacteria found in the tissues of patients with IBD but not in the controls; (3) to identify potential virulence factors associated with cultured bacteria.

Methods: 84 biopsy specimens were collected from 15 controls, 13 patients with Crohn's disease (CD) and 19 patients with ulcerative colitis (UC) from a population-based case–control study. Ribosomal intergenic spacer analysis (RISA) was conducted to identify unique DNA bands in tissues from patients with CD and UC that did not appear in controls.

Results: RISA followed by DNA sequencing identified unique bands in biopsy specimens from patients with IBD that were classified as Escherichia coli. Targeted culture showed a significantly ($p<0.05$) higher number of Enterobacteriaceae in specimens from patients with IBD. The B2+D phylogenetic group, serine protease autotransporters (SPATE) and adherence factors were more likely to be associated with tissues from patients with UC and CD than with controls.

Conclusions: The abundance of Enterobacteriaceae is 3–4 logs higher in tissues of patients with IBD and the B2+D phylogenetic groups are more prevalent in patients with UC and CD. The B2+D phylogenetic groups are associated with SPATE and adherence factors and may have a significant role in disease aetiology.

Inflammatory bowel disease (IBD) is a collective term for UC and CD.[1-3] These diseases are chronic inflammatory diseases of the digestive tract, potentially leading to severe inflammation, ulceration and obstruction, the end point of which may be surgical resection.[1-3] The high incidence of IBD in the Western world is not explained by simple genetic drift of a susceptible human genome, prompting the hypothesis that environmental factors are important in disease aetiology.[4 5]

These diseases are thought to be a result of recognition of a microbial antigen(s) by a dysfunctional immune system in a genetically predisposed host.[6 7] Many bacteria have been linked to IBD, but specific bacteria may have been missed because not more than 30% of the microbial diversity in the gut can be cultured.[8-12] Cultivation-independent methods now allow simultaneous surveys of microbial diversity.[11 12] We maintain that culture-independent surveys of microbial diversity should form a prelude to the targeted cultivation of bacteria. However, it is important to emphasise that, wherever possible, microorganisms identified using culture-independent methods, as being associated with disease, should be cultured so that their virulence mechanisms can be evaluated.

Several bacteria have been implicated in the aetiology of IBD, the most prominent among these being Mycobacterium paratuberculosis. Other bacteria that have been associated are members of the Enterobacteriaceae, Helicobacter pylori and Bacteroides species.[2 3] In this paper, we describe the use of ribosomal intergenic spacer analysis (RISA)[13] of biopsy tissue to identify bands that were consistently associated with tissue from patients with IBD. This allowed us to use highly focused cultivation methods, including resuscitation methods, to specifically culture Enterobacteriaceae.

MATERIALS AND METHODS

Study subjects

We utilised 84 biopsy specimens from 15 controls, 13 patients with Crohn's disease (CD) (3 with ileal disease, 6 with ileocolonic disease and 4 with isolated colonic disease) and 19 patients with ulcerative colitis (UC) (3 with proctitis, 8 with left-sided colitis and 8 with pancolitis) from a population-based case–control study undertaken at the University of Manitoba, Winnipeg, Manitoba, Canada (table 1) as described previously.[14]

In brief, a population-based study refers to a process by which selection of study subjects proceeds by accounting for bases related to various factors such as lifestyle (eg, smoking), geographical location (eg, urban vs rural), age, sex or ethnicity. In IBD research, it is challenging to obtain untainted biopsy controls because endoscopy is normally performed on persons only when it is clinically required. The controls were true controls in the sense that the subjects voluntarily submitted to endoscopy and were drawn from the same population-based study. No antibiotics were prescribed to any of the subjects during the 6 weeks before the colonoscopy.

Colonoscopy and biopsies

Following standard oral Fleet Phospho-soda (CB Fleet Company, Lynchburg, Virginia, USA) treatment, biopsy specimens were taken from the caecum and the rectum. In subjects with a previous caecal resection, biopsies were obtained from the right colon distal to the ileocolonic anastomosis. All biopsy

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Abbreviations: CD, Crohn's disease; IBD, inflammatory bowel disease; PAI, pathogenicity island; RFLP, restriction fragment length polymorphism; RISA, ribosomal intergenic spacer analysis; SPATE, serine protease autotransporters; UC, ulcerative colitis

Table 1 Biopsy samples used in this study*

Site	Controls (15 subjects, 28 tissues)		CD (13 patients, 27 tissues)		UC (19 patients, 29 tissues)		IBD (32 patients, 56 tissues)	
	End	Hist	End	Hist	End	Hist	End	Hist
Rectum	0(13)	0(13)	5(13)	5(13)	9(10)	9(10)	14(23)	14(23)
Caecum	0(15)	0(15)	4(9)	6(9)	2(15)	4(15)	6(24)	10(24)
Colon	0(0)	0(0)	3(5)	3(5)	2(4)	2(4)	5(9)	5(9)

CD, Crohn's disease; End, endoscopic examination; Hist, histological examination; UC, ulcerative colitis.
*Endoscopic and histological examination was made on biopsy specimens, and only histologically positive samples were considered inflamed. Total numbers of biopsies for each group are presented in parentheses, and the numbers not in parentheses represent inflamed biopsies.

samples were snap frozen in liquid nitrogen and stored at −70°C. Biopsy specimens were subject to standard histological staining with H&E for evaluation of inflammation. A site was considered inflamed if it had histological evidence of inflammation and uninflamed if it was histologically normal.

