

## INTESTINAL MICROFLORA

# Identification and quantitation of mucosal and faecal desulfovibrios using real time polymerase chain reaction

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**Background:** Desulfovibrios produce sulphide, which is toxic to colonic epithelial cells. These bacteria have previously been linked to ulcerative colitis. Traditional methods of culturing these organisms are slow, and often unreliable, while molecular approaches are either non-quantitative or lack sensitivity.

**Aims:** To develop a sensitive method for quantitating desulfovibrios in stools and biopsy tissue, and to investigate the effects of age and disease on these bacteria.

**Methods:** Rectal biopsies were taken from 10 colitis patients and 10 healthy controls. Stool samples were obtained from 10 healthy infants (mean age 1.01 (0.18) years), 10 healthy young adults (26.7 (1.2) years), and 10 healthy elderly people (71.7 (1.2) years). Primers were designed and developed for analysing *Desulfovibrio* populations in the bowel using real time polymerase chain reaction (PCR).

**Results:** The PCR primers were highly specific for desulfovibrios. Large numbers (approximately  $10^6$ – $10^7$ /g) occurred in biopsies in colitis patients and healthy subjects, and no disease related differences were observed. Measurements of mucosal desulfovibrios over 12 months showed marked changes in some patients. Infants ( $10^6$ – $10^7$ /g) and elderly people ( $10^7$ – $10^8$ /g) had significantly higher numbers of desulfovibrios in stools compared with young adults ( $10^5$ /g).

**Conclusions:** Real time PCR analysis of desulfovibrios was an efficient and accurate method for studying these potentially harmful microorganisms. Desulfovibrios were ubiquitous in the bowel, irrespective of age. As rectal mucosae were heavily colonised in health and disease, if these bacteria play a role in colitis, some host defect, possibly in sulphide detoxication pathways or in bacterial antigen handling, is required for manifestations of pathogenicity.

Evidence from animal models and the study of patients with ulcerative colitis (UC) implicates the intestinal microbiota in disease aetiology. However, no specific pathogen has been identified as being causal, and UC is widely thought to result from a genetically determined but abnormal immune response to bacterial species in the normal gut microflora.<sup>1</sup>

Sulphate reducing bacteria (SRB) of the genus *Desulfovibrio* can occur in large numbers in faeces<sup>2</sup> and have been linked to UC.<sup>3</sup> It has been reported<sup>4</sup> that healthy people have a lower prevalence of SRB in faeces (24%) than inflammatory bowel disease (IBD) patients (68%) while other investigations, using culturing methods, have shown that mucosa associated SRB were present in 92% of UC tissues and in 52% of non-IBD subjects.<sup>5</sup> However, SRB were found to be present in all of the biopsies studied when they were analysed by conventional polymerase chain reaction (PCR).

SRB in the large bowel reduce sulphate to sulphide, which is toxic to colonic epithelial cells.<sup>6</sup> Sulphide inhibits butyrate metabolism in colonocytes,<sup>7,8</sup> inhibits phagocytosis and bacterial killing,<sup>9</sup> and induces hyperproliferation and metabolic abnormalities in epithelial cells, similar to those observed in UC.<sup>10</sup> In humans, untreated UC patients were observed to have significantly higher faecal sulphide excretion than healthy controls, while 5-ASA, the main drug used to treat UC, was reported to inhibit sulphide production.<sup>11</sup>

Conventional culture based methods for studying SRB are time consuming due to slow bacterial growth rates,<sup>12</sup> and like other methods of culture, do not necessarily provide an accurate assessment of the types and numbers of bacteria present in a biological sample.<sup>5,13,14</sup> However, it has been shown that variable regions of 16S ribosomal RNA (rRNA) gene sequences provide the basis for development of specific

primers/probes that can be used for culture independent analyses of bacterial populations.<sup>15–20</sup>

The purpose of this study was to develop specific real time PCR primers for rapid, sensitive, high throughput determinations of intestinal desulfovibrios, based on analysis of 16S rRNA genes. These bacteria may play an important role in gut health, and because luminal communities are known to change with age<sup>14</sup> and distinct bacterial populations have been reported to occur on the gut epithelium,<sup>21</sup> the occurrence of desulfovibrios in different age groups and on mucosal tissue was investigated.