DNA extraction for RISA analysis
Tissue samples were suspended in 150 μl lysis buffer (10 mM Tris-HCl, pH 8.0; 5 mM EDTA, pH 8.0; 4 M guanidinium isothiocyanate, pH 7.5; 50 g sarcosyl/l, 2.5 g SDS/l, 5 g sodium citrate/l and 5 g Triton X-100/l). 300 μl of chloroform and Tris-saturated phenol (pH 6.9) were added to each tube. The samples were placed at −20°C for 1 h. Subsequently, samples were centrifuged in microfuge tubes at 4°C for 20 min at 10 000 g. Supernatants were transferred to fresh tubes. Isopropanol was added to 1/4 volume of the supernatants and the mixtures loaded on to silica-cellulose membranes in columns. Samples were allowed to filter through the membrane by gravity. The membranes were washed twice with 300 μl of 95% ethanol (by gravity). DNA was eluted with 400 μl hot (about 75°C) Tris-EDTA buffer (by gravity) and precipitated with two portions of 95% ethanol. The resulting pellets were suspended in 25 μl 0.5× Tris-EDTA buffer (pH 8.0) and stored at −20°C until further analysis.

Table 2 lists the primer sequences used for RISA and the amplified intergenic transcribed spacers between the 16S and 23S rDNA.[13] PCR products were subjected to electrophoresis using 2% agarose. DNA fragments found only in patients with UC and CD were purified from agarose gels, cloned into the pCR2.1-TOPO TA vector (Invitrogen, Carlsbad, California, USA) and sequenced. Standard bioinformatics analysis was used to taxonomically classify the sequence fragment.

Bacterial cultures
Once RISA analysis had determined that the bacteria that appeared in patients with UC and CD and not in controls were E coli, targeted bacterial cultivation was carried out. All cultivation of the bacteria was done with untreated biopsy tissue, and no procedures were used to wash the tissues or to remove the mucus. To ensure that as many E coli cells as possible were cultured, resuscitation in buffered peptone water was performed, followed by decimal dilution and culturing on chromogenic E coli/coliform medium (Oxoid CM0956; Oxoid, Nepean, Ontario, Canada). Resuscitation was done by incubation of the biopsy specimen in 1 ml of 100 mM buffered peptone water for 16 h at 37°C. Ten μl droplets were pipetted (Maxipettor, Eppendorf; Eppendorf AG, Hamburg, Germany) onto media, allowed to dry, and then inverted and incubated at 37°C. After 18 h, E coli (purple colonies) and non-E coli coliforms (blue, pink and white colonies) were counted separately.

Colour differentiation on chromogenic agar is a good first approximation of E coli identity. Five putative E coli (purple colonies) were picked from each positive tissue and subcultured

in Luria-Bertani broth, then recultured on E coli/coliform medium, and tested for reactivity to indole, methyl red, Vogues Proskauer and citrate utilisation to differentiate E coli from non-E coli. The 16S rDNA gene sequences for all the non-E coli were determined using standard primers (table 2).

DNA extraction from bacterial cultures
For DNA extraction from cultures, 1 ml suspensions of each culture were centrifuged and pellets were suspended in lysis buffer, and subsequently mixed with chloroform and Tris-saturated phenol. All other steps were the same as for the extraction of DNA from tissue samples.

Molecular analysis of bacterial cultures
Table 2 lists all primer sequences used for molecular analysis of bacterial cultures. Primers were either modified from published primers or newly designed for the purposes of our studies. PCR was done by standard methods after optimisation for specificity of primer pairs by adjusting annealing temperature and salt concentration. Amplified products were run on agarose gels as described above.

Statistical analysis
For statistical analysis of data, we applied a χ^2 test based on the Mantel–Haenszel method (Epi Info V.6.04).

RESULTS
A total of 84 biopsies were collected from 15 controls, 13 patients with CD and 19 patients with UC (table 1). In most cases, more than one biopsy specimen was obtained from multiple sites, or from adjacent sites from the same subject. DNA was extracted from each biopsy sample and subjected to RISA analysis (fig 1). We were able to identify bands (~450 bp) that were consistently present in approximately 70% of patients but in <30% of controls (fig 1). The bands in the controls were also of a much lower density than those from tissues of patients with IBD. Five bands from tissues of patients with IBD were retrieved from the gel, sequenced, aligned with Genbank sequence and found to be E coli.