## MATERIALS AND METHODS

### Bacterial strains

The following bacteria, obtained from the National Collection of Industrial and Marine Bacteria (Aberdeen, UK), the National Collection of Type Cultures (London, UK), Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), American Type Culture Collection (Manassas, Virginia, USA), and laboratory stock cultures at the University of Dundee (DUN-), were used to evaluate primer efficacy: *Faecalibacterium prausnitzii* ATCC 27768, *F prausnitzii* NCIMB 13872, *Bifidobacterium catenulatum* NCIMB 702246, *Bif adolescentis* NCIMB 702231, *Bif bifidum* NCIMB 702715, *Bif breve* NCTC 11815, *Eubacterium limosum* DUN-112, *Eub rectale* DUN-128, *Eub aerofaciens* DUN-207, *Eub cylindroides* DUN-119, *Clostridium clostridioforme* DUN-120, *C malenominatum* DUN-102, *C perfringens* NCTC 8346,

**Abbreviations:** IBD, inflammatory bowel disease; rRNA, ribosomal RNA; PCR, polymerase chain reaction; SRB, sulphate reducing bacteria; UC, ulcerative colitis

*C. butyricum* NCIMB 7423, *C. bifementans* DUN-156, *C. perfringens* NCTC 8533, *C. sordellii* DUN-113, *C. tetani* NCTC 5404, *C. difficile* NCTC 11223, *C. histolyticum* DSM 2158, *Desulfovibrio desulfuricans* NCIMB 12833, *Dsv vulgaris* NCIMB 8303, *Dsv desulfuricans* NCIMB 8307, *Desulfococcus multivorans* NCIMB 12965, *Desulfobacter vibrioformis* NCIMB 13525, *Dsb vibrioformis* NCIMB 13525, *Desulfotomaculum ruminis* DSM 2154, *Dsm ruminis* NCIMB 8452, *Dsm ruminis* (Professor GR Gibson, University of Reading, UK), *Bacteroides fragilis* NCTC 9343, *B. ovatus* DUN-203, *B. thetaiotaomicron* NCTC 10582, *B. vulgatus* DUN-116, *Escherichia coli* ATCC 11775, *E. coli* NCTC 9001, *Enterococcus faecalis* ATCC 51299, *Ent. faecium* NCTC 12202, *Lactobacillus plantarum* DUN-145, *L. acidophilus* DSM 20079, *Peptostreptococcus anaerobius* DUN-167, *Shigella sonnei* DUN-198, *S. flexneri* DUN-200, *Salmonella typhimurium* DUN-217, and *Veillonella parvula* NCTC 12140.

### Subjects and specimens

Rectal biopsies were taken from 10 UC patients and 10 normal controls who had not received any bowel preparation. Diagnosis of UC was confirmed in all cases at colonoscopy, and by histology. All UC patients had actively inflamed mucosae categorised as either mild or moderately severe. All UC biopsies were of inflamed tissue. Biopsies were collected aseptically and frozen immediately at  $-80^{\circ}\text{C}$  in sterile Eppendorf tubes. Stools were obtained from 30 healthy subjects, comprising 10 infants, 10 young adults, and 10 elderly people. Clinical features, numbers, age, and gender of the subjects are shown in table 1. Informed consent was obtained for these investigations which were approved by the Tayside Committee on Medical Research Ethics, Dundee.

### Primer design

Primer Premier for Windows version 5.0 (Premier Biosoft International, Palo Alto, California, USA) was used for searching, aligning, editing, and handling primers for collected bacterial sequences. The Ribosomal Database Project was used for screening specific primer sets. *Desulfovibrio* specific primers were designed on the basis of 16S rRNA gene sequences, available at the National Center for Biotechnology Information databases. A wide range of bacterial species was tested to confirm the specificity of the developed primers to target bacteria using conventional PCR. The forward primer sequence was DSV691-F: 5'-CCGTAGATATCTGGAGGAACATCAG-3', and the reverse primer sequence was DSV826-R: 5'-ACATCTAGCATC CATCGTTTACAGC-3'. Designed primers were purchased from Invitrogen Life Technologies (Paisley, UK).

### DNA extraction from bacterial cultures

Intestinal isolates and a range of culture collection type strains were used as controls in testing specificities of the PCR primers. SRB were grown in Postgate's medium B in Universal bottles,<sup>12</sup> and other organisms were cultured on Wilkins-Chalgren agar plates in an anaerobic chamber (atmosphere  $\text{N}_2$  80%;  $\text{CO}_2$  10%;  $\text{H}_2$  10%) at  $37^{\circ}\text{C}$ . Cell pellets from liquid media or bacterial colonies from plate swabs were resuspended in 450  $\mu\text{l}$  of sterile water and 50  $\mu\text{l}$  of lysozyme (50 mg/ml), which was added to digest bacterial cell walls. Reaction mixtures were incubated in a water bath at  $37^{\circ}\text{C}$  for 30 minutes. Proteinase K solution 1 (25  $\mu\text{l}$  of 20 mg/ml), 50  $\mu\text{l}$  of 20% sodium dodecyl sulphate, 500  $\mu\text{l}$  of  $\text{H}_2\text{O}$ , and 350 mg glass beads (0.1 mm diameter) were then added, and the mixture bead beaten before and after incubation in a water bath at  $70^{\circ}\text{C}$  for 10 minutes. Total DNA was obtained after centrifugation at 5000  $g$  (three minutes).