Resuscitation of biopsies by resuspension in buffered peptone water, incubation for 16 h at 37°C, and subsequent plating on chromogenic agar allowed growth of coliform bacteria in over 90% of biopsy tissues. Chromogenic agar enriches for predominantly coliform bacteria, but only lactose-fermenting bacteria (E coli) turn purple. We could culture purple colonies in only 46.7% of control subjects, 69.2% of patients with CD and 63.2% of patients with UC, even though non-E coli coliforms (white and pink colonies) could be cultured from most biopsies (table 3).

Serial dilution and plating on chromogenic agar allowed for quantification of total coliforms and E coli (fig 2). The numbers of E coli (4×10^2/ml) and non-E coli coliforms (6.3×10^5/ml) cultured were higher (p<0.05) in CD and UC than in controls.

Table 2 Primers used

Primer sequences	Target sequences	References
Autotransporters		
SPATE1 5′ GAGGTCAACAACCTGAACAAACGTATGGG	The genes encoding SPATE	This study
SPATE2 5′ CCGGCACGGGCTGTCACTTTCCAG		
E coli phylogenetic groups		
ChuAf 5′ CGGACGAACCAACGGTCAGGAT	The *chuA* gene is required for haemtransport in *E coli* O157:H7	Modified from Clermont *et al* [5]
ChuAr 5′ TGCCGCCAGTACCAAAGACACG		
Yjaf 5′ CGTGAAGTGTCAGGAGACGCTGC	The *yjaA* gene coding for protein of unknown function	Modified from Clermont *et al* [5]
Yjar 5′ TGCGTTCCTCAACCTGTGACAAACC		
Tsp1 5′ GGGAGTAATGTCGGGGCATTCAG	Tsp encodes for a putative DNA fragment (TSPE4.C2) in *E coli*	Modified from Clermont *et al* [5]
Tsp2 5′ CATCGCGCCAACAAAGTATTACGCAG		
E coli toxins		
Cnff 5′ AGTACTGACACTCACTCAAGCCGC	Cytotoxic necrotising factors (Cnf1 and Cnf2)	This study
Cnfr 5′ GCAGAACGACGTTCTTCATAAGTATCACC		
IpgDf 5′ CGACTTCTCTTCTGACGCCGAC	*ipgD* gene modulates entry of bacteria into epithelial cells	This study
IpgDr 5′ CAACATTCCTCCAGCCTAAGCCC		
Vt1f 5′ CGCATAGTGGAACCTCACTGACGC	Verocytotoxin 1	This study
Vt1r 5′ CATCCCCGTACGACTGATCCC		
Vt2f 5′ CGGAATGCAAATCAGTCGTCACTCAC	Verocytotoxin 2	This study
Vt2r 5′ TCCCCGATACTCCGGAAGCAC		
HlyAf 5′ TGCAGCCTCCAGTGCATCCCTC	The *hlyA* gene encoding α haemolysin	
HlyAr 5′ CTTACCACTCTGACTGCGATCAGC		
STaf 5′ GTGAAACAACATGACGGGAGG	Heat-stable enterotoxin 1	This study
STar 5′ ATAACATCCAGCACAGGCAGG		
STbf 5′ GGGGTTAGAGATGGTACTGCTGGAG	Heat-stable enterotoxin 2	This study
STbr 5′ GACAATGTCCGTCTTGCGTTAGGAC		
LTf 5′ CCGTGCTGACTCTAGACCCCCA	Heat-labile enterotoxin LT	This study
LTr 5′ CCTGCTAATCTGTAACCATCCTCTGC		
Eaef 5′ CCAGGCTTCGTCACAGTTGCAGGC	The *eae* gene coding for intimin present in AEEC strains	This study
Eaer 5′ CGCCAGTATTCGCCACCAATACC		
Enterotoxigenic Bacteroides fragilis (ETBF)		
Bf1F 5′ GTTAGTGCCCAGATGCAGGATGCGG	The genes coding for *Bacteroides fragilis* enterotoxins (Bft1, Bft2 and Bft3)	This study
Bf2F 5′ GAACTCGGTTTATGCAGTTCATGGACTG		
Bf3R 5′ TGGGTTGTAGACATCCCACTGGCTT		
Bf4R 5′ GGATACATCAGCTGGGTTGTAGACATCCC		
E coli adhesins		
PapF 5′ CCGGCGTTCAGGCTGTAGCTG	The genes coding for pathogenicity islands (PAI I, PAI II)[16] and *sfp* gene cluster	This study
PapR 5′ GCTACAGTGGCAGTATGAGTAATGACCGTTA		
PAI1 5′ TAGCTCAGACGCCAGGATTTTCCCTG		
pai2 5′ CCTGGCGCCTGCGGGCTGACTATCAGGG		
BmaEf 5′ CTAACTTGCCATGCTGTGACAGTA	The *bmaE* gene for M-agglutinin subunit; Afa-8 gene cluster	Martin *et al* [17]
BmaEr 5′ TTATCCCCTGCGTAGTTGTGAATC		
Sfaf 5′ CGGAGGAGTAATTACAAACCTGGCA	S-fimbrial adhesins encoded by *sfaD* to *sfaE*	Martin *et al* [17]
Sfar 5′ CTCCGGAGAACTGGGTGCATCTTAC		
Afaf 5′ TATGGTGAGTTGGCGGGGATGTACAGTTACA	AfaE-3 gene cluster	This study
Afar 5′ CCGGGAAAGTTGTCGGATCCAGTGT		
AIDA1 5′ TATGCCACCTGGTATGCCGATGAC	The *aidA* gene coding *E coli* AIDA-I adhesin in DAEC strains	This study
AIDA2 5′ ACGCCCACATTCCCCCAGAC		
AggRf 5′ GAGTTAGGTCACTCTAACGCAGAGTTG	The *aggR* gene for adhesin of aggregative adherence fimbria I	This study
AggRr 5′ GACCAATTCGGACAACTGCAAGCATCTAC		
Ag43F 5′TGACACAGGCAATGGACTATGACCG	The *agn43* gene coding for antigen involved in *E coli* autoaggregation	This study
Ag43R 5′GGCATCATCCCGGACCGTGC		
Flagella and type 1 fimbrae typing		
Primer1 5′CAAGTCATTAATAC(A/C)AACAGCC	The *fliC* genes coding for flagellin proteins	Machado *et al* [18]
Primer2 5′GACAT(A/G)TT(A/G)GA(G/A/C)ACTTC(G/C)GT		
FimHf 5′ CTGGTCATTCGCCTGTAAAACCGCCA	The *fimH* gene encoding FimH subunit of type 1 pili	This study
FimHr 5′ GTCACGCCAATAATCGATTGCACATTCCCT		
Ribosomal DNA-based primers		
ITSF 5′ GTCGTAACAAGGTAGCCGTA	16S-23S rRNA intergenic transcribed spacers	Cardinale *et al* [13]
ITSReub 5′ GCCAAGGCATCCACC		
27f 5′ AGAGTTTGATCMTGGCTCAG	Conserved 16S rDNA used to amplify ribosomal genes	Lane[19]
342r 5′ CTGCTGCSYCCCGTAG		

SPATE, serine protease autotransporter.

There was a poor correlation ($r = 0.22$) between the site of inflammation and the presence of *E coli*, and data were pooled by subject.

Five purple colonies were picked from all agar plates (total 135 colonies) that showed *E coli* growth. Taxonomic identity of the *E coli* was confirmed by reactivity to indole, methyl red,
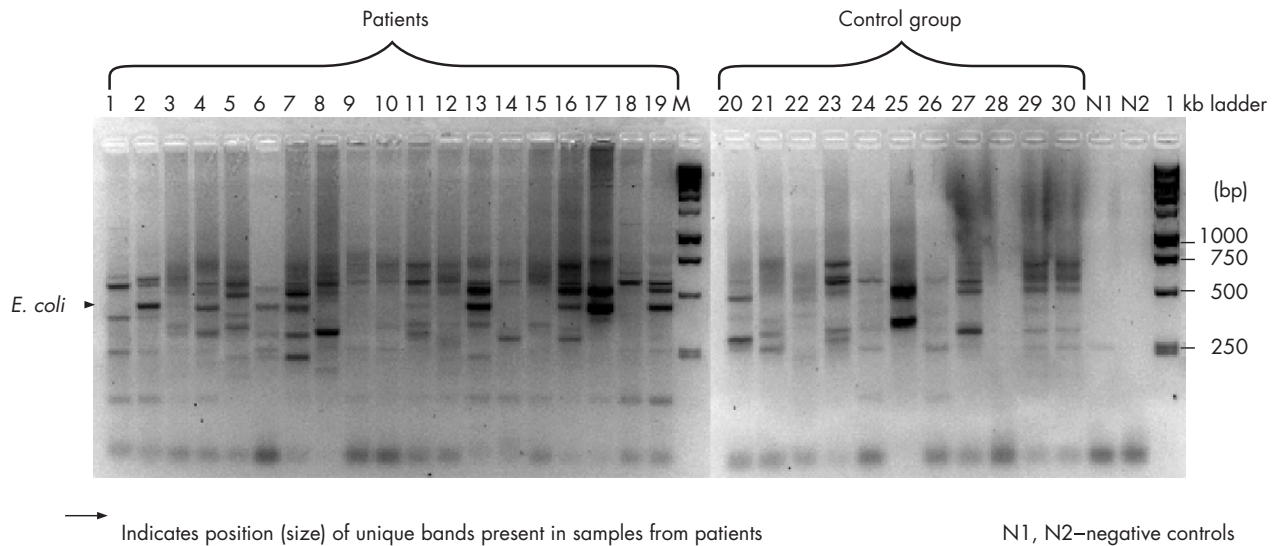
Patients Control group

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 M   20 21 22 23 24 25 26 27 28 29 30 N1 N2   1 kb ladder

E. coli ►

(bp)
— 1000
— 750
— 500
— 250

→ Indicates position (size) of unique bands present in samples from patients   N1, N2—negative controls

Figure 1 Ribosomal intergenic spacer analysis of biopsy samples from tissues of patients with inflammatory bowel disease and control tissues.