### DNA extraction and purification from biopsy material and stools

Frozen tissue sections were extracted using QIAmp DNA spin columns (Qiagen Ltd, West Sussex, UK). Briefly, biopsy specimens (2–5 mg) were suspended in lysis buffer and lysozyme solutions before incubating at  $55^{\circ}\text{C}$ . Proteinase K, buffer ATL, and ethanol were sequentially added, and the mixture bead beaten before and after incubation at  $70^{\circ}\text{C}$ . Bacterial cell lysates were obtained by centrifugation at 5000  $g$  (three minutes).

Stool samples were extracted for bacterial DNA using a Qiagen Stool Kit. Frozen stool samples were weighed in 200 mg quantities, and 1.4 ml of ASL buffer (preheated at  $95^{\circ}\text{C}$ ) was added. Mixtures were bead beaten for two minutes before and after heating at  $95^{\circ}\text{C}$  (five minutes). Supernatants containing bacterial DNA were collected after centrifugation at 5000  $g$  (two minutes). Inhibitex tablets were used to remove faecal debris, and bacterial cell lysis was completed by adding 25  $\mu\text{l}$  of proteinase K solution and 600  $\mu\text{l}$  of buffer AL, and incubating at  $70^{\circ}\text{C}$  for 10 minutes.

Biopsy and faecal DNA lysates were adsorbed onto minicolumn membranes, washed, and eluted according to their respective Qiagen kit procedures. The purified DNA was eluted in 200  $\mu\text{l}$  of elution buffer.

### Amplification in conventional PCR

For conventional PCR, a Techne Genius PCR machine (Techne Ltd, Duxford, UK) was used. PCR consisted of 35 cycles of initial DNA denaturation step at  $95^{\circ}\text{C}$  (one minute), followed by annealing at  $62^{\circ}\text{C}$  (one minute), and elongation at  $72^{\circ}\text{C}$  (45 seconds). The procedure was completed with a

**Table 1** Characteristics of patients and subjects used in this study

	Biopsies		Stools		
	UC patients (n = 10)	Controls (n = 10)	Infants (n = 10)	Adults (n = 10)	Elderly (n = 10)
Age (y)					
Range	28–65	19–76	22 days–23 mo	20–35	64–83
Mean (SEM)	50.2 (2.8)	50.8 (4.7)	1.01 (0.18)	26.7 (1.2)	71.7 (1.2)
Sex (M/F)	4/6	4/6	5/5	3/7	0/10
UC duration (y)*	9.2 (1.6)	–	–	–	–
Therapy for UC					
None	2	–	–	–	–
Balsalazide	2	–	–	–	–
Salazopyrine	2	–	–	–	–
Minocycline	1	–	–	–	–
Asacol	3	–	–	–	–

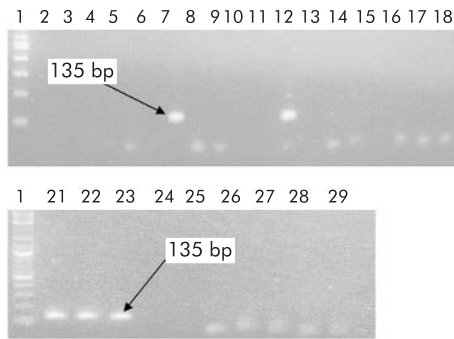
\*Values are mean (SEM).  
UC, ulcerative colitis.

**Table 2** Alignment of sequences of amplimers of desulfovibrios with corresponding regions of 16S rRNA genes of related species\*†