Vogues Proskauer and citrate utilisation. There were also few polymorphisms in the RISA product, confirming that isolates were predominantly clonal by subject, irrespective of site. We selected 150 colonies of non-*E coli* (five per biopsy) of pink, blue and white colonies from plates and were able to confirm that none of these colonies were *E coli* by checking for reactivity to indole, methyl red, Vogues Proskauer and citrate utilisation. The identities of these colonies were determined by 16S-rDNA sequence analysis (table 3).

E coli comprises four phylogenetic groups (A, B1, B2 and D) with virulent types typically belonging to groups B2 and D.[15] These groups can be identified by a simple PCR procedure of the *chu*A and *yja*A genes and a cryptic DNA fragment (table 2). Clermont *et al*[15] indicated that these groups could be identified in a single multiplex PCR for all DNA targets, but we did not get reproducible results. We modified the primers (table 2) and the PCR conditions and were able to generate highly reproducible results from a suite of pathogenic and non-pathogenic *E coli* in our laboratory (data not shown). The abundance of the pathogenic B2+D groups was significantly ($p = 0.04$) greater in IBD than in controls (table 4).

E coli strains were also assayed for the presence of serine protease autotransporter proteins (SPATE). Alignments were made of a prominent group (Vat, Sat, Pic, EspI) of SPATE nucleic acid sequences, and primers to conserved regions were designed to amplify targets which, when digested with *Hae*III, were diagnostic of the different groups of SPATE. The identity of each SPATE was confirmed by sequence analysis. One new SPATE, Pic-like, was identified by sequence analysis and alignment with known SPATE. When all SPATE-positive *E coli* isolates were totalled, patients with IBD had a higher number of SPATE-positive isolates than the controls (table 4). Interestingly, this "new" SPATE has actually previously been identified in the enteropathogenic *E coli* E22 genome (Genbank: AAJV01000028), but no functions were assigned.

PCR analyses of *E coli* isolates were conducted for a range of adhesins commonly found in pathogenic *E coli* (tables 3 and 4). Isolates were positive only for *agn43* and *aidA*, and gene clusters coding for AfaE-3, F1C, Sfa, PAI I and Sfp. No *E coli* positive for *cnf1*, *cnf2*, *eae*, *hlyA*, *ipgB*, *aggR*, *bmaE* or genes coding for verotoxins, heat-stable and heat-labile toxins were found in biopsy specimens of patients with UC or CD.

χ^2 test based on the Mantel–Haenszel method (table 5) was performed to verify relationships between disease, B2+D genotype, SPATE toxins and adhesins. We observed a significant difference ($p = 0.04$) in the number of *E coli* isolates from the B2+D genotype for both patients with UC and those with CD (table 5). The relationship between IBD and an adhesin ($p = 0.07$) or $\geqslant 2$ adhesins ($p = 0.03$) was significant, but not with a SPATE and an adhesin ($p = 0.15$). The relationship between the B2+D genotype and SPATE(s), adhesion(s) or APEC was significant ($p<0.05$).

DISCUSSION

Culture-independent methods only provide evidence of the presence and abundance of a bacterium in a sample; they do not provide functional information. The most powerful method of understanding the function of a bacterium is to culture it and to study its physiology. The logic in our decision to culture the Enterobacteriaceae was to (a) use a culture-independent method to identify DNA bands that are present in tissues of patients with IBD but not in controls, (b) cut the bands out of the gel and sequence them, (c) identify the bacterial species on the basis of the sequence composition, (d) specifically culture the bacterial group identified from the sequence information and (e) investigate potential virulence factors in the cultured bacteria. The RISA analysis (fig 1) we used provided the rationale for culturing *E coli* and other Enterobacteriaceae.

The numbers of Enterobacteriaceae in our biopsy tissues were 3–4 logs higher in the tissues of patients with IBD than in the controls (fig 2). Martin *et al*[17] found a significant increase in the numbers of *E coli* only in specimens of patients with CD, but not in those with UC, after a mucin-releasing step with dithiothreitol. Darfeuille-Michaud *et al*[20] isolated *E coli* in higher numbers from tissues of patients with IBD than from controls, but did not enumerate the bacteria on the epithelial tissue with techniques to determine numbers in a range >1 log. Mylonaki *et al*[21] used fluorescence microscopy to show that the numbers of *E coli* were high in the rectal tissue of patients with UC but not of controls, but this methodology did not allow for decimal enumeration.

Conte *et al*[22] showed that Gram-negative bacteria, including *E coli*, have increased by 3–4 logs in tissues of patients with IBD, a result strikingly similar to ours. It is thus clear that the

Table 3 Microbial tissue phenotypes encountered from control tissues and tissues of patients with inflammatory bowel disease*

Disease	Patient	*E coli*† group	Non-*E coli*‡	SPATE	*E coli* adhesins	*E coli* H-type	*E coli* type 1 fimbriae
Control	41A	ND	*Enterobacter sp* *Bacteroides fragilis* Bft1§	ND	–	–	–
Control	43A	B2		Vat	Ag43, PAI I	H7	Bovine
Control	48B	B2		Vat, Pic-like	Sfp¶	H4	APEC
Control	50A	B2		Vat, Sat, Pic	Ag43	H1	APEC
Control	58	ND	*Enterobacter flavescens*	ND	–	–	–
Control	59B	ND	*Klebsiella oxytoca*	ND	–	–	–
Control	73A	A	*Klebsiella sp*	ND	AIDA-I	H12	Bovine
Control	69A	ND	*Enterococcus sp* *Bacteroides fragilis* Bft1	ND	–	–	–
Control	76	ND	*Escherichia fergusonii* *Staphylococcus epidermidis* *Staphylococcus capitis* *Bacteroides fragilis* Bft2§	SigA, Sat††, SepA	–	–	–
Control	80	ND	*E fergusonii* *Klebsiella sp*	SigA, Sat††, SepA	–	–	–
Control	81	ND	*Enterococcus durans*	ND	–	–	–
Control	87	ND	*Klebsiella sp*	ND	–	–	–
Control	90	A		ND	AIDA-I, Ag43	H11	Bovine
Control	17B	D		ND	Ag43	H4	UPEC
Control	88	A	*Klebsiella sp*	ND	AIDA-I	H21	Bovine
CD	15B	B2		Vat, Pic-like	Ag43	H4	APEC
CD	79A	B2		Vat, Sat, Pic	Ag43	H1	APEC
CD	91A	B1	*Acidovorax sp*	ND	AIDA-I	H21	Bovine
CD	118	ND	*Staphylococcus auricularis*	ND	–	–	–
CD	120A	D		EspI	PAI I, AIDA-I, Ag43	H52	Bovine
CD	124A	ND	*E fergusonii* *Enterococcus faecium* *Bacteroides fragilis* Bft1	SigA, Sat††, SepA	–	–	–
CD	125B	ND	*Klebsiella oxytoca*	ND	–	–	–
CD	126C	B2		ND	AIDA-I, Ag43	H39	Bovine
CD	132	B2		Sat	AfaE-3, Ag43, AIDA-I	H4	Bovine
CD	137	ND	*Klebsiella sp*	ND	–	–	–
CD	138	ND	*Staphylococcus aureus* *Pseudomonas putida* *Klebsiella sp*	ND	–	–	–
CD	146	B2		Vat	Sfa**, Ag43, PAI I	H7	APEC
CD	149	B2		Vat, Pic	Sfa, Ag43, PAI I	H5	APEC
CD	128	B2	*E fergusonii*	Vat, SigA, Sat††, SepA	Sfa	H7	UPEC
UC	117A	D		ND	AIDA-I, Ag43	H18	Bovine
UC	119A	ND	*Enterococcus faecium*	ND	–	–	–
UC	121	B1		ND	ND	H10	Bovine
UC	122	ND	*Enterococcus faecalis* *Bacteroides fragilis* Bft1	ND	–	–	–
UC	127A	B2	*Enterobacter cloacae*	Vat, Sat, Pic	F1C	H7	Bovine
UC	130	ND	*Bacillus licheniformis* *Bacillus pumilus*	ND	–	–	–
UC	131A	B2	*E fergusonii* *Bacteroides fragilis* Bft2	Vat, Sat††, Pic, SigA, Sat, SepA	Ag43, PAI I	H10	APEC
UC	133A	ND	*Klebsiella sp*	ND	–	–	–
UC	135	B2	*Klebsiella oxytoca*	Pic	AIDA-I, Ag43	H31	Bovine
UC	136	B2	ND	Vat	Ag43, PAI I	H7	Bovine
UC	139	D	ND	ND	AIDA-I	H6	APEC
UC	140A	ND	*Enterococcus sp*	ND	–	–	–
UC	141	B2	ND	ND	Sfa, Ag43, PAI I	H7	APEC
UC	142	B2	ND	Vat	AfaE-3, Ag43	H5	APEC
UC	143	B2	ND	Sat, Pic-like	AfaE-3, Ag43	H4	APEC
UC	145B	B2	ND	Vat, Pic-like	Ag43	H4	Bovine
UC	147	B2	*Klebsiella sp*	Vat	Sfa, Ag43	H5	APEC
UC	134A	ND	*Klebsiella sp*	ND	–	–	–