Species	Accession	Forward primer	Reverse primer	Position	Position
<b>Desulfovibrios</b>		<b>C CGT AGAT AT CT GGAGGAA CAT CAG</b>	<b>ACATCT AGCAT CCAT CGT T TACAGC</b>		
<i>Desulfovibrio</i> spp	AF0566089	GGCAT CT AT AGACCT CCT T GT AGAC	T GT AGAT CGT AGGT AGCAAAT GT CG	677	812
<i>Desulfovibrio aminophilus</i>	AF067964	GGCAT CT AT ATACCT CCT T GT CGAC	T GT CGAT CGT AGGT AGCAAAT GT CG	688	823
<i>Desulfovibrio oryzae</i>	AF273083	GGCAT CT AT AGACCT CCT T GT AGAC	T GT CGAT CGT AGGT AGCAAAT GT CG	696	831
<i>Desulfovibrio desulfuricans</i>	AF354664	GGCAT CT AT AGACCT CCT T GT AGAC	T GT AGAT CGT AGGT AGCAAAT GT CG	691	826
<i>Desulfovibrio gigantis</i>	AF418170	GGCAT CT AT AGCCCT CCT T GT CGGC	T GT CGAT CT GAGGT AGCAAAT GT CG	698	833
<i>Desulfovibrio vulgaris</i>	AF418179	GGCAT CT GT AGACCT CCT T GT AGTC	T GT CGAT CGT AGGT AGCAAAT GT CG	695	830
<i>Desulfovibrio intestinalis</i>	AJ295680	GGCAT CT AT AGACCT CCT T GT AGTC	T GT AGAT CGT AGGT AGCAAAT GT CG	567	702
<i>Desulfovibrio magneticus</i>	D43944	GGCAT CT AT AGCCCT CCT T GT CGGC	T GT CGAT CGT AGGT AGCAAAT GT CG	698	833
<i>Desulfovibrio gigas</i>	M34400	<b>AAACCTCAGAGACCT CCT T AGAATC</b>	T RT CNAT CGT AGGT AGCAAAT GT CG	435	839
<i>Desulfovibrio fairfieldensis</i>	U2221	<b>CCCACTCT AT AGACCT CCT T GATGTC</b>	T CT AGAT CGT AGGT AGCAAAT GT CG	677	812
<i>Desulfovibrio gabonensis</i>	U31080	GGCAT CT AT AGCCCT CCT T GT CGTC	T GT CGAT AGGAGGT AGCAAAT GT CG	653	788
<i>Desulfovibrio basitii</i>	U53462	GGCAT CT AT AGACCT CCT T GT CGTC	T GT CGAT CT TAGGT AGCANNNGT CG	658	793
<i>Desulfovibrio gracilis</i>	U53464	GGCAT CT AT AGACCT CCT T GT CGTC	T GT CGAT CT TAGGT AGCAAAT GT CG	658	793
<i>Desulfovibrio caledonensis</i>	U53465	GGCAT CT AT AGACCT CCT T GT CGGC	T GT CGAT ATTAGGT AGCAAAT GT CG	658	793
<i>Desulfovibrio termithidis</i>	X87409	GGCAT CT AT AGACCT CCT T GT AGTC	T GT CGAT CT TAGGT AGCAAAT GT CG	676	811
<i>Desulfovibrio vietnamensis</i>	X93994	GGCAT CT AT AGACCT CCT T GT CGGC	T GT CGAT CT TAGGT AGCAAAT GT CG	676	811
<i>Desulfovibrio aespoensis</i>	X95230	GGCAT CT AT AGACCT CCT T GT CGGC	T GT CGAT ATTAGGT AGCAAAT GT CG	682	817
<i>Desulfovibrio africanus</i>	X99236	GGCAT CT AT AGCCCT CCT T GT CGTC	T GT CGAT CT TAGGT AGCAAAT GT CG	676	811
<i>Desulfovibrio halophilus</i>	X99237	GGCAT CT AT AGCCCT CCT T GT CGGC	T GT CGAT CT TAGGT AGCAAAT GT CG	673	808
<i>Desulfovibrio indonensis</i>	Y09504	GGCAT CT AT AGCCCT CCT T GT CGTC	T GT CGAT AGGAGGT AGCAAAT GT CG	698	833
<i>Desulfovibrio alaskensis</i>	Y11984	GGCAT CT AT AGACCT CCT T GT CGCC	T GT CGAT CT TAGGT AGCAAAT GT CG	677	812
<i>Desulfovibrio zosteriae</i>	Y18049	GGCAT CT AT AGACCT CCT T GT CGTC	T GT AGAT CT TAGGT AGCAAAT GT CG	682	817
<i>Desulfovibrio longreachensis</i>	Z24450	GGCAT CT AT AGACCT CCT T GT AGTC	T GT CGAT CT TAGGT AGCAAAT GT CG	673	808
<b>Other SRB genera</b>					
<i>Desulfohalobium</i> <i>ruminis</i>	M34418	GGCAT CT AT <b>AGACCT CCT T T T CGTC</b>	T GT CGAT CGT CT GT AGCAAAT GT CG	956	1091
<i>Desulfohalobium</i> sp.	M34416	GGCAT CT AT <b>AGACCT CCT T GT CGGA</b>	<b>CTGCAGACCAAGCGCGGAAAGTAA</b>	712	826
<i>Desulfohalobium</i> <i>biacutus</i>	AJ277887	GGCAT CT AT <b>AGACCT CCT T GT CGGC</b>	<b>TCGAGACCTCTCGGACCGCTGGAGCCG</b>	947	1518
<i>Desulfohalobium</i> <i>propionicus</i>	M34410	GGCAT CT AT <b>AGCCCT CCT T T T GGC</b>	<b>GGTAGACTCTTAGGTAGCAAATGTCTCG</b>	708	843
<i>Desulfohalobium</i> <i>vibriobarmis</i>	U12254	GGCAT CT AT <b>AGCCCT CCT T GT GGC</b>	<b>TCGGGTGATGGTAGCAAACGGAAAC</b>	694	1284
<i>Desulfohalobium</i> <i>multivorans</i>	M34405	<b>CGCAT CT AT AGT CCT CCT T GT GGC</b>	<b>CCTCTAGCGANGGGANNCTATGTCG</b>	713	None

\*Nucleotides that are different from the target sequence for each organism are shown in underlined bold face. Primer alignment was done using Primer Premier Software.

†Sequence position according to 16S rRNA gene in *E. coli*.