APEC, avian pathogenic *E coli*; ND, *E coli* not detected; PAI, pathogenicity island; SPATE, serine protease autotransporters; UPEC, uropathogenic *E coli*.
*A total of 84 biopsy specimens from 47 subjects were used. Data are not shown by site, and were pooled because the correlation between bacterial species and site, within and between subjects, was low (r = 0.22).
†A total of 28 *E coli* strains and 34 non-*E coli* were picked and assessed.
‡Identity was determined by sequencing the 16S rDNA between with 27f and 342r.
§Bft1 and Bft2 are enterotoxins produced by *Bacteroides fragilis* detected from tissue by the nested-PCR method.
¶sfp is a gene cluster of sorbitol-fermenting enterohaemorrhagic *E coli* O157:H⁻.
**sfa gene coding for S-fimbriae minor subunit of *E coli* UTI89.
††Sat gene coding for autotransporter toxin Sat identified in *Escherichia fergusonii*.

numbers of Enterobacteriaceae do increase on the epithelial tissue of patients with IBD, irrespective of the differences in techniques used. The major difference in our study was that we resuscitated tissue in buffered peptone water to ensure that bacteria, even at very low numbers, were given the maximum chance to grow. This was done because *E coli* in environmental samples enter into the viable but non-culturable state, making them difficult to grow without a resuscitation step.[23]
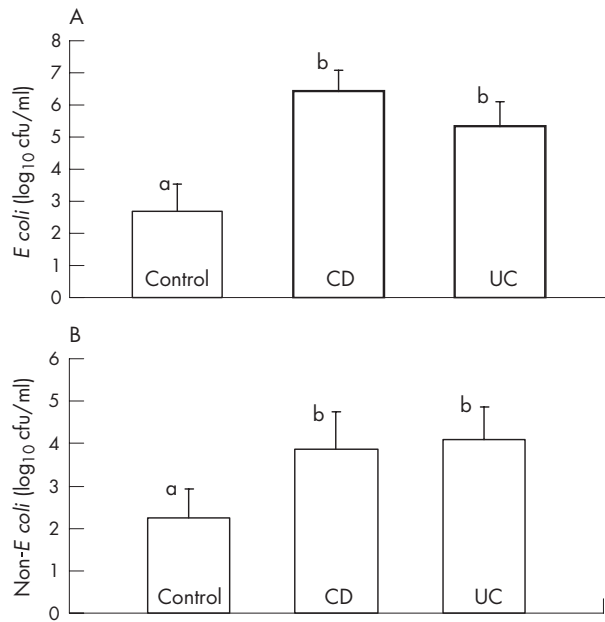
Figure 2 Proportions of (A) *E coli* and (B) non-*E coli* in biopsy tissues from controls, and patients with ulcerative colitis (UC) and Crohn's disease (CD).

Clermont *et al*[15] developed a method to type pathogenic *E coli* using *chu*A, a gene required for haem transport in enterohaemorrhagic O157:H7 *E coli*, *yja*A, a gene identified in *E coli* K-12 but which has no known function, and TSPE4.C2, a cryptic fragment that was identified from subtractive libraries. These genes, when applied to 230 isolates, determined that type B2, and to a lesser extent type D, included virulent extraintestinal strains of *E coli,* but the prevalence of B2 and D in gastrointestinal isolates was not determined. *E coli* from stool samples from a range of geographically separate normal healthy human subjects determined that non-pathogenic groups A and B1 were most prevalent, while group D made up only 15% and group B2 11% of isolates.[24] A significant relationship ($p < 0.05$) between the B2 genotype of "resident", or adherence factor carrying *E coli* in infants[25] and in human colonic cells of adults[26], has been shown. We found a significant relationship ($p = 0.04$) between IBD and the B2+D genotype (table 5). Our data suggest a significant relationship between the presence of a SPATE ($p = 0.02$) or an adhesin ($p = 0.04$) in B2+D-positive *E coli* strains.

The "resident" population that Nowrouzian *et al*[25] referred to are strains that have adherence factors, in particular P fimbriae that promote adherence to enterocytes. This definition is of course somewhat arbitrary because there are a large number of cell factors that promote adhesin to intestinal tissue. *E coli* from our controls had relatively few adhesins (table 4), while IBD strains had a higher prevalence of Ag43, AIDA-I, Sfa, AfaE-3 and PAI I. Ag43 is a surface adhesin that promotes bacterial biofilm formation due to cell-to-cell aggregation,[27] Sfa is one of a class of S-fimbrial adhesins,[16] and AIDA-I is an adhesin-like protein.[16] Martin *et al*[17] measured adherence and invasion of IBD-derived *E coli* in tissue culture, and showed that *E coli* strains isolated from patients with CD possessed haemagglutinating ability to all red cells regardless of blood group. We did not measure adherence and invasion in a cell culture assay but all *E coli* isolates, both from tissues of patients with IBD and controls, were negative for the presence of *bmaE* gene encoding M-agglutinin.