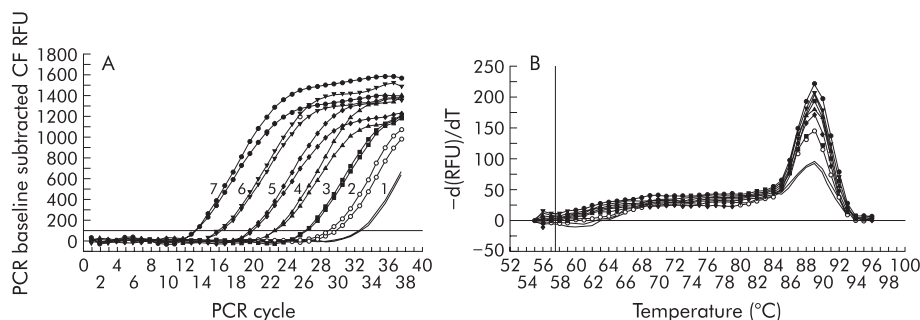


**Figure 1** Specificity of the desulfovibrio polymerase chain reaction (PCR) assay. Electrophoresis was done on an agarose (2% w/v) gel with PCR products obtained with primer pair DSV691-F and DSV826-R, and genomic DNA from various bacterial templates. DNA ladder (100 bp) (lane 1), *Bacteroides fragilis* NCTC 9343 (lane 2), *Faecalibacterium prausnitzii* ATCC 27768 (lane 3), *Clostridium difficile* NTCC 11223 (lane 4), *C. histolyticum* DSM 2158 (lane 5), *Lactobacillus acidophilus* DSM 20079 (lane 6), *E. coli* ATCC 11775 (lane 7), *Desulfovibrio desulfuricans* NCIMB 12833 (lane 8), *Bifidobacterium catenulatum* NCIMB 702246 (lane 9), *Bif. bifidum* NCIMB 702715 (lane 10), *Bif. breve* NCTC 11815 (lane 11), *Faecalibacterium prausnitzii* NCIMB 13872 (lane 12), *Desulfovibrio vulgaris* NCIMB 8303 (lane 13), *Eubacterium limosum* DUN-112 (lane 14), *C. clostridioforme* DUN-120 (lane 15), *C. malenominatum* DUN-102 (lane 16), *Eubacterium aerofaciens* DUN-207 (lane 17), *Clostridium perfringens* NCTC 8346 (lane 18), *Desulfovibrio desulfuricans* NCIMB 12833 (lane 21), *Dsv vulgaris* NCIMB 8303 (lane 22), *Dsv desulfuricans* NCIMB 8307 (lane 23), *Desulfotomaculum ruminis* NCIMB 8452 (lane 24), *Desulfococcus multivorans* NCIMB 12965 (lane 25), *Desulfobacter vibrioformis* NCIMB 13525 (lane 26), *Dsb vibrioformis* NCIMB 13525 (obtained as DNA) (lane 27), *Desulfotomaculum ruminis* DSM 2154 (lane 28), *Dsm ruminis*-Reading (lane 29), negative control (lane 30). PCR positivity is indicated by the presence of a 135 bp PCR product.

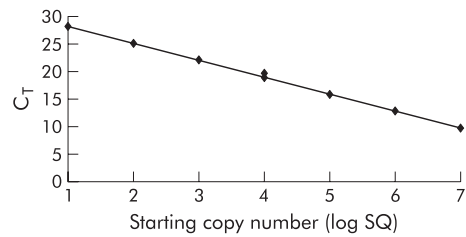
final elongation step at 72°C (five minutes). Determinations of optimum temperature were done using a Mastercycler Gradient PCR machine (Eppendorf, Hamburg, Germany).

### Purification and cloning of PCR products

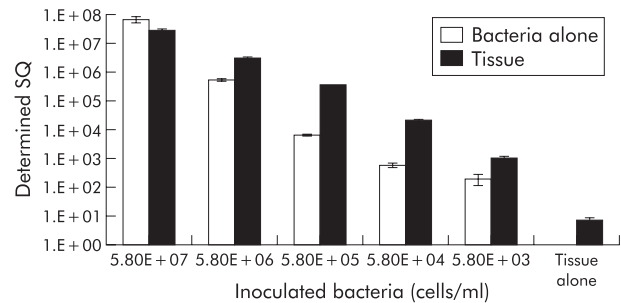
Reaction products (amplicons) were purified using QIAquick spin PCR purification kit (Qiagen) according to the manufacturer's instructions. PCR products were eluted in 30 µl. Purified PCR products were then used for sequencing and cloning. PCR products were ligated into the pGEMT Vector, as specified by Promega (Madison, USA). Competent *E. coli* JM109 cells were transformed with ligation products by heat shock at 42°C. LB medium supplemented with ampicillin (200 µg/ml) was used for cloning and subculture. Cells



**Figure 2** Post-amplification melt curve analysis of desulfovibrios. Fluorescence of SYBR Green with the progress of amplicon cycles is shown in (A). Values are duplicates of different dilutions of the plasmid used as standard. 16S rRNA gene copy numbers ranging from  $\log_{10}$  1.0 to 7.0 are shown in duplicate. The rate of change of fluorescence against temperature is shown in (B). The peak ( $T=89$  (1)) is specific to the genus *Desulfovibrio*. Symbols and lines correspond to amplifications shown in (A). PCR, polymerase chain reaction; CF, curve fit; RFU, relative fluorescent unit.



**Figure 3** Quantitation of desulfovibrios. A standard curve for desulfovibrios was constructed with 10-fold dilution of plasmid from  $10^1$ – $10^7$  copies. Threshold cycle ( $C_T$ ) is plotted against the starting copy number. Values on the line represent the result of duplicates. The equation for the curve is:  $C_T = -3.425 \log SQ + 33.16945$  ( $r=0.997$ ). The efficiency of the determination was calculated to be 97%.

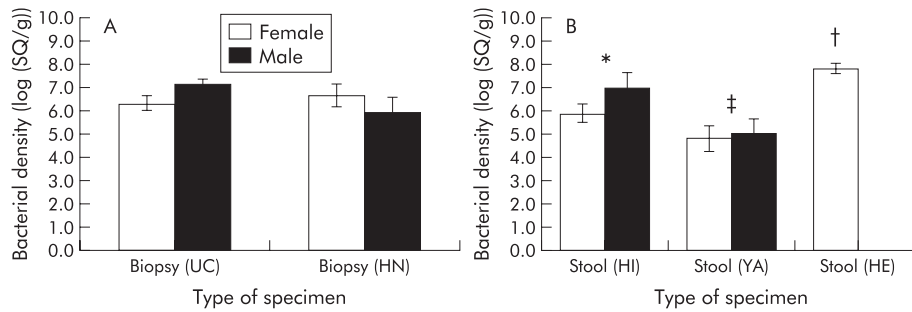


**Figure 4** Recovery of desulfovibrio DNA added to tissue samples by real time polymerase chain reaction (PCR). The target bacterium (*Desulfovibrio desulfuricans*) was added in 10-fold dilutions from a culture grown in Postgate's medium B to colonic tissue. The tissue was cut into six subsamples and each was treated with the bacterial preparation before extraction of genomic DNA. Means of triplicate copy number of 16S rRNA genes were plotted against bacterial cell numbers (mean (SEM)). A sample without added sulphate reducing bacteria was used to determine whether the original tissue samples contained desulfovibrios.

containing the correct plasmid insert were confirmed with conventional PCR and sequencing.

### Calculation of starting 16S rRNA gene copy numbers

Plasmid DNA was purified using the Wizard Plus SV Minipreps DNA Purification Kit (Promega). Miniprep concentrations were determined by electrophoresis and comparison of band strengths against molecular marker DNA (2 log DNA Ladder N3200L; New England Biolabs Ltd, Herts, UK). DNA concentrations were then converted into 16S rRNA gene copy numbers.



**Figure 5** Quantitation of desulfovibrios from biopsies (A) and different stool specimens (B). Bacterial density is expressed as copy number of 16S rRNA genes for desulfovibrios (SQ) per g of biopsy or stool. Biopsies from patients with ulcerative colitis (UC) and biopsies from healthy normal adults (HN), and faeces from healthy infants (HI), healthy young adults (YA), and healthy elderly people (HE) are shown. Values are mean (SEM) for each group. Significant difference, irrespective of sex: \* $p=0.008$  compared with stool YA; † $p=0.021$  compared with stool HI; and ‡ $p=0.000$  compared with stool HE.

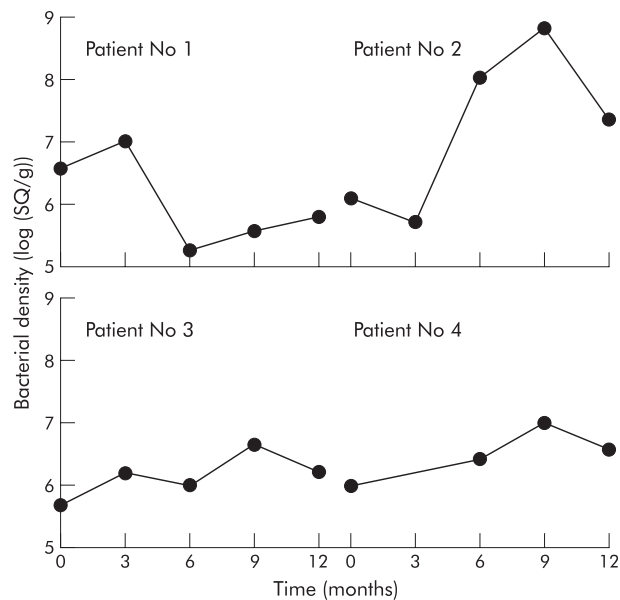
### Real time PCR

Quantitation of bacterial DNA was performed using an iCycler Real-Time PCR Detection System (BioRad, Hercules, California, USA). DNA melting curves were used to monitor product specificities. Detection was based on fluorescence resonance energy transfer, with a SYBR Green 490 fluorophore. The cycle number at which the signal was first detected correlated with the original concentration of DNA template, and the starting copy number of amplicons was inversely proportional to the real time threshold cycle.