A novel finding of this study is the higher prevalence of *E coli* from the B2+D phylogenetic group in tissues of patients with

Table 4 Distribution of virulence features of *E coli* isolated from patients with inflammatory bowel disease and controls

Item*	Control (15 subjects, 28 tissues)	UC (19 patients, 27 tissues)	CD (13 patients, 29 tissues)
Group			
A	3 (20)†	0	0
B1	0	1 (5.3)	1 (7.7)
B2	3 (20)	9 (47.4)	7 (53.8)
D	1 (6.7)	2 (10.5)	1 (7.7)
B2+D	4 (26.7)	11 (57.9)	8 (61.5)
SPATE			
Vat	3 (20)	7 (36.8)	4 (30.8)
Pic	1 (6.7)	3 (15.8)	3 (23.1)
Sat	3 (20)	4 (21.05)	3 (23.1)
Pic-like	1 (6.7)	2 (15.4)	1 (7.7)
EspI	0	0	1 (7.7)
SepA	2 (13.3)	2 (10.5)	1 (7.7)
SigA	2 (13.3)	2 (10.5)	1 (7.7)
Total	5 (33.3)	9 (47.4)	7 (53.8)
Adhesins			
Ag43‡	4 (26.7)	9 (47.4)	7 (53.8)
AfaE3	0	2 (10.5)	1 (7.7)
AIDA-I	3 (20)	3 (15.8)	4 (30.8)
F1C	0	1 (5.3)	0
Sfa	0	3 (15.8)	2 (15.4)
PAI I	1 (6.7)	3 (15.8)	3 (23.1)
Sfp	1 (6.7)	0 (5.3)	0
Total	7 (46.6)	12 (63.2)	8 (61.5)

CD, Crohn's disease; UC, ulcerative colitis.
*Table 2 gives the functions of each of the genotypic characteristics.
†Values in parentheses represent the frequency of items in patients as a percentage.
‡Antigen 43 (Ag43) is a self-recognising surface adhesin found in most *Escherichia coli* strains.

IBD. Moreover, SPATE[28] and adhesins, that have been primarily associated with *E coli* isolated from urinary tract infections, were also more prevalent in *E coli* isolates from patients with IBD. SPATE are a unique class of transporters found in the Enterobacteriaceae that direct their own transport across the outer cell membrane. These proteins have been implicated in virulence but their precise role is not known. We believe that the functional properties of SPATE make them potentially important in IBD because they exhibit functions like degradation of the barrier function of the gut, and cleavage of proteins in the enterocyte, all of which are phenotypes associated with IBD.[28] For example, Vat, Pic and Pic-like have haemaglutinin, mucinase and elastase activity,[29][30] Sat has cytotoxic effects[31–33] on cells as well as elastase activity,[27–30] and EspP cleaves

Table 5 Statistical relationship between the presence of virulence factors in patients with inflammatory bowel disease and control subjects

Disease	Virulence factor	p Value
IBD	≥2 adhesins	0.03*
IBD	B2+D	0.04*
IBD	Adhesins	0.07
IBD	SPATE(s)+adhesin(s)	0.15
Genotype	**Virulence feature**	**p Value**
B2+D	SPATE(s)+adhesin(s)	0.002**
B2+D	Adhesins	0.04*
B2+D	SPATE	0.02*
B2+D	APEC	0.04*

APEC, avian pathogenic *E coli*; IBD, inflammatory bowel disease; SPATE, serine protease autotransporters.
*$p < 0.05$, **$p < 0.01$.

lipoproteins.[27–30] More recently, functional studies on SPATE have demonstrated that Pic and Sat can cleave coagulation factor V, potentially linking it to haemorrhagic events in the gut. Additionally, Pic is thought be involved in colonisation of *E coli* to intestinal tissue.[34]

One means by which SPATE may be involved in promoting inflammation could be as an accessory protein in pathogenicity islands (PAI). EspC, a SPATE from enteroaggregative *E coli*,[35] and the non-SPATE Tsh autotransporter[36] have been associated with PAI. Bidet *et al*[37] suggested that PAI I$_{J96}$ were associated with *hra*, *hlyA*, *cnf*1 and *pap*. We determined the prevalence of *hlyA* and *cnf*1 and cnf2 in our isolates but they were not present. When we designed primers that were conserved for a range of PAI, only 23.1% of CD and 15.8% of UC were positive. If these were the only PAI, then <50% of our SPATE would be associated with PAI. Further work is needed to investigate the possibility of the association of other PAI with SPATE.

One might argue that IBD is caused by a dysfunctional immune system that results in an increase in *E coli* on the gut tissue, and that *E coli* has nothing whatever to do with initiating inflammation in UC or CD. If this was true then the genotypes of *E coli* isolated from tissue should be a consequence of random colonisation of gut mucosa. Consequently, the distribution of these genotypes should be equivalent among IBD and control tissues. This is not the case, with IBD having more ($p = 0.04$) putatively pathogenic bacteria than controls. What we do not know at this point is whether the increase in certain types of *E coli* in IBD is a consequence of inflammation, or the cause. One way forward is to describe the *E coli* population before inflammation sets in—for example, obtain biopsy tissue that ranges along the colon, distal and proximal to UC lesions.

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