Analysis of PCR amplification and melting curves was made using iCycler Optical System Software, version 3.0 (BioRad). Conditions included one cycle of 95°C for three minutes followed by 38 cycles of denaturation (95°C) and annealing (30 seconds) at 62°C. The melt curve analysis was run for 43 repeats, increasing by 1°C. Plasmid standards and samples were simultaneously assayed in duplicate.

### Bacterial recovery from tissue material

Bacterial recovery tests were conducted to validate DNA extraction and the analysis techniques. Serial dilutions of *Dsv desulfuricans* ( $10^3$ – $10^7$  bacterial cells/ml) were made in anaerobic phosphate buffered saline and added to colonic



**Figure 6** Stability of desulfovibrio populations on the rectal mucosa in four patients with ulcerative colitis.

tissue cut into six subsections to resemble six similar samples of biopsy material. Total DNA was extracted using the Qiagen Tissue Kit procedure. Desulfovibrio DNA recoveries from treated and untreated tissue were then compared with five dilutions of the bacteria with no added tissue.

### Statistical analysis

Data analysis was done using the SPSS v9.0 Statistics Package (SPSS, Chicago, USA). Copy numbers of 16S rRNA genes per mg of sample were transformed into logarithms and normally distributed data were subjected to statistical analysis. One way analysis (Tukey multiple comparison) of variance was used for comparison of bacterial densities between different specimens. The  $\chi^2$  test was used for determining associations of bacteria detection and types of samples.

### Chemicals

Unless stated otherwise, all chemicals were obtained from Sigma (Poole, Dorset, UK). Bacteriological culture media were purchased from Oxoid Ltd (Basingstoke, Hampshire, UK).

## RESULTS

### Development of real time PCR for enumerating intestinal desulfovibrios

A highly specific PCR primer set was developed for quantitating intestinal desulfovibrios in stools and biopsy tissues (table 2). *Desulfovibrio desulfuricans*, which had no nucleotide mismatch with the primers, and *Dsv vulgaris*, which had a mismatch of two nucleotides with the primer pair, showed strong PCR positivity.

The two strains of *Dsv desulfuricans* and *Dsv vulgaris* yielded a single electrophoretic band (135 bp amplicon) when amplified by conventional PCR. Colonic bacteria such as bacteroides, clostridia, bifidobacteria, enterococcus, escherichia, faecalibacterium, eubacteria, shigella, salmonella, veillonella, peptostreptococcus, and lactobacillus gave no bands (fig 1). Other SRB known to be present in the human large intestine, in lower numbers than desulfovibrios (desulfotomaculum, desulfobacter, desulfococcus), were tested for specificity of the primers. After 35 cycles of PCR amplification (annealing temperature 54°C) only desulfovibrios were detected (fig 2A) at about 20 ng of genomic DNA. Temperature gradient PCR showed an optimum annealing temperature of 62°C while no false positive amplifications were found over a wide range of annealing temperatures. As indicated by real time PCR, the negative derivative of fluorescence versus temperature ( $-d(RFU)/dT$ ), over the course of PCR product melt (fig 2B), generated a specific peak for desulfovibrios at 89 (1)°C. The average

efficiency of real time PCR from several experiments was found to be 97% (fig 3).

#### Recovery of desulfovibrio DNA from tissue sections

The effect of tissue material on recovery of desulfovibrio DNA is shown in fig 4. Untreated tissue sections harboured desulfovibrios and consequently, with the exception of the  $10^7$  inoculum, bacterial DNA was extracted from tissue in higher amounts than in controls. Results showed a good correlation between numbers of added bacteria and DNA recoveries from tissue, demonstrating high efficiencies in the extraction methods and real time PCR assay procedures. Irrespective of the amount of added bacteria, different tissue sections had variable bacterial densities due to differences in tissue size.

#### Measurements of desulfovibrios in stools and biopsies

Quantitation of intestinal desulfovibrios in UC and healthy rectal biopsies, and stools from three different population age groups is shown in fig 5A and B. In principle, the sensitivity of the method was about 10 bacteria, but median values were between  $10^5$  per gram of sample in healthy young adults to  $10^8$  in the elderly. The lowest prevalence of desulfovibrios was observed in faecal samples from healthy young adults (7/10) whose stools also had the lowest numbers of desulfovibrios. These organisms were found in 8/10 normal biopsy samples and 9/10 UC biopsies. Eight of the 10 UC patients were receiving therapy, and no real differences were seen in SRB counts (no therapy,  $n=2$ , 6.9 (0.08)/g biopsy; balsalazide,  $n=2$ , 7.1 (0.22); salazopyrine,  $n=2$ , 6.9 (0.35); minocycline,  $n=1$ , 6.59; Asacol,  $n=3$ , 6.1 (0.67)). There were also no significant differences in SRB counts between UC and healthy rectal biopsies. To assess the stability of mucosal desulfovibrios, bacteria were studied over a 12 month period in four UC patients (fig 6). Results showed great variations in numbers in two of the patients, over several orders of magnitude, whereas populations were stable in the other two individuals.

All stools from healthy elderly people and infants contained desulfovibrios, and individuals in these groups also had the highest numbers of SRB in their faeces. The  $\chi^2$  test showed that these associations were statistically significant (Pearson  $\chi^2 = 16$ ;  $p = 0.003$ ). Comparisons made between the mean values of bacterial cell population density and one way analysis of variance showed significant differences. The main difference related to the high levels of desulfovibrios in stool samples from healthy elderly people. However, there were no significant differences between males and females in the groups containing both sexes, with the exception of slightly higher male counts than female values in UC biopsy tissue which were just below statistical significance ( $p = 0.068$ ).

#### DISCUSSION

Desulfovibrios are the predominant SRB in the human large intestine.<sup>4, 22</sup> In addition to their links with IBD,<sup>3-7</sup> these bacteria are involved in a number of important processes in the bowel, including hydrogen disposal and other nutrient cross feeding reactions.<sup>2, 23-25</sup> In view of their metabolic significance in the gut, it is important that reliable technologies are available for their detection in faeces and mucosal tissue.

Investigating the human colonic microbiota using culturing techniques has certain limitations, such as the requirement for samples to be processed quickly, the need for specialised anaerobic techniques, and low cell recoveries, while bacterial isolations and identifications are time consuming and prohibitive for large scale studies. However, current developments in molecular analysis have overcome

many of these restrictions, and the design and employment of carefully evaluated primers is proving to be of value in identifying bacteria in complex microbial communities.<sup>26</sup> Nevertheless, probes developed for a particular group of bacteria may also detect unrelated organisms.<sup>20, 27, 28</sup>

The PCR primers developed here specifically detected and quantitated desulfovibrios in biopsy material and faeces. The method provided a highly sensitive means of identifying and quantitating these organisms, and facilitated high throughput bacterial analysis of biological samples. Tests using a wide range of gut microorganisms showed that the desulfovibrio primers did not target other bacteria (table 2, fig 1), including other intestinal SRB.<sup>29</sup> Real time PCR analysis had no secondary peaks or peaks corresponding to primer dimers (fig 2A) while the plasmid derived PCR amplicon was successfully diluted to copy numbers in the order of units, without loss of linearity (fig 3). Lysis of the cells by heat treatment,<sup>30, 31</sup> mechanical disruption, and enzymic and chemical extraction favoured recovery of bacterial DNA.

Real time PCR showed that desulfovibrios were ubiquitous in faeces, irrespective of age (fig 5B). However, there was an age related association with respect to numbers of these bacteria in stools. In particular, faeces from older people (64-83 years) showed significantly higher numbers of desulfovibrios compared with their younger counterparts (age range 20-35 years). This may reflect differences in sulphate availability through diet or endogenous sources, such as chondroitin sulphate and mucins,<sup>32</sup> as well as being a reflection of slower intestinal transit times in older people because SRB are relatively slow growing bacteria and this would facilitate proliferation of these organisms.<sup>3</sup> The occurrence of desulfovibrios in infants was very surprising because young children have immature developing microbiota<sup>33</sup> while SRB have a specialised mode of energy generation that makes them dependent on other intestinal microorganisms for their growth requirements.<sup>34</sup>

As well as occurring in high numbers in faeces, desulfovibrios heavily colonised mucosal tissue (fig 5A, fig 6). Although rectal biopsies from UC patients had generally higher numbers of desulfovibrios than healthy people, the differences were not statistically significant. This would suggest that if these bacteria play a role in UC, some host defect, possibly in sulphide detoxication pathways or in bacterial antigen handling, is required for disease to occur. Once inflammatory processes have been initiated, or the mucosal antibacterial response compromised, desulfovibrios may produce antigens that could damage the integrity of epithelial cells,<sup>5</sup> in addition to sulphide. However, sulphide is thought to be the single most important cytotoxic bacterial metabolite where inhibition of butyrate oxidation in epithelial cells has been demonstrated in UC patients.<sup>35, 36</sup>

Interestingly, although the patient numbers involved in the present investigation were low, anti-inflammatory drugs, which have previously been linked to inhibition of faecal SRB in UC,<sup>37</sup> appeared to have little effect on mucosal populations, which may reflect the inherent resistance of bacteria growing in biofilms to antimicrobial substances.<sup>38</sup> The potential link between mucosal SRB and UC is intriguing. Studies are now needed to determine how these biofilm communities interact with host immunity and metabolic processes in the mucosa.

